NOVEL BIOTECHNOLOGICAL APPROACH FOR THE PRODUCTION OF CHITIN AND DE-ICING AGENTS

by

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DEDICATION

To my beloved grandmother, parents and brother whom have been a source of inspiration, guidance and support throughout my entire life

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LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviations

A	Adenine
$\bar{A}$	Absorbance
AC	Acetic acid
ADP	Adenosine diphosphate
AHA	American Heart Association
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BOD	Biological oxygen demand
BPST	2-(2-benzothiazolyl)-3-(4-phthalhydrazidyl)-5-styryl-2H-tetrazolium chloride
BT	Blue tetrazolium
C	Cytosine
CFU	Colony forming units
CMA	Calcium magnesium acetate
CNS	Central nervous system
COD	Chemical oxygen demand
CTC	5-cyano-2,3-ditolyl tetrazolium chloride
CV	Coefficient of variation
D	Dextro
DA	Degree of acetylation
DF	Degrees of Freedom
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DS	Demineralized shells
EDX	Energy Dispersive X-ray
EG	Ethylene glycol
ESEM	Environmental Scan Electron Microscope
FAO	Food and Agriculture Organization
FHWA	Federal Highway Administration
G	Guanine
INT	2-p-iodophenyl-3-p-nitrophenyl-5-phenyl-2H-tetrazolium chloride
L	Levo
LA	Lactic acid
MDS	Microbially deproteinized shells
MF	Microfiltration
MFGM	Milk fat globule membrane
MLD	Minimum lethal dose
MOE	Ministry of the Environment
MS	Mean of Squares
MSNF	Milk solids-non-fat
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide

NBT	Nitroblue tetrazolium
NOD	Nitrogen oxygen demand
NT	Neotetrazolium
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PER	Protein efficiency ratio
PG	Propylene glycol
PHB	Poly-3-hydroxybutyrate
P_i	Phosphate
PVC	Polyvinyl chloride
RNA	Ribonucleic acid
RO	Reverse Osmosis
SCP.	Single cell protein
SmF	Submerged fermentation
SMP	Skimmed milk powder
SS	Sum of Squares
SSF	Solid-state fermentation
T	Thymine
TBP	Tributylphosphate
TF	Triphenyl formazan
TKN	Total Kjeldahl Nitrogen
TNBT	Tetranitroblue tetrazolium
TRB	Transportation Research Board
TS	Total solids
TTC	Triphenyltetrazolium chloride
TV	Tetrazolium violet
U	Uracil
UF	Ultrafiltration
UV	Ultraviolet
WPC	Whey protein concentrate

Symbols

$(NH_4\text{-}N)_c$	Ammonium nitrogen of the recovered chitin
$(NH_4\text{-}N)_s$	Ammonium nitrogen of the sample
$(Org.\text{-}N)_c$	Organic nitrogen of the recovered chitin
$(Org.\text{-}N)_s$	Organic nitrogen of the sample
A	Arrhenius constant
$\bar{A}_{484}$	Absorbance measured at 484 nm
$\bar{A}_{492}$	Absorbance measured at 492 nm
A_b	Surface area of the UV reactor bottom
A_c	Annular section area
AC	Ash content
A_{iw}	Surface area of inside wall of the UV reactor
A_{ow}	Surface area of outside wall of the UV reactor

A_t	Surface area of the UV reactor top
a_w	water activity factor
A_w	Surface area of the UV reactor outer wall
C	Concentration of the substrate
Ca	Calcium concentration in the fouling material
C_{ga}	Galactose concentration
CH_c	Chitin content
C_{in}	Substrate concentration
C_{pe}	Specific heat of the cheese whey effluent
C_{TTC}	TTC concentration
D	Dose
d_b	Thickness of the UV reactor bottom
D_h	Hydraulic diameter
D_i	Inner diameter
D_o	Outer diameter
d_t	Thickness of the UV reactor top
E	Activation energy
E_a	Activation energy
E_d	Deactivation energy
f	Denotes that the properties are obtained at film temperature
F	Flow rate
F	Fat concentration in the fouling material
Fe	Concentration of iron in the fouling material
g	Gravitational acceleration
G	Gap size through which the cheese whey is flowing
Gr_f	Grashof number
Gr_{fb}	Grashof number for the UV reactor bottom
Gr_{fit}	Grashof number for the UV reactor top inner surface
Gr_{fot}	Grashof number for the UV reactor top outer surface
Gr_fPr_f	Rayleigh number
Gr_{fw}	Grashof number for the UV reactor wall
H_G	Rate of heat gain per unit volume
h_{ib}	Convective heat transfer coefficient between the inner surface of the UV reactor bottom and the cheese whey influent
h_{it}	Convective heat transfer coefficient between the inner surface of the UV top and the air entrapped inside the UV reactor
h_{iw}	Convective heat transfer coefficient between the cheese whey and the UV reactor inside wall
h_{ob}	Convective heat transfer coefficient between the outer surface of the UV reactor bottom and ambient air
h_{ot}	Convective heat transfer coefficient between the outer surface of the UV reactor top and ambient air
h_{ow}	Convective heat transfer coefficient between the UV reactor outside wall and the ambient air
H_t	Total heat gain per unit volume
I	Intensity

k	Decay rate constant
K	Concentration of potassium in the fouling material
k_a	Activation rate constant
K_b	Thermal conductivity of the bottom material
K_D	Equilibrium distribution coefficient
k_d	Deactivation rate constant
K_i	Inhibition concentration
K_{in}	Inhibition constant
K_m	Michaelis-Menten constant
K_t	Thermal conductivity of the top material
K_w	Thermal conductivity of the wall material
L_b	Characteristic length, diameter of the UV reactor bottom
L_t	Characteristic length, diameter for disc
L_w	Characteristic length, height of the reactor
MC	Moisture content
MC_e	Amount of moisture lost through evaporation with the exhaust gas
MC_i	Initial moisture content
MC_m	Contribution of metabolic heat production to moisture content
MC_{net}	Net moisture content
m_e	Mass flow rate of the effluent
Mg	Concentration of magnesium in the fouling material
N	Number of viable organisms present in the cheese whey
Na	Concentration of sodium in the fouling material
P	Concentration of phosphorous in the fouling material
pH	Medium pH
Pr	Protein concentration in the fouling material
PR_c	Protein content
Pr_f	Prandtl number
q	Volumetric flow rate
q_b	Rate of heat lost through the UV reactor bottom
q_e	Rate of heat lost with the effluent
q_l	Rate of heat generated by the UV lamp
q_t	Rate of heat lost through the UV reactor top
q_w	Rate of heat lost through the UV reactor wall
R	Gas constant
R^2	Coefficient of determination
Re	Reynolds number
r_i	Inner radius of the UV reactor
r_o	Outer radius of the UV reactor
S	Concentration of sulfur in the fouling material
t	time
T	Absolute temperature
T_a	Laboratory ambient temperature
T_f	Film temperature
TF_a	Triphenyl formazan yield under aerobic conditions
TF_{an}	Triphenyl formazan yield under anaerobic conditions

T_{fb}	Film temperature for the UV reactor bottom
T_{fit}	Film temperature for the UV reactor top inner surface
T_{fot}	Film temperature for the UV reactor top outer surface
T_{fw}	Film temperature for the UV reactor wall
T_i	Inlet cheese whey temperature
t_I	Incubation time
T_I	Incubation temperature
T_{ia}	Temperature of the UV reactor headspace
T_{it}	Temperature of the inside surface of the UV reactor top
TKN_c	Total Kjeldahl nitrogen of the recovered chitin
TKN_s	Total Kjeldahl nitrogen of the sample
T_m	Mean temperature of the cheese whey
T_o	Outlet cheese whey temperature
T_{ob}	Temperature of the outside surface of the UV reactor bottom
T_{ot}	Temperature of the outside surface of the UV reactor top
T_{ow}	Temperature of the outside surface of the UV reactor wall at mid height
T_s	Temperature of the lamp socket surface
T_{w1}	Temperature of the outside surface of the UV reactor opposite to the inlet
T_{w2}	Temperature of the outside surface of the UV reactor at mid height
T_{w3}	Temperature of the outside surface of the UV reactor opposite to the outlet
U_b	Overall heat loss coefficient through the UV reactor bottom
U_t	Overall heat transfer coefficient of the UV reactor top
U_w	Overall heat transfer coefficient of the UV reactor wall
V	Working volume of the UV reactor
W	Weight of fouling material
W_a	Weight of the ash
W_c	*A. niger* dry cell weight
W_c	Weight of recovered chitin
W_{cw}	*A. niger* wet cell weight
W_{ds}	Weight of the dry sample
W_s	Weight of sample
W_{ws}	Weight of the wet sample
β	Volume coefficient of expansion
υ	Volumetric rate of reaction
υ	Kinematic viscosity
υ_m	Mean velocity
υ_{max}	Maximum reaction rate

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CONTRIBUTIONS TO KNOWLEDGE

The contributions of this thesis to knowledge in the field of biotechnology can be summarized as follows:

1. An efficient on-line sterilization method for cheese whey using ultraviolet radiation was proposed.
   a. It has been demonstrated that ultraviolet radiation can be used for the sterilization of highly turbid material such as cheese whey if a properly designed photoreactor is used.
   b. It has been demonstrated that fouling can be a major limitation in the use of tubular UV reactors when used for an extended period of time due to heat generation.
   c. The mechanism of UV lamp fouling has been documented.
   d. Recommendation for the enhancement of the sterilization efficiency of cheese whey using ultraviolet radiation was proposed.

2. New information on triphenyltetrazolium chloride (TTC) for biomass quantification was provided.
   a. The nonezymatic reduction of TTC at high temperatures and/or pH values was documented.
   b. The reduction of the red color intensity of formazan solutions in the presence of copper due to the formation of triphenyl formazan copper complex has been demonstrated.
   c. The effect of *Aspergillus niger* cell age on the enzymatic reduction of TTC has been demonstrated.
   d. The presence of residual dehydrogenase activity after autoclaving of *A. niger* was documented.
   e. The effect of the presence of oxygen on the reduction of TTC has been documented.
   f. The contribution of shrimp shells to the reduction of TTC has been documented.

3. A novel biotechnological approach for the use of two potent pollutant waste materials (cheese whey and shrimp shells) for the production of three value-added products (salts of organic acids, chitin and protein liquor was proposed.
      a. The effects of initial galactose concentration, particle size, initial pH and autoclaving on the deproteinization of shrimp shells using *A. niger* were demonstrated.
      b. The inhibitory effect of lactic acid on the growth of *A. niger* and proteases production was documented.
      c. The effectiveness of organic acids (lactic and acetic) for the demineralization of shrimp shells was documented.
      d. The production of de-icing agents from the demineralization process of shrimp shells using acetic acid was demonstrated.

ABSTRACT

This study proposed the use of two potent pollutant waste materials (cheese whey and shrimp shells) for the production of three value-added products: salts of organic acids (useful as de-icing agents), chitin (for use in the medical, biomedical and pharmaceutical industries) and protein liquor (useful as protein supplement in animal feed). The study was carried out in four phases.

In the first phase, the applicability of on-line sterilization of cheese whey using ultraviolet radiation (UV) was investigated. It was found that despite the high turbidity of the cheese whey, UV radiation can be used for cheese whey sterilization if the proper flow thickness and residence time are used. A residence time of 0.8 h was required to completely destruct a microbial population of 5.95×10^6 cells/mL using tubular UV reactors of 6 mm gap size. Fouling was found to be a major limitation in tubular UV reactors when used for extended period of time as a result of lamp heat generation. Adsorption and direct ion exchange were the most likely fouling mechanisms. Recommendations were made for the design of an efficient on-line sterilization system for cheese whey.

In the second phase, the applicability of tetrazolium salt triphenyltetrazolium chloride (TTC) for biomass quantification was investigated. For dehydrogenase activity measurements, the pH of the samples and the incubation temperature should not be higher than 9 and 60 °C in order to ensure that the TTC reduction is caused only by the biochemical reaction. The presence of copper in the medium caused a reduction in the red color intensity due to the formation of formazan copper complex. The optimum TTC-test conditions for biomass quantification of *Aspergillus niger* in liquid growth medium were TTC concentration of 20 g/L, a pH of 9, a temperature of 55 °C, and incubation time of 3 h and anaerobic conditions. The age of *A. niger* cells affected the TF yield. Shrimp shells contributed to the reduction of the TTC and therefore TTC-test can be used for *A. niger* quantification during solid-state fermentation of shrimp shells with some limitations.

In the third phase, a biotechnological approach was used for the deproteinization of shrimp shells using the fungus *Aspergillus niger* in solid-state fermentation. *A. niger* was able to produce proteases in the presence of shrimp shells and galactose as a carbon source, thus causing the deproteinization of the shrimp shells. An initial galactose concentration of 20% w/w, ground shells, autoclaved shells were found to be optimum for the deproteinization process. Using lactic acid for pH adjustment inhibited the growth of *A. niger* and the protease production. Moistening shrimp shells with buffered medium at low pH may enhance the deproteinization process.

In the forth phase, the applicability of using lactic and acetic acids for the demineralization of microbially deproteinized shells was investigated. The effectiveness of lactic and acetic acids for the demineralization of shrimp shells was comparable to that of hydrochloric acid. Retention time of 2 h and shells to acid ratio of 1:20 were found satisfactory. Under these conditions, the total minerals and calcium removal efficiencies were 97.4 and 99.11% and 86.36 and 85.33% for lactic and acetic acids, respectively. The use of acetic acid for the demineralization process is preferred since acetate salts have excellent de-icing capabilities.

1. INTRODUCTION

Each year, the seafood processing industry generates huge amount of solid wastes, which have become a major environmental concern to the main producing countries due to the high perishability and the bulky nature of the waste material (Hall and De Silva, 1992; Hall et al., 1994; and Zakaria et al., 1998). Environmental and economic pressures also are being brought to bear on shellfish producers to reduce the waste from the processing plants (Hall and Reid, 1995). Shellfish waste is considered a rich source of many valuable products including: chitin, protein, pigments (e.g. astaxanthin), and flavor compounds. However, chitin, a high versatile biopolymer, is the most valuable component of the waste material (Zakaria et al., 1998). The number, importance and variety of industrial uses (e.g. water treatment, pulp and paper, biomedical devices and therapies, cosmetics, biotechnology, agriculture, food science and membrane technology) of chitin and its derivatives are growing rapidly (Dunn et al., 1997).

All crustacean shells (e.g. shrimp, crab, lobster, etc.) contain considerable amount of chitin and are considered the most important chitin source for commercial use (Gagné and Simpson, 1993; Abdullah, 1995; Kim and Lee, 1995; Sophanodora and Hutadilok, 1995; and Subasinghe, 1995). However, chitin present in the crustacean waste is associated with proteins, inorganic material (mainly calcium carbonate) and lipids including pigments. The traditional method of chitin extraction from crustacean waste involves the use of strong acid and alkali (e.g., HCl and NaOH), for demineralization and deproteinizaton, respectively (Kong, 1975; Gagné and Simpson, 1993; Bustos and Healy, 1994; Hall et al., 1994; and Zakaria et al., 1998). This method has many disadvantages: (a) it yields end product with variable and inconsistent physiochemical properties, (b) it results in effluent wastewater, which creates disposal problems, (c) it wastes the other valuable components present in the waste material and (d) the process costs are fairly high. The high price of technical grade chitin along with the environmental problems associated with its purification precludes its widespread industrial use (Muzzarelli, 1990). Therefore, a less expensive environmentally friendly method for chitin extraction from crustacean waste is needed (Kim and Lee, 1995; and Sophanodora and Hutadilok, 1995).

Several studies proposed the use of biotechnological approaches such as lactic acid bacterial fermentation for demineralization (Hall and De Silva, 1992; Hall et al., 1994; and Hall and Reid, 1995) and proteolytic enzymes for deproteinization either by using proteolytic microorganisms, which secret extracellular proteases (Shimahara et al., 1982; Cano-Lopez et al., 1987; Shimahara and Takiguchi, 1988; Gagné and Simpson, 1993; Bustos and Healy, 1994a; Bustos and Healy, 1994b; Wang and Chio, 1998; and Teng et al., 2001), or by adding purified proteolytic enzymes (Takeda and Abe, 1962; Takeda and Katsuura, 1964; Wang and Chio, 1998) for the extraction of the chitin from crustacean waste. However, deproteinization by enzymes is not as effective as living microbes since: (a) presence of microorganisms provides a constant and gradual increase of protease throughout the fermentation period and (b) microorganisms can grow in the interstices of shell waste structure and allow for closer contact between the enzyme producing site and the protein substrate (Bustos and Healy, 1994; and Teng et al., 2001). Nonetheless, chitin extraction using biotechnological approaches appears to be a good alternative to the traditional chemical approach since: (a) it is considered less expensive compared to the traditional method, (b) it results in an effluent that is less harmful to the environment and (c) it preserves the natural state of the biopolymer.

Cheese whey is a by-product from cheese or casein manufacture (Zall, 1992; and Yang et al., 1993). The total world production of cheese whey was ~ 139.6 million metric ton in 2000 (FAO, 2001). Discarding cheese whey as untreated effluent creates huge disposal problems due to its high biological oxygen demand (BOD) (~ 40000 mg/L) and chemical oxygen demand (COD) (~ 60000 mg/L) (Hall and Adams, 1988; and Siso, 1996). The actual market trends point to a gradual increase in cheese production that generates large amount of liquid whey per year (Yang et al., 1992; and Siso, 1996). Cheese whey contains lactose, protein, salts, lactic acid and traces of vitamins (Ghaly and Tango, 2000). Protein and lactose are the most valuable components of whey. However, protein recovery does not solve the BOD problem associated with the remaining lactose in effluent. Whey lactose can be utilized for the production of organic acids (e.g. lactic, acetic and propionic acids) using microbial fermentation. Salts of organic acids such as

potassium acetate, calcium magnesium acetate, sodium lactate are useful for de-icing/anti-icing practices.

The use of de-icing/anti-icing agents is necessary in parts of the world where temperature fall below 0° C. In these regions, winter precipitation in the form of snow and ice can cause traffic accidents and paralyze movement of goods and people thus causing closure of business and government offices which results in significant economic costs (Hanneman, 1992). It has been found that 90% of the cost can be avoided by the simple expedient de-icing of roads (Sills and Blakeslee, 1992). Moreover, traffic accidents can be reduced by 75% when icy and snowy roads are salted to restore bare pavement. Flight safety can also be insured by applying large amount of de-icing/anti-icing chemicals in airports (Fritzsche, 1999). De-icing agents are classified into three main groups: (a) inorganic salts, (b) organic salts and (c) organic compounds. Inorganic salts, especially sodium chloride, are the most popular de-icers because of their effective snow ice control, low cost in comparison to other de-icing chemicals, availability, and simple handling, storing, and applying techniques (Gales and Vander-Meulen, 1992; Menzies, 1992; Demers, 1992; and Yang et al., 1992). However, over the years the heavy use of inorganic salts led to many negative side effects including damage to motor vehicles, infrastructure, and the natural environment. Marynowski et al. (1985) stated that the economic argument for using sodium chloride is weakened if the cost of the environmental damages caused by using it has been taken into consideration. Table 1.1 shows some incidents of environmental damage that occurred as a consequence of de-icing chemicals application. Therefore, an environmentally friendly cost effective ice control chemical is needed in order to both reduce the environmental impact and the application cost at the same time. The use of organic salts as de-icing/anti-icing agents offers many advantages over inorganic salts (Ghaly et al., 2001; and Mahmoud and Ghaly, 2002). They have a low BOD and are less harmful to vegetative, aquatic and mammalian life. The melting point of organic salt-water mixtures is much lower than inorganic salts. In addition, organic salt compounds are biodegradable and hence cause less accumulation of salts in the environment.

Table 1.1. Environmental damage incidents caused by the addition of de-icing agents.

City	De-icing Agent	Environmental Damage	Reference
Minnesota	Sodium Chloride	Incidents of vegetation injury were reported during 1950's. Death and removal of nearly 14,000 trees have been reported.	Fritzsche (1992)
Ontario	Sodium Chloride	Fruit crops damage has been reported.	Fritzsche (1992)
Lake Tahoe Basin	Sodium Chloride	Thousands of roadside conifers have been destroyed.	Fritzsche (1992)
Massachusetts	Sodium Chloride	Contamination of one-third of the municipal drinking water supplies has been reported.	Fritzsche (1992)
First Sister Lake in Ann Arbor, Michigan	Sodium Chloride	Complete DO depletion in bottom waters was reported as a result of increased sodium and chloride concentrations, 55 mg/L and 136 mg/L respectively.	Fritzsche (1992)
North America	Sodium Chloride	More than one-half corrosion damage to highway structures had been reported.	Fritzsche (1992)
Main	Sodium Chloride	Chloride concentrations of 5-more than 100 mg/L and 1.4-221 mg/L in small ponds and farm ponds respectively have been reported.	Moran et al. (1992)
Near Madison, Wisconsin	Sodium Chloride	Chloride concentrations had been quadrupled between 1959 and 1962 in five lakes.	Moran et al. (1992)
England	Urea	Two fish kill occurrences has been reported.	Sills and Blakestee (1992)
Missouri (Lambert Field, St. Louis)	Ethylene Glycol	Fish kill at Lambert Field has been reported	Sills and Blakestee (1992)

However, at the present time organic salts are more costly than sodium chloride and are, therefore, too expensive for widespread use, even though some studies have shown that the savings in infrastructure replacement costs can offset all of its material cost (Moran et al., 1992). Using organic salts, as deicers, will be widely accepted if its production cost can be reduced. Current commercial production of organic acids is exclusively by petrochemical routes. In recent years, many attempts to produce low-cost organic acids from fermentation of glucose, woody biomass and cheese whey had been proposed. Fermentation produced organic acids, from low cost substrate such as cheese whey, could be used to manufacture low cost organic salts that can be used as organic biodegradable noncorrosive de-icing/ anti-icing salts. Tango and Ghaly (2002) proposed a process for the production of high lactic acid concentration (75.6 g/L) from cheese whey using a continuous lactic acid fermentation system in which immobilized packed bed of *Lactobacillus helveticus* was applied. Yang et al. (1999) proposed also a process for the production of high acetic acid concentration (75.0 g/L) from cheese whey fermentation using recycle fed-batch immobilized coculture of *Lactococcus lactis* and *Clostridium formicoaceticum*.

The current study proposes the use of two potent pollutant waste materials (cheese whey and shrimp shells) for the production of three value-added products: salts of organic acids (useful as de-icing/anti-icing agents), chitin (useful for the medical, biomedical and pharmaceutical industries) and protein liquor (useful as ingredient in animal feed). A biotechnological approach was used to recover chitin from shrimp shell waste using microorganisms that secret extracellular proteolytic enzymes for deproteinization and lactic acid/ acetic acid for demineralization. The final products of the proposed process would include chitin, calcium lactate/acetate and protein liquor. The proposed process would provide multiple economic and environmental benefits by: (a) reducing the production cost of anti-icing/de-icing agents and chitin and its derivatives, (b) reducing the pollution problems associated with the disposal of cheese whey, shellfish waste and waste from the chitin extraction process which uses inorganic chemicals, (c) eliminating the environmental problems associated with the use of inorganic de-icing/anti-icing

agents and (d) enhancing the economy of the dairy and fishery industries by converting these waste products into highly profitable value-added products.

2. OBJECTIVES

The study aimed at the development of a novel bio-process for the production of three value-added products (calcium lactate/acetate as environmentally friendly de-icing/anti-icing agents, chitin as a high commodity biopolymer and protein liquor as a high potential animal feed additive) using two potent waste materials (cheese whey and shrimp shells). The specific objectives were:

1. To investigate the effectiveness and reliability of UV on-line cheese whey sterilization technique.
2. To study the applicability of the use of the tetrazolium salt triphenyltetrazolium chloride (TTC) for biomass quantification and to establish the optimum test conditions for *Aspergillus niger* quantification.
3. To design and construct a solid-state fermentation system for chitin purification from shrimp shell waste.
4. To study the various parameters that affect deproteinization of shrimp shells using the fungus *Aspergillus niger* in drum solid-state fermentation bioreactor.
    a. Addition of galactose at various initial concentrations
    b. Particle size of the shrimp shells
    c. Initial pH
    d. Autoclaving of the shrimp shells
5. To study the effect of various parameters that affect the demineralization of the microbialy deproteinized shrimp shells using lactic and acetic acids.
    a. Acid type and demineralization conditions
    b. Retention time
    c. Shells to acid ratio

3. SCOPE OF WORK

This work was carried out in four phases:

Phase 1: On-line sterilization technique

The ability of ultraviolet radiation to sterilize cheese whey, and the performance of three tubular UV reactors (consisted of a low pressure UV lamp enclosed in a quartz tube submerged in a chamber, through which the fluid flows) having different gap sizes (18, 13, and 6 mm) at various flow rates (3.84, 1.92, 1.28, 0.96, 0.76, 0.64 L/h) and residence times (0.25, 0.50, 0.75, 1.00, 1.50, and 2.00 h) were investigated. Heat generation by the low pressure UV lamp and various heat losses through the UV reactor were determined. UV lamp fouling during on-line cheese whey sterilization was investigated.

Phase 2: TTC-Test

The applicability of using the tetrazolium salt Triphenyltetrazolium chloride (TTC) for biomass quantification was investigated. The effects of temperature and pH on the chemical reduction (non-enzymatic reduction) of TTC were investigated using two different waste materials (cheese whey and municipal solid waste compost) having different composition and different moisture content. Ten different incubation temperatures (40, 50, 60, 70, 75, 80, 85, 90, 95 and 100° C) were studied at 2 h incubation period and a pH of 7 (a preliminary experiment showed that no chemical reduction of TTC could be observed at pH of 7). Also, thirteen pH values (1.5, 3.0, 4.5, 6.0, 9.0, 9.5, 10.0, 10.5, 11.0, 11.5, 12.0, 12.5 and 13.0) and three different incubation periods (1, 2 and 3 h) were tested at incubation temperature of 40° C (a preliminary experiment showed that no chemical reduction of TTC could be observed at or below this temperature). The effect of the presence of six heavy metals (copper, potassium, zinc, sodium, calcium and nickel) on color intensity of formed triphenyl formazan (TF) was also studied.

The optimum TTC-test conditions (TTC concentration, pH, temperature, incubation time and presence/absence of O_2) for biomass quantification of the fungus *Aspergillus niger* was developed in liquid growth medium. 16 TTC concentrations (2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0, 22.5, 25.0, 27.5, 30.0, 32.5, 35.0, 37.5 and 40.0 g/L), 3 pH values (7, 8 and 9), three incubation temperatures (25, 40 and 55 °C) and 14 incubation periods (15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330 and 360 min) were tested. The effect of *A. niger* cell age on TF yield was also investigated. Standard curves for *A. niger* quantification using the optimum test conditions (TTC concentration, pH, temperature and incubation time) were generated in the absence and presence of O_2. The effectiveness of the developed TTC–test for measuring the microbial growth of *A. niger* during solid-state fermentation of shrimp shells was investigated. The effects of amount of alcohol used for TF extraction, filtration of the extracted TF solution and autoclaving of shrimp shells on TF yield and contribution of shrimp shells to TF yield were investigated. An attempt to develop a standard curve in the presence of shrimp shells was done.

Phase 3: Development and Testing of Solid-State Drum Bioreactor for Deproteinization of shrimp shells

The experimental setup consisted of a system of three small drum type bioreactors each has a working capacity of 200 g intact shrimp shells or 400 g ground shrimp shells, 11 thermocouples, an aeration system, a humidifier and a data acquisition system for monitoring temperature during the course of the deproteinization process. The effect of initial galactose concentration, shrimp shells particle size, initial pH and autoclaving on the temperature, pH and moisture content of the shrimp shells, residual galactose concentration in the shrimp shells, carbon dioxide concentration in the exhaust gas, protease activity and protein and chitin concentrations in the shrimp shells during the deproteinization process. Three initial galactose concentrations (10, 20 and 30 w/w), two particle sizes (intact versus grinded), two initial pH values (8.64 and 7.00) and autoclaving were studied.

Phase 4: Demineralization of Microbially Deproteinized Shrimp Shells

The applicability of using organic acids (lactic and acetic) for the demineralization of microbially deproteinized shells was investigated. The effect of acid type (hydrochloric, lactic and acetic acids) and demineralization conditions (A and B) were studied. Condition A included a ratio of 1 g shells to 50 mL acid, a temperature of 100 °C and a retention time of 1 h. Condition B included a ratio of 1 g shells to 10 mL acid, a temperature of 24 °C (room temperature) and a retention time of 6 h. The effects of two retention times (2 and 6 h) on the demineralization of microbially deproteinized shells using lactic and acetic acids in the ration of 1 shells to 10 mL acid at room temperature (24 °C) were also studied. Finally, the effects of shells to acid ratios (1:10, 1:20 and 1:30 g shells: mL acid) on the demineralization of microbially deproteinized shells using lactic and acetic acids at room temperature for 2 h were studied.

4. LITERATURE REVIEW

4.1 Cheese Whey

Whey, a by-product from cheese or casein manufacture, originates from mammal's milk, as do it's permeate (Zall, 1992; and Savant and Torres, 2000). It has been characterized as the fluid obtained after separating the coagulum from whole milk, cream or skim milk. Generally, most whey comes from cheese making, but some of it results from the production of casein. Whey from cheese is mostly made from milk processed with rennet or acid produced by microbial fermentation methods where the degree of acidity in the process depends on cheese variety. However, some kinds of cheese use a combination of enzymes and microbial processes.

4.1.1 Types of Cheese Whey

Cheese whey falls into two major types depending into its acidity: sweet whey and acid whey. However, whey acidity will change and sweet whey can ultimately become acidic as culture organisms continue to grow unless their growth is retarded by heating or cooling practices. Sweet whey results from the manufacture of dairy products (cheddar cheese) that principally use rennet-type enzymes at a pH of about 5.6 or more. Acid whey results as the by-product of dairy processes (cottage and soft cheese) where the coagulum is formed by acidification at a pH of about 5.1 or below. In addition to having lower pH, higher lactic acid and lower lactose contents, acid whey has substantially higher calcium and phosphorous contents caused by the solubilization of calcium-phosphate complex of casein micelle at the acidic pH range used for the acid coagulation of the casein (Silva and Yang, 1995; and Siso, 1996; and Francis, 2000).

4.1.2 Composition of Whey

Whey, a by-product of cheese manufacturing, represents about 85 – 95% of the milk volume and retains about 55% of the milk nutrients (Singh and Ghaly, 1985; and Siso, 1996). It is a dilute liquid containing lactose, minerals (e.g., calcium, magnesium, phosphorus), noncasein protein (except glycomacropeptide), and traces of milk fat and

vitamins. Cheese whey contains approximately 6% total solids of which over 70% is lactose. It contains also appreciable quantities of other components, such as lactic acid (0.05% w/v) and citric acids, non-protein nitrogen compounds (urea and uric acid), and B group vitamins (Tang et al., 1988; Yang et al., 1992; Zall, 1992; Siso, 1996; Huang and Yang, 1998; and Savant and Torres, 2000). Table 4.1 shows the composition of both acid and sweet wheys. The protein and lactose contents are 13.4 and 12.5% and 75.0 and 67.4% of the dry weight, respectively. The mineral content in sweet whey (7.3%) is less than that of the acid whey. The lower lactose content in acid whey powder results from the lactose being converted into lactic acid, while the higher mineral level is due to solubilization of calcium by acidification. Cheese whey salts are comprised of NaCl and KCl (more than 50%), calcium salts (primarily phosphate) and magnesium. Tables 4.2 and 4.3 show the composition of some whey products and the mineral and vitamin content of liquid and dry whey.

Protein and lactose are the most valuable components of whey. Protein has the greatest value for the following reasons: (a) the biological value of whey protein is superior to most other proteins and its biological value exceeds even that of whole egg protein, (b) whey proteins have proportionately more sulfur- containing amino acids (e.g., cysteine and methionine) than casein and the sulfur amino acid content of whey proteins is higher than that of whole-milk proteins (1.35% versus 0.36%) as reported by Siso (1996), (c) the protein efficiency ratio (PER) value of whey protein is high (3.5) compared to standard casein (2.8), (d) whey protein represents about 20% of the milk proteins, the most abundant of which are beta-lactoglobuline (50%), alpha-lactalbumin (12%), immunoglobulins (10%), serum albumin (5%) and proteose peptones (0.23%) of total whey protein (Siso, 1996; Caessens et al., 1999; and Savant and Torres, 2000), (e) whey proteins are globular proteins with a limited number of disulfide bonds (Kinsella and Whitehead, 1989), and (f) whey proteins denaturalized rapidly at temperatures above 70° C. Major whey proteins exhibit thermostability to structural unfolding in the order α-lactalbumin < albumine < immunoglobulin < β-lactoglobulin (Cheryan, 1998). Francis (2000) reported that about 50 – 60% of the whey protein can be coagulated by heat (Table 4.4). However, during the manufacture of whey protein concentrate (WPC),

Table 4.1. Characteristics and composition of cheese whey (Cheryan, 1986; and Yang et al., 1999).

Composition (%)	Sweet Whey[a]		Acid Whey[b]	
	Fluid	Dried	Fluid	Dried
Moisture	93 - 94	3.5	93.5 – 94.0	4.0
Total solids	6.0 – 7.0	96.5	6.0 - 6.5	96.0
Lactose	4.2 – 5.0	75.0	4.2 – 4.9	67.4
Protein (N×6.38)	0.7 – 0.9	13.4	0.7 – 0.8	12.5
Fat	0.1 - 0.5	0.8	0.1	0.6
Lactic acid	0.05	0.2	0.04	4.2
Ash	0.5 – 0.6	7.3	0.5 – 0.7	11.8
Calcium	0.047	0.7	0.103	1.1
Potassium	0.161	0.7	0.143	1.9
Sodium	0.054	2.1	0.048	2.3
Magnesium	0.008	1.3	0.010	1.1
pH value	5.6 – 6.3		4.0-4.7	

[a] from cheddar cheese manufacturing
[b] from cottage cheese manufacturing

Table 4.2. Composition of whey products (Kosaric and Asher, 1985).

Product	Component (%)				
	Lactose	Protein	Fat	Ash	Moisture
Condensed whey	38.5	7.0	2.4	4.0	48.1
Dried Sweet whey	73.5	12.9	0.9	8.0	4.5
Dried acid whey	66.5	13.0	-	10.2	3.2
Modified whey solids	56.6	36.0	3.3	2.4	1.7

Table 4.3. Minerals and vitamins presented in liquid and dried whey (per 100 g) (Kosaric and Asher, 1985).

Component	Unit	Liquid whey	Whey Powder
Calcium	mg	51.00	646.00
Phosphorous	mg	53.00	589.00
Iron	mg	0.10	1.40
Vitamin A	I.U.	10.00	50.00
Thiamin	mg	0.03	0.50
Riboflavin	mg	0.14	2.51
Niacin	mg	0.10	0.80
Food energy	cal	26.00	349.00

Table 4.4. Thermal characteristics of the whey protein fraction (Francis, 2000).

Protein	Denaturation Temperature (°C)	Heat Precipitation Stability
α-Lactalbumin	61	Quite stable[a]
β-Lactoglobulin	82	Labile
Serum albumin	66	Very labile
Immunoglobulins	72	Extremely labile
Proteose-peptones		Heat stable
Other (caseins glycoproteins)		

[a] due to its renaturation characteristics and despite its low denaturation temperature.

permeate with a high lactose content is formed as a by-product. Therefore, protein recovery does not solve the BOD problem associated with the remaining lactose in effluent.

Cheese whey lactose is also important for the following reasons: (a) it is the major component (70 - 80%) of whey solids, (b) it is considered a low cost carbon source for fermentation processes, (c) it is the component responsible for the high BOD and COD and converting it to value-added products helps in reducing the adverse environmental impact of cheese whey waste material, and (d) many value added products such as organic acids, single cell protein, ethanol, methane and biopolymers could be produced from cheese whey lactose.

4.1.3 Cheese Whey Production

The total world production of all kinds of cheese in 2000 was 16,045,795 metric tons of which 4,057,450 (25.3%) and 351,000 (2.2%) metric tons are produced in USA and Canada, respectively (FAO, 2001). Typically, every 10 kg of milk produces 1.3 kg of cheese and 8.7 kg of whey (Kosikowski, 1979; Cheryan, 1986; Hall and Adams, 1988; and Morr, 1992). Table 4.5 shows the estimated whey production in the world countries during the period 1995 - 2000. Actual market trends point to a gradual increase in cheese production that generates more than 139,598,417 metric tons of which 35,299,815 and 3,053,700 metric tons are produced in USA and Canada, respectively. The estimated lactose content is about 6×10^6 tons.

4.1.4 Environmental Impact of Cheese Whey Disposal

Since the first commercialization of cheese manufacture, whey has been considered a waste product (Pearce, 1992). Only in the last two decades of environmental awareness and economic competitiveness has the concept arisen of whey as a valuable co-product of cheese manufacturing. Although several possibilities for cheese whey exploitation have been assayed over the last 50 years, approximately 50% of the world cheese whey production is disposed in sewer systems, water bodies and/or land (Cheryan, 1986; Siso, 1996 and Cristiani-Urbina et al., 2000). Few uses have been found for acid whey because

Table 4.5 Annual cheese whey production for cheese producing countries during the period 1995-2000 (FAO, 2001).

Country	Cheese Production (metric tonns)					
	1995	1996	1997	1998	1999	2000
Afghanistan	155,817	175,392	196,533	222,372	244,296	244,269
Albania	86,130	132,240	97,440	97,440	99,180	99,180
Algeria	9,092	9,092	9,092	9,091	9,092	9,091
Angola	8,169	8,970	9,640	10,466	10,449	10,449
Argentina	3,210,300	3,271,200	3,871,500	3,654,000	3,697,500	3,758,400
Armenia	78,213	75,255	77,430	84,286	85,808	81,354
Australia	2,039,280	2,296,800	2,479,500	2,566,500	2,679,600	2,679,600
Austria	1,046,758	1,042,356	1,105,257	1,172,473	1,190,447	1,190,447
Azerbaijan	33,930	15,660	10,440	9,222	7,613	7,830
Bangladesh	8,700	8,700	8,700	8,700	8,700	8,700
Belarus	311,843	343,259	444,631	535,085	495,117	476,064
Belgium-Luxembourg	636,866	650,690	680,410	654,936	565,500	565,500
Bhutan	374	374	374	374	374	374
Bolivia	58,795	58,795	58,795	58,795	58,795	58,794
Bosnia and Herzegovina	117,450	117,450	117,450	121,800	95,700	75,690
Botswana	20,297	20,619	26,613	20,054	19,262	19,262
Brazil	198,360	288,405	291,450	334,950	334,950	334,950
Bulgaria	708,763	660,434	575,027	622,920	504,600	504,600
Canada	2,726,980	2,707,919	3,116,601	3,059,094	3,071,100	3,053,700
Chile	413,389	425,230	438,584	463,084	458,186	458,185
China	1,606,890	1,610,370	1,658,220	1,510,320	1,612,110	1,701,285
Colombia	443,700	443,700	443,700	443,700	443,700	443,700
Costa Rica	49,503	49,068	46,180	46,632	50,199	50,199
Croatia	155,782	169,989	187,224	167,284	164,909	164,908
Cuba	126,150	126,150	126,150	126,150	126,150	126,150
Cyprus	47,415	47,981	48,807	38,628	38,976	38,976
Czech Republic	832,399	1,021,589	1,108,102	1,244,883	1,262,283	1,272,723
Denmark	2,707,440	2,603,040	2,528,220	2,516,910	2,523,000	2,566,500
Dominican Rep.	21,750	21,750	21,750	21,750	21,750	21,750

Table 4.5 Continued.

Country	Cheese Production (metric tonns)					
	1995	1996	1997	1998	1999	2000
Ecuador	61,840	61,909	62,744	63,014	63,014	63,162
Egypt	2,990,625	3,229,875	3,480,000	3,714,900	4,038,975	4,038,975
El Salvador	22,446	22,446	22,446	22,446	22,446	22,446
Eritrea	1,618	1,931	2,245	2,506	2,558	2,140
Estonia	139,200	122,583	159,210	150,510	134,850	134,850
Ethiopia	30,015	31,320	32,625	33,930	33,930	33,930
Finland	832,312	824,734	765,043	807,908	805,620	805,620
France	13,858,935	13,965,797	14,108,059	14,384,467	14,577,894	14,511,165
Georgia	3,872	2,410	1,235	409	818	818
Germany	12,353,530	13,025,292	13,554,905	13,652,553	13,595,751	14,407,504
Greece	1,952,619	2,014,276	2,051,382	2,013,650	2,108,462	2,084,520
Guatemala	100,050	96,570	96,570	96,570	96,570	96,570
Honduras	66,294	70,183	75,560	74,646	75,499	75,499
Hungary	724,388	771,116	791,048	772,873	804,750	804,750
Iceland	30,233	30,702	31,964	32,277	32,277	31,842
Iran	1,668,425	1,661,561	1,656,515	1,733,057	1,851,865	1,851,865
Iraq	157,905	240,155	257,938	263,201	264,350	266,098
Ireland	715,575	871,740	858,690	855,210	943,950	800,400
Israel	800,687	820,697	825,369	827,614	841,542	841,542
Italy	8,541,869	8,565,394	8,254,778	8,808,498	8,539,154	8,793,194
Japan	916,884	947,900	992,148	1,077,191	1,075,833	1,098,323
Jordan	34,548	37,654	33,991	30,685	20,054	19,575
Kazakhstan	118,477	89,018	65,607	51,530	54,810	60,465
Kenya	1,314	1,766	1,653	1,827	1,827	1,827
Kyrgyzstan	33,060	18,270	18,270	23,551	24,464	29,101
Latvia	85,260	84,260	100,137	95,570	97,518	88,740
Lebanon	140,079	151,458	129,978	182,604	183,492	178,981
Lithuania	194,010	265,350	332,340	428,910	342,780	468,060
Macedonia	14,747	12,963	11,606	13,067	13,398	13,398
Malta	1,166	2,453	2,453	2,453	2,453	2,453

Table 4.5 Continued.

Country	Cheese Production (metric tonns)					
	1995	1996	1997	1998	1999	2000
Mauritania	16,739	16,661	17,339	17,287	15,660	15,660
Mexico	1,098,384	1,069,160	1,128,990	1,132,949	1,203,732	1,290732
Moldova	31,877	28,536	42,308	44,622	44,588	43,065
Mongolia	8,926	8,195	10,336	10,544	11,484	11,484
Morocco	61,056	61,422	63,284	65,372	66,773	67,129
Myanmar	244,427	250,978	255,502	260,200	266,324	333,793
Namibia	609	609	609	609	609	609
Netherlands	5,916,661	5,986,061	6,029,100	5,550,600	5,616,720	6,003,000
New Zealand	1,717,119	1,993,048	2,206,651	2,311,025	1,861,800	2,262,000
Nicaragua	62,762	67,373	64,328	68,782	98,397	98,397
Niger	117,546	118,129	118,129	118,129	118,129	118,129
Nigeria	62,675	59,586	54,888	57,629	60,509	60,508
Norway	736,238	748,391	757,631	790,847	747,330	747,330
Oman	3,506	3,567	3,732	3,732	3,732	3,732
Panama	55,497	59,543	58,351	76,151	82,650	81,536
Peru	50,138	53,427	46,928	52,374	52,661	52,661
Poland	3,081,279	3,455,292	3,872,718	4,116,057	4,142,070	3,872,370
Portugal	575,348	576,819	594,950	612,750	632,995	633,360
Romania	542,010	511,995	363,660	416,034	429,780	447,180
Russian Federation	4,148,865	3,723,600	3,366,900	3,349,500	3,158,100	3,166,800
Slovakia	335,777	361,798	395,232	472,323	478,091	473,837
Slovenia	137,799	147,117	159,906	182,248	183,718	183,718
South Africa	327,764	343,815	311,147	330,174	313,200	313,200
Spain	1,444,200	1,426,800	1,453,640	1,620,419	1,525,754	1,525,754
Sudan	1,413,750	994,845	1,013,115	1,064,010	1,288,905	1,307,175
Sweden	1,122,300	1,104,900	1,026,600	1,138,004	1,161,119	1,161,119
Switzerland	1,194,467	1,210,831	1,184,940	1,173,456	1,179,772	1,351,110
Syrian Arab Rep.	570,311	653,187	751,515	840,037	764,791	770,167
Tajikistan	25,926	8,178	36,888	48,285	52,200	57,637
Tanzania	15,660	15,660	16,530	17,400	17,400	17,400
Tunisia	54,114	55,854	55,854	55,854	55,854	55,854

Table 4.5 Continued.

Country	Cheese Production (metric tonns)					
	1995	1996	1997	1998	1999	2000
Turkey	1,215,990	1,229,884	1,157,639	1,148,939	1,138,447	1,138,447
Turkmenistan	20,880	15,660	15,660	13,311	13,485	13,816
Ukraine	763,790	609,905	502,999	548,100	548,100	580,725
UK	3,079,800	3,210,300	3,279,900	3,184,200	3,271,200	2,984,100
USA	30,393,015	31,553,334	31,711,500	32,437,950	34,199,265	35,299,815
Uruguay	220,615	214,551	243,296	251,865	237,684	255,084
Uzbekistan	188,355	139,635	171,173	163,778	204,015	148,770
Venezuela	845,127	850,512	814,825	867,938	771,499	771,499
Yemen	81,101	83,233	87,644	92,455	95,100	95,187
Yugoslavia	91,959	117,728	154,590	117,885	100,050	100,050
Zambia	8,491	7,926	5,951	6,168	6,455	6,734
Zimbabwe	18,270	18,270	18,270	18,270	18,270	18,270
World	126,553,663	129,610,590	132,185,477	134,887,775	136,768,672	139,598,417

of its high ash content and low pH. Acid whey is not rich in certain nutrients, which are required to promote cell growth during its utilization by fermentation. Its high organic content requires costly waste treatment.

Cheese whey is a high strength organic waste product, which contains variable amounts of lactic acid, lactose, organic and ammonium nitrogen and can create disposal problems in regions where there are a large number of cheese producers (Hall and Adams, 1988). Whey is extremely potent pollutant, containing about 30 000 - 50 000 mg/L biochemical oxygen demand (BOD) and 60 000 - 80 000 mg/L chemical oxygen demand (COD) (Morr, 1992; Yang et al., 1992; Mawson, 1994; Huang and Yang, 1998; and Mawson, 1994; Siso, 1996 and Cristiani-Urbina et al., 2000). A large amount of cheese whey is dumped into streams, rivers and seas, and this possesses a significant environmental hazard due to the high biological oxygen demand of whey. The microbial degradation of cheese whey is accompanied by the removal of oxygen from waters, which renders the water incapable of supporting other forms of aquatic life such as fish. In addition, the nutrient present in the whey may contribute to eutrophication. The continuous discharge

of whey onto land also can endanger the chemical and physical structure of the soil, reduce crop yields and lead to a serious groundwater pollution problems (Ghaly and Singh, 1985; Ghaly et al., 1988; and Marwaha and Kennedy, 1988). Moreover, disposing cheese whey into sewer systems disturbs the biological processes of the wastewater treatment plants, is becoming costly to treat and most municipalities disallowed its disposal into their sewer systems. About 50% and 17% of the cheese whey produced in USA and Canada respectively is dumped into sewers (Yang et al., 1992; and Ghaly et al., 1988).

4.1.5 Cheese Whey Utilization

In most parts of the world whey utilization is still a serious problem (Zall, 1992). Cheese production is at the same time increasing world wide, which complicates a full whey utilization program. Generally, whey processing needs to be economically feasible in order to invest capital and resources into a program to utilize the dilute liquor. Figure 4.1 illustrates most of the utilization systems being looked at today (Siso, 1996). It is obvious that there are many solutions to the whey utilization problem. Choosing the optimum method for whey processing requires the study of market conditions, disposal costs of effluent waste streams as well as energy requirements and costs (Singh and Ghaly, 1985). Figure 4.2 shows various processes and products associated with whey utilization/disposal reported by Yang et al. (1992).

4.1.5.1 Human Food. Whey components are emerging as high-value dairy ingredients with potential use in functional foods. The new discoveries of the biological functions of dairy ingredients, along with advances in developing economical separation and demineralization technologies for bioactive components of whey, provide the dairy industry with exciting opportunities in nutrition and health markets. Table 4.6 shows the different functions of cheese whey in different foods. However, there are several factors that interfere with the use of cheese whey in human-food products. These are: (a) the high concentration of mineral salts causes excessive saline taste which constitutes a problem in using the whey in dietetic or baby foods, (b) the small protein/sugars ratio along with the low sweetening power of lactose (only 40% when compared to sucrose), and (c) the low

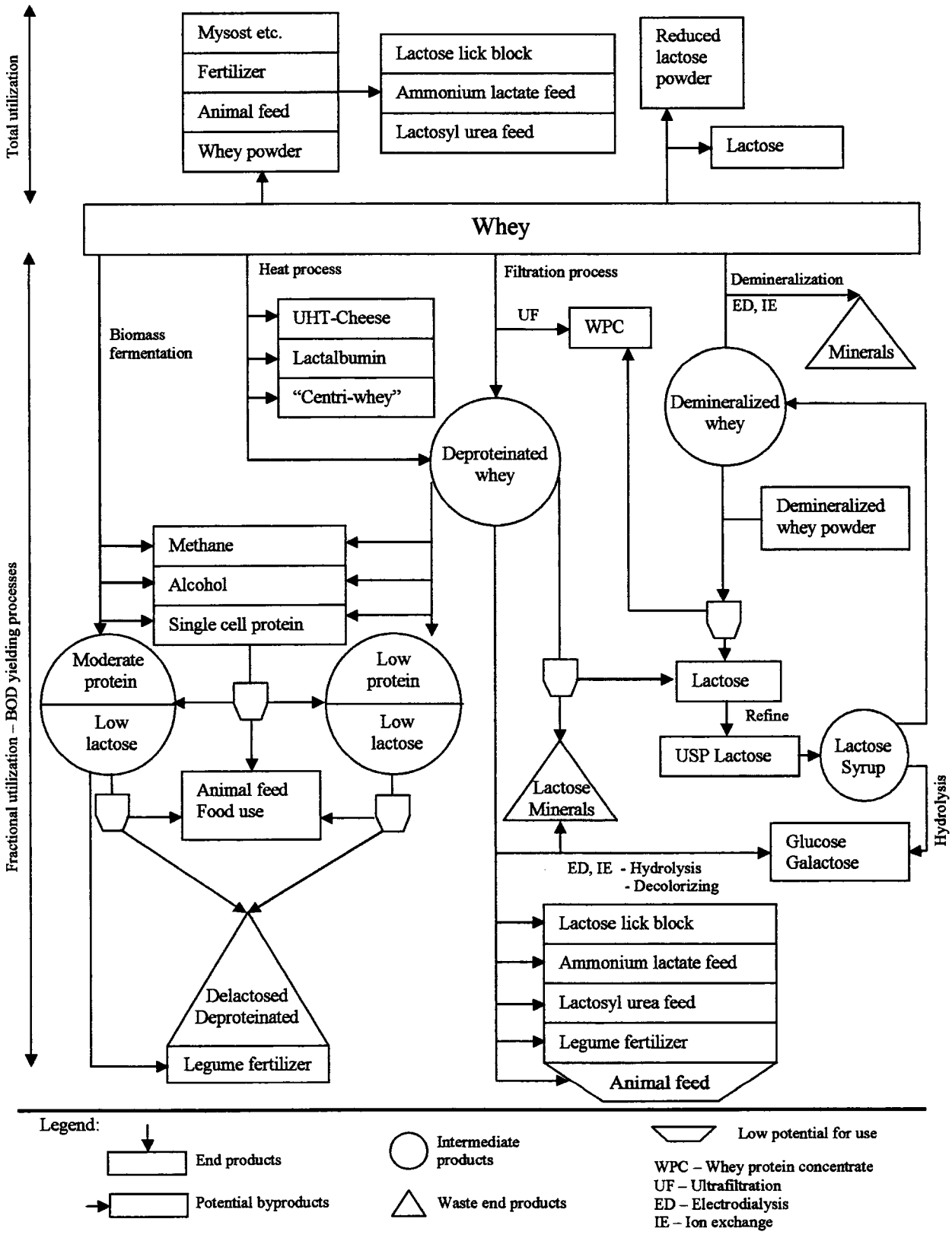


Figure 4.1. Whey utilization systems (Zall, 1992).

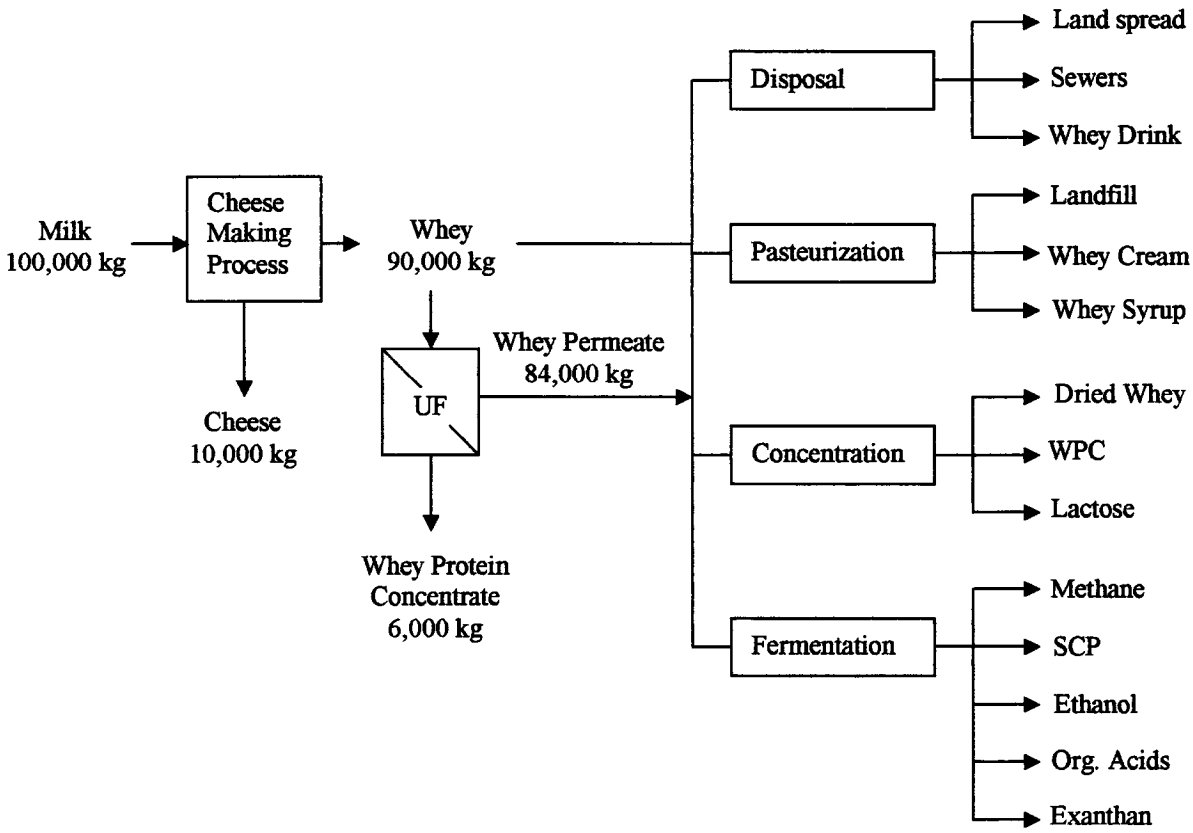


Figure 4.2. A wide spectrum of products from various whey processing (Yang et al., 1992).

Table 4.6. The uses and functions of whey in various foods (Kosaric and Asher, 1985).

Food	Whey solids used (%)	Function
Baked goods – sweet goods, bread, crackers	3.0 (of flour wt.)	Flavor, texture, shorter dough time, improved keeping quality, color.
Dry mixes	10.0	Tenderizing, color, flavor.
Ice cream sherbet	2.7	Flavor, acid and fruit stability.
Confections	10	Flavor, body, moisture.
Frosting, icing	6.0	Retention, whipping properties.
Jams, apple butter	4.0	Flavor.
Batter mix (for frying)	5.0	Color, flavor.
Whey soy beverage, citrus flavor	6.0	Flavor, lactic acid.
Processed cheese food	10.0	Body flavor.

solubility of lactose (Jelen, 1992; and Siso, 1996). However, about 50% of the total world cheese whey production is now treated and transformed into various food products, of which, about 45% has been reported to be used directly in liquid form, 30% in the form of powder cheese whey, 15% as lactose and delactosed byproducts, and the rest as cheese whey protein concentrates (Siso, 1996).

The economics of production of whey-based food products for direct consumer use is not greatly affected by the quantity of whey available for processing, but influenced favorably by the local market conditions, innovative marketing approaches and various retail forces (Jelen, 1992). In many dairy products, whey is utilized to replace egg products and part of the non-fat dry milk. Probably the largest use of whey in dairy product is in ice cream (Kosaric and Asher, 1982 and Kosaric and Asher, 1985). U.S. Federal regulations permit the use of whey in ice cream for up to 25% of the non-fat milk used. Another large dairy use for whey is in cheese products such as pimento and ricotta

cheeses, dairy spread, dairy imitation sour cream, flavored dips, whipping cream, yogurt, desserts, and frozen dessert. Whey is also used in the manufacture of bakery products, confectioneries, infant formula products, and meat products (Kosaric and Asher, 1982; Morr, 1992; and Tamime and Robinson, 1999). Table 4.7 summarizes different food products that could be obtained from sweet and acid whey.

Table 4.7. Food product applications for whey products (Morr, 1992).

Sweet whey production applications	Acid whey production applications
Ice cream and ice cream coatings, toppings and syrups.	Fruit flavored beverages and fermented dairy products.
Sweet rolls, bread, crackers and cakes.	Cheese, cheese powders, dips and spreads.
Icings, candy coatings, fudge, caramels and chocolate.	Crackers, bread and bakery products.
Margarine, cheese foods, gravy mixes and snack foods.	Salad dressings.
Fruit juices and beverages	Sherbets
Soups, infant foods and puddings.	Sausage and miscellaneous processed meat products.
Meat products.	

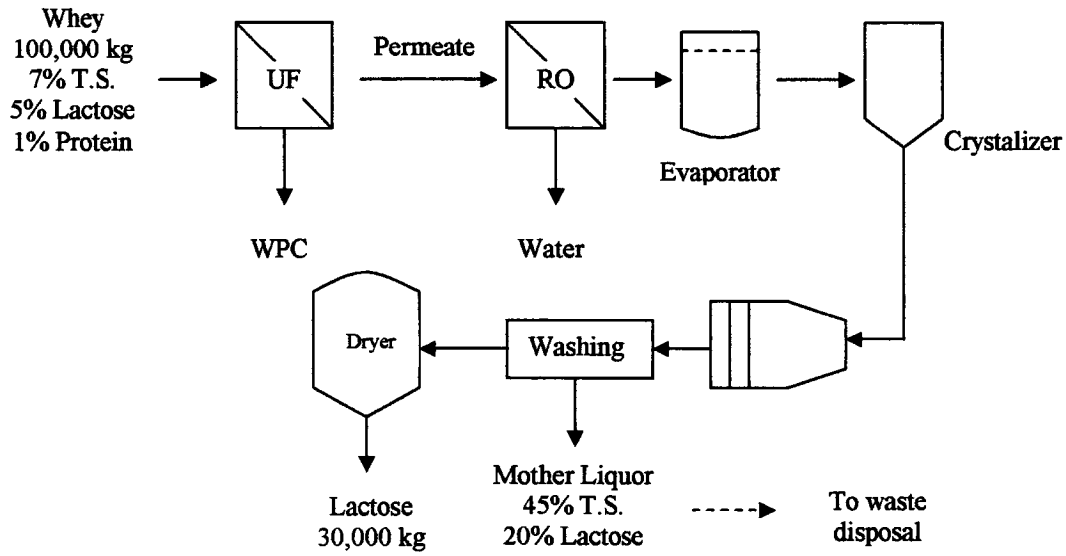
4.1.5.1.1 Whey Protein Concentrates: Whey proteins make up 15% to 22% of the total milk proteins and have ability to enhance texture and stability of emulsions, foams and gels in food products (Tang et al., 1988; Yang et al., 1992; Jain et al., 1991; Huffman, 1996; Yang et al., 1999; and Savant and Torres, 2000). It is a white to light cream-colored product with a bland, clean flavor and can be defined as the product derived from milk whey containing 50, 65 or 80% of denatured or undenatured protein. Whey protein concentrate has enjoyed a worldwide market, with a significant dollar value, because of its excellent functional properties and nutritional values. Whey protein concentrates

(WPC) have been used to fortify the yogurt mix at a level up to 30% without affecting the quality of the product (Tamime and Robinson, 1999).

There are several industrial methods suitable for selectively concentrating whey. The non-protein constituents are removed by physical separation techniques such as precipitation, filtration, or dialysis. The most commonly used methods nowadays are ultrafiltration and diafiltration, because of their advantages of cost reduction, high processing speed, the absence of denaturation or protein-structure modification, and the fact that the protein concentrate is free of salts, thereby making it suitable for all kinds of human foods, even dietetic or baby foods (Siso, 1996). In the ultrafiltration process low-molecular weight compounds such as lactose, minerals, non-protein nitrogen and vitamins are filtered from the whey to the permeate, and the proteins are concentrated in the retentate. After sufficient removal of nonprotein constituents from pasteurized whey the material is subjected to drying. Safe and suitable pH-adjusting ingredients may be used to adjust the acidity of WPC.

4.1.5.1.2 Lactose: Milk sugar lactose (4-O-ß-D-galatopyranosyl-D-glucose) can be isolated and purified from whey permeate by crystallization as shown in Figure 4.3 (Siso, 1996; and Yang et al., 1999). The isolated lactose is usually used as a supplement in baby milks and as an excipient for pharmaceutical products. However, the lactose recovery yield from whey permeate is low (only about 60%), and the waste stream (mother liquor) from the crystallization process contains high salts (>20%), high lactose (~20%) and consequently high BOD. Because of the high salt content, this mother liquor has limited applications and generally requires costly treatment. Lactose has a relatively weak marketing potential for several reasons: (a) a great portion of the world population cannot digest lactose (lactose malnutrition) due to a lack of the enzyme lactase which is required for breaking down the lactose onto glucose and galactose, (b) sweetness produced by lactose is very low, thus it increases the calorie content of the food products without contributing to the functional sweetening effect, (c) excessive amounts of lactose in ice cream and condensed milk causes undesired crystallization which develops a sandy

structure, and (d) lactose has poor solubility (about 20 g per 100 g water at room temperature) (Kosaric and Asher, 1982 and Kosaric and Asher, 1985).



UF Ultrafiltration
RO Reverse Osmosis
T.S. Total Solids

Figure 4.3. A process flowchart showing production of WPC and lactose from whey (Yang et al., 1999).

4.1.5.1.3 Whey Powder: The quality of fresh cheese whey could be maintained for a longer period of time in the form of whey powder, facilitating manipulation and transport (Siso, 1996). The manufacture of whey powder includes preheating, concentration, flash cooling, precrystallization, spray drying, and cooling in a vibrated fluid bed. There are different kinds of whey powder such as condensed whey, complete acid or sweet whey powders, demineralized whey powder, delactose whey powder, deproteinized whey and fat enriched whey.

Whey powder could be used in several human foods such as ice creams, baked goods, cakes, sauces, milky derivatives, meat, beverages, infant formula products, etc. (Morr,1992). Up to 25% of the milk solids-non-fat (MSNF) content of ice cream and other dairy-based frozen desserts can be replaced with dried whey without adversely

altering their quality attributes (Morr, 1992). Demineralized whey powder increases the viscosity and decreases the saltiness of ice cream mix compared with mixes prepared with sweet whey powder. The amount of acid whey in the formulation must be controlled to avoid imparting off-flavor defects resulting from minerals and other whey components. Siso (1996) stated that when powdered cheese whey used to sweetened ice creams only a small portion should be added, because whey reduces protein content and if a high proportion is added which is required to obtain the sweet flavor the protein/sugar ratio required by law may be unbalanced. Moreover, in high concentrations, lactose crystallizes and, therefore, the texture of the product is notably worsened. Whey protein powder has been used to fortify yogurt mix; the replacement of skimmed milk powder (SMP) by whey-caseinate blends at 50% reduced the cost of manufacture and the yogurt was acceptable. The recommended level of addition of whey powder to the yogurt mix is around 1-2 %, since higher levels can impart an undesirable whey flavor (Tamime and Robinson, 1999).

4.1.5.1.4 Beverages: Milk nutrients could be returned into the human food chain by utilizing liquid whey in beverages manufacture (Siso, 1996). Whey beverages are in general inexpensive products but the general problem for its development is the whey flavor, which must be masked (Singh and Ghaly, 1985). Many types of beverages could be produced from cheese whey. These include non-carbonated beverages, fruit-flavored beverages, yogurt-flavored whey beverages, carbonated beverages, whey wine, fruit-flavored wine beverages, and milk-like beverages. Cheese whey beverages are typically pasteurized and bottled to provide up to 6 months' shelf-life at room temperature. Whey-based beverages contain 80-90% whey plus added citric acid, flavoring, sugar and vitamin C (Table 4.8). Patil and Gupta (1982) stated that increasing whey protein concentration in beverages increases the total solids, thus decreasing the acceptability of the beverages significantly.

Ben-Hassan (1991) reported that whey is suitable for the manufacture of beer like beverages because: (a) it contains materials similar to the colloids of beer wort and it has a high salt content, (b) some constituents in whey develop caramel-like flavor after

prolonged heating under pressure, which are similar to the taste and odor of cured malt, and (c) lactose is only slightly sweet so it does not alter the taste of the finished beverages. Singh and Ghaly (1985) reported that under proper processing conditions some wine-like flavor could be developed from whey. However, raw whey does not contain sufficient lactose for conversion into alcohol to make table wine. Fortifying whey with glucose, sucrose or grape juice to create a richer fermentable substrate is a plausible alternative. Some researchers have developed a process to convert whey into lightly carbonated, flavored alcoholic beverages. The product depended heavily on added dextrose; about 22% of dextrose was added to the deproteinized whey (Kosaric and Asher, 1985).

Table 4.8. Composition of some whey-based drinks (Kosaric and Asher, 1985).

Ingredients	Weight (kg)		
	Strawberry drink	Lemon drink	Chocolate drink
Stabilizer	0.02	0.01	0.02
Cream (35 % fat)	2.59	2.59	-
Sugar	2.27	2.27	2.27
Non-fat dry milk	2.72	-	-
Flavor	0.08	0.07	-
Liquid whey	39.01	-	38.56
Whey concentrate	-	40.82	-
Whey powder	-	-	3.63
Chocolate	-	-	0.91

Considerable progress has been made in developing non-alcoholic beverages from whey. Most of the non-alcoholic whey beverages are prepared by mixing pasteurized and deodorized whey with the appropriate proportion of water, fruit juice concentrate, flavourings, colour, and preservatives. Ben-Hassan (1991) reported that a drink based on cheddar cheese whey with shelf-life of at least 14 days at 22° C was prepared at Mississippi State University by mixing whey, sugar, orange concentrate, citric acid, and other ingredients (pH of 3.8 and a total solids content of 16.5%). The study showed

76.5% acceptability for this formulated drink. Moreover, a frozen whey concentrate was developed and used as a punch base at various dilutions with ginger ale. The product, in which at least half of the liquid was fresh sweet whey, was similar to frozen orange juice concentrate and was claimed to have no whey flavor. Patil and Gupta (1982) developed a soy-whey powder beverage with high acceptability score the physical and chemical properties of the powder is shown in Table 4.9.

Table 4.9. Physical and chemical characteristics of soy-whey beverage powders (Patil and Gupta, 1982).

Component	Average value	Units
Moisture	1.66	%
Protein	21.48	%
Fat	10.16	%
Ash	4.54	%
Carbohydrates	62.16	%
Crude Fiber	0.31	%
Calcium	379.50	mg
Iron	4.20[a]	mg
Non-protein nitrogen	0.41	%
Free fat	63.40	% of total fat
Free fatty acids	0.17	%
Viscosity	7.64	CP
Solubility index	13.13	mL
Bulk density	0.42	g/mL
Trypsin inhibitor activity	Nil	-
Sensory score	7.50	-

[a] Including fortified iron 0.75 mg/100g

4.1.5.2 <u>Animal Feed</u>. Cheese-whey surplus can be supplied without any treatment into drinking waters for animals (Siso, 1996). It provides high-quality proteins, lactose, calcium, phosphorus, sulfur and water-soluble vitamins. However, high trucking costs

limit the use of liquid whey for animal feeding to only within an economic radius of 30-40 kilometers (Marwaha and Kennedy, 1988; and Ghaly, 1988).

Swine rely primarily on the action of mucosal lactase to hydrolyze lactose, whereas lactose is metabolized by rumen microorganisms in the rumen and does not reach the small intestine in significant quantities. Barba et al. (2001) stated that many pig farms use whey without any treatment as feed but lactose in whey is hardly metabolized by animals and consequently other inexpensive available carbohydrates are preferred, unless the cost of whey is extremely small or negative. The hydrolysis of lactose reportedly improves the nutritional value of whey and deproteinized whey for pigs and other animals that are lactose tolerant. Ruminants can consume as much as 30% of their dry matter requirements in the form of liquid whey, but pigs are limited to an amount of whey equivalent to 20% of their dry matter intake (Morr, 1992).

Liquid whey concentrates, dried whey, UF whey permeate, whey 'lick blocks' and mother liquor remaining after lactose crystallization and removal from whey are also available for feeding animals. Ammonium lactate, produced by fermenting whey or UF permeate by the 'Lacto-Whey' process can also be used as animal feed. Whey powders such as: condensed whey, complete acid or sweet whey powders, demineralized whey powder, delactosed whey powder, deproteinized whey, and fat-enriched whey can also be used in animal feeding as a mixture with molasses or soya flour (Sico, 1996).

4.1.5.3 Fertilizers. Farmland irrigation is one of the known cheese whey disposal methods since it contributes both water and nutrients to the soil that promote plant growth. 3.8 thousand liters of whey contains minerals equivalent to 5.9 kg potassium sulphate, 36 kg ammonium sulphate, 12 kg super phosphate and 6.8 kg calcium carbonate (Marwaha and Kennedy, 1988; and Siso, 1996). This whey disposal approach works well for near neutral to slightly alkaline soils. However in acid soils, the added whey promotes soil compaction, resulting in inhibition of soil microorganism's propagation that biodegrade the added whey nutrients. Spraying is the preferred method for applying whey to the soil since it avoids the formation of stagnant pools that inhibit the ability of the soil

microorganisms to biodegrade the added nutrients. Generally, the excess application of whey results in damage to the physical and chemical structure of the soil and hence reduces the crop yield (Ghaly et al., 1988; and Marwaha and Kennedy, 1988). An application rate of about 740 kg of nitrogen per hectare from cheese whey, which equals to 48,000 litres of liquid whey per hectare, is recommended (Ghaly et al., 1988). Exceeding these limits poses a threat to environmental pollution from run-off and off-odor production. Moreover, the water-soluble nutrients may also be leached and cause ground water contamination.

4.1.5.4 Energy. Recently, the rise in the cost of petroleum resulted in an increased interest in the development of renewable energy sources as an alternative to fossil energy supply. Cheese whey is one of these renewable resources that could be converted to biogas and alcohol by anaerobic fermentation.

4.1.5.4.1 Biogas: Biogas (or bio-methane) is the gas produced during the anaerobic fermentation process. It contains about 50 % - 78 % methane, 25 % - 45 % carbon dioxide, along with small amounts of other gases such as hydrogen sulphide, hydrogen, carbon monoxide and nitrogen (Ramkumar, 1991). It is colorless gas mixture and possesses a slight odor due to the presence of hydrogen sulphide. Biogas burns with a bluish flame and has a calorific value between 20 and 26 MJ per cubic meter as shown in Table (4.10).

The practical application of anaerobic digestion to high strength, high volume wastes such as whey required the development of digesters in which the microorganisms converting the waste to methane could be retained in the reactor. The process is mediated by a complex association of several groups of anaerobic and facultative-anaerobic bacteria operating within rigid thermodynamic constraints. Organic matter is first hydrolysed and fermented to short-chain volatile fatty acids, carbon dioxide and hydrogen. Fatty acids are then degraded by the hydrogen-producing acetogenic bacteria to acetate, carbon dioxide, and hydrogen, which serve as the major substrates for methane production by the methanogens (Mawson, 1994). Anaerobic digestion produces

methane that can be directly used as a source of energy in situ. Switzenbaum et al. (1979) stated that this process is quite important for cheese producers as the production of biogas from the cheese plant can offset the use of fossil fuels by up to 40%. The average anaerobic methane generation for cheese whey was found to be 23.4 L CH_4/L cheese whey and the resulted biogas contains 77.5% CH_4 (Demirer et al., 2000).

Table 4.10. Comparison of calorific values of biogas with other fuel gases (Ramkumar, 1991).

Gas	Calorific values (MJ/m^3)
Biogas	20 - 26
Butane	107 - 126
Coal gas	16 - 19
Methane	33 - 40
Natural gas	38 - 82
Propane	81 - 97

However, several researchers have reported instability of the anaerobic digestion process for cheese whey caused by operational parameters such as temperature and pH and inhibitors. Volatile organic acids are formed, as the lactose in the whey is readily broken resulting in low pH, which inhibits the methane-forming microbes and hence decreases the biogas yield. It is, thus, recommended that the pH of the digester contents must be controlled in order to improve the biogas yield from the process (Ghaly and Pyke, 1988).

4.1.5.4.2 Alcohol: The anaerobic fermentation process of cheese whey to produce alcohol is becoming of increasing interest (El-Taweel and Ghaly, 1991). The process includes fermentation of cheese whey by strains of yeast such as *Kluyveromyces marxianus* (*Kluyveromyces fragilis*) to yield a beer containing approximately 18-20 g/L ethanol. By-products such as glycerol, esters, higher alcohols and organic acids are also formed. Continuous fermentation, fed-batch fermentation, cell-recycling and cell immobilization have been applied to ethanol production from cheese whey. In a batch fermentation, *K. fragilis* utilizes more than 95% of the lactose with a conversion efficiency of 80-85% of

the theoretical value of 0.538 kg ethanol/ kg lactose consumed (Mawson, 1994 and Siso, 1996).

Ethanol produced from cheese whey could be used as a fuel, an industrial solvent, or in beverage production.The production of ethanol from nonconcentrated cheese whey is not economically feasible since the levels of ethanol produced reach about 2%, which makes the distillation process too expensive (Tin and Mawson, 1993). Mawson (1994) stated that lactose concentration up to 100-120 g/L could reduce the cost of ethanol production significantly.

4.1.5.5 Chemicals. Some researchers reported on the use of cheese whey for the production of several chemicals such as organic acids, biodegradable plastics, de-icers and anti-icers, bacteriocins and single cell protein.

4.1.5.5.1 Organic Acids: Cheese whey has been extensively studied as a fermentation substrate for the production of organic acids such as lactic, propionic and acetic acids (Huang and Yang, 1998). Lactic acid has been produced from whey in developing countries, and it has been proved to be an economically feasible process that also reduces the biological oxygen demand (BOD) of the substrate (Arasaratnam et al., 1996). Mostafa (1996) stated that whey is more suitable substrate for lactic acid production than pure lactose since it contains all the essential nutrients. Boyaval and Corre (1987); Anderson et al. (1986); Blanc and Goma (1989) and Jain et al. (1991) reported that Propionibacteria could be used for the production of propionic acid in whey based media with high yield. In recent years many attempts have been made to produce a low-cost acetic acid from fermentation of cheese whey (Tang et al., 1988; Huang and Yang, 1998 and Balasubramanian et al., 2001). No homoacetogen can directly ferment cheese whey lactose to acetate. A fermentation co-culture consisting of homolactic and homoacetic bacteria must be used.

4.1.5.5.2 Biodegradable Plastics: Degradable plastic is a plastic designed to undergo a significant change in its chemical structure under specific environmental conditions

resulting in a loss of some properties (Narayan and Pettigrew, 1999). Biodegradable plastic is a degradable plastic in which the degradation results from the action of naturally occurring microorganisms such as bacteria, fungi and algae. To be biodegradable, a plastic needs to have chemical groups at the ends of its polymer chains that are susceptible to oxidation by microbial enzymes.

Poly(3-hydroxybutyrate), PHB, is an energy and carbon reserve material synthesized by numerous bacteria and has attracted much attention as a candidate for biodegradable and biocompatible plastic material (Lee et al., 1997; and Dhanasekar et al., 2001). It is the most widespread and best characterized polymer, which has material properties similar to polypropylene and has a wide range of clinical applications since implanting PHB in mammalian tissues, would not be toxic. These applications include: surgical pins, sutures, staple, swass, wound dressing and bone replacement and plates. The environmentally friendly PHB polymer can be efficiently produced from environmentally polluting whey (Lee et al., 1997; and Wong and Lee, 1998). Recombinant *E. coli* strains and *Azotobacter vinelandii* are able to accumulate good amount of PHB when growing on cheese whey and either ammonium sulfate or peptone along with yeast extract as nitrogen source as shown in Table 4.11 (Dhanasekar et al., 2001).

Table 4.11. Effect of nitrogen source on PHB production (Dhanasekar et al., 2001).

Nitrogen source	Total cell mass (mg/L)	PHB (%dry weight)	Cell protein (mg/mL)
Yeast extract	1.1	90	0.33
Yeast extract + ammonium sulfate	1.4	75	0.35
Yeast extract + peptone	1.59	83	0.27
Ammonium sulfate + peptone + yeast extract	1.5	84	0.24

4.1.5.5.3 De-Icers and Anti-Icers: Organic salts (Deicers and anti-icers) such as sodium lactate, potassium acetate and calcium magnesium acetate could be produced by biodegradation of waste materials such as cheese whey. Generally, using organic salts

have many advantages as they have a low BOD and are less harmful to vegetative aquatic and mammalian life. The melting point of organic salt-water mixtures can be much lower than that of inorganic salts. In addition, organic salt compounds are biodegradable; therefore cause less accumulation of salts in the environment.

Fermentation produced acetic acid could be used to manufacture low cost calcium magnesium acetate (CMA) as an organic biodegradable noncorrosive deicing salt (Yang et al., 1992; Yang et al., 1999; and Talabardon, 2000). A new process to develop low-cost calcium magnesium acetate (CMA) and potassium acetate (KA) deicers from cheese whey has been reported by Yang et al. (1999). The new process involves the fermentation of cheese whey in fibrous bed reactors using anaerobic mixed culture of homolactic and homoacetic bacteria to produce acetic acid from lactose. The acetic acid is extracted, recovered, and separated from the fermented broth. Deicing tests have shown that CMA from cheese whey fermentation has an equal or slightly better ice penetration rate than that of commercial CMA.

Yang et al. (1992) also developed a new reasonable anaerobic fermentation process to produce CMA from cheese whey. Their fermentation process involves two stages, the first for homolactic fermentation and the second for homoacetic fermentation. After fermentation the whole broth containing acetate can be directly concentrated by evaporation and dried to make CMA (Figure 4.4). If the acetate concentration is not high enough or pure CMA is required, the acetic acid can be recovered and concentrated by using two-step extraction process.

Cost analysis showed that using zero-cost raw material such as cheese whey for CMA production could be reasonable. CMA could be produced from cheese whey at a production cost of \$0.09 – 0.15/Lb or less, which is less than 30 percent of the current market price for commercial CMA. Production of acetate from currently unused liquid whey could provide 0.77 million tonnes per year of low-cost CMA and potassium acetate (KA) for highway and airport runway deicing materials (Yang et al., 1992; and Yang et al., 1999).

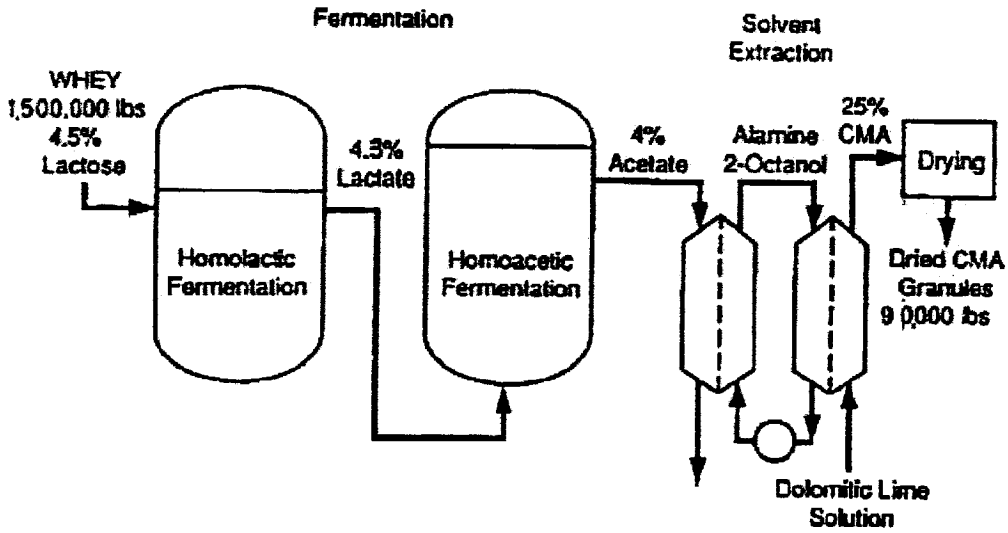


Figure 4.4. A fermentation-extraction process for CMA production from whey lactose (Yang et al., 1992).

4.1.5.5.4 Bacteriocins: In recent years, interest in bacteriocins has grown considerably because they are potentially useful for preventing the growth of spoilage and pathogenic bacteria such as *Listeria monocytogenes* and *Clostridium botulinum*. Among several bacteriocins, nisin is the only one that is produced commercially by microbial fermentation and it is used as a food preservative in processed cheese and canned food to prevent growth of *Clostridia* and other pathogens. Uses of nisin to control spoilage have also been identified in beer, wine, alcohol production and low-pH foods such as salad dressing. Nisin is a lanthionine-containing peptide antibiotic, or lantibiotic. Lantibiotics exhibit heat stability and strong anti-microbial activity because they have intramolecular mono-sulphide bridges such as lanthionine and 3-methyl-lanthionine. It is also heat-stable at acidic pH and has a broad anti-microbial spectrum against Gram-positive bacteria. Recently, there has been increased interest in the production of bacteriocin from whey permeate since certain lactic acid bacteria strains can grow and produce appreciable amounts of bacteriocin in this medium (Flores and Alegre, 2001).

4.1.5.5.5 Single Cell Protein: The term single cell protein (SCP) refers to dried cell of microorganisms such as algae, actinomycetes, bacteria, yeast, molds and higher fungi (with yeast being the predominant microorganism) grown in large scale culture systems

for use as protein source in human food, animal feed, or pharmaceutical products (Kosaric and Asher, 1982; Litchfield, 1983 and Mawson, 1994). At present, food grade yeasts (e.g., *S. cerevisiae*, *C. utilis*, and *K. fragilis*) have a position in the food market for their flavor enhancement characteristics (Singh and Ghaly, 1985).

Zall (1992) and Siso (1996) stated that whey can be used for the production of single cell protein. The *K. fragilis* strains have been widely studied for the production of yeast biomass from whey. However, a biomass yield for fed-batch culture of *T. Cremoris* and *C. utilis* has been proved to be greater than those reported for *K. fragilis* in a study by Cristiani-Urbina et al. (2000). *Kluyveromyces lactis*, *K. fragilis* and *Torulopsis bovina* are usually grown in cheese whey permeate since these microorganisms cannot metabolize proteins in the whole cheese whey and also the proteins promotes yeast flocculation causing fermentation inhibition. Both batch and continuous processes for the production of biomass from cheese whey have been developed but the major industrial processes have favored continuous operation (Mawson, 1994). The biomass produced could be recovered by centrifugation prior to further processing and drying, or the whole fermenter contents could be dried without preconcentration. Using cheese whey to produce single cell protein can reduce its pollution potential. Ghaly and Singh (1989) reported that employing aerobic fermentation of cheese whey using *Kluyveromyces fragilis* reduced the total oxygen demand by 42%, the soluble chemical oxygen demand by 65%, the total solids by 53% and the ammonium nitrogen by 90%.

4.2 Shellfish Waste

4.2.1 Types and Composition of Shellfish Waste

Shellfish are aquatic animals, such as a mollusk (e.g. clams, oysters, and snails) and crustacean (e.g. crabs and shrimp), having a shell or shell-like external skeleton (exoskeleton). Shellfish waste includes shells, heads, viscera and adhered meat. In General shellfish waste contains many valuable components such as chitin, protein, minerals, pigments, enzymes and fat. The relative amount of these constituents varies with the species, season, age, gender and the technology of processing (Green and

Kramer, 1984 and Hansen and Illanes, 1994). Table 4.12 shows chitin, protein and calcium contents of wastes from different shellfish sources.

Table 4.12 Typical values of chitin, protein and calcium content[a] of various shellfish wastes (Zakaria, 1997).

Source of waste	Chitin (%)	Protein (%)	Calcium (%)	Reference
Crawfish	14.2	32.2	18.1	Chen and Meyers (1983)
Crawfish	15.9	35.8	12.3	No et al. (1989)
Shrimp	17.0	41.9	ns[a]	Shahidi and Synowiecki, (1991)
Shrimp	21.4	27.9	16.0	Sabry (1992)
Shrimp shells	27.2	22.8	11.1	Barratt and Montano (1986)
Shrimp heads	11.1	53.5	7.2	Barratt and Montano (1986)
Scampi waste[c]	12.1	32.8	20.8	Zakaria et al. (1997)
Nylon shrimp	17.0	ns[a]	ns[a]	Hansen and Illanes (1994)
Yellow and red prawn	14.0	ns[a]	ns[a]	Hansen and Illanes (1994)
Crab and king crab	15 - 30	ns[a]	ns[a]	Hansen and Illanes (1994)
Krill	25.0	ns[a]	ns[a]	Hansen and Illanes (1994)

[a] Values are calculated on dry matter basis
[b] ns = not stated
[c] includes heads (thorax) and claws

Chitin is considered the most valuable component present in shellfish waste for several reasons: (a) it is the major component of shellfish waste, (a) it is the second most abundant natural biopolymer on earth after cellulose, (d) it is closely related to cellulose in structure and (e) it has a wide range of applications. The relative percentage of chitin found among crustacean species varies from one species to another (Rhazi et al., 2000). Even among the same species of crustacean there is a significant difference in the chitin content depending on age, gender and season. Different shrimp have been found to have between 17 and 30% chitin content (Rhazi et al., 2000; and Shahidi and Synowiecki, 1992). Table 4.13 shows the chitin characteristics of shrimp and crab.

Table 4.13. Characteristics of shrimp and crab chitin (Shahidi and Synowiecki, 1992).

Specification	Units	Shrimp	Crab
Moisture	%	2.50 ± 0.3	0.50 ± 0.30
Nitrogen	%, db[a]	6.20 ± 0.1	6.50 ± 0.10
Ash	%, db[a]	0.10 ± 0.0	0.10 ± 0.00
Free amino groups	μmole /g, db[a]	55.30 ± 1.0	45.90 ± 0.50
Protein residue	%, db[a]	2.10 ± 0.0	0.50 ± 0.00
Specific gravity	g/cm^3, 16.35 mesh	0.06 ± 0.0	0.20 ± 0.01
Particle size	16.35% after 30s grinding	74.70 ± 0.7	53.20 ± 1.20
Hunter L value[b]	-	83.50 ± 1.1	91.80 ± 0.30

[a] db = dry basis
[b] Color measurement method (L value of 0 represents pure black and 100 pure white)

Zakaria (1997) stated that the high protein content reported in shellfish waste comes from the adhered meat, meat in the claws and protein from head. In addition, the exoskeleton also contains a small amount of protein. The nutritional value of shellfish waste protein is high because the essential amino acids are significantly higher than the standards, and sulfur amino acids are higher than the soybean meal (Hansen and Illanes, 1994).

The mineral concentrations of crab and shrimp wastes are illustrated in Table 4.14. The major mineral component of shellfish waste is calcium. Hansen and Illanes (1994) reported that shellfish waste material usually consists of more than 90% calcium carbonate. The exoskeleton of crustaceans also contains carotenoid pigments (Table 4.15), mainly astaxanthin and astaxanthin ester (Hansen and Illanes, 1994; and Guillou et al., 1995). However, the pigments in shellfish waste are highly unstable to temperature and chemicals, which makes their extraction a very critical process (Hansen and Illanes, 1994).

Shellfish waste contains many valuable enzymes. Olsen et al. (1990) isolated four types of enzymes from the effluent of shrimp processing plant. Those included alkaline phosphate, hyaluronidase (carbohydrate degrading enzyme), β -N-acetylglucosaminidase

Table 4.14. Minerals concentration[a] of shrimp and crab discards (Shahidi and Synowiecki, 1992).

Mineral	Units	Shrimp	Crab
Ca	%	15.30	14.95
Na	%	2.05	0.95
K	%	0.20	0.25
Mg	%	0.95	0.92
P	%	1.66	2.88
Sr	%	0.22	0.23
Mn	ppm	29.00	13.00
Fe	ppm	82.00	70.00
Cu	ppm	13.00	6.00
Zn	ppm	21.00	17.00
As	ppm	27.00	24.00
Ba	ppm	54.00	20.00

[a] Values are calculated on dry matter basis

Table 4.15. Composition of carotenoids (as % of total) of shrimp and crab discards (Shahidi and Synowiecki, 1992).

Carotenoid	Shrimp	Crab[a]
Astaxanthin	3.95 ± 0.19	21.16 ± 1.15
Astaxanthin monoester	19.72 ± 0.19	5.11 ± 0.23
Astaxanthin diester	74.29 ± 0.38	56.57 ± 1.60
Astacene	-	3.26 ± 0.47
Leutein	-	8.24 ± 0.30
Zeaxanthin	0.62 ± 0.05	4.64 ± 0.76
Unidentified	-	0.22 ± 0.05

[a] Soft-shelled crabs contain 2.66 ± 0.73% cathaxanthin

and chitinase. Jiang et al. (1992) reported the existence of trypsin-like and chymotrypsin-like enzymes in grass shrimp (*Penaeus monodon*). Bustos et al. (1999) also reported the presence of highly stable trypsin-like enzymes in krill processing wastewater. Peberdy (1999) reported that the heads of shrimp contain digestive enzymes including chymotrypsin-like proteases. Kristinsson and Rasco (2000) stated that among the major enzymes of fish viscera and digestive tract are proteases such as trypsin, chymotrypsin and pepsin. Lysosomal proteases and catheptic enzymes, present in fish muscle cells, have also a proteolytic effect. Fats are largely derived from viscera and vary from 0% in clean crab-leg shells to about 5% or more in some shrimp and lobster species and 10% or more in krill wastes (Hansen and Illanes, 1994).

4.2.2 Shellfish Waste Production

Solid wastes generated by the seafood processing industry range from about 30 to 85% of the landed fish depending on the fishery. Worldwide the production of crustacean waste has been estimated as about 1.2 million tones on a dry basis (Hall and De Silva, 1992; and Teng et al., 2001). Over 70% of the raw material of crabs and sea spiders are discarded as waste during processing into frozen or canned meat. The global shrimp processing industry yields large quantities of waste. Nearly, 35% of the raw shrimp material is discarded as waste when processed into head-less shell products. Removing the shell increases the total waste production up to 40-49% (Balogun and Akegbejo-Samsons, 1992; Sophanodora and Hutadilok, 1995; and Subasinghe, 1995). Crab processing also generates about 75 – 85% waste (Green and Mattick, 1979). Table 4.16 shows the annual landings of crustaceans during the period 1995 - 1999 for the world, Canada, and the Atlantic Region.

4.2.3 Environmental Impact of Shellfish Waste Disposal

The high tonnages of shellfish waste produced annually have become a major environmental concern to the main producing countries, as they constitute a serious environmental hazard through their decomposition when they are not properly disposed of (Balogun and Akegbejo-Samsons, 1992; and Hall and De Silva, 1992). The main problem associated with shellfish waste is its high perishability under normal ambient

Table 4.16. Annual landings of various shellfish (FAO, 2001).

Species	Region	Production (MT)				
		1995	1996	1997	1998	1999
Clams, Cockles, Arkshells	World	2,732,260	2,700,257	2,758,294	3,089,498	3,557,347
	Canada	34,409	34,444	35,972	34,429	36,835
	Atlantic Region	29,740	30,266	32,360	30,341	32,535
Freshwater Molluscs	World	606,851	573,016	520,777	593,187	563,814
	Canada	N/A[a]	N/A	N/A	N/A	N/A
	Atlantic Region	N/A	N/A	N/A	N/A	N/A
Horseshoe Crab etc.	World	926	1,598	2,607	3,252	2,397
	Canada	N/A	N/A	N/A	N/A	N/A
	Atlantic Region	N/A	N/A	N/A	N/A	N/A
Krill	World	118,714	101,708	82,508	81,216	103,318
	Canada	N/A	N/A	N/A	N/A	N/A
	Atlantic Region	N/A	N/A	N/A	N/A	N/A
Lobster, Spiny-rock Lobster	World	221,263	210,709	233,867	216,841	228,942
	Canada	40,508	39,369	40,079	41,030	43,087
	Atlantic Region	40,508	39,369	40,079	41,030	43,087
Marine Molluscs	World	2,514,851	2,299,986	2,705,464	2,682,397	2,800,060
	Canada	2,688	2,019	3,326	2,020	2,134
	Atlantic Region	1,121	683	521	185	663
Mussels	World	1,374,013	1,323,732	1,376,016	1,627,765	1,688,855
	Canada	13,131	17,979	20,612	25,193	28,904
	Atlantic Region	13,131	17,979	20,612	25,193	28,904

Table 4.16. Continued.

Species	Region	Production (MT)				
		1995	1996	1997	1998	1999
Oysters	World	3,242,612	3,223,162	3,264,692	3,699,688	3,869,144
	Canada	10,031	10,121	7,357	11,061	12,510
	Atlantic Region	4,771	4,641	3,707	5,761	6,710
Scallops, Pectens	World	1,663,493	1,770,996	1,746,948	1,396,990	1,519,373
	Canada	68,707	60,154	66,077	63,082	57,950
	Atlantic Region	68,683	60,012	66,054	63,061	57,920
Shrimp, Prawns	World	3,291,423	3,420,696	3,602,774	3,815,018	4,021,521
	Canada	63,139	65,708	82,111	113,112	120,103
	Atlantic Region	54,582	56,316	76,911	107,909	115,903
Squat-lobster	World	76,330	62,201	70,705	79,223	84,030
	Canada	0[b]	0	0	0	0
	Atlantic Region	0	0	0	0	0
Squids, Cuttlefishes, Octopi	World	2,875,477	3,103,994	3,417,407	2,633,303	3,373,496
	Canada	1,107	9,019	15,983	2,083	345
	Atlantic Region	999	8,822	15,775	1,944	313

[a] Data Not Available
[b] Some species caught, but is less than the unit of measure

conditions as a result of putrefaction by protein-degrading bacteria such as *Pseudomonads* resulting in the formation of ill-smelling and toxic compounds (Hall et al., 1994; Zakaria et al., 1998). The compounds produced as a consequence of bacterial spoilage include: (a) trimethylamine, which has fishy or ammoniacal smell, (b) sulphur compounds which give a smell of bad eggs, (c) indole, which has faecal smell, and (d) carbonyl compounds which give rancid or fruity smells (Hobbs, 1982).

The major disposal methods for shellfish waste include agricultural land application, offshore disposal and landfilling. The shellfish waste is nutritionally valuable for crops; but the waste must be turned under rapidly after application in order to minimize the odor problem. The odor problem is very significant when shellfish waste is used as a fertilizer in regions close to residential areas. Offshore dumping of shellfish waste causes odor problems, floating debris and increased water turbidity. Dumping at the sea is a controversial subject and environmental groups strongly oppose any dumping at sea, regardless of the source of waters or method used (Green and Mattick, 1979). It causes eutrophication and high load of biological oxygen demand. Many problems have become associated with landfill operations including running out of available land, gas formation from decaying waste, leachates contaminating groundwater and pathogens-borne problems. Currently, the amount of organic matter that goes to landfills is significantly reduced and hence landfilling shellfish waste might not be an option in the future (Healy et al., 2003).

4.2.4 Shellfish Waste Utilization

The environmental impacts of traditional methods of disposal of shellfish waste as well as the strengthening of environmental regulations in many countries and the growing interest in natural materials have created interests in developing alternative methods of disposal while considering the economic potential of waste utilization (van Ornum, 1992; Hansen and Illanes 1994; Hall and Reid, 1995; and Subasinghe, 1995). Hansen and Illanes (1994) reported that during the last two decades, the shellfish industry has experienced a significant expansion, making crustacean waste materials available in concentrated areas and in much larger quantities. Balogun and Akegbejo-Samsons (1992)

stated that shellfish wastes can be processed into products of high nutritional value if their collection is organized and centralized.

However, some problems associated with the recovery of shellfish waste components include: (a) the availability of regular supply of the raw material is restricted by the seasonal nature of the material as well as the distributed nature of the processing industries in many countries, (b) the relatively bulky nature of the waste material and its high perishability makes it difficult to transport, and (d) the physical differences among species requires different designs of the processing plants (McGahren et al., 1984; Hansen and Illanes 1994; Subasinghe, 1995; and Teng et al., 2001).

4.2.4.1 Food and Feed. Chitin, protein and pigments are the valuable constituents in shellfish waste from the standpoint of the food and feed industry (Ayangbile, 1989). Shellfish waste contains chitin, residual protein (45%) and internal organs. If lipids and pigments are extracted from shellfish meal, a high quality protein can be obtained for human consumption. Adhered muscle and viscera could be recovered to produce shellfish food snacks, which are also good for human consumption. Chitin and chitosan purified from shellfish waste could be used as a functional ingredient in foods (Hansen and Illanes, 1994).

Shellfish-processing waste represents a valuable resource, which could be used for animal feed. Shellfish meal is usually used as a supplement in diets for chicken. Shellfish pigments are also used in broiler chicken feed to give a desirable color to egg yolk in laying hens and quails (Chawan and Gerry, 1974). Shrimp processing waste could be converted into shrimp by-product meals as an alternative source of animal protein for use in livestock. Barratt and Montano (1986) prepared shrimp silage from the waste material by adding organic acids since the acidic conditions allow the enzymes in the waste to break down the protein forming liquid protein. They stated that this shrimp silage could be mixed with other substances such as malt wastes, rice husk, corn, bananas and other carbohydrates rich substances and once it is dried it could be introduced in poultry and cattle feed. Cosio et al. (1982) proposed a method for the pretreatment of shrimp-

processing waste for chitin bioconversion to produce single cell protein (yeast protein) acceptable as a protein component for animal feed. The shellfish waste could be deproteinized and demineralized to obtain chitin. Chitinase is then used to hydrolyze the chitin to the monomer N-acetyl glycosylamine, which serves as a substrate for the production of single cell protein (Wilke et al., 1976). Ayangbile (1989) studied the performance and carcass characteristics of beef cattle fed three levels of crab waste-straw silage (0, 15, and 30% dry basis). Their results showed that feeding crab waste-straw silage did not adversely affect the rate of gain, feed efficiency and carcass quality and hence crab waste silage can be used as a protein roughage supplement for ruminants.

Shellfish meal could also be used as a supplement in diets for fish. Astaxanthin is usually used in fish feed for salmonid species since it enhances the red coloration of the flesh and favors consumer acceptability (Meyers and Bligh, 1989; and Healy et al., 2003). Shrimp silage prepared by Barratt and Montano (1986) could also be introduced in shrimp feed. Rhishipal and Philip (1998) isolated four marine yeasts that are able to generate single cell protein when growing on prawn-shell waste. After biotransformation of prawn-shell waste using the isolated yeast strains the protein content of the final product reached 60.6 – 70.4%. This single cell protein could find wide application in aquaculture especially as a feed supplement, which would be economically viable and cost effective.

4.2.4.2 Fertilizers. Shellfish waste is high in organic material and thus it could be used directly as a fertilizer. Aung and Flick (1980) reported restriction of the excessive vegetative growth of tomato plant when adding crab-processing waste at 10 to 40 g per 3.5 kg of sand medium. This might be due to the existence of calcium in the crab shells and the nitrogen is not in excess that causes excessive growth.

Composting of shellfish wastes is a valid alternative to landfilling or offshore disposal. Chitinous-based composts have the potential to control fungal diseases and root parasitic nematodes because of the disease suppressive nature of chitin derivatives. Roy et al. (1997) developed a novel two-phase method for the production of shrimp waste-based compost. They claimed that their compost has significantly higher levels of disease

suppressive chitin derivatives than commercially available compost. Labrie et al. (2001) concluded that two-phase composting process of chitin waste-based composts promotes the proliferation of gram-positive bacteria antagonistic to oomycete plant pathogens. Hountin et al. (1995) also reported that peat moss-shrimp waste compost is beneficial for the growth of barely on a loamy sand soil. Murphy et al. (2000) used a peat-based substrate amended with shellfish waste to grow wild strawberry (*Fragaria vesca*). Their results showed positive interactions between the plant mycorrhizal inoculant (which was used to increase establishment and stimulate plant growth) and shellfish waste amended substrate and demonstrated plant resistance to the root disease *Phytophthora fragariae*.

Wang et al. (1999) purified a new antifungal compound (pafungin) from culture broth of *Pseudomonas aeruginosa* K-187 grown aerobically in a shellfish waste-containing medium (shrimp and crab shell powder). The new antifungal displayed growth inhibition characteristics of many pathogenic fungi (it affected all 39 fungi strains tested). It was also able to suppress *Fusarium oxysporum* that causes damping-off disease.

4.2.4.3 Chemicals. Subasinghe (1995) reported that chitin, as valuable product with wide range of applications, could be recovered from shellfish waste. The conventional process of chitin recovery from shellfish waste includes treatment by acid and alkali to remove minerals and proteins, respectively. Biotechnological approaches also could be used for chitin recovery from shellfish waste. Assuming 50% conversion of waste into chitin, the global production potential could reach 45 000 MT. Chitin could also be processed into chitosan (chitin deacetylated form), which is a high valuable commodity with wide range of applications in the medical and pharmaceutical industries.

Shrimp shells could be used for the production of amino acids and glucosamine by hydrothermal decomposition of protein and chitin present in the waste material (Quitain et al., 2001). Amino acids have a wide range of applications in pharmaceuticals (treatment of various diseases), food products (taste enhancement), animal nutrition and cosmetic industries. Glucosamine is also used in medicines (helps in rebuild damaged joints, tendons, cartilage, and soft tissue) and food industry. The amino acids are

produced from hydrolysis of proteins originally present in the shrimp shells while glucosamine is produced by deacetylation of shrimp shell chitin to chitosan followed by hydrolysis. Quitain et al. (2001) concluded that approximately 70 mg of amino acids/ g of dry shrimp shell could be obtained at a temperature of 523 K in 60 min. However, they did not detect any glucosamine due to its deamination and recommended further investigations to control deamination and hydrolysis of chitin using some additives in order to produce glucosamine from shrimp shells.

4.2.4.4 Energy. Hydrogen gas offers large potential benefits in terms of reduced emissions of pollutants and greenhouse gases and diversified primary energy supply. Like electricity, hydrogen is a premium-quality energy carrier, which can be used with high efficiency and zero emissions (Ogden, 1999). Evvyernie et al. (2001) reported that marine biomass such as shrimp and crab shells could be used to produce hydrogen gas as a source of green energy. They also stated that *Clostridium paraputrificum* M-21 is a potent hydrogen producer. It is capable of solubilizing and fermenting the shellfish waste material. However, the hydrogen productivity of *C. paraputrificum* M-21 from shellfish wastes should be improved in order for the process to be economically feasible.

4.3 Organic Acids

4.3.1 Lactic Acid

Lactic acid is a product of lactose fermentation (milk sugar). It is present in sour milk, koumiss, leban, yogurt, and cottage cheese and is also naturally produced in the muscles by oxidation of glucose during intense activity. Although it can be prepared by chemical synthesis, production of lactic acid by fermentation of glucose and other sugars is a less expensive method (Bu'lock and Kristiansen, 1987). The approximate world annual production of lactic acid is 50,000 t (Datta and Tsai, 1997).

4.3.1.1 Physical and Chemical Properties. Lactic acid ($C_3H_6O_3$) is the smallest chemical molecule with an asymmetric carbon. Chemically, It exists in two forms L(+) (Levo) and D(-) (Dextro) as well as in a racemic mixture (Figure 4.5). The lactic acid of

commerce is usually an optically inactive racemic mixture of the two isomers. The L(+) form is naturally present in animal and human tissues as well as in numerous food products (meat, milk products, pickles, beer, etc.). Lactic acid can condense with itself to form esters such as lactoyl-lactic acid and dilactide since it contains both a carboxyl and a hydroxyl group (King and Cheetham, 1987). When a solution of lactic acid is evaporated, part of the lactic acid is converted to these compounds and to polymers containing more than two lactic acid units, resulting in somewhat viscous non-crystallising syrup.

$$CH_3 - CHOH - COOH$$

(a) Lactic acid formula

L-(+)-Lactic acid D-(-)-Lactic acid

(b) Lactic acid structure

Figure 4.5. Lactic acid formula and structure.

Pure and anhydrous lactic acid is white crystalline solid with a low melting point of about 54 °C and could be obtained by special methods such as molecular distillation techniques. However, due to difficulties in preparation of the pure and anhydrous acid, it is rarely found in this form and thus, commercial lactic acid generally appears in the form of concentrated aqueous solutions (Holten et al., 1971; and Bu'lock and Kristiansen, 1987). Good quality solutions are colorless, odorless, and miscible with water or ethanol.

Table 4.17 shows some of the physical and chemical characteristics of lactic acid aqueous solutions.

4.3.1.2 Production Process. Industrial manufacture of lactic acid was first established in 1881 in the USA, and in 1895 in Germany. Generally, the preparation of lactic acid falls into two groups: biochemical and chemical formations (Holten et al., 1971).

4.3.1.2.1 Chemical Processes: In the chemical formation processes, lactic acid is formed in a variety of chemical reactions (Figure 4.6). These chemical reactions include: (a) hydrolysis of derivatives of lactic acid, e.g. esters or nitrile, or liberation from salts, (b) hydrolysis of other 2-substituted propionic acids, (c) decarboxylation of certain derivatives of 2-methylmalonic acid, (d) reduction, (e) oxidation, and (f) rearrangement and disproportionation. Either of these chemical processes leads to DL-lactic acid.

Lactonitrile is the most popular source of lactic acid production (Holten et al., 1971). Lactic acid can be produced synthetically by hydrolysis of lactonitrile which itself is produced either as a by-product of acrylonitrile manufacture or by the reaction of acetaldehyde with hydrogen cyanide (King and Cheetham, 1987). The governing chemical reaction for lactic acid production from lactonitrile is as follows:

$$CH_3CHOHCN + 2H_2O \longrightarrow CH_3CHOHCOOH + NH_3 \qquad (4.1)$$

Pure lactic acid could be chemically obtained by decomposing calcium lactate with zinc carbonate, whereby sparingly soluble and readily crystallizable zinc lactate is obtained (Martin and Cooke, 1952). The pure zinc lactate is then decomposed by H2S, filtered from the zinc sulfide and evaporated in an enameled vacuum apparatus in order to obtain clear, pure lactic acid. This process can be described by the following equations:

$$\underset{\text{Calcium Lactate}}{Ca(C_3H5O_3)_2} + ZnCO_3 \longrightarrow \underset{\text{Zinc Lactate}}{Zn(C_3H_5O_3)_2} + CaCO_3 \qquad (4.2)$$

$$\underset{\text{Zinc Lactate}}{Zn(C_3H_5O_3)_2} + H_2S \longrightarrow ZnS + 2\underset{\text{Lactic Acid}}{(CH_3CHOHCOOH)} \quad (4.3)$$

Table 4.17. Some chemical and physical properties of L (+) and DL-lactic acid (Holten et al., 1971).

Property	Description
Appearance	White crystalline solid
Melting point	
D(-) or L(+)	52.8-54° C
DL (Varies with composition)	16.8-33° C
Boiling point (DL)	122° C (1.6-2 kPa)
Vapor pressure	1.8 kPa (@ 120° C)
Density	1.1989 g/mL (20° C) for aqueous solution of 85.32% by weight
Heat of combustion	-1344.6 kJ/mole (Crystalline L-(+)-Lactate) -1356.6 kJ/mole (liquid @ 25° C)
Molecular weight of pure substances	90.08
Viscosity (centipoises)	1.67 (24.35% @ 25° C) 3.09 (45.48% @ 25° C) 6.96 (64.89% @ 25° C) 28.50 (85.32% @ 25° C)
Heat of fusion	11.34 kJ/mole (racemic acid) 16.87 kJ/mole (L-(+)-Lactic acid)
Heat of vaporization	83.3 kJ/mole (@ 120° C)
Heat of formation	-694.4 kJ/mole (Crystalline L-(+)-Lactate) -682.3 kJ/mole (liquid @ 25° C)
Absolute entropy	142.3 J/° C.mole (crystalline lactate @ 25° C) 192.1 J/° C.g (liquid lactic acid @ 25° C)
Heat of solution	7819 J/mole (crystalline L-(+)-lactic acid @ 25° C)
Heat capacity	1.4 J/° C.g (crystalline lactic acid @ 25° C) 2.3 J/° C.g (liquid lactic acid @ 25° C)
Dielectric constant	20.90 @ 15° C
Dissociation constant	1.387×10^{-4} (25° C)
pH of aqueous solution	Highly corrosive (pH = 0 @ 25° C for 88.6% by weight lactic acid solution)
Solubility	
In water	Completely miscible
In other common materials	Miscible in ethanol and ethyl ether Insoluble in hydrocarbons (solubility in benzene is less than 1%) Insoluble in chloroform

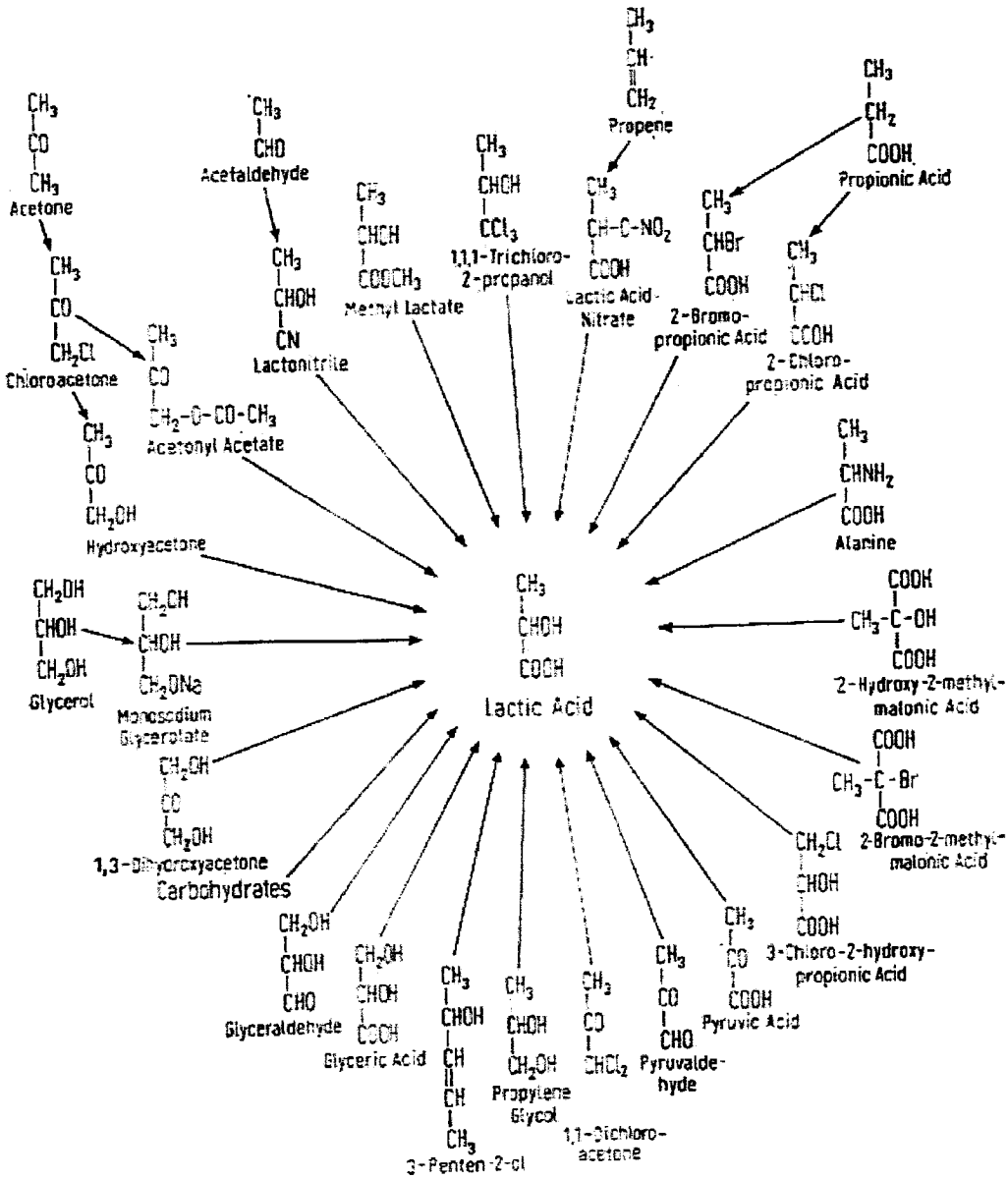


Figure 4.6. Routes for the chemical formation of lactic acid (Holten et al., 1971).

Pure lactic acid could also be obtained by distilling syrupy commercial lactic acid with superheated steam at 140 - 160° C from a calcium chloride bath. The concentrated distillate is then decolorized by sunlight or animal charcoal. However, 20 – 30% of the lactic acid is lost due to the formation of lactic anhydride during this period.

4.3.1.2.2 Fermentation Processes: About 50% of the lactic acid world production is by fermentation (Ohleyer et al., 1985; Cheryan and Mehaia, 1986; and von Frieling and

Schügerl, 1999). The practical significance of lactic acid fermentation is that it has the advantage of being able to produce optically pure lactic acid, it utilizes renewable carbon sources and it could be used in lactic acid production on an industrial scale (Holten et al., 1971; Cotton et al., 2001; and Demirci et al., 2003).

Generally, two different types of lactic fermentation from carbohydrates are known as shown in Figure 4.7: (a) homolactic fermentation which is called pure lactic and (b) heterolactic fermentation which is called mixed lactic.

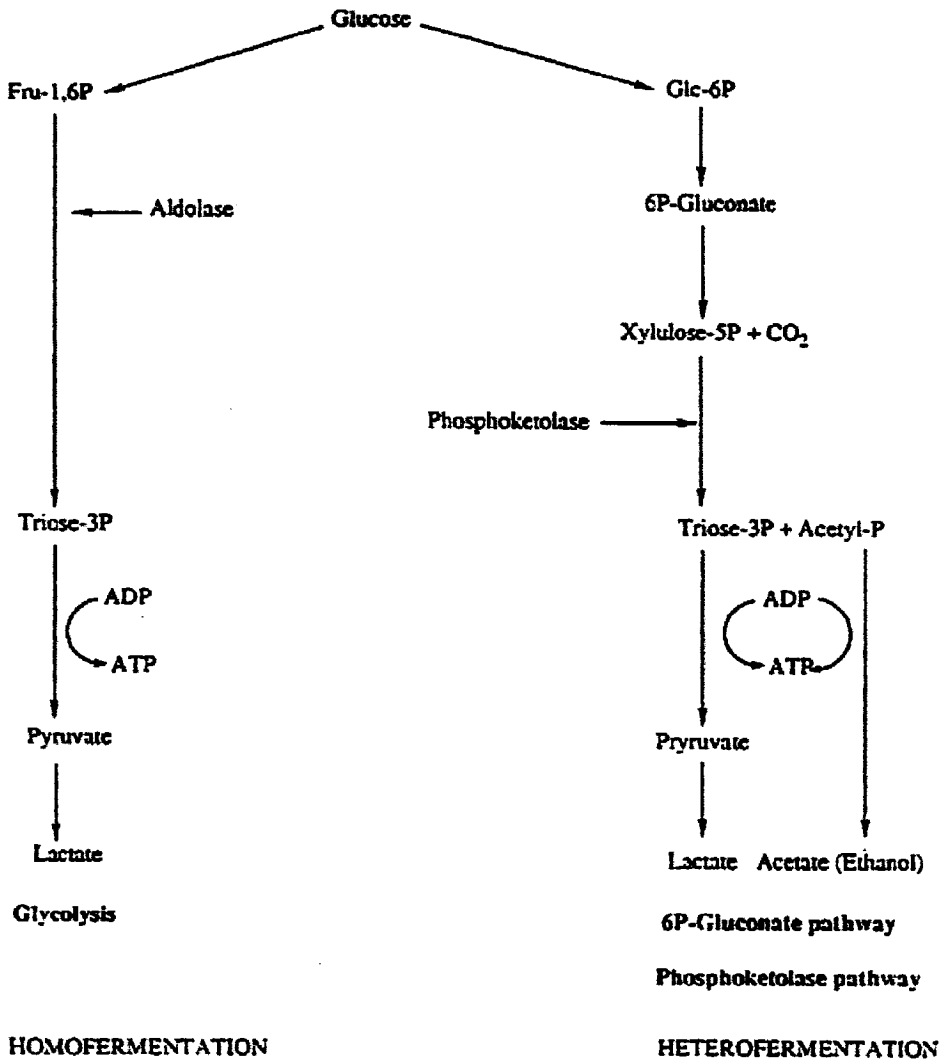


Figure 4.7. The homo- and heterofermentation pathways (Adams and Moss, 1995).

Homolactic fermentation is considered a characteristic of microorganisms, which form lactic acid predominantly. This type of fermentation is performed by the homolactic Lactobacteriaceae. It is anaerobic exothermic reaction and the yield of lactic acid may be more than 90% with some Lactobacillus species. Homolactic bacteria, e.g. *Lactobacillus bulgaricus* and *L. delbrückii* are commonly used for lactic acid production (Bu'lock and Kristiansen, 1987; and King and Cheetham, 1987).

The substrate may be lactose (whey), glucose (or glucose syrups), or sucrose, either pure or as beet molasses. *L. delbrückii* is used at temperatures of up to 50 °C and *L. bulgaricus* up to about 44 °C. The latter must be used to ferment whey because *L. delbrückii* is not able to ferment lactose. Disaccharides are hydrolysed to hexoses, which are catabolized via the Embden-Meyerhof path to pyruvate, which is finally reduced to L (+)-Lactic acid by lactate dehydrogenase. Under some conditions, the DL-Lactic acid is produced, possibly by action of racemase. The fermentation route using *Lactobacillus delbrückii* is said to produce about 3% of D(-) acid in addition to 97% of the L(+) isomer. Contaminant organisms, if present, e.g. *Lactobacillus plantarum*, may elaborate enzymes causing racemisation resulting in the production of a much greater portion of the D(-) acid. *Lactobacillus Lactis* produces only the D(-) isomer.

However, the normal fermentation medium needs besides the carbohydrate, inorganic nutrients (to supply nitrogen, phosphate, potassium, etc.), organic nitrogen sources (e.g. corn steep liquor, malt sprouts, or yeast extract) are often used, and additional vitamins may be added. pH control is also necessary to insure a satisfactory rate of fermentation.

At lower pH values, the fermentation is progressively slower and at pH of 4.5 no lactic acid is formed. The fermentation generally takes 6 – 7 days and yields in the range 80 – 90 g lactic acid per 100 g carbohydrate. Generally, the homolactic fermentation proceeds according to the following equation:

$$\text{Glucose} + 2\text{ADP} + 2\text{P}_i \longrightarrow 2\text{Lactic acid} + 2\text{ATP} \qquad (4.4)$$

In heterolactic fermentation, considerable amounts of other fermentation products are formed besides the production of lactic acid e.g. acetic acid, ethanol, formic acid and carbon dioxide. This process is performed by the heterolactic Lactobacteriaceae and also by various other bacteria, e.g. *Staphylococcus*, *Bacillus*, *Actinomyces* and *Salmonella* species as well as by *Rhizopus* species among the fungi. The heterolactic Lactobacteriaceae investigated most thoroughly are *Lactobacillus plantarum* and *Leuconostoc mesenteroides*. They degrade glucose anaerobically to lactic acid, ethanol, and carbon dioxide with the aid of enzymes belonging to the hexose monophosphate shunt, according to the following equation:

$$\text{Glucose} + 2\text{ADP} + 2\text{P}_i \longrightarrow 2\text{Lactic acid} + \text{Ethanol} + \text{CO}_2 + 2\text{ATP} \quad (4.5)$$

Lactic acid has been produced from whey in developing countries, and has been proved to be an economically feasible process while reducing the wastage of biological oxygen demand substrate (Arasaratnam et al., 1996; Tango and Ghaly, 1999; Tango and Ghaly, 2002; and Ghaly et al., 2003). Mostafa (1996) reported that whey is more suitable substrate for lactic acid production than pure lactose due to the fact that whey substrate contains all essential nutrients. Prescotts et al. (1990) reported that *Lactobacillus* and sometimes *Streptococcus* are popularly used for lactic acid production from cheese whey. *Lactobacillus helveticus* seems to be the most productive lactic acid bacteria that could ferment cheese whey lactose. It is superior in terms of fermentation rate, complete lactose utilization, its tolerance against lactic acid concentration and it produces a racemic (D and L-lactic acid. *Lactobacillus helveticus* is a homofermentative, thermophilic and acidophilic bacterium, which can grow in conditions inhibitory for most contaminants (Aeschlimann and von Stockar, 1989; Aeschlimann et al., 1990; and Tango and Ghaly, 1999). This bacterium grows in a temperature range of 35 – 42 °C with optimum temperature of 37 °C and optimum pH of 5.5 for batch fermentations and 6.3 for immobilized fermentation (Roy et al., 1986; Aeschlimann and von Stockar, 1989; and Kulozik and Wilde, 1999). Table 4.18 and 4.19 show the some data reported in the literature for batch and continuous lactic acid production from cheese whey, respectively.

Table 4.18. Batch fermentation for lactic acid production.

Substrate	Bioreactor	Microorganism	Immobilization material	Production rate g/L.h	Yield %	Reference
Whey	Batch	Lactobacillus helveticus	-	1.34	61	Tango and Ghaly (1999)
Whey	Batch	Lactobacillus helveticus	Alumina beads Plastic rings Ceramic rings	1.5 0.9 2.2	69 57 92	Mahmoud and Ghaly (2002)
Whey permeate	Batch	L. bulgaricus S. lactis	κ-Carrageenan-locust bean gum gel	1.7 1.3	-[a]	Audet et al. (1988)
Deproteinized whey	Extractive batch Batch	Lactobacillus delbrueckii	κ-Carrageenan beads	11.7[c] 6.4[c]	-[a]	Yabannavar and Wang (1991)
Whey	Repeated batch	Coimmobilized Lactobacillus casei and Lactococcus lactis	Ca-alginate κ-Carrageenan Agar Polyacrylamide	0.86 0.79 0.78 0.62	97 93 98 83	Roukas and Kotzekidou (1991)
Whey	Recycle batch	Lactobacillus casei subsp. Rhamnosus	Foam glass pora-bact A beads	4.3-4.6	90	Senthuran et al. (1997)
Deproteinized whey	Fed batch	Coimmobilized Lactobacillus casei and Lactococcus lactis	Ca-alginate beads	1.96[b]	72.3	Roukas and Kotzekidou (1998)

[a] Not presented
[b] Estimated from reported data
[c] g/Lgel.h

Table 4.19. Continuous fermentation for lactic acid production.

Substrate	Bioreactor	Microorganism	Immobilization material	Production rate g/L.h	Yield %	Reference
Whey	Continuous	Lactobacillus helveticus	Alumina beads	5.2	85.5	Tango and Ghaly (2002)
Whey	Continuous	Lactobacillus helveticus	Alumina beads Plastic rings Ceramic rings	3.4 1.9 3.9	94.9 56.3 94.9	Mahmoud and Ghaly (2002)
Salt whey permeate	Continuous	Strain isolated from Egyptian cheddar cheese	Agarose beads	6.12[b]	68[b]	Zayed and Winter (1995)
Whey	Continuous packed tubular reactor	Lactobacillus casei	Agar gel beads	7.28 9.36 (with recycle)	-[a]	Mostafa (1996)
Deproteinized whey	Continuous	Coimmobilized Lactobacillus casei and Lactococcus lactis	Ca-alginate beads	7	70	Roukas and Kotzekidou (1996) Silva and Yang (1995)
Acid whey	Continuous fibrous-bed	Lactobacillus helveticus	Fibrous matrix of cotton terry cloth and stainless steel mesh	2.6-7	95	Norton et al. (1994a) Norton et al. (1994b)
Whey permeate	Continuous	Lactobacillus helveticus	κ-Carrageenan-locust bean gum gel beads	28.5	-[a]	Roy et al. (1987)
Whey permeate	Continuous (two-stages)	Lactobacillus helveticus	κ-Carrageenan-locust bean gum gel beads	13.5	-[a]	
Whey permeate	Continuous	Lactobacillus helveticus	Ca-alginate beads	2.6	82	

[a] Not presented
[b] Estimated from reported data
[c] g/Lgel.h

4.3.1.3 Applications. Generally, lactic acid is used as a food additive and as an industrial chemical because of two important properties: (a) it is very stable to heat, thus it tolerates all heat treatments such as those in bread baking, and (b) it undergoes self-esterification, resulting in the formation of lactoyllactic acid and eventually higher linear polyesters in its aqueous solutions (Holten et al., 1971). At the end of their life-cycle, Galactic's lactic acid and lactates undergo a bio-degradation occurrence leaving only H_2O and CO_2. Therefore, they are re-entering the natural lifecycle without leaving behind harmful pollutants. The industrial production of lactic acid utilizes homolactic fermentation whereas in the production of lactic acid foodstuffs heterolactic fermentation is of great importance.

Pharmaceutical and food industries show preference for the L(+) lactic acid and lactates because the D(-) form is not metabolized by the human body.

About one half of the total world production of lactic acid was for food uses (as acidulent and preservative). Kaufman et al. (1996), Roukas and Kotzekidou (1996), and Silva and Yang (1995) reported that lactic acid is a specialty chemical used in the food industry for the manufacture of cheese, pickles, and yogurt. About one fifth of the total world production was processed to produce calcium- and sodium-stearoyl-2-lactylate (for backing), the rest was used in the pharmaceutical industry (dietary calcium source, blood coagulant) or in technical applications (biodegradable plastics) (Bu'lock and Kristiansen, 1987; Cheryan and Mehaia, 1986; Jeantet et al., 1996; Wang et al., 1995; and von Frieling and Schügerl, 1999). Lactic acid is also produced commercially for use in leather tanning and textile dyeing, in making plastics, synthesis of other organic acids, acetaldehyde, solvents, inks, ethanol and lacquers (Bu'lock and Kristiansen, 1987).

4.3.2 Acetic Acid

Acetic acid is an important organic acid in the chemical industry. Historically, all acetic acid was prepared by sugar fermentation to ethyl alcohol and subsequent oxidation to acetic acid by microorganisms to produce vinegar. Currently, acetic acid is produced by chemical synthesis since the traditional vinegar fermentation process suffers from low

product yield and high-energy cost. However, the exhaustion of fossil fuel will sooner or later force the use of fermentation process of renewable substrates. Acetic acid is an important organic chemical, with an annual production rate of 4.68 billion pounds in 1995 in the United States and a projected 3% annual growth rate in market demand (Huang and Yang, 1998; and Talabardon et al., 2000).

4.3.2.1 Physical and Chemical Properties. Acetic acid (CH_3COOH) is a colorless, watery liquid with a strong irritating vinegar odor. Table (4.20) summarizes some of the chemical and physical properties of acetic acid.

4.3.2.2 Production Processes. Much of the present world output of acetic acid comes from petroleum, while microbial oxidation of ethanol to acetic acid in the so-called quick vinegar process has long been known (Bu'lock and Kristiansen, 1987). However, the chemical routes for the acetic acid production are considered very expensive processes. Recently, newer processes (biochemical routes) have been under development for the manufacture of acetic acid by anaerobic fermentation of cellulosic and other renewable resources (Tang et al., 1988; Huang and Yang, 1998; and Talabardon et al., 2000).

4.3.2.2.1 Chemical Processes: Most of current commercial production of glacial acetic acid is exclusively by petrochemical routes (Bu'lock and Kristiannsen, 1987; Huang and Yang, 1998; and Balasubramanian et al., 2001). Liquid-phase oxidation of butane is currently used for acetic acid manufacture with acetic acid yield of about 75%. Butane is charged to the reactor at high pressure (1000-5500 kPa, 50-250 °C), with a solution of cobalt, manganese, or chromium acetate. Air at 5 kg per kg butane is bubbled through this solution (Environment Canada, 1985). The process of acetic acid production from butane can be described by the following equation:

$$C_4H_{10} + O_2 \longrightarrow CH_3CO_2H + \text{by-products (other acids, aldehydes, and carbon oxides)} \quad (4.6)$$

Table 4.20. Some chemical and physical properties of acetic acid (Environment Canada, 1985).

Property	Description
Appearance	Clear, colorless liquid
Physical state @ 15° C, 1 atm.	Solid
Melting point	16.604° C – 16.75° C
Boiling point	117.87° C
Vapor pressure	1.57 kPa (20° C)
Density	1.0492 g/mL (20° C)
Flammability	Gives off flammable vapor at high temperatures.
Autoignition temperature	464° C
Burning rate	1.6 mm/min
Heat of combustion	-874.5 kJ/mole (25° C)
Combustion product	Carbon dioxide and water
Explosiveness	Vapor forms explosive mixtures with air
Molecular weight of pure substances	60.05
Viscosity	1.183 mPa.s (20° C), 0.018 mPa.s (40° C) 1.2 mPa.s (20% @ 25° C) 1.7 mPa.s (40% @ 25° C) 2.1 mPa.s (60% @ 25° C) 3.1 mPa.s (80% @ 25° C)
Liquid interfacial tension with air	27.57 mN/m (20° C)
Liquid interfacial tension with water	38 mN/m (20° C)
Latent heat of fusion	11.74 kJ/mole (at melting point)
Latent heat of vaporization	23.69 kJ/mole (at boiling point)
Heat of formation	-484.3 kJ/mole (25° C)
Entropy	159.9 J/mole.K
Ionization potential	10.66 eV
Heat of solution	-1506 J/mole (25° C)
Heat capacity	
Constant pressure (C_p)	
Liquid	122.68 J/(mole. ° C) (14.4° C)
Vapor	301.99 J/(mole. ° C) (124° C)
Solid	88.77 J/(mole. ° C) (1.5° C)
Constant volume (C_v)	107 J/(mole. ° C) (25° C)
Critical pressure	5786 kPa
Critical temperature	321.6° C
Coefficient of thermal expansion	1.071×10^{-3}/° C (20° C)
Thermal conductivity	0.158 W/(m.K) (20° C)
Saturation concentration	38 g/m^3 (20° C), 63 g/m^3 (30° C)
Dielectric constant	2.665 (-10° C), 6.170 (20° C)

Table 4.20. Continued.

Property	Description
Dissociation constant	1.845×10^{-5} (25° C)
Specific conductance	0.060 cm^2/ohm (100%, 25° C) 2.19 cm^2/ohm (97.6%, 25° C) 111 cm^2/ohm (90.75%, 25° C)
Diffusivity	0.1064 cm^2/s (in air, 0° C), 1.24×10^{-5} cm^2/s (in water, 25° C)
pH of aqueous solution	Highly corrosive at dilute concentrations, somewhat less so at glacial strength 2.4 (1N @ 25° C)
Evaporation rate	0.45 g/(m2.s) (galacial @ 20° C, wind 4.5 m/s)
Solubility	
In water	Completely soluble in all properties
In other common materials	Miscible in ethanol and diethyl ether, insoluble in carbon disulphide. Miscible in acetone and benzene.

Another way of producing acetic acid is that of using methanol and carbon monoxide as feedstock in a methanol carboxylation process. In this reaction, methanol and carbon monoxide are passed over an iodide-based catalyst (nickel iodide, copper or cobalt with iodine, etc.). The governing equation for this process is as follows:

$$CH_3OH + CO \xrightarrow{\text{Catalyst}} CH_3CO_2H \qquad (4.7)$$

The major disadvantages of the chemical production acetic acid are: (a) the need for high temperatures and high pressures, (b) the need for strong agitation, (c) the risk of explosion, (d) the high cost of catalyst and (e) the dependence on nonrenewable resources.

4.3.2.2.2 Fermentation Processes: In recent years, many attempts to produce a low-cost acetic acid from anaerobic fermentation of glucose, woody biomass, cheese whey and other renewable resources had been tried (Huang and Yang, 1998). Nearly 100% of the substrate carbon can be recovered in the product acetic acid by anaerobic fermentation

(Tang et al., 1988). Cellulose and hemicellulose in lignocellulosic biomass are the two most abundant renewable sources of carbon for fermentation to industrially useful chemicals (Balasubramanian et al., 2001). The pathways involved in acetic acid accumulation are quite complex as summarized in Figure 4.8 (Bu'lock and Kristiansen, 1987). Cellulosic wastes are hydrolysed to glucose by cellulases elaborated by anaerobic bacteria. In practice, the theoretical yield of three moles acetic acid from one mole of glucose is often approached, without the production of hydrogen and carbon dioxide.

Several anaerobic bacteria can convert carbohydrates to acetic acid by homofermentation. These bacteria include *Clostridium thermoaceticum*, *C. formicoaceticum*, *C. aceticum*, *Acetobacterium woodii* and *A. kivui*. Balasubramanian et al. (2001) reported that homoacetate anaerobic organisms such as *Clostridium thermoaceticum* convert glucose and xylose to acetic acid with a theoretical weight yield of 100%. All the bacteria mentioned previously cannot ferment lactose but *C. formicoaceticum* can readily convert lactate to equal amounts of acetate at a pH near 7 and mesophilic temperatures.

The production of acetic acid from biomass could follow many routes including biomass to methanol to acetic acid, biomass to acetic acid by wet-air oxidation, anaerobic digestion of biomass to produce acetic acid, and sugars fermentation (Marynowski et al., 1985). In case of using the route of biomass to methanol to acetic acid, it has been found that methanol production from biomass via thermal gasification process indicates that methanol by this route is not likely to be competitive with methanol produced from fossil fuel feedstocks. Noncatalytic wet-air oxidation can convert biomass to acetic acid in certain conditions of temperature, pressure, and reaction time. Using this route would result in low yields of acetic acid because of further oxidation to formic acid, CO_2, and H_2O. Therefore, this route is not technically or economically competitive with other routes. Anaerobic digestion of biomass using mixture cultures of bacteria is used to produce a methane-rich fuel gas. Some of the intermediate products in the process consist of a number of organic acids including acetic acid. But in this, case the yield of acetic acid is very low and the recovery from the mixture is too expensive.

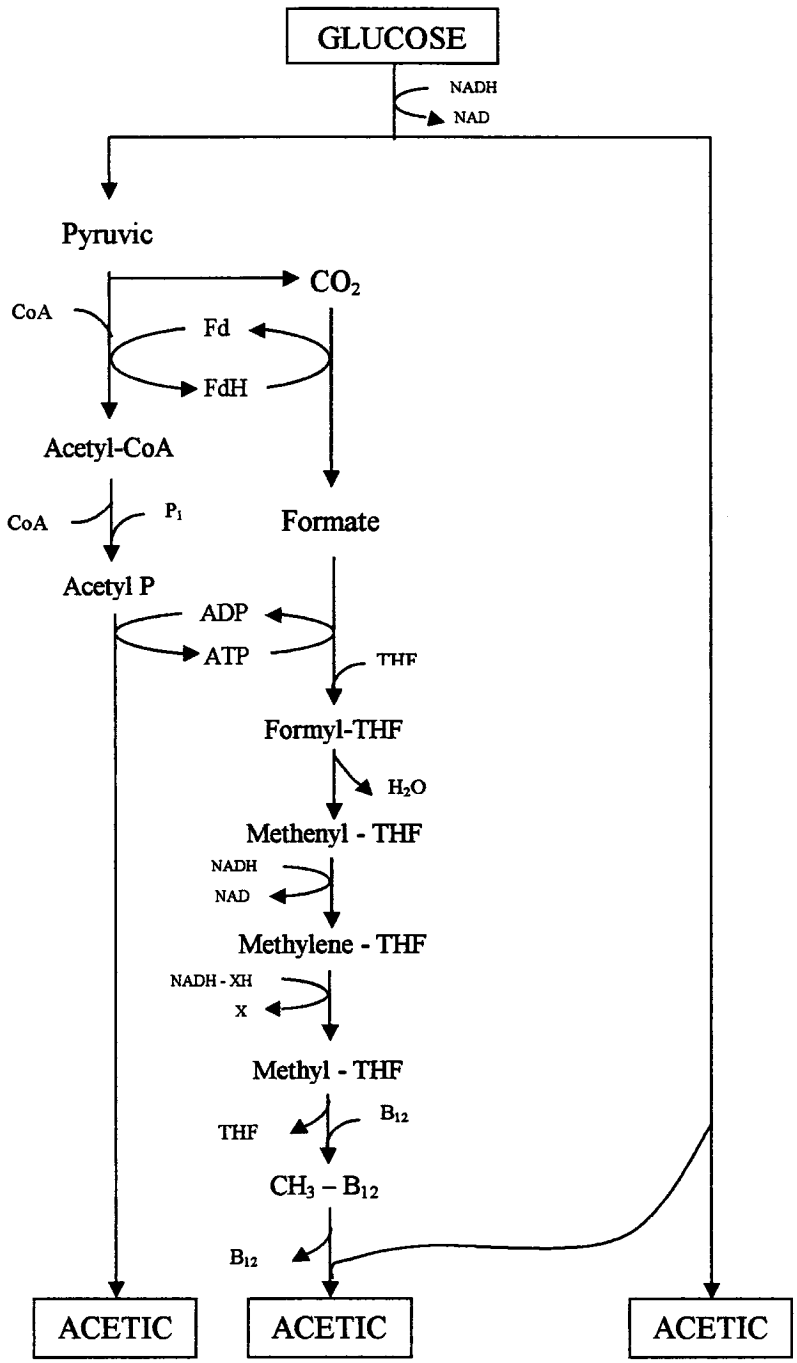


Figure 4.8. Routes to Acetic acid from glucose (Bu'lock and Kristiansen, 1987).

Monosaccharides and disaccharides produced from sugar crops, starch crops, lignocellulosic materials (wood or field crop residues) are potential feedstocks for the production of acetic acid from biomass. There are two fermentation routes, which offer

significant near-term potential for the production of acetic acid in high yield from sugars. The first route is used commercially for vinegar production and could be represented by the following equations:

$$\underset{180\ kg}{C_6H_{12}O_6} \xrightarrow[Yeast]{\textit{S. cerevisae}} \underset{92\ kg}{2C_2H_5OH} + \underset{88\ kg}{2CO_2} \qquad (4.8)$$

$$\underset{92\ kg}{2C_2H_5OH} + 2O_2 \xrightarrow[(bacteria)]{\textit{A. aceti}} \underset{120\ kg}{2CH_3COOH} + \underset{36\ kg}{2\ H_2O} \qquad (4.9)$$

Ethyl alcohol is oxidized to acetic acid by microorganisms of the genus Acetobacter in the presence of air. In an industrial scale (quick Vinegar process), the alcohol-bearing aqueous substrate is allowed to trickle over wood shavings carrying the microorganisms or like carriers having a large surface area exposed to ambient air. In another industrial method, the microorganisms are cultured while submersed in the substrate. Air is passed through the substrate in bubbles. Both commercial processes are essentially batch processes, although the liquid substrate may be circulated during fermentation in the quick vinegar process (Gutcho, 1973). However, Tang et al. (1988) reported that the conventional vinegar fermentation suffers from low product yield and high-energy cost, and thus cannot compete with the oil-based synthetic process.

The second route is preferable economically to the first one because of its much higher yield of acetic acid (Marynowski et al., 1985). In practice, the theoretical yield of three mole of glucose is often approached, without the production of hydrogen and carbon dioxide (Bu'lock and Kristiansen, 1987). The second route can be represented by the following equations:

$$\underset{180\ kg}{C_6H_{12}O_6} \xrightarrow{\textit{C. thermoaceticum}} \underset{180\ kg}{3CH_3COOH} \qquad (4.10)$$

$$\underset{150\ kg}{C_6H_{12}O_6} \xrightarrow{\textit{C. thermoaceticum}} \underset{150\ kg}{3CH_3COOH} \qquad (4.11)$$

In case of using cheese whey as a fermentation substrate, no homoacetogen can directly ferment lactose to acetate, and a coculture fermentation consisting of homolactic and homoacetic bacteria, in which the homolactic and homoacetic bacteria sequentially convert lactose to lactate and then to acetate, should be used. However, the fermentation process must be supplemented with nutrients unless the fermentation and the conversion of acetate will be slow. Balasubramanian et al. (2001) stated that acetic acid can be produced from whey permeate using mixed cultures of *C. formicoaceticum* and *S. lactis* when provided with appropriate levels of growth nutrients and an optimal pH of 7.6. Yeast extract and trypticase are expensive to be used in large-scale production of acetate, therefore, inexpensive nutrient supplements, such as corn steep liquor, industrial-grade yeast and casein hydrolysates could be used instead. It has been found that efficient acetate production from whey lactose could be achieved with sweet whey permeate and acid whey supplemented with small amount of inexpensive corn steep liquor or industrial-grade yeast and casein hydrolystates using fibrous-bed bioreactor operated in batch mode. Table 4.21 shows some of the results reported in the literature for acetic acid production from cheese whey fermentation.

3.2.3 Applications. Acetic acid is an important raw material in the chemical industry (Tang et al., 1988). Acetic acid could be used to produce many chemicals including vinyl acetate polymer, cellulose acetate, terephthalic acid/dimethylterephthalate, acetic acid esters, acetic anhydride, chloroacetic acid, and photochemicals (Tang et al., 1988; and Balasubramannian et al., 2001). In 1982, 33% of the Canadian domestic production was used for the manufacture of vinyl acetate, 29% was exported, and 26% was used in the manufacture of cellulose acetate (Environment Canada, 1985). In addition acetic acid could be used to produce acetate deicers, including calcium magnesium acetate (CMA) as an organic biodegradable noncorrosive road deicer and as additive to coal-fired combustion units to control sulfur emission and potassium acetate and sodium acetate as airport runway deicers (Yang et al., 1993; Huang and Yang, 1998; and Talabardon et al., 2000).

Table 4.21. Acetic acid production from cheese whey fermentation.

Substrate	Bioreactor	Microorganism	Immobilization material	Production rate gL.h	Yield %	Reference
Sweet whey permeate	Batch	Coculture of Streptococcus lactis and Clostridium thermoaceticum	-	0.38[b]	95	Tang et al. (1988)
Sweet whey permeate	Fed batch	Coculture of Lactococcus lactis and Clostridium formicoaceticum	Spiral-wound terry cloth	0.18	90	Huang and Yang (1998)
Sweet whey permeate	Continuous	Coculture of Streptococcus lactis and Clostridium thermoaceticum	Spiral-wound fibrous matrix	-[a]	95	Yang et al. (1992)
Milk permeate	Recycled batch	Coculture of Clostridium thermolacticum and Moorella thermoautotrophica	Fibrous bed bioreactor (piece of cotton towel)	0.54	96	Talabardon et al. (2000)
Acid whey	Batch	Coculture of Lactococcus lactis and Clostridium formicoaceticum	Fibrous bed bioreactor (spiral-wound terry cloth)	0.24	90	Yang et al. (1999)

[a] Not presented
[b] estimated from reported data

4.3.3 Extraction

The recovery of organic acids from fermentation broths is a big concern. Many researchers had suggested several methods for organic acids recovery including: filtration, dialysis, electrodialysis, ion exchange resin, reverse osmosis, and liquid-liquid extraction, with liquid-liquid extraction being the most commonly used extraction method (Friedman and Gaden, 1980; King and Cheetham, 1987; Schlicher and Cheryan, 1990; Namura et al., 1991, Hano et al., 1993; and Vaccari et al., 1993). Liquid-liquid extraction of organic acid consists of two steps: (a) extracting organic acid from fermentation broth (aqueous phase) into organic phase (organic solvent with carrier compounds) and (b) stripping extracted organic acid into another aqueous phase (stripping solution).

Generally, the used solvent in liquid-liquid extraction should have high extractive capacity, low viscosity, and low toxicity to the microorganisms (Chen and Lee, 1997). Amine carriers as well as phosphine oxides could be used for organic acids extraction with high distribution coefficient. However, the degree of extraction and the distribution coefficients are higher with the amine carriers than with the phosphine oxide carrier (von Frieling and Schügerl, 1999). The distribution coefficient also decreases with increasing temperature and pH.

4.3.3.1 Lactic Acid. Lactic acid is a strong inhibitor during the fermentation process even with pH control. In-situ removal (extractive fermentation) of lactic acid is one of the promising downstream processes because: (a) it has low cost compared to other separation methods, (b) both reactor productivity and product yield is improved since the removal of the fermentation product reduces product inhibition and shifts the metabolic pathway to favor product formation and (c) extractive fermentation eliminates the use of alkali in pH control and thus reduces the wastes from fermentation process (Yabannavar and Wang, 1991; Yang et al., 1992; Solichien et al., 1995; Chen and Lee, 1997; and Iyer and Lee, 1999). One disadvantage of extractive fermentation is that it usually involves the use of a water immiscible solvent, which is usually toxic to cells. Solvent toxicity could occur at the phase level or at the molecular level. Phase toxicity could be

eliminated by using hydrophobic membrane or immobilized systems (immobilized cells are more resistant to solvent toxicity than free cells). Solichien et al. (1995) reported that there has been increasing interest in using membranes in extractive fermentations in order to increase the transfer area, reduce emulsification, and reduce the toxic effects of the extractant on the microorganisms. Yabannavar and Wang (1991) stated that the direct contact between the immiscible solvent phase and the cells is reduced by cell immobilization, which results in minimizing toxicity to cells. Demirci et al. (2003) reported that plastic-composite-support (containing 50% wt/wt polypropylene, 53% wt/wt ground soybeanhulls, 5% wt/wt yeast extract, 5% wt/wt soybean flour and 5% wt/wt bovin albumin) biofilm reactors for lactic acid fermentation using *Lactobacillus casei* eliminates the toxic effect from solvent leakage into the fermentation medium from liquid-liquid extractive integrated fermentation. Molecular level toxicity could also be reduced by the use of essentially water insoluble solvents (von Frieling and Schügerl, 1999).

For extraction of carboxylic acids, tertiary amides have been reported to be more suitable than the primary and secondary amines (Chen and Lee, 1997). High molecular weight aliphatic amines, such as Alamine 336 (trioctyl amine), are effective in dilute carboxylic acids extraction. Alamine 336 is a water-insoluble, saturated, straight chain tertiary amine, with alkyl groups of C_8-C_{10}. The extraction capacity of Alamine can be greatly enhanced by mixing it with a polar solvent such as water insoluble alcohols (Chen and Lee, 1997). Long-chain alcohols serve well for this purpose since they are less toxic to microorganism. It can interact with the acid-amine complex through hydrogen bonding and make the complex more stable in the solvent phase. Alamine 336 and water insoluble alcohols are extremely viscous; therefore, the addition of a diluent such as kerosene is necessary to reduce the solvent viscosity. However, the disadvantage of using kerosene is the formation of a third phase with amine carriers, but this could be avoided by the addition of modifiers. Chen and Lee (1997) and Iyer and Lee (1999) found that a solvent mixture of 20% Alamine, 40% oleyl alcohol, and 40% kerosene is most effective in the extraction of lactic acid. Demirci et al. (2002) combined the two steps of liquid-liquid extraction together in an emulsion liquid membrane-extraction system in which stripping

solution is emulsified in organic solvent containing carrier compound and surfactant. They recommended an optimum emulsion system for lactic acid extraction consisting of 91% v/v iso-octan, 5% v/v TBP (tributylphosphate), 4% v/v span-80 and internal aqueous phase of 1.2 M Na_2CO_3. Yabannavar and Wang (1991) studied the effect of extractive fermentation on lactic acid production using 15% Alamine 336 in oleyl alcohol. The bioreactor operation resulted in a higher productivity (12 g/L gel h) compared to that of the control fermentation (7 g/L gel h).

4.3.3.2 Acetic Acid. In order eliminate the product inhibition in acetic acid fermentation, it may be beneficial to integrate the fermentation and extraction process to remove the acetic acid from the reactor while it is being produced (Yang et al., 1992). The extraction step will require a packed column extractor to provide multi contacts necessary to recover 99%of the acetic acid present in the fermentation broth. The acetic acid present in the extractant can be easily stripped by back-extraction with a small amount of an alkaline (dolime) solution. A simple mixture-settler can be used for back-extraction and reaction to form calcium magnesium acetate (CMA). Yang et al. (1999) reported that low cost potassium acetate (KA) could be produced from acetic acid fementation broth. In this case a concentrated KOH solution would be used in the back-extraction step to produce 50% weight/volume (wt/v) potassium acetate solution.

Bu'lock and Kristiansen (1987) stated that the manufacture of acetic acid by anaerobic fermentation yields an aqueous solution in which the product concentration is rather low and solvent extraction (using 20% trioctylphosphine oxide in kerosene) could be used to recover the product. Organic basis (amines and trioctyl phosphine oxide) can provide much higher equilibrium distribution coefficients (K_D) for extraction of acetic acid than are obtainable with conventional solvents (ethyl acetate and diethyl ketone) (Ricker et al., 1979). Organic basis take the advantage of the acidic nature of acetic acid and can thereby provide higher values of K_D. Diluent serves to provide suitable physical properties (viscosity, density, etc.) for the extraction process. In case of amines, it can produce a much higher K_D at an intermediate solvent composition than occurs for either the pure extractant or the pure diluent. Secondary amines provide high K_D values. A K_D

value of 160 was obtained with Adogen 283-D in 2-ethyl-1-hexanol. Adogen 283-D consists of two branched-chain, isomeric alkyl groups of 13 carbon atoms. Of the tertiary amines, Alamine 336 and Adogen 364, having average chain lengths of 9 carbons, appeared to give the higher K_D values.

4.4 De-Icing and Anti-Icing

There are two distinct snow and ice control strategies that make use of chemical freezing-point depressants: de-icing and anti-icing. These strategies differ in their fundamental objectives (Gilfillan, 2001; and Ketcham et al., 1996). De-icing involves the spreading of solid or spraying of liquid de-icing agents on already formed ice and collected snow. The principle of de-icing depends on the fact that pure water freezes at 0 °C while water with dissolved salt freezes at a temperature below 0 °C. When a de-icing compound is applied the ice melts creating a brine solution with a freezing point lower than that of pure water. Anti-icing on the other hand involves spraying of a liquid or spreading of a solid de-icing agent directly on the pavement or aircrafts before snow or ice are present, thus lowering the freezing point at which snow/ice bonds to the pavement or aircraft. The mechanism of anti-icing involves two processes: pre-wetting and direct application. The pre-wetting process is the addition of liquid chemicals to existing salt before they are spread on a surface. This helps reduce the ice build-up, and decreases the amount of salt and aggregate needed. The direct application process is the application of a liquid chemical to the bare surface before an expected snowfall.

Generally, de-icing and anti-icing practices are necessary in parts of the world where temperature fall below 0 °C (Figure 4.9), as snow and ice can cause traffic accidents and can paralyze movement of goods and people. Sometimes, snowstorms are able to close business and government offices thereby resulting in significant economic cost. It has been found that 90% of the costs can be avoided by the simple expedient of deicing of roads. Also traffic accidents can be reduced by 75% when icy and snowy roads are salted to restore bare movement (Hanneman, 1992).

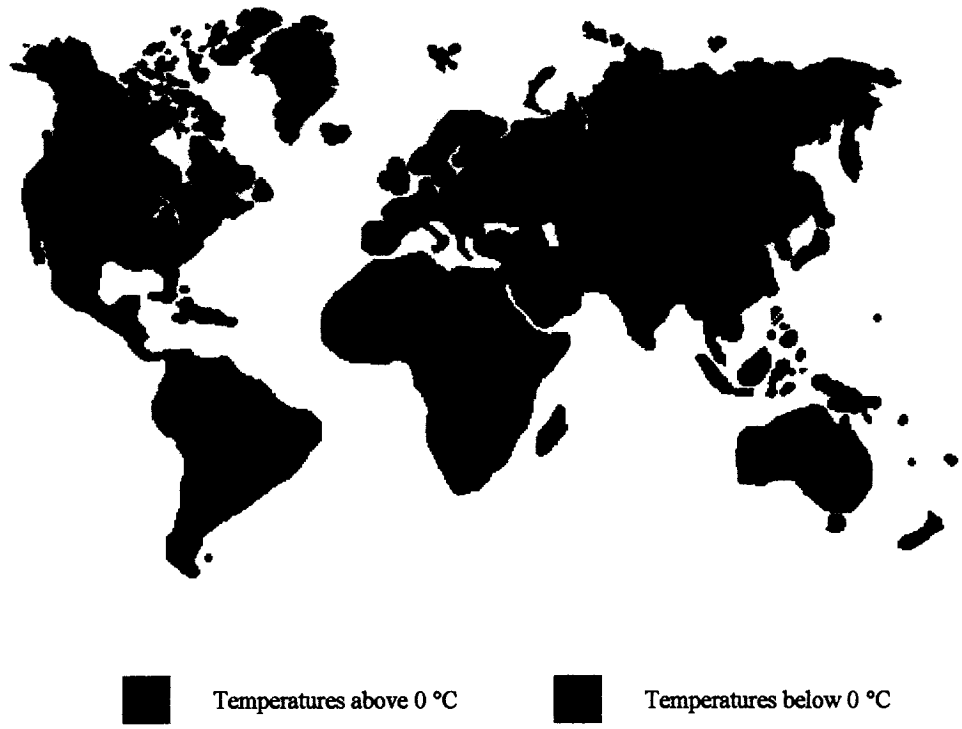


Figure 4.9. Cold regions of the world during the month of January.

4.4.1 Types of De-icing/Anti-icing Agents

The main function of the chemicals used as de-icing/anti-icing agents is to lower the freezing temperature of water and hence prevent forming a strong bond between the snow or ice and the pavement surface in winter (Iverson et al., 1997; and Bang and Johnston, 1998). Generally, airfield pavement deicers fall into two main categories: solid and liquid. The choice between these two categories depends on a wide range of local factors including: (a) the temperatures, (b) the types of precipitation encountered and (c) the preferences of airfield maintenance personnel. However, liquid deicers are sometimes used in conjunction with solid deicers as a wetting agent during periods of high wind to help the solid particles adhere to the pavement.

A number of different chemical freeze point depressants are available for anti-icing and de-icing practices, and can be divided into three main groups: inorganic salts, organic salts and organic compounds (Table 4.22). The composition and performance of such

Table 4.22. Classification,uses, advantages and disadvantages of some de-icing/anti-icing chemicals (Gales and VanderMeulen, 1992; Moran et al., 1992; Ketcham et al., 1996; McKenna et al., 1996; Iverson et al., 1997; Bang and Johnston, 1998; Pillard and DuFresne, 1999; Gilfillan, 2001; and Ritter, 2001).

Chemical	Formula	Usage	Advantages	Disadvantages
Inorganic Salts				
Sodium Chloride	NaCl	Runways/Roads	(a) It is very inexpensive, (b) It is easy to handle, store and apply, (c) It needs relatively low concentration to perform, (d) It is soluble in water, and (e) It can work as a chemical de-icer down to -12.22 °C.	(a) It is highly corrosive, and (b) It has highly damaging effect to the environment.
Calcium Chloride	$CaCl_2$	Runways	(a) It has a very low freezing point, (b) It attracts moisture (hygroscopic) and releases heat (exothermic) as it melts which enhances its melting ability, and (c) It offers number of benefits compared to sodium chloride.	(a) It is highly corrosive (more corrosive than sodium chloride), (b) It is not readily soluble, (c) It has larger adverse environmental effects than sodium chloride, and (d) It is expensive (eight times more expensive than sodium chloride)
Potassium Chloride	KCl		It is considered environmentally safe, and is a safe product to use around vegetation (since it is a common fertilizer)	(a) It is highly corrosive, (b) It is not as effective as many other de-icers and fails to work at temperatures below –9.44 °C, and (c) It needs to be used at high rate since its de-icing ability is slow

Table 4.22. Continued.

Chemical	Formula	Usage	Advantages	Disadvantages
Magnesium Chloride	$MgCl_2$	Runways	(a) It has a low freezing point, (b) It has high ice melting capacity (40% greater than $CaCl_2$), and (d) It attracts moisture and releases energy as it dissolve which enhances its melting ability	(a) It is highly corrosive, (b) It foams easily and (c) It is not readily soluble.
Organic Salts				
Urea	NH_2CONH_2	Runways/Roads	It is less (since it does not contain chloride) corrosive and safe for use on concrete containing rebar and around steel structures.	(a) It is likely to burn foliage, (b) It is expensive, (c) It affects aquatic life, (d) It is not as effective as many other de-icers and fails to work at temperatures below –9.44 °C, and (e) It needs to be used at high rate since its de-icing ability is slow.
Sodium Formate	HCOONa	Runways/Roads	Its performance is similar to that of sodium chloride in terms of de-icing speed, temperature range, and endurance of deicing effect.	(a) It is expensive (it costs 13 times as much as sodium chloride), and (b) it causes comparable environmental problems due to the existence of sodium ions

Table 4.22. Continued.

Chemical	Formula	Usage	Advantages	Disadvantages
Calcium Magnesium Acetate	$CaMg_2(CH_3COO)_6$	Runways	(a) It is considered environmentally safe, (b) It has good de-icing ability, (b) It adheres to snow and ice better than sodium chloride, preventing spraying of the chemical, and (d) It works at low temperatures (-9.44 °C) when sodium chloride and most de-icing salts lose their effectiveness	(a) It is very expensive (20 times as much as sodium chloride), and (b) needs to be used at high rate of application.
Organic Materials				
Ethylene and Propylene Glycols	$C_3H_8O_2/C_2H_6O_2$	Aircraft	(a) They are non-corrosive liquids, (b) They are very effective in snow and ice removal especially for aircrafts and (c) They can lower the freezing point of water to -50 °C.	(a) It is highly toxic to mammals, (b) It has high biological oxygen demand and (c) It is much more expensive than salt.
Methanol	CH_3OH	Roads	(a) It is non-corrosive and (b) It works much rapidly than sodium chloride and at a much lower temperature.	(a) It is more expensive than sodium chloride (5.5 times), (b) It is flammable, and (c) It evaporates after few hours from application requiring reapplication

Table 4.22. Continued.

Chemical	Formula	Usage	Advantages	Disadvantages
Other mixture				
Sodium Chloride/ Potassium Chloride Blend	-	Roads	(a) It offers the benefit of reducing sodium chloride exposure and the environmental damage caused from using it, and (b) It enhances the de-icing capability of potassium chloride.	It is not as effective as sodium chloride alone.
Sodium Chloride/Urea Blend	-	Roads	(a) It offers the benefit of reducing sodium chloride exposure and the environmental damage caused from using it, and (b) It enhances the de-icing capability of urea.	It is not as effective as sodium chloride alone.
Sodium Chloride/ Calcium Chloride Blend	-	Roads	(a) It is more effective than sodium chloride again, (b) Less expensive than using calcium chloride, (c) in its liquid form, it could be used as a prewet deicer for better de-icing process and cost effectiveness.	(a) It causes high environmental problems, (b) It is corrosive, and (c) proper mixing ratios cause handling problems
Brines	-	Roads	They are liquid and hence no delay in reaction time for melting and hence ice bonds dissolve faster.	It has the same environmental impacts as sodium chloride.
Sodium Acetate/ Sodium Formate Mixture	-	Roads	(a) It is relatively harmless to aquatic life and roadside vegetation and (b) At low concentrations in soil it is likely to act as a fertilizer.	It contains sodium and hence has much of the environmental impact of sodium chloride.

chemicals are usually described in terms of specific gravity, eutectic temperature (the temperature at which a given solution will freeze based on chemical concentration) and concentration. A deicing agent is known to perform more effectively if it has a lower molecular weight with greater solubility (Bang and Johnston, 1998). Moran et al. (1992) reported that the critical variables in defining the melting characteristics of each de-icing chemical are temperature and time. The order of some de-icing chemicals in terms of their initial melting rate (0-30 min) at all temperatures is: calcium chloride, sodium chloride, sodium chloride/urea, sodium chloride/potassium chloride, potassium chloride, and urea at all temperatures.

4.4.1.1 Inorganic Salts. Inorganic salts such as sodium, calcium, potassium and magnesium chlorides are commonly used for ice control practices. There are two types of brines available as liquid deicers: natural brines and commercial brines. Commercial brine is a mixture of sodium chloride or calcium chloride and water. Natural brine is a by-product of oil and gas production containing sodium, calcium, magnesium, or potassium chlorides. Generally, ice bond dissolves faster with liquid deicers due to a larger, more uniform surface contact area (Moran et al., 1992).

Sodium chloride could be produced by three processes: (a) rock salt is mined by conventional hard rock mining equipment and techniques, (b) solar salt is produced by the evaporation of sea water and may contain only a small amount of impurities, and (c) evaporated or solution or vacuum salt, a very pure form made by drying under vacuum the solution resulting from injection of water into deep underground deposits. Naturally occurring rock salt is the mineral halite, and usually contains between 1 percent and 4% impurities, mostly gypsum, shale, dolomite, and quartz (Ketcham et al., 1996). Sodium chloride works as a chemical deicer down to -12.22 °C by dissolving in the presence of water into separate sodium (Na^+) and chloride (Cl^-) ions. The resulting brine has a much lower freezing temperature than salt-free water and has the ability to break the bond between the ice and the pavement (Gales and VanderMeulen, 1992).

Calcium chloride has a very low freezing point, and is faster and more effective than sodium chloride, however, it is highly corrosive and not readily soluble. Calcium chloride attracts moisture and dissolves quickly to activate rock salt melting action. It also releases heat as it melts to speed salt melting rate (Gales and VanderMeulen, 1992; Moran et al., 1992; Gilfillan, 2001; and Ketcham et al., 1996). Calcium chloride offers a number of benefits, which make it more attractive than sodium chloride: (a) calcium ions poses no known health threat in drinking water, (b) it is a faster and more effective deicer, (c) it outperforms sodium chloride in the first 30 minutes after application, but if time lapses longer than 30 minutes the performance is almost the same, and (d) it melts up to eight times as much ice as does sodium chloride alone within the first 30 minutes at –6.67 °C following application. The most important advantage of calcium chloride over sodium chloride is its ability to retain its deicing ability at low temperatures (0 to -15 °C), while sodium chloride losses its effectiveness at temperatures lower than -9.44 °C (Gales and VanderMeulen, 1992; and Moran et al., 1992).

Mixtures of sodium chloride and calcium chloride are usually used for de-icing on very cold days (Iverson et al., 1997). The mixture is used as a prewet deicer for better de-icing process and cost effectiveness. However, at low concentrations of the mixture, the liquid could freeze in the application equipment, while at too high concentration mixtures, salts could form solid phase in the storage tank. Therefore, proper mixing ratios should be used in order to prevent such problems and reduce unnecessary use of expensive de-icing chemicals. The presence of calcium chloride in the mixture increases its effectiveness, and hence reducing the number or applications necessary during storms saving manpower, equipment and material costs.

Potassium chloride is a common fertilizer and a common icemelter that is often described as a safe product to use around vegetation. It has irregular shape, off white or white crystals. Generally, it is considered environmentally safe. However, it is not as effective as many other de-icers and fails to work at temperatures below –9.44 °C. It also needs to be used at a slightly higher rate of application. Potassium chloride is also highly corrosive. Sometimes sodium chloride is added to potassium chloride in order to increase

its deicing capability. Although this blend is not as effective as sodium chloride alone and its use may be considered as a method of reducing sodium chloride exposure (Moran et al., 1992).

Magnesium chloride has low freezing point and its ice melting capacity is about 40% greater than $CaCl_2$. It attracts and retains moisture and releases energy as it dissolves increasing the melting rate. However, it is highly corrosive, foams easily and is not readily soluble (Gilfillan, 2001; and Ketcham et al., 1996).

4.4.1.2 Organic Salts and Materials. In addition to inorganic salts there are number of organic salts available for runway and road de-icing practices such as: urea, calcium magnesium acetate, potassium acetate, sodium acetate, sodium formate, calcium magnesium propionate, sodium lactate, and magnesium lactate. Generally, using organic salts in de-icing and anti-icing practices have many advantages: (a) they have a low biological oxygen demand (BOD) and are less harmful to vegetation and aquatic and mammalian organisms, (b) the melting point of organic salt-water mixtures can be much lower than that of inorganic salts, and (c) organic salt compounds are biodegradable, therefore, cause less accumulation of salts in the environment (Ghaly et al., 2001; and Mahmoud and Ghaly, 2002).

Urea is a common de-icer and one of the preferred ice melters for airports. It is made from ammonia and carbon dioxide and is used primarily as a nitrogen-based fertilizer. Urea is less likely to burn foliage and it does not contain chlorides, therefore, it is less corrosive and safer for use on concrete containing rebar and around steel structures. However, it is not as effective as many other de-icers, it fails to work at temperatures below –9.44 °C and it is expensive and needs to be used at a slightly higher rate of application (Gales and VanderMeulen, 1992; and Moran et al., 1992). Moran et al. (1992) reported that sodium chloride/urea blend is sometimes used to increase the deicing capability of urea and at the same time to reduce sodium chloride exposure.

Sodium formate performance is similar to sodium chloride in terms of de-icing speed, temperature range, and endurance of deicing effect. It is noncorrosive in respect to steel and appears to cause no more damage to concrete than sodium chloride. However, it costs 13 times as much as sodium chloride and causes comparable environmental problems due to the existence of sodium ions (Gales and VanderMeulen, 1992). A mixture of equimolar Na-acetate and Na-formate showed an ability to lower the water freezing point, penetrate ice and lower the strength of bonding between the ice and the pavement (Bang and Johnston, 1998). This mixture proved to be an effective deicer and the chemical compounds in the mixture are readily oxidized biologically. Biodegradation of the mixture during transport is rapid enough to dilute the concentration of organic salts in typical highway runoffs. This alternative highway deicer is relatively harmless to aquatic life and roadside vegetation. Moreover, at lower concentrations in soil this mixture is likely to act as a fertilizer for plants.

Alkaline salts of carboxylic acids, particularly acetate, had been found to have good deicing ability (Ketcham et al., 1996; Bang and Johnston, 1998; and Jin et al., 2002). Recently calcium magnesium acetate (CMA) was identified as an environmentally favorable de-icer. However, the major disadvantage of CMA is its relatively high production costs, which is mainly attributed to the acetic acid. Schenck (1986) reported that the long-term ability of CMA to break the ice-to-roadway surface bond is nearly as effective as sodium chloride. However, researchers in Washington State found that CMA was slower acting by 20 minutes than sodium chloride, but works well when mixed with sand, which helps to adhere the chemical to the road surface until melting action began (Ernst et al., 1985). Generally, CMA adheres to snow and ice better than sodium chloride, preventing spraying of the chemical from traffic movement. It is most effective when applied at the beginning of a snowstorm. It works best at about -3.89 °C but has been shown to work in low temperatures below -9.44 °C where sodium chloride loses its effectiveness.

Methanol is commonly used as a roadway liquid deicer. It works much more rapidly as salt and to a much lower temperature (Gales and VanderMeulen, 1992). However, this

benefit lasts only for few hours as methanol evaporates and requires reapplication. Methanol is more expensive than salt (approximately 5.5 times). Generally, methanol was identified as potentially acceptable, non-corrosive alternative deicing chemicals (Dunn and Shenk, 1980).

Ethylene glycol (EG) and propylene glycol (PG) based de-icing/anti-icing fluids are commonly used for aircrafts and are generally very effective in snow and ice removal (Pillard and DuFresne, 1999). Ethylene glycol is a colorless, slightly viscous liquid with a freezing point of -13 °C, and can lower the freezing point of water to about -50 °C, depending on dilution. However, it has acute toxicity to mammals and has led to the death of animals that have licked up the antifreeze. When ingested, it depresses the central nerve system and can be fatal to humans even in small quantities (Ritter, 2001). Propylene glycol is also a colorless, viscous liquid at room temperature. It does not have a true freezing point, but becomes glasslike at -51 °C. It can lower the freezing point of water to about -60 °C and is essentially nontoxic. Generally, propylene glycol based fluids are preferable to ethylene glycol based fluids from the toxicological standpoint (Sills and Blakeslee, 1992; McKenna et al., 1996; and Ritter, 2001). Sills and Blakeslee (1992) reported that glycols are highly biodegradable in soil and aquatic systems and have high biological oxygen demand, which threaten oxygen dependent aquatic life in receiving waters. The 5-day biological oxygen demands at 20 °C are about 400,000-800,000 mg/L and 1000,000 mg/L for ethylene and propylene glycols, respectively.

4.4.2 Environmental and Health Impacts of De-icing Activities

The quantitative data on the amount of deicer application in airports shows that a large amount is used to insure flight safety (Sills and Blakeslee, 1992). The heavy use of deicing chemical causes serious environmental problems: (a) it destroys the roadside vegetation, (b) it degrades aquatic ecosystems, (c) it pollutes groundwater and domestic drinking water supplies, and (d) it damages highway infrastructure (Fritzsche, 1992). The problem is that these consequences are often cumulative and long lasting. Moreover, the biodegradation of some deicing chemicals such as glycols, urea and other organic constituents may result in objectionable odor due to the release of aldehydes. The

depletion of oxygen from the deicer-laden runoff in retention basins may also result in the anaerobic formation and release of highly odorific substances including mercaptans and hydrogen sulfide from glycol degradation.

4.4.2.1 Impact on Soil Quality. Sodium chloride has the most damaging effect among the known deicing agents to the soil quality. Moran et al. (1992) stated that the effect of sodium chloride on soil quality depends on: (a) the amount and frequency of application, (b) the amount of precipitation, (c) the length of time the ground is frozen, (d) soil porosity and chemistry, and (e) the closeness of the soil to the roadway. If sodium chloride is applied to wet surfaces it will dissolve, resulting in runoff to nearby drainage ways and soils (Gales and VanderMeulen, 1992). Plows and wind can also spray the salt more than 30 m from the road. Sodium and chloride ions are quite soluble and hence could be transported readily by water over and through roadside soils (Fritzsche, 1992). The horizontal movement is affected by the soil permeability and the season of the year. Less permeable or frozen soils favor runoff and, therefore, there is greater horizontal salt movement over these soils. Moran et al. (1992) reported that de-icing salts could be found as far down as 7 cm in soil, which means that deicing salts can be absorbed by frozen soils.

Like soil particles, chloride ions are negatively charged. The negative charge and small size make chloride ions very mobile through the soil column. Other than contributing to soil salinity, chloride has little impact on soil characteristics. In contrast, larger sodium ions are positively charged and are less mobile through soil. Negatively charged soil particles adsorb sodium cations from soil water by cation exchange. Under normal conditions, calcium and magnesium ions occupy most cation exchange sites. However, when present in unnaturally high concentrations, sodium can displace naturally occurring calcium and magnesium cations. Sodium's stripping calcium and magnesium is the most damaging road salt soil effect. Because of the soil's high sodium content, organic and inorganic particles normally present in pores through the soil profile are dispersed and flushed down the soil column. This decreases the soil's permeability and aeration, resulting in compact soil, which increases overland flow, surface runoff, and erosion. The

increased erosion in turn results in nutrient and heavy metal transport from the roadside to surface waters (Gales and VanderMeulen, 1992; and Fritzsche, 1992). Sodium formate could also damage soil structure in the same way since it contains sodium ions. The use of any chloride salts as well as natural brines has similar effects on soil quality as sodium chloride (Moran et al., 1992).

Using calcium magnesium acetate for ice control practices was found to have little or no negative effect on soils (Moran et al., 1992). Calcium magnesium acetate movement over and through roadside soil is influenced by the same physical factors that influence salt dispersion (Fritzsche, 1992). However, calcium magnesium acetate affects soil differently because: (a) the acetate anion is much less mobile in soil than the chloride anion, (b) acetate, the most abundant organic acid found in nature, is readily degraded by soil microorganisms (half-life of acetate is less than 2 days at 7 °C) and, therefore, not conserved, and (c) acetate degradation produces carbon dioxide, water, and bicarbonate and results in metal carbonate precipitation, which increase soil pH and decrease heavy metal solubility. However, metals mineralization in soil by co-precipitation with the oxides, hydroxides, and carbonates formed by acetate degradation are considered a benefit, especially where vehicle emissions have produced high metal levels in roadside soils. Calcium and magnesium ion mobility in soil is limited because calcium and magnesium cations have high affinities for the cation exchange sites on soil particles and calcium precipitates to form calcium carbonate (limestone). In contrast to sodium cations, calcium magnesium cations increase the soil permeability and aeration as a result of organic and inorganic flocculation by calcium and magnesium, which create pore space. The use of urea also has no damaging effect to soil quality. In contrast, urea is a form of nitrogen, which may actually help improving soil quality. However, it is likely to burn foliage.

4.4.2.2 Impact on Vegetation. The effect of deicers on grass, trees, shrubs, and plants has long been a factor in the decision of which product to use. Vegetation damage, primarily due to the application of de-icing/anti-icing chemicals, can occur through two mechanisms: (a) the direct salt accumulation on foliage and branches due to vehicle-

produced splash and spray of vegetation adjacent to the treated area particularly within about 30 m, and (b) the indirect distribution of salt-laden water from the root zone to the plant tissues (Gales and Vander Meulen, 1992; McFarland and O'Reilly, 1992; Moran et al., 1992; Fritzsche, 1992; and TRB, 1991). Generally, all de-icing chemicals are capable of harming grass, trees, shrubs, and plants, although many of chemical deicers are routinely used in agriculture. Calcium chloride has found use as a calcium source for a number of fruits, vegetables, and soils. Magnesium chloride, potassium chloride, sodium chloride and urea are added to soil to impart nutrients. However, when de-icing chemicals is used properly, there is little chance of vegetation damage. To avoid harm to vegetation: (a) deicers must be used sparingly, only the quantity enough to break the bond between the snow/ice and pavement could be used, (b) care must be taken to plow or shovel the deicer treated snow and ice away from sensitive vegetation and (c) when considering planting vegetation salt tolerant trees, shrubs, grass, and plants must be considered (Gales and Vander Meulen, 1992).

Salt particles deposition on foliage and twigs add weight to these parts, which may result in physical breakdown of the plant or burn and kill the affected parts. However, the majority of damage is most likely due to the intake of salt ions through roots. Generally, salt affects plants by: (a) increasing the osmotic pressure of the soil solution and thus cause interference with some plants' abilities to absorb water and significantly increase water stress during spring and summer, (b) accumulation of toxic concentrations of salt ions in plant tissues, and (c) unbalance the mineral composition of the soil thus alter the nutrition to the plant, and (d) sodium can raise the pH to levels harmful to plants within about 14 m of the area of treatment (Gales and VanderMeulen, 1992; Moran et al., 1992). Injury due to salt is typified first by inhibition of general growth, followed by specific injuries to foliage and limbs, and, in some cases plant death. The effect of salt in vegetation causes the following clear physiological symptoms: leaf scorch, late summer coloration, early fall defoliation, reduced shoot growth, and dying twigs and branches in the crown. Concentrations of either sodium or chloride are harmful to vegetation. However, chloride is thought to be more harmful than sodium as it tends to accumulate in

plant tissues over a long period of time, causing osmotic stress, which can lead to dehydration.

The degree of vegetation damage due to salt concentrations depends on many factors including: (a) extent of exposure and closeness to area of application, (b) species and age of the plant, (c) temperature, (d) light, (e) humidity, (f) wind, (g) soil water, (h) soil texture and drainage, and (i) precipitation. Exposure to high levels of salt causes stunted and sometimes abnormal growth. Vegetation exists directly on the side of the road are more susceptible to soil and water contamination due to the leaching of de-icing salts. Different species of grass, trees, and shrubs have different salt tolerances as shown in (Table 4.23). Most grasses, woody trees and shrubs are fairly adaptable to high concentrations of sodium and chloride while conifers are the least tolerant of deicing salt. Generally, younger plants are more susceptible to damage due to salt exposure. The effects of salt are compounded by higher temperatures, which cause increased dehydration through foliage, faster movement through foliage, faster movement of salt to plant, and increased salt absorption through roots. The exposure to direct light increases the rate of dehydration whereas high humidity lowers the rate of dehydration and, therefore, helps alleviate water stress. Exposure to wind may increase the rate of dehydration (more damage is always on the windward side of the trees than elsewhere). As salinity increases, the soil water available to plants decreases. The ability of soil to retain salt is partially determined by soil texture and drainage characteristics (coarse-textured soils are quickly leached of salt, and steep slopes may not absorb salt because of rapid runoff). Rainfall can flush salt deposits from foliage and dilute salt solutions in soil water causing less damage, but on the other hand, it can transport salts, via surface runoff, to roadside soils.

Potassium and calcium chlorides also cause vegetation damage as sodium chloride due to the presence of chloride ions. However, the percentage of chloride by weight in potassium chloride is less than in both sodium chloride and calcium chloride which make potassium chloride less harmful to the environment (Moran et al., 1992). Calcium magnesium acetate on the other hand is much less damaging to plants than sodium

Table 4.23. Salt tolerance of common trees, shrubs, and grasses (Moran et al., 1992).

Name	Tolerance
Deciduous trees:	
Thornless Honey Locust	High
Paper Birch	High
White Oak	High
White Ask	High
Cottonwood	High
White Birch	Moderate
Sugar Maple	Moderate
RedBud	Moderate
Green Ash	Low
Tulip Poplar	Low
Red Oak	Low
Red Elm	Low
American Elm	Low
Deciduous Shrubs:	
Privet	High
Honeysuckle	High
Forsythia	Moderate
Weigela	Moderate
Spirea	Low
Rose	Low
Evergreen Trees and Shrubs:	
Pfitzer Juniper	High
Creeping Juniper	High
Adam's Needle	High
Jack Pine	High
White Spruce	Moderate
White Fir	Moderate
Douglas Fir	Moderate
White Pine	Moderate
Norway Spruce	Moderate
Canadian Hemlock	Low
Grasses:	
Kentucky 31 Fescue	High
Red Fescue	Moderate
Bromegrass	Moderate
Kentucky Bluegrass	Low

chloride. Fritzsche (1992) reported that seventeen of eighteen species of deciduous and evergreen trees that showed damage by salt showed little or no effect after exposure to

calcium magnesium acetate. In the contrary, calcium magnesium acetate can enhance plant growth by improving soil permeability and providing needed calcium and magnesium. This may be valuable characteristic in areas where heavy salt use has resulted in soil compaction. Moran et al. (1992) reported that plants can withstand CMA concentrations of up to 2500 mg/L for root exposure and spraying CMA directly on plants has no topical damage either to leaves or to twigs. The author also reported that the application of urea has no negative impact on vegetation.

Bang and Johnston (1998) stated that a mixture of equimolar Na-acetate and Na-formate causes minimal toxicity to roadside vegetation such as herbaceous (e.g. sunflowers, beans, and lettuce) and woody plants (e.g. pine seedlings). On the other hand low concentrations of the de-icer (2g/kg soil) seem to work as a fertilizer promoting the yield of biomass. However, Gales and VanderMeulen (1992) reported that sodium formate may contributes to roadside vegetation burn. Pillard and DuFresne (1999) studied the toxicity of ethylene glycol and propylene glycol as well as two formulated glycol aircraft de-icing/anti-icing fluids to four plant species: lettuce (*Lactuca sativa*), perennial ryegrass (*Lolium perenne*), a green alga (*Selenastrum capricornutum*), and duckweed (*Lemna minor*). They found that both formulated glycol aircraft de-icing/anti-icing fluids and pure glycols were toxic to the tested species, however, there were substantial differences in the response to the tested materials.

4.4.2.3 Impact on Water Quality. The primary source of groundwater and surface water contamination by de-icing salts is highway runoff (Moran et al., 1992). Another source of water contamination by de-icing salts is poorly protective storage stockpiles. Hanneman, (1992) reported that the improper salt storages are responsible for about 80% of the environmental problems caused by road salts. However, TRB (1991) stated that in recent years greater attention to salt storage practices has reduced the incidence of storage-related contamination. Therefore, the major source of water salt contamination is the highway runoff. Salt also enters surface water indirectly when detained in stormwater drains and roadside catchments or by groundwater transport.

4.4.2.3.1 Ground Water: Much of the salt applied to roadways during ice control practices enters ground water due to the fact that salt has high solubility and low adsorption affinity (Fritzsche, 1992). Generally, highway salt enters ground water in several ways: (a) runoff from highways can flow from the pavement into unlined ditches and infiltrate surrounding soil, (b) road salt applied during snowstorms is plowed off the roadway and paved shoulder, (c) when the resulting snowbanks melt, the melt water, together with dissolved salt, can migrate through soil and move to the water table (Moran et al., 1992; and TRB, 1991). Reservoirs and wells located near salt-storage sites or highways receiving frequent and heavy salt treatments are the most susceptible to road salt infiltration (Menzies, 1992). However, this susceptibility will depend on many factors, including salting intensity, soil type, topography, climatic conditions, and water volume and dilution. The impact of salt use on water quality can be cumulative because the mean residence time of salt in groundwater may be greater than 1 year. Fritzsche (1992) stated that sodium and chloride in well water increases alkalinity and harness. Due to its mobility, the chloride ion is highly conserved; almost all the chloride applied to the road will pass through the soil and accumulate in groundwater and surface water. Chlorides are naturally present in ground water supplies at concentrations less than 10 mg/L (Jones et al., 1992). Moran et al. (1992) reported that among 20 wells analyzed for chloride concentrations, three wells, which were not in the vicinity of a road, had chloride concentrations of less than 1 mg/L while eight of the remaining showed significant chloride concentrations, ranging from 250 to a maximum of 1,8080 mg/L. Gales and VanderMeulen (1992) reported that numerous private wells along highways had been contaminated by road deicing salt. In some wells, the chloride concentration exceeded 250 mg/L and sodium concentration exceeded 75 mg/L. The authors also stated that five wells, which normally had chloride concentrations between 6 and 12 mg/L, were found to have chloride concentrations ranging from 18.5-1345 mg/L due to runoff from a stockpile entering a leaky storm sewer and contaminating groundwater. The Ontario Ministry of the Environment (MOE, 1979) reported elevated chloride concentrations in 53 wells among 134 private wells located along highways and other regional roads in Ottawa (MOE, 1979). The elevated chloride concentrations were attributed to road deicing salt applications. McArthur (1974) attributed elevated chloride and sodium concentrations in

selected Township of Georgina, Ontario, wells to de-icing salt applied to County Road 21. Joy (1979) studied the sodium concentrations in private well water supplies close to Metro Toronto. The study revealed that due to pollution of aquifer from road salting, more than 50% of the tested wells had sodium concentrations exceeding the recommended value (20 mg/L), 19% had concentrations greater than 100 mg/L, and 6% had levels above 250 mg/L.

Although Fritzsche (1992) reported that calcium and magnesium increases water harness, Bang and Johnston (1998) reported that the impact of calcium magnesium acetate on ground water is negligible. The same characteristics that limit calcium magnesium acetate movement in soil greatly reduce its ground water impact. Calcium and magnesium have high affinities for cation exchange sites on soil particles and are easily assimilated. Moreover, Moran et al. (1992) stated that calcium magnesium acetate is more hygroscopic than sodium chloride and hence is less susceptible to leaching.

4.4.2.3.2 Surface Water: Fresh water naturally contains low salt concentrations. Average chloride concentrations in fresh water lakes and rivers range from 0 – 100 mg/L, and most concentrations are lower than 20 mg/L (TRB, 1991). Anon (1980) reported that depending on sodium source and geography of the area, sodium concentrations in surface water throughout Canada normally vary from less than 1 mg/L to greater than 300 mg/L. Sodium concentrations in some small Ontario lakes was found to vary from 0.8 to 1.8 mg/L while mean concentrations reported from the Great Lakes range from 1.3 mg/L for lake superior to between 12 and 13 mg/L for Lake Ontario. Chloride ions are persistent and may not be removed from aqueous environments by chemical, physical or biological processes (Hochstein and Sills, 1989). Chloride persistent along with its mobility is considered the most characteristics that affect surface water quality due to de-icing practices.

During and after storms and during spring melts, highway runoff may contain high concentrations of sodium and chloride. Elevated levels of sodium and chloride from salting operations may cause lake density stratification, which upsets the natural balance

and prevents seasonal mixing resulting in dissolved oxygen (DO) depletion (Gales and VanderMeulen, 1992; Moran et al., 1992; and Fritzsche, 1992). Fritzsche (1992) reported that evidence gathered over 20 years indicates that 96% of the sodium increased in New York's Mohawk River Basin can be attributed to road salt. Chloride concentrations higher than 10,000mg/L have been reported in Ontario and Wisconsin during early spring thaws near large roadside snowbanks (TRB, 1991). Fritzsche (1992) stated that in urban areas, chloride concentrations attributable to road salting as high as 18,200 mg/L, have been measured in snowmelt runoff. However, the high concentrations of salt in runoff are quickly diluted depending on size and type of surface water when they enter larger streams. In Wisconsin, the chloride concentrations in spring runoff were found to be higher than 10,000 mg/L, while the maximum concentration in adjacent surface waters was only 45 mg/L (TRB, 1991). Smaller roadside streams and creeks are more likely to be affected. However, the magnitude of the impact depends on factors such as water flow, salting intensity, precipitation, type of highway drainage system, topography, and natural drainage patterns. Lakes and ponds, unlike small streams and creeks, are often recharged by a large and varied watershed (including ground water), which increases dilution and complicates identifying specific sources of chloride and sodium. In Maine chloride concentrations in small roadside ponds varied from less than 5 to more than 100 mg/L for reasons related to road salt usage. A survey of ten farm ponds in Maine in 1967 revealed chloride concentrations ranging from 1.4 to 221 mg/l (Moran et al., 1992). Ponds close to busy roadways doubled or tripled their chloride concentrations in winter months. Also in five lakes near Madison, Wisconsin chloride concentrations quadrupled between 1959 and 1962 due to salt exposure.

The only potential impact of calcium magnesium acetate to surface waters is related to the biodegradation of acetate ion. Microorganisms consume oxygen as they degrade organic substances like acetate. This oxygen consumption creates a biochemical oxygen demand (BOD) that can cause localized oxygen depletion. However, acetate will not create BOD problems because soil and soil microbes capture and degrade much of the applied acetate, about 70% of the applied acetate every 3 meters from the treated road surface, thus preventing its transport in runoff. Also the dilution of acetate allows for

acetate biodegradation without significant oxygen depletion. CMA causes localized oxygen depletion in surface water as a result of the acetate ion's biodegradation only at concentrations greater than 100 mg/L according to the following equation (Connolly et al., 1990; and Fritzsche, 1992):

$$\underset{\text{Acetate}}{CH_3COO^-} + 2O_2 + H^+ \longrightarrow 2CO_2 + 2H_2O \qquad (4.12)$$

Gales and VanderMeulen (1992) stated that methanol does not create oxygen demand problems when carried into streams as it is highly evaporated. The urea used in runway deicers degrades by hydrolysis to ammonia a process that depends on the soil enzyme urease, and then the ammonia is converted to nitrate by nitrifying soil microorganisms (Sills and Blakeslee, 1992; and Turnbull and Bevan, 1995). The process of urea degradation does not occur in low temperatures thus urea in deicer runoff would not be expected to significantly contribute to the BOD_5 but would contribute to the longer term NOD (nitrogen oxygen demand) of airport stormwater under near-freezing conditions. The urea is converted to ammonium, nitrite and nitrate according to the following equations:

$$\underset{\text{Urea}}{CO(NH_2)_2} + 2H_2O \xrightarrow{\text{Urease}} 2NH_4^+ + CO_3 \qquad (4.13)$$

$$2NH_4^+ + 3O_2 \xrightarrow{\text{Nitrosomosase}} 2NO_2^- + 4H^+ + 2H_2O \qquad (4.14)$$

$$2NO_2^- + O_2 \xrightarrow{\text{Nitrobacter}} 2NO_3^- \qquad (4.15)$$

4.4.2.4 <u>Impact on Aquatic Life</u>. Generally, the impacts of salt concentrations from highway deicing on water life are thought to be minor (TRB, 1991). Whereas high and sustained chloride concentrations in surface waters (more than 1000 mg/L) have been linked to growth changes in some plankton. The extreme chloride concentrations that are harmful to fish (400 - 12000 mg/L) are rarely generated by highway deicing. However, Gales and VanderMeulen (1992) reported that chloride concentration as low as 250 mg/L can increase the production of aquatic plants (including algae) and may be part of a

chemically induced reduction of diversity in aquatic plants. Salt has a high density, which makes it sinks to the bottom causing accumulation of chloride levels in the lower depths (density stratification) of lakes (Moran et al., 1992). The change in water density causes oxygen depletion in the bottom of the lake, which can be detrimental to plant and fish life. Prolonged periods of reduced oxygen can result in increased nutrient loading at the stream or lack bottom, which in turn increase spring and summer algal growth that may further deplete dissolved oxygen. Generally, few incidents of this extreme effect have been reported. Salt additives on the other hand, such as sodium ferrocyanide and sodium hexametaphosphate endanger water biota (Moran et al., 1992). When sodium ferrocyanide decomposes, cyanide ions are released into the environment, they cause fish poisoning. The decomposition of sodium hexametaphosphate releases phosphorus, which causes damage to the aquatic life.

In case of small streams that have low flow rates, the effect of road salt is often encountered. Salt in highway runoff is responsible for the increase of water acidity as the chloride accumulates (Bang and Johnston, 1998). If the use of chloride salts in de-icing practices continued, a population shift towards less desirable salt-tolerant aquatic species may be encountered in some lakes (Hochstein and Sills, 1989). Demers (1992) examined the effects of elevated chloride levels on the aquatic macroinvertebrates inhabiting four small Adirondack stream and concluded that in small streams with low flow rates, significant increases in chloride concentration as a result of de-icing salt could change the composition of benthic macroinvertebrate communities. A less diverse community with reduced evenness appears to be a potential result of increased salt concentration.

Very little chronic toxicity data exist for ethylene and propylene glycols or glycol formulations to aquatic species. Based on available data, the relative acute toxicities of ethylene glycol (EG) and propylene glycol (PG) are very similar. LC_{50}s (the concentration of a chemical which kills 50% of a sample population) values for freshwater and marine fish invertebrates and algae range from 7,900 - 111,000 mg/L for EG and from 10,000 - 79,700 mg/L for PG range. The acute toxicities of commercial ethylene glycol and propylene glycol aircraft de-icer/anti-icer formulations are also

similar across groups of organisms encompassing producer (algae) and consumer (daphnids, minnows) species. Sills and Blakeslee (1992) stated that a fish kill linked to ethylene glycol was reported downstream from the stormwater discharge at Lambert Field, St. Louis, Missouri. In order to protect aquatic life from ethylene glycol and propylene glycol, chronic exposure conditions were calculated as 68 mg/L and 191 mg/L, respectively, based on direct toxicity only and not on secondary toxicity due to oxygen depletion.

Calcium magnesium acetate has little or no effect on phytoplankton growth (Fritzsche, 1992). However, eutrophic lakes may experience a slight decrease in algae concentration with calcium magnesium acetate use, where the added acetate allows bacteria to out compete algae for nutrients. Moran et al. (1992) recommended that CMA not to be used in areas where receiving waters would have less than a 100:1 dilution ratio since CMA decomposes in water causing depletion to the dissolved oxygen content of the aquatic system. The equimolar mixture of sodium acetate and sodium formate also has low toxicity to aquatic animals (Bang and Johnston, 1998). The toxicity level of such mixture to rainbow trout is relatively low even under high concentrations of the deicer. The measured LC_{50} of the mixture was found to be comparable to that of CMA (16.1 g/L vs. 17.5 g/L).

Urea has relatively low toxicity to aquatic life, but the ammonia formatted from urea has relatively high acute toxicity, with reported LC_{50} values generally in the range of 1-10 mg/L (Sills and Blakeslee, 1992; and Turnbull and Bevan, 1995). The Canadian government has proposed a maximum allowable limit for ammonia-nitrogen in storm water discharges at 1.0 mg/L. Nitrate, on the other hand, has nutrient properties, which stimulates eutrophication. Two fish kill occurrences attributed to discharges of urea from airports and subsequent ammonia formation has been reported in England. Gales and VanderMeulen (1992) reported that urea could promote unwanted plant growth and algal blooms in water bodies, which disturbs aquatic life.

4.4.2.5 Impact on Human. Both sodium and chloride ions in salt can degrade drinking water (Gales and VanderMeulen, 1992; and Fritzsche, 1992). Increasing sodium concentrations in drinking-water reservoirs has public health implications, such as exacerbation of hypertension, congestive heart failure, renal diseases, cirrhosis of the liver, and toxemias of pregnancy. Public health officials have proven that reduced sodium intake reduces blood pressure in persons with hypertension. While a national standard for sodium in drinking water does not exist, several recent studies indicate that sodium levels between 100 and 125 mg/L adversely impact public health. The American Heart Association (AHA) recommends, and many communities have instituted, a 20 mg/L limit for sodium in drinking water (Gales and VanderMeulen, 1992; and Fritzsche, 1992). Chloride ions present no known hazard to human health in lower concentrations. However, most people can taste chloride in drinking water at about 200 mg/l. Moran et al. (1992) and Jones et al. (1992) reported that the U.S. Public Health Service has set the recommended chloride concentration level in portable water at 25 mg/L and maximum safe level at 250 mg/L.

Use and misuse of ethylene glycol (EG) in commercial products are responsible for a number of human deaths yearly from acute exposures. Human case studies reporting death caused by exposure to EG have not been found in the scientific literature. However, the acute oral toxicity of EG in humans is well documented, and follows a defined series of stages including central nervous system (CNS) dysfunction with severe metabolic acidosis, cardiopulmonary failure, and acute renal failure. While the stages of acute EG poisoning in humans have been well recorded, the stages may overlap and death can result at any stage. The lowest reported minimum lethal dose (MLD) of EG is 1.57 g/kg, and accidental and intentional deaths have been attributed to ingestion of EG. No acute inhalation exposure studies to EG in humans have been reported, although EG caused upper respiratory irritation, slight headaches, and lower backaches in volunteers exposed to as much as 140 mg/m^3 (37 mg/kg-day) for 30 days. Dermal contact with EG produced mild to no skin irritation, and minimal sensitization potential. Sills and Blakeslee (1992) reported that an ethylene glycol chronic criterion to protect humans was calculated as 70 mg/L (human drinking water resources).

Acute propylene glycol (PG) toxicity, in contrast to that of EG, is not well defined but includes CNS effects (e.g., CNS depression) and lactic acidosis from high doses. No deaths from oral, dermal, or inhalation exposure to PG have been reported. Acute oral exposure to high-dose PG has resulted in hematological effects. No acute toxicities have been reported from the inhalation of PG vapors or aerosols. Mild skin irritation has been reported from dermal contact with PG in various patch tests, and the ability of PG to cause allergic sensitization in humans remains debatable. Sills and Blakeslee (1992) reported that propylene glycol surface water criteria to protect humans under chronic exposure were calculated as 730 mg/L (humans, drinking water sources). Generally, propylene glycol based de-icers are less toxic than ethylene glycol based de-icers to mammals (Pillard and DuFresne, 1999).

Nitrate formatted from urea presents concerns for contamination of drinking water supplies (Sills and Blakeslee, 1992). Excessive nitrate exposure by humans impairs the ability of blood hemoglobin to transport oxygen (methemoglobinemia) resulting in "blue baby syndrome" in infants. The federal drinking water standard to protect from this effect is 10 mg/L as nitrate-nitrogen, or 10 mg/L total nitrogen from nitrate and nitrite. Methanol as a deicer can affect the ambient air quality with its vapor, which can be toxic when consumed or encountered in an enclosed space (Gales and VanderMeulen, 1992). Also sodium formate can react with car exhaust to form deadly formic acid.

4.4.2.6 Corrosion. Corrosion is most often caused by oxygen combining with steel and other metals when moisture is present. The corrosion process is accelerated when the moisture includes salt (Gales and VanderMeulen, 1992; and Menzies, 1992). The chloride ions in salt destroy natural protective films in metal surfaces and increase the conductivity of water, which both induces and accelerates electrochemical corrosion. De-icing salts have been cited as a major cause of vehicle corrosion or rust. Salt is especially damaging to bridge decks because the chloride ions from salt, along with moisture, can migrate through fine cracks and pores in the deck surface, causing the rusting of reinforcing steel bars embedded in the concrete. As these bars corrode, the rust product expands and generates pressure on the surrounding concrete, which causes cracking and

spalling of the deck surface. In turn, these damaged areas provide free access to additional salt, which accelerates the destructive processes already caused by moisture, freeze-thaw, vibrations, and impact loadings from traffic. While this damage seldom affects the structural integrity of the deck, it causes extensive delaminations and plot-holing of the deck surface, which can seriously degrade ride quality. In addition to decks, other components of bridges that are potentially vulnerable to salt damage include concrete girders and piers and exposed steel framing, bearings, and joint devices. Leaky decks and salt splash and spray often cause salt damage to these other bridge components from adjacent roadways.

Parking structures can also be contaminated and damaged by salt dropped from parked cars. The damage is similar to that of bridge decks. The rust product on the steel expands and exerts pressure, causing surrounding concrete to crack and crumble, forming delamination and potholes on the concrete surface.

Sodium formate is non-corrosive with respect to steel due to the absence of chloride ions and appears to cause no more damage to concrete than salt (Gales and VanderMeulen, 1992). Uric acid and hydrofluoric acid are more effective for de-icing practices but are far more corrosive than other deicing materials. Marynowski et al. (1985) reported that the Federal Highway Administration (FHWA) identified calcium magnesium acetate (CMA) and methanol as potentially acceptable, noncorrosive alternative de-icing chemicals. In order to minimize corrosion, Iverson et al. (1997) stated that corrosion-inhibitor-added deicing salts or salt substitutes could be used. However, it has been found that corrosion-inhibitor-added deicing salts and salt substitutes do not have enough low freezing temperatures to be effective to control ice under low temperatures.

4.4.3 Factors Affecting Choosing Deicers

The selection of particular product for the deicing and anti-icing of runways and other paved areas has traditionally been a function of local meteorological conditions, available equipment and personnel cost. Add to these factors that with increasing regulatory emphasis on the control of storm water contamination, airports must now also incorporate

environmental factors into the selection process. There are several de-icing chemicals in the market, which have different properties and can be utilized for specific winter maintenance demand (Gilfillan, 2001). The most important properties are eutectic temperature, effective temperature, concentration value, solubility and corrosiveness. Eutectic temperature is especially important when looking at cost efficiency and maximum volume utilization. Effective temperature measures the lowest temperature in which the cost of the application is justified by the results obtained. Concentration value is optimized when the lowest concentration percentage can be used to return the highest result measurement. Solubility and corrosiveness probably have the greatest and most immediate effect on roadside ecosystems.

4.4.4 Economics and Practical Consideration of Using Deicers

Icy roads can increase fuel consumption by as much as 50 percent, as drivers tend to spin their car wheels harder to drive the vehicle. Snow and ice also can delay emergency services causing higher death rates from medical conditions and higher fire and criminal losses. Also vehicle accidents are highest for snow-packed roads. Each year a huge amount of money is spent on highway snow and ice control programs. The most important element of these programs is chemical de-icing, which represents about one-third of winter maintenance expenditure.

Sodium chloride is the most popular deicer because it is an effective snow and ice control agent, inexpensive to purchase (US $30-40/ton) in comparison to all other deicing chemicals, readily available, and easy to handle, store, and apply (Demers, 1992; Gales and VanderMeulen, 1992; Menzies, 1992; Moran et al., 1992; and Yang et al., 1992). Large amount of sodium chloride is applied each winter in order to control ice and snow. Over the years, however, evidence has grown linking the widespread use of sodium chloride with many negative side effects, including damage to motor vehicles, infrastructure, and the natural environment resulting in indirect costs. Ingraham (1988) reported that the total cost of corrosion repair in the United States each year is about US $200 billion. Monetary losses to vehicles, bridges, and other highway structures in Michigan alone have been estimated in the millions of dollars annually (Gales and

VanderMeulen, 1992). Hudson (1987) reported that although a ton of road salt costs much less than any other de-icing chemicals, it causes more than US $1400 in damage. If the cost of the environmental damages (maintenance of roadside vegetation, removal of dead trees and shrubs, replanting, decrease in property values, corrosive damage to structures and vehicles) caused by using salt has been taken into consideration it will be found that the economic argument for using sodium chloride is weakened (Marynowski et al., 1985; and Moran et al., 1992).

Calcium chloride is much more expensive than sodium chloride (US $ 200/ton). Since calcium chloride is not significantly better as a deicer, except at very low temperatures, and also still poses the same environmental risks, the use of calcium chloride is not justified. Potassium chloride is not significantly more expensive than sodium chloride but it is not a very effective deicer. Potassium chloride/ sodium chloride blends would be reasonably inexpensive and are moderately effective, but negative impact to the environment still exists. Urea is the most expensive deicer. Urea mixed with sodium chloride would still be more expensive than sodium chloride, but the lessened adverse effects to the environment may justify the cost. Natural brines, an industrial by-product, would be inexpensive but they require very different storage facilities and equipment requirements than solid salts.

At the present time, CMA is 20 times more costly than sodium chloride, it costs about US $600-700/ton. It is too expensive for wide spread use, even though some studies have shown that all of its material cost can be offset by the savings in infrastructure replacement costs. Using CMA as a de-icer will be widely accepted if its production costs can be reduced to US $300-400/ton or less (Ministry of Transportation of Ontario, 1989; and Yang et al., 1992). The high cost of (CMA) is mainly attributed to the acetic acid used to produce CMA since it comprises about 70% of the total production cost (Dionysiou et al., 2000). Acetic acid for industrial purposes is obtained principally from natural gas; however, many attempts have been made to ferment biomass as an alternate rout to producing acetic acid or CMA. It has been found that CMA production from cheese whey could be reasonable since the CMA production costs could be easily

reduced to US $0.20 – 0.33/kg or less by using zero-cost raw material such as whey (Yang et al., 1992). Dionysiou et al. (2000) proposed an extractive crystallization process for the production of low cost efficient CMA de-icer using acetic acid produced by a biochemical process. They claimed that their process leads to a lower cost than the commercial processes and the CMA produced has better applicability than the currently produced commercial CMA. Jin et al. (2001) also recommended a new process for the production of calcium magnesium acetate de-icer. The new process involves the use of organic waste (rice hull, Welsh onion (onion waste after extraction of valuable matters), carrots, and beef suet) for the production of low cost acetic acid by wet oxidation and the conversion of acetic acid to calcium magnesium acetate by mixing it with Ca/Mg source (oyster shells and industrial Mg wastes).

4.5 Chitin and Chitosan

The terms chitin and chitosan do not refer to specific compounds; however, they refer to two ranges of copolymers containing two monomers: anhydro-N-acetyl-D-glucosamine and anhydro-D-glucosamine (Roberts, 1995). Anhydro-N-acetyl-D-glucosamine is predominant in chitin while anhydro-D-glucosamine is predominant in chitosan. The degree of acetylation (DA) is the critical parameter for defining chitin and chitosan. Generally, the term chitosan is used when the polymer has a DA less than 60%.

4.5.1 Chitin Sources

Chitin could be found in a variety of species in both the animal and plant kingdoms. It is present in amounts varying from trace quantities up to about 40% of the body weight of the organism. Chitin could be found in the exoskeletons, gastro-intestinal linings and the respiratory tracts of arthropods, the largest phylum of the animal kingdom, which includes insects and arachnids (spiders, scorpions, etc.). It is also present in crustacea (shrimp, crab, lobster, etc.), cephalopods (squid, cuttlefish, etc.), gastropods (mollusks) and in some annelids (segmented worms) but it does not occur in vertebrates. Chitin is also found in the cell wall of most fungi and the extracellular fibers of algae (diatoms). Generally, chitin is seldom found in a pure state except in the case of diatom. It is usually

found as a part of a very complex system with other sugars, proteins, minerals and polyphenols (Kong, 1975; Gagné and Simpson, 1993; Abdullah, 1995; and Subasinghe, 1995).

Insect chitin is of intuitive importance because of its lack of calcium carbonate. It is present in the exoskeleton, the lining of the fore and hindguts and the reproductive ducts (Retnakaran, 1995). The amounts of chitin in insects vary with species; the species that has softer cuticle contain more chitin than the species that has hard cuticles. In general, a few species of insects have more than 10% of their body weight chitin. The low percentage of chitin of the insect body weight tends to make insects unattractive source of chitin, add to that the fact that it is difficult to rear commercial quantities of insects (Kong, 1975).

Chitin is present in some body parts of a number of mollusks such as clams, scallops, oysters, squids and cuttlefish. However, the chitin content of the calciferous shell of mollusks is also low compared to that of crustacean waste. Fungi synthesize chitin in their bodies. The fungal chitin could be found in the cell wall. The chitin fungal source is always less than 20% based on the dry fungal weight. Fungi seem to be the next likely potential source of chitin (Kong, 1975). The main advantages of fungal chitin are its consistent composition and its availability all over the year. Moreover the fungal chitin does not require demineralization step (Roberts, 1992).

The crustacean waste is the most important chitin source for commercial use due to its high chitin content and ready availability (Gagné and Simpson, 1993; Abdullah, 1995; and Subasinghe, 1995). The typical amount of chitin extractable from crustacean waste is 30% of the waste. It is estimated that shrimp and prawn waste contains from 10 – 55% chitin based on the dry basis (Subasinghe, 1995). In addition to chitin crustacean wastes are also considered a rich source of protein, pigments (e.g. astaxanthin), and flavor compounds. However, chitin is the most valuable component and forms a significant fraction of the waste products of the seafood industry (Zakaria et al., 1998; Healy et al., 2003).

4.5.2 Chitin Production

Chitin and chitosan are versatile environmentally friendly modern materials. The estimated annual production of chitin is 10 to 100 billion metric tonnes (Gooday, 1991; and Rattanakit et al., 2002). Japan and USA are the main chitin and chitosan producers in the world. India, Italy, and Poland come next (Brzeski, 1987). In year 1999 the total production of chitin containing aquatic organisms were 1,608,042, 1,138,146, 471,393, 300,754 and 24,323 metric tonnes in Japan, USA, India, Italy and Poland, respectively (FAO, 2001).

4.5.3 Chitin and Chitosan Properties

Chitin is a naturally occurring high molecular weight linear homopolysaccharide composed of N-acetyl-D-glucosamine residues in ß(1-4) linkage. It is a biodegradable and biocompatible natural polymer. The chitin chain length varies among and between species. In crab, there are between 5000 and 8000 residues in the chitin polymer while in yeast the polymer is only 100 residues long. It is probably the second most abundant polysaccharide in nature, next to cellulose particularly in marine invertebrates, insects, fungi and algae (van Ornum, 1992; Kong, 1975; McGahren et al., 1984; Roberts, 1992; Gagné and Simpson, 1993; Vincendon, 1994; Kim and Lee, 1995; Retnakaran, 1995; Place, 1996; and Rhazi et al., 2000). This abundance mainly attributed to the widespread occurrences of animals and microbes in which chitin forms skeletal elements, as well as to the intrinsically stable chemical structure of chitin.

Chitin is a white, amorphous solid with a flaky or fibrous nature. It forms extended fibers, indigestible by vertebrate animals, similar to those of cellulose. It differs from cellulose only in the replacement of the hydroxyl group at C-2 with an acetylated amino group (Kong, 1975; McGahren et al., 1984; and Roberts, 1992). Chitin exists in three forms, α-chitin, ß-chitin, and γ-chitin. α-chitin is prevalent in the fibrils of arthropods and crustaceans where extreme hardness is required and characterized by an antiparallel arrangement of adjacent chains. ß-chitins prevalent in marine diatoms and the chains in this form are parallel. γ-chitin has two of three chains parallel and the third antiparrallel, a form that has been detected in several marine organisms (Place, 1996). ß-chitin, and γ-

chitin are found where flexibility and toughness are required. Generally, most chitin forms are tough, highly hydrophobic, insoluble in most ordinary solvents, and relatively inert to biodegradation. Rhazi et al. (2000) stated that α-chitin has strong intermolecular hydrogen bonding compared to ß-chitin. Therefore, ß-chitin has higher solubility and swelling than α-chitin because of the weaker intermolecular hydrogen bonding.

Chitin is insoluble in water, dilute acids, dilute and concentrated alkalis and most organic solvents because of the rigid crystalline structure formed through hydrogen bonds originated from an amide linkage of N-acetylglucosamine residue (van Ornum, 1992; Vincendon, 1994; Kim and Lee, 1995; and Tokura and Nishi, 1995). The chains associated together by very strong hydrogen bonding between the >N-H groups of one chain and the >C=O group of the adjacent chain. Concentrated aqueous acids (hydrochloric, sulfuric and phosphoric) and anhydrous formic acids dissolve the polymer but hydrolyze it at the same time (Kong, 1975; Kawamura, 1995; and Place, 1996). Vincendon (1994) reported that phosphoric acid is a useful solvent for chitin at room temperature that produces highly viscous solution. Chitin can easily be regenerated with a good yield (80%) from phosphoric acid solutions. Shirai et al. (1995) reported that calcium chloride dehydrate saturated methanol was found to be a good solvent system for chitin.

Chitosan is the deacetylated form of chitin (Gagné and Simpson, 1993). It is a poly[β-(1→ 4)-2-amino-2-deoxy-D-glucopyranose]. It could be prepared from chitin by extreme treatment with alkali (35 – 55% base). It is a white odorless substance. The hydrogen bond of chitosan is not so tight enough to form the rigid crystalline structure like that of chitin, and therefore, it has good solubility and reactivity (Tokura and Nishi, 1995). Chitosan is soluble in dilute organic acids and in hydrochloric acid and nitric acid in the range of 0.15-1.10%, but insoluble in organic solvents and at pHs above 6.5. It is also not soluble at any concentrations of sulphuric acid and is marginally soluble in ortho-phosphoric acid at 0.5% concentration (Kong, 1975; and van Ornum, 1992). The chemical structures of cellulose, chitin and chitosan are shown in Figure 4.10. The only structural difference between the three materials is at carbon 2.

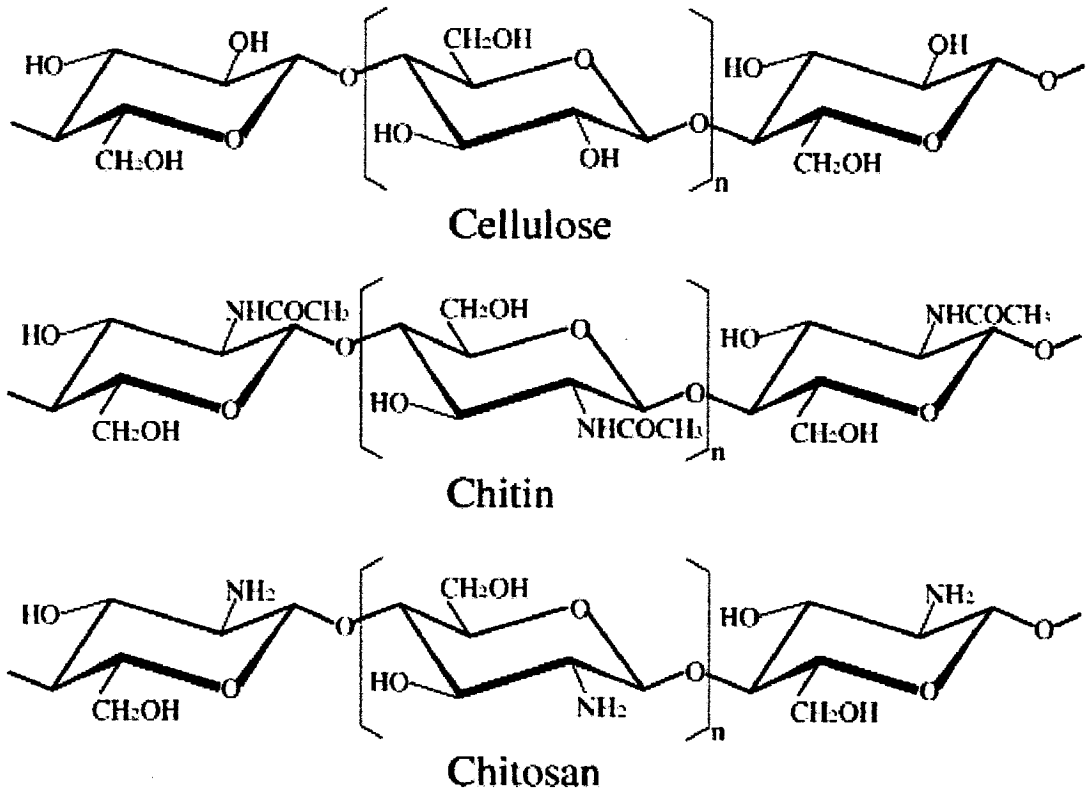


Figure 4.10. Structures of cellulose, chitin and chitosan (Shahidi and Synowiecki, 1992).

4.5.4 Chitin and Chitosan Applications

Chitin and chitin derivatives have been used in virtually every significant segment of the economy. The number, importance and variety of industrial uses are growing rapidly. Brzeski (1987) reported that the potential applications of chitin, chitosan and their derivatives have been estimated at over 200. However, there is no large-scale industrial use of chitin due to its insolubility. While chitosan, the deacetylated derivative of chitin, which could be found infrequently in nature, is more tractable material than chitin since it is readily soluble in dilute acid solutions (McGahren et al., 1984; and Kim and Lee, 1995).

Chitin and its derivative chitosan could be used as a permeability control agent for enhancing oil recovery from depleted wells, a bonding agent, a paper-sizing agent, a chelating agent for metal ions, a chromatographic support, an encapsulation agent for pharmaceuticals, a thickner of cosmetic, a natural flocculant for wastewater treatment, in

controlled release of pesticides and in coating of lithographic paper,. It could also be used for animal feed, lumber improvements, seed treatment in agriculture, and the treatment of drinking water and water used in food processing industry. Moreover it could be converted into various highly valuable materials used for both medical and health care purposes such as lenses, surgical suture, and cosmetics (Kong, 1975; McGahren et al., 1984; Brzeski, 1987; van Ornum, 1992; Gagné and Simpson, 1993; Bustos and Healy, 1994; Abdullah, 1995; Hirano, 1995; Sophanodora and Hutadilok, 1995; Yarmo et al., 1995; Zakaria et al., 1998; and Healy et al., 2003).

In the area of water treatment, a study by Kawamura (1995) suggested that natural biopolymers such as chitosan appeared to work over a wide range of turbid water and could be viable substitutes for synthetic products as coagulants. The study showed that chitosan can be as effective as or more effective than commercially available synthetic organic polymers as a coagulant or filtration aid. Another study by Zakaria et al. (1995) investigated the potential of chitosan as a chemical agent in the treatment of raw water, domestic effluent and palm oil mill effluent. They reported that chitosan partially coagulates and flocculates suspended and dissolved substances in raw water, domestic effluent and palm oil mill effluent. They also found that the turbidity of the raw water and wastewaters were significantly reduced by chitosan treatment, and concluded that chitosan concentration of 30-40 ppm, combined with alkaline pH was found to be optimum for raw water treatment, while a slightly higher chitosan concentration and alkaline pH conditions are optimum for domestic effluent treatment. Jais et al. (1995) reported that the optimum treatment condition for effluent from rubber coagulation industry using chitosan is chitosan concentration of 20 ppm at pH of 4.8-6.0. They concluded that the treatment with chitosan reduced the turbidity, the color, chemical oxygen demand (COD), and biological oxygen demand (BOD). Samah et al. (1995) studied the ability of chitosan for peatland water, the natural colored water, which is derived from peat soils, treatment. The results showed that chitosan treatment performed better than commercial coagulant-flocculants and also did not require other chemicals and pH adjustment. They concluded that chitosan is a potential biopolymer capable of treating peatwater into drinking water.

Chitin and chitosan have a great potential in biomedical applications and as animal feed additives (Hirano, 1995). In oral administration, chitin and chitosan and their oligosaccharides are almost nontoxic and digestible. Chitosan suppresses the serum cholesterol level and hypertension, while chitin makes better bacterial flora and stimulates lactose digestion in animal intestines. In intravenous administration, they induce lyozyme in animal serum and tissues, and inhibit tumor cell growth. In implant administration into animal tissues, they are digested and accelerate animal wound healing and hemostatic activity. Moreover, chitosan inhibits the growth of bacteria and fungal pathogens. However, Yarmo et al. (1995) reported that the addition of protein chitin to chicken feed in the range between 0.25 – 0.75% w/w had no significant effect on chickens' weight, number of eggs laid, mortality rate, and feed intake. They suggested that adding the protein chitin in a percentage greater than 1% w/w might have a significant effect.

4.5.4 Preparation of Chitin from Shellfish Waste

Chitin and its derivative chitosan could be prepared from all crustacean shells, which is considered the richest source of chitin for commercial use (Kim and Lee, 1995; Luyen, 1995; and Sophanodora and Hutadilok, 1995). Chitin is commercially extracted from shrimp shells either by chemical or biotechnological approaches and chitosan is the polymer obtained from N-deacetylation of chitin. Chitin present in the crustacean waste is associated with proteins, inorganic material (mainly calcium carbonate), and lipids including pigments. Therefore, chitin purification passes through four steps: (a) the grinding of the shells to a uniform particle size, (b) protein separation (deproteinization), (c) mineral removal (demineralization), and (d) elimination of pigments and lipids (Figure 4.11). A fifth step (deacetylation) is to be added in order to obtain chitosan.

The traditional method (chemical approach) of chitin purification is known for years. This method includes mechanical grinding and then alternative acid and alkali (e.g., HCl and NaOH) treatment for calcium carbonate and proteins removal, respectively, leaving a residue of relatively pure chitin (Muzzarelli, 1977; Kong, 1975; Roberts, 1992; Gagné and Simpson, 1993; Bustos and Healy, 1994; Hall et al., 1994; and Zakaria et al., 1998).

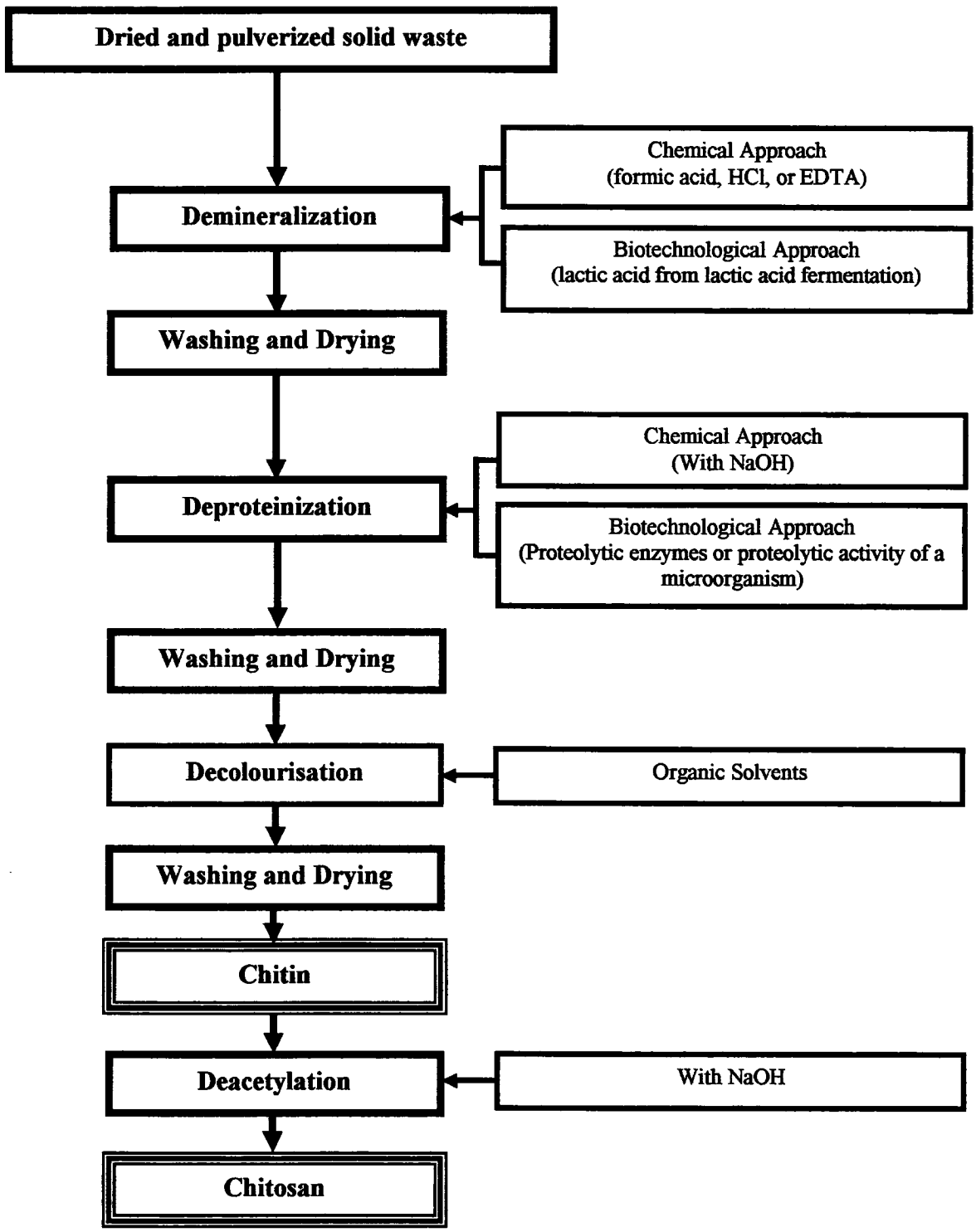


Figure 4.11. Preparation of chitin and chitosan from shellfish waste (Ghanem et al., 2003).

However, there are several disadvantages of such method including: (a) the chemicals used are corrosive chemicals and could cause a partial deacetylation of chitin and hydrolysis of the polymer, which result in lower molecular weight end product with variable and inconsistent physiochemical properties, (b) these chemicals also cause disposal problems and detoxification and neutralization of the effluent wastewater may be necessary, (c) the presence of sodium hydroxide in the deproteinization liquid reduces its value and other useful compounds such as proteins, carotenoids and enzymes are discarded and (d) it is a fairly expensive method (transportation and handling, chemical treatment and generation of potentially polluting wastes are the most important factors that increase the process cost) (Kong, 1975; Brine and Austin, 1981; Shimahara and Takiguchi, 1988; Roberts, 1992; Gagné and Simpson, 1993; Bustos and Healy 1994a,b; Hall et al., 1994; Joensen and Villadsen, 1994; Zakaria et al., 1998; and Healy et al., 2003; and Beaney et al., 2005). Therefore, in order to increase chitin use and reduce emission of pollutants, a less expensive environmentally friendly method for chitin extraction is required.

The deproteinization step plays an important role in the chitin extraction process, since the protein content of the final product should be below 5% in order to optimize the quality and achieve higher market price of the product. Moreover, the carotenoprotein present in crustacean shell is a valuable product for the fish farming industry, therefore, it is important to recover the protein in high yield with a good quality (Bustos and Healy, 1994). The most common practice for the removal of protein from the shell waste is to dissolve it in NaOH at elevated temperature for several hours (chemical approach). However, there is a wide range of chemical concentrations, temperature, reaction time and shell to chemical ratio that may be employed (Table 4.24).

The decalcification in the production of chitin from shellfish waste is a costly process. HCl appears to be the most commonly used chemical in the demineralization of shell waste. There is also a wide range of conditions that was used in the literature for the demineralization step (Table 4.25). The demineralization takes place when the acid reacts with the calcium carbonate releasing carbon dioxide according to the following equation

Table 4.24. Conventional methods of deproteinization of shellfish waste from different sources (No and Meyers, 1995).

Source	Chemical	Conditions			Reference
		Temperature (°C)	Time (h)	Ratio (w/v)	
Lobster	1N NaOH	100	12 × 5[a]	1:5.5	Hackman (1954)
Lobster	5% NaOH	80 - 85	0.5 × 2[a]	-	Blumberg et al. (1951)
Lobster	10% NaOH	ambient	72[c]	-	Whistler and BeMiller (1962)
Lobster	10% NaOH	100	2.5	1:50	Horowitz et al. (1957)
Krill	3.5% NaOH	90 - 95	2	1:10	Anderson et al. (1978)
Krill	3.5% NaOH	25	2	-	Brzeski (1982)
Krill	3% KOH	95	2	1:10	Synowiecki et al. (1981)
Shrimp	3% NaOH	100	1	-	Bough et al. (1978)
Shrimp	1% NaOH	65	1	1:10	Wu and Bough (1978)
Shrimp	1% KOH	90	2	1:20	Shahidi and Synowiecki (1991)
Shrimp	4% NaOH	100	1	-	Suryanarayana Rao et al. (1987)
Shrimp	1M NaOH	70	6	1:15	Percot et al. (2003)
Crawfish	3.5% NaOH	65	2	1:10	No et al. (1989)
Cuttlefish	10% NaOH	103 – 105	6 × 2[a]	excess	Okafor (1965)
Crab	1N NaOH	80	3 × 2[a]	-	Mima et al. (1983)
Crab	2% KOH	90	2	1:20	Shahidi and Synowiecki (1991)
Crab	1N NaOH	50	6	-	Brine and Austin (1981a)
Crab	5% NaOH	90	2	-	Broussignac (1986)[d]
Prawn	5% NaOH	Reflux	2	1:15 – 20	Kamasastri and Prabhu (1961)
Prawn	5% NaOH	100	0.5	1:1	Moorjani et al. (1975)
Prawn	0.5% NaOH	100	0.5	2:3	Madhavan and Ramachandran Nair (1974)
Prawn	15% NaOH	65	3	1:10	Beaney et al. (2005)
Squid	1N NaOH	100	10 – 12	-	Cho and Lee (1980)
Squid	2M NaOH	ambient	overnight	1:10	Kurita et al. (1993)
Squid	2M NaOH	100	4[b]	1:10	Kurita et al. (1993)

[a] times of repeated alkali treatment
[b] second extraction
[c] fresh NaOH solution was used for soaking each day
[d] when chitosan is final product

(Percot et al., 2003):

$$CaCO_3 + 2HCl \rightarrow CO_2 \uparrow + CaCl_2 + H_2O \quad (4.16)$$

Kong (1975) reported that Xanthation technique could be applied for chitin extraction from shellfish waste. This technique includes reacting the crustacean shells with appropriate mixture of carbon disulfide and caustic soda, and yields soluble chitin in the form of xanthates. The calcium carbonate in the shells remains insoluble and hence could be separated. After separating the calcium carbonate, chitin could be regenerated by hydrolyzing the xanthate in an acidic medium.

The negative impacts of the traditional methods of chitin purification had attracted much attention to find an alternative environmentally friendly method. The biotechnological approach in which enzymes or microbial activity is employed seems to be a promising alternative. Bustos and Healy (1994b) stated that from the economical and environmental point of view, the microbial approach for chitin purification seems to be the most realistic and cost-effective alternative method for crustacean shell waste processing. In addition, Beaney et al. (2005) stated that despite the incomplete purification of chitin, biological processes could be considered more environmentaly friendly approach for the production of chitin and its derivitavies. Generally, the advantages of using the biotechnological approach include: (a) harsh chemicals are not used, (b) it is more economical when considering energy and waste disposal costs, (c) it preserves the natural state of chitin, and (d) it results in deproteinization liquid which could be used for protein recovery purposes (Gagné and Simpson, 1993).

Recently, many researchers have proposed deproteinization of crustacean waste using proteolytic enzymes either by using proteolytic bacteria which secret extracellular proteases or by adding proteolytic enzymes (Table 4.26). Wide range of proteolytic enzymes has been studied including papain, trypsin, pepsin, tuna proteinase, bacterial proteinase, and chymotrypsin. The use of proteolytic enzymes is a simple and inexpensive alternative to the traditional methods for chitin purification. Bustos and

Table 4.25. Conventional methods of demineralization of shellfish waste from different sources (No and Meyers, 1995).

Source	Chemical	Conditions			Reference
		Temperature (° C)	Time (h)	Ratio (w/v)	
Lobster	2N HCl	ambient	5	1:9	Hackman (1954)
Lobster	2N HCl	0	48	1:5.5[b]	Hackman (1954)
Lobster	5% HCl	70	2 × 2[a]	-	Blumberg et al. (1951)
Lobster	37% HCl	-20	4	-	Whistler and BeMiller (1962)
Krill	0.6N HCl	ambient	2	1:22	Anderson et al. (1978)
Krill	3.5% HCl	20	1.5	1:4	Brzeski (1982)
Krill	22% HCl	ambient	2	1:10	Synowiecki et al. (1981)
Shrimp	1N HCl	ambient	0.5	3× [c]	Bough et al. (1978)
Shrimp	0.5N HCl	ambient	-	1:11	Wu and Bough (1978)
Shrimp	2.5% HCl	20	1	1:10	Shahidi and Synowiecki (1991)
Shrimp	5% HCl	ambient	-	-	Suryanarayana Rao et al. (1987)
Shrimp	8% HCl	30	8	1:10	Cosio et al. (1982)
Shrimp	1.75N acetic acid	ambient	12	1:15	Gagné and Simpson (1993)
Shrimp	1N HCl	ambient	0.5	1:6	Sophanodora and Hutadilok (1995)
Shrimp	0.25M HCl	ambient	0.25	1:40	Percot et al. (2003)
Crawfish	1N HCl	ambient	0.5	1:15	No et al. (1989)
Cuttlefish	10% HCl	ambient	192	excess	Okafor (1965)
Crab	1N HCl	ambient	12 × 2[a]	-	Mima et al. (1983)
Crab	2.5% HCl	20	1	1:10	Shahidi and Synowiecki (1991)
Crab	1N HCl	20	3	-	Brine and Austin (1981a)
Crab	5% HCl	ambient	24	-	Broussignac (1986)
Prawn	5% HCl	ambient	2	1:15 – 20	Kamasastri and Prabhu (1961)
Prawn	5% HCl	ambient	1	1:2	Moorjani et al. (1975)
Prawn	1.25% HCl	ambient	1	-	Madhavan and Ramachandran Nair (1974)
Prawn	1M HCl	ambient	2	1:15	Beaney et al. (2005)
Squid	2N HCl	10	12	excess	Cho and Lee (1980)
Squid	1M HCl	ambient	overnight	1:10	Kurita et al. (1993)

[a] times of repeated acid treatment

[b] second extraction

[c] threefold excess to stoichiometric amount of calcium carbonate in the dry hulls

Table 4.26. Deproteinization of shellfish waste using the biochemical approach.

Waste	Enzyme	Conditions				Protein Removal (%)	Residual Protein (%)	Reference
		Temperature (°C)	pH	Time	Ratio (w/w)			
Shrimp waste	Chymotrypsin	40	8.0	72 h	7:1000	-	1.3	Gagné and Simpson (1993)[a]
Shrimp waste	Papain	38	8.7	72 h	10:1000	-	2.8	
Shrimp waste	Atlantic cod trypsin[b]	4	7.7	24 h	25 mg%	81	-	Cano-Lopez et al. (1987)
Shrimp waste	Bovine trypsin[b]					65	-	
Shrimp shells[d]	Protease of *Pseudomonas aeruginosa* K-187	37	-	5 days	-	82	-	Wang and Chio (1998)
Shrimp heads[d]						81	-	
Shrimp shells[e]						55	-	
Shrimp heads[e]						60	-	
Prawn shells	*Pseudomonas maltophilia*	-	-	6 days	-	82	-	Bustoss and Healy (1994)
Prawn shells	Purified microbial protease					64	-	

Table 4.26. Continued.

Waste	Enzyme	Conditions				Protein Removal (%)	Residual Protein (%)	Reference
		Temperature (°C)	pH	Time	Ratio (w/w)			
Prawn shells	*Pseudomonas maltophilia*	30	7	3 days	-	95	1.1	Shimahara et al. (1982)
Prawn shells	*Pseudomonas maltophilia*	30	7	24 h	-	-	1	Shimahara and Takiguchi (1988)
Prawn shells	BAFP-202[c]	30	7	16 days	-	81.7	-	Bustos and Healy (1994a)
Prawn shells	BAFP-202[c]	30	7.5	6 days	-	82	-	Bustos and Healy (1994b)
Crab shells	Protease extracted from powder of pyloric caeca of striped marlin	-	-	20h	-	-	2	Takeda and Abe (1962)
Crab shells	Bacterial Proteinase (Pronase-P)	60	7	30h	-	-	1	Takeda and Katsuura (1964)
	Papin	37.5	5.5					
	Protease extracted from pyloric caeca of tuna	40	8.6					
Shrimp shells	*Aspergillus niger*	30	6.5	4 days	-	-	3.3 ± 0.3	Teng et al. (2001)

[a] optimized using RSM (response surface methodology) approach.
[b] with the presence of EDTA in the extraction medium
[c] containing *A. oryzae*, *Streptococcus faecium*, *Pediococcus pentosaseus* and *B. subtilis*
[d] solid-phase fermentation
[e] liquid phase fermentation

Healy (1994) proposed a deproteinisation method for chitin extraction from demineralized prawn shell waste using different proteolytic microorganisms (*Pseudomonas maltophilia*, *Bacillus subtilis*, *Streptococcus faecium*, *Pediococcus pentosaseus* and *Aspergillus oryzae*). They achieved over 80% deproteinization and concluded that higher molecular weight chitin could be achieved by microbial methods than chemical methods and the protein generated is less degradable than that of the traditional chemical treatment and hence allow higher protein recovery. Bustos and Michael (1994) reported that deproteinization by enzymes is not as effective as living microbes. Teng et al. (2001) reported that the presence of microorganisms provides a constant and gradual increase of protease throughout the fermentation period, which is not possible in the case of placing a fixed amount of enzyme. Bustos and Healy (1994) also reported that microorganisms can grow in the interstices of shell waste structure and allow for closer contact between the enzyme producing site and the protein substrate. Microorganisms seem to be the best alternative to harsh chemical treatment for deproteinization of shellfish waste. Wang and Chio (1998) stated that cooking and high drying temperatures cause lower protein removal from shellfish waste because of Millard-type nonenzymatic reaction, which renders the proteins (due to reaction with sugars and other compounds) resistant to protease treatment. They conclude that solid-state fermentation achieves higher protein removal efficiency than liquid/solid or liquid fermentation and that the deproteinization activity of *Pseudomonas aeruginosa* k-187 is better than that of proteolytic bacterium *Pseudomonas maltophilia* reported in many studies. Pandey (2003) reported that the exact reason for higher production rate of solid-state fermentation compared to submerged fermentation is not well known, however, the logical explanation might be that in solid-state fermentation microbial cultures are closer to their natural habitat which probably increases their activity.

Teng et al. (2001) proposed a cost-effective method for good quality chitin recovery from shrimp shells and fungal mycelia. Their method included the use of proleolytic enzymes, which are secreted from certain chitin-containing fungal strains (*Aspergillus niger*; strains 0576, 0307 and 0474), to deproteinize the shrimp shells. The hydrolyzed proteins from the shrimp shells in turn worked as a nitrogen source for the cultivation of fungi,

accumulating fungal mycelia, which are an additional source of chitin. They obtained < 5% residual protein in the chitin isolates from the shrimp shells; while the residual protein in the fungal chitin isolates was higher and ranged between 10 – 15%, which required final deproteinization.

Joensen and Villadsen (1994) developed an environmentally friendly low cost chitin production process. They combined two waste products, shrimp waste and fish silage of entrails, to produce saleable products including long chained chitin, high quality oil with astaxanthin, a liquid phase of partially hydrolysed protein and ashes. The production process includes the removal of protein and oil extraction by the crude enzymes in the fish guts, and the use of sulfuric acid for demineralization allowing the ash contents to be separated as gypsum which results in a protein phase with low ash content. They concluded that the total variable cost using their developed process to produce chitosan from shell waste is about one-third of traditional methods.

Hall and De Silva (1992), Hall et al. (1994), Hall and Reid (1995), Kungsuwan et al. (1996), Healy et al. (2003) and Beaney et al. (2005) proposed the use of lactic acid bacterial fermentation for the stabilization of shrimp waste and separation of its components (protein, chitin and pigments). The lactic acid bacteria produce lactic acid, which lowers the pH preventing the shellfish waste from spoilage. The acid also dissolves the calcium carbonate and under these conditions native enzymes breakdown the protein (autolysis). Much of the calcium and protein in the shrimp waste could be removed in the fermentation, saving the use of alkali/acid (smaller volumes and lower concentrations) in the downstream processing of the chitin and shortening the reaction time at the same time.

Hall and Reid (1995) used 1N sodium hydroxide (2:1 w/v) at 24h retention time and 1N HCl (2:1 w/v) at 1h retention time for chitin recovery after fermentation. They also used 10% household hypochlorite for a few minutes for chitin bleaching.

Hall and De Silva (1992) reported that adding lactic acid bacteria and carbohydrate to tropical prawn waste allowed acidification and proteolysis and the protein rich liquor was also easily separated from the insoluble chitin.

Kungsuwan et al. (1996) used lactic acid bacteria as starter for shrimp head fermentation for the production of protein hydrolysate for developing various natural health products. They reported that the chitin remained in the solid part could be further extracted and converted to chitosan or any other derivatives.

Healy et al. (2003) reported that the anaerobic fermentation of shellfish waste using lactic acid bacteria in combination with proteolytic enzyme producing bacteria is an effective method for breaking down shell waste and isolating the valuable components contained within the shell structure (chitin, protein and pigments). In the study a microbial mixture of *Lactobacillus plantarum*, *Lactobacillus salivarius*, *Streptococcus faecium* and *Pediococcus acidilactici* was used to ferment prawn shell waste. The fermentation achieved 90.99% calcium removal on dry basis and allowed for the production of pigmented liquor containing peptides, amino acids and only a few intact proteins. The yield of chitin obtained on a dry basis was 20.43% w/w.

Fresh shell waste is recommended to use for the purification process in order to preserve both proteins and pigments. Hall et al. (1994) and Healy et al. (2003) reported that drying prawn shells causes the loss of the majority of its pigmentation due to the strong affinity for oxygen of the carotenoid compounds and degradation of some proteins. Also Teng et al. (2001) reported that autoclaving of shrimp shells causes deproteinization (56.06±3.14%), releasing hydrolyzed proteins into the medium. Hall and Reid (1995) stated that for chitin purification using lactic acid fermentation, a good mixing is necessary for two reasons: (a) prevent pockets of high pH material, which can support the growth of spoilage organisms, and (b) help in the removal of carbon dioxide generated in the fermentation, which is able to prevent good contact between the fermentation broth and the solid waste material giving good conditions for spoilage. However, continuous

mixing for the removal of all carbon dioxide is not preferred as lactic acid bacteria favor the anaerobic conditions.

4.6 Ultraviolet Radiation Sterilization

4.6.1 UV Radiation Divisions

Ultraviolet (UV) radiation is the electromagnetic energy that is located in the electromagnetic spectrum (100–400 nm) at wavelengths between those of X-rays and visible light (Harm, 1980; Cairns, 1996; Diamant, 1996; O'Brien et al., 1996 and Miller et al., 1999). UV spectrum exists in three forms as shown in Figure 4.12: (a) UV-A which extends from 315-400 nm and is also called near UV or long UV, (b) UV-B which extends between 290-315 nm and (c) UV-C which extends between 100-290 nm and is also called far UV or short UV (Meulemans, 1987; Cairns, 1996; and Miller et al., 1999).

4.6.2 Sources of UV Radiation

UV radiation is formed on hot surfaces such as the sun, which is considered the largest natural source of this radiation (Diamant, 1996). The most common sources of UV radiation, which are commercially available, are low and medium mercury arc lamps. However, other lamp systems including broad-band pulsed xenon, narrow-band excimer, pulsed electric fields and those which use advanced oxidation processes are available (O'Brien et al., 1996). Generally, the lamp consists of tightly sealed tube of UV transmitting material (usually quartz) with electrodes at both ends (usually tungsten with a mixture of alkaline earth metals). The tube is filled with a small amount of mercury and an inert gas (usually argon) as shown in Figure 4.13a. An electric arc is established by applying a high voltage across the electrodes. The established electric arc facilitates the promotion of electrons within the mercury atoms to higher energy orbital. UV radiation is then emitted from the lamp when the electrons return to their ground state. After original establishment, the electric arc can be maintained by a comparatively low voltage and therefore a transformer (ballast) should be used in order to regulate the voltage applied across the electrodes. Ballasts may be characterized as either electromagnetic or electronic as shown in Figure 4.13b. However, electronic ballasts can be more compact,

have greater electrical efficiency, reduce system cost, and can operate at various power settings. Operation of the lamp at a higher AC frequency increases lamp output and extends lamp life (O'Brien et al., 1996). There are two types of mercury arc lamps: low pressure and high pressure (Figure 4.14).

Low pressure mercury arc lamps have an internal mercury partial pressure of 10^{-3} mbar and a total vapor pressure of approximately 2 mbar. The output spectrum of low pressure mercury arc lamps is characterized by several emission lines in the visible range (15% of the total light emitted), which is responsible for the pale blue color, and two in the UV range, 254 nm and 185 nm (85% of the total light emitted) as shown in Figure 4.14 a. For this type of lamps, the conversion efficiency of the electrical energy to UV radiation is approximately 35-40%. However, recently, high output low pressure mercury arc lamps have been developed and is characterized by a relatively high UV output (about twice that of a conventional low pressure arc mercury lamp) and retaining the monochromatic character. Tchobanoglous and Burton (1991) reported that at present, low pressure mercury lamps are the principle means for generating UV energy for the purpose of disinfection. This is mainly because of the fact that 85% of the light output is nearly monochromic at a wavelength of 253.7 nm, which is within the optimum range for germicidal effects (the 253.7 nm mercury line has an effectiveness of about 85% of the maximum effectiveness at 260-265 nm) (Figure 4.15) (Meulemans, 1987; Diamant, 1996; O'Brien et al., 1996; Tchobanoglous and Burton, 1991; and Darby et al., 1993).

Medium pressure arc mercury lamps have an internal pressure in the range of 2-5 bars. They are characterized by high UV output and a strongly polychromatic output spectrum (200-300 nm) as shown in Figure 4.14 b. A medium pressure UV lamp contains far less mercury than a low pressure UV lamp, however, it operates at higher pressures and temperatures, which allow the mercury to emit radiation at much higher intensity and over a wider range of wavelengths (O'Brien et al., 1996). The high output of this kind of lamps allowed for the development of UV disinfection systems based on relatively small number of lamps compared to low-pressure mercury arc lamps. However, the decrease in system size as well as and the complexity associated with medium pressure lamps are

accompanied by substantially higher lamp costs as well as relatively low lamp efficiency. O'Brien et al. (1996) reported that because of the higher intensity obtained by using medium pressure UV lamps, it can be used with waters with low transmittance (50% or less). However, because of the wide range of radiation emitted, less power is used for the production of germicidal wavelengths.

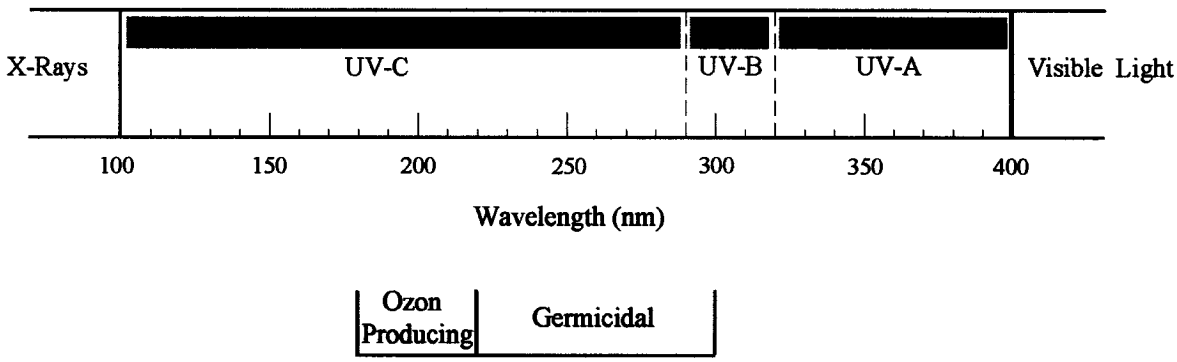
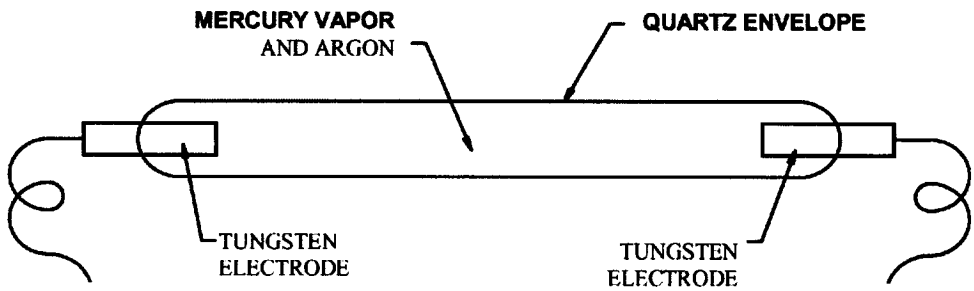
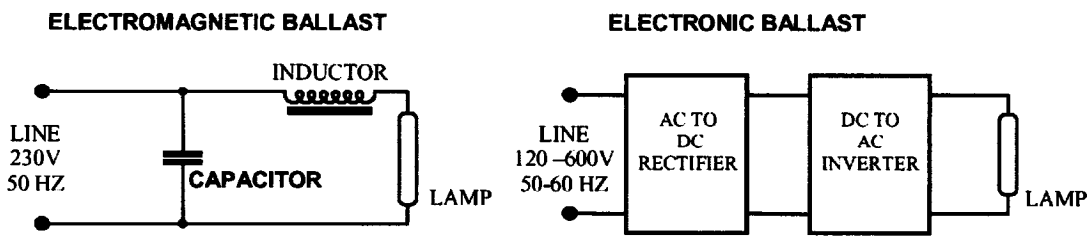


Figure 4.12. Common UV divisions.

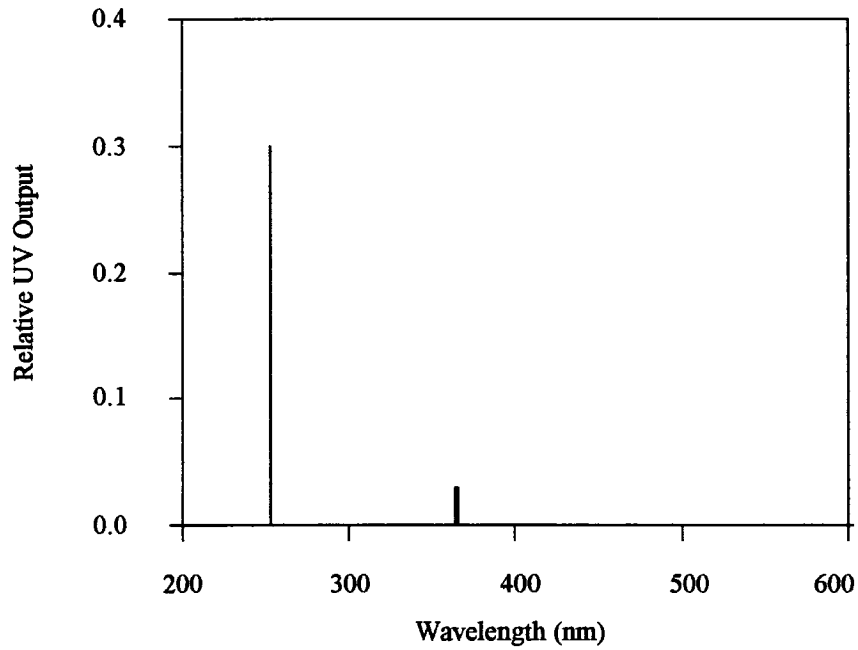


(a) Lamp Components

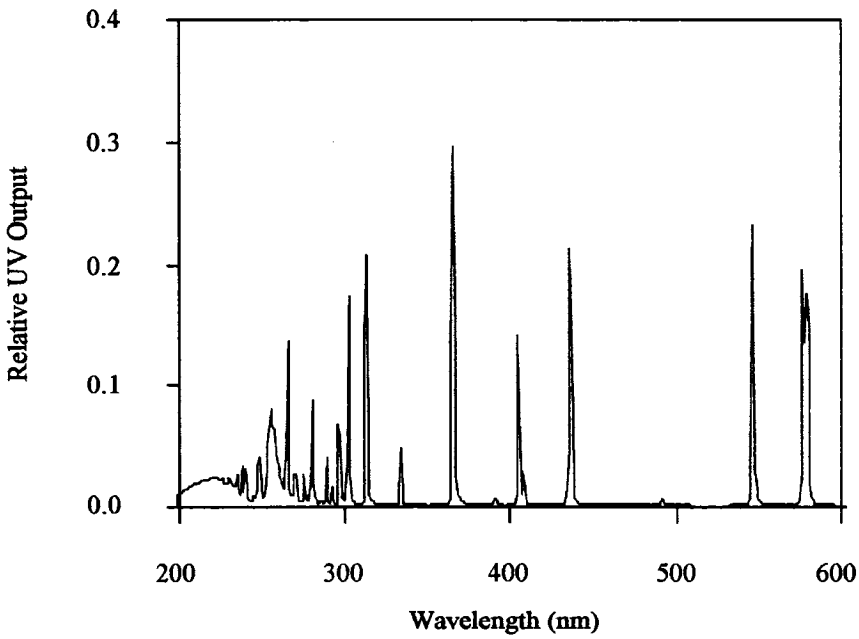


(b) Ballast Design

Figure 4.13. Mercury arc lamp components and ballast design (Trojan, 2003).



(a) Low pressure mercury arc lamp



(b) Medium pressure mercury arc lamp

Figure 4.14. Spectral output for mercury arc lamps (Trojan, 2003).

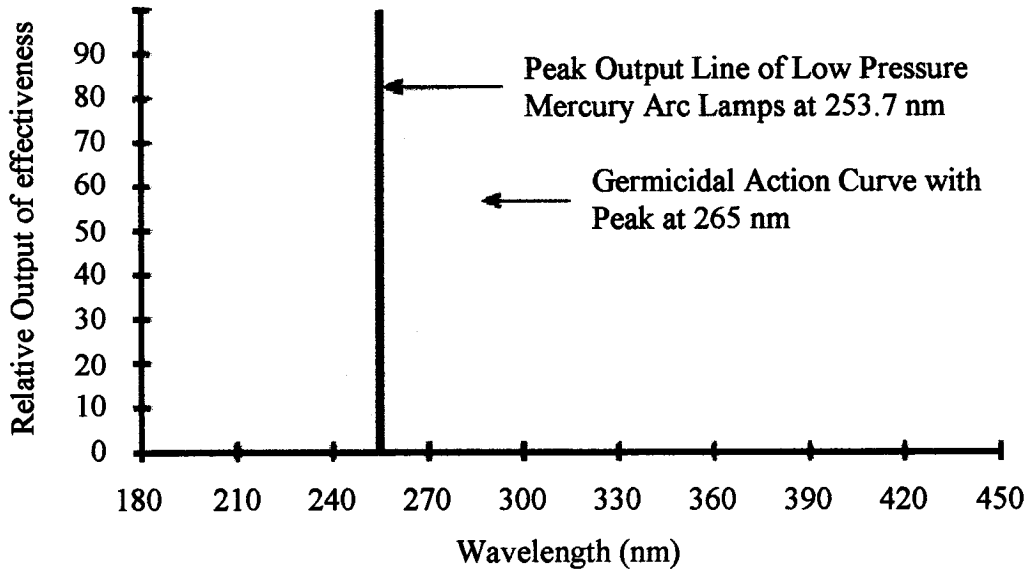


Figure 4.15. Low pressure UV lamp output versus Germicidal effectiveness (Light Sources, 2003).

Several other alternatives have been applied in disinfection applications, of which the most important are those based on pulsed UV and excimer lamp technologies. Pulsed UV system deliver high intensity pulses of polychromatic UV radiation (200-300 nm). To date, these systems have had limited applications, but some have shown some promise in terms of antimicrobial efficacy. Excimer lamps were also used in systems for microorganism's control. They produce monochromatic radiation by applying an electric potential across a dielectric barrier, between which is contained a mixture of rare gases. The combination of gasses dictates the characteristic wavelength of the emission. Therefore, the output of these lamps is tunable to the wavelength of interest. Excimer lamps are able to operate at much lower surface temperatures than other UV sources, which considered an advantage in terms of fouling behavior in some applications.

4.6.3 Mechanism of UV Germicidal Effect

The germicidal effectiveness of the UV radiation depends on its wavelength. The short-wave UV is the most dangerous since it can be absorbed in living tissues and causes irreversible changes in the structure of proteins, thus destructing the tissue (Diamant, 1996). The germicidal effect of UV radiation is basically based on photochemical

reactions of biomolecules in the microorganism (Blatchley III and Peel, 2001). In order to produce a photochemical reaction and consequently the germicidal effect of UV radiation, the radiation has to hit one or more targets such as DNA, RNA, or enzymes inside the microorganisms (Meulemans, 1987; Darby et al., 1993; and Miller et al., 1999). Harm (1980) reported that the biological effectiveness of ultraviolet radiation is basically due to its absorption by nucleic acids (DNA and RNA), and to a much lesser extent by proteins and other biologically important molecules. The total effect of the UV radiation depends on the total energy supplied (dose). Depending on the received UV dose, the photochemical reaction can result in inhibited growth or in total inactivation of the microorganism (Meulemans, 1987). High UV doses damage cell membrane and cause the biological death of the cell. While, low doses cause what is called clonogenic death, in which a photochemical transformation of pyrimidine bases, that produce links between successive thymines on a DNA strand, takes place to form dimmers. The cells that have these DNA coding errors cannot replicate (Harris et al., 1987; Darby et al., 1993; and McDonald et al., 2000).

The deoxyribonucleic acid (DNA) contains the entire cell's hereditary information mainly in the sequence of the sub-unit nucleotides along the polymer chain. Nucleotides of the DNA or RNA are composed of: (a) phosphoric acid, (b) ribose or deoxyribose (five-carbon sugar as shown in Figure 4.16), and (c) a heterocyclic nitrogenous base, usually derived from either purine or pyrimidine (Figure 4.17). DNA exists in the form of two helical long chain polymers (Figure 4.18). The two strands of the double helix are held together by hydrogen bond between the base pairs (adenine with thymine and cytosine with guanine) (Bailey and Ollis, 1986; and McDonald et al., 2000).

Nucleotide nitrogenous bases in DNA and RNA (usually the purine derivatives, adenine (A), and guanine (G) and the pyrimidine derivatives, thymine (T), cytosine (C), and uracil (U)), are the absorbing components within the nucleic acids. The common maximum absorption is in the 260-265 nm region. The absorption characteristics of RNA is similar to that of DNA, However the UV-induced alternations in case of RNA are of much lesser biological consequence than in case of DNA except for the biological

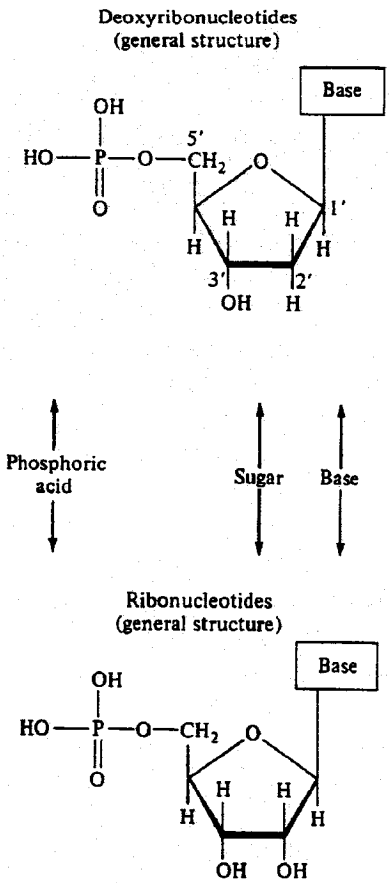


Figure 4.16. The general structure of ribonucleotides and deoxyribonucleotides (Bailey and Ollis, 1986).

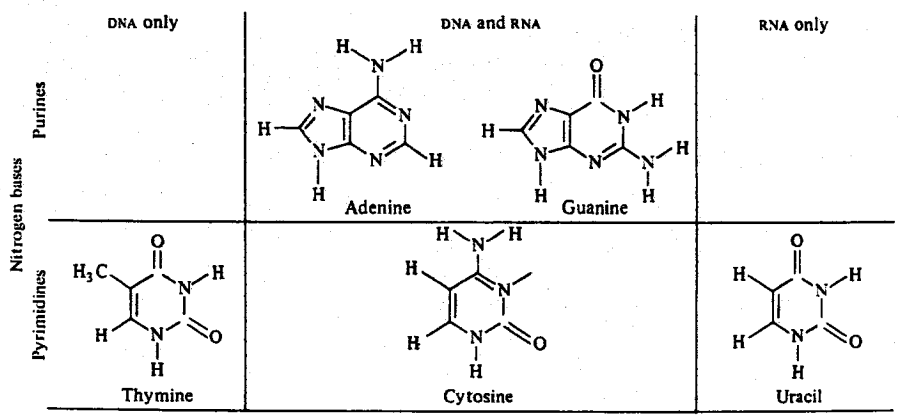


Figure 4.17. The five nitrogenous bases found in DNA and RNA (Bailey and Ollis, 1986).

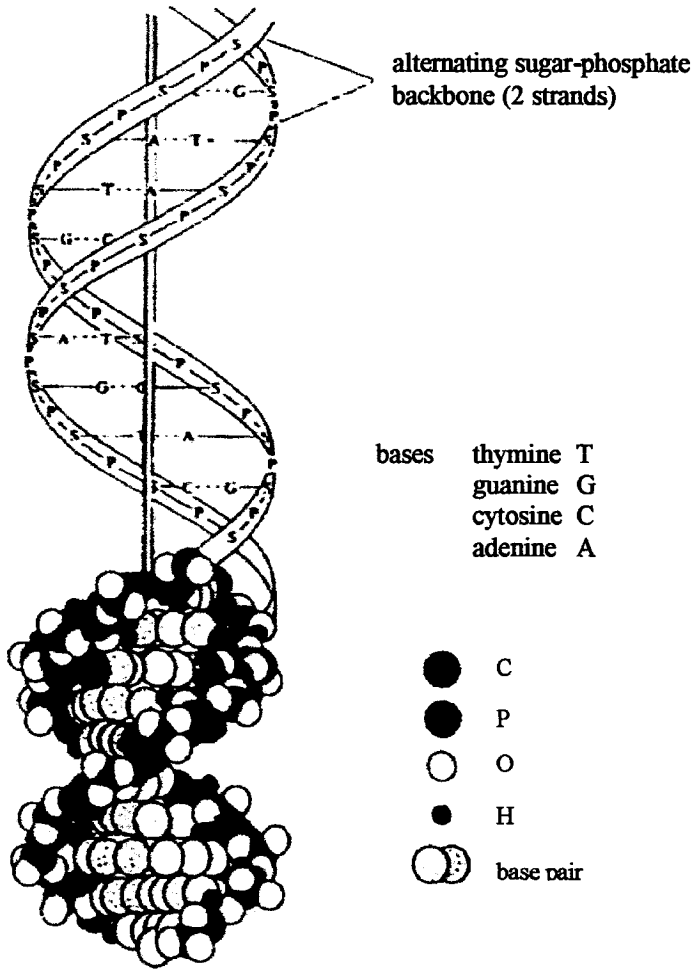


Figure 4.18. Typical DNA double helix strands (McDonald et al., 2000).

systems in which RNA is the only genetic material, as in some animal and plant viruses as well as in a few bacteriophages. The reason is the uniqueness of the DNA as a genetic material while RNA, in the form either messenger RNA, transfer RNA, or ribosomal RNA exists in many copies and can be replaced as long as the information of their production (carried by the DNA) is unaffected (Harm, 1980). Although both purine and pyrimidine are strong absorbers in the far UV region, their contributions to the biological effects are different. Under identical irradiation conditions pyrimidines undergo photodecomposition at a much higher rate than purines. The main types of photoproducts in irradiated DNA are: (a) cyclobutyl type dimmers, (b) Pyrimidine adducts, (d) spore photoproduct (formed only under special conditions), and (e) DNA-protein crosslinks (Figure 4.19). The absorption of UV by one of two adjacent thymines causes the

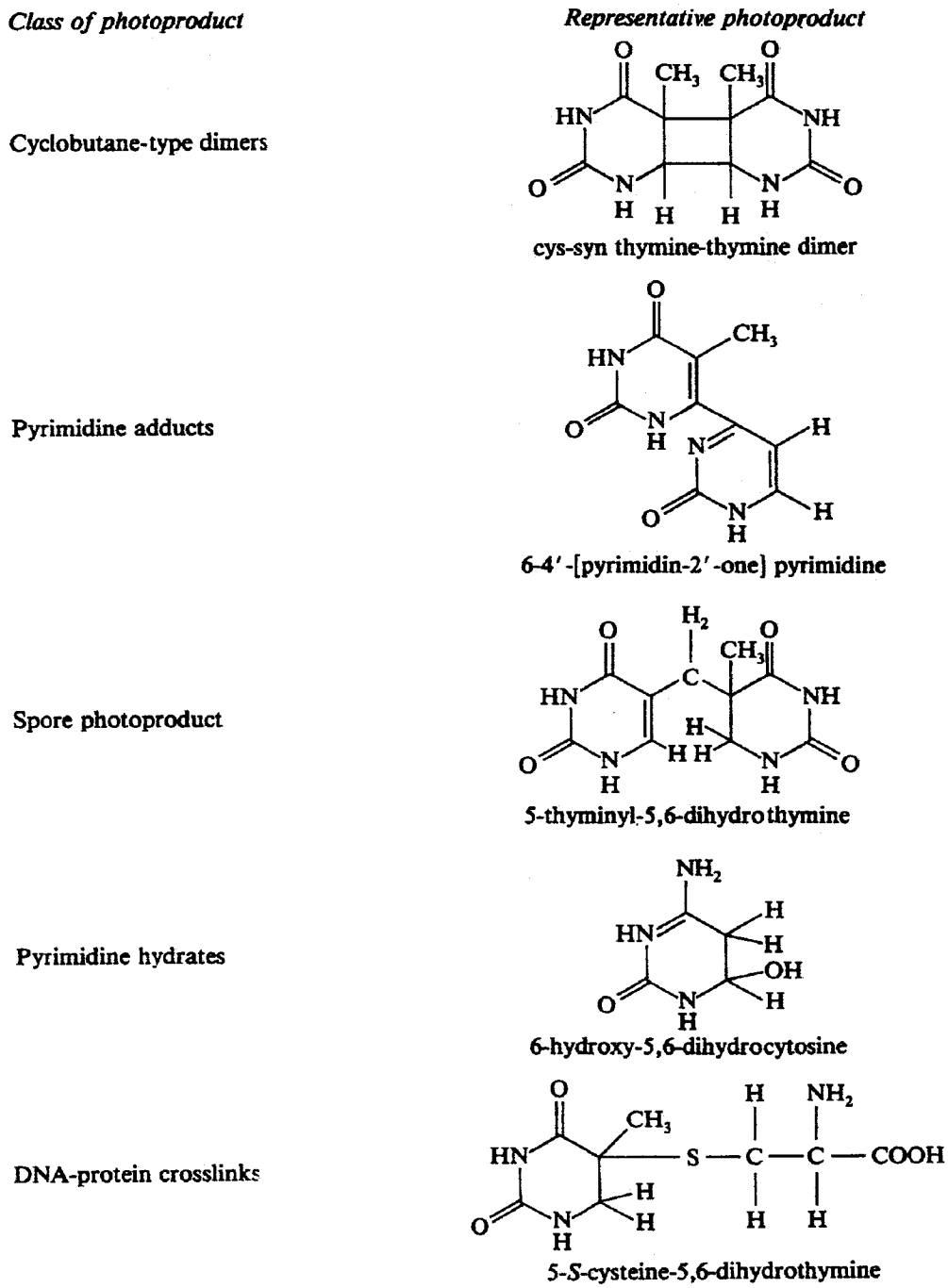


Figure 4.19. Examples of DNA photoproducts formed in UV-irradiated cells (Harm 1980).

formation of a chemical bond between the two thymines (Figure 4.20). Hence, thymine dimmer is formed, which is very stable and contains a non-replicable genetic locus in the structure of the DNA. Sometimes, another indirect mechanism causes the photo-production of OH-peroxy, and hypoperoxy radicals in the cellular fluids and provides reactive oxidants that attack ligand membranes, proteins, enzymes and DNA. These radicals have cytotoxic effects on cell respiration and metabolism (McDonald et al., 2000).

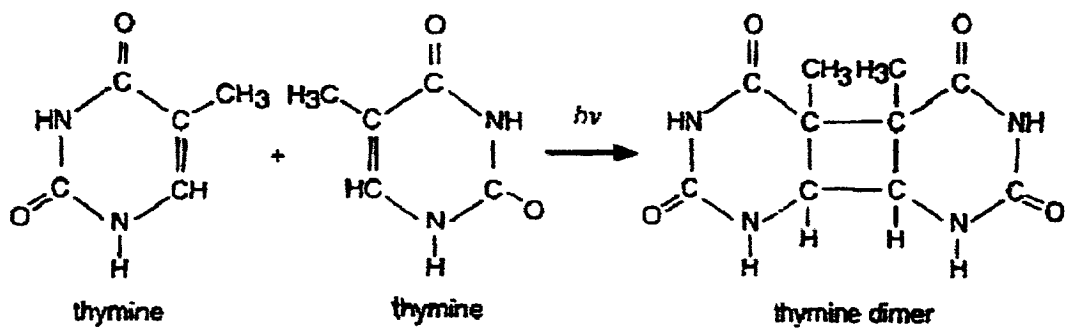


Figure 4.20. Photochemical dimerization of two thymine bases (McDonald et al., 2000).

Absorption of most proteins in the far-UV region (from 240 – 300nm) is much lower than that of nucleic acid solutions of equal concentration and is of minor consequence. Moreover, proteins are present in cells in a large number of identical copies and hence photochemical alternations in a fraction of them may still allow the biological function to get through (Harm, 1980). Sometimes, biological substances with unsaturated bonds, other than nucleic acids and proteins, may be involved in the effect of UV radiation.

4.6.4 UV Sterilization Applications

Due to its germicidal effect, UV radiation has been extensively used in many sterilization applications such as: treatment and disinfection of drinking water and wastewater (Harris et al., 1987; Meulemans 1987; Qualls et al., 1989; Darby et al., 1993; Diamant, 1996; Oppenheimer et al., 1997; Lin et al., 1999; and McDonald et al., 2000), water sterilization in aquaculture facilities (Liltved and Landfald, 1993; and Abraham and Palaniappan, 2000), medical devices sterilization (Khomich et al., 1998), air disinfection (Miller and

Macher, 2000; Kowalski et al., 2000; and VanOsdell and Foarde, 2002) and polymeric materials sterilization (Fischbach et al., 2001).

4.6.5 Factors Affecting UV Sterilization Performance

The most important factors that affect the UV sterilization performance are: (a) the emission spectrum of the UV-source, (b) the UV dose, which is the quantity of germicidal UV energy and (c) the characteristic of the medium to be sterilized.

4.6.5.1 UV Spectrum. The germicidal effectiveness of the UV radiation depends on its wavelength since the absorbance of biomolecules in the organism, mainly DNA, relays on the UV radiation wavelength. UV light in the spectrum 180 to 320 nm has the greatest germicidal effect and can inactivate microorganisms such as protozoa, bacteria, molds, yeasts, virus, fungi and algae, with an optimum effect at 260-265 nm (Harris et al., 1987; Meulemans, 1987; Corbitt, 1990; McDonald et al., 2000; and Blatchley III and Peel, 2001). Diamant (1996) reported that small deviation from the optimal wavelength, results in a sharp decrease in efficiency, regardless of the direction of the deviation. The 254 nm and 275 nm emissions are approximately 85% as effective as 265 nm emission (Cairns, 1996). Figure 4.21 shows Comparison between microbial UV action and DNA UV absorbance.

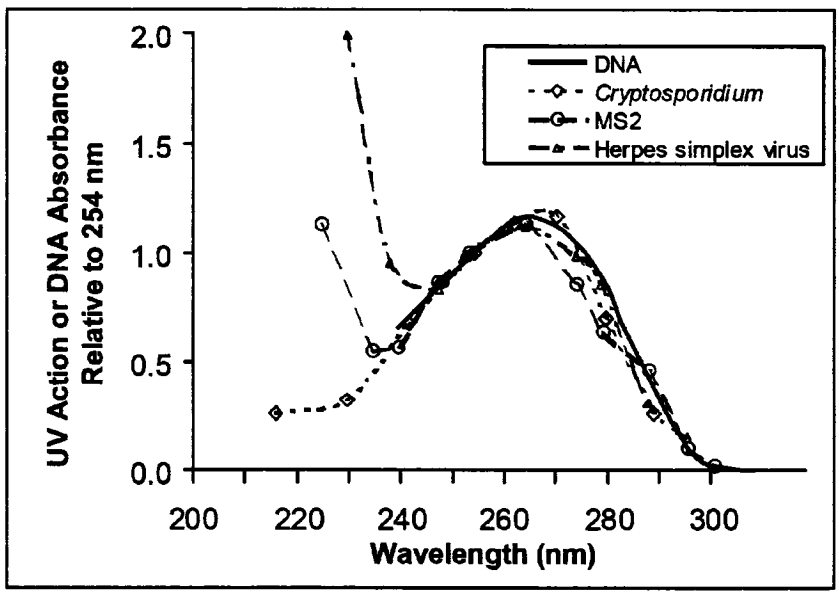


Figure 4.21. Microbial UV action and DNA UV absorbance (EPA, 2003).

4.6.5.2 UV Dose. The dose can be defined as the average intensity of the germicidal UV energy times the exposure time according to the following equation (Harris et al., 1987; Meulemans, 1987; Darby et al., 1993; O'Brien et al., 1996; and Andreadakis et al., 1999):

$$D = I \times t \tag{4.17}$$

Where:

D is the dose ($mW.s/cm^2$).

I is the intensity of the germicidal UV energy (mW/cm^2).

t is the exposure time (s).

The UV radiation dose must be sufficient to cause the germicidal effect. The UV dose required for germicidal effect varies with the chemical composition of the fluid to be treated as well as the species of the organism (Harris et al., 1987; Meulemans, 1987; Darby et al., 1993; and O'Brien et al., 1996). Gram positive bacteria with a thick capsule of mureine are more difficult to destroy than gram negative bacteria with thin capsule. Spore forming bacteria are also more difficult to destroy because of the very thick capsule of the spore. Algae need hundred times more energy than bacteria, while viruses are comparable with that of bacteria.

4.6.5.3 Medium Characteristics. Medium characteristics have a high effect on the disinfection performance. The most important medium characteristics that can affect UV disinfection performance are: UV transmittance of medium at 253.7 nm, suspended solids, turbidity, particle size distribution and color (Corbitt, 1990; Darby et al., 1993 Andreadakis et al., 1999; and Blatchley III and Peel, 2001). The transmittance of the fluid to be sterilized is one of the most important factors that could affect the UV sterilization performance since it indicates the amount of UV radiation that will pass through the fluid (Murray and Baur, 1996). Darby et al. (1993) stated that UV absorbance at 253.7 nm is the water quality parameter that has been used most often to predict UV disinfection efficiency. UV radiation is absorbed as part of the disinfection process but some of the radiation could also be absorbed by soluble organic and inorganic compounds as well as

suspended particulates in the fluid to be treated and hence the average intensity within the reactor is decreased, thus affecting the sterilization performance. As UV radiation moves through solid material, it may be absorbed, scattered, or pass through the material unobstructed (Figure 4.22). As a result, the intensity of light reaching a particular point within a solid containing medium is a function of the quantity of UV light that passes through solid material unobstructed plus the quantity of light coming from all directions as a result of scattering (Loge et al., 1999).

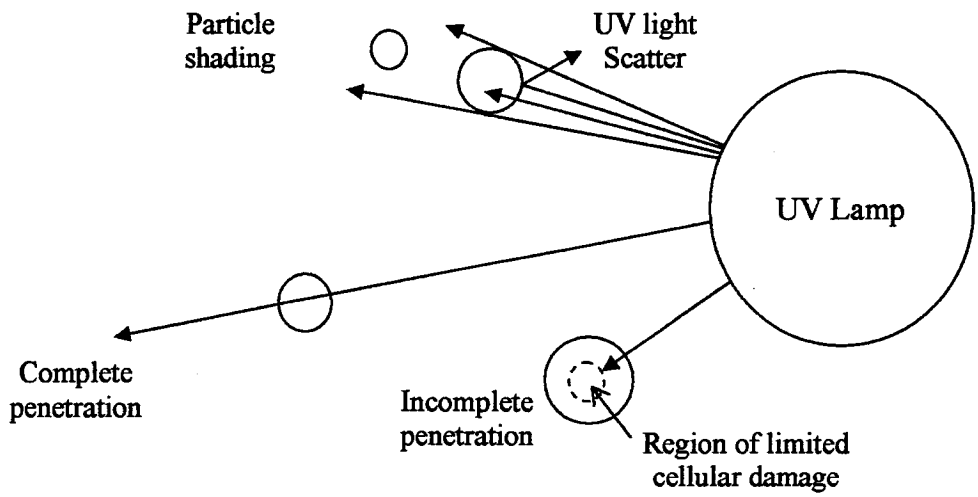


Figure 4.22. Effects of particles on UV disinfection (Darby et al., 1993).

Radiation produces electromagnetic energy which spreads in waves that move in straight lines in all directions from the source. Therefore, impurities such as suspended solids can block the radiation and waste the rays (Diamant, 1996). Tchobanoglous and Burton (1991) stated that ultraviolet radiation is not an effective disinfectant for wastewaters that contain high solid concentrations. Diamant (1996) and Andreadakis et al. (1999) reported that the existence of high suspended solids significantly increases the UV dose required for disinfection. However, Qualls et al. (1983) reported that clay merely scatter UV radiation and, therefore, do little to inhibit the performance. Generally, suspended particles in wastewater effluents can play two roles in UV disinfection: (a) absorb and

scatter the UV light, and (b) shield bacteria which become practically protected from the UV radiation (Qualls et al., 1983; Harris et al., 1987; and Diamant, 1996).

4.6.6 Fouling

The UV intensity is affected by factors including characteristics of the lamp itself, reactor geometry, and fouling characteristics of the medium. Mercury arc lamps are usually enclosed in a UV high transmitting material, (commonly quartz), in order to permit their immersion in aqueous streams and at the same time not to affect the radiation intensity reaching the stream. A potentially serious problem in UV disinfection systems, which represents a performance limitation in photochemical reactors, is the accumulation of fouling materials at the quartz liquid interface. Harris et al. (1987), Darby et al. (1993), Blatchley III et al. (1996), Diamant (1996), McDonald et al. (2000) and Sheriff and Gehr (2001) reported that fouling materials are efficient absorbers of UV radiation and hence affects the disinfection process efficiency by reducing the UV intensity in the treated medium. A new clean quartz sleeve is supposed to have 90-95% transmittance, but as material accumulates on its surface the transmittance is considerably reduced over time (Harris et al., 1987).

Lin et al. (1999) and Sheriff and Gehr (2001) reported that fouling is a function of time and that fouling rate is governed by several factors including geometry of fouling surfaces, velocity, surface temperature, and liquid composition. Cairns (1996) reported that more volume of liquid per lamp results in more UV absorbing organics and inorganics which could foul the lamp sleeve and the fouling rates could potentially increase. Several phenomena can explain the accumulation of fouling material on quartz surfaces in UV reactors. One such phenomenon is the direct ion exchange. Quartz is a hydrophilic surface due to the presence of amphoteric surface groups. These groups express a net negative surface charge at near neutral or alkaline pH. The dissociation of silanol (≡SiOH) groups on the quartz surface is responsible for the surface charge and provides surface sites available for a wide range of reactions including adsorption, complexation and precipitation (Stumm, 1992). Another explanation is the precipitation as a result of inverted solubility. The solubility of some compounds decreases with

increasing temperature. Therefore, the thermal output of UV lamps can enhance precipitation of compounds with inverted solubilities. This phenomenon was observed with many metal-ligand combinations. Blatchley III (1997) reported that energy utilization of a typical 65 W low pressure mercury lamp has been reported to be 50% and therefore 50% of the input power is converted into UV radiation and the rest is converted into heat.

Lin et al. (1999) conducted a study in order to characterize inorganic fouling on quartz surfaces in photoreactors by chemical analyses of fouling materials and of corresponding wastewaters. Their results showed that the fouling materials were predominantly amorphous inorganics and was of heterogenic distribution. The majority of the metals (cations) present in the fouling materials were iron, aluminum and calcium. While, the predominant anions found in the fouling materials were carbonate, sulfate, hydroxide, chloride and phosphate. They concluded that the primary mechanism responsible for the inorganic fouling was thermally-induced precipitation of inorganic constituents and impaction of preexisting colloidal particles. They showed that direct ion exchange reactions with the quartz surfaces were of negligible importance and that in the absence of UV radiation and heat from the lamp the inorganic fouling was substantially diminished for most of the inorganic constituents.

Andreadakis et al. (1999) reported that UV lamp fouling as a function of time follows S-type curve. Initially a lag period in which no fouling took place is obtained and then fouling decreases significantly for a period of time after which the UV system disinfection performance fails. They stated that this behavior is similar to chemical precipitation kinetics in which the initial period represents the time required for nucleation and then the formation of precipitation takes place. They attributed lamp fouling to formation of inorganic scale with high calcium content. However, Oppenheimer et al. (1997) described the relationship between percentage of fouling and time elapsed since lamp cleaning by a linear function with coefficient of determination (R^2) of 0.966.

Sheriff and Gehr (2001) investigated the effect of heat and/or UV radiation on inorganic fouling of low pressure UV lamp using recirculating and flow through configurations. Their study showed no observed fouling over extended period of time (12 days) with the recirculation flow regime. While with the flow through, fouling associated with a decrease in UV irradiance was observed. They reported that Iron (III) is one of the main inorganic constituents that cause lamp fouling (UV intensity reduction of 50% within one day for Fe concentration over 2.7 mg/L). They also stated that the existence of phosphorous increases the fouling rate (UV intensity reduction of 80% in one day for Fe concentration of 10.6 mg/L and in 5 hours for mixture of Fe and P in a mass ratio of 5:5). In this study the fouling was attributed to particle sedimentation rather than thermally induced precipitation, and therefore it was concluded that in order to reduce precipitation fouling, adequate flow needs to be maintained and/or the UV system should be designed to maximize shear effects. They reported that fouling has been associated with high hardness, high grease and oil content, a variety of multi-cationic species such as Ca, Mg, Al, Fe, and the presence of microorganisms. Harris et al. (1987) reported that the scale build up on quartz sleeves in wastewater disinfection systems is usually comprised of calcium, magnesium and iron.

4.7 Solid-State Fermentation

4.7.1 Definition of Solid-State Fermentation

The term solid-state fermentation (SSF) could be defined as the fermentation process in which microorganisms are grown and products are formed on the surfaces of solid substances in the absence (or near absence) of free water (Knapp and Howell, 1980; Lonsane et al., 1985; Tengerdy, 1985; Mudgett, 1986; Ghildyal et al., 1992; Pandey, 1992; Lu et al., 1998; Sangsurasak and Mitchell, 1998; Pandey et al., 1999, van de Lagemaat and Pyle, 2001; and Pandey, 2003). However, the substrate must posses enough moisture to support the growth and metabolism of the microbes. The major difference between microbial growth in submerged fermentation and solid-state fermentation is that all the substrate is equally accessible in case of submerged fermentation while much of the substrate is initially inaccessible in case of solid-state

fermentation. During the course of fermentation the net amount of accessible substrate will always decline for submerged fermentation and either decline, increase or remain constant at different stages of growth for solid-state fermentation (Knapp and Howell, 1980).

Mudgett (1986) described solid substrates as gas-liquid-solid mixtures in which an aqueous phase is intimately associated with solid surfaces in various states of sorption and its contact with a gas phase continuous with the external gas environment. The gas liquid interface provides a boundary for gas exchange and heat transfer while the solid phase provides a source of nutrients for the culture microorganisms. Generally, it has been claimed that solid-state fermentation has higher yield in comparison to submerged fermentation (SmF). Pandey (2003) reported that the exact reason for that is not well known. However, solid-state fermentation simulates the growth of microorganisms in their natural habitat and probably their activity is increased.

Mudgett (1986) stated that the unique characteristic of solid-state fermentation is their ability to provide a selective environment at low moistures for mycelial organisms that produce a variety of extracellular enzymes (surface bound or free) and can grow at high nutrient concentrations near solid surfaces. These organisms include a large number of filamentous fungi and a few bacteria such as the actinomycetes, in addition to at least one strain of *Bacillus*. Although some bacteria, which are involved in composting and ensiling, could grow in solid-state fermentation, bacteria and yeast usually require the presence of free water and have comparatively high water activity (Tengerdy, 1985; Pandey, 1992; and Pandey, 2003). Therefore, filamentous fungi are the most suitable organism for solid-state fermentation. Lonsane et al. (1985) stated that the growth characteristics of filamentous fungi as well as their penetration ability into the solid substrate tissue make them the microbes of choice for solid-state fermentation applications. Bacteria, yeast and single cell fungi lack the ability of deep penetrating the solid substrate tissue.

Filamentous fungi grow on solid surfaces. They have a long tubular body, which grows alongside the solid particle. They use the available surface liquid film as a source of moisture and nutrients and penetrate cracks and pores of the substrate using special appendages looking for more nutrients. Sometimes the growing part of the filament produces several powerful enzymes and branches at certain intervals helping the fungus to spread from one surface to another. However, this characteristic limits space usage since the branching filaments form a very loosely woven web, with only 8-10% of the available space filled with cells and thus causing low biomass productivity of most solid-state fermentation processes (Tengerdy, 1985; and Pandey, 2003).

4.7.2 Advantages and Disadvantages of Solid-State Fermentation

Solid-state fermentation presents number of advantages over submerged fermentation: (a) it has lower energy requirements, (b) it is considered environmentally friendly because it resolves the problem of solid waste disposal, (c) it requires small bioreactor volume because of the limited water usage, (d) the low moisture content required for this type of fermentations reduces the risk of contamination during the fermentation process, (e) it has high productivity because the conditions for growth of some microorganisms such as fungi are similar to those in natural habitats, (f) foaming problem do not occur in solid-sate fermentation (g) it produces less wastewater, (h) it reduces downstream processing and products may be incorporated directly into animal feed.

However, a number of disadvantages is also reported for solid-state fermentation: (a) in case of using continuous agitation, solid-state fermentations require high power input, (b) solid-state fermentation requires high spore inocula, (c) sometimes, it requires substrate pretreatment such as particle size reduction, (d) heat build up is a common problem, (e) it is difficult to control fermentation parameters such as temperature, aeration and moisture content, (f) it is difficult to quantifying the growth of microorganisms during the fermentation process, (g) there are limited types of organisms that could be used as a result of the low moisture content, (h) in case of high moisture content, there is risk of contamination at the start of the fermentation process and (i) process feasibility is hard to attain with scale-up due to lack of knowledge about fundamental processes especially

transport and microbial phenomena and about the effects of design and operation of bioreactors on fermentation performance (Hesseltine, 1972; Han and Anderson, 1975; Knapp and Howell, 1980; Rathbun and Shuler, 1983; Lonsane et al., 1985; Mudgett, 1986; Smith et al., 1986; Daubresse et al., 1987; Kumar and Lonsane, 1987; Pandey, 1991a; Ghildyal et al., 1994; Chakraborty et al., 1995; Gupte and Madamwar, 1997; Stuart et al., 1999; van de Lagemaat and Pyle, 2001; Rattanakit et al., 2002; Viniegra-González et al., 2003; Pandey, 2003; and Patel et al., 2004).

4.7.3 Applications of Solid-State Fermentation

Rathbun and Shuler (1983), Lonsane et al. (1985), Pandey (1992), van de Lagemaat and Pyle (2001) and Rattanakit et al. (2002) stated that solid-state fermentation has been used since ancient time for the production of several types of fermented foods, composting, enzymes, other extracellular metabolites and for the bioconversion of organic wastes into value-added products. Solid-state fermentation is considered one of the oldest and the most economic methods for food preservation (Knapp and Howell, 1980; Steinkraus, 1983; Tengerdy, 1985; Chakraborty et al., 1995; Pandey et al., 1999; and Viniegra-González et al., 2003). According to Pandey et al. (1999), enzymes produced from solid-state fermentation have several applications in various industries such as food, pharmaceutical, dairy, detergent, brewing and leather as shown in Table 4.27. Proteolytic enzymes account for nearly 60% of the industrial market in the world. Substrates that are traditionally used in such a type of fermentation include various agricultural products such as rice, wheat, millet, barley, corn and soya beans. Agricultural, forest and food-processing wastes are considered nontraditional substrates (Steinkraus, 1983 and Tengerdy, 1985). A large number of microorganisms including bacteria, yeast and fungi have been used for the production of different groups of enzymes (Pandey et al., 1999). Table 4.28 illustrates some of the microbial cultures employed for the production of different enzymes in solid-state fermentation systems. van de Lagemaat and Pyle (2001) investigated the production of fungal enzyme tannase from the waste product coffee pulp willing to produce value added product while addressing an environmental problem.

Table 4.27. Industrial applications of enzymes produced by solid-state fermentation processes (Pandey et al., 1999).

Process	Enzyme
Enzyme-assisted ensiling	Fungal cellulases and hemicellulases
Bioprocessing of crops and crop residues	Fungal cellulases and hemicellulases
Fibre processing (retting)	Fungal pectinases, cellulases, hemicellulases
Food supplement	Amylases, proteases, lipases, cellulases, hemicellulases
Biopulping	Xylanases
Directed composting	Hydrolytic enzymes
Soil bioremediation	Laccases, ligninases
Post-harvest residue decomposition	*Trichoderma harzianum* cellulases
Biopesticide	*T. harzianum* cellulase for helper function

Table 4.28. Microbial cultures and substrate materials employed for production of several enzymes in solid-state fermentation systems (Pandey et al., 1999).

Substrate	Microorganisms	Enzyme
Bagasse	*Trichoderma reesei* + *Aspergillus phoenicus*	Cellulases
Coconut coir pith	*A. niger*	Cellulases, β-glucosidase
Grapevine trimming dust	*Cerrena unicolor*	Cellulase, xylanase, ligninases
Rice husk	*Penicillium citrinum*	Cellulases
Rice husk	*Mesophilic fungi* (10 species)	Cellulase (FP)
Tea production waste	*Cerrena unicolor, Coriolus hirsulus, Pleurotus ostreatus*	CMCase, xylanase, laccase
Wheat bran	*A. niger*	Cellulase, xylanase
Cellulose, starch	*T. viride, A. niger*	Cellulase, amylase
Ligno-cellulosic materials	*Lentinula edodes*	Various enzymes
Bagasse	Strains of *Basidiomycetes*	Cellulase, ligninase
Cellulosics	*T. reesei*	cellulase
Sweet sorghum silage, wheat straw	*Gliocladium* sp., *Trichoderma* sp., *Penicillium* sp.	Cellulase, xylanase
Agro-wastes	*A. niger*	Cellulase, β-glucosidase
Agro-wastes	*T. reesei*	Cellulase
Sugar beet pulp	*P. capsalotum*	Polysaccharide degrading enzymes
Wheat bran + rice straw, spent wheat bran	*Trichoderma* sp. *Botrius* sp. *A. ustus, Sporotrichum pulverulentum*	Cellulase, β-glucosidase, xylanase
Bagasse	*Polyporus* sp.	Cellulase, ligninase
Wheat straw	*Neutrospora crassa*	CMCase, β-glucosidase
Rice straw, spent wheat bran	*Botritis* sp., *A .ustus, S. pulverulentum*	β-glucosidase, xylanase
Wheat bran	*T. reesei, S. pulverulentum*	Cellulase
Wheat straw + wheat bran	*T. harziasum*	Cellulase
Cellulosic wastes	*T. reesei*	Cellulase
Agro-wastes	*Spiecelium roseam*	CMCase

Table 4.28. Continued.

Substrate	Microorganisms	Enzyme
Bagasse, wheat bran, rice bran	*Aspergillus* sp.	Cellulase, β-glucosidase
8 ligno-cellulosic substances	*Streptomyces* sp.	Cellulase
Argo-wastes	*Pestalotiopsis versicolor*	Cellulase
Wheat bran	*T. reesei*	Cellulase
Cellulosic wastes	*T. viride*	Cellulase
Argo-wastes	*Trichoderma* sp.	Cellulase
Wheat bran	*T. reesei*	Cellulase
Saw-dust + wheat bran	*T. koningii*	Cellulase
Grapevine cutting waste	*C. unicolor*	Cellulases, xylanase, laccase
Palm oil mill waste	*A. niger*	Cellulases, xylanase
Cellolosics	*T. viride*	Cellulases
Bagasse	*A. ellipticus, A. fumigatus*	Cellulases, β-glucosidase
Wheat bran	*T. reesei*	Cellulases
Sago hampas	*P. sajor-caju*	Cellulases, xylanase, laccase
Sweet sorghum pulp, wheat straw	Ligninolytic fungal cultures	Cellulases, xylanase
Cassavn waste	*T. harzianum*	Cellulases, xylanase
Steam pre-treated willow	*T. reesei*	Cellulases
Wheat bran	*T. reesei*	CMCase
Sweet sorghum	*Gliocladium* sp	Cellulases, xylanase
Wheat straw	*T. reesei*	Cellulases
soyhull	*Phanerochaete chrysosporium*	Cellulases
Paddy straw	*T. reesei*	Cellulases
Wheat straw	*Lentinula edodes*	Cellulases
Sweet sorghum silage	*T. reesei, A. niger*	Cellulases, xylanase
Cellulosic wastes	*A. oryzae*	Cellulases, β-glucosidase
Bagasse	*T. reesei, A. niger, A. phoenicus*	Xylanase
Wheat straw	*Phlebia radiate, P. eryngii*	Xylanase, laccase, aryl-alcohol oxidase
Wheat bran	*Bacillus licheniformis*	Xylanase
Wheat bran + bagasse	*A. niger*	Xylanase
Wheat bran defined media	*P. chrysosporium*	Xylanase, β-xylosidase
Wheat straw	*P. sajor-caju*	Xylanase, cellulase, β-glucosidase
Rice straw + cotton stalks + kenaf	*L. edodes*	Xylanase, laccase, β-xylosidase
Lingo-cellulosics	Bacterial isolate B693	Xylanase
Rice straw, soybean hull, wheat bran, birchwood, oat spelt, kraft pulp, beet pulp	*A. sojae*	Xylanase, α-arabinofuranosidase
Wheat/rice straw, bagasse, rice husk	*Melanocarpus albomyces*	Xylanase, acetoesterase
Wheat bran, apple pomace, sugar beet pulp, wheat straw	*Chaetomium globusum, A. niger*	Xylanase
Rice straw, corn hull, corncobs, bagasse, wheat bran	*A. fumigatus*	Xylanase, xylosidase

Table 4.28. Continued.

Substrate	Microorganisms	Enzyme
Wheat straw	*P. sanguineous*	Xylanase, CMCase, catecholoxidase
Wheat bran	*Thermomyces lanuginosa (Humicola lanuginosa) Thermoscus*	Xylanase
Wheat bran	*Humicola sp.*	Xylanase
Apple pomace	*A. niger, A. fumigatus, T. viride*	Xylanase, CMCase
Coffee processing plant waste	*Thermomonospora sp.*	Xylanase, cellulose, α-arabinofuranosidase, β-xylosidase
Solka floc, wheat bran, barley straw, oat straw, wheat straw	*Talaromyces emersonii*	Xylanase
Wheat bran	*A. awasori*	β-xylosidase
Wheat bran	*T. harzianum*	β-xylosidase
Wheat bran	*A. terreus*	β-xylosidase
Wheat bran	*A. fumigatus*	β-xylosidase, β-glucosidase
C-lignin (perlite)	*P. ostreatus*	Laccase
Wheat straw	*Pleurotus sp.*	Laccase, Mn-peroxidase
Bagasse	*P. chrysosporium* and 44 species of *Basidiomycetes*	Laccase, Li-peroxidase, Mn-peroxidase
Synthetic media	*P. floridae*	Laccase, Li-peroxidase, Mn-peroxidase
C-lignin labeled wheat straw	*P. chrysosporium* and four species of *Pleurotus*	Mn-peroxidase, laccase, aryl-alcohol oxidase
Synthetic media	*P ostreatus*	Mn-peroxidase, laccase, catalase
Wheat straw	*Phlebia radiate*	Laccase, Li-peroxidase, Mn-peroxidase
Bagasse	*Trametes versicolor*	Laccase, Mn-peroxidase,
Bagasse	*Flammulina velutipes*	Phenol oxidase
Wheat straw	*Pleurotus sp.*	Laccase, aryl-alcohol oxidase
Wood chips	*P. chrysosporium*	Laccase, Li-and Mn-peroxidase
Wheat straw	*Penus tigrinus, Phlebia radiata*	Laccase, Li-peroxidase, Mn-peroxidase
Cotton stocks	*P. chrysosporium, P. ostreatus*	Laccase
Saw dust	*Rigidoporus lignosis*	Mn-peroxidase
Wheat straw	*Penus tigrinus*	Laccase, Mn-peroxidase
Wheat straw, kraft lignin, cellulose powder	*T. versicolor, P. ostreatus*	Laccase, Li-peroxidase, Mn-peroxidase
Bagasse	*Polyporus sp.*	Laccase
Soybean floor, sunflower flour, coffee husk	*Penicillium citrinum*	Protease
Soybean meal	*Bacillus amyloliquefaciens*	Alkaline protease
Wheat bran	*A. niger*	Acid protease
Wheat bran	*Bacillus licheniformis*	Neutral protease, α-amylase
Rice bran	*Rhizopus oligosporus*	Acid protease

Table 4.28. Continued.

Substrate	Microorganisms	Enzyme
Rice bran	*R. oligosporus*	Protease
Wheat bran	*B. amyloliquefaciens*	Protease
Wheat bran	*Pseudomonas. sp.*	Alkaline protease
Polyurethane foam	*A. oryzae*	Alkaline protease
Wheat bran + bean cake	*A. niger*	Acid protease
Wheat bran, soybean, rice, rice bran	*R. oligosporus*	Protease
Wheat bran	*A. niger*	Acid protease
Wheat bran + rice bran	*T. koningii*	Alkaline protease
Aspen wood	*P. chrysosporium*	Acid protease
Wheat bran, rice bran, corn bran, rice hull	*A. oryzae*	Protease
Wheat bran	*A. flavus*	Alkaline protease
Sweet potato residue	*A. niger*	Acid protease
Wheat bran	*A. flavus*	Alkaline protease
Wheat bran	*Actinomycetes* strains	Protease
Waster hair	*Streptomyces sp.*	Protease
Peanut press cake	*Neurospora sitophila, R. oligosporus*	Lipase
Wheat bran	Several filamentous fungi	Lipase
Coconut oil cake	*Candida rugosa*	Lipase
GYP medium	*A. oryzae*	Lipase
Amberlite	*R. delemer*	Lipase
Rice bran, wheat bran	*Candida sp.*	Lipase
Wheat bran	*P. candidum*	Lipase
Rice bran	*C. rugosa*	Lipase
Wheat bran	*A. niger*	Polygalacturonase
Coffee pulp	*A. niger*	Polygalacturonase
Citrus waste	*A. foetidus*	Polygalacturonase
Apple pomace	*A. foetidus*	Polygalacturonase
Wheat bran	*A. oryzae*	α-galactosidase
Wheat bran	*A. niger*	α-galactosidase
Soybean cake residue	*A. oryzae*	β-galactosidase
Wheat bran	*A. fonsecaeus*	β-galactosidase
Wheat bran	*Rhizomucor sp.*	β-galactosidase
Wheat bran	*Kluyveromyces lactis*	β-galactosidase
Polystyrene	*Vibrio costicola*	ι-glutaminase
Wheat bran, rice husk, saw dust, coconut oil cake	*Vibrio costicola*	ι-glutaminase
Starch waste	*B. megaterium*	β-amylase
Wheat bran	*Pycnoporus sanguineus*	α-amylase
Banana waste	*Aeromonas caviae*	α-amylase
Polyurethane foam	*A. oryzae*	α-amylase
Wheat bran	*A. kawachi*	α-amylase
Banana waste	*B. subtilis*	α-amylase
Corn flour, wheat flour, potato, sweet potato	*Saccharomycopsis capsularia*	α-amylase, glucoamylase
Wheat bran	*B. coaglans*	α-amylase

Table 4.28. Continued.

Substrate	Microorganisms	Enzyme
Wheat bran	*B. licheniformis*	α-amylase
Rice bran	*A. oryzae, A. niger*	α-amylase, glucoamylase
Wheat bran	*A. niger*	Glucoamylase
Urethane foam	*A. oryzae*	Glucoamylase
Wheat bran	*A. awamori*	Glucoamylase
Rice bran, soybean meal	*A. niger*	Glucoamylase
Rice bran, defatted soybean meal	*A. niger*	Glucoamylase
Copra waste	*A. niger*	Glucoamylase
Wheat bran	*Rhizopus* sp.	Glucoamylase
Rice bran + cassava starch + rice hulls	*Aspergillus* sp.	Glucoamylase
Rice	*Amylomyces roaxii*	Glucoamylase
Cassava	*Rhizopus* sp.	Glucoamylase
Rye meal + beet pulp	*A. oryzae, A. awamori*	Glucoamylase, α-amylase
Cassava starch/kappa carrageenum	*R. oligosporus*	Glucoamylase
Chicory roots, wheat bran	*Staphylococcus* sp. *Kluyveromyces marxiamus*	Inulinase
Canola meal	*A. ficuum, A. carbonarius*	Phytase
Wheat bran + tannic acid	*R. oryzae*	Tannase
Sugar beet pulp	*T. reesei*	α-ι-arabinofuranosidase
Wheat bran	*Acremonium strictum*	Glucooligosaccharide oxidase
Wheat straw	*Pencillium pinophilum*	Feruloyl-para-coumareyl, esterase

4.7.4 Solid-State Fermentation Parameters

Pandey et al. (1999) and Pandey (2003) stated that the selection of process parameters and their optimization are important in solid-state fermentation particularly for enzymes production. These parameters include: (a) selection of suitable substrate and culture organism, (b) age and size of the inoculum, (c) period of cultivation, (d) pretreatment of the substrate, (e) moisture content and water activity, (f) pH, (g) temperature control and removal of the metabolic heat, (h) maintenance of uniformity, (i) aeration, (j) supplementation of nutrients such as N, P and trace elements or supplementation of additional carbon source and inducers and (k) extraction of product and its purification, etc.

4.7.4.1 Pretreatment of Substrate. Sometimes, natural solid substrates require sort of pretreatment such as size reduction, cooking/sterilization, nutrient supplementation, and

chemical or enzymatic hydrolysis in order to make their chemical constituents more accessible and their physical structure more susceptible to mycelial penetration (Lonsane et al., 1985; Mudgett, 1986; Mitchell et al., 1992; and Pandey et al., 1999).

4.7.4.1.1 Particle Size: Particle size of the substrate is very important since it affects the surface area to volume ratio as well as the packing density. The surface area to volume ration determines the fractions of the substrate, which is initially accessible to the microorganisms. This is of a great importance when the substrate is to be preserved by the microbial action. Loose packing densities are important in solid-state fermentations in order to enable diffusion of oxygen (Mitchell et al., 1992; Zakaria, 1997; and Pandey et al., 1999).

Generally, smaller particle sizes provide a larger surface area for microbial attack, heat transfer and gas exchange and higher surface nutrient concentrations and shorter pathways for diffusion of nutrients. However, smaller particle sizes result in closer packing densities of the substrate and reduction of the void space between particles, which tends to reduce the area for heat transfer and gas exchange with the surrounding atmosphere resulting in a lower respiration/aeration efficiency, unless the particles can be sufficiently agitated to provide a high degree of particle separation (Mudgett, 1986; Krishna and Chandrasekaran; and Pandey et al., 1999). Therefore, a lower and upper limit of particle size should be addressed according to heat transfer and gas exchange limitation and nutrient transfer limitation, respectively. Particle size reduction methods (such as milling, grinding, rasping and chopping) are costly (Mudgett, 1986; and Zakaria, 1997).

The largest size which allows an acceptable fermentation is probably the best and this is basically varied for different systems Mudgett (1986). Pandey (1991b) found an increase in glucoamylase enzyme productivities by *Asperigillus niger* with substrate (commercial wheat bran) of mixed particle sizes in the range of smaller than 180 μm to larger than 1.4 mm. Kumar and Lonsane (1987) reported increase in Gibberellic acid production in coarse wheat bran probably because of larger void fraction and better oxygen transfer and

CO_2 removal. Krishna and Chandrasekaran (1996) reported an optimum particle size of 400 μm for the production of α-amylase from banana waste using *Bacillus subtilis* in solid-state fermentation. However, Chakraborty et al. (1995) reported no difference in acid protease production for different wheat bran sizes. Zakaria (1997) studied the effect of particle size on lactic acid solid-state fermentation for chitin purification from shellfish waste and reported that mincing shellfish waste (6 mm average diameter) promotes good fermentation resulting in substantial level of chitin purification and that large particle sizes promotes the growth of spoilage organisms.

During solid-state fermentation, a reduction in the volume of the substrate (substrate shrinkage and voidage decrease) might take place as a result of: (a) compression of the mass, (b) changes of the physical structure of the substrate and (c) reduction in the water activity as a result of water evaporation and utilization of some of the substrate components by the microbes. Ghildyal et al. (1993) reported a reduction of 10-15% of the substrate after 60 h.

4.7.4.1.2 Cooking/Sterilization: Most bacterial species require high moisture content (greater than 40%). Therefore, most solid-state fermentations are less susceptible to bacterial contamination and require no sterilization. However, in fermentations at higher moisture levels, mild thermal processes may be required for sterilization of the solid substrate (Mudgett, 1986).

Chakraborty et al. (1995) studied the effect of different wheat-bran sterilization times (20, 40 and 60 min) on the production of acid protease from *Asperigillus niger*. They found that 40 min sterilization at 103.4 kPa gave better microbial growth and acid protease production as a result of the easy availability of starch. Lonsane et al. (1985) reported that sterilization may change the texture of the substrate.

Rathbun and Shuler (1983) stated that cooking of soybeans in an autoclave for 10 min at 120 °C sterilized the beans and at the same time destroyed the trypsin inhibitor inherent in the beans that is believed to adversely affect the growth of the mold during tempeh

fermentation. Maziero and Zadrazil (1994) studied the effect of different heat pretreatment of wheat straw on the microbial activity and colonization of different edible mushrooms. They reported good growth and high speed of colonization on sterilized wheat straw. They also stated that heat pretreatment at 60-90 °C of cereal straw is sufficient for commercial cultivation processes.

Krishna and Chandrasekaran (1996) studied the effect of cooking time (0-120 min) and temperature (100-130 °C) on the production of α-amylase from Banana waste using *Bacillus subtilis* during solid-state fermentation. They obtained a maximum production of α-amylase when the substrate was cooked for 60 min at 121 °C and reported that an either increase or a decrease in both cooking time and cooking temperature significantly reduce the yield. Gupte and Madamwar (1997) reported improvement in the production of celluloytic enzymes by a coculture of *Aspergillus ellipticus* and *Aspergillus fumigatus* grown on sugarcane bagasse in solid-state fermentation when steam-treat the substrate.

Teng et al. (2001) conducted a study on chitin production using *Aspergillus niger* grown on shrimp shells in solid-state fermentation. They reported that autoclaving of shrimp shells prior to the fermentation process causes degree of deproteinization (56.06±3.14%), releasing hydrolyzed proteins into the medium. Wang and Chio (1998) reported that high cooking temperatures causes low protein removal efficiency during deproteinization of shrimp and crab shells with the protease of *Pseudomonas aeruginosa* K-187. They explained this as a result of the Maillard reaction associate with cooking at high temperatures that renders the proteins resistance to deproteinization.

4.7.4.1.3 Nutrient Supplementation: Pandey et al. (1999) reported that substrates that provide all the needed nutrients to the culture organisms are considered ideal substrates. However, in some cases some of the nutrients may be available in suboptimal quantities and/or even absent. In this case, therefore, the substrate should be supplemented externally with nutrients. Nutrient supplementation initiates biomass production, induces enzyme synthesis, provides balanced growth conditions, or in some cases prolongs secondary metabolite production Mudgett (1986).

Chakraborty et al. (1995) reported enhancement of acid protease production from *Aspergillus niger* grown on wheat bran in solid-state fermentation when the substrate was supplemented with different organic nitrogen sources such as casiton, casein, peptone and trader's protein. However, they stated that the addition of different carbon sources such as D-glucose, sucrose, maltose, lactose and fructose inhibited the protease production indicating catabolite repression on protease syntheses. Pandey (1990b) stated that addition of trace elements as well as nitrogen increase the yield of glucoamylase from wheat bran during solid-state fermentation. He also reported further enhancement in the enzyme production by adding 5% and 10% (dw) whole corn (yellow) flour to the substrate. Kumar and Lonsane (1987) stated that adding soluble starch to commercial wheat bran enhances the accumulation of Gibberellic acid in solid-state fermentation to an extent of 3.5 times.

Teng et al. (2001) reported that shrimp shells are able to provide the fungus *A. niger* with its nitrogen, minerals and trace nutrients requirements. However, a carbon source such as D-glucose or any other suitable carbon source should be added to the fermentation medium. Rattanakit et al. (2002) realized increase in chitinase activity during solid-state fermentation of shellfish waste using *Aspergillus* species when replacing part of the shellfish waste substrate with corncob (40%). They stated that the enhancement in the chitinase activity could be due to improvement in conditions or supply of certain nutrient(s). They also reported that the addition of ammonium sulfate (optimum of 0.1%) as a nitrogen source increased the enzyme production while the addition of peptone, yeast extract, and urea had a repressive effect.

Rodriguez et al. (1985) stated that the growth of *Aspergillus niger* grown on citrus peel was limited when no nitrogen source was added to the substrate. However, addition of ammonium sulfate has a significant effect on the specific growth rate.

Smith et al. (1986) realized enhancement in fungus growth as well as protein production during solid-state fermentation of cassava using *Sporotrichum pulverulentum* when supplementing the substrate with mineral salts solution of high ionic strength.

4.7.4.1.4 Chemical and Enzymatic Hydrolysis: Han and Anderson (1975) studied the effect of hydrolysis on fermentation of ryegrass straw using *Candida utilis*, *Aureobasidium pullulans* and *Trichoderma viride* for the production of nutritional valuable animal feed. They compared substrate hydrolysis by 0.5 N H_2SO_4 at different temperatures (121, 100 and 25 °C) and found that the best results in terms of increasing protein and straw digestibility were obtained when *C. utilis* was grown on straw preheated at 121 °C.

Gupte and Madamwar (1997) examined the effect of pretreatment with alkalis such as 2% $Ca(OH)_2$ and 1M and 5M NaOH and acids such as 1N and 5N HCL and H_2SO_4 on the enzymatic production of coculture of *Aspergillus ellipticus* and *Aspergillus fumigatus* grown on sugarcane bagasse. They reported that bagasse pretreated with 2% (w/v) calcium hydroxide gave the maximum enzymatic production.

Patel et al. (2004) reported significant increase in bacterial growth as a result of enzymatic hydrolysis of oats for the production of probiotics using *Lactobacillus plantarum* in solid-state fermentation. They stated that the enzymatic hydrolysis increased nutrient accessibility by breaking down the polysaccharides into oligosaccharides, disaccharides and monomers and proteins into amino acids and short peptides.

4.7.4.2 <u>Age and Size of Inoculum</u>. It is important to optimize the inoculum density in solid-state fermentation since low inoculum density allows for the growth of undesirable organisms while high inoculum density causes depletion of the substrate, which is necessary for product formation Mudgett (1986). Spores are usually used in inoculum development or as seed in solid-state fermentation processes (Bapat et al., 2003). The required spore quantity ranges from 10^6 to 10^7 spores per milliliter and depends on the type of fermentation as well as the product. For laboratory-scale processes, spores are produced on slants, Petri dishes or Ferbach flasks with suitable chemically defined media. Large-scale production of spores can be achieved using natural or inert support materials. Sometimes vegetative inoculum is required for solid-state fermentation because the spore

production of some fungal strains is limited on plate cultures (Krishna and Nokes, 2001). Yadav (1988) reported no remarkable effect on the bioconversion performance during solid-state fermentation when using different types of inocula (broth culture, mycelial fragment suspension, agar plugs and grain cultures).

Contradicting results were reported in the literature regarding the effect of inoculum size and age on the fermentation process. Krishna and Chandrasekaran (1996) reported that inoculum to substrate ratio (v/w) had a significant effect on α-amylase production from banana waste using *Bacillus subtilis* in solid-state fermentation. They reported an optimum inoculum size of 10 % and stated that inoculum ratios less or higher than the optimum ratio significantly affect the enzyme yield. Nair and Duvnjak (1990) studied the effect of different vegetative inoculum concentrations of *Aspergillus ficuum* on the reduction of phytic acid content of canola meal in solid-state fermentation. They reported increase in phytic acid hydrolysis rate with the increase in inoculum concentration. Raimbault and Alazard (1980) reported rapid initial growth when using high concentrations of inoculum, however, the growth was limited after 30 h fermentation time. By a microscopic examination of the product after 30 h, they found a large number of non germentating spores. Pandey (1990b) also reported no significant difference in the overall amyloglucsidase production when using high concentrations (10% and 15%) of inoculum, however, the initial rate of enzyme production was higher. They reported a suitable inoculum concentration of 5% (w/w, wet wt of inoculum dough/ wet wt of substrate) for the production of amyloglucsidase from wheat bran using *Aspergillus niger*. Krishna and Nokes (2001) reported no significant effect of vegetative inoculum size of *Aspergillus niger* on phytase production during solid-state fermentation of mixture of wheat bran and full-fat soybean flour for fermentation times higher than 48 h. they also reported no significant effect of inoculum age on phytase yield at maximum production periods. Chakraborty et al. (1995) reported no significant change in acid protease production from *Aspergillus niger* grown in solid-state fermentation occurred as a result of culture age variation.

4.7.4.3 Moisture Content. Moisture is the most critical factor for the growth of microorganisms in solid-state fermentation since water supply is always limited. Rattanakit et al. (2002) stated that water content in solid-state fermentations is an important parameter for both microbial growth and enzyme production and that the control of moisture level within a relatively narrow range is necessary for optimizing solid-state fermentation processes. Generally, in solid-state fermentation, water exists in three states: (a) monolayer region, in which water is tightly bounded to the solid surface, (b) multilayer region, in which water is less tightly bounded and (c) free water. The control of moisture level, which is specific for each microbe-substrate system, within a relatively narrow range is, therefore, necessary for optimizing solid-state fermentation processes. Low moisture content causes low accessibility of nutrients resulting in poor microbial growth while high moisture content can cause aggregation of the solid substrate and decreases substrate porosity and hence prevents oxygen penetration, which help bacterial contamination (Knapp and Howell, 1980; Pandey, 2003; and Asagbra, 2005). Hesseltine (1965) stated that successful solid-state fermentation can be achieved by the proper moisture content, which allows mold growth but not contaminating bacteria. A properly moistened substrate should have enough water to facilitate dissolution and mass transfer of nutrients and oxygen and at the same time the inter-particle channels left free to facilitate oxygen diffusion and heat dissipation. In some cases moisture also regulates product formation; higher moisture favors the formation of certain product while lower moisture favors the formation of other product (Tengerdy, 1985). Hesseltine (1972) stated that the optimal level of moisture content is specific for each microbial species and probably for each strain as well. Lonsane et al. (1985) reported that moisture content in solid-state fermentation ranges from 30 to 75%. Chakraborty et al. (1995) reported on the significant effect of moisture content of the substrate on production and realized a confound effect of both temperature and moisture content probably because of the effect of temperature on evaporation.

Moisture content is usually expressed as water activity factor (a_w). Water activity can be defined as the relative humidity of the gaseous atmosphere in equilibrium with the substrate (Oriol et al., 1988; and Pandey, 1992). Most bacterial species grow at higher

water activity values while filamentous fungi and yeasts can grow at lower water activity values (usually in the range of 0.6-0.7). Generally, the microorganisms that are capable of growing and carrying out their metabolic activities at lower water activity values are suitable for solid-state fermentation. Smith and Onions (1994) and Ray (1996) reported a water activity of 0.77 at temperature of 35 °C for *A. niger*. Krishna and Chandrasekaran (1996) reported optimum initial moisture content of 70% for the production of α-amylase from banana waste using *Bacillus subtilis* during solid-state fermentation. Rattanakit et al. (2002) reported optimum moisture content of 58 – 65% for the production of chitinase using a fungus belongs to *Aspergillus* grown on shrimp shellfish waste and stated that the productivity declined significantly at water content of 50% or below. Lonsane et al. (1985) reported optimum water content of 46 to 56% for *Aspergillus niger* and stated that the productivity decreased significantly at water content less than 37% or more than 67%. Ghildyal et al. (1994) reported that an initial moisture content of 51% was optimum for *A. niger* when grown on wheat bran. Asagbra et al. (2005) reported an optimum moisture content of 68% for the production of tetracycline by *Streptomyces* strains during solid-state fermentation of peanut shells. Grajek (1988) stated that small variation around the optimal water activity value could result in significant reduction in the growth and metabolism of fungi.

4.7.4.4 Temperature. Temperature is a significant parameter in solid-state fermentation (Knapp and Howell, 1980; and Mudgett, 1986). It is one of the most important environmental parameters that affects the growth and activities of microorganisms (Zakaria, 1997). However, during solid-state fermentation temperature is hard to control because of the difficulty in measuring the temperature as a result of the low moisture content of the substrate (Mudgett, 1986).

During solid-state fermentation and due to the microbial activity (respiration or oxidation of sugars) a large amount of heat is generated in the fermentation solids, thus causing heat accumulation (Grajek, 1988; Saucedo-Castañeda et al., 1990; Pandey, 1990a; Pandey, 1990b; Pandey, 1992; and Ghildyal et al., 1993). Thermal characteristics of organic material as well as the low moisture content required for solid-state fermentation

create difficult conditions for heat transfer. About 2511.48 kJ/kg solids of heat generation have been reported in several solid-state fermentations for the production of enzymes, biomass, and acids (Aidoo et al., 1982).

Several authors reported temperature gradient in bioreactors. Pandey (1990b) reported an increase in the substrate temperature up to 41 °C during solid-state fermentation of wheat bran in flask using *A. niger* for the production of glucoamylase enzyme. Ghildyal et al. (1993), Ghildyal et al. (1994) and Pandey (2003) reported that, in case of packed bed bioreactors under some operating conditions, temperatures in the middle of the bed can reach about 20 °C higher than the temperature of the inlet air. Rathbun and Shuler (1983) reported 3 °C/cm temperature gradient during active mold growth with bed depth as low as 6.35 cm during tempeh production from soybeans using *Rhiopus oligosporus*. Saucedo-Castañeda et al. (1990) reported an axial temperature gradient of 0.17 °C/cm and a radial temperature gradient of 5 °C/cm in their packed-bed bioreactor during the fermentation of cassava using *A. niger*. Several reasons can explain the temperature gradient in solid-state fermentations: (a) low thermal conductivity of the substrate, (b) low moisture content, (c) heat entrapment in the small voids between the solid substrate particles, (d) low porosity of the moist solid substrate bed and (e) pressure drop in the flow of cooling air in case of packed bed reactors.

Heat build-up during fermentation causes evaporation of water, stops vegetative growth and induces protective, but nonproductive sporulation of moulds (Tengerdy, 1985). Generally, the heat accumulation causes variations in the growth of the microorganism, spore formation and germination, and product formation (Pandey, 2003). Therefore, heat removal is a major consideration in the design of solid-sate fermentation bioreactors (Mitchell, et al., 2000). In submerged fermentation heat removal is most likely done by a water jacket. However, in case of solid-state fermentation this might not be the best solution since the solid substrate usually has poor thermal properties, which may also cause a steep temperature gradient even with a water jacket. A cooling coil embedded in the bed is considered a better solution compared to water jacket in this case. In drum bioreactors, drum rotation as well as substrate loading significantly affect substrate

temperature build up. Grajek (1988) stated that in practice only air can be used for both temperature and humidity control during solid-state fermentation and therefore the amount of air required is usually higher than that is usually required for microbial respiration.

4.7.4.5 pH. Most organisms will retain viability at pH 7, but cells will remain viable over a range of several pH units (Mittal, 1992). Several organisms produce different metabolites at different pH conditions, and in such cases better pH control is needed for optimum yield. Filamentous fungi vary in their pH requirements. However, most common fungi grow well over the pH range of 3 to 7, although some species such as *A. niger* can grow at pH 2 and below (Smith and Onions, 1994).

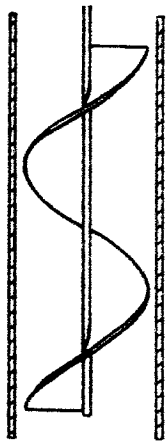
An optimum pH in the range of 4.5 to 6.5 and a maximum pH in the range of 9.8 to 10.5 have been reported for *A. niger*. Teng et al. (2001) stated that when growing *A. niger* with shrimp shell powder, the fungus releases extracellular protease, which causes the deproteinization of the shells and the release of hydrolyzed proteins in the fermentation medium. The fungus is able to utilize the hydrolyzed proteins as a nitrogen source for growth causing reduction in the pH of the fermentation medium. They reported a pH reduction between 3 and 6 during the fermentation. O'Donnell et al. (2001) stated that *A. niger* normally tends to quickly acidify its medium to a pH between 2 and 3 during the first two days and remain there afterwards when grown without pH control.

Low pH is considered an advantage during the purification of chitin from shrimp shells since: (a) it promotes the proteolytic hydrolysis of the protein associated with the shells by the secreted extracellular protease and (b) it enhances the demineralization process of the shells to a certain degree. O'Donnell et al. (2001) reported that biomass profile of *A. niger* does not change significantly from pH 3 to pH 6 (maximum biomass of 5.1 g/L at pH of 3 vs. 4.4 g/L at pH of 6). However, the biomass concentration (1.9 g/L) was reduced significantly at pH of 7.

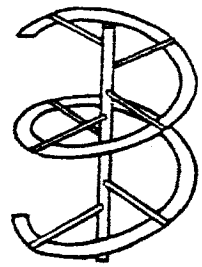
The protease activity on the other hand is strongly affected by pH. O'Donnell et al. (2001) stated that protease activity is the highest at pH of 3 with a large decline in activity when the pH reached 6. Chakraborty et al. (1995) reported marginal increase in acid protease production of *Aspergillus niger* grown on wheat bran in solid-state fermentation when increasing the initial pH of the medium from 4.0 – 6.0.

4.7.4.6 Agitation. In solid-state fermentation processes, agitation has the advantages of: (a) effectively distributing the inoculum, (b) maintaining homogeneity, (c) preventing the formation of mycelial pellets, (d) improving aeration, (e) facilitating heat transfer and hence preventing localized overheating of the substrate, (f) facilitating uniform distribution of any additives introduced into the bioreactor during the fermentation process and (g) depending on the type of the substrate agitation might enhance or prevent the agglomeration of solid substrate (Knapp and Howell, 1980; Lonsane et al., 1985; Mudgett, 1986; Mitchell et al., 1992; Zakaria, 1997; and van de Lagemaat and Pyle, 2001). Hesseltine (1972) investigated the importance of agitation for the production of secondary metabolites in solid-state fermentations. Hall and Reid (1995) emphasize on the importance of mixing during chitin purification from shrimp shells using lactic acid fermentation. They stated that a good mixing is necessary to: (a) prevent pockets of high pH material, and (b) help in the removal of carbon dioxide generated in the fermentation and hence preventing the growth of spoilage organisms. Paddle impellers are successfully used in submerged fermentations. However, in case of solid-state fermentation paddle impellers fail to provide sufficient mixing since they do not provide axial mixing. More complex designs of mixing shafts such as helical ribbon, helical screw or a combination between helical ribbon and screw are required for solid-state fermentation in order to provide axial mixing of the solid substrate (Figure 4.23).

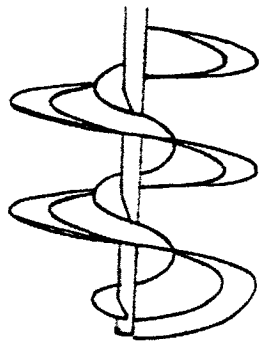
The requirements for agitation in solid-state fermentation depend on number of factors including: the type of the process, the reactor design and the product concerned. Solid-state processes can be static, periodically agitated or continuously agitated (Mitchell et al., 1992). Periodic agitation is functioning to replenish the inter-particle space with fresh air and also affects the growth of microorganisms and hence the production of metabolic



(a) a helical screw with a draft tube



(a) a helical ribbon



(c) a combination of helical ribbon and screw

Figure 4.23. Types of mixers used in solid-state fermentation (Zakaria, 1997).

heat. The frequency of mixing required depends on substrate characteristics such as particle size and shape. Enzymes production is greatly enhanced by agitation. However, processes involving filamentous organisms will require the use of intermittent rather than continuous agitation to prevent damage and disruption of mycelial attachment to solids by shearing. Tengerdy (1985) also reported that helical screw agitator is a promising design since it avoids substrate compaction and at the same time does not damage the mycelia by shearing and provides throughout mixing. van de Lagemaat and Pyle (2001) reported on the deleterious effect of mixing in a fermenter rotating horizontally. They stated that rotation rate as low as 0.7 rpm caused lower biomass and product yield compared to static culture. On the contrary, Aidoo et al. (1984) questioned the

importance of mycelial damage in affecting growth in agitated solid-state fermentation systems. They claimed that agitation enhances growth initially leading to excess heat accumulation which is the main factor responsible for negatively affecting the fermentation process.

4.7.4.7 Aeration. Molds are aerobic. They also can grow as microaerobes. Ghildyal et al. (1992) reported that low oxygen concentration adversely affect enzyme production during solid-state fermentation. Aeration is also one of the most important parameters for both microbial growth and removal of heat build up in the fermentation medium. Ghildyal et al. (1994) reported that heat build up is very high in case of low air flow rates compared to high flow rates. They stated that in case of using 750 g of wheat bran in packed bed fermentor (150 mm diameter and 345 mm height), low flow rate (5 L/min) resulted in temperature gradient of 0.79 °C/cm, while high flow rate (25 L/min) reduced the gradient to 0.13 °C/cm.

Higher air flow rates might decrease the temperature gradient but might also negatively affect the productivity as a result of: (a) low microbial activity (spore germination, growth, metabolism and sporulation), (b) channeling of the substrate and (c) drying of the substrate due to high evaporation rate. Ghildyal et al. (1994) reported a reduction of moisture content from 51% to 22% when using air flow rate of 25 L/min in packed bed fermentor (150 mm diameter and 345 mm height) filled with 750 g wheat bran. Pandey and Radhakrishnan (1992) studied the effect of aeration rate (0.10, 0.12, 0.15 L/min) on glucoamylase enzyme production during solid-state fermentation using packed-bed bioreactor of 930 mL and packing density of 0.35 g/mL. They reported an optimum aeration rate of 0.1 L/min. Grajek (1988) reported minimum aeration rate of 3 to 4 m^3 cooling air/h.kg medium and of 1 to 2 m^3 cooling air/h.kg medium in order to remove the metabolic heat generated during the fermentation process and at the same time keep the optimum moisture content conditions for mesophilic and thermophilic fungi, respectively.

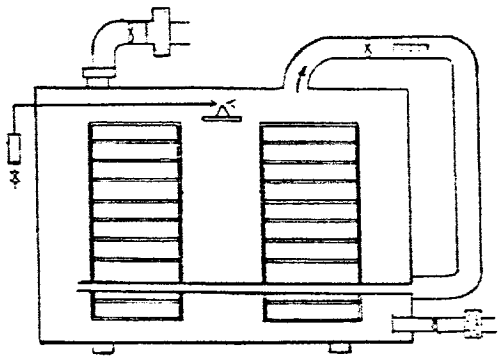
4.7.5 Solid-State Fermentation Bioreactors

As in any fermentation process, the bioreactor is responsible for attaining the optimal environmental conditions for the growth and activity of the culture organism, which carry out the biological reaction (Pandey, 1991a). Generally, the bioreactor should have several characteristics: (a) it should be constructed from non-toxic material that can withstand hydraulic and pneumatic pressures and chemical corrosion, (b) it has to provide proper aeration, agitation, and ports for charging, sampling and discharging, (c) cooling system may be required to remove metabolic heat and (d) it should be able to operate under aseptic conditions. Although, extensive studies have been made on liquid or submerged state fermentation bioreactors, limited information is available for solid-state fermentation bioreactors.

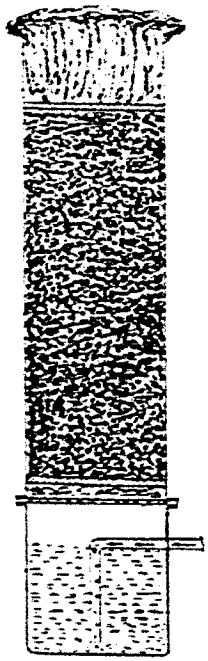
Most solid-state fermentations involve batch culture since the effective implementation of fed-batch or continuous techniques at large scale requires the application of automated solids-handling techniques. Solid-state fermentation bioreactors include tray, packed bed, stirred and drum fermenters (Figure 4.24). Pandey (1991a) summarized some types of solid-state fermentation bioreactors that were used for several fermentation processes as shown in Table 4.29.

4.7.5.1 Tray Bioreactor. Tray type bioreactors consist of wooden, metallic (usually aluminum or iron) or plastic shallow trays arranged one above the other with suitable gap size. The bottom part of each tray is usually perforated in such a way that it holds the substrate material and at the same time allows aeration of the undersurface of the substrate. The tray arrangement is placed in a chamber in which the environmental conditions are controlled. The temperature of the substrate is usually controlled by circulating warm or cool air as required.

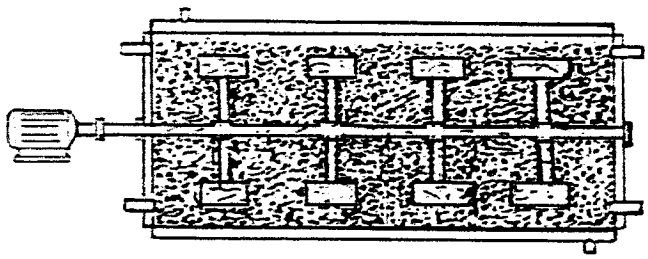
Tray type bioreactor is the simplest type of solid-state fermentation bioreactors. However, it needs a large operational area, is labor intensive, is difficult to be automated and is described as high cost bioreactor (Lonsane et al., 1985; Pandey, 1991a; and Ghildyal et al., 1994). Tray type bioreactors have been used since ancient time for the



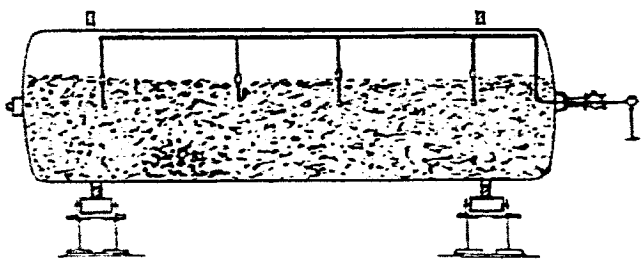
Tray bioreactor



Packed-bed bioreactor



Stirred bioreactor



Drum bioreactor

Figure 4.24. Solid-state fermentation bioreactors (Mitchell et al., 1992).

Table 4.29. Types of solid-state fermentation bioreactors (Pandey, 1991a).

Type	Process	Microorganism
Wooden trays	Koji fermentation	*A. oryzae*
Rotating drum	Composting of garbage	-
Rotating drum	Koji fermentation	*A. oryzae*
Rotating drum	Enzyme production	*A. oryzae*
Fernbach flask	Aflatoxins production Fungal spores Enzyme production	*A. oryzae*
Horizontal vessel (4 chambered)	Ochratoxin production	*A. Ochraceus*
Horizontal drum (3 chambered)	Continuous, using feedlot wastes and corn	-
Cement mixer	Corn fermentation	-
Cement mixer	Straw fermentation	*Trichoderma, Candida*
Bread making blender	Protein enrichment of starchy materials	*A. niger*
Horizontal vessel with paddle	Koji fermentation	*A. oryzae*
Valmic bags (polyethylene porous films)	Mycotoxins	*A. flavus*
Fixed-bed differential reactor	Spore production	*P. roqueforti*
Stainless steel box kiln	Protein enrichment	*T. viridae*
Aluminium trays	Enzyme production	*A. niger*
Packed bed column	Enzyme production	*A. niger*
Packed bed column	Heat transfer simulation	*A. niger*
Tubular	Ethanol, feed protein	*Saccharomyces cerevisiae*
Pits	Feed protein	*Coprinus fimetarius*
Pot fermenter	Feed protein	*Chaetomium cellulolyticum*
Hollow shaft horizontal	Fungal protein	*T. reesei* *Endomycopsis fibuliger*

production of traditional fermented foods such as soy sauce Koji (in Japan and South East Asia), Miso (in Japan, China, Taiwan, The Philippines, Indonesia and the Orient) and tempeh (in Indonesia, New Guinea and Surinam). Smith et al. (1986) designed a tray fermenter in which the bottoms were made of stainless steel screen to provide better gas exchange. Pandey (1990b) used tray type fermenter for the production of glucoamylase enzyme from cooked wheat bran mixed with whole corn flour and mineral solution using *A. niger* in solid-state fermentation. Ghildyal et al. (1992) used a tray bioreactor to study the relationship between gaseous concentration gradient and enzymatic activity at different bed depths of different initial substrate bed heights during the production of

amyloglucosidase production by *A. niger* grown on mixture of wheat bran and corn flour. Daubresse et al. (1987) used tray bioreactor for protein enrichment of cassava in solid-state fermentation using *Rhizopus oryzae*.

4.7.5.2 Packed Bed Bioreactors. The packed bed bioreactor consists of a glass or a plastic column in which both ends are enclosed. The column is filled with the substrate and supported on a perforated plate through which air is admitted. In this type of fermenters, temperature can be controlled by water circulation through a water jacket surrounding the column or by placing the whole column in a temperature controlled water bath (Lonsane et al., 1985; and Pandey, 1991a). Packed bed bioreactors are relatively simple while allowing better process control compared to tray bioreactor (Mitchell et al., 1992). However, difficulties may arise while emptying the bioreactor at the end of the fermentation process (Lonsane et al., 1985). Ghildyal et al. (1994) mentioned that packed-bed bioreactors with forced aeration allow the bioreactor to operate in a higher substrate bed thickness with much lower temperature and oxygen concentration gradients than tray bioreactors, which leads to an increase in the fermentation process productivity.

Rodriguez et al. (1985) used a packed bed bioreactor to carry out solid-state fermentation of citrus peel by the fungus *A. niger*. Gonzalez et al. (1988) used packed-column reactor for the production of penicillin by solid sate fermentation. Pandey and Radhakrishnan (1992) developed a horizontal packed-bed bioreactor (length/diameter 3:1, volume 930 mL) for the production of glucoamylase enzyme from wheat bran in solid-state fermentation using *A. niger*. Ghildyal et al. (1994) used a packed-bed solid-state fermentor to study the effect of temperature gradient on the performance of a fermentation process in which the enzyme amyloglucosidase is produced from wheat bran using the fungus *Aspergillus niger*. Rathbun and Shuler (1983) used packed-bed bioreactor to study the temperature gradient during the production of tempeh from soybeans using *Rhiopus oligosporus*. Lu et al. (1998) designed a multi-layer packed-bed reactor for citric acid production from kumara (a starch containing root crop) in solid-state fermentation using *Aspergillus niger*. They claimed that their designed multi-layer

packed bed reactor improved the mass transfer significantly compared to a single-layer packed-bed with equal substrate loading.

4.7.5.3 Stirred Bioreactors. There are two types of stirred bioreactors horizontal and vertical. The only difference between stirred type bioreactor and packed bed column is that mixing is provided by an internal mixing shaft either continuously or intermittently (Mitchell et al., 1992). Saucedo-Castañeda et al. (1990) stated that the use of stirred bioreactors can reduce system heterogeneity in comparison to packed bed bioreactors. However, due to the abrasion encountered in rotating systems and its negative effect on mold development, stirred type bioreactors were not extensively used in case of solid-state fermentation processes.

Viesturs et al. (1981) conducted a solid-state fermentation of wheat straw by *Chaetomium cellulolyticum* and *Trichoderma lignorum* using horizontal Plexiglas stirred bioreactor in which a paddle like stirrer used to mix the substrate by scraping it off the wall of the bioreactor. Durand and Chereau (1988) used a pilot scale vertical stirred bed reactor for single cell protein production by solid-state fermentation of raw sugar beet pulp using *Trichoderma viride*. Bandelier et al. (1997) also used a pilot-scale vertical stirred reactor for the production of gibberellic acid by fed-batch solid-state fermentation using *Gibberella fujikoroï* grown on wheat bran. Xue et al. (1992) carried out solid-state fermentation for the production of microbial protein in a rectangular stirred bioreactor using *Aspergillus tamarii* grown on sugar beet pulp. Fernández et al. (1996) conducted solid-state fermentation for the production of gibberellic acid by *Gibberella fujikuroi* in a stirred bioreactor. Sato et al. (1988) carried out solid-state ethanol fermentation in a continuously-stirred bioreactor using *Charomyces cerevisiae* grown on corn grits.

4.7.5.4 Drum Bioreactors. Drum bioreactors consist of a drum container provided with air inlet and outlet and mounted horizontally on a system of rollers that acts as support and rotating device (Lonsane et al., 1985). The drum is rotated around its central axis to cause tumbling motion of the substrate. The rotary motion can be introduced either continuously or intermittently. Internal baffles may be introduced in order to enhance the

mixing effect (Kargi and Curme, 1985). Pandey (1991a) claimed that the growth of microorganisms in this type of bioreactors is considered to be rapid and more uniform than in tray bioreactor as a result of better mass and heat transfer. However, handling difficulties may be arising with this type of bioreactor and agglomeration of substrate particles into balls might take place as a result of the tumbling effect (Lonsane et al., 1985). Zakaria et al. (1998) conducted a solid-state fermentation in a rotating drum bioreactor for chitin purification from scampi waste using lactic acid fermentation.

Stuart et al. (1999) concluded that the effects of operational variables on the performance of solid-state fermentation in drum bioreactors are mediated by their effects on transport phenomena (such as mixing, shear and heat and mass transfer within the substrate bed). Pandey (1991a) stated that the extent of mixing by drum rotation depends on the details of fermentation and low rotation rates are usually applied (1-15 rpm). The effect of rotation rate on production reported in the literature is contradictory. Kargi and Curme (1985), Larroche and Gros (1986), Silman (1980) and Stuart et al. (1999) reported reduced fermentation productivity and sporulation at high rotational speed, presumably due to the deleterious effects of shear. Shear forces may separate penetrative hyphae from surface mycelia or may pull them out of the substrate material. Silman (1980) also reported that aerial hyphae can be crushed onto the substrate surface during agitation causing sporulation inhibition. However, Lindenfelser and Ciegler (1975) reported highest productivity at the highest rotational speed. Aidoo et al. (1984) reported that drum rotation significantly improves aeration of the substrate, which in turn causes rapid microbial growth. Aidoo et al. (1984) and Stuart et al. (1999) suggested that the adverse effect of shear on the fungal growth can be outweighed by improvements in aeration and heat removal during drum rotation.

5. ON-LINE STERILIZATION OF CHEESE WHEY USING ULTRAVIOLET RADIATION

5.1 Introduction

Due to environmental awareness and economic competitiveness of the dairy industry, cheese whey has been extensively studied in the last two decades as a low cost carbon source fermentation substrate for producing value-added products. These value-added products include lactose (Kosaric and Asher, 1982), single cell protein (Mahmoud and Kosikowski 1982; Sandhu and Waraich, 1983; and Ghaly et al., 1992), whey protein concentrate (Tang et al. 1988; Yang et al., 1992; Zall, 1992; and Jain et al. 1991), methane (Switzenbaum et al., 1979; Ghaly, 1989; Mawson, 1994; and Demirer et al., 2000), alcohols (Mahmoud and Kosikowski, 1982; and Ghaly and El-Taweel, 1995), organic acids (Anderson et al., 1986; Boyaval and Corre, 1987; Tang et al., 1988; Blanc and Goma, 1989; Jain et al., 1991; Huang and Yang, 1998; Arasaratnam et al., 1996; Mostafa, 1996; Balasubramanian et al., 2001; and Ghaly et al., 2004), biopolymers (Tao, 1990; Lee et al., 1997; Dhanasekar et al., 2001; and Fitzpatric et al., 2003), human food (Kosaric and Asher, 1982; and Tamime and Robinson, 1999), whey beverages (Kosaric and Asher, 1982; and Patil et al., 1982), whey powder (Siso, 1996), animal feed (Morr, 1992; Siso, 1996; and Barba et al., 2001), fertilizers (Marwaha and Kennedy, 1988; Ghaly et al., 1985; and Siso, 1996) and de-icers and anti-icers (Yang et al., 1992; Huang and Yang, 1998; Talabardon, 2000; and Ghaly et al., 2001). However, in order to produce a pure fermentation product, a culture of a certain organism (or organisms) must be grown in a sterilized nutrient medium. Although, steam and dry heat sterilization techniques have been used successfully for various media (Khomich et al., 1998; and Joslyn 2001), such high temperature techniques are known to denaturize cheese whey protein (Bailey and Ollis, 1986; Fennema, 1996; Cheryan, 1998; Francis, 2000; and Anema and Li, 2003).

Currently, a pasteurization technique, developed by Ghaly and El-Taweel (1995) for cheese whey sterilization is being used to avoid protein precipitation. This pasteurization

process involves heating the cheese whey to 70 °C for 45 minutes in a water bath, cooling it suddenly to 0 °C for 30 minutes using a rapid cool refrigerated circulating bath and then keeping it at room temperature (20 °C) for 24 hours for the spores to germinate. These processes of alternate heating, cooling and warming are repeated three times to ensure complete destruction of microorganisms. Although this pasteurization technique is very effective, it is also very tedious, time consuming and cannot be used for on-line sterilization. Therefore, it is necessary to find more convenient alternative technique for on-line sterilization of cheese whey.

UV radiation in the spectrum 180 to 320 nm has a known germicidal effect and can inactivate microorganisms such as protozoa, bacteria, yeasts, fungi, algae and virus, with an optimum effect at 260-265 nm (Harris et al., 1987; Meulemans, 1987; Corbitt, 1990; McDonald et al., 2000; and Blatchley III and Peel, 2001). Due to this germicidal effect, UV radiation has been extensively used in many sterilization applications such as: air disinfection (Miller and Macher, 2000; Kowalski et al., 2000; and VanOsdell and Foarde, 2002), disinfection of drinking water (Meulemans 1987; Diamant, 1996; McDonald et al., 2000; and Christensen and Linden, 2003), treatment of municipal wastewater (Harris et al., 1987; Qualls et al., 1989; Darby et al., 1993; Oppenheimer et al., 1997; Lin et al., 1999; and McDonald et al., 2000), sterilization of water used in aquaculture facilities (Liltved and Landfald, 1993; and Abraham and Palaniappan, 2000), sterilization of medical devices (Khomich et al., 1998) and sterilization of polymeric materials (Fischbach et al., 2001). Wide sources of UV are available including: low-pressure mercury glow discharges, medium-pressure mercury discharges, pulsed xenon arc discharge, xenon excimer, and submerged arc (O'Brien et al., 1996). However, low-pressure mercury lamps are the principle means for generating UV energy for the purpose of disinfection (Tchobanoglous and Burton, 1991). This is mainly because of the fact that 85% of the light output is nearly monochromic at a wavelength of 253.7 nm, which is within the optimum range for germicidal effects. The 253.7 nm mercury line has an effectiveness of about 85% of the maximum effectiveness at 260-265 nm (Meulemans, 1987; Diamant, 1996; O'Brien et al., 1996; Tchobanoglous and Burton, 1991; and Darby et al., 1993). Mercury arc lamps are usually enclosed in a UV high transmitting material

(commonly quartz) in order to permit their immersion in aqueous streams. Cheese whey sterilization using UV radiation is an attractive alternative to pasteurization as: (a) it shortens the sterilization time, (b) it is considered an inexpensive technique, and (c) it can be used on-line during continuous mode fermentations.

However, if on-line sterilization of cheese whey using ultraviolet radiation is to be used, several problems, which may represent performance limitations, should be investigated. Cheese whey has high turbidity, low transmittance and high percentage of solids, which may reduce the sterilization efficiency by shielding the microorganisms or absorbing part of the UV radiation. Corbitt (1990) and Blatchley III and Peel (2001) reported that turbidity, color, and/or organic compounds affect the effectiveness of UV disinfection process due to the limited penetrating ability of the UV radiation. Qualls et al. (1983) reported that suspended particles in wastewater effluents can negatively affect the disinfection process. Lamp fouling is another limitation since fouling material can block the light causing reduction in the sterilization efficiency. Harris et al., 1987; Darby et al. (1993); Blatchley III et al. (1996); Diamant (1996); McDonald et al. (2000); and Sheriff and Gehr (2001) reported that the accumulation of fouling materials at quartz liquid interface is a potentially serious problem in UV disinfection systems.

5.2 Objectives

The aim of this study was to investigate the effectiveness and reliability of UV on-line cheese whey sterilization technique. The specific objectives were to:

(a) study the ability of ultraviolet radiation to sterilize cheese whey.
(b) evaluate the effects of the flowing material thickness (reactor gap size) and the flow rate on the performance of tubular UV reactors.
(c) investigate the susceptibility of UV lamp to fouling during online cheese whey sterilization.
(d) determine the characteristics and composition of the fouling material.
(e) determine the heat generated by the UV lamp and calculate the various heat losses through the UV reactor.

(f) investigate the effect of gap size as well as lamp heat generation on the extent of lamp fouling.

(g) determine the lamp fouling mechanism.

5.3 Experimental Apparatus

The experimental setup consisted of 6 mm thick Plexiglas continuously stirred feed tank of 10 L volume, variable speed modular peristaltic pump with Masterflex® precision tubing (Digi-Staltic, Masterflex®, Model No. 7525-30, with Head Model No. 7024-20, and Tubing No. 6404-24, Barnant Company, Division of Cole-Parmer Instrument. Co., Barrington, Illinois, USA), UV reactors, and 3 mm thick Plexiglas overflow tank of 25 L volume (Figure 5.1). Three tubular type UV reactors having different gaps (between the outer surface of the UV lamp quartz sleeve and the inner surface of the UV reactor casing) were studied in a continuous operation mode.

A 380 mm arc length low-pressure mercury lamp enclosed in a 21 mm diameter (OD) quartz tube was used in this study with the three UV reactors. The first UV reactor (Figure 5.2a) has a 1.1 L working volume and a 3 mm thick stainless steel chamber of 455 mm length and 62.5 mm inside diameter, giving a gap size of 18 mm. The second UV reactor (Figure 5.2b) has a 0.598 L working volume and a 1.34 mm thick stainless steel chamber of 467 mm length and 47.4 mm inside diameter, giving a gap size of 13 mm. The third UV reactor (Figure 5.2c) has a 0.201 L working volume and 4.3 mm thick stainless steel chamber of 475 mm length and 33 mm inside diameter, giving a gap size of 6 mm. The outer stainless steel chambers were designed in such a way that ensured no leakage of ultraviolet radiation from the system. An inlet port was located at the bottom part of each reactor and was connected to a peristaltic pump, which pumped the cheese whey from the feed tank. The outlet port was located at the top of each reactor and was connected to the overflow tank

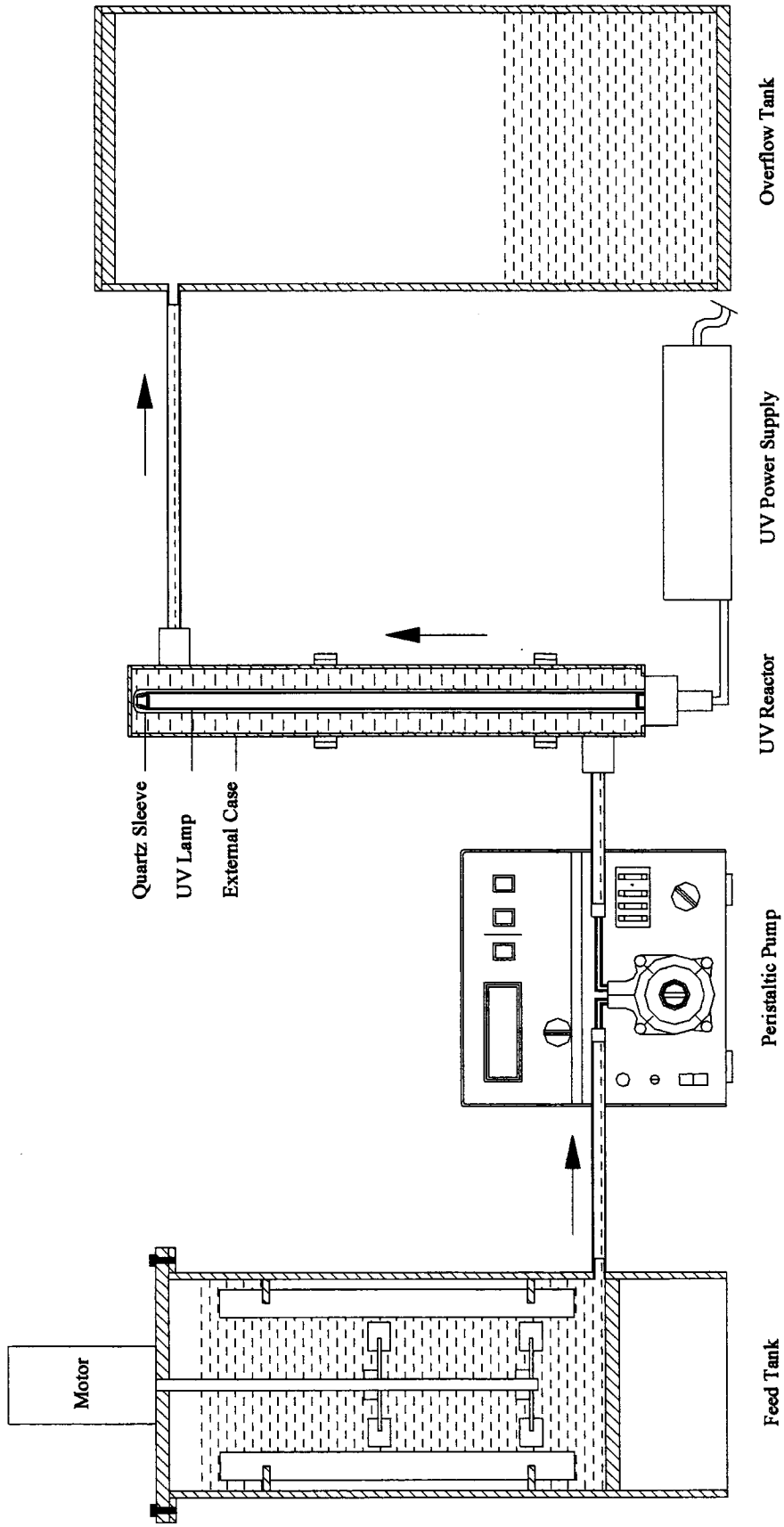
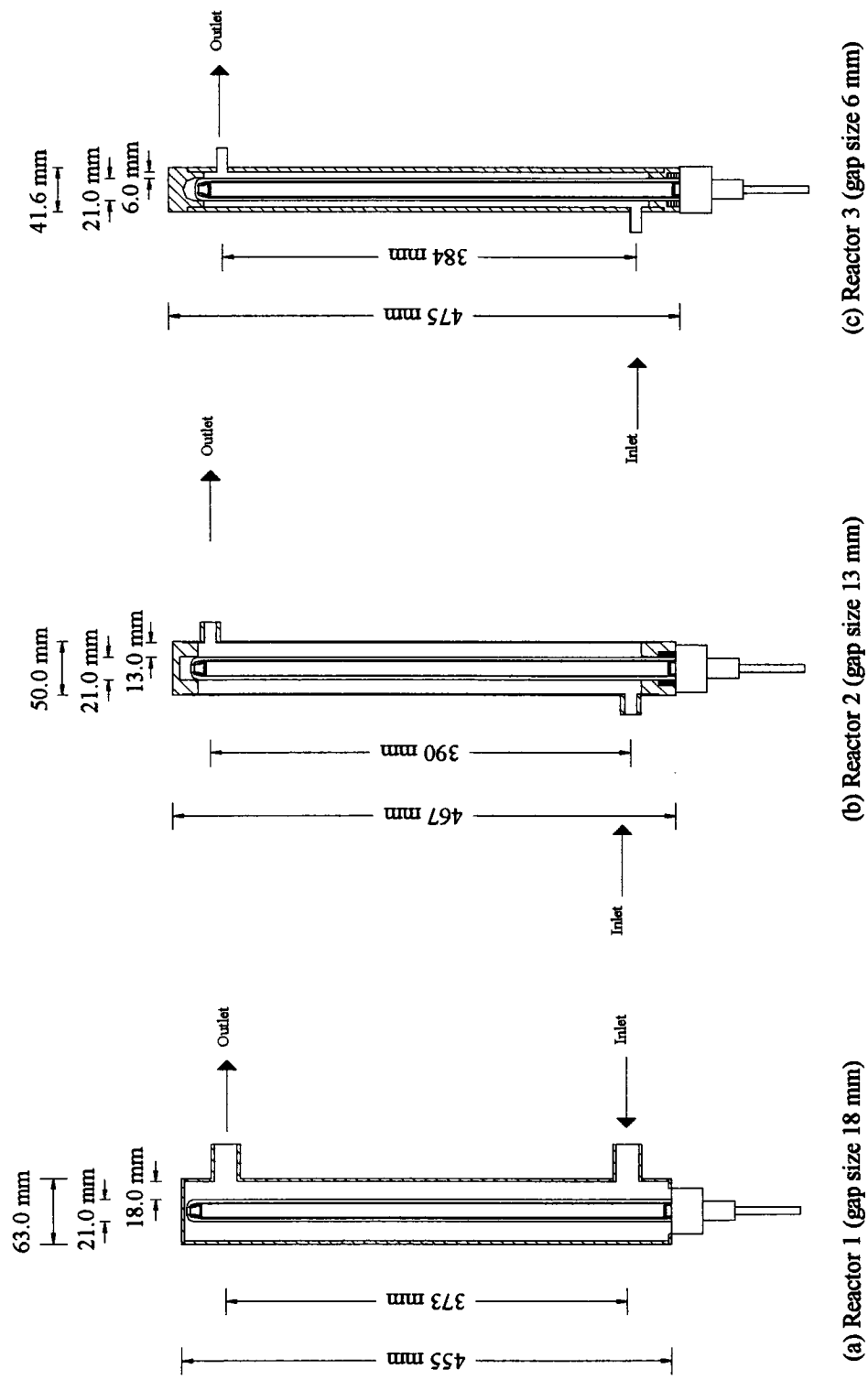


Figure 5.1. Experimental setup.



(a) Reactor 1 (gap size 18 mm) (b) Reactor 2 (gap size 13 mm) (c) Reactor 3 (gap size 6 mm)

Figure 5.2. The UV reactors used in this study.

5.4 Experimental Material

The cheese whey was obtained from Farmer's Cooperative Dairy Plant in Truro, Nova Scotia in 60 L plastic containers. The containers were sealed and transported to the Biotechnology Laboratory, where they were stored in a large freezer at -25 °C until used. Some characteristics of the cheese whey used in this study are shown in Table 5.1. Prior to using the cheese whey in this study, it was allowed to completely thaw at room temperature. The thawed cheese whey was transferred to a storage tank and provided with continuous mixing at room temperature (20 °C). In order to investigate the effectiveness of UV radiation for cheese whey sterilization, the cheese whey was left in the storage tank for 72 h in order to increase the microbial population. Then, it was placed in several 4 L bottles and stored in the fridge at (4 °C) until used in the experiment.

5.5 Experimental Design

Three sets of experiments were conducted in this study. In the first set of experiments, the performances of three tubular UV reactors were evaluated at different flow rates (3.84, 1.92, 1.28, 0.96, 0.76, 0.64 L/h). The studied flow rate values were calculated based on the assumption that the effluent from the UV reactor has to supply four continuous mode bioreactors (used for fermentation), each with a 4.8 L working volume, and allow for a hydraulic retention time in the range of 5 to 30 hours. Table 5.2 shows the bioreactor hydraulic retention times and corresponding flow rates and the calculated flow rates and residence times for each UV reactor. The hydraulic diameter for annular space and the mean velocity of the medium were calculated for each UV reactor using the following equations (Cengel, 2003):

$$D_h = D_o - D_i \tag{5.1}$$

$$\upsilon_m = \frac{q}{A_c} = \frac{q}{1/4\pi(D_o^2 - D_i^2)} \tag{5.2}$$

Table 5.1. Some characteristics of the cheese whey used in this study.

Characteristics	Measured value	Units
Total solids	68300	mg/L
Fixed solids	6750	mg/L
Percent fixed solids	10	%
Volatile solids	61550	mg/L
Percent volatile solids	90	%
Suspended solids	25160	mg/L
Fixed solids	230	mg/L
Percent fixed solids	1	%
Volatile solids	24930	mg/L
Percent volatile solids	99	%
Total Kjeldahl nitrogen	1560	mg/L
Ammonium nitrogen	260	mg/L
Percent ammonium nitrogen	17	%
Organic nitrogen	1300	mg/L
Percent organic nitrogen	83	%
Total chemical oxygen demand	81050	mg/L
Soluble chemical oxygen demand	68050	mg/L
Percent soluble chemical oxygen demand	85	%
Insoluble chemical oxygen demand	13000	mg/L
Percent insoluble chemical oxygen demand	15	%
Biological oxygen demand (BOD_5)	52220	mg/L
Lactose	50000	mg/L
Fat	4700	mg/L
Protein	9953	mg/L
Elements		
Magnesium	94	mg/L
Calcium	429	mg/L
Manganese	1	mg/L
Potassium	1670	mg/L
Sodium	684	mg/L
Iron	42	mg/L
Phosphorus	154	mg/L
Sulfur	483	mg/L
Others	3193	mg/L
pH	5.00	

Table 5.2. The bioreactor hydraulic retention times (HRT) and corresponding flow rates and the calculated flow rates and residence times for the UV reactors.

Bioreactors		UV Reactors			
HRT	Flow Rate[a]	Flow Rate[b]	Residence Time (h)		
(h)	(L/h)	(L/h)	Reactor (1)[c]	Reactor (2)[d]	Reactor (3)[e]
5.0	0.96	3.84	0.286	0.156	0.052
10.0	0.48	1.92	0.573	0.311	0.105
15.0	0.32	1.28	0.859	0.467	0.157
20.0	0.24	0.96	1.146	0.623	0.209
25.0	0.19	0.76	1.447	0.787	0.264
30.0	0.16	0.64	1.719	0.934	0.314

[a] Calculated on the basis that the bioreactor working volume is 4.8 L
[b] Calculated on the basis that the outflow from the UV-reactor is equal to the inflow of four bioreactors of 4.8 L working volume each
[c] 1.100 L volume and 18 mm gap size
[d] 0.598 L volume and 13 mm gap size
[e] 0.201 L volume and 6 mm gap size

Table 5.3. The tested residence times and the corresponding flow rates of the UV reactors.

Residence Time	Flow Rate (L/h)		
(h)	Reactor (1)	Reactor (2)	Reactor (3)
0.25	4.40	2.39	0.80
0.50	2.20	1.20	0.40
0.75	1.47	0.80	0.27
1.00	1.10	0.60	0.20
1.50	0.73	0.40	0.13
2.00	0.55	0.30	0.10

Where:

D_h Hydraulic diameter (cm)

D_o Outer diameter (cm)

D_i Inner diameter (cm)

υ_m Mean velocity (cm/h)

q Volumetric flow rate (cm^3/h)

A_c Annular section area (cm^2)

In the second set of experiments, the performances of the three tubular UV reactors were evaluated at different retention times (0.25, 0.50, 0.75, 1.00, 1.50, and 2.00 h) in order to eliminate the confound effects of residence time and the thickness of the flowing medium. Table 5.3 shows the residence time and the corresponding flow rate for each UV reactor.

In the third set of experiments, the susceptibility of lamp fouling during on-line sterilization of cheese whey using ultraviolet radiation was studied at 2 hours residence time, which was proved from the first two sets of experiments to be effective for cheese whey sterilization. This gave flow rates of 0.55, 0.30, 0.10 L/h for the first (18 mm gap size), second (13 mm gap size) and third (6 mm gap size) UV reactors, respectively.

5.6 Experimental Procedure

For the first and second set of experiments, the UV reactors including the UV lamp and all accessories (feeding tank, tubing and overflow tank) were cleaned with laboratory detergent and tap water and then chemically sterilized using 2% sodium metabisulfite solution (prepared by dissolving 20 g sodium metabisulfite ($Na_2S_2O_5$) in 1 L deionized-distilled water) to eliminate any microbial contamination. The UV reactor and all the accessories were then washed with hot water several times in order to remove any chemical traces. The system was left to cool down for 15 min, and then the UV reactor was operated for 5 minutes while it was empty in order to eliminate any possible contamination before treating the cheese whey. These sterilization procedures were

carried out before each run. The cheese whey was pumped through the UV reactors with the required flow rates using the variable speed modular peristaltic pump. Samples of UV treated cheese whey were collected from the outlet of each UV reactor in disposable sterile test tubes for direct plate count analyses.

For the third set of experiments, prior to each run, the UV lamp was carefully washed with a piece of cotton using tap water and laboratory detergent, and then rinsed thoroughly with de-ionized distilled water. Raw cheese whey was pumped through the UV reactors with the required flow rates using the variable speed modular peristaltic pump. The reactors were operated for 48 hours, during which the temperatures were measured using thermocouples and a data logger (Model 4702-5E, Cole-Parmer, Chicago, Illinois) at 9 different locations as shown in Figure 5.3: (a) the inlet cheese whey temperature (T_i), (b) the outlet cheese whey temperature (T_o), (c) the temperature of the outside surface of the UV reactor opposite to the inlet (T_{w1}), (d) the temperature of the outside surface of the UV reactor at mid height (T_{w2}), (e) the temperature of the outside surface of the UV reactor opposite to the outlet (T_{w3}), (f) the temperature of the outside surface of the UV reactor top (T_{ot}), (g) the temperature of the UV reactor headspace (T_{ia}), (h) the temperature of the lamp socket surface (T_s), and (i) the laboratory ambient temperature (T_a). The accumulated power input consumption was also measured using power meter (True RMS Power Multimeter, Model 382860, ExTech Instrument, Waltham, MA, USA) and recorded every 20 minutes. After the 48 hour operating period, the lamp was carefully removed from the reactor for visual inspection. The fouling materials were then scraped and placed in small plastic containers. The containers were sealed with Parafilm and kept in the refrigerator at 4 °C until needed for analysis.

5.7 Experimental Analyses

The transmittance of raw cheese whey to UV radiation (in the germicidal range of 180 – 320 nm) was measured using a spectrophotometer (Spectronic 601, Fisher Scientific Montreal, Quebec, Canada) at 10 different wavelengths (195, 216, 240, 253, 254, 265, 270, 300, 315, and 330 nm). De-ionized distilled water was used as 100 % transmittance.

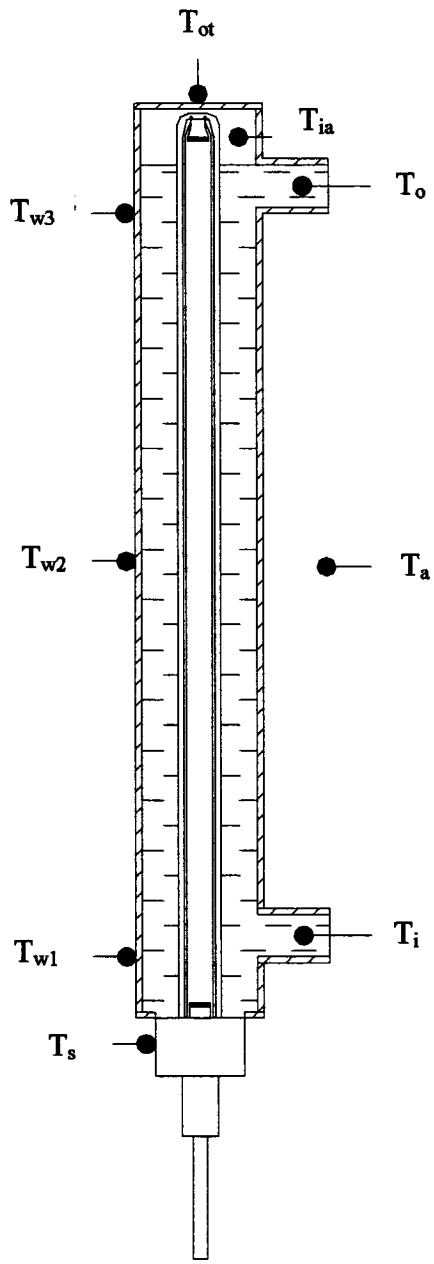


Figure 5.3. Locations of temperature measurements.

The turbidity of cheese whey was measured according to the procedures described in the Standard Methods for the Examination of Water and Wastewater (APHA, 1985). A turbidimeter (HACH 2100P, HACH Company, Loveland, Colorado, USA) was used for the turbidity measurements. Because of the range of the turbidimeter, the cheese whey had to be diluted. Therefore, six dilutions (1:10, 1:20, 1:40, 1:60, 1:80, and 1:100) were performed to investigate the effect of dilution on the turbidity results.

The particle size distribution of the cheese whey solids was measured using laser diffraction (Droplet and Particle Sizer, Series 2600C, Malvern Instruments, Worcestershire, England). Because the laser diffraction has three different ranges (1.2 – 118.4, 8.4 – 188.0, and 5.8 – 564 μm), the test was performed in these ranges. A sieve apparatus (Tyler, Combustion Engineering Canada Inc., Ontario, Canada) was used with the appropriate wire mesh sieve to remove the large particles from the cheese whey. For the first range (1.2 – 118.4 μm), a 50 mL sample of the cheese whey was sieved using a 0.09 mm wire mesh sieve to remove particles larger than 90 μm. For the second range (1.9 – 188 μm), a 50 mL sample of the cheese whey was sieved using a 0.18 mm wire mesh sieve to remove particles larger than 180 μm. For the third range (5.8 – 564 μm), a 100 mL sample of the cheese whey was sieved using a 0.5 mm wire mesh sieve to remove particles larger than 500 μm. The filtered cheese whey samples were then pumped through the particle sizer The sieved particles were washed from the sieves with deionized-distilled water and filtered under suction using coarse porosity filter paper (Whatman No. 40 Filter Paper, Fisher Scientific Co. Pittsburgh, PA). The filter paper, along with the removed particles, was dried in an oven (Isotemp Oven, Model 655F, Fisher Scientific, Montreal, Quebec, Canada) at 105 °C for 24 hours The weight of the removed particles for each range was measured.

Untreated and UV treated cheese whey samples from the first two sets of experiments were analyzed for their microbial count. The visible growth was counted as colony forming units per milliliter (CFU/mL) according to the procedure described in the Standard Methods for the Examination of Dairy Products (APHA, 1972). A plate count

agar (DIFCO, 0751-17-2), which contained 5 g of bacto yeast extract, 10 g of bacto tryptone, 2 g of dextrose and 15 g of agar all dissolved in 1 L de-ionized distilled water, was prepared, sterilized at 121 °C and 103.4 kPa for 30 minutes in an autoclave (Model No. STM-E, Market Forge Sterilmatic, New York, USA), and then poured into several Petri dishes. The Petri dishes were allowed to solidify and then kept in a fridge at 4 °C until ready for use. Serial dilutions of 10^{-1} - 10^{-10} were prepared from the samples. An aliquot of 0.1 mL of each of the dilutions was placed on the plate count agar in the Petri dish (in triplicate) and gently swirled using a flame sterile glass hockey stick until consistently spread on the agar surface. The Petri dishes were then incubated at 37 °C in an incubator (Fisher Isotemp*, Cat. No. 11-680-626, Fisher Scientific, Whitby, Ontario, Canada) until visible growth was observed.

Both raw cheese whey and fouling materials were analyzed for protein, fat and trace elements (magnesium, calcium, manganese, potassium, sodium, iron, sulfur, phosphorous). The solid, lactose and nitrogen contents, chemical oxygen demand, biological oxygen demand and pH analyses were also performed on the raw cheese whey. The moisture content and the total weight of fouling materials were obtained.

The solids, moisture, chemical oxygen demand and biological oxygen demand analyses were performed according to the procedures described in the Standard Methods for the Examination of Water and Wastewater (APHA, 1985). Lactose concentration was determined using a sugar analyzer (YSI Model 27, Yellow Spring, Ohio, USA). Ammonium nitrogen and total Kjeldahl nitrogen were measured using KJELTEC AUTO 1030 Analyzer (Fisher Scientific, Montreal, Quebec, Canada). Calculations of protein concentrations were based on a Kjeldahl factor of 6.38 (cheese whey proteins contain about 15.67% nitrogen) according to Cheryan (1998), Francis (2000) and van der Ven et al. (2001).The fat content was obtained according to the method described by Bligh and Dyer (1959). The method depended on the extraction of lipids from the material using ratios of chloroform: methanol: water of 1:2:0.8 and 2:2:1.8 before and after dilution, respectively. The extracted lipids were then regenerated in a rotary evaporator (Flash-Evaporator, Buchler Instruments, Fort Lee, NJ, USA) and weighed.

Quantitative trace element analyses (magnesium, calcium, manganese, potassium, sodium, and iron) were done using an Atomic Absorption Spectrophotometer (SpectrAA 55B, Varion, Mulgrave, Victoria, Australia) in the Minerals Engineering Center, Dalhousie University, Halifax, Nova Scotia. The samples were first digested with hydrochloric, nitric, hydrofluoric and perchloric acids (30, 10, 10 and 5 mL/g sample, respectively) in a closed vessel at a temperature of 100 °C and then the elements were determined by flame atomic absorption with detection limit of 1 ppm. Sulfur was determined with Leco Sulfur analyzer along with Leco Induction Furnace (Leco Corporation ST. Joseph, HI, USA). Phosphorous was determined as P_2O_5 by a colorimetric method using spectrophotometer with micro flow-thru system (Spectoronic 100, Bausch & Lomb Incorporation, Rochester, New York, USA) at 430 nm.

Visualization and microscale elemental analyses of both surfaces (the inner surface which was facing the lamp and the outer surface which was facing the flow) of the fouling materials obtained from the three UV reactors were performed using Environmental Scan Electron Microscope (ESEM) and Energy Dispersive X-ray (EDX) analyses at the Bedford Institute of Oceanography, Halifax, Nova Scotia.

5.8 Heat Balance

In order to evaluate the performance of the on-line UV sterilization system, a through heat balance must be performed on the system. The heat balance was performed on the entire UV reactor during the continuous sterilization of the cheese whey. It included: (a) the heat generated by the UV lamp, (b) the heat lost with the cheese whey effluent, (c) the heat lost through the UV reactor top, (d) the heat lost through the UV reactor bottom and (e) the heat lost through the UV reactor wall.

$$q_l = q_e + q_t + q_b + q_w \tag{5.3}$$

Where:

q_l is the rate of heat generated by the UV lamp (kJ/h)

q_e is the rate of heat lost with the effluent (kJ/h)

q_t is the rate of heat lost through the UV reactor top (kJ/h)

q_b is the rate of heat lost through the UV reactor bottom (kJ/h)

q_w is the rate of heat lost through the UV reactor wall (kJ/h)

Figure 5.4 shows the various heat transfer components of the reactor. The values of q_e, q_t, q_b and q_w can be calculated from the following equations:

$$q_e = m_e C_{pe} (T_i - T_o) \quad (5.4)$$

$$q_t = U_t A_t (T_{ia} - T_a) \quad (5.5)$$

$$q_b = U_b A_b (T_i - T_a) \quad (5.6)$$

$$q_w = U_w A_w (T_m - T_a) \quad (5.7)$$

Where:

A_b is the surface area of the UV reactor bottom (m^2)

A_t is the surface area of the UV reactor top (m^2)

A_w is the surface area of the UV reactor outer wall (m^2)

C_{pe} is the specific heat of the cheese whey effluent (kJ/kg.K)

m_e is the mass flow rate of the effluent (kg/h)

T_a is the air ambient temperature (K)

T_i is the inlet temperature of the cheese whey (K)

T_{ia} is the temperature of the air inside the headspace (K)

T_m is the mean temperature of the cheese whey (K)

T_o is the outlet temperature of the cheese whey (K)

U_b is the overall heat loss coefficient through the UV reactor bottom ($kJ/m^2.h.K$)

U_t is the overall heat transfer coefficient of the UV reactor top ($kJ/m^2.h.K$)

U_w is the overall heat transfer coefficient of the UV reactor wall ($kJ/m^2.h.K$)

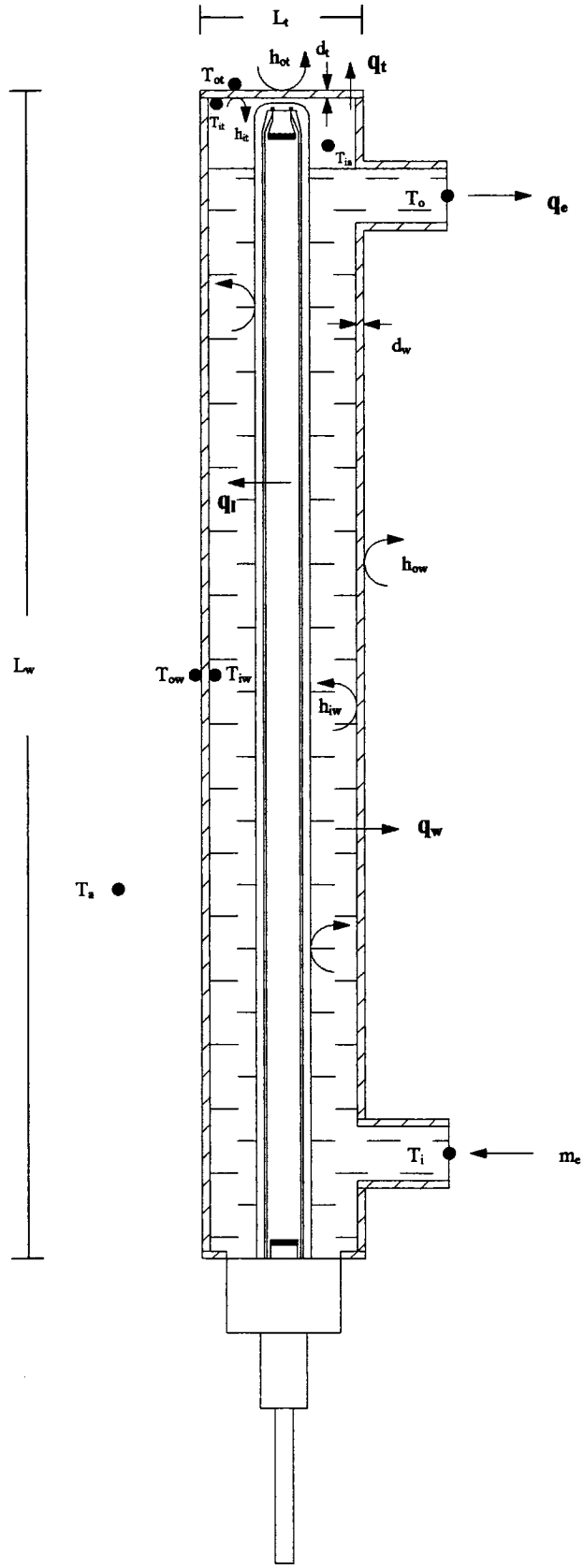


Figure 5.4. Heat transfer components.

The overall heat transfer coefficients of the UV reactor bottom (U_b), wall (U_w) and top (U_t) can be calculated as follows (Holman, 2002):

$$U_b = \frac{1}{\left(\frac{d_b}{K_b}\right)+\left(\frac{1}{h_{ob}}\right)+\left(\frac{1}{h_{ib}}\right)} \tag{5.8}$$

$$U_w = \frac{1}{\left[\left(\frac{A_{ow}}{A_{iw}}\right)\left(\frac{1}{h_{iw}}\right)+\frac{A_{ow}\ln(r_o/r_i)}{(2\pi K_w L_w)}\right]+\left[\frac{1}{h_{ow}}\right]} \tag{5.9}$$

$$U_t = \frac{1}{\left(\frac{1}{h_{it}}\right)+\left(\frac{d_t}{K_t}\right)+\left(\frac{1}{h_{ot}}\right)} \tag{5.10}$$

Where:

A_{ow} is the surface area of outside wall of the UV reactor (m^2)

A_{iw} is the surface area of inside wall of the UV reactor (m^2)

d_b is the thickness of the UV reactor bottom (m)

d_t is the thickness of the UV reactor top (m)

h_{ib} is the convective heat transfer coefficient between the inner surface of the UV reactor bottom and the cheese whey influent ($kJ/m^2.h.K$)

h_{it} is the convective heat transfer coefficient between the inner surface of the UV top and the air entrapped inside the UV reactor ($kJ/m^2.h.K$)

h_{iw} is the convective heat transfer coefficient between the cheese whey and the UV reactor inside wall ($kJ/m^2.h.K$)

h_{ob} is the convective heat transfer coefficient between the outer surface of the UV reactor bottom and ambient air ($kJ/m^2.h.K$)

h_{ot} is the convective heat transfer coefficient between the outer surface of the UV reactor top and ambient air ($kJ/m^2.h.K$)

h_{ow} is the convective heat transfer coefficient between the UV reactor outside wall and the ambient air ($kJ/m^2.h.K$)

K_b is the thermal conductivity of the bottom material (kJ/m.h.K)

K_t is the thermal conductivity of the top material (kJ/m.h.K)

K_w is the thermal conductivity of the wall material (kJ/m.h.K)

L_w is the characteristic length, height of the reactor (m)

r_i is the inner radius of the UV reactor (m)

r_o is the outer radius of the UV reactor (m)

The heat transfer from the cheese whey to the inner surface of the UV reactor bottom and wall is by forced convection, while that from the outer surface to the air is by natural convection. Because the heat transfer coefficients due to forced convection (h_{ib} and h_{iw}) are very large compared to those of the natural convection (h_{ob} and h_{ow}), the terms ($1/h_{ib}$) in equation (5.8) and (A_{ow}/A_{iw})($1/h_{iw}$) in equation (5.9) will be very small and can be neglected. Thus, the overall heat transfer coefficients of the UV reactor bottom (U_b) and wall (U_w) can be rewritten as follow:

$$U_b = \frac{1}{\left(\frac{d_b}{K_b}\right)+\left(\frac{1}{h_{ob}}\right)} \tag{5.11}$$

$$U_w = \frac{1}{\left[\frac{A_{ow}\ln(r_o/r_i)}{(2\pi K_w L_w)}\right]+\left[\frac{1}{h_{ow}}\right]} \tag{5.12}$$

For laminar flow ($10^4<Gr_fPr_f<10^9$), the convective heat transfer coefficients (h_{ob}), (h_{ow}), (h_{ot}) and (h_{it}) can be calculated as follows (Holman, 2002):

$$h_{ob} = 0.59\left(\frac{T_{ob}-T_a}{L_b}\right)^{0.25} \tag{5.13}$$

$$h_{ow} = 1.42\left(\frac{T_{ow}-T_a}{L_w}\right)^{0.25} \tag{5.14}$$

$$h_{ot} = 1.32\left(\frac{T_{ot} - T_a}{L_t}\right)^{0.25} \quad (5.15)$$

$$h_{it} = 1.32\left(\frac{T_{ia} - T_{it}}{L_t}\right)^{0.25} \quad (5.16)$$

Where:

Gr_f is the Grashof number

Pr_f is the Prandtl number

Gr_fPr_f is the Rayleigh number

L_b is the characteristic length, diameter of the UV reactor bottom (m)

L_t is the characteristic length, diameter for disc (m)

T_{ia} is the temperature of air entrapped in the headspace of the UV reactor (K)

T_{it} is the temperature of the inside surface of the UV reactor top (K)

T_{ob} is the temperature of the outside surface of the UV reactor bottom (K)

T_{ot} is the temperature of the outside surface of the UV reactor top (K)

T_{ow} is the temperature of the outside surface of the UV reactor wall at mid height (K)

For turbulent flow ($Gr_fPr_f>10^9$), the convective heat transfer coefficient (h_{ob}), (h_{ow}), (h_{ot}) and (h_{it}) can be calculated as follows (Holman, 2002):

$$h_{ob} = 1.31\ (T_{ob} - T_a)^{0.33} \quad (5.17)$$

$$h_{ow} = 1.31\ (T_{ow} - T_a)^{0.33} \quad (5.18)$$

$$h_{ot} = 1.52\ (T_{ot} - T_a)^{0.33} \quad (5.19)$$

$$h_{it} = 1.52\ (T_{ia} - T_{it})^{0.33} \quad (5.20)$$

The values of Prandtl number (Pr_f) can be obtained from Standard Tables (Holman, 2002) while those of Grashof number (Gr_f) can be calculated using the following expression for the UV reactor bottom (Gr_{fb}), wall (Gr_{fw}), top outer surface (Gr_{fot}) and top inner surface (Gr_{fit}), respectively (Holman, 2002):

$$Gr_{fb} = \frac{g\beta(T_{ob} - T_a)L_b^3}{\upsilon^2} \tag{5.21}$$

$$Gr_{fw} = \frac{g\beta(T_{ow} - T_a)L_w^3}{\upsilon^2} \tag{5.22}$$

$$Gr_{fot} = \frac{g\beta(T_{ot} - T_a)L_t^3}{\upsilon^2} \tag{5.23}$$

$$Gr_{fit} = \frac{g\beta(T_{ia} - T_{it})L_t^3}{\upsilon^2} \tag{5.24}$$

Where:

g	is the gravitational acceleration (m/s^2)
f	denotes that the properties obtained at film temperature (T_f)
β	is the volume coefficient of expansion (1/K)
υ	is the kinematic viscosity (m^2/s)

For ideal gases the volume coefficient of expansion (β) may be calculated as the reciprocal of the absolute temperature ($1/T_f$) of the gas while for other fluids it may be obtained from Standard Tables (Holman, 2002). The film temperatures for the UV reactor bottom (T_{fb}), wall (T_{fw}) and top outer and inner (T_{fot} and T_{fit}) surfaces can be calculated by the following equations, respectively:

$$T_{fb} = \frac{(T_{ob} + T_a)}{2} \tag{5.25}$$

$$T_{fw} = \frac{(T_{ow} + T_a)}{2} \tag{5.26}$$

$$T_{fot} = \frac{(T_{ot} + T_a)}{2} \tag{5.27}$$

$$T_{fit} = \frac{(T_{ia} + T_{it})}{2} \tag{5.28}$$

5.9 Results

5.9.1 Cheese Whey Physical Characteristics

Table 5.4 shows the average turbidity of the original cheese whey, using several dilutions (1:10, 1:20, 1:40, 1:60, 1:80, and 1:100). The results showed that the average turbidity of the cheese whey was 4317 ± 208 NTU with a coefficient of variation of 4.8%. Cheese whey transmittance to UV radiation in the range 195 - 315 nm was zero as shown in Figure 5.5. At longer wavelength (330 nm), the cheese whey transmittance was only 0.1%. The cheese whey particle size distribution results are shown in Table 5.5. Figure 5.6 shows the cheese whey particle size distribution as constructed from the results presented in Table 5.5. About 80% of the particles were smaller than 100 μm .

5.9.2 Effect of Flow Rate

Raw cheese whey with initial population of 5.95×10^6 cells/mL was used to study the effect of flow rate (0.96, 0.48, 0.32, 0.24, 0.19, and 0.16 L/h) on the performance of the three tubular UV reactors. The results are shown in Table 5.6. The results are the average of three replicates. The survival curves for the UV reactors are shown in Figure 5.7.

5.9.3 Effect of Residence Time

The residence time (t) is calculated as follows:

$$t = \frac{V}{F} \tag{5.29}$$

Where:

F is the flow rate (L/h)

V is the working volume of the UV reactor (L)

A different batch of cheese whey with initial microbial population of 340×10^6 cells/mL was used to study the effect of residence time (0.25, 0.50, 0.75, 1.00, 1.5, and 2.00 L/h) on the performance of the three tubular UV reactors. The results are shown in Table 5.7. Figure 5.8 shows the survival curves for the UV reactors.

Table 5.4. Cheese whey turbidity at different dilutions[a].

Sample	Dilution	Turbidity (NTU)	
		Diluted	Raw[b]
1	1:10	462	4620
2	1:20	227	4540
3	1:40	106	4240
4	1:60	70.1	4206
5	1:80	52.1	4168
6	1:100	41.3	4130
Average			4317

[a] Standard Deviation = 208 NTU, Coefficient of Variation = 4.8%
[b] Values are calculated for the original sample

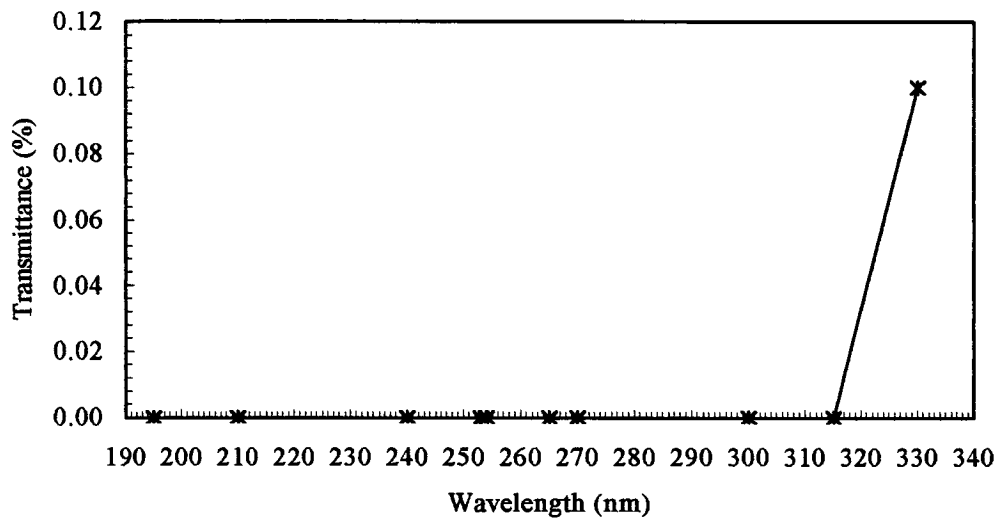


Figure 5.5. Transmittance of cheese whey to UV light at different wavelengths.

Table 5.5. Measured cheese whey particle size distribution.

5.8 – 564 μm lense[a]			1.9 – 188 μm lense[b]			1.2 – 118 μm lense[c]		
Size (μm)	Under	In band (%)	Size (μm)	Under	In band (%)	Size (μm)	Under	In band (%)
500.0	100.0	0.0	180.0	100.0	0.5	90.0	100.0	1.7
487.0	100.0	0.3	162.0	99.5	1.4	88.1	98.3	1.9
420.0	99.7	0.6	140.0	98.1	2.1	76.0	96.4	2.5
362.0	99.1	1.2	121.0	96.0	2.8	65.6	93.9	3.1
312.0	97.9	1.8	104.0	93.2	3.1	56.6	90.8	3.4
270.0	96.1	2.6	89.9	90.1	3.4	48.8	87.4	3.4
233.0	93.5	3.1	77.5	86.7	3.5	42.1	84.0	3.3
201.0	90.4	3.0	66.9	83.2	3.4	36.3	80.7	2.6
173.0	87.4	2.0	57.7	79.8	3.2	31.3	78.1	2.3
149.0	85.4	1.2	49.8	76.6	3.0	27.0	75.8	2.6
129.0	84.2	1.3	42.9	73.6	2.8	23.3	73.2	3.3
111.0	82.9	1.8	37.1	70.8	2.8	20.1	69.9	3.3
95.9	81.1	2.6	32.0	68.0	2.5	17.4	66.6	2.9
82.7	78.5	3.3	27.6	65.5	2.1	15.0	63.7	2.6
71.4	75.2	3.4	23.8	63.4	2.3	12.9	61.1	2.9
61.6	71.8	3.3	20.5	61.1	2.8	11.1	58.2	3.2
53.1	68.5	3.0	17.7	58.3	3.0	9.6	55.0	4.1
45.8	65.5	2.6	15.3	55.3	3.1	8.3	50.9	4.3
39.5	62.9	2.3	13.2	52.2	2.7	7.2	46.6	3.8
34.1	60.6	2.3	11.4	49.5	2.4	6.2	42.8	2.9
29.4	58.3	2.6	9.8	47.1	2.3	5.3	39.9	3.4
25.4	55.7	2.6	8.5	44.8	2.6	4.6	36.5	5.0
21.9	53.1	2.5	7.3	42.2	3.2	4.0	31.5	6.7
18.9	50.6	2.0	6.3	39.0	3.9	3.4	24.8	6.4
16.3	48.6	1.8	5.4	35.1	4.1	3.0	18.4	4.7
14.1	46.8	2.0	4.7	31.0	4.0	2.6	13.7	3.6
12.1	44.8	1.8	4.1	27.0	4.4	2.2	10.1	2.1
10.5	43.0	1.9	3.5	22.6	4.3	1.9	8.0	0.9
9.0	41.1	2.0	3.0	18.3	3.6	1.6	7.1	0.6
7.8	39.1	2.7	2.6	14.7	3.3	1.4	6.5	0.4
6.7	36.4	3.6	2.2	11.4	3.0	1.2	6.1	6.1
5.8	32.8	32.8	1.9	8.4	8.4	-	-	-

[a] Particles > 500 μm were filtered before analysis

[b] Particles > 180 μm were filtered before analysis

[c] Particles > 90 μm were filtered before analysis

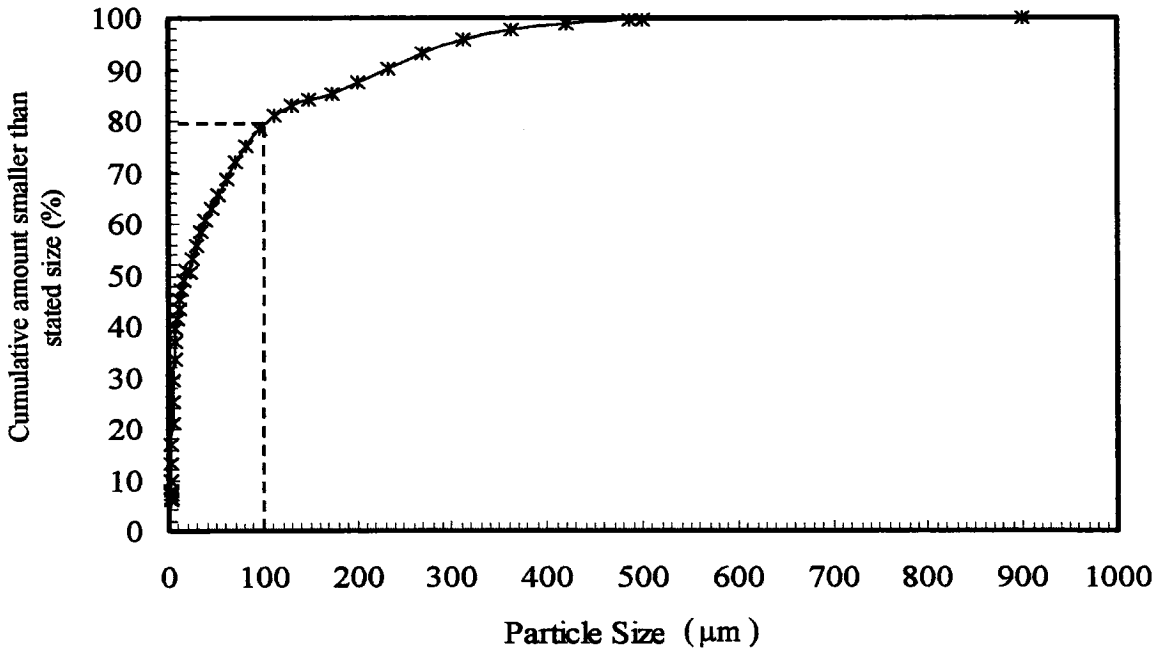


Figure 5.6. Particle size distribution curves for cheese whey.

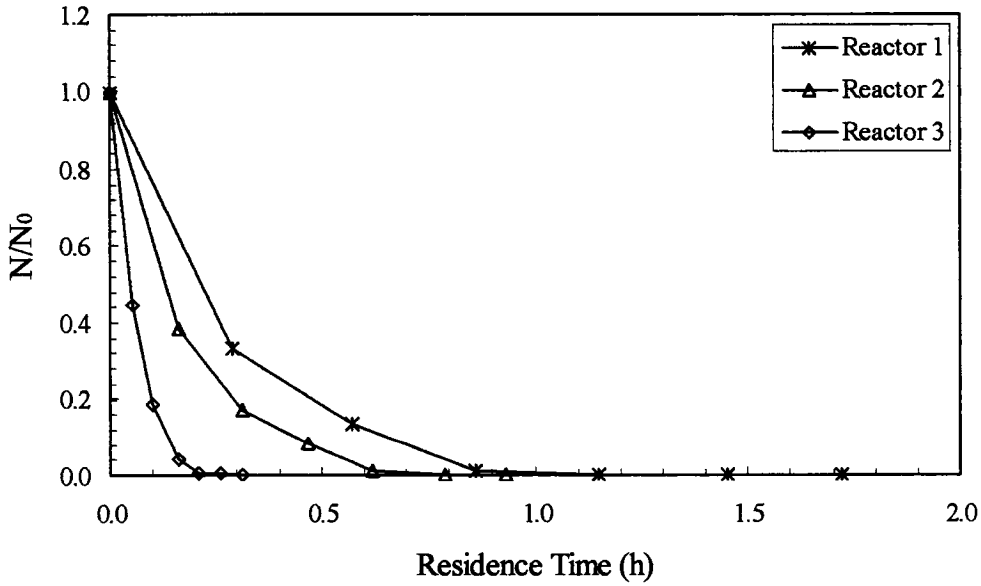


Figure 5.7. Survival curves for the three UV reactors as affected by residence time.

Table 5.6. The effect of flow rate on the cell survival ratio and removal efficiency of the three UV reactors[a].

Flow Rate (L/h)	UV Reactor (1)				UV Reactor (2)				UV Reactor (3)			
	Residence Time (min)	Cell No. ($\times 10^6$ cells/mL)	Survival Ratio	Removal Efficiency (%)	Residence Time (min)	Cell No. ($\times 10^6$ cells/mL)	Survival Ratio	Removal Efficiency (%)	Residence Time (min)	Cell No. ($\times 10^6$ cells/mL)	Survival Ratio	Removal Efficiency (%)
3.84	17.19	1.980	0.332773	66.72	9.34	2.300	0.386555	61.34	3.14	2.670	0.448739	55.13
1.92	34.38	0.790	0.132773	86.72	18.69	1.020	0.171429	82.86	6.28	1.120	0.188235	81.18
1.28	51.56	0.065	0.010924	98.91	28.03	0.500	0.084034	91.60	9.42	0.233	0.039160	96.08
0.96	68.75	0.005	0.000840	99.92	37.38	0.054	0.009076	99.09	12.56	0.039	0.006555	99.34
0.76	86.84	0.004	0.000672	99.93	47.21	0.010	0.001681	99.83	15.87	0.016	0.002689	99.73
0.64	103.14	0.003	0.000504	99.95	56.06	0.003	0.000504	99.95	18.84	0.014	0.002353	99.76

[a] The results are the average of three replicates
Coefficient of variation ranged from 2.7 – 18.9%
Initial cells population = 5.95×10^6 Cells/mL

5.9.4 Visual Inspection of Fouling Material

Visual inspection of the lamps after 48 hours of operation at 2 hours residence time revealed a large amount of fouling material surrounding the quartz tube surface as shown in Figure 5.9. Figure 5.10 shows parts of the fouling materials scraped from the quartz sleeve surface. Because of the large thickness of fouling material accumulated on the quartz surface, most of the fouling material, which accumulated on the lamp, was scraped by the reactor opening while trying to get the lamp out of the outer stainless steel chamber (Figure 5.11). The outer surface (the side facing the flow) of the fouling materials was creamy-white in color while the inner surface (the side facing the lamp) was yellowish-brown. Figure 5.12 shows an example of ESEM photographs for the outer and inner surfaces of the fouling materials obtained from the second UV reactor. The inner surface of the fouling materials was much smoother than the outer surface. Figure 5.13 represents the EDX analyses of both surfaces of the fouling materials obtained from the three UV reactors. The EDX analysis revealed almost the same elemental composition (C, O, Na, Al, P, S, Cl, K, Ca and P) for all the fouling materials obtained from the three UV reactors.

5.9.5 Composition of Fouling Material

The cheese whey used in this study contained about 9,953 mg/L (1%) protein, 4,700 mg/L (0.47%) fat and 6,750 mg/L (0.68%) minerals (94, 429, 1, 1670, 684, 42, 154, 483 and 3193 mg/L magnesium, calcium, manganese, potassium, sodium, iron, phosphorus, sulfur and others, respectively) as shown in Table 5.1. The fouling materials obtained from the three UV reactors were found to contain protein, fat and minerals. Table 5.8 shows the protein, fat and minerals composition of the fouling materials.

5.9.6 UV Lamp Heat Generation and Measured Temperature

As a result of UV lamp heat generation, the measured temperatures increased gradually with time reaching the steady state after 220 minutes. Figure 5.14 shows the measured values of the temperature at the nine different locations for the three UV reactors during the operating period (48 h). The effects of gap size on the observed steady state temperatures are shown in Figure 5.15. The results from the heat balance showed that the

Table 5.7. The effect of residence time on the cell survival ratio and removal efficiency of the three UV reactors[a].

Residence Time (min)	UV Reactor (1)			UV Reactor (2)			UV Reactor (3)		
	Cell No. ($\times 10^6$ cells/mL)	Survival Ratio	Removal Efficiency (%)	Cell No. ($\times 10^6$ cells/mL)	Survival Ratio	Removal Efficiency (%)	Cell No. ($\times 10^6$ cells/mL)	Survival Ratio	Removal Efficiency (%)
15	160.000	0.470588	52.94	80.000	0.235294	76.47	0.980	0.002882	99.71
30	65.000	0.191176	80.88	20.000	0.058824	94.12	0.000	0.000000	100.00
45	15.800	0.046471	95.35	6.700	0.019706	98.03	0.000	0.000000	100.00
60	3.200	0.009412	99.06	2.890	0.008500	99.15	0.000	0.000000	100.00
90	0.006	0.000018	99.99	0.001	0.000002	99.99	0.000	0.000000	100.00
120	0.003	0.000009	99.99	0.000	0.000000	100.00	0.000	0.000000	100.00

[a] The results are the average of three replicates
Coefficient of variation ranged from 0.0 – 13.6%
Initial cells population was 340×10^6 Cells/mL

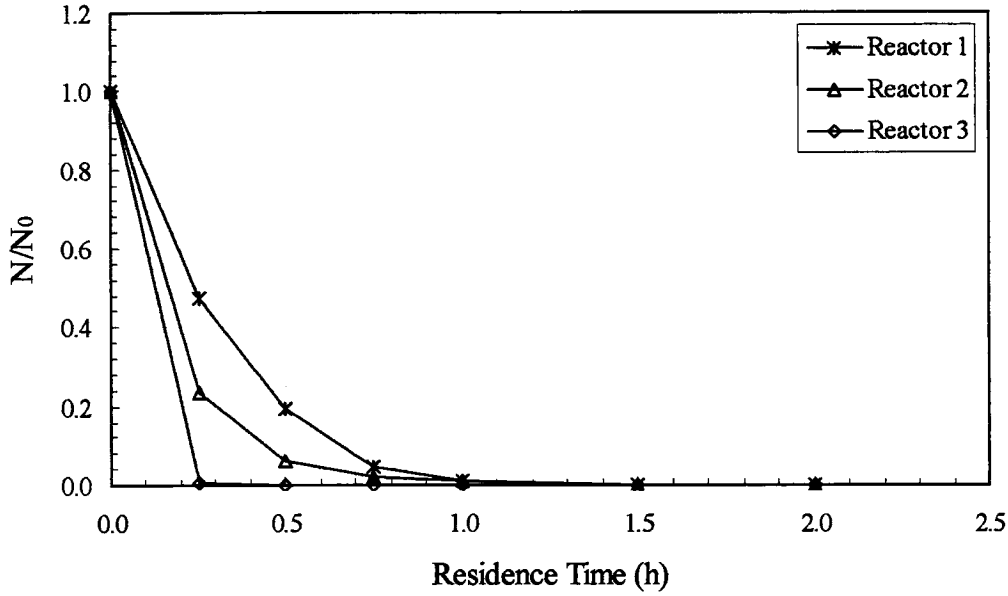
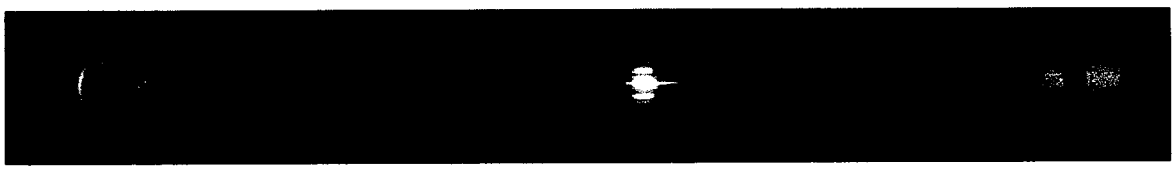


Figure 5.8. Survival curves for the three UV reactors as affected by residence time.

Table 5.8. Composition of fouling materials obtained from the three UV reactors.

Constituent (g/kg)	UV Reactor		
	1[a]	2[b]	3[c]
Protein	108.6000	121.8000	132.0000
Fat	21.5000	23.6000	28.2000
Minerals			
Magnesium	0.1790	0.2050	0.2420
Calcium	0.5860	0.7760	1.1480
Manganese	0.0040	0.0090	0.0100
Potassium	3.3000	3.4700	4.0100
Sodium	1.0700	1.1200	1.6800
Iron	0.1300	0.2290	0.6630
Phosphorous	2.2900	2.9130	3.2400
Sulfur	2.5000	3.2000	5.2000

[a] 18 mm gap size with 1.100 L working volume and 0.55 L/h flow rate
[b] 13 mm gap size with 0.598 L working volume and 0.30 L/h flow rate
[c] 6 mm gap size with 0.201 L working volume and 0.10 L/h flow rate



(a) Clean lamp

(b) Lamp after treatment using the first reactor

(c) Lamp after treatment using the second reactor

(d) Lamp after treatment using the third reactor

Figure 5.9. The UV lamp before and after treatment of the cheese whey with ultraviolet radiation using the three UV reactors.

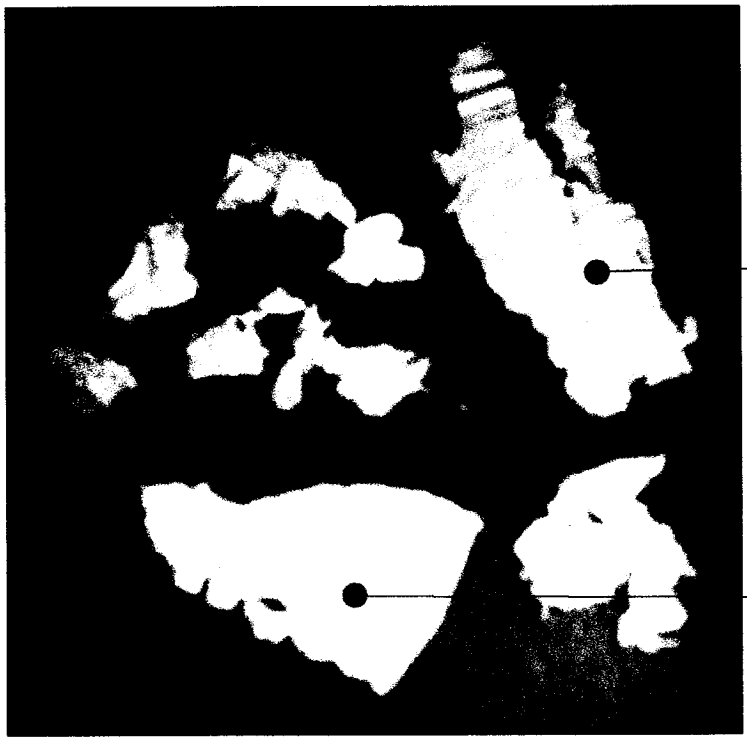


Figure 5.10. Fouling material scraped from the lamp surface of the third UV reactor after operating for 48 hours at 2 h residence time.

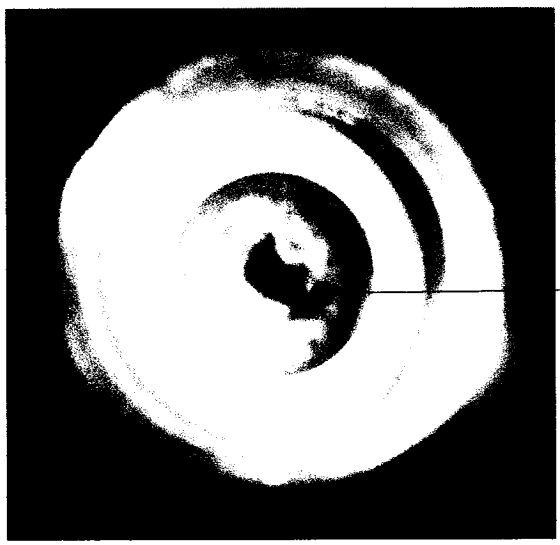
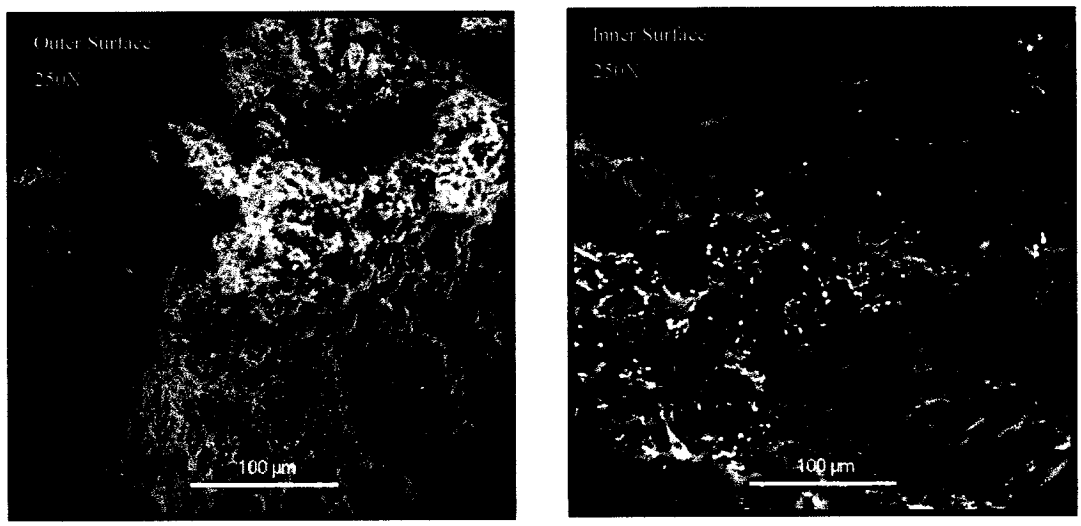
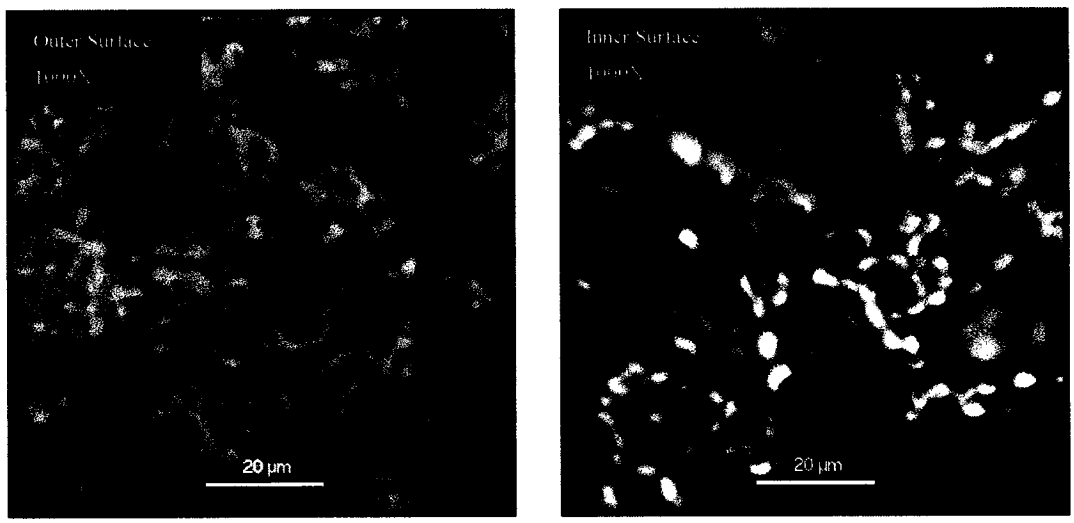


Figure 5.11. Fouling material scraped by the reactor opening and remained inside the UV reactor.

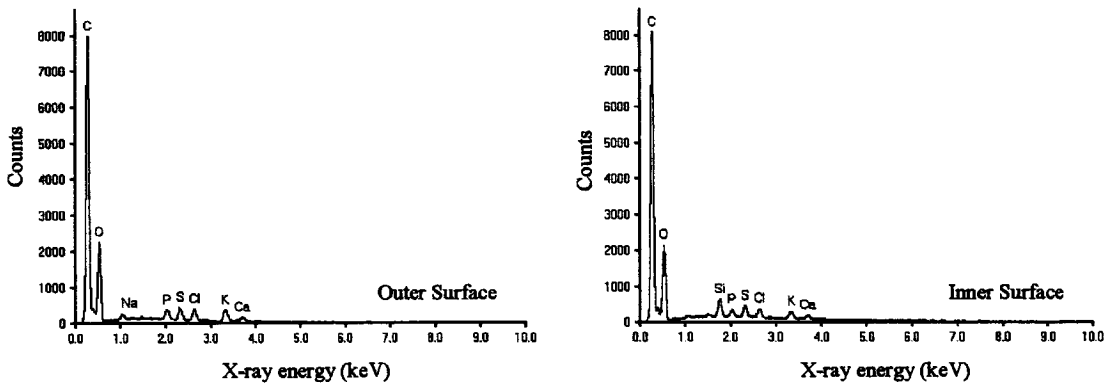


(a) 250X magnification

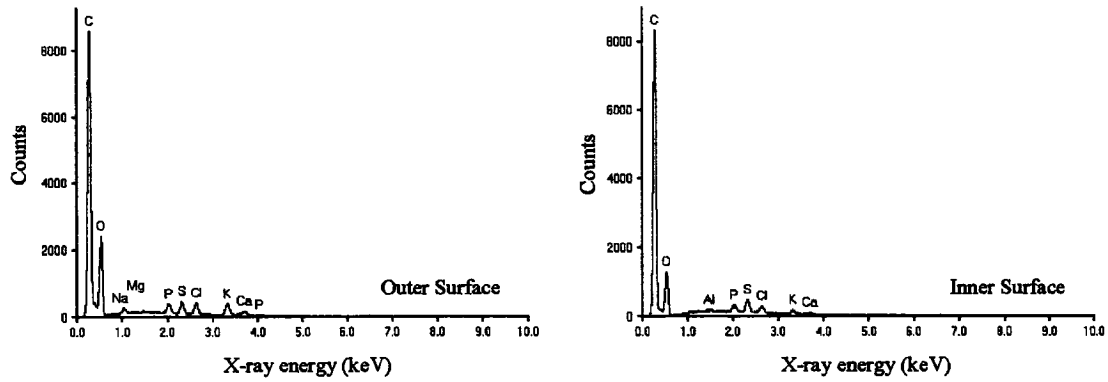


(b) 1000X magnification

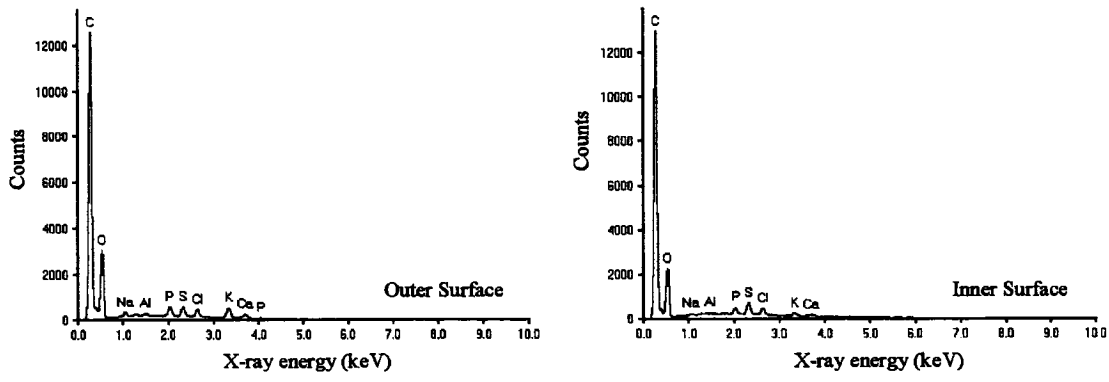
Figure 5.12. ESEM visualization of outer and inner surfaces of the fouling material obtained from the quartz surface in the second UV reactor.



(a) Reactor 1

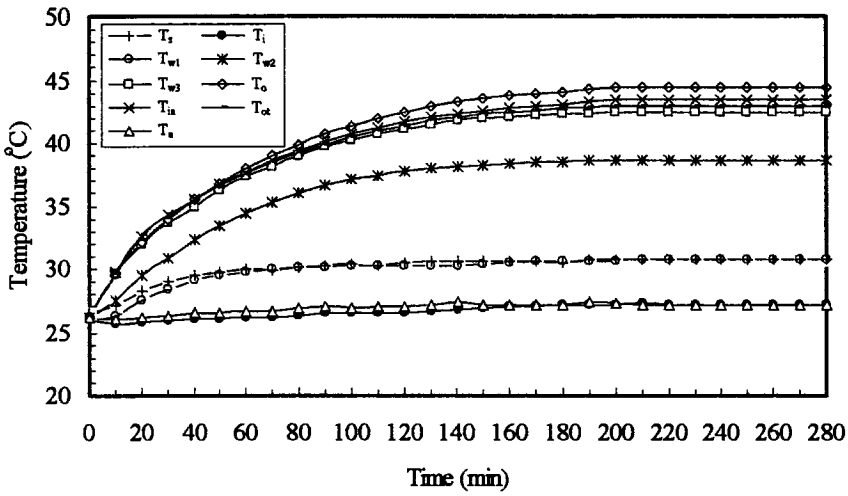


(b) Reactor 2

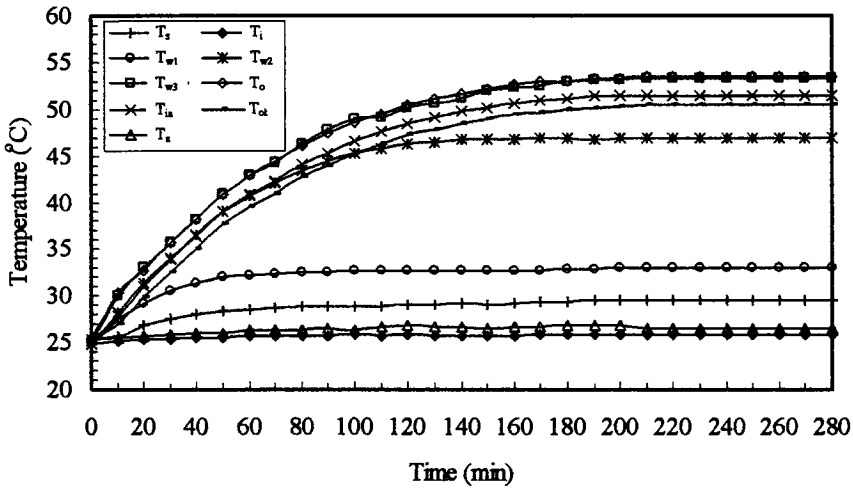


(c) Reactor 3

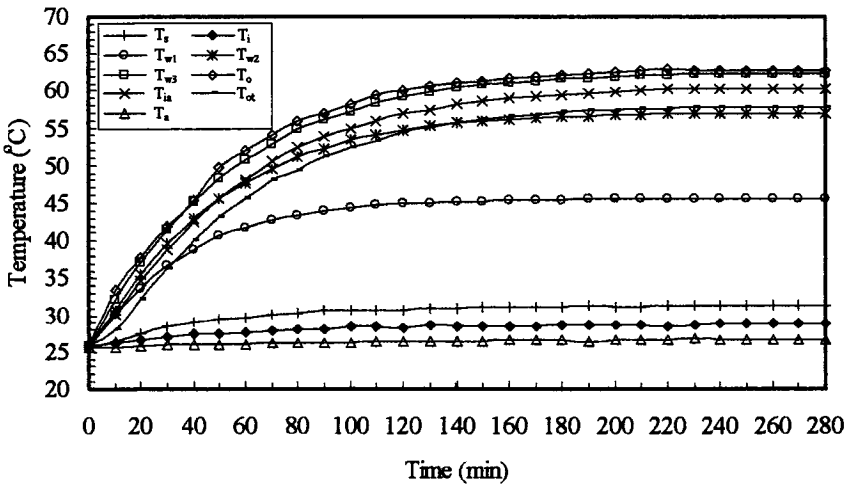
Figure 5.13. Elements identified in the fouling materials obtained from the three UV reactors (EDX analysis).



(a) First UV Reactor (18 mm gap size)



(b) Second UV Reactor (13 mm gap size)



(c) Third UV Reactor (6 mm gap size)

Figure 5.14. The temperature change with time at the 9 different locations.

lamp heat generation was 50.482, 47.708 and 31.912 kJ/h for the first (18 mm gap size), second (13 mm gap size) and third (6 mm gap size) reactors, respectively

5.9.7 Extent of Lamp Fouling

The results showed that the amount of fouling material increased with the decrease in the flow thickness (gap size). The highest amount of fouling material was observed with the smallest UV reactor (6 mm gap size). The total wet weight of the fouling materials was found to be 14.4187, 15.3099, 25.2548 g for the first (18 mm gap size), second (13 mm gap size) and third (6 mm gap size) UV reactor, respectively. The average moisture content of the fouling materials was 82.9 ± 2.2 % (CV = 2.7%).

5.9.8 Heat Balance

The heat losses through the entire UV reactor were calculated during both transient and steady state stages for the three UV reactors. The heat transfer coefficients were taken from ASHRAE (1989) and Holman (2002). Throughout the whole calculations, the heat losses through the UV reactor bottom were neglected for two reasons: (a) the cheese whey inlet temperature was similar to the ambient air temperature (25.6-27.0 °C vs. 26.4-26.9 °C) and (b) the area of the UV reactor bottom was very small. Therefore, the heat balance on the entire UV reactor (equation 5.3) can be rewritten as follows:

$$q_l = q_e + q_t + q_w \tag{5.30}$$

The calculated Rayleigh numbers ($Gr_f Pr_f$) at the average film temperatures for both the UV reactor wall and the UV reactor top of the three UV reactors were less than 10^9. Therefore, the flow was considered laminar and equations 5.14, 5.15 and 5.16 were used to calculate the convective heat transfer coefficients between the UV reactor outside wall and the ambient air, between the outer surface of the UV reactor top and the ambient air and between the inner surface of the UV reactor top and the air entrapped inside the UV reactor headspace, respectively. Table 5.9 shows the values of the different parameters used in the calculations. Table 5.10 shows the calculated values of the various heat transfer coefficients during the transient and the steady state heat transfer stages.

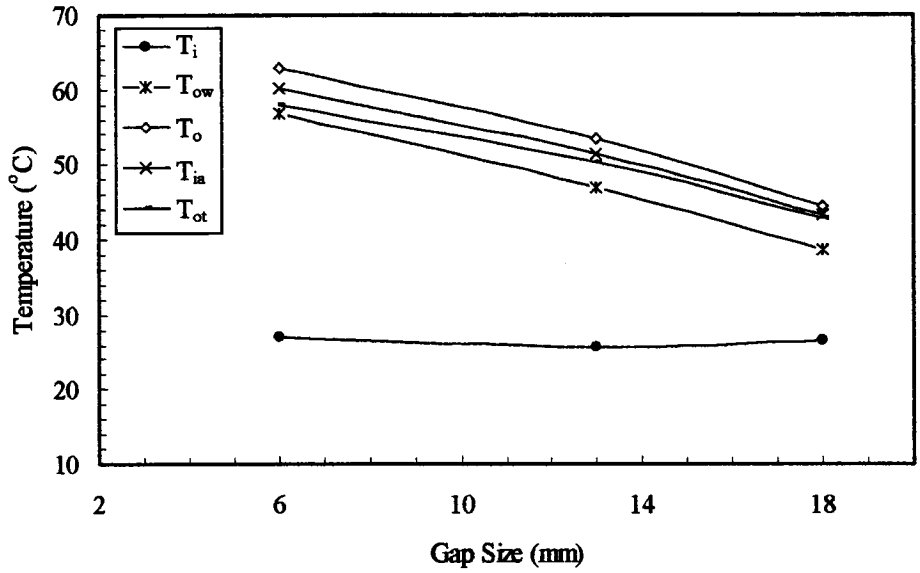


Figure 5.15. The steady state temperatures at different gap sizes.

Table 5.9. Values of various parameters at steady state conditions.

Parameter	Values			Units
	Reactor 1	Reactor 2	Reactor 3	
A_{iw}	893.39	695.42	492.44	cm^2
A_{ow}	900.54	733.56	620.78	cm^2
A_t	31.17	19.63	13.59	cm^2
C_{pe}	4.174	4.174	4.174	kJ/kg.°C
d_t	5.00	7.00	15.00	mm
d_w	3.00	1.34	4.30	mm
g	9.81	9.81	9.81	m/s^2
K_t	47.60	47.60	47.60	W/m.K
K_w	47.60	47.60	47.60	W/m.K
L_t	63.00	50.00	41.60	mm
L_w	455.00	467.00	475.00	mm
m_e	0.55	0.30	0.10	Kg/h
r_i	31.25	23.70	16.50	mm
r_o	31.50	25.00	20.80	mm
T_a^a	26.90	26.40	26.30	°C
T_i^a	26.50	25.60	27.00	°C
T_o^a	44.50	53.40	62.80	°C
T_m^a	35.50	39.50	44.90	°C
T_{ia}^a	43.40	51.50	60.20	°C
T_{it}^a	43.20	51.00	60.00	°C
T_{ot}^a	43.00	50.40	57.80	°C
T_{ow}^a	38.6	46.90	56.90	°C

[a] At steady state

Table 5.10. Heat transfer coefficients during the transient and steady state stage of the three UV reactors.

Coefficient (W/m^2 °C)	Time (min)																	
	40			80			120			160			200			220[a]		
	(1)[b]	(2)[c]	(3)[d]	(1)[b]	(2)[c]	(3)[d]	(1)[b]	(2)[c]	(3)[d]	(1)[b]	(2)[c]	(3)[d]	(1)[b]	(2)[c]	(3)[d]	(1)[b]	(2)[c]	(3)[d]
h_{ow}	2.68	3.09	3.58	3.01	3.49	3.97	3.13	3.61	4.10	3.17	3.64	4.15	3.16	3.64	4.17	3.20	3.66	4.02
U_w	2.68	3.09	3.58	3.01	3.49	3.96	3.13	3.60	4.09	3.17	3.64	4.14	3.16	3.64	4.17	3.20	3.65	4.02
h_{ot}	4.55	4.85	5.65	4.94	5.62	6.42	5.13	5.93	6.73	5.22	6.09	6.84	5.24	6.14	6.89	5.28	6.18	6.92
h_{it}	1.48	1.57	1.64	1.48	1.57	1.64	1.48	1.57	1.64	1.48	1.57	1.64	1.48	1.57	1.64	1.76	3.08	5.93
U_t	1.12	1.19	1.27	1.14	1.99	1.31	1.15	2.03	1.32	1.15	2.05	1.32	1.16	2.06	1.33	1.32	2.06	3.19

[a] At steady state stage
[b] (1) First UV reactor (18 mm gap size)
[c] (2) Second UV reactor (13 mm gap size)
[d] (3) Third UV reactor (6 mm gap size)

5.10 Discussion

5.10.1 Cheese Whey Physical Characteristics

Generally, turbidity can be defined as the decrease in the transparency of a solution as a result of the presence of suspended and some dissolved substances causing incident light to be scattered, reflected, and attenuated rather than transmitted in straight lines, the higher the intensity of the scattered or attenuated light the higher the value of turbidity (Tchobanoglous and Burton, 1991). Turbidity is usually expressed in nephelometric turbidity units (NTU), which is defined as the intensity of light at a specific wavelength scattered or attenuated by suspended particles or absorbed at a specified angle (usually 90°) from the path of the incident light compared to a synthetic chemically prepared standard (APHA. 1985; and Ziegler, 2002).

The results indicated that whey is a highly turbid waste material. The high turbidity of the cheese whey is attributed to the presence of suspended solid particles. The cheese whey used in this study contains about 68300 mg/L total solids of which 25160 mg/L (36.84%) are suspended solids. Hwang and Damodaran (1994) and Hwang and Damodaran (1995) reported that turbidity of the cheese whey is mainly the result of light scattering caused by the presence of the micron-sized milk fat globule membrane fragments (MFGM). The cheese whey used in this study contained about 4700 mg/L fat. The average turbidity of the cheese whey used in this study was about 4317 NTU. A previous study by Savant and Torres (2000) reported a turbidity of about 1536 NTU for the supernatant of centrifuged cheddar cheese whey. No values were reported for the turbidity of raw cheese whey in the literature.

Cheese whey has a very poor transmittance to UV radiation in the germicidal range (0.0% for all the tested wavelengths except for 330 nm which had transmittance of 0.1%). The results indicated that most of the radiation in the UV germicidal range is absorbed or scattered, rather than transmitted through the cheese whey medium as a result of the high turbidity. Shaban et al. (1997), Keller et al. (2003) and Otaki et al. (2003) reported that

UV disinfection is considered to be inefficient with water of high turbidity because of the inhibition of light penetration.

The results of the particle size distribution showed that the cheese whey used in this study contained large solid particles. About 80% of the total solids were of 100 μm size or less. Particles that are larger than 100 μm accounted for about 20% of the total solids. Generally, cheese whey contains about 4.4-5.0% lactose, 0.1-0.5% fat, 0.6-0.9 protein, 0.5-0.9% minerals (e.g. calcium, magnesium, and phosphorous), and 0.2% lactic acid (Cheryan and Merin, 1980; and Francis, 2000). The size distribution of these cheese whey components is illustrated in Figure 5.16. Cheryan (1998) reported that whey usually contains small quantities of fat in the form of small globules of 0.2-1 μm and casein as fine particulates of 5-100 μm . From the analysis, it seems that there were some particles (20%) that have larger size than the common cheese whey components. This might be due to coagulation of some of the whey components such as proteins and casein. Proteins and casein are known to coagulate at acidic conditions. Generally, at high pH proteins remain soluble, while at low pH they often aggregate (Francis, 2000). Cheryan (1998) stated that the solubility of proteins is generally lowest at the isoelectric point and increases as the pH is adjusted away from the isoelectric point. The isoelectric points of whey proteins range from 4.7 to 6.8 (Eigel et al., 1984). Macleod et al. (1995) reported that the isoelectric point of β -Lactoglobulin (55% of the total whey protein) is pH of 5.2. At pH of 5.2 β -Lactoglobulin reaches its maximum particle size (34 μm). Because the cheese whey was left for three days at room temperature (18 – 21 °C) in order to increase the microbial population before running the experiment, the acidity of the cheese whey was increased (pH = 4.5) as a result of lactose conversion to lactic acid.

Particles in wastewater are known to affect the performance of UV radiation disinfection process either by absorbing the light or shielding the microorganisms. Qualls et al. (1983) evaluated the effects of exclusion of various particle sizes on coliforms disinfection in wastewater and found that filtered samples were more effectively disinfected than the corresponding unfiltered samples. Loge et al. (1999) stated that neither size of a particle

nor distance of a targeted organism from the perimeter of a particle alone dictates the likelihood of inactivating a targeted organism, but particle porosity is of a great effect.

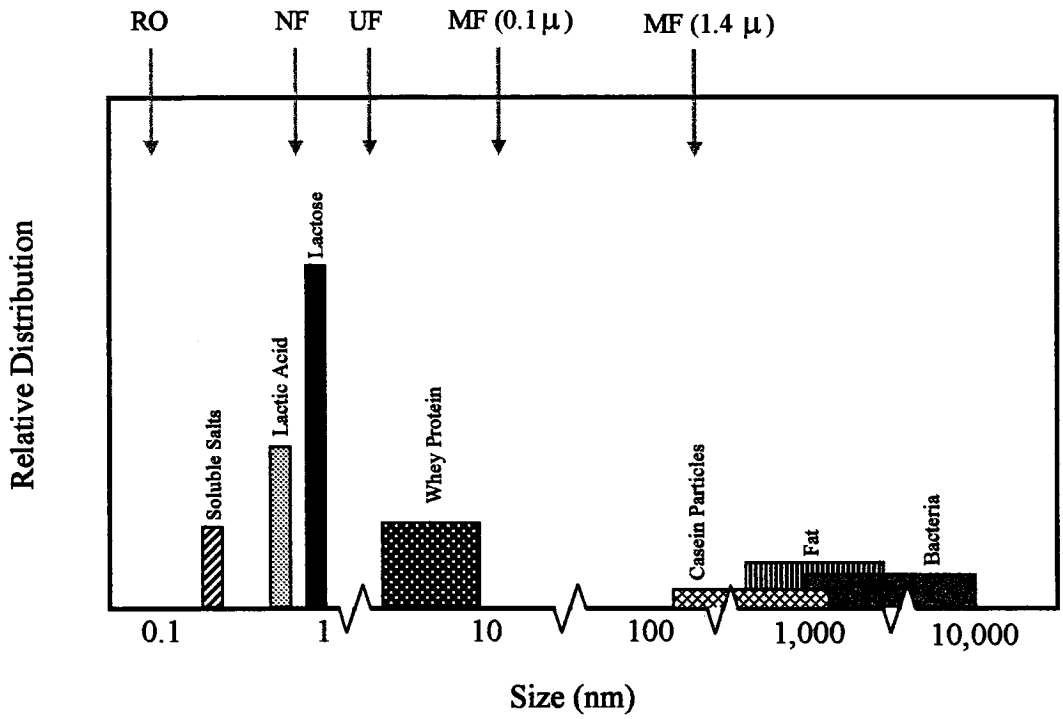


Figure 5.16. Size distribution of components in cheese whey (Cheryan, 1998).

Since low transmittance, high turbidity, and large solid particles content of the cheese whey were expected to affect the UV germicidal performance as a result of the reduction of the penetration ability of the UV radiation and shielding the microbial cells, it was believed that high residence time (low flow rates) and thin flow thickness (gap size) may enhance the UV sterilization process.

5.10.2 Effect of Flow Rate

Reynolds number calculations were performed using the following equation:

$$Re = \frac{\upsilon_m D_h}{\nu} \tag{5.31}$$

Where:

Re is the Reynolds number

ν is the kinematic viscosity (cm^2/h)

The results indicated that the flow was laminar in the three UV reactors (Re = 0.6 – 33.0) as a result of the fairly low flow rates tested in this study. Generally, the flow is laminar when Reynolds number is less than 4000 and is turbulent when Reynolds number is greater than 4000 (Cengel, 2003). Therefore, no turbulence mixing was achieved in the three UV reactors and residence time and flow thickness were the two main parameters that controlled the effectiveness of the sterilization process.

The results from the flow rate experiments showed that UV radiation was capable of reducing the microbial population in the cheese whey by 55.13 – 99.95%, depending on the flow rate. The removal efficiency was 66.72, 61.34, and 55.13% at the highest flow rate (3.84 L/h) and increased to 99.95, 99.95, and 99.76% at the lowest flow rate (0.64 L/h) for the first (18 mm gap size), second (13 mm gap size) and third (6 mm gap size) UV reactors, respectively. The flow rate of 0.64 L/h corresponded to a residence time of 103.14, 56.06 and 18.84 min for the first (18 mm gap size), second (13 mm gap size) and third (6 mm gap size) UV reactors, respectively. Longer residence times increased the probability of the scattered radiation hitting the microbial cells and causing the germicidal effect. The scattered light is supposed to destruct cells in the same way as the transmitted light. Moreover, organic particles are highly porous and consequently create pathways for light penetration (Loge et al., 1999). The results also indicated that there was a combined effect of residence time and gap size. Smaller gap size could increase the probability of the UV radiation reaching the cells.

Analysis of variance (α = 0.05) was performed on the number of viable cells after treatment using the SAS System, 5th ed. (SAS Institute Inc., Cary, North Carolina) as shown in Table 5.11. Table 5.12 shows the results of Duncan's Multiple Range test. The analysis showed that the gap size and the flow rate have significant effects on the number of viable cells remaining after UV treatment (P = 0.0001). The analysis also showed a

Table 5.11. Analysis of variance of the results obtained from the flow rate set of experiments.

Source	DF	SS	MS	F-Value	P
Total	53	39.6985			
Model	17	39.1898	2.3053	163.17	0.0001
Gap Size	2	0.4457	0.2229	15.77	0.0001
Flow Rate	5	38.0097	7.6019	538.06	0.0001
Interaction	10	0.7344	0.0730	5.20	0.0001
Error	36	0.5086	0.0141		

DF = Degrees of Freedom
SS = Sum of Squares
MS = Mean of Squares
R^2 = 0.987
CV = 19.77%

Table 5.12. Differences among the levels of gap size and flow rate.

Parameter	Number of Observations	Viable Cells ($\times 10^6$ cells/mL)	Duncan Grouping[a]
Gap Size (mm)			
6	18	0.68178	A
13	18	0.64783	A
18	18	0.47433	B
Flow rate (L/h)			
3.84	9	2.31667	A
1.92	9	0.97667	B
1.28	9	0.26600	C
0.96	9	0.03267	D
0.76	9	0.00922	D
0.64	9	0.00667	D

[a] Means with the same letter are not significantly different from each other at 95% confidence level.

significant interaction between the two parameters (P = 0.0001). At 95% confidence level, the means of viable cell concentration in the reactors having 6 and 13 mm gap sizes were not significantly different from one another but were significantly different from the mean of the viable cell concentration in the reactor having 18 mm gap size. The levels of flow rate in the range 3.84 – 0.96 L/h were significantly different from one another. Decreasing the flow rate below 0.96 L/h did not have any significant effect on the viable cells remaining in the reactor.

From the survival curves, it is obvious that the relation between the survival ratio and the residence time is exponential. Survival curves are semilog plots of the ratio of the concentration of surviving organisms to the initial number versus time. They are commonly used to present inactivation data and to interpret the inactivation kinetics of microorganisms (Harm, 1980; and Weavers and Wickramanayake, 2001). The destruction of microbial cells present in the cheese whey by ultraviolet radiation could be described with the following first order equation:

$$-\left(\frac{dN}{dt}\right) = kN \tag{5.32}$$

Where

N is the number of viable organisms present in the cheese whey.

t is the sterilization time (h).

k is the decay rate constant (h^{-1}).

For a given set of operating conditions, the decay rate (k) is dependent on the effective irradiance of the UV radiation and the sensitivity of the microorganisms (Meulemans, 1987). In this study, the decay rate increased with the decrease in the thickness of the flowing column (gap size). The values of the decay rate were found to be 4.94, 7.62, and 20.90 h^{-1} for the first (18 mm gap size), second (13 mm gap size) and third (6 mm gap size) UV reactors, respectively.

The decimal reduction time, which is defined as the time necessary to reduce the microbial population by 1-log or 90% at a given radiation intensity was found to be equal to 27.98, 18.13, and 6.60 min for the first, second and third UV reactors, respectively. On this basis, it would require over 195.86, 126.91, and 46.20 min (3.3, 2.1, and 0.8 h) to destroy the whole initial population in the cheese whey used in this set of experiments using the first (18 mm gap size), second (13 mm gap size), and third (6 mm gap size) UV reactors, respectively.

5.10.3 Effect of Residence Time

The results indicated that for a given residence times, the gap size through which the cheese whey is flowing has affected the sterilization efficiency: the lower the gap size, the higher the sterilization efficiency. At 15 min residence time the removal efficiency was 52.94, 76.47, and 99.71% for the first (18 mm gap size), second (13 mm gap size) and third (6 mm gap size) UV reactors, respectively. A removal efficiency of 100% was obtained at residence time of 30 and 120 minutes with the third (6 mm gap size) and the second (13 mm gap size) UV reactors, respectively. However, 100% removal efficiency was never achieved with the first UV reactor (18 mm gap size) as the fluid depth exceeded the limits of irradiated zone. Blatchley III and Peel (2001) reported that when the fluid depth exceeds the limits of irradiated zone, a portion of the flow will be minimally irradiated and hence causes poor efficiency.

Analysis of variance ($\alpha = 0.05$) was performed on the number of viable cells remaining in the UV reactor using the SAS System, 5th ed. (SAS Institute Inc., Cary, North Carolina) as shown in Table 5.13. Table 5.14 shows the results of Duncan's Multiple Range test. The analysis showed that the gap size and the residence time have significant effects on the number of viable cells remaining after UV treatment ($P = 0.0001$). The analysis also showed a significant interaction between the two parameters ($P = 0.0001$). At 95% confidence level, the means of viable cell concentration with reactors having 6, 13 and 18 mm gap sizes were significantly different from one another. The levels of residence time in the range 0.25 – 1.00 h were significantly different from one another. Increasing the residence time above 1.0 h did not seem to have any significant effect.

Table 5.13. Analysis of variance of the results obtained from the residence time set of experiments.

Source	DF	SS	MS	F-Value	P
Total	53	90129.9308			
Model	17	89862.7858	5286.0462	712.34	0.0001
Gap Size	2	14821.2763	7410.6382	998.64	0.0001
Flow Rate	5	44885.2368	8977.0474	1209.73	0.0001
Interaction	10	30156.2728	3015.6272	406.38	0.0001
Error	36	267.1450	7.4207		

DF = Degrees of Freedom
SS = Sum of Squares
MS = Mean of Squares
R^2 = 0.997
CV = 13.81%

Table 5.14. Differences among the levels of gap size and residence time.

Parameter	Number of Observations	Viable Cells ($\times 10^6$ cells/mL)	Duncan Grouping[a]
Gap Size (mm)			
6	18	0.1633	A
13	18	18.2651	B
18	18	40.6682	C
Residence Time (h)			
0.25	9	80.327	A
0.50	9	28.333	B
0.75	9	7.500	C
1.00	9	2.030	D
1.50	9	0.002	D
2.00	9	0.001	D

[a] Means with the same letter are not significantly different from each other at 95% confidence level.

From the survival curves of the residence time experiments, it was found that the decay rates for the first (18 mm gap size), second (13 mm gap size) and third (6 mm gap size) reactors were 5.40, 7.07, 23.40 h^{-1}, respectively. The difference between the values of the decay rate obtained from the flow rate and residence time sets of experiments ranged from 7% to 10%, which is reasonably within the experimental error.

5.10.4 Combined Effects of Residence Time and Gap Size

The relationship between the decay rate and gap size is shown in Figure 5.17. This relationship could be presented by the following equation ($R^2 = 0.99$):

$$k = 41.26\, e^{-0.122G} \tag{5.33}$$

Where:

G is the gap size through which the cheese whey is flowing (mm).

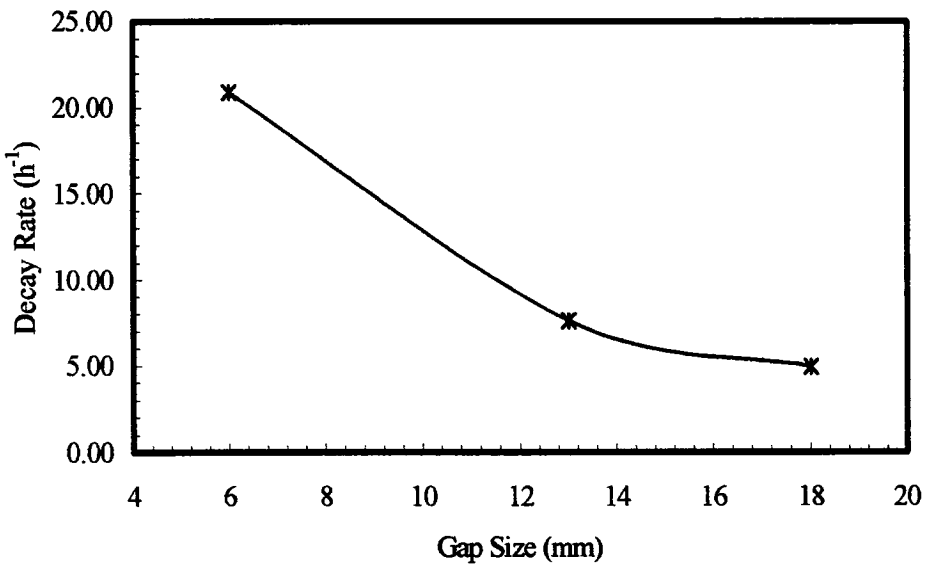


Figure 5.17. The decay rate as affected by the gap size.

By substituting equation (5.33) in equation (5.32), the combined effects of gap size and residence time on the microbial survival ratio could be described by the following equation ($R^2 = 0.98$):

$$\frac{N}{N_0} = e^{-(41.26e^{-0.122G})t} \tag{5.34}$$

5.10.5 Fouling Material Characteristics

The whole lamp was completely surrounded with a rubber-like material, the morphology of which was homogeneous and consistent for the three UV reactors. However, the inner surface (the side facing the lamp) of the fouling material looked much smoother than the outer surface (the side facing the flow). The outer surface of the fouling material was creamy-white in color while the inner surface was yellowish-brown because it was exposed to higher temperature than the outer surface that was subjected to cooling by the flowing cheese whey. As obtained from this study and as reported in the literature (Zall, 1992; and Savant and Torres, 2000), cheese whey is known to contain about 5% lactose and 1% protein. Morales and van Boekel (1998) reported that milk and milk-based systems darken when increasing the intensity of heat because of Maillard reaction and sugar caramelization. Maillard reaction is a complex network of chemical reactions in which reducing sugars react with proteins or amino acids (Fox et al., 1983; and Morales and van Boekel, 1998). Sugar caramelization is the reaction in which sugars, when exposed to high temperature conditions, undergo similar reactions (on their own) similar to the Maillard reaction but without interaction with amino compounds (Nursten, 1986). Since high heat intensity (temperature > 120 °C) and extreme pH conditions (3 > pH > 9) are required to cause sugar caramelization (Morales and van Boekel, 1998), the difference in color between the inner and outer layers observed in this study is most likely due to the Maillard reaction rather than the sugar caramelization since the highest steady state temperature recorded was 62.8 °C and the cheese whey pH did not go below 4. Finot et al. (1981) reported that whey is much more sensitive to Maillard reaction than whole milk because it is rich in lactose and has soluble proteins. The Maillard reaction products include volatile compounds of low molecular mass, non-volatile colored

compounds of intermediate molecular mass and brown substances of high molecular mass. Factors such as water activity, temperature, pH and chemical composition of the material affect the velocity of Maillard reaction (Finot et al., 1981; Fox et al., 1983; and Nursten, 1986). As with any chemical reaction, the Maillard reaction proceeds much faster at high temperatures. Color change is an important and obvious characteristic of the extent of the Maillard reaction and the color may range from pale yellow to very dark brown depending on the extent of the reaction (Nursten, 1986).

5.10.6 Chemical Composition of Fouling Materials

5.10.6.1 Fat Content. The raw cheese whey used in this study contained about 0.47% fat, which is within the range of 0.1-0.5% reported by Cheryan and Merin (1980). The fouling materials obtained from the first (18 mm gap size), second (13 mm gap size) and third (6 mm gap size) UV reactors contained about 2.15, 2.36, 2.82% fat, respectively (Table 5.15). Although the percentage of fat in the fouling materials increased slightly with decreasing the UV reactor gap size, the amount of fouling material deposited on the quartz surface increased significantly with the decrease in gap size. Consequently, the total amount of fat obtained in the fouling material was 0.3100, 0.3613 and 0.7122 g for the first (18 mm gap size), second (13 mm gap size) and third (6 mm gap size) UV reactors, respectively. These amounts represented about 0.25, 0.53 and 3.17% of the total fat of 124.08, 67.68 and 22.46 g passed through the first (18 mm gap size), second (13 mm gap size) and third (6 mm gap size) UV reactors during the whole operating period (48 h), respectively.

Whey lipids are mainly phospholipids (3-sn-phosphatidylcholine; common name is lecithin), which derived from milk fat globule membrane (MFGM) fragments that remain dispersed in a stable colloidal state (Hwang and Damodaran, 1994; Hwang and Damodaran, 1995; and Fennema, 1996). The high negative surface charge density resulting from the phosphate groups of the bilayer membrane as well as the high hydrophilicity of the membrane surface and the electrostatic repulsion between the membrane particles is responsible for the colloidal stability of MFGM fragments in the cheese whey. The relationship between phosphorous and fat in the fouling material

Table 5.15. Fat content[a] of the raw cheese whey and the fouling materials obtained from the three studied UV reactors after 48 hours of operation.

UV Reactor	Gap Size (mm)	Reactor Volume (L)	Flow Rate (L/h)	Cumulative Cheese Whey (L)	Cumulative Cheese Whey Fat[b] (g)	Fouling Material (g)	Fat Content of the Fouling Material[c] (g/kg)	Cumulative Fouling Material Fat (g)
1	18	1.100	0.55	26.4	124.08	14.4187	21.5	0.3100
2	13	0.598	0.30	14.4	67.68	15.3099	23.6	0.3613
3	6	0.201	0.10	4.8	22.46	25.2548	28.2	0.7122

[a] Values are presented on wet basis
[b] The raw cheese whey contains 0.47% fat (4700 mg/L)
[c] Obtained from the chemical analysis

(Figure 5.18) can be described by the following equation ($R^2 = 0.99$).

$$F = 96.0 - 0.0605P + 0.000012P^2 \quad (5.35)$$

Where:

F is fat concentration in the fouling material (g/kg)

P is phosphorous concentration in the fouling material (mg/kg)

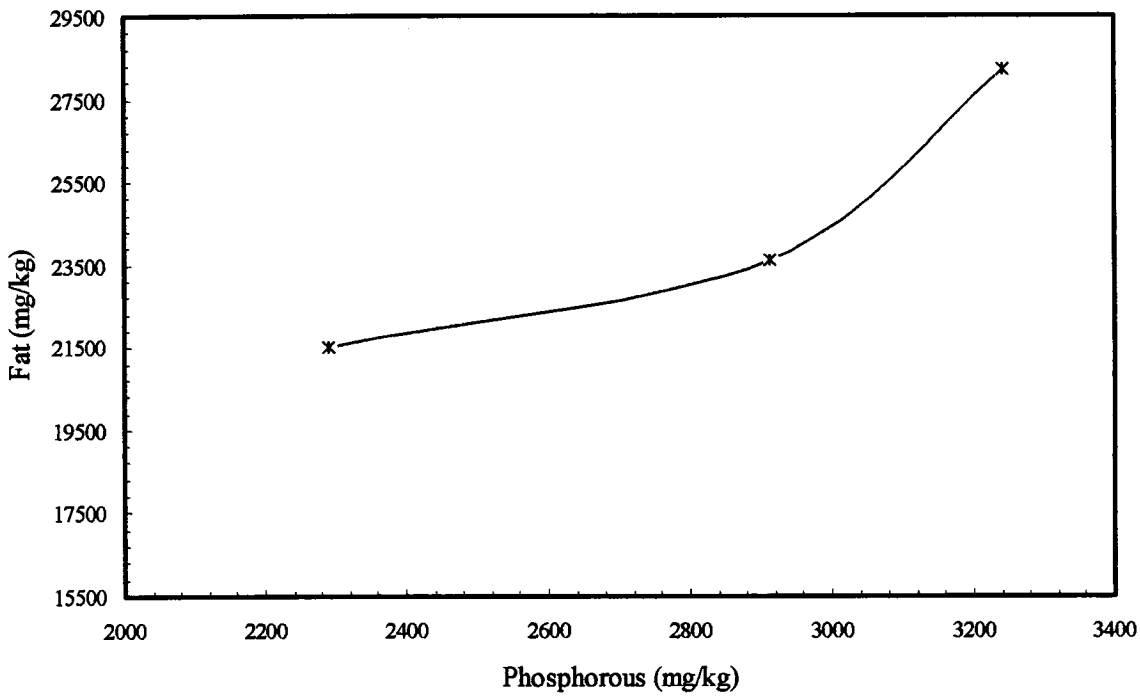


Figure 5.18. The relationship between phosphorous and fat in the fouling material.

5.10.6.2 Protein Content. The raw cheese whey (93% water) contained about 9,953 mg/L protein, which represented about 1.0% of the raw cheese whey (14.3% of the dry matter). Cheryan and Merin (1980) and Francis (2000) reported that whey protein constitutes approximately 0.6-1.0% of the whey (about 9-14% of the dry matter). The protein content of the fouling materials was about 10.9, 12.2 and 13.2% for the first (18 mm gap size), second (13 mm gap size) and third (6 mm gap size) UV reactors, respectively (Table 5.16). The percentage of protein in the fouling materials increased slightly with decreasing the UV reactor gap size. The total amount of protein lost from

Table 5.16. Protein content[a] of the raw cheese whey and the fouling materials obtained from the three studied UV reactors after 48 hours of operation.

UV Reactor	Gap Size (mm)	Reactor Volume (L)	Flow Rate (L/h)	Cumulative Cheese Whey (L)	Cumulative Cheese Whey Protein[b] (g)	Fouling Material (g)	Protein Content of the Fouling Material[c] (g/kg)	Cumulative Fouling Material Protein (g)
1	18	1.100	0.55	26.4	262.7539	14.4187	108.6	1.5659
2	13	0.598	0.30	14.4	143.3203	15.3099	121.8	1.8647
3	6	0.201	0.10	4.8	47.7734	25.2548	132.0	3.3336

[a] Values are presented on wet basis (Protein Content = Total Kjeldahl Nitrogen (TKN) × 6.38)
[b] The raw cheese whey contains 1560 mg/L TKN (9953 mg/L or 1%)
[c] Obtained from the chemical analysis

the raw cheese whey over the 48 hours operating time was 0.60, 1.30 and 7.0% of the total protein present in the cheese whey passed through the first (18 mm gap size), second (13 mm gap size) and third (6 mm gap size) UV reactors, respectively.

Whey proteins represent 20% of the total proteins found in milk and consist predominantly of β-Lactoglobulin (50%), α-Lactalbumin (12%), Immunoglobulins (10%), bovine serum albumin (5%), and Proteose-peptones (0.23%) (Savant and Torres, 2000). Bailey and Ollis (1986) stated that proteins contain 50-55% carbon, 6-7% hydrogen, 20-23% oxygen, 12-19% nitrogen, and 0.2-3.0% sulfur. Cheryan, (1998) reported that proteins are a major foulant because of: (a) the multiplicity of functional groups, (b) the charge density within the protein molecule, (c) the varying degrees of hydrophobicity, and (d) the complex secondary and tertiary structure that allows a protein to interact with other components and materials. However, all these properties are affected by pH, ionic strength, shear, heat treatment, and other environmental factors. Calcium in ionic form acts as a bridge between the lamp surface and protein leading to faster protein fouling. The relationship between calcium and protein in the fouling material (Figure 5.19) can be described by the following equation ($R^2 = 0.99$).

$$Pr = 33.9 + 0.171Ca - 0.000075Ca^2 \quad (5.36)$$

Where:

Pr is protein concentration in the fouling material (g/kg)

Ca is calcium concentration in the fouling material (mg/kg)

5.10.6.3 <u>Minerals Content</u>. The mineral composition of the cheese whey and fouling materials are shown in Table 5.17. The results showed that the raw cheese whey used in this study contained about 0.4% minerals (0.0094% magnesium, 0.0429% calcium, 0.0001% manganese, 0.1670% potassium, 0.0684% sodium, 0.0042% iron, 0.0154% phosphorous, 0.0483% sulfur and 0.0443% others). These results were comparable to those reported by Cheryan (1986) and Kosaric and Asher (1982). Siso (1996) reported

that cheese whey salts are mainly comprised of NaCl and KCl (more than 50%), calcium salts (primarily phosphate) and magnesium.

Cheryan (1998) reported that mineral salts are important foulants since they can bind directly to surfaces by charge. The total mineral content of the fouling materials were 1.02, 1.19 and 1.62% for the first (18 mm gap size), second (13 mm gap size) and third (6 mm gap size) UV reactors, respectively. The fouling material obtained from the first UV reactor (18 mm gap size) contained 0.0179, 0.0586, 0.0004, 0.3300, 0.1070, 0.0130, 0.2290, 0.2500%, that obtained from the second UV reactor (13 mm gap size) contained 0.0205, 0.0776, 0.0009, 0.3470, 0.1120, 0.0229, 0.2913 and 0.3200 and that obtained from the third UV reactor (6 mm gap size) contained 0.0242, 0.1148, 0.0010, 0.4010, 0.1680, 0.0663, 0.3240 and 0.5200% of Mg, Ca, Mn, K, Na, Fe, P and S, respectively. The cheese whey passed through the first (18 mm gap size), second (13 mm gap size) and third (6 mm gap size) UV reactors lost about 0.10, 0.07, 0.38, 0.11, 0.09, 0.17, 0.81 and 0.28%, 0.23, 0.19, 0.69, 0.22, 0.17, 0.58, 2.01 and 0.70% and 1.35, 1.41, 6.25, 1.26, 1.29, 8.28, 11.07 and 5.66 of the Mg, Ca, Mn, K, Na, Fe, P and S, respectively.

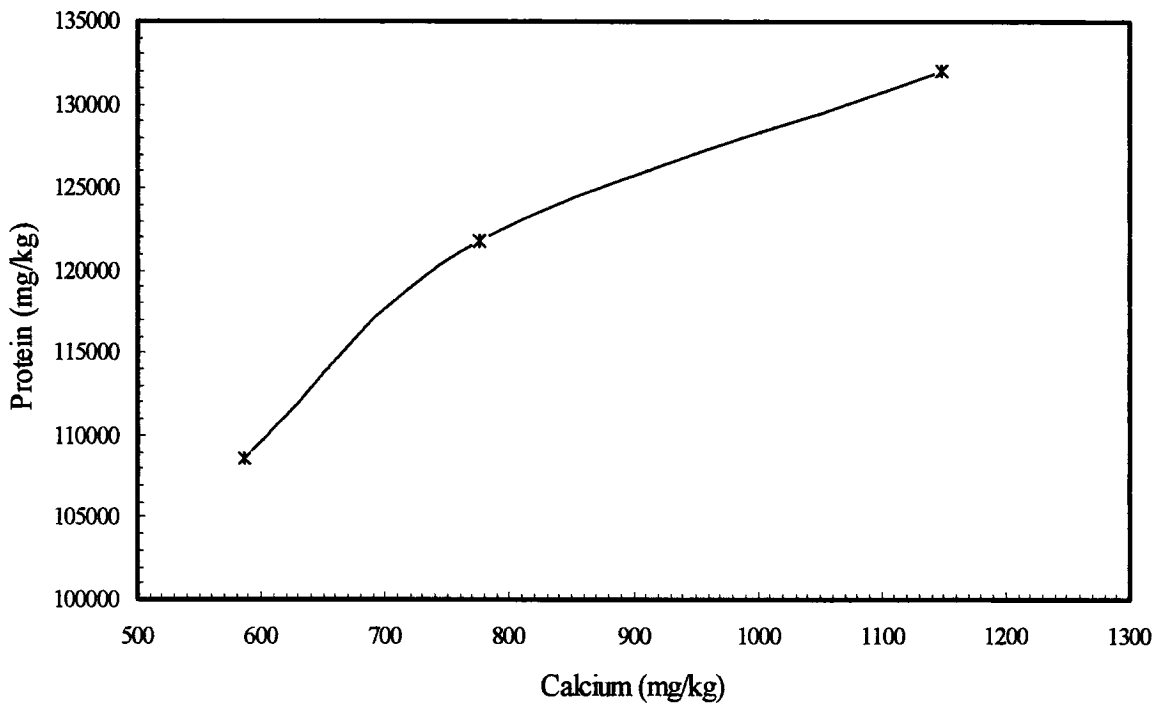


Figure 5.19. The relationship between protein and calcium in the fouling material.

Table 5.17. Elements present in the raw cheese whey used in this study.

Element	Cheese Whey[a]	Cumulative Cheese Whey[b]			Fouling Material[a]			Cumulative Fouling Materials[c]		
		Reactor (1)	Reactor (2)	Reactor (3)	Reactor (1)	Reactor (2)	Reactor (3)	Reactor (1)	Reactor (2)	Reactor (3)
	(mg/L)	(mg)	(mg)	(mg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg)	(mg)	(mg)
Mg	94	2481.6	1353.6	451.2	179	205	242	2.6	3.1	6.1
Ca	429	11325.6	6177.6	2059.2	586	776	1148	8.4	11.9	29.0
Mn	1	26.4	14.4	4.8	4	9	10	0.1	0.1	0.3
K	1670	44088.0	24048.0	8016.0	3300	3470	4010	47.6	53.1	101.2
Na	684	18057.6	9849.6	3283.2	1070	1120	1680	15.4	17.1	42.4
Fe	42	1108.8	604.8	201.6	130	229	663	1.9	3.5	16.7
P	154	4065.6	2217.6	739.2	2290	2913	3240	33.0	44.6	81.8
S	483	12751.2	6955.2	2318.4	2500	3200	5200	36.0	49.0	131.3

[a] Calculated based on 2 hours residence time and 48 hours operation period.

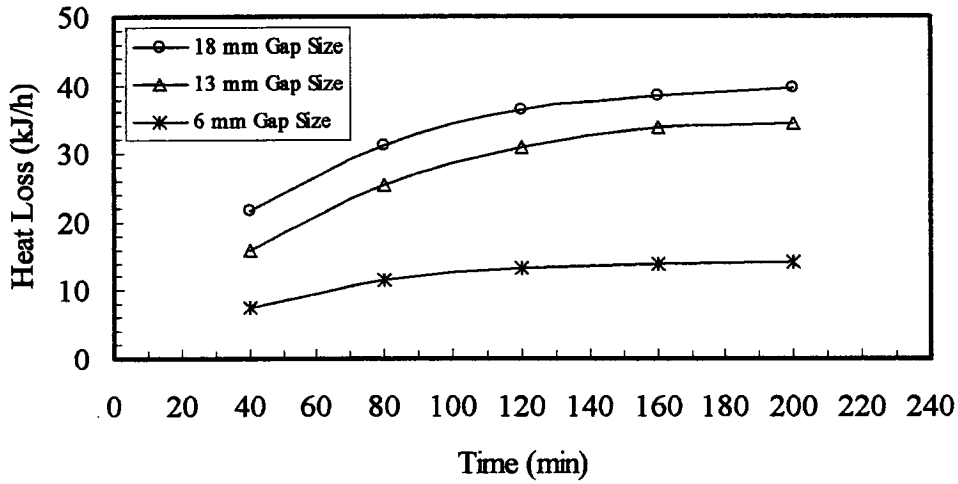
[b] Obtained from the chemical analysis

[c] Calculated on the basis that the total amount of fouling material obtained after 48 hours of operation was 14.4187, 15.3099 and 25.2548 g for the first (18 mm gap size), second (13 mm gap size) and third (6 mm gap size) UV reactors, respectively

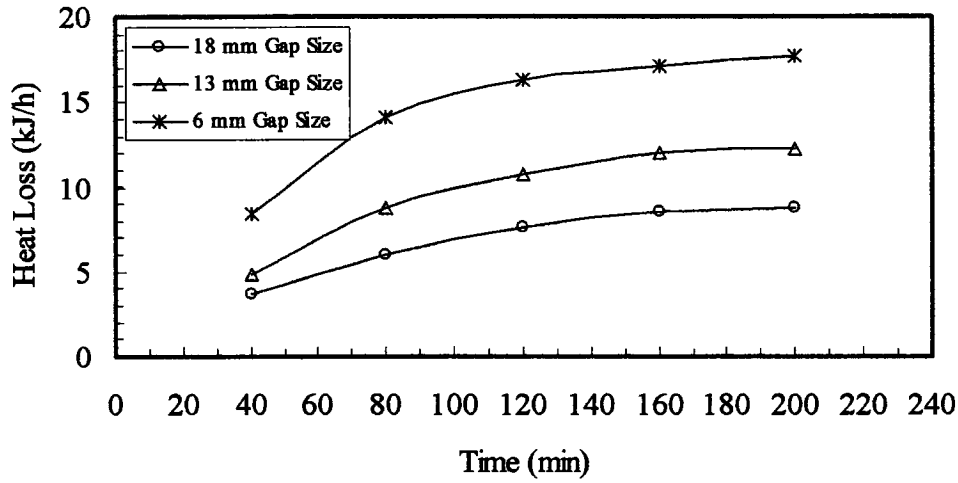
5.10.7 Heat Balance

Figure 5.20 presents the different heat losses for the three UV reactors during the transient stage. Overall, the heat losses with the cheese whey effluent, through the UV reactor wall and through the UV reactor top increased gradually with time for the three UV reactors during the transient stage. However, the amount of heat lost with the effluent decreased with the decrease in the gap size as a result of the decrease in flow rate (the residence time was kept constant at 2 h for the three UV reactors). On the other hand, the heat losses through the UV reactor wall increased with the decrease in gap size. As the gap size decreased the average cheese whey temperature inside the UV reactor (T_m) increased and consequently the difference between the cheese whey temperature (T_m) and the ambient air temperature (T_a) increased allowing for more heat transfer by conduction through the UV reactor wall. In the case of heat losses by conduction through the UV reactor top, even though there was a temperature difference between the air entrapped inside the reactor headspace (T_{ia}) and the ambient temperature (T_a), the surface area of the top disc (A_t) through which the heat was transferred had a big effect on the amount of heat lost. The area of the top disc of each of the three UV reactors was 31.17, 19.63 and 13.59 cm^2 for the first (18 mm gap size), second (13 mm gap size) and third (6 mm gap size) UV reactors, respectively. The amount of heat transferred by conduction through the UV reactor top increased with the decrease in gap size from 18 mm to 13 mm. A further decrease in the gap size (6 mm) did not affect the amount of heat transferred by conduction through the UV reactor top as a result of the small surface area (A_t). The amount of heat transferred by conduction through the UV reactor top was the same for the first (18 mm gap size) and third (6 mm gap size) UV reactors as the increase in the temperature was compensated for by the decrease in the surface area.

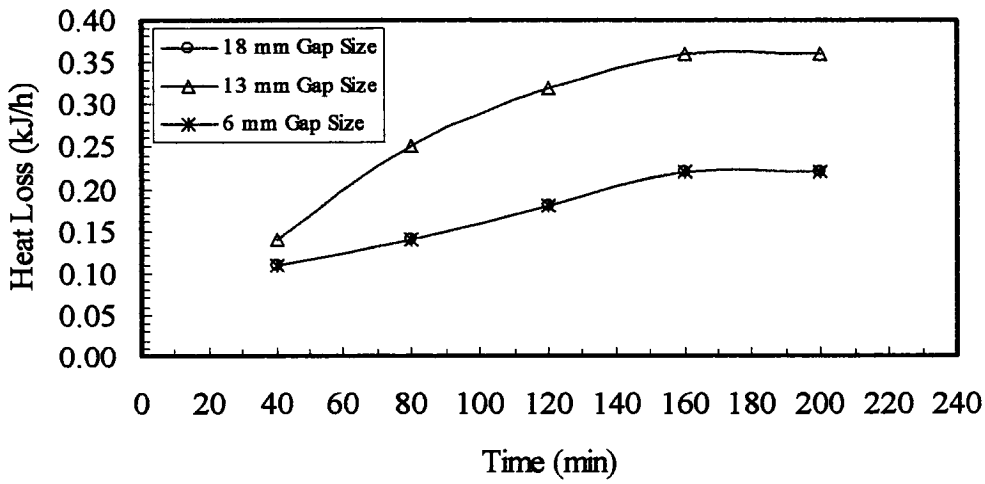
The measurements of the input power to the UV system are illustrated in Figure 5.21. Table 5.18 presents the calculated values of the various steady state heat losses. Table 5.19 shows the distribution of the total energy input to the UV system. For the first UV reactor (18 mm gap size), the results showed that most of the heat generated by the lamp (81.9%) was lost with the cheese whey effluent, while a small amount was lost through the UV reactor wall and top (17.6 and 0.5%, respectively). For the second UV reactor (13 mm gap size), the amount of heat lost with the cheese whey effluent decreased as a



(a) Heat losses with the effluent



(b) Heat losses through the UV reactor wall



(c) Heat losses through the UV reactor top

Figure 5.20. The calculated heat losses for the three UV reactors during the transient stage.

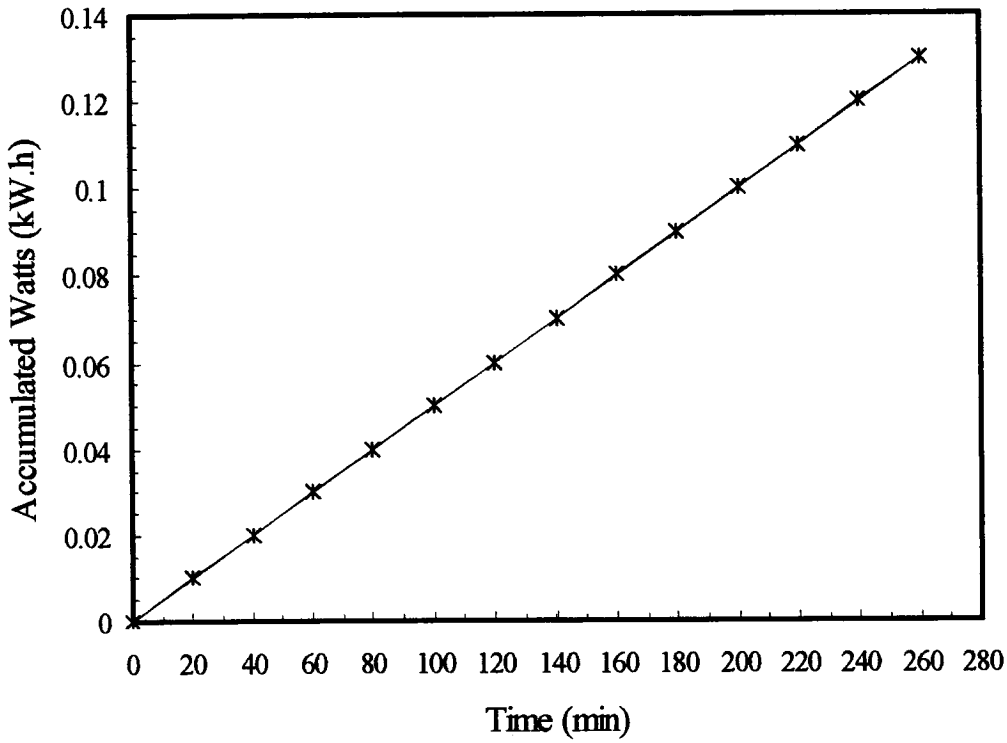


Figure 5.21. The accumulated input power of the ballast.

consequence of the decreased flow rate, but still accounts for most of the heat losses (73.0%) compared to the heat lost through the UV reactor wall and top (26.5 and 0.5%, respectively). In the case of the third UV reactor (6 mm gap size), a significant amount of the heat was lost through the UV reactor wall compared to that lost with the cheese whey effluent (53.6% vs. 45.6%), while a very small amount of heat was lost through the UV reactor top (0.8%).

The power measurements showed that the UV system receives 108 kJ/h of power. According to the manufacturer, approximately 18 kJ/h of the power input are lost in the ballast. Part of the remaining 90 kJ/h is converted to ultraviolet radiation at wavelength of 257.3 nm, while another part is radiated at other wavelengths and was ultimately converted to heat. Some of the radiated ultraviolet radiation is consumed in photochemical reactions and the rest is also converted to heat. The total radiation that was converted to heat was calculated from the heat balance (Table 5.18). The fraction of energy converted to heat decreased with the decrease in gap size; 50.482, 47.708 and

31.912 kJ/h for the first (18 mm gap size), second (13 mm gap size) and third (6 mm gap size) UV reactors, respectively. As a result, the effective radiation, which was consumed in photochemical reaction increased with the decrease in gap size; 39.518, 42.292 and 58.088 kJ/h for the first (18 mm gap size), second (13 mm gap size) and third (6 mm gap size) UV reactors, respectively.

Table 5.18. Calculated values of the various heat losses during the steady state stage.

Heat Losses	First Reactor (18 mm gap Size)		Second Reactor (13 mm gap size)		Third Reactor (6 mm gap size)	
	kJ/h	%	kJ/h	%	kJ/h	%
q_e	-41.323	81.9	-34.811	73.0	-14.943	46.8
q_w	-8.914	17.6	-12.643	26.5	-16.716	52.4
q_t	-0.245	0.5	-0.254	0.5	-0.253	0.8
q_l[a]	-50.482	100.0	-47.708	100.0	-31.912	100.0

[a] Heat generated by the lamp (q_l) = total heat losses during steady state ($q_e + q_w + q_t$)

Table 5.19. Distribution of the energy input during the steady state stage.

Energy	First Reactor (18 mm gap Size)		Second Reactor (13 mm gap size)		Third Reactor (6 mm gap size)	
	kJ/h	%	kJ/h	%	kJ/h	%
Total Energy Input	108.000	100.0	108.000	100.0	108.000	100.0
Lost in the Ballast[a]	18.000	16.7	18.000	16.7	18.000	16.7
Converted to Heat[b]	50.482	46.7	47.708	44.2	31.912	29.5
UV radiation	39.518	36.6	42.292	39.1	58.088	53.8

[a] The value is given by the manufacturer
[b] The values were calculated from heat balance

5.10.8 The Extent of Fouling

The results showed that the amount of fouling material (82.9 ± 2.2% moisture content) deposited on the quartz sleeve surface increased with the decrease in gap size; 14.4187, 15.3099, 25.2548 g on wet basis for the first (18 mm gap size), second (13 mm gap size) and third (6 mm gap size) UV reactors, respectively. Cairns (1996) reported that more flowing liquid volume per lamp results in more UV absorbing organics and inorganics

which could foul the lamp sleeve. Although, the total amount of cheese whey passed through the UV reactors during the course of this study decreased with the decrease in gap size (26.4, 14.4 and 4.8 L for 18, 13 and 6 mm gap sizes, respectively), the amount of fouling material increased. This may be explained as a result of the effect of the heat generated by the lamp on the cheese whey constituents and their reactivity with the quartz sleeve surface. Table 5.20 shows the calculated values of output heat per unit volume of the flowing cheese whey attained in this study. Smaller gap size resulted in a smaller fluid thickness, thus causing higher heat gain per unit volume and thus higher temperatures of the flowing fluid. Blatchy III and Peel (2001) stated that the flowing fluid in UV systems acts as a coolant that continuously remove the heat generated by the lamp, the higher the volume the greater the amount of heat removed. Lin et al. (1999) and Sheriff and Gehr (2001) reported that fouling is a function of time and is governed by several factors including: geometry of fouling surfaces, velocity, surface temperature, and liquid composition.

Table 5.20. The output heat per unit volume of the flowing cheese whey at 2 h residence time.

UV Reactor	Gap Size (mm)	Reactor Volume (L)	Flow Rate (L/h)	Rate of Heat Gain[1] (kJ/h)	Rate of Heat Gain per Unit Volume[2] (kJ/h.L)	Heat Gain per Unit Volume[3] (kJ/L)
1	18	1.100	0.55	50.482	45.893	91.785
2	13	0.598	0.30	47.708	79.779	159.027
3	6	0.201	0.10	31.912	158.766	319.120

[1] Values were calculated from the heat balance
[2] Rate of heat gain divided by the UV reactor volume
[3] Rate of heat gain divided by the flow rate

The results showed that the amount of heat generated by the UV lamp decreased with the decrease in the gap size. However, when taking the UV reactor volume (1.100, 0.598 and 0.201 L for the first, second and third UV reactors, respectively) and the rate of flow introduced to each UV reactor into consideration (0.55, 0.30 and 0.10 L/h for the first, second and third UV reactors, respectively), it was found that the rate of heat gain per unit volume (H_G) and the total heat gain per unit volume (H_t) of the flowing cheese whey

increased with the decrease in the gap size. In this study, the rate of heat gain (H_G) is defined as the amount of heat generated by the UV lamp divided by UV reactor volume, while the total heat gain (H_t) is defined as the amount of heat generated by the lamp divided by the flow rate introduced to the UV reactor. The results showed that the calculated values of the rate of heat gain were 45.893, 79.779, 158.776 kJ/h.L, while those of the total heat gain were 91.785, 159.027, 319.120 kJ/L for the first (18 mm gap size), second (13 mm gap size) and third (6 mm gap size) UV reactors, respectively. The relationships between the UV reactor gap size (G) and each of the flow rate (F), the weight of fouling material (W), the total heat gain per unit volume (H_t) and the cheese whey effluent temperature followed polynomial expressions ($R^2 = 0.99$) as shown in the following equations and illustrated in Figure 5.22.

$$F = 67.857 - 5.3571\,G + 1.7857\,G^2 \qquad (6 \leq G \leq 18) \qquad (5.37)$$

$$W = 41.855 - 3.3879\,G + 0.1035\,G^2 \qquad (6 \leq G \leq 18) \qquad (5.38)$$

$$H_t = 517.59 - 37.789\,G + 0.7852\,G^2 \qquad (6 \leq G \leq 18) \qquad (5.39)$$

$$T_o = 68.016 - 0.6507G - 0.0364G^2 \qquad (6 \leq G \leq 18) \qquad (5.40)$$

The direct relationship between the weight of accumulated fouling material (W) and the total heat gain per unit volume of the flowing cheese whey (H_t) can be expressed by the following polynomial expression ($R^2 = 0.99$) and as illustrated in Figure 5.23.

$$W = 16.34 - 0.0407\,H_t + 0.0002\,H_t^2 \qquad (5.41)$$

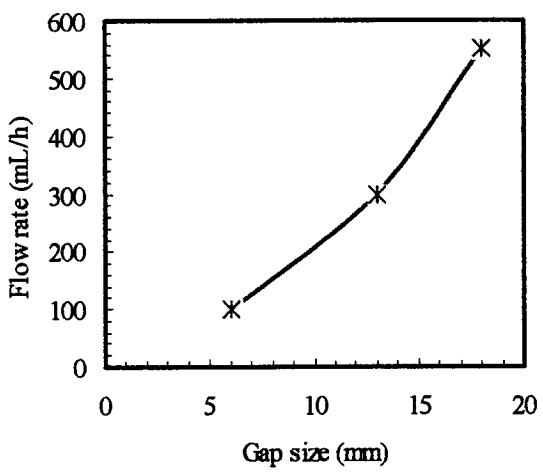
Where:

F is the flow rate (mL/h)

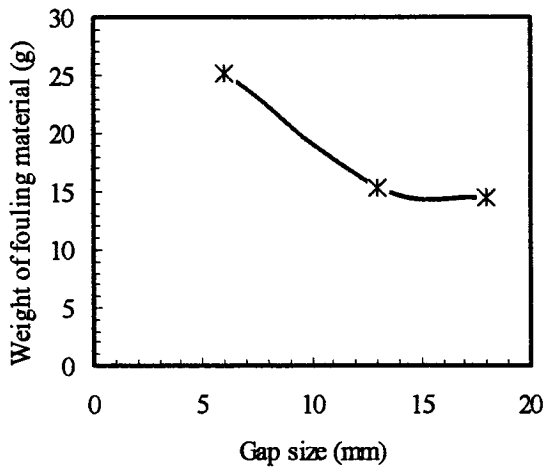
H_t is total heat gain per unit volume of the flowing cheese whey (kJ/L)

W is the weight of fouling material accumulated on the quartz sleeve (g)

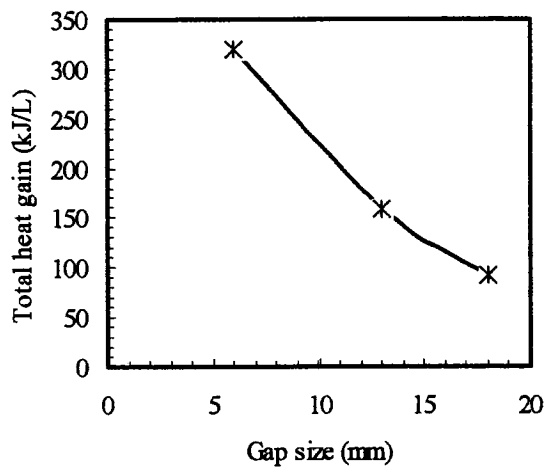
G is the UV reactor gap size (mm)



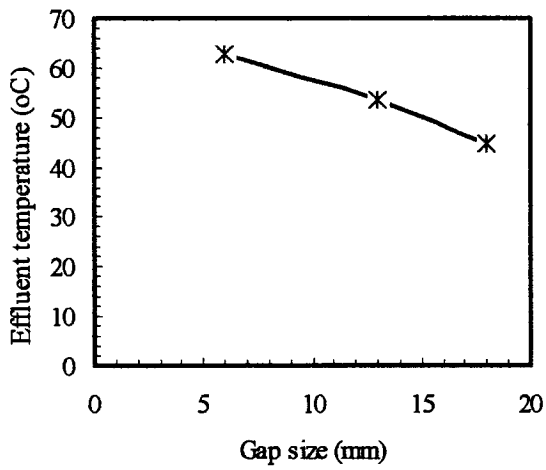
(a) Flow rate



(b) Weight of fouling material



(c) Total heat gain



(d) Effluent temperature

Figure 5.22. Effect of gap size.

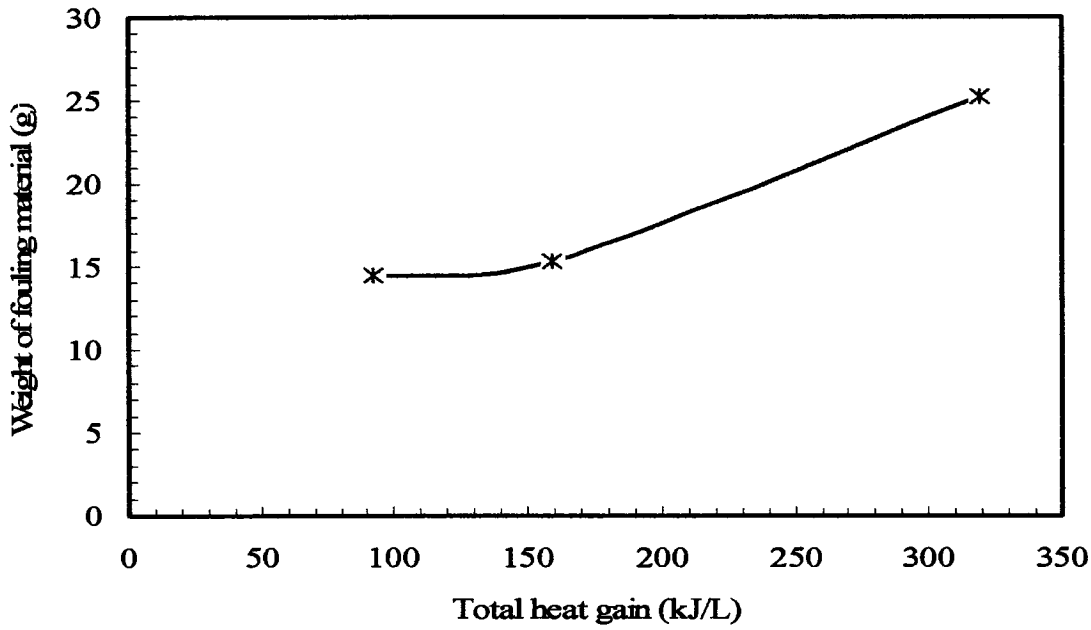
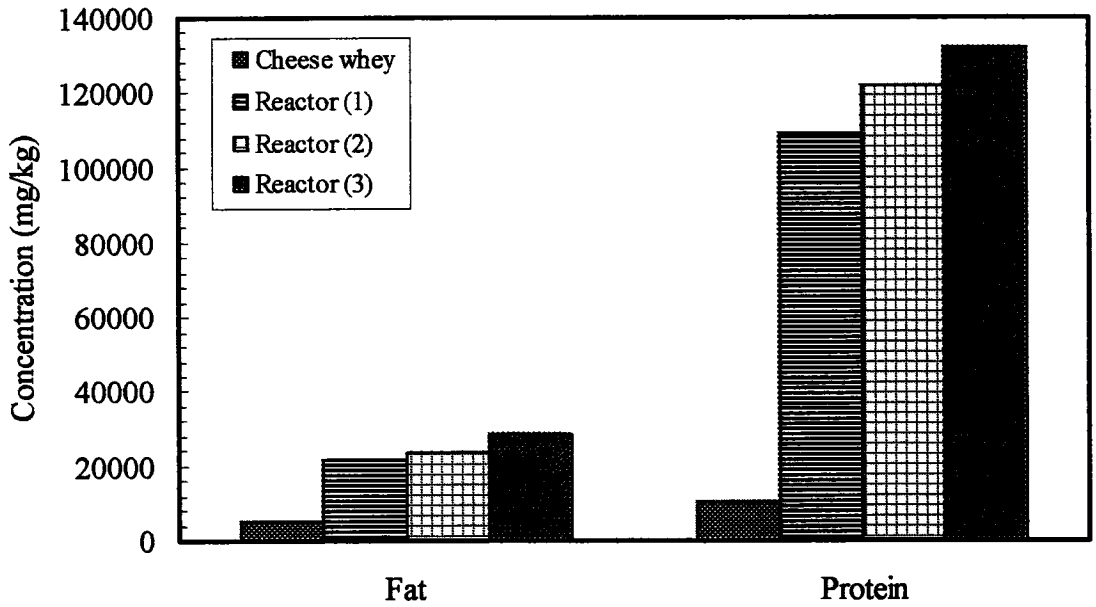


Figure 5.23. Weight of fouling material as a function of the total heat gain per unit volume of the flowing cheese whey.

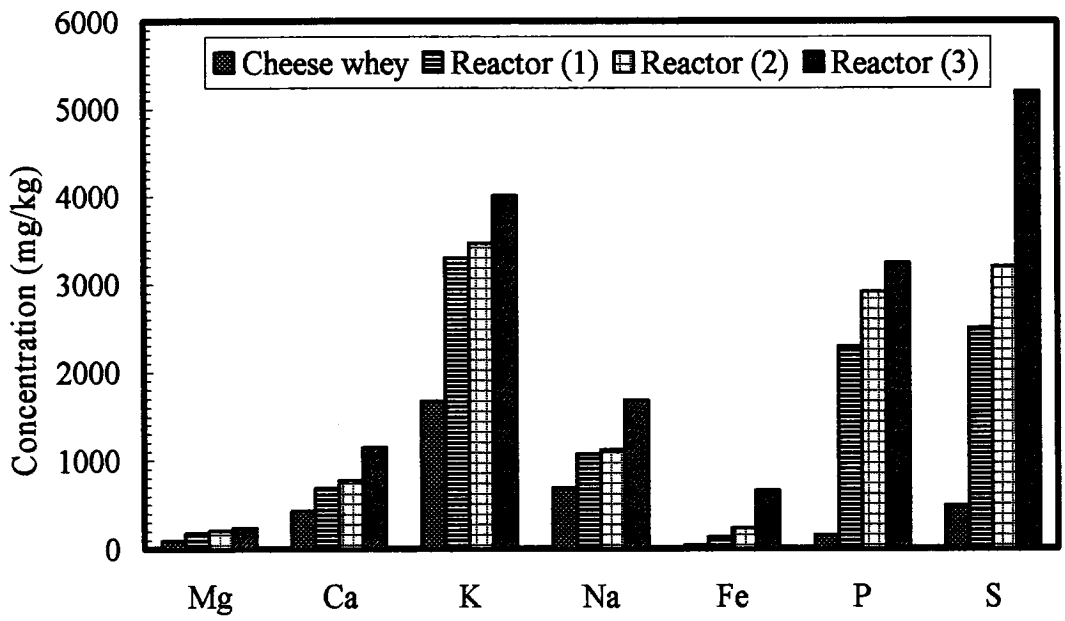
5.10.9 Mechanism of Lamp Fouling

The fouling material was found to contain 63.51-77.19% protein, 12.57-16.49% fat and 6.51-9.47% minerals on dry weight bases, depending on the reactor gap size. The concentrations of fat, protein and minerals in the fouling material increased with the decrease in the flow thickness (gap size) as shown in Figure 5.24.

The accumulation of fouling material on the quartz surface in UV reactors is caused by: (a) direct ion exchange, (b) thermally induced precipitation, and (c) particle sedimentation (Andreadakis et al., 1999; Lin et al., 1999; and Sheriff and Gehr, 2001). The UV reactors used in this study were tubular type reactors with vertical flow. Therefore, precipitation due to inverted solubility and particle sedimentation could not induce lamp fouling in this case. Thus, fouling in this study was most likely due to direct ion exchange. However, the high temperature and low pH attained in this study enhanced fouling by making the constituents of the cheese whey more soluble and reactive. Quartz is a hydrophilic surface due to the presence of amphoteric surface groups, which express



(a) Fat and protein



(b) Minerals

Figure 5.24. Fat, Protein and Minerals found in raw cheese whey and fouling materials obtained from the three UV reactors.

a net negative surface charge at near neutral or alkaline pH. The dissociation of silanol (≡SiOH) groups on the quartz surface is responsible for the surface charge and provides surface sites available for a wide range of reactions including: adsorption, complexation and precipitation (Stumm, 1992).

Cheryan (1998) stated that pH can affect protein in two ways: (a) the solubility of a protein is generally the lowest at the isoelectric point and increases as pH is adjusted away from the isoelectric point and (b) the interaction between proteins and some materials such as Ca also changes with pH. The isoelectric points of whey proteins range from 4.7 to 6.8 (Eigel et al., 1984). Macleod et al. (1995) reported that the isoelectric point of β-lactoglobulin (55% of the total whey protein) is pH of 5.2. According to Fennema (1996), the isoelectric point of α-lactalbumin (12% of the total whey protein) is 4.8. At high pH, proteins remain soluble, mainly because of the predominance of negative charges and their repulsion (Francis, 2000). pH reduction results in increasing the total positive charge of the proteins. In the pH range of 5.0 – 5.5, immunoglobulin is positively charged (Thomas et. al, 1992).

Temperature also has an effect on proteins since the adsorption of protein molecules in the temperature range of 30-60 °C generally increase with the increase in temperature (Cheryan, 1998). Calcium is a major cause of fouling in dairy streams and decreasing the pH increases the amount of soluble calcium, which can interact and bind to negatively charged groups on the surface of interest by electrostatic or charge effects (Cheryan, 1998). Calcium phosphate has an inverse solubility and its solubility decreases with the increase in temperature.

No studies have been conducted in the literature on lamp fouling during UV sterilization of cheese whey. However, several studies have been carried out on water and wastewater disinfection using ultraviolet radiation. Andreadakis et al. (1999) attributed lamp fouling to formation of inorganic scale with high calcium content. Harris et al. (1987) reported that the scale build up on quartz sleeves in wastewater disinfection systems is usually comprised of calcium, magnesium and iron. Lin et al. (1999) conducted a study in order

to characterize inorganic fouling on quartz surfaces in photoreactors by chemical analyses of fouling materials and of corresponding wastewaters. They reported that the majority of the metals (cations) present in the fouling materials were iron, aluminum and calcium, while the predominant anions found in the fouling materials were carbonate, sulfate, hydroxide, chloride and phosphate. They concluded that the primary mechanism responsible for the inorganic fouling was thermally-induced precipitation of inorganic constituents and impaction of preexisting colloidal particles. Sheriff and Gehr (2001) investigated the effect of heat and/or UV radiation on inorganic fouling of low-pressure UV lamps. They found that iron (III) is one of the main inorganic constituents that cause lamp fouling. They also stated that the existence of phosphorous increases the fouling rate and attributed fouling to particle sedimentation rather than thermally induced precipitation. They reported that fouling has been associated with high hardness, high grease and oil content, a variety of multi-cationic species such as Ca, Mg, Al, Fe, and the presence of microorganisms. The cheese whey used in the present study contained Mg, Ca, K, Na, Fe, P and S and the concentration of these elements in the fouling material increased with decreasing gap size. The amount of the fouling material can be described as a function of the concentrations of these elements by the following equation (R^2 = 0.99):

$$W = 0.118Ca + 0.085Mg + 0.065Na + 0.037Fe + 0.001P - 0.0131K - 0.041S \qquad (5.42)$$

Where:

W	is the weight of fouling material accumulated on the quartz surface (g)
Ca	is the concentration of calcium in the fouling material (mg/kg)
Mg	is the concentration of magnesium in the fouling material (mg/kg)
Na	is the concentration of sodium in the fouling material (mg/kg)
Fe	is the concentration of iron in the fouling material (mg/kg)
P	is the concentration of phosphorous in the fouling material (mg/kg)
K	is the concentration of potassium in the fouling material (mg/kg)
S	is the concentration of sulfur in the fouling material (mg/kg)

High correlations between the weight of the fouling material and the concentration of each of Ca, Mg, Na, Fe, P, K, and S were also obtained. Although positive relationships between the weight of the fouling material and the concentration of each of potassium and sulfur were realized, the negative sign in equation (5.43) may indicate that these elements might interfere with the accumulation of other elements thus reducing the net weight of fouling material. This phenomenon is worth investigating.

5.11 Conclusions

The following conclusions were made from this study:

1. Because of its high solids content (68300 mg/L of which 36.84% is suspended solids), cheese whey is considered a high turbid (4317 NTU) waste material that has a very poor transmittance (~ 0%) to UV radiation in the germicidal range (195-330 nm).
2. Despite the high turbidity of the cheese whey, UV radiation can be used for cheese whey sterilization if the proper flow thickness and residence time are used.
3. The sterilization efficiency was governed by both residence time and flow thickness.
   (a) The number of viable cells remained after UV treatment decreased with the decrease in flow rate and/or the flow thickness.
   (b) Microbial decay rates of 4.94, 7.62, and 20.9 h^{-1} were achieved using tubular UV reactors of 18, 13 and 6 mm gap size, respectively.
   (c) A residence time of 3.3, 2.1, and 0.8 h would be required to completely destruct a microbial population of 5.95×10^6 cells/mL using tubular UV reactors of 18, 13 and 6 mm gap size, respectively.
4. Fouling is a major limitation in UV tubular reactors.
   (a) Large amount of fouling material (14.42-25.25 g) was realized on the quartz sleeve surface after a short period of time (48 h).
   (b) The fouling material completely surrounded the quartz sleeve surface and could render the UV radiation incapable of reaching the microbial cell.

(c) The extent of fouling was strongly influenced by the flow thickness and lamp heat generation.

(d) The amount of accumulated fouling material on the quartz sleeve surface increased with decreasing flow thickness as a consequence of the increased heat gain by the flowing cheese whey (14.4187, 15.3099 and 25.2548 g for 18, 13 and 6 mm flow thickness, respectively).

5. The effective radiation of low pressure UV mercury arc lamp that was consumed in photochemical reaction was affected by the flow thickness.

   (a) The effective radiation increased with the decrease in flow thickness (39.518, 42.292 and 58.088 kJ/h for 18, 13 and 6 mm flow thicknesses, respectively).

   (b) The heat generation decreased with the decrease in flow thickness (50.48, 47.71 and 31.91 kJ/h for 18, 13 and 6 mm flow thicknesses, respectively).

   (c) The heat gain per unit volume of the flowing cheese whey increased with the decrease in flow thickness ((91.785, 159.027 and 319.120 kJ/h for 18, 13 and 6 mm gap sizes, respectively).

6. The fouling materials had a moisture content of 82.9 ± 2.2% and contained 63.51-77.19% protein, 12.57-16.49% fat and 6.51-9.47% minerals on dry bases.

   (a) The concentrations of fat, protein and minerals increased with the decrease in the flow thickness.

   (b) Fat concentration in the fouling material correlated with the phosphorous concentration whereas protein concentration in the fouling material correlated with the calcium concentration.

7. Adsorption and direct ion exchange were the most likely fouling mechanisms. However, the high temperature and low pH attained in this study enhanced fouling by making the constituents of the cheese whey more soluble and reactive.
8. In order to use ultraviolet radiation for on-line sterilization of cheese whey effectively, the fouling problem should be resolved.

5.12 Recommendations

5.12.1 Turbidity Reduction

Cheese whey turbidity reduction is expected to enhance the penetration ability of the UV radiation and thus increase the sterilization efficiency. It is also expected to reduce the residence time required to cause the killing effect and at the same time larger flow thickness can be used. However, keeping the valuable cheese whey constituents such as lactose sugar and protein as a nutrient source for the culture microorganisms should be taken into consideration. Hwang and Damodaran (1994) and Hwang and Damodaran (1995) stated that the main reason for the cheese whey turbidity is the light scattering by fat globule membrane. Hwang and Damodaran (1995) reported 95% reduction in the cheese whey turbidity by selectively precipitating fat globules using chitosan under certain conditions. Insoluble complex between chitosan and fat globules is formed and can be removed by centrifugation. The chitosan can be recycled by extraction from the chitosan-fat globule complex with organic solvents and the recovered lipids, which are mainly phospholipids, could be used as a value-added product in food, cosmetics and pharmaceutical products. Cheese whey turbidity reduction and its effect on sterilization efficiency using ultraviolet radiation are worth studying. Figure 5.25 presents a recommended cheese whey on-line sterilization process in which the sterilization efficiency is expected to be enhanced. The recommended sterilization process consists of: (a) a reactor in which fat precipitation takes place in order to reduce the cheese whey turbidity, (b) a UV reactor in which cheese whey sterilization takes place and (c) a bioreactor(s) in which cheese whey fermentation is carried out. The validity of this process should be investigated. Both the sterilization efficiency and the treated cheese whey characteristics should be tested.

5.12.2 Reactor Design

UV lamp fouling was the main problem with tubular type UV reactors. Different designs should be investigated in order to enhance the sterilization process and overcome the fouling problem. The new designs should offer: (a) small flow thickness, (b) turbulent flow or internal mixing, (c) separation of the cheese whey from the lamp surface and (d)

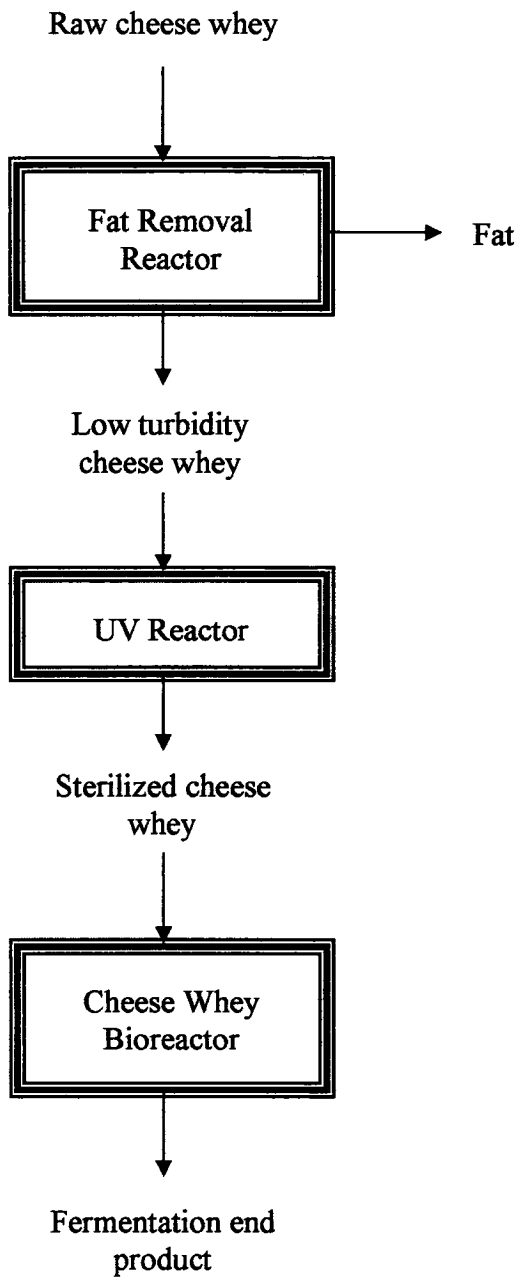


Figure 5.25. Suggested on-line sterilization process of cheese whey.

lamp cooling. Figure 5.26 presents a recommended reactor design. The cheese whey is to pass through small diameter UV transparent coiled tubing around which UV lamps are placed. A UV lamp can also be placed in the center of the coiled tubing in order to increase the radiation intensity and enhance the sterilization process. Cooling air can be introduced into the system. This new design is expected to enhance the sterilization process and overcome the fouling problem.

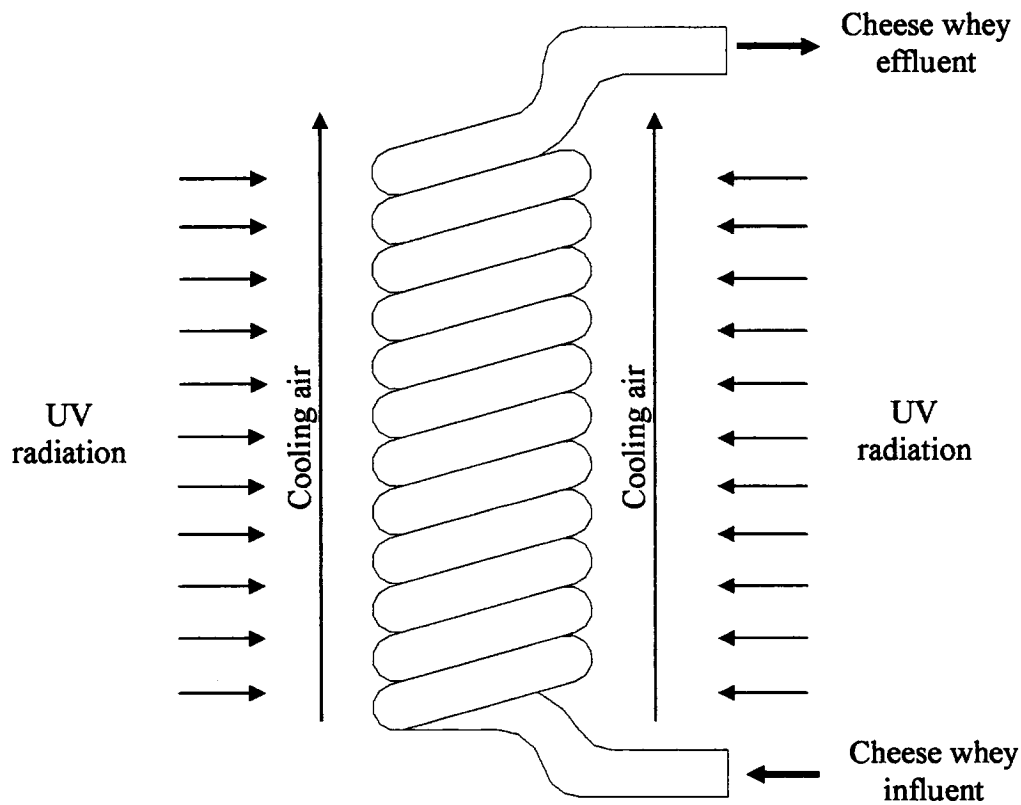


Figure 5.26. Suggested UV reactor design.

6. DEHYDROGENASE ACTIVITY AND MICROBIAL QUANTIFICATION

6.1 Introduction

Growth rates of microorganisms during submerged fermentations have been measured using various techniques such as: (a) gravimetric methods, (b) direct microscopic count, (c) plate count, (d) measuring the change in absorbance, and (e) determination of cell constituents. However, these methods have some drawbacks. The gravimetric, optical and direct microscopic count methods are not accurate and lead to error, since the suspension includes both living and dead cells as well as organic matter particulates. The plate count technique, in which enumeration of colony forming unit (CFU) is determined, takes at least 72 hours to perform and is considered to be very tedious, time consuming and labor intensive. The methods that depend on the determination of cell constituents are very expensive. During solid-state fermentations especially for those in which a filamentous fungus is used, microorganisms are intimately bound to the solid matrix (Durand et al. 1988), which makes direct methods for biomass quantification for monitoring and control of bioreactors very difficult. Indirect methods such as (a) alkali extraction of mycelial protein from the cellulose-fungal complex, (b) determination of ATP, (c) determination of glucosamine, (d) plate counting of samples blended with water, (e) sieving the sample through an appropriate sieve, (f) determination of extracellular laccase, (g) estimation of CO_2 evolution and (h) determination of O_2 uptake were reported in the literature for biomass quantification during solid-state fermentation (Lonsane et al., 1985). Methods that are based on measuring the dehydrogenase activity, using a dye as an electron acceptor, seem to be more accurate, simpler, less time consuming, less expensive and more suitable for biomass estimation during solid-state fermentation. Dehydrogenase activity is caused by a wide group of endocellular enzymes. These enzymes are present in all living cells and are essential in catalyzing the biological oxidation of organic compounds. They transfer hydrogen and electrons through a chain of intermediate electron carriers to oxygen as a final electron acceptor, which then combine with them

and form water (Ruhling and Tyler, 1973; Rogers and Li, 1985; and Chander and Brookes, 1991).

Tetrazolium salts are a large group of heterocyclic organic compounds. The most commonly used tetrazolium salts are stable, water soluble and range in color from clear white through various shades of clear yellow and brown. These salts are characterized by several important features: (a) they can be easily reduced, (b) they form highly colored insoluble products, which are called formazans, upon reduction and (c) their formazans are non auto-oxidizable (Altman, 1976). The highly colored nature of formazans allows them to be conveniently quantified by visible light absorption. Several tetrazolium salts have been reported in the literature to be effective for the assessment of dehydrogenase activity and viability. Table 6.1 shows the most commonly used tetrazolium salts, their systematic names, their trivial names, their empirical formula and their molecular weights.

Measuring the dehydrogenase activity by tetrazolium salts involve the incubation of the sample in which the dehydrogenase activity is to be measured with tetrazolium salt in the presence of electron donating substrate under certain conditions. The mechanism of measuring the dehydrogenase activity includes the oxidation of organic matter, in the presence of dehydrogenase enzymes, to produce a hydrogen atom. A hydrogen acceptor such as tetrazolium salt is then used to measure the enzyme activity. Since coupling the oxidation of the substrate with the reduction of the tetrazolium salt (colorless) gives a highly colored formazan, this dye can be extracted by a solvent and the color intensity be measured colorimetrically using a spectrophotometer at the maximum spectra of the formazan solvent system (Griebe et al., 1997). Table 6.2 shows the absorption maxima and the molar extinction coefficients for the visible peaks of formazans in solution. Generally, one unit of dehydrogenase activity is the amount of enzyme which would reduce 1 μmole tetrazolium salt to 1 μmole of its formazan per minute.

The reducibility differs among different tetrazolium salts. Altman (1976) ordered the reducibility of the most common tetrazolium compounds as follows: INT > MTT >

Table 6.1. Most commonly used tetrazolium salts.

Empirical Formula	Molecular Weight	Systematic Name	Trivial Name	References
$C_{19}H_{15}ClN_4$	334.80	2,3,5-triphenyl-2H-tetrazolium chloride	TT or TTC	Friedel et al. (1994)
$C_{19}H_{13}ClIN_5O_2$	505.70	2-p-iodophenyl-3-p-nitrophenyl-5-phenyl-2H-tetrazolium chloride	INT	Friedel et al. (1994) and Gong (1997)
$C_{23}H_{17}ClN_4$	384.86	3α-naphthyl-2,5-diphenyl-2H-tetrazolium chloride	TV Tetrazolium violet	Altman (1976)
$C_{18}H_{16}BrN_5S$	414.34	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide	MTT	van Ophem et al. (1991) and Stentelaire et al. (2001)
$C_{24}H_{16}ClN_7O_2S$	501.96	2-(2-benzothiazolyl)-3-(4-phthalhydrazidyl)-5-styryl-2H-tetrazolium chloride	BPST	Altman (1976)
$C_{38}H_{28}Cl_2N_8$	667.57	3,3'-(4,4'-biphenylylene)-bis-(2,5-diphenyl-2H-tetrazolium chloride)	NT Neotetrazolium	De Jong et al. (2001)
$C_{40}H_{32}Cl_2N_8O_2$	727.63	3,3'-(3,3'-dimethoxy-4,4'-biphenylylene)-bis-(2,5-diphenyl-2H-tetrazolium chloride)	BT Blue tetrazolium	Tengerdy et al. (1967) and Altman (1976)
$C_{40}H_{30}Cl_2N_{10}O_6$	817.63	3,3'-(3,3'-dimethoxy-4,4'-biphenylylene)-bis-(2-p-nitrophenyl-5-phenyl-2H-tetrazolium chloride)	NBT Nitroblue tetrazolium	Saito (1993) and Chikamori et al. (1998)
$C_{40}H_{28}Cl_2N_{12}O_{10}$	907.63	3,3'-(3,3'-dimethoxy-4,4'-biphenylylene)-bis-(2,5-p-nitrophenyl-2H-tetrazolium chloride)	TNBT Tetranitroblue tetrazolium	Selyakh et al. (1990)

Table 6.2. Absorption characteristics of fully reduced formazans in solution (Altman, 1976).

Formazan of	Absorption maximum (nm)	Molar extinction coefficient	Solvent
TNBT	600	38,500	Ethanol dimethylformamide mixture (1:1)
NBT	605	40,200	Nitrobenzene
NT	560	41,000	Dimethylformamide
BT	600	48,100	Dimethylformamide
MTT	505	17,400	Dimethylformamide
MTT-Co^{2+}	635	37,200	Dimethylformamide
INT	465	17,400	Ethanol dimethylformamide mixture (1:1)
BPST	485	17,400	Dimethylformamide + 1% 0.5N HCl
TV	510	13,000	Dimethylformamide
TTC	483	21,000	Ethanol

TNBT > NBT > NT > BT > TV > TTC. This sequence was similar to that found by Altman (1969): BPST > MTT > INT > TNBT > NBT > NT > BT > TV > TTC. It was also mentioned that the activities of TTC, TV, BT and NT are affected by the presence of oxygen while those of MTT, INT, TNBT and NBT are totally unaffected by oxygen (Altman, 1976). For example, one electron reduction of triphenyl tetrazolium chloride produces triphenyl tetrazolium chloride free radical. Under anaerobic conditions, this radical is further reduced, by the addition of another electron, to triphenyl formazan. In the presence of oxygen, the TTC radical reduces molecular oxygen to the superoxide radical $O_2^{\cdot}$ and itself becomes oxidized back to TTC.

The dehydrogenase activity has been used for measuring the microbial metabolic activity in soils (Lenhard, 1956; Casida et al. 1964; Klein et al., 1971; Casida, 1977; Chendrayan et al., 1979; Farini et al., 1988; Chander and Brookes, 1991; Rossel and Tarradellas,

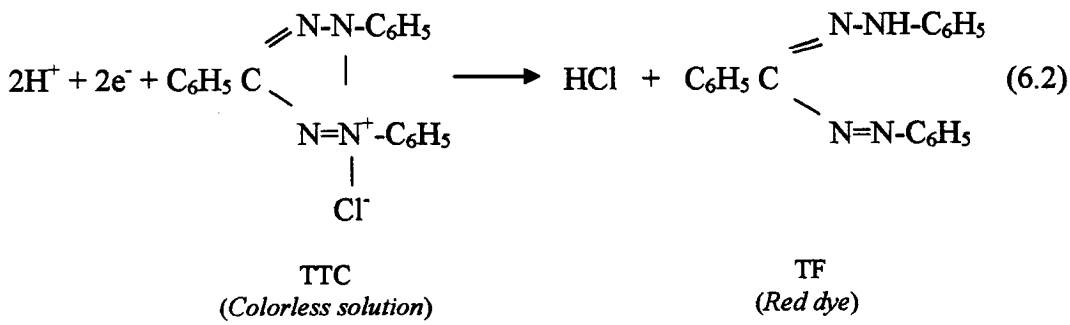
1991; Friedel et al., 1994; and Gong, 1997), sludges (Lenhard et al., 1964), and lake sediments (Muntean et al., 1999). Ghaly et al. (1989) used the dehydrogenase activity to measure the activity of microbial population in swine manure. Ghaly and Ben-Hassan (1993), Ghaly and El-Taweel (1995) and Tango and Ghaly (1999) used the dehydrogenase activity as an indicator for the microbial growth during the production of alcohol, single cell protein, and lactic acid from cheese whey, respectively. Bratcher et al. (1993), Tomita and Konno (1998) and Vujanovic et al. (2000) used the dehydrogenase activity for seeds viability determination. Sutherland and Cohen (1983), El-Hamalawi and Erwin (1986), An and Hendrix (1988), Jiang and Erwin (1990), Meier and Charvat (1993), Walley and Germida (1995) and Stentelaire et al. (2001) used the dehydrogenase activity for fungal spores viability assessment.

The triphenyltetrazolium chloride (TTC) has been widely used as a direct measurement of microbial growth (Tengerdy et al., 1967; Skujins, 1973; Ghaly and Ben-Hassan, 1993; Ohara and Saito, 1995; and Gabrielson et al., 2002) whereas MTT has been widely used for fungal spores viability assessment (Stentelaire et al. 2001). Figure 6.1 shows the chemical structure of TTC. Equations (6.1) and (6.2) describe the reactions of TTC (Lenhard et al., 1964):

(a) Biological oxidation of organic compounds:

$$\underset{\text{Substrate}}{RH_2} \xrightarrow{\text{Dehydrogenase}} \underset{\text{Oxidized Substrate}}{R} + 2H^+ + 2e^- \qquad (6.1)$$

(b) Chemical reduction of tetrazolium salts:



TTC (*Colorless solution*) TF (*Red dye*) (6.2)

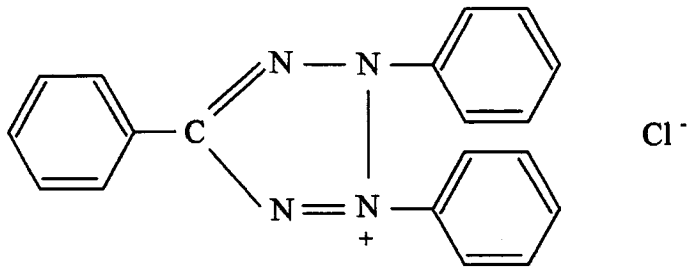


Figure 6.1. Chemical structure of 2,3,5-triphenyl-2H-tetrazolium chloride (TTC).

Since the dehydrogenase activity method measures enzymatic activity, the modification of some parameters of the reaction such as pH and temperature should lead to significant improvement of the assay (Stentelaire, 2001). Farini et al. (1988) noted that medium pH, incubation temperature, quantity of tetrazolium salt and incubation period are critical parameters that affect the accuracy of dehydrogenase activity measurements using tetrazolium salts. In addition, the enzymatic activities are significantly different for different microorganisms (Stentelaire, 2001). Metabolic and respiratory activities as well as cell wall permeability are specific to each microorganism and hence the dehydrogenase activity measurement procedure should be optimized for each microorganism in order to get a sensitive assay. Although measuring the dehydrogenase activity using the TTC test depends on the biochemical reduction of the TTC, some authors observed a chemical reduction of TTC at high temperature and pH values (Lenhard et al., 1964; Casida et al., 1964; and Mahmoud and Ghaly, 2004). It is, therefore, necessary to investigate the sensitivity of the test to temperature and pH in order to produce credible results when measuring the microbial activity.

6.2 Objectives

The specific objectives were to:

1. Investigate the effects of temperature, pH, water content of the medium and presence of heavy metals on the chemical reduction of triphenyletetrazolium chloride (TTC) to triphenyl formazan (TF) in liquid and solid media and determine the significance of nonenzymatic reduction of TTC to TF.

2. Investigate the applicability of the TTC-test for quantifying the growth of the fungal species *Aspergillus niger* in liquid media.
   (a) Determine the optimum conditions for the test (pH, temperature, TTC concentration and incubation time).
   (b) Study the effects of presence of O_2 and cell age on biochemical reduction of TTC to TF.
3. Investigate the effectiveness of the developed TTC test for measuring the microbial growth of *Aspergillus niger* during solid-state fermentation of shrimp shells.
   (a) Study the effects of filtration and amount of alcohol on the TF yield.
   (b) Determine the contribution of dead cells and shrimp shells to TF yield.

6.3 Experimental Materials

6.3.1 Cheese Whey

The cheese whey was obtained from Farmers Cooperative Dairy Plant in Truro, Nova Scotia, Canada. For the chemical reduction experiments, the cheese whey was sterilized at 121 °C and 103.4 kPa for 30 minutes in an autoclave (Model No. STM-E, Market Forge Sterilmatic, New York, USA) to ensure the absence of any microorganisms in the liquid waste. Samples were plated on plate count agar and incubated at 37 °C to confirm the absence of any microbes. The autoclaved cheese whey was then stored in a refrigerator at 4 °C until needed. Some characteristics of cheese whey are shown in Table 5.1. The solids, nitrogen, chemical oxygen demand, potassium, sulfur and phosphorus analyses were performed according to the procedures described in the Standard Methods for the Examination of Water and Wastewater (APHA, 1985). Lactose concentration was determined using a sugar analyzer (YSI Model 27, Yellow Spring, Ohio, USA).

6.3.2 Municipal Solid Waste Compost

The municipal solid waste compost was obtained from the late mesophilic stage of the 25,000 T/year New Era Farms Compost Facility in Halifax, Nova Scotia, Canada. The compost material was sterilized at 121 °C and 103.4 kPa for 30 minutes in an autoclave (Model No. STM-E, Market Forge Sterilmatic, New York, USA). The autoclaved

compost was kept at room temperature for 24 h for spores to germinate. The process was repeated three times in order to ensure that all the microorganisms and their spores had been killed. Samples were plated on plate count agar and incubated at 37 °C to confirm the absence of microbes. The autoclaved compost was then stored in a refrigerator at 4 °C until needed. Some characteristics of the compost material are shown in Table 6.3. These analyses were performed by the Phillips Analytical Services, Dartmouth, Nova Scotia.

6.3.3 Shrimp Shells

Northern Pink Shrimp (*Pandalus borealis)* shell waste was obtained from Ocean Nutrition Ltd. of Bedford, Nova Scotia. The material came from a shell processing plant in Mulgrave, Nova Scotia. These shrimp were caught in the fall of 2001, as part of the offshore Northern Shrimp Fishery by a vessel owned by Clearwater Fine Foods Inc., and then individually quick-frozen on board the vessel. They were stored frozen in this manner until arrival at a cooking/peeling plant operated by St. Anthony Seafoods Ltd., St. Anthony, Newfoundland where they were cooked in boiling salt water for 10 minutes. Following cooking, the shrimp were sent to automated peeling machines where the shell and meat portions were separated. The shell material was collected and dried in large kiln dryers in Mulgrave. The shrimp shells used in this study were stored at about -25 °C in the storage facility (Associated freezers of Canada, Dartmouth, Nova Scotia) till needed. Before use in the experiments, the shrimp shell waste was sterilized at 121 °C and 103.4 kPa for 30 minutes in an autoclave (Model No. STM-E, Market Forge Sterilmatic, New York). The autoclaved shrimp shell waste was kept at room temperature for 24 h for spores to germinate. The process was repeated three times in order to ensure that all the microorganisms and their spores had been killed. Samples were plated on plate count agar and incubated at 37 °C to confirm the absence of microbes. The autoclaved shrimp shell waste was then stored in a refrigerator at 4 °C until needed. Table 6.4 shows some of the characteristics of the shrimp shells used in the study.

6.3.4 Microorganisms

Aspergillus niger (ATCC 6275) were obtained from the American Type Culture

Table 6.3. Compost analyses[a].

Parameters	Valance	Value	Units
Moisture	-	0.10	%(w)
Total Carbon	-	100.00	mg/kg
Total Kjeldahl Nitrogen	-	100.00	mg/kg
Elements			
Aluminum (Al)	+3	10.00	mg/kg
Antimony (Sb)	+3, +5, -3	2.00	mg/kg
Arsenic (As)	+3, +5, -3	2.00	mg/kg
Barium (Ba)	+2	5.00	mg/kg
Beryllium (Be)	+2	5.00	mg/kg
Boron (B)	+3	5.00	mg/kg
Cadmium (Cd)	+2	0.30	mg/kg
Chloride (Cl)	+1, +5, +7, -1	1.00	mg/L
Chromium (Cr)	+2, +3, +6	2.00	mg/kg
Cobalt (Co)	+2, +3	1.00	mg/kg
Copper (Cu)	+1, +2	2.00	mg/kg
Iron (Fe)	+2, +3	20.00	mg/kg
Lead (Pb)	+2, +4	0.50	mg/kg
Manganese (Mg)	+2	2.00	mg/kg
Mercury (Hg)	+1, +2	0.01	mg/kg
Molybdenum (Mo)	+6	2.00	mg/kg
Nickel (Ni)	+2, +3	2.00	mg/kg
Phosphorus (P)	+3, +5, -3	1.00	mg/kg
Potassium (K)	+1	100.00	mg/kg
Selenium (Se)	+4, +6, -2	2.00	mg/kg
Silver (Ag)	+1	0.50	mg/kg
Strontium (Sr)	+2	5.00	mg/kg
Thallium (Tl)	+1, +3	0.10	mg/kg
Uranium (U)	+3, +4, +5, +6	0.10	mg/kg
Vanadium (V)	+2, +3, +4, +5	2.00	mg/kg
Zinc (Zn)	+2	2.00	mg/kg
pH		1.00	-

[a] All results are expressed on a dry weight basis.

Table 6.4. Some characteristics[a] of shrimp shells used in this study.

Characteristics	Measured value	Units
Moisture content	4.79 ± 0.03	%
Ash content	31.73 ± 0.02	%
Total Kjeldahl nitrogen	64265	mg/kg
Ammonium nitrogen	3492	mg/kg
Percent ammonium nitrogen	5.4	%
Organic nitrogen	60773	mg/kg
Percent organic nitrogen	94.6	%
Protein nitrogen	49339	mg/kg
Percent protein nitrogen	76.8	%
Chitin nitrogen	11434	mg/kg
Percent chitin nitrogen	17.8	%
Chitin[b]	165907	mg/kg
Protein[c]	308369	mg/kg
Fat	22000	mg/kg
Total carbon	305000	mg/kg
Total organic carbon	176000	mg/kg
Elements		
Magnesium	6150	mg/kg
Calcium	142000	mg/kg
Manganese	116	mg/kg
Potassium	871	mg/kg
Sodium	3480	mg/kg
Iron	308	mg/kg
Phosphorus	22400	mg/kg
Sulfur	4700	mg/kg
Silicon	794	mg/kg
Aluminum	250	mg/kg
Copper	24	mg/kg
Others	136207	mg/kg
pH	8.64	

[a] Characteristics are presented on dry basis.
[b] calculated as chitin nitrogen × 14.51
[c] calculated as protein nitrogen × 6.25

Collection (Rockville, Maryland) as freeze dried cells. The freeze dried cells were revived in sterilized 6 mL of 0.1% peptone water prepared by dissolving 1 g bacto-peptone (Difco, Detroit, Michigan, USA) in 1 L deionized-distilled water and then sterilized in an autoclave (Model No. STM-E, Market Forge Sterilmatic, New York) at 121 °C and 103.4 kPa for 30 minutes). The microbial culture was kept in the peptone water for 24 hours at room temperature (24 °C).

The rehydrated *A. niger* was transferred to potato dextrose broth (PDB), which contained Infusion from 200 g Potatoes (4 g/L) and 20 g/L Bacto Dextrose. A spore stock suspension was obtained by growing the fungus on potato dextrose agar (PDA), which contained Infusion from 200 g Potatoes (4 g/L), 20 g/L Bacto Dextrose and 15 g/L Bacto Agar at room temperature (25 °C) for 4 days. The conidia were harvested from the surface by adding sterilized deionized distilled water containing 0.01% (v/v) Tween 80 and scraping the surface with a sterilized spatula. The spore concentration was obtained using direct standard plate count method. The prepared suspension was then stored in the refrigerator at about 4 °C till needed.

6.3.5 Reagents

The chemicals used in performing the dehydrogenase activity tests included: 0.05 M tris (hydroxymethyl) aminomethane hydrochloride ($NH_2C[CH_2OH]_3.HCl$) buffer solution (pH ~ 8.0), glucose and 2:3:5 triphenyltetrazolium chloride salt (TTC). The tris buffer was used to control the pH of the samples. The 0.05 M buffer was prepared by dissolving 6.057 g tris (hydroxymethyl) aminomethane ($C_4H_{11}NO_3$) in 20 mL 1.0 N HCl and bringing the solution to 1.0 L with distilled-deionized water. The glucose served as an immediately-bioavailable carbon supply to enhance the ability of microorganisms to reduce the TTC within the incubation period (Klein et al., 1971). The TTC were used as a colorimetric indicator. Several TTC solutions were prepared. The TTC glucose solution was prepared by adding either 0.25, 0.50, 0.75, 1, 1.25, 1.50, 1.75, 2.00, 2.25, 2.50, 2.75 or 3.00 g triphenyl tetrazolium chloride ($C_{19}H_{15}N_4Cl$) depending on the required concentration and 1 g glucose ($CH_2OH[CHOH]_4CHO$) to a 100 mL volumetric flasks and

bringing the solution to 100 mL with distilled-deionized water. The TTC solution is sensitive to light and was, therefore, kept in the dark at near 0 °C.

6.4 Experimental Procedure

6.4.1 TTC Standard Curve

In order to determine the total formazan (TF) production from the reduction of triphenyl tetrazolium chloride, a standard curve was developed from solutions of TF and ethyl alcohol with different concentrations. First, a standard solution of 0.0002 M triphenyl formazan was prepared by dissolving 0.03 g triphenyl formazan ($C_6H_5N{:}NC[C_6H_5]{:}NNHC_6H_5$) in 500 mL ethyl alcohol. Then a set of 9 solutions with triphenyl formazan concentration of 0.2, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 μmol/50 mL (1.2, 3.0, 6.0, 9.0, 12.0, 15.0, 18.0, 21.0 and 24.0 μg/ mL) were prepared. Finally, the absorbances of the prepared solutions were measured using spectrophotometer (Spectronic 601, Fisher Scientific, Montreal, Quebec, Canada) at a wavelength of 484 nm and plotted against the known TF concentrations (μmol/mL) as shown in Figure 6.2. A blank sample (pure ethyl alcohol) was used to zero the spectrophotometer. The following linear relationship between the triphenyl formazan and the absorbance was obtained, with a coefficient of determination (R^2) value of 0.99:

$$TF = 0.0603\ \bar{A}_{484} \tag{6.3}$$

Where:

TF is the triphenyl formazan concentration (μmol / mL).

$\bar{A}_{484}$ is the absorbance measured at 484 nm.

6.4.2 TTC Chemical Reduction Test Procedures

The procedures described by Ghaly and Ben-Hassan (1993) were used after slight modifications. First, four centrifuge tubes (50 mL capacity) with screw caps were used (three sample replicates and one control). An amount of 5 mL liquid medium (cheese whey) or 0.5 g solid medium (compost) was transferred to each tube and 2.5 mL of the

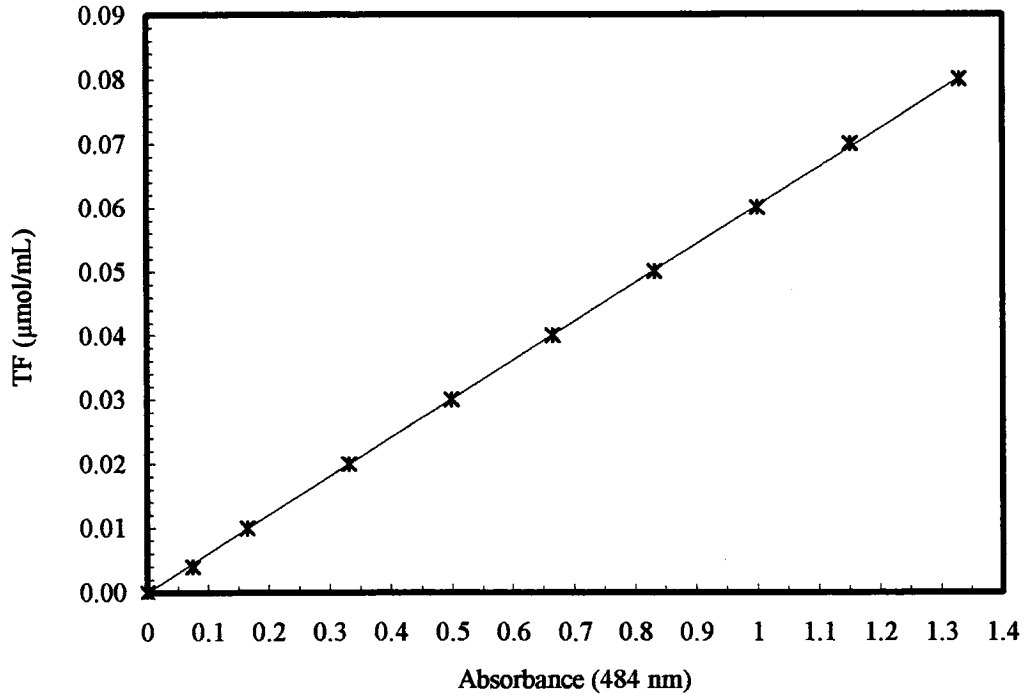


Figure 6.2. Triphenyl formazan standard curve.

tris buffer solution were added. The pH was then adjusted to the required value using either 1N sodium hydroxide or 1N hydrochloric acid. An aliquot of 0.5 mL of TTC/glucose solution (TTC concentration of 5 g/L) was transferred to each of the three sample tubes whereas an aliquot of 0.5 mL of sterilized distilled-deionized water was transferred to the control tube. The tubes contents were mixed using a vortex mixer (Sybron Maxi Mix model M-16715, Thermolyne Corporation, Dubuque Iowa, USA) for 30 seconds and then incubated at the assigned temperatures and incubation times. After the incubation period, 5 mL of ethanol were added and the tubes were shaken using the vortex mixer, and then centrifuged at 2150 rpm (975 ×g) for 20 minutes. The suspension was transferred to a graduated cylinder. Two separate extractions of the retained solids, each with 5 mL ethyl alcohol, were done. The supernatant was transferred to the graduated cylinder and the combined suspension was made up to 25 mL with addition of ethyl alcohol. Finally, the suspension was filtered using a membrane micro-filter having 0.45 µm pore size (MILLIPORE, No. HVLP04700, Fisher Scientific, Montreal, Quebec, Canada). This filtration step was added to the procedure described by Ghaly and Ben-

Hassen (1993) since it was realized that the high turbidity of the wastes used in the analysis could give a misleading readings. The absorbance of the filtrate was then measured at 484 nm using a spectrophotometer (Spectronic 160, Fisher Scientific Montreal, Quebec, Canada). The blank sample was used to zero the spectrophotometer. The formazan contents were then obtained from the standard curve.

6.4.3 *Aspergillus niger* Sample Preparation

Initially, samples were prepared by inoculating a specific volume of liquid growth medium (4.7 mL potato dextrose broth for each sample) with a certain amount (0.3 mL for each sample) of spores in solution of 70×10^6 CFU/mL. Prior to applying the TTC-test, the prepared samples were incubated for 60 h at room temperature in order to allow for spores germination. However, difficulties in attaining the same cell concentration in all samples led to the modification of this procedure. Unless otherwise specified, *A. niger* vegetative cells were allowed to grow in liquid growth media for 4-5 days, after which the fungal cells formed clumps, which were then harvested and placed between two filter papers in order to remove excess liquid media before use in the tests. The moisture content of the fungal clumps was determined and the results were corrected and presented on dry basis for the experiments that was conducted in the absence of shrimp shells.

6.4.4 *A. niger* Dehydrogenase Activity Test Procedures

Each sample was transferred to a 50 mL centrifuge tube with screw caps. An amount of 2.5 mL of the tris buffer solution was added to each tube and the pH was then adjusted to the required value using either 1N sodium hydroxide or 1N hydrochloric acid. An aliquot of 0.5 mL of TTC/glucose solution with the desired concentration was transferred to each tube. For the control tubes, an aliquot of 0.5 mL of sterilized distilled-deionized water was transferred to each tube instead of the TTC/glucose solution. The contents of the tubes were mixed using a vortex mixer (Sybron Maxi Mix model M-16715, Thermolyne Corporation, Dubuque Iowa, USA) for 30 seconds and then incubated at the desired temperature for the assigned incubation time.

In the case in which dehydrogenase activity of *A. niger* alone (no shrimp shells) was to be measured, the tubes were centrifuged after the incubation period at 2150 rpm (975 ×g) for 20 minutes in order to separate the fungal cells from the liquid media. The supernatant was discarded and 2.5 mL of ethyl alcohol were added to the remained fungal cells. The tubes were shaken using the vortex mixer, and then centrifuged at 2150 rpm (975 ×g) for 20 minutes. The suspension was transferred to a test tube. One more extraction of the retained fungal cells with 2.5 mL ethyl alcohol was done. The supernatant was transferred to the test tube and the combined suspension was shaken. Finally, the absorbance of the combined suspension was measured at 484 nm using a spectrophotometer (Spectronic 160, Fisher Scientific Montreal, Quebec, Canada). The blank sample was used to zero the spectrophotometer. The formazan contents were then obtained from the standard curve.

In the case in which the dehydrogenase activity of *A. niger* was to be measured in the presence of shrimp shells, unless otherwise mentioned, samples had to be filtered under suction through filter paper (Whatman No. 40 filter paper, Fisher Scientific Co., Pittsburgh, PA) after the incubation period in order to separate the solids from the liquid media. The filtrate was discarded and the separated solids were transferred to a centrifuge tube for triphenyl formazan extraction. Two washes with ethyl alcohol (5 mL unless otherwise specified) were applied. After each wash the tubes were centrifuged and the contents were filtered through a membrane micro-filter having 0.45 μm pore size (MILLIPORE, No. HVLP04700, Fisher Scientific, Montreal, Quebec, Canada). The filtrate was collected and the absorbance was measured at 484 nm using a spectrophotometer (Spectronic 160, Fisher Scientific Montreal, Quebec, Canada). The formazan contents were then obtained from the standard curve.

6.5 Experimental Protocol

6.5.1 TTC Chemical Reduction

Two sets of experiments were conducted using sterilized liquid (cheese whey) and solid (municipal solid waste) wastes. The first set was used to investigate the sensitivity of the

TTC-Test to temperature changes while the second set was used to determine the sensitivity of the TTC-Test to pH changes. A third set of experiment was conducted using standard solutions of 0.0002 M triphenyl formazan to investigate the effect of heavy metals on the color intensity of TF. All the laboratory ware used in this study were sterilized at 121°C and 103.4 kPa for 30 minutes in an autoclave (Model No. STM-E, Market Forge Sterilmatic, New York, USA).

6.5.1.1 Temperature Sensitivity Experiment. For the temperature sensitivity experiment, 10 different incubation temperatures (40, 50, 60, 70, 75, 80, 85, 90, 95, and 100 °C) were studied. Three replicates were carried out. The samples were incubated in an oven (Isotemp Oven, Model 655F, Fisher Scientific, Montreal, Quebec) at the desired temperatures. The pH of the samples was maintained at 7.00 (since no chemical reduction of TTC took place at or below a pH of 7 in this study). The TF yield was determined after a 2-hour incubation period of the liquid and solid samples using a spectrophotometer (Spectronic 601, Fisher Scientific Montreal, Quebec). It was also decided to investigate whether evaporation of water during incubation at high temperatures has any significant effect on the TF yield.

6.5.1.2 pH Sensitivity Experiment. For the pH sensitivity experiment, the total formazan content was determined at 9 alkaline pH values (9.0, 9.5, 10.0, 10.5, 11.0, 11.5, 12.0, 12.5, and 13.0) and 4 acidic pH values (1.5, 3.0, 4.5, 6.0) and three different incubation periods (1, 2, and 3 hours). Three replicates were carried out. The samples were incubated in an oven (Isotemp Oven, Model 655F, Fisher Scientific, Montreal, Quebec) at a controlled temperature of 40° C (since chemical reduction of TTC was not observed at/or below 40 °C in this study). The pH was measured using a pH meter (Model 805MP, Fisher Scientific, Montreal, Quebec). The TF yield was measured using a spectrophotometer (Spectronic 601, Fisher Scientific Montreal, Quebec).

6.5.1.3 Heavy Metals Experiment. From the results of the initial experiment, it was suspected that the heavy metals present in the compost samples may have decreased the color intensity of TF as a result of a reaction between the heavy metals and the triphenyl

formazan. To proof this assumption, the effect of heavy metals on the triphenyl formazan yield was tested. Standard solutions of 0.0002 M triphenyl formazan were incubated in the presence of different concentrations of heavy metals and the TF color intensity was measured. Seven different concentrations (0, 1, 5, 10, 20, 50, and 100 mg/L) of six heavy metals (copper, potassium, zinc, sodium, calcium and nickel) were studied. Salts of cupric nitrate ($Cu(NO_3)_2.3H_2O$), potassium sulfate (K_2SO_4), zinc sulphate ($ZnSO_4.7H_2O$), sodium sulfite (Na_2SO_3), calcium sulfate ($CaSO_4.2H_2O$), and nickel sulfate ($NiSO_4.6H_2O$) were used to prepare copper, potassium, zinc, sodium, calcium, and nickel solutions with different concentrations, respectively. An aliquot of 6 mL of the triphenyl formazan standard solution was incubated with 3 mL of each the heavy metal solutions (or 3 mL distilled water for the control) for 2 hours at 40 °C. The pH of the mixture was measure before incubation using a pH meter (Model 805MP Fisher Scientific, Montreal, Quebec) and found to be close to neutral. After the incubation period, ethyl alcohol was added to bring up the mixture to 25 mL and then the absorbance of TF was measured at 484 nm using spectrophotometer (Spectronic 601, Fisher Scientific Montreal, Quebec).

6.5.2 Measuring Dehydrogenase Activity of *Aspergillus niger* Using TTC

Preliminary experiments conducted with *A. niger* vegetative cells according to the procedures described by Ghaly and Ben-Hassan (1993) showed that little color was developed when samples were incubated for 2 h with TTC/glucose solution of 5 g/L concentration at a pH of 7.0 and a temperature of 24 °C. It was, therefore, necessary to investigate the effects of TTC concentration, pH, temperature, incubation time, cell age and presence/absence of O_2 on the development of the red dye (triphenyl formazan). Four sets of experiments (Table 6.5) were conducted using *A. niger* vegetative cells previously grown in liquid media. The first set of experiments was used to investigate the effects of four parameters (TTC concentration, pH, temperature and incubation time) on TF yield. The second set of experiments was used to investigate the effects of TTC concentration and cells age on TF yield at the optimum pH, temperature and incubation time. The third set of experiments was used to study the effect of presence and absence of O_2 on the reaction rate at the optimum TTC concentration, pH and temperature. The fourth set of experiments was used to investigate the effect of the presence/absence of O_2 on the TF

yield and to develop standard curves for *A. niger* quantification using the optimum test conditions (TTC concentration, pH, temperature and incubation time).

Table 6.5. Experimental design for *A. niger* study without shrimp shells.

Experiments	Parameters Studied
First Set	• TTC concentration • pH • Temperature • Incubation time
Second Set[a]	• TTC concentration • Cell age
Third Set[b]	• Incubation time • N_2/O_2
Fourth Set[c]	• N_2/O_2 • Cell concentration

[a] at optimum pH, temperature and incubation time
[b] at optimum TTC concentration, pH and temperature
[c] at optimum TTC concentration, pH, temperature and incubation time

All the laboratory ware used in these tests was sterilized at 121 °C and 103.4 kPa for 30 minutes in an autoclave (Model No. STM-E, Market Forge Sterilmatic, New York). pH measurements were performed using a pH meter (Model 805MP, Fisher Scientific, Montreal, Quebec). Incubation at the desired temperature took place in an oven (Isotemp Oven, Model 655F, Fisher Scientific, Montreal, Quebec). The absorbance was measured at 484 nm using a spectrophotometer (Spectronic 601, Fisher Scientific Montreal, Quebec).

6.5.2.1 Effects of TTC Concentration, pH, Temperature and Incubation Time. In a preliminary experiment, no color was developed at acidic conditions (pH < 6.0) when samples were incubated at room temperature with TTC concentration of 5 g/L for 2 h.

Also, the results of the chemical reduction of TTC experiments showed that TTC can be chemically reduced at pH $\geq$ 9.5 and temperature $\geq$ 75 °C. In this set of experiments, four factors (TTC concentration, pH, temperature and incubation time) with three levels each (5, 10 and 15 g/L for TTC concentration, 7, 8 and 9 for pH, 25, 40 and 55 °C for temperature and 1.5, 3 and 4.5 h for incubation period) were investigated. The experiment was designed as 3^4 factorial experiment with two replicates. This resulted in 162 tests. Samples of the mold were prepared by inoculating 4.7 mL potato dextrose broth with 0.3 mL spores solution and were incubated for 60 h at room temperature for the spores to germinate. The solution with vegetative cells was used in the TTC test. The TF yield was determined for each experimental run.

6.5.2.2 Effects of TTC Concentration and Cell Age. In this set of experiments, the effect of TTC concentration (2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0, 22.5, 25.0, 27.5 and 30.0 g/L) on color development was studied at the optimum test conditions (a pH of 9, a temperature of 55 °C and an incubation period of 3 h) using *A. niger* vegetative cells from two cultures of different age (4 and 12 days). For each sample tested, 0.2 g (wet basis) of *A. niger* vegetative cells, which was placed between two filter papers to remove excess liquid media, was added to 4.7 mL potato dextrose broth and the TTC-test procedure was performed. Two replicates were carried out.

6.5.2.3 Reaction Rate. In this set of experiments, the effect of 14 incubation periods (15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330 and 360 min) on color development were studied at the optimum test conditions (a TTC concentration of 20 g/L, a pH of 9.0 and a temperature of 55 °C) under aerobic (O_2 environment) and anaerobic (N_2 environment) conditions. One set of sample tubes were flushed with nitrogen gas for 5 minutes before incubation whereas aerobic conditions were maintained in the other set. TTC/glucose solution was added to the tubes after flushing with nitrogen. For each sample tested, 0.2 g (wet basis) of *A. niger* vegetative cells (4 days old) was added to 4.7 mL potato dextrose broth. The TF yield was determined after each incubation period. Two replicates were carried out for each experiment.

6.5.2.4 *A. niger* Standard Curve in the Presence and Absence of O_2. In this set of experiments, the TTC-test was performed for four *A. niger* cell masses (0.025, 0.050, 0.075 and 0.1 g) having an age of 4 days under both anaerobic (N_2 environment) and aerobic (O_2 environment) conditions at the optimum test conditions (a TTC concentration of 20 g/L, a pH of 9, a temperature of 55 °C and an incubation period of 3 h). One set of sample tubes were flushed with nitrogen gas for 5 minutes before incubation whereas aerobic conditions were maintained in the other set. TTC/glucose solution was added to the tubes after flushing with nitrogen. Samples were prepared by adding the desired weight of *A. niger* vegetative cells to 4.7 mL potato dextrose broth. Samples were tested in duplicates. A plot of TF yield versus cell mass was developed and an equation describing the best fit was obtained.

6.5.3 Measuring Dehydrogenase Activity of *Aspergillus niger* in the Presence of Shrimp Shells Using TTC

After the TTC-test was optimized, it was necessary to investigate the effectiveness of the test in quantifying *A. niger* in the presence of shrimp shells. Five sets of experiments (Table 6.6) were conducted using *A. niger* vegetative cells, previously grown in liquid media, mixed with shrimp shells at optimum test conditions (a TTC concentration of 20 g/L, a pH of 9, a temperature of 55 °C, an incubation period of 3 h and absence of O_2). The first set of experiments was used to study the effect of filtration. The second set of experiments was used to study the effect of the amount of alcohol used for extraction per wash. The third set of experiments was used to determine the contribution of shrimp shells to the red color. The fourth set of experiments was used to study the effect of autoclaving on TF yield. The fifth set of experiments was used to develop a standard curve for *A. niger* in the presence of shrimp shells.

6.5.3.1 Effect of Filtration. This set of experiments was conducted to determine TF yield for four *A. niger* vegetative cell masses (0.025, 0.050, 0.075 and 0.1 g) in the presence of shrimp shells under the optimum test conditions (a TTC concentration of 20 g/L, a pH of 9, a temperature of 55 °C, an incubation period of 3 h and absence of O_2). Samples were prepared by mixing the desired *A. niger* vegetative cells weight with 1 g shrimp shells.

Then, 4.7 mL of potato dextrose broth was added to the mixture. After the incubation period, TF was extracted using two washes with 2.5 mL alcohol for each wash. The absorbance of the extract was measured before and after filtration through a membrane micro-filter having 0.45 μm pore size (MILLIPORE, No. HVLP04700, Fisher Scientific, Montreal, Quebec, Canada). The TTC-test procedure was performed on two replicates from each sample.

Table 6.6. Experimental design for *A. niger* study with shrimp shells[a].

Experiments	Parameters Studied
First Set	• Cell concentration • Filtration
Second Set	• Cell concentration • Amount of alcohol per wash
Third Set	• Cells with and without shrimp shells
Fourth Set	• Cells with and without shrimp shells • Autoclaving
Fifth Set	• Cell concentration • Autoclaving

[a] at optimum TTC concentration, pH, temperature, incubation time and in the absence of O_2

6.5.3.2 Effect of Amount of Alcohol per Wash. While extracting the TF in the first set of experiments, it appeared that the amount of alcohol used (2.5 mL/wash) was absorbed by the shells. This experiment was, therefore, performed to study the effect of the amount of alcohol added on TF yield. The TTC test was performed on *A. niger* three cell masses (0.025, 0.050 and 0.075 g cells mixed with 1 g shells) in duplicates under the optimum test conditions (a TTC concentration of 20 g/L, a pH of 9, a temperature of 55 °C, an incubation period of 3 h and absence of O_2). After the incubation period, samples were filtered under suction through filter paper (Whatman No. 40 filter paper, Fisher Scientific

Co., Pittsburgh, PA) and the TF was extracted from the separated solids using two washes with ethyl alcohol (1 group of samples with 2.5 mL/wash and the other with 5 mL/wash). After each wash the tubes were centrifuged and the contents were filtered through a membrane micro-filter (MILLIPORE, No. HVLP04700, Fisher Scientific, Montreal, Quebec, Canada).

6.5.3.3 Contribution of Shrimp Shells to TTC Reduction. During the previous experiments, it was observed that some color was also extracted from the shrimp shells. This experiment was, therefore, performed to determine the contribution of shrimp shells to the red color. The TTC test was performed on three samples (0.1 g cells, 1 g shells and 0.1 g cells mixed with 1 g shells) in duplicates under the optimum test conditions (a TTC concentration of 20 g/L, a pH of 9, a temperature of 55 °C, an incubation period of 3 h and absence of O_2). After the incubation period, samples were filtered under suction through filter paper (Whatman No. 40 filter paper, Fisher Scientific Co., Pittsburgh, PA) and the TF was extracted from the separated solids using two washes with ethyl alcohol (5 mL/wash). After each wash the tubes were centrifuged and the contents were filtered through a membrane micro-filter (MILLIPORE, No. HVLP04700, Fisher Scientific, Montreal, Quebec, Canada).

6.5.3.4 Effect of Autoclaving. An attempt to develop standard curve for *A. niger* in the presence of shrimp shells under the optimum test conditions (a TTC concentration of 20 g/L, a pH of 9, a temperature of 55 °C, an incubation period of 3 h and absence of O_2) using two washes with 5 mL alcohol each was performed. Four *A. niger* cell masses (0.025, 0.050, 0.075 and 0.1 g) were tested in the presence of shrimp shells. The variability of the standard curve results was high. Therefore, in order to improve the quality of the standard curve data, it was decided to grind the shrimp shells (to provide more homogeneous samples) and to study the effect of autoclaving of the shrimp shells on the color yield at optimum test conditions (a TTC concentration of 20 g/L, a pH of 9, a temperature of 55 °C and an incubation period of 3 h). Shrimp shells as well as *A. niger* cells were autoclaved three times in an autoclave (Model No. STM-E, Market Forge Sterilmatic, New York, USA) in three consecutive days for 45 min each. The TTC test

was performed on two sets of experiments with three samples each (0.1 g cells, 1 g shells, 0.1 g cells mixed with 1 g shells). One set was autoclaved and each set was ran in duplicates. After the incubation period, samples were filtered under suction through filter paper (Whatman No. 40 filter paper, Fisher Scientific Co., Pittsburgh, PA) and the TF was extracted from the separated solids using two washes with ethyl alcohol (5 mL each). After each wash, the tubes were centrifuged and the contents were filtered through a membrane micro-filter (MILLIPORE, No. HVLP04700, Fisher Scientific, Montreal, Quebec, Canada). Plate count of autoclaved and unautoclaved samples was also carried out in triplicates according to the procedures described in the Standard Method for the Examination of Dairy Products (Messer et al., 1985).

6.5.3.5 *A. niger* Standard Curve. Four cell masses of *A. niger* vegetative cells (0.025, 0.050, 0.075 and 0.1 g) were mixed with either 1 g of unautoclaved shrimp shells or 1 g of autoclaved shrimp shells. The tubes were flushed with nitrogen gas for 5 minutes and the test was performed at optimum test conditions (a TTC concentration of 20 g/L, a pH of 9, a temperature of 55 °C and an incubation period of 3 h). After the incubation period, samples were filtered under suction through filter paper (Whatman No. 40 filter paper, Fisher Scientific Co., Pittsburgh, PA) and the TF was extracted from the separated solids using two washes with ethyl alcohol (5 mL each). After each wash, the tubes were centrifuged and the contents were filtered through a membrane micro-filter (MILLIPORE, No. HVLP04700, Fisher Scientific, Montreal, Quebec, Canada). The TF yield was determined in duplicates. A plot of TF yield versus cell mass was developed for shrimp shells with and without autoclaving. A best fit was obtained to represent the *A. niger* standard curve in the presence of shrimp shells.

6.6 Experimental Analyses

6.6.1 Plate Count

For the chemical reduction experiments a standard plate count agar was used while for *A. niger* experiments potato dextrose agar was used. Plate count agar (DIFCO, 0751-17-2) contained 5 g bacto yeast extract, 10 g bacto tryptone, 2 g dextrose and 15 g agar all

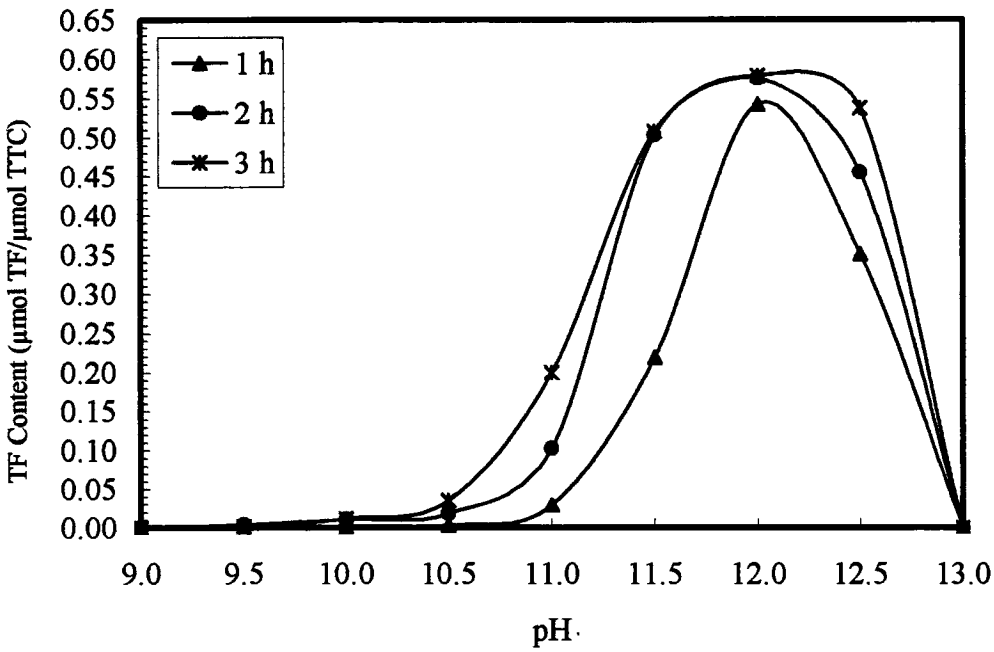
dissolved in 1 L. Potato dextrose agar contained infusion from 200 g potatoes (4 g), 20 g Bacto dextrose and 15 g Bacto agar all dissolved in 1 L. Both agars were sterilized at 121 °C and 103.4 kPa for 30 minutes in an autoclave (Model No. STM-E, Market Forge Sterilmatic, New York, USA), and then poured into several Petri dishes. The Petri dishes were allowed to solidify and then kept in a fridge at 4 °C until ready for use. Serial dilutions of 10^{-1} - 10^{-10} were prepared from the samples. An aliquot of 0.1 mL of each of the dilutions was placed on the agar in the Petri dish (in triplicates) and gently swirled until consistently spread on the agar surface. The Petri dishes were then incubated at room temperature until visible growth was observed (≈ 48 h). The visible growth was counted as colony forming units per milliliter (CFU/mL) according to the procedure described in the Standard Method for the Examination of Dairy Products (Messer et al., 1985).

6.7 Results

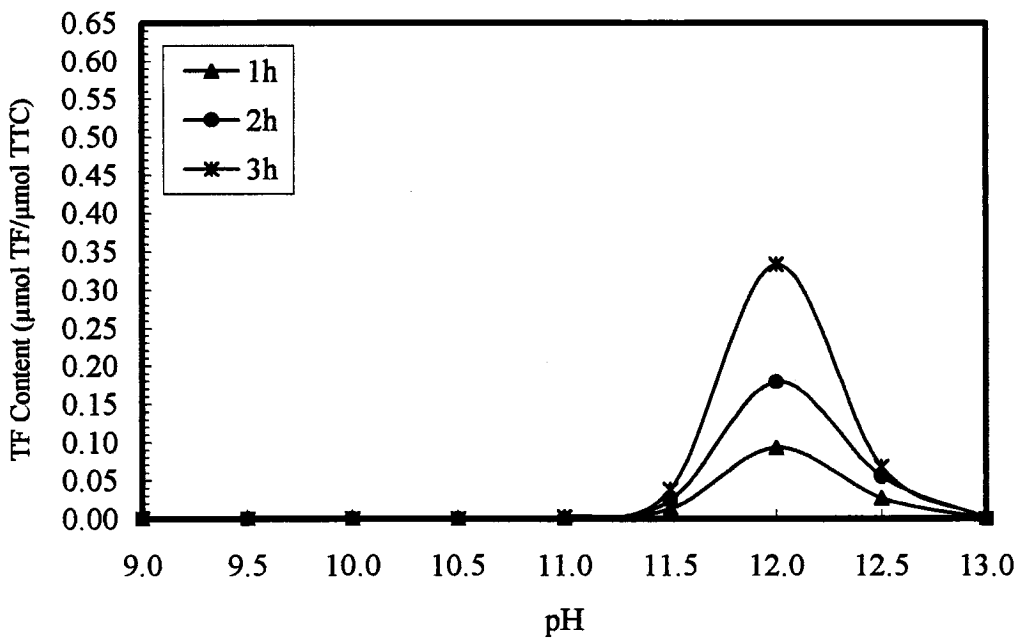
6.7.1 Chemical Reduction of TTC

6.7.1.1 pH Sensitivity. Autoclaved cheese whey and compost material were used to test the sensitivity of chemical reduction of the TTC to the pH of the medium. The triphenyl formazan yield was measured at 13 pH values (nine alkaline pH values and 4 acidic pH values) and 3 different incubation times. The effect of pH on the TTC reduction at the different incubation times and pHs and the incubation temperature of 40 °C are illustrated in Figure 6.3. The values are the average of three replicates. The coefficient of variation was very small (ranged from 2.0 to 12.5%).

6.7.1.2 Temperature Sensitivity. Autoclaved cheese whey and compost material were used to test the sensitivity of chemical reaction of the TTC to incubation temperature. The triphenyl formazan yield was measured at 10 different incubation temperatures after a 2-hour incubation time. The effect of incubation temperatures on the TTC reduction is shown in Figure 6.4.



(a) Cheese whey



(b) Compost

Figure 6.3. Triphenyl formazan at different pHs and incubation times.

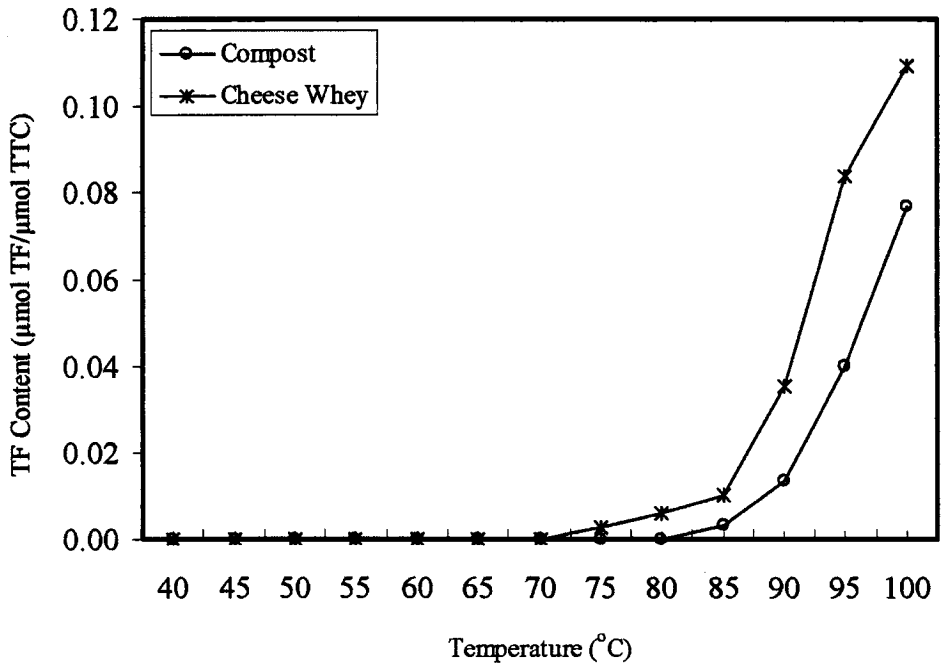


Figure 6.4. Total triphenyl formazan content at different incubation temperatures.

The water losses during the incubation are presented in Table 6.7. No significant weight losses were observed during the incubation time since the tubes were screw capped before incubation. The highest percentage of weight loss was 3.21% at 100° C. This small reduction in the sample water content did not seem to have any significant effect on the reaction rate or the TF yield.

Table 6.7. Sample weight[a] reduction during the incubation period.

Temperature	Weight Losses	
(°C)	(g)	(%)
40	0.0009	0.01
50	0.0040	0.05
60	0.0074	0.09
70	0.0158	0.20
75	0.0223	0.42
80	0.0329	0.55
85	0.0449	0.85
90	0.0671	1.23
95	0.1077	1.61
100	0.2571	3.21

[a] Initial sample weight ≈ 8 g.

6.7.1.3 Effect of Heavy Metals on the Color Intensity of TF. The results shown in Figure 6.5 indicated that copper was the only heavy metal (among the tested heavy metals) that was able to change the color intensity of the triphenyl formazan obtained from compost samples. The presence of copper at different concentrations resulted in reductions of the measured absorbance by 6.6, 54.4, 95.5, 98.9, 99.3, and 99.6% for 1, 5, 10, 20, 50, 100 mg/L concentrations, respectively. Visual observations showed that by the end of the incubation period (2 h) for low copper concentrations, the solution color remained red but with lower intensity, while at moderate and high copper concentrations the color turned violet and then disappeared completely.

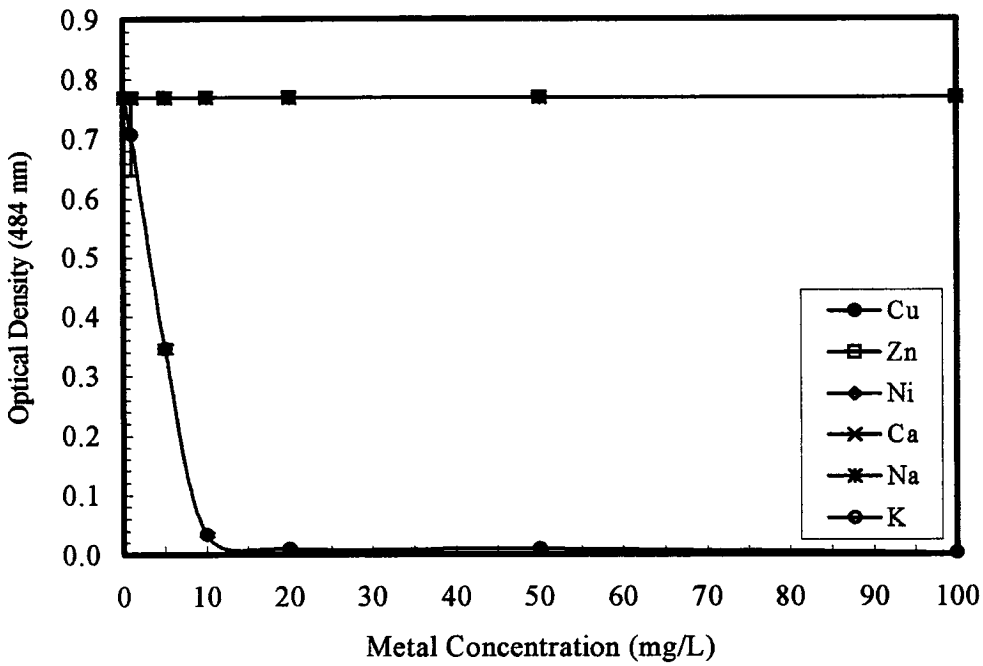


Figure 6.5. Effect of heavy metal concentration on the absorbance of TF.

6.7.2 Measuring Dehydrogenase Activity of *A. niger* using TTC

6.7.2.1 Effects of TTC Concentration, pH, Temperature and Incubation Time. Table 6.8 shows triphenyl formazan yield and TTC consumption resulting from the dehydrogenase activity of *A. niger* vegetative cells under various TTC concentrations, pH values, temperatures and incubation times. The values are the average of two replicates. The coefficient of variation ranged from 0.2 to 38.7%.

Table 6.8. Effects of incubation time, temperature, pH and TTC concentration on formazan formation during the dehydrogenase activity measurements of *A. niger*.

Time (h)	Temperature (°C)	pH	TTC (g/L)	TTC added[a] (µmol)	TTC added[a] (µg)	TF produced[b] (µmol)	TF produced[b] (µg)	TTC consumed (%)
1.5	25	7	5	7.47	2243.99	0.0045	1.36	0.06
			10	14.94	4487.98	0.0068	2.04	0.05
			15	22.40	6728.96	0.0134	4.03	0.06
		8	5	7.47	2243.99	0.0124	3.71	0.17
			10	14.94	4487.98	0.0194	5.84	0.13
			15	22.40	6728.96	0.0249	7.47	0.11
		9	5	7.47	2243.99	0.0191	5.75	0.26
			10	14.94	4487.98	0.0217	6.52	0.15
			15	22.40	6728.96	0.0270	8.11	0.12
	40	7	5	7.47	2243.99	0.0093	2.81	0.13
			10	14.94	4487.98	0.0161	4.85	0.11
			15	22.40	6728.96	0.0291	8.74	0.13
		8	5	7.47	2243.99	0.0185	5.57	0.25
			10	14.94	4487.98	0.0568	17.07	0.38
			15	22.40	6728.96	0.0814	24.45	0.36
		9	5	7.47	2243.99	0.0507	15.22	0.68
			10	14.94	4487.98	0.0794	23.87	0.53
			15	22.40	6728.96	0.1426	42.84	0.64
	55	7	5	7.47	2243.99	0.0164	4.94	0.22
			10	14.94	4487.98	0.0421	12.63	0.28
			15	22.40	6728.96	0.0576	17.30	0.26
		8	5	7.47	2243.99	0.0517	15.53	0.69
			10	14.94	4487.98	0.1247	37.45	0.83
			15	22.40	6728.96	0.1741	52.30	0.78
		9	5	7.47	2243.99	0.1208	36.27	1.62
			10	14.94	4487.98	0.1465	44.02	0.98
			15	22.40	6728.96	0.2819	84.68	1.26

[a] Total TTC added during the dehydrogenase activity measurements (calculated based on TTC molecular weight of 334.8 g/mol and 0.5 mL TTC glucose solution added to each sample)

[b] Total amount of TF extracted (calculated based on TF molecular weight of 300.4 g/mol and the usage of 5 mL alcohol for extraction)

Table 6.8. Continued.

Time (h)	Temperature (°C)	pH	TTC (g/L)	TTC added[a] (µmol)	TTC added[a] (µg)	TF produced[b] (µmol)	TF produced[b] (µg)	TTC consumed (%)
3.0	25	7	5	7.47	2243.99	0.0078	2.35	0.11
			10	14.94	4487.98	0.0154	4.62	0.10
			15	22.40	6728.96	0.0213	6.39	0.10
		8	5	7.47	2243.99	0.0204	6.11	0.27
			10	14.94	4487.98	0.0274	8.24	0.18
			15	22.40	6728.96	0.0431	12.95	0.19
		9	5	7.47	2243.99	0.0308	9.24	0.41
			10	14.94	4487.98	0.0672	20.20	0.45
			15	22.40	6728.96	0.0855	25.68	0.38
	40	7	5	7.47	2243.99	0.0234	7.02	0.31
			10	14.94	4487.98	0.0351	10.55	0.24
			15	22.40	6728.96	0.0828	24.86	0.37
		8	5	7.47	2243.99	0.0390	11.73	0.52
			10	14.94	4487.98	0.0674	20.24	0.45
			15	22.40	6728.96	0.1125	33.78	0.50
		9	5	7.47	2243.99	0.0983	29.53	1.32
			10	14.94	4487.98	0.1467	44.06	0.98
			15	22.40	6728.96	0.2573	77.30	1.14
	55	7	5	7.47	2243.99	0.0314	9.42	0.42
			10	14.94	4487.98	0.0730	21.92	0.49
			15	22.40	6728.96	0.1229	36.91	0.55
		8	5	7.47	2243.99	0.0742	22.28	0.99
			10	14.94	4487.98	0.0853	25.63	0.57
			15	22.40	6728.96	0.1500	45.06	0.67
		9	5	7.47	2243.99	0.0962	28.89	1.29
			10	14.94	4487.98	0.1658	49.81	1.11
			15	22.40	6728.96	0.2805	84.28	1.25

[a] Total TTC added during the dehydrogenase activity measurements (calculated based on TTC molecular weight of 334.8 g/mol and 0.5 mL TTC glucose solution added to each sample)

[b] Total amount of TF extracted (calculated based on TF molecular weight of 300.4 g/mol and the usage of 5 mL alcohol for extraction)

Table 6.8. Continued.

Time (h)	Temperature (°C)	pH	TTC (g/L)	TTC added[a] (μmol)	TTC added[a] (μg)	TF produced[b] (μmol)	TF produced[b] (μg)	TTC consumed (%)
4.5	25	7	5	7.47	2243.99	0.0110	3.31	0.15
			10	14.94	4487.98	0.0205	6.16	0.14
			15	22.40	6728.96	0.0262	7.88	0.12
		8	5	7.47	2243.99	0.0250	7.52	0.34
			10	14.94	4487.98	0.0461	13.86	0.31
			15	22.40	6728.96	0.0577	17.34	0.26
		9	5	7.47	2243.99	0.0473	14.22	0.63
			10	14.94	4487.98	0.0529	15.90	0.35
			15	22.40	6728.96	0.1461	43.88	0.65
	40	7	5	7.47	2243.99	0.0375	11.28	0.50
			10	14.94	4487.98	0.0577	17.34	0.39
			15	22.40	6728.96	0.0764	22.96	0.34
		8	5	7.47	2243.99	0.0998	29.98	1.34
			10	14.94	4487.98	0.1195	35.91	0.80
			15	22.40	6728.96	0.2311	69.42	1.03
		9	5	7.47	2243.99	0.2019	60.64	2.70
			10	14.94	4487.98	0.2359	70.87	1.58
			15	22.40	6728.96	0.4301	129.20	1.92
	55	7	5	7.47	2243.99	0.0390	11.73	0.52
			10	14.94	4487.98	0.0939	28.21	0.63
			15	22.40	6728.96	0.1387	31.20	0.46
		8	5	7.47	2243.99	0.1233	37.04	1.65
			10	14.94	4487.98	0.1058	31.79	0.71
			15	22.40	6728.96	0.1975	59.32	0.88
		9	5	7.47	2243.99	0.1530	45.96	2.05
			10	14.94	4487.98	0.2985	89.66	2.00
			15	22.40	6728.96	0.4556	136.85	2.03

[a] Total TTC added during the dehydrogenase activity measurements (calculated based on TTC molecular weight of 334.8 g/mol and 0.5 mL TTC glucose solution added to each sample)

[b] Total amount of TF extracted (calculated based on TF molecular weight of 300.4 g/mol and the usage of 5 mL alcohol for extraction)

6.7.2.2 Effects of TTC Concentration and Cell Age. Figure 6.6 shows the effect of TTC concentration on the triphenyl formazan yield during the dehydrogenase activity measurement of *A. niger* vegetative cells of two different ages (4 and 12 days old) under the optimum conditions of pH (9), temperature (55 °C) and incubation time (3 h). The values are the average of two replicates and are presented on dry weight basis. The coefficient of variation ranged from 1.99 to 30.17%.

6.7.2.3 Reaction Rate. Figure 6.7 shows the effect of incubation time on the triphenyl formazan yield during the dehydrogenase activity of *A. niger* vegetative cells measured under optimum conditions of TTC concentration (20 g/L), pH (9) and temperature (55 °C) in the presence and absence of O_2. The values are the average of two replicates and are presented on dry weight basis. The coefficient of variation ranged from 1.04 to 12.13%.

6.7.2.4 *A. niger* Standard Curve in the Presence and Absence of O_2. Figure 6.8 shows the triphenyl formazan yield during the dehydrogenase activity measurement of *A. niger* vegetative cells of different cell masses under the optimum conditions of TTC concentration (20 g/L), pH (9), temperature (55 °C) and incubation time (3 h) for both aerobic and anaerobic conditions. The values are the average of two replicates and are presented on dry weight basis. The coefficient of variation ranged from 1.72 to 16.02%.

6.7.3 Measuring Dehydrogenase Activity of *A. niger* in the Presence of Shrimp Shells using TTC

6.7.3.1 Effect of Filtration. Figure 6.9 shows the relationship between *A. niger* vegetative cells mass (in the presence of shrimp shells) and triphenyl formazan yield during the dehydrogenase activity measurements under optimum conditions of TTC concentration (20 g/L), pH (9), temperature (55 °C), incubation time (3 h) and absence of oxygen. TF was extracted by two alcohol washes of 2.5 mL each. Values are the average of two replicates. The coefficient of variation ranged from 0.19 to 30.93%.

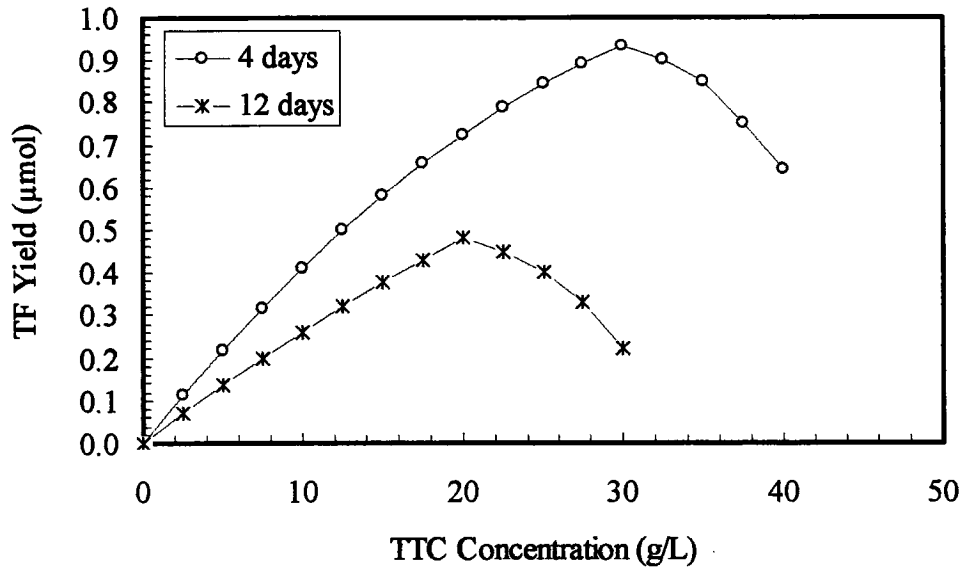


Figure 6.6. Effect of TTC concentration and cell age on triphenyl formazan yield during the dehydrogenase activity measurement of *A. niger* under optimum conditions of pH (9), temperature (55 °C) and incubation time (3 h).

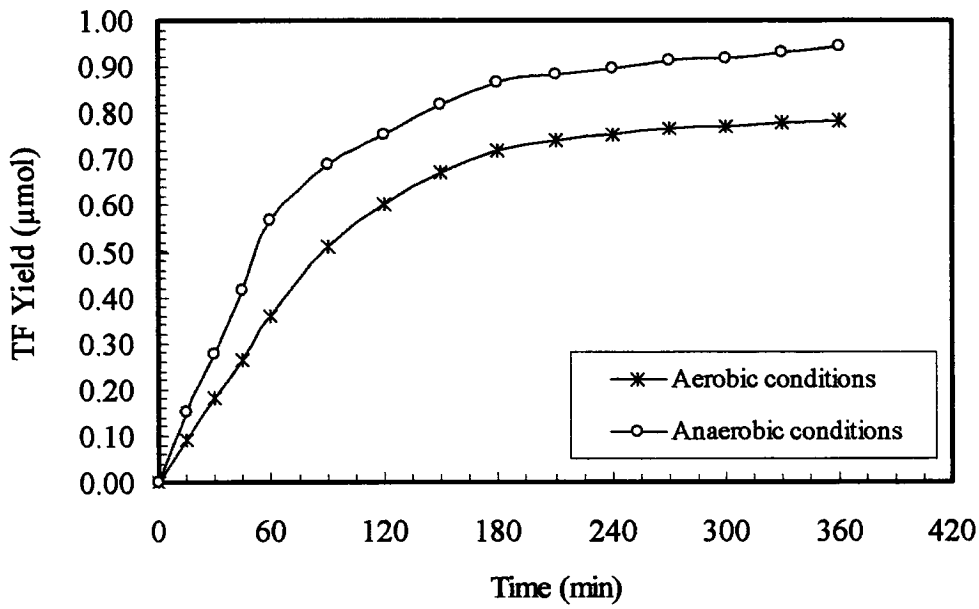


Figure 6.7. Effect of incubation time and O_2 on the triphenyl formazan yield measured under optimum conditions of TTC concentration (20 g/L), pH (9) and temperature (55 °C).

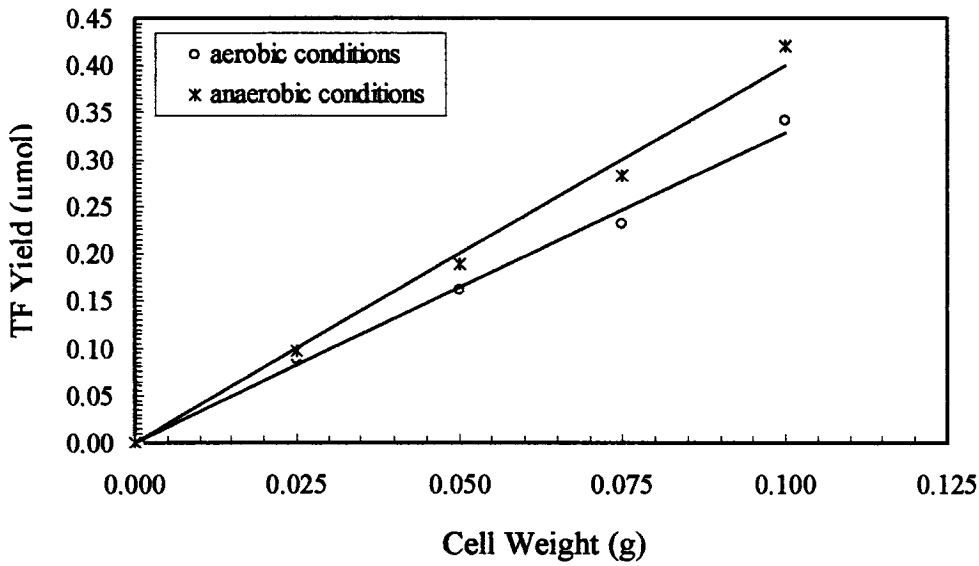


Figure 6.8. Correlation between total formazan yield and the mass of *A. niger* vegetative cells in liquid media under optimum conditions of TTC concentration (20 g/L), pH (9), temperature (55 °C) and incubation time (3 h).

6.7.3.2 Effect of the Amount of Alcohol per Wash. Figure 6.10 shows the effect of the amount of alcohol used for extraction on the TF yield for three A. *niger* cell masses (0.025, 0.050 and 0.075g) in the presence of shrimp shells under optimum conditions of TTC concentration (20 g/L), pH (9), temperature (55 °C), incubation time (3 h) and absence of oxygen. TF was extracted using two washes and the extract was filtered through a membrane micro-filter. Values are the average of two replicates. The coefficient of variation ranged from 0.92 to 4.04%.

6.7.3.3 Contribution of Shrimp Shells to TTC Reduction. Figure 6.11 shows the color developed when the TTC-test was performed on 0.1 g of *A. niger* vegetative cells, 0.1 g of *A. niger* vegetative cells mixed with 1 g shrimp shells and 1 g of shrimp shells under optimum conditions of TTC concentration (20 g/L), pH (9), temperature (55 °C), incubation time (3 h) and absence of oxygen. TF was extracted by two alcohol washes (5 mL each) and the extract was filtered through a membrane micro-filter having 0.45 μm pore size. Values are the average of two replicates. The coefficient of variation ranged

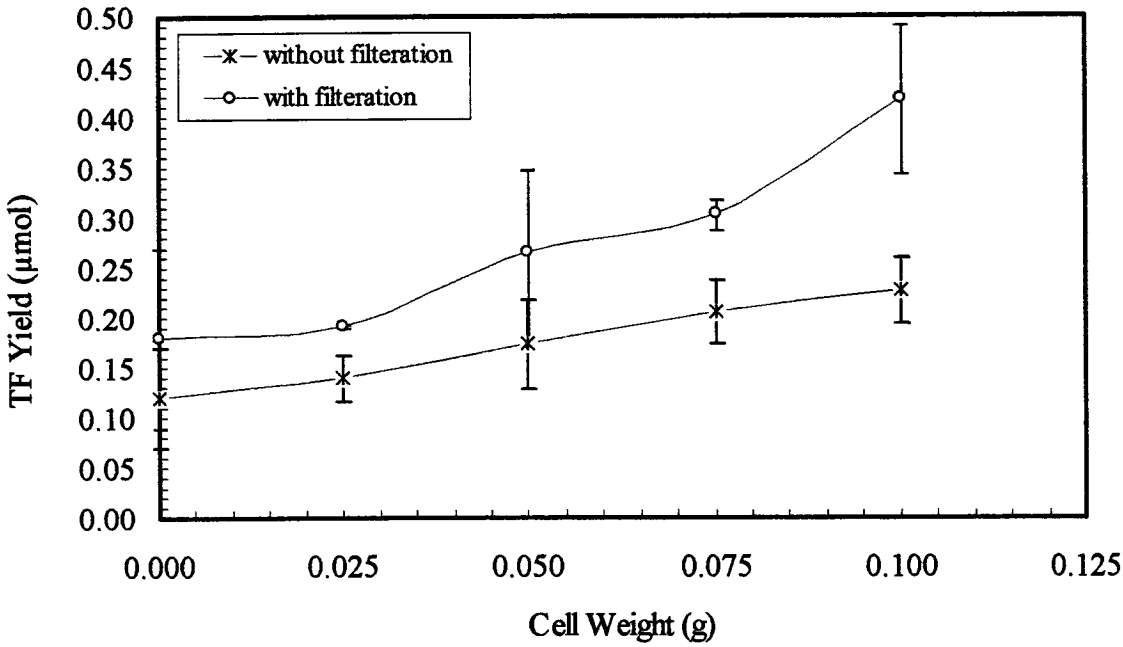


Figure 6.9. Effect of Filtration on TF yield measurements of *A. niger* in the presence of shrimp shells under optimum conditions of TTC concentration (20 g/L), pH (9), temperature (55 °C), incubation time (3 h) and absence of oxygen.

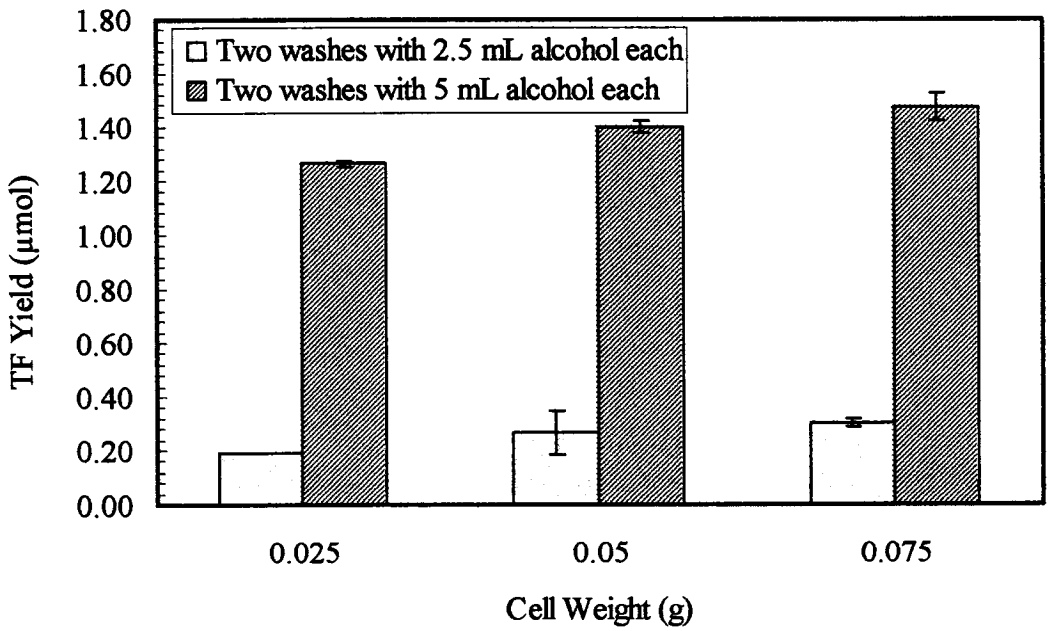


Figure 6.10. Effect of alcohol volume used for TF extraction on the total formazan yield under optimum conditions of TTC concentration (20 g/L), pH (9), temperature (55 °C), incubation time (3 h) and absence of oxygen.

from 2.45 to 24.77%.

6.7.3.4 Effect of Autoclaving. Figure 6.12 shows the effect of autoclaving on the color development when the TTC-test was performed on *A. niger* vegetative cells, shrimp shells and *A. niger* vegetative cells mixed with shrimp shells under optimum conditions of TTC concentration (20 g/L), pH (9), temperature (55 °C), incubation time (3 h) and absence of oxygen. Values are the average of two replicates. The coefficient of variation ranged from 11.70 to 24.73%. Plate count of autoclaved shrimp shells revealed no growth after an incubation period up to three days at room temperature (~24 °C) whereas plate count of shrimp shells without autoclaving showed the existence of 4 bacterial species and 1 fungal species (Figure 6.13). The number of indigenous microorganisms on shrimp shells used in this study was about 67×10^2 CFU/ g shells.

6.7.3.5 *A. niger* Standard Curve. Figure 6.14 shows the relationship between *A. niger* vegetative cell weight mixed with 1 g shrimp shells (autoclaved or unautoclaved) and triphenyl formazan yield under optimum conditions of TTC concentration (20 g/L), pH (9), temperature (55 °C), incubation time (3 h) and absence of oxygen. Values are the average of two replicates. The coefficient of variation among the replicates ranged from 3.1 to 40.0%.

6.8 Discussion

6.8.1 Chemical Reduction of TTC

6.8.1.1 Effect of Heavy Metals on the Color Intensity of the TF. Many studies reported a decrease in the TF accumulation from TTC when measuring the dehydrogenase activity in heavy metal (e.g. Pb, Cu, Ni, Cd, and Zn) contaminated soils (Ruhling and Tyler, 1973; Doelman and Haanstra, 1979; Brookes et al., 1984; Rogers and Li, 1985; Reddy et al., 1987; and Reddy and Faza, 1989). These studies claimed that the reduction in the TF yield was due to the effect of heavy metals on the soil microbes and hence on the dehydrogenase activity in the soil, thus resulting in less TF accumulation. These studies did not, however, identify which individual metals were causing the inhibition.

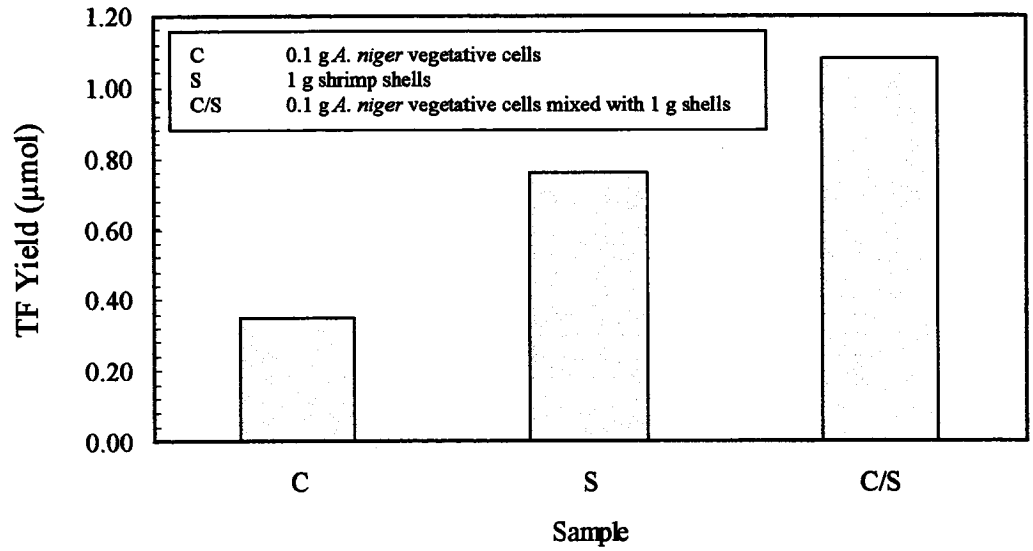


Figure 6.11. TF yield of *A. niger* vegetative cells and shrimp shells under optimum conditions of TTC concentration (20 g/L), pH (9), temperature (55 °C), incubation time (3 h) and absence of oxygen.

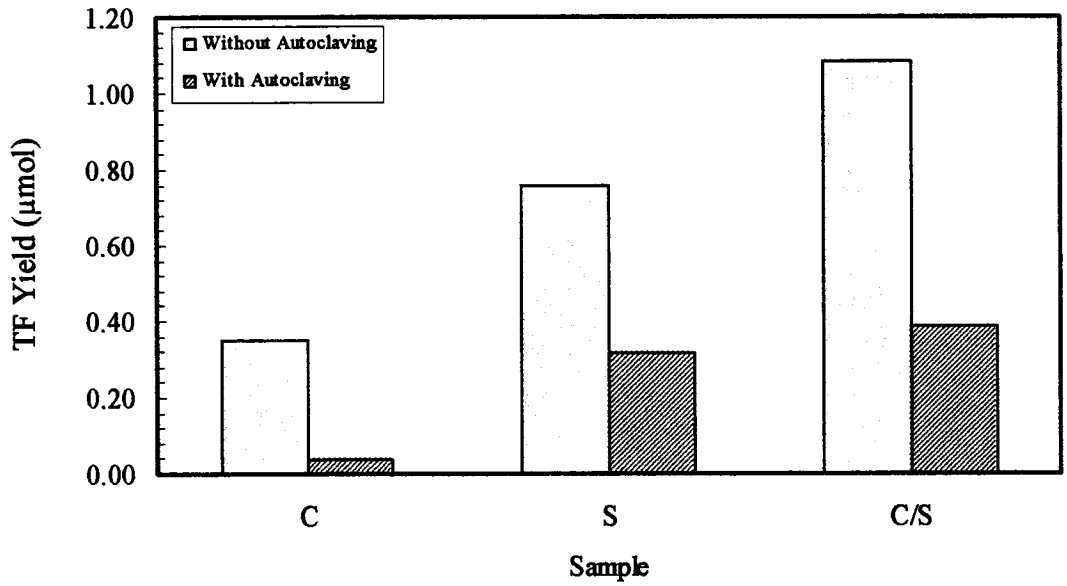
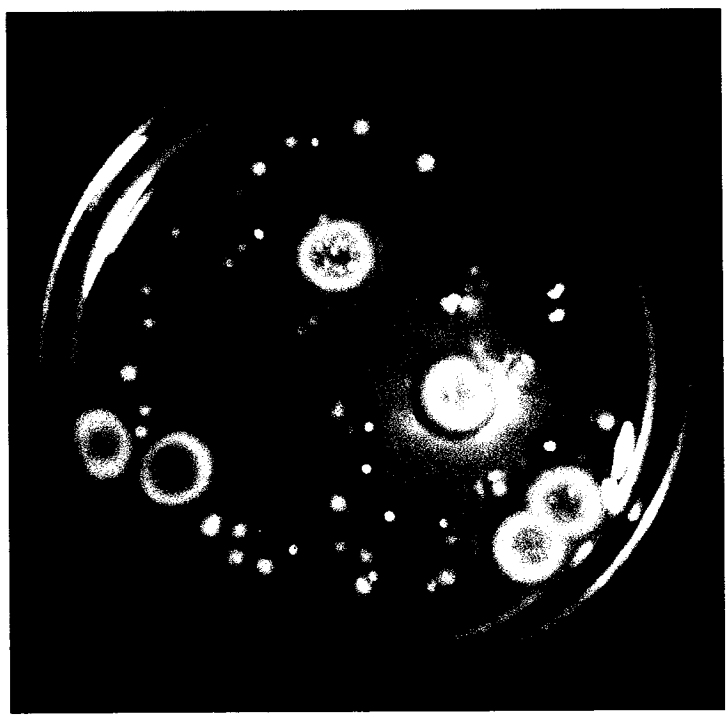


Figure 6.12. TF yield of autoclaved and unautoclaved *A. niger* vegetative cells and shrimp shells under optimum conditions of TTC concentration (20 g/L), pH (9), temperature (55 °C), incubation time (3 h) and absence of oxygen.

(a) with autoclaving



(b) without autoclaving

Figure 6.13. Indigenous microorganisms in shrimp shells.

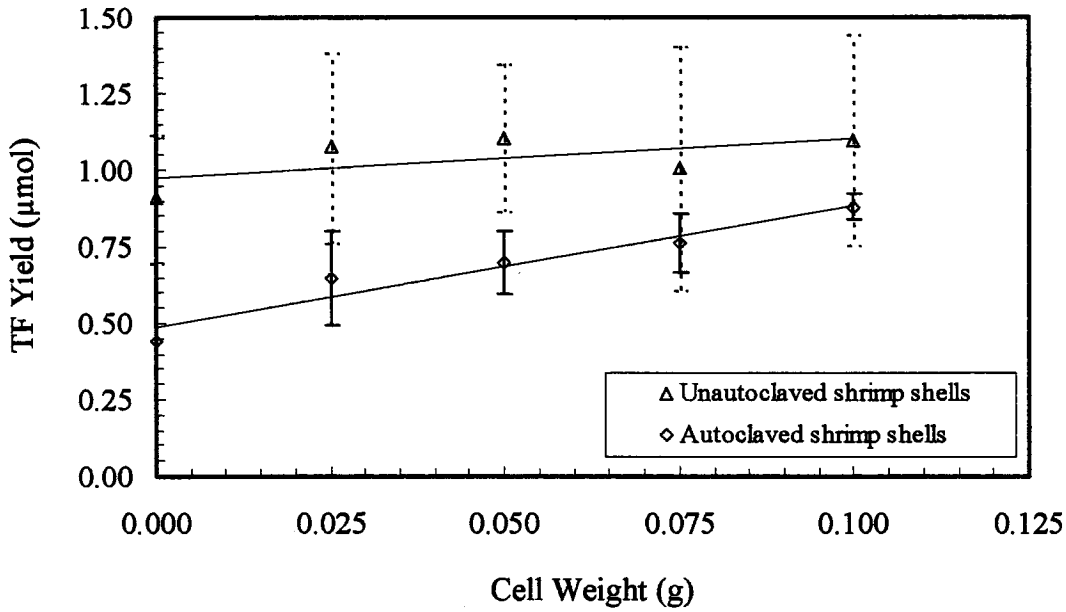
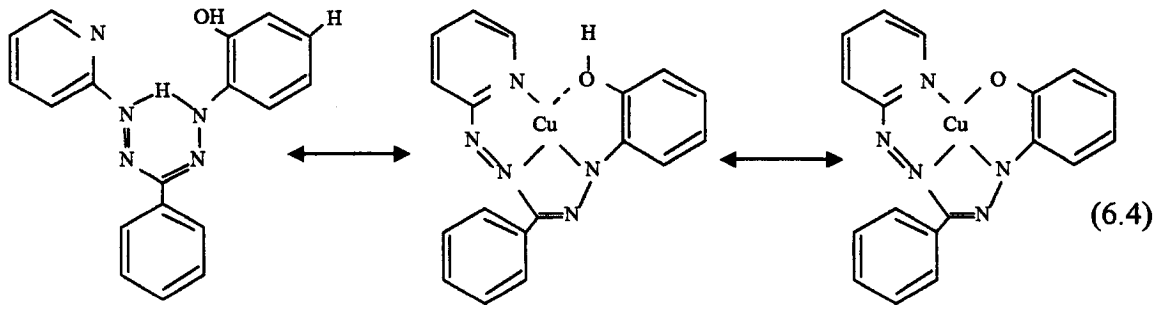


Figure 6.14. Relationship between total formazan yield and the mass of *A. niger* vegetative cells mixed with 1 g shrimp shells under optimum conditions of TTC concentration (20 g/L), pH (9), temperature (55 °C), incubation time (3 h) and absence of oxygen.

However, Chander and Brookes (1991) dismissed those claims and suggested that non-biological reactions between triphenyl formazan and heavy metals could have interfered with the dehydrogenase assay. They studied the effect of Ni, Cd, Zn, and Cu on TF yield and observed more than 95% decrease in the absorbance of the triphenyl formazan in the absence of any microbial activity and the presence of copper with concentrations of 0-20 mg/L. They concluded that the presence of Cu (not the other metals) prevented the development of the red color of TF from the TTC. The results obtained from this study indicated that the presence of copper in the compost sample reduced the absorbance of TF present in the sample significantly.

Honda et al. (1993) stated that formazan compounds tend to react with transition metal ions to form stable metal complexes resulting in bathochromic color change. They reported a color change of complexes (*o*-PHF = 1-(2-hydroxyphenyl)-3-phenyl-5-(2-pyridyl)formazan) with pH changes due to structural change of the metal- *o*-PHF

complexes (red in acidic conditions and blue in neutral to alkaline conditions) as shown in the following equation:



(6.4)

(*o*-PHF)
[Red]

Cu(II)(*o*-PHF)
@ acidic conditions
[Red]

Cu(II)(*o*-PHF)
@ neutral to alkaline conditions
[Blue]

6.8.1.2 <u>TTC Reaction Sensitivity to pH</u>. The results showed that changes in the pH of the sample affected the chemical reduction of the triphenyltetrazolium chloride to triphenyl formazan for both liquid and solid wastes. While no chemical reduction was observed in acidic media (pH < 7), high pH values caused the TTC to be reduced chemically.

In case of liquid wastes (Figure 6.3a), no chemical reduction was detected at pH values lower than 9.5. The amount of reduced triphenyltetrazolium chloride (triphenyl formazan) increased with increasing the pH reaching its maximum value at pH of 12. The TF yield then decreased when the pH value was higher than 12 and the reduction of TTC was completely hindered at a pH of 13. For a given pH, the results also showed that TF yield increased with increasing the incubation period. However, the effect of the incubation period at the optimum pH value (pH of 12) was not significant (the percentage of TTC reduction was 54.3, 57.5, and 57.8% after 1, 2, and 3 hours of incubation, respectively) but was significant at pH values lower or higher than 12. The effect of pH on the TTC chemical reduction for a given incubation period can be described by the following equation, with a coefficient of determination (R^2) of 0.81.

$$TF = e^{\left(701-204pH+19.3pH^2-0.598pH^3+2.6t-0.492t^2\right)} \qquad (9.5 \leq pH \leq 13) \qquad (6.5)$$

Where:

TF is the triphenyle formazan content (μmoleTF/μmoleTTC).

pH is the pH value.

t is the incubation period (h).

In case of solid waste (Figure 6.3b), the results showed that the TTC chemical reduction started at higher pH value compared to liquid waste (11.0 vs. 9.5). No chemical reduction of TTC was observed at pHs lower than 11.0. The amount of formazan yield increased with increasing the pH till it reached its peak at a pH of 12.0, after which the reduction of TTC decreased with increases in pH and was completely hindered at a pH of 13. The effect of incubation period at the optimum pH (pH 12) was very significant in this case. At pH of 12, the percentage of reduced TTC was 9.3, 17.9, 33.4% after 1, 2, 3 hours incubation, respectively. However, at pH values higher or lower than 12, the effect of incubation period was less significant. The effect of pH on the TTC chemical reduction can be described by the following equation with a coefficient of determination (R^2) of 0.96.

$$TF = e^{\left(-665+110pH-4.55pH^2+0.597t\right)} \qquad (11 \leq pH \leq 13) \qquad (6.6)$$

The results obtained from the TTC pH sensitivity test indicated that the amount of TF produced was much higher in case of liquid medium (cheese whey) than in case of solid medium (compost). The highest percentage of reduction in the TTC (at pH of 12) was 57.8 and 33.4% for cheese whey and compost, respectively. Furthermore, the TTC chemical reduction in a liquid medium was observed at a lower pH than that of solid medium (9.5 for cheese whey vs. 11.0 for compost). Water content seems to play a major role in the TTC chemical reaction. The lower amount of TF produced in the case of municipal solid waste compost might also be due to the interaction between the heavy metals present in the compost and the triphenyl formazan under the tested pH values. The

compost analysis (Table 6.3) showed that there are some traces of heavy metals including copper (2.0 mg/kg) present in the waste material.

6.8.1.3 TTC Reaction Sensitivity to Temperature. The results obtained in this study indicated that the triphenyltetrazolium chloride (TTC) could be reduced chemically to triphenyl formazan (TF) at high incubation temperatures. The nonenzymatic reduction of TTC started at temperatures higher than 70 °C and 80 °C for the cheese whey and compost, respectively. The amount of TF produced increased with increasing the incubation temperature. The total formazan yield in case of cheese whey samples was higher than that in case of compost samples at any given incubation temperature. This is due to the high moisture content in cheese whey, which enhanced the TTC chemical reaction. It could also be explained as a result of chemical reactions between the triphenyl formazan and some of the heavy metals present in the compost material especially the copper.

The amount of TF produced at 100 °C was 0.109 μmoleTF/μmoleTTC (0.24 mg) for cheese whey and 0.077 μmoleTF/μmoleTTC (0.17 mg) for compost. A study by Casida et al. (1964) showed a TTC chemical reduction at temperatures higher than 65 °C. The authors also found that the total formazan content measured in sterile soil samples incubated at 37, 65, 80, and 100 °C, were 0.28, 0.08, 0.72, and 14.28 mg, respectively. These TF values are much higher than those produced in this study.

It is well known that the velocity of a chemical reaction is a function of temperature. Chemical reaction rates increase with rises in temperature by a factor of between 0 and 1 with each 10 °C rise in temperature. The effect of temperature is usually described by the following Arrhenius relationship (Wiseman, 1985):

$$k = Ae^{-E/RT} \tag{6.7}$$

Where:

k is the reaction rate (h^{-1}).

A is the Arrhenius constant (h^{-1}).

E is the Activation Energy (J/mol).

R is the gas constant (8.314 J/mol.K).

T is the absolute temperature (K).

The increase in TTC chemical reduction rate with the increase in incubation temperature (from 70 – 100 °C) in cheese whey samples can be described by the following Arrhenius equation, with coefficient of determination (R^2) of 0.98.

$$TF = 57.15 \times 10^{22} e^{-168,807.5/RT} \qquad (70\ ^\circ C < T \leq 100\ ^\circ C) \qquad (6.8)$$

Where:

T is the incubation temperature (K).

Similarly, the increase in TTC chemical reduction rate with the incubation temperature (from 80 – 100 °C) in compost samples can be described by the Arrhenius equation, with coefficient of determination (R^2) of 0.98.

$$TF = 2.77 \times 10^{32} e^{-239,102.3/RT} \qquad (80\ ^\circ C < T \leq 100\ ^\circ C) \qquad (6.9)$$

The activation energy is the minimum energy that should be possessed by the reacting molecules in order for the reaction to take place, the larger the activation energy the more temperature sensitive is the reaction. Wiseman (1985) reported that for enzyme catalyzed reactions, the activation energy is normally in the range of 16,736-83,680 J/mol. Fogler (1999) stated that for first order gas phase reactions, the activation energy could be assumed to be 300,000 J/mol. Spiegel (1999) reported activation energy values for whey protein aggregation of 400,000 J/mol and 80,000 J/mol in low and high temperature ranges, respectively. Hy et al. (1994) reported activation energy values in the range of 50,000 – 70,000 J/mol for BOD removal for anaerobic wastewater treatment using a biofilter. Mizuno et al. (2000) reported activation energy values for supercritical water

oxidation of municipal solid waste at temperature range of 673 – 823 K of 97,200 and 130,800 J/mol for the reduction of total organic carbon and ammonium ion, respectively. Caldwell (1993) reported activation energy values in the range of 115,060 – 334,720 J/mol for TTC reduction by cucumber leaf discs. In this study, the activation energy for the TTC chemical reduction in cheese whey and municipal solid waste compost was found to be 168,808 and 239,102 J/mol, respectively.

6.8.2 Measuring Dehydrogenase Activity of *A. niger* using TTC

6.8.2.1 Effects of TTC Concentration, pH, Temperature and Incubation Time. The results showed that the TTC concentration, pH, temperature and incubation time all affected the triphenyl formazan (TF) yield during the dehydrogenase activity measurement of *A. niger* vegetative cells using the TTC-Test. The amount of TF formed during the test increased with the increase in each parameter within the studied ranges. Analysis of variance was performed on the formazan concentration (μmol/mL) using SAS System (5th Ed, SAS Institute Inc., Cary, North Carolina) as shown in Table 6.9.

The Duncan's Multiple Range test was also performed on the data to test the differences among the levels of each parameter as shown in Table 6.10. The analysis showed that the TTC concentration, pH, temperature and incubation time all had significant effects on the formazan concentration (P = 0.0001). There were also significant interactions between the parameters (P = 0.0001) as shown in Figure 6.15. The levels of each of the four parameters (TTC concentration, pH, temperatures and incubation period) were significantly different from one another at 95% confidence. Equation 6.10 describes the effects of TTC concentration, pH, temperature and incubation time on TF yield (μmol) ($R^2 = 0.92$):

$$TF = 6.384 \times 10^{-7} e^{(0.0973C_{TTC} + 0.679pH + 0.168T - 0.00149T^2 + 0.297t_i)} \qquad (6.10)$$

Where:

TF is the TF yield (μmol)

C_{TTC} is the TTC concentration (g/L)

T is the incubation temperature (°C)

t_I is the incubation time (h)

Table 6.9. Analysis of variance.

Source	DF	SS	MS	F-Value	P
Total	161	4787.8683			
Model	80	4762.5219	59.5315	190.25	0.0001
C_{TTC}	2	699.6380	349.8190	1117.92	0.0001
pH	2	1286.8033	643.4016	2056.13	0.0001
T	2	1076.2404	538.1202	1719.68	0.0001
t_I	2	472.0135	236.0068	754.21	0.0001
C_{TTC} * pH	4	204.5133	51.1283	163.39	0.0001
C_{TTC} * T	4	160.2992	40.0748	128.07	0.0001
C_{TTC} * t_I	4	48.1151	12.0288	38.44	0.0001
pH * T	4	267.7266	66.9316	213.89	0.0001
pH * t_I	4	195.1633	48.7908	155.92	0.0001
T * t_I	4	122.7424	30.6856	98.06	0.0001
C_{TTC} * pH * T	8	47.4259	5.9282	18.95	0.0001
C_{TTC} * pH * t_I	8	61.8320	7.7290	24.70	0.0001
C_{TTC} * T * t_I	8	11.4468	1.4309	4.57	0.0001
pH * T * t_I	8	71.8236	8.9779	28.69	0.0001
C_{TTC} * pH * T * t_I	16	36.7385	2.2962	7.34	0.0001
Error	81	25.3464	0.3129		

C_{TTC} = TTC concentration (g/L)
pH = medium pH
T_I = Incubation temperature (° C)
t_I = Incubation period (h)
R^2 = 0.995
CV = 10.19%

Table 6.10. Differences among the levels of the TTC concentration, pH, temperature and incubation time.

Parameter	Number of Observations	TF concentration (μg/mL)	Duncan Grouping
TTC concentration (g/L)			
15	54	8.26	A
10	54	4.96	B
5	54	3.25	C
pH			
9	54	9.21	A
8	54	4.87	B
7	54	2.39	C
Temperature (°C)			
55	54	8.16	A
40	54	6.31	B
25	54	2.00	C
Incubation time (h)			
4.5	54	7.77	A
3.0	54	5.03	B
1.5	54	3.67	C

Means with the same letter are not significantly different from each other at 95% confidence level.

To test the sensitivity of each parameter, a sensitivity analysis was carried out on equation 6.10. When testing a given parameter, the values of the other parameters were kept at their respective base values (optimum values) while the value of the tested parameter were varied within the range studied (Table 6.11). The results of the sensitivity analysis (Figure 6.16) showed that the TF yield was more sensitive to changes in temperature followed by pH, TTC concentration and incubation time. TF yield increased by 334.30, 288.82, 164.48 and 143.65% when the temperature, pH, TTC concentration and incubation time was increased from 25 to 55 °C, from 7 to 9, from 5 to 15 g/L and from 1.5 to 4.5 h, respectively.

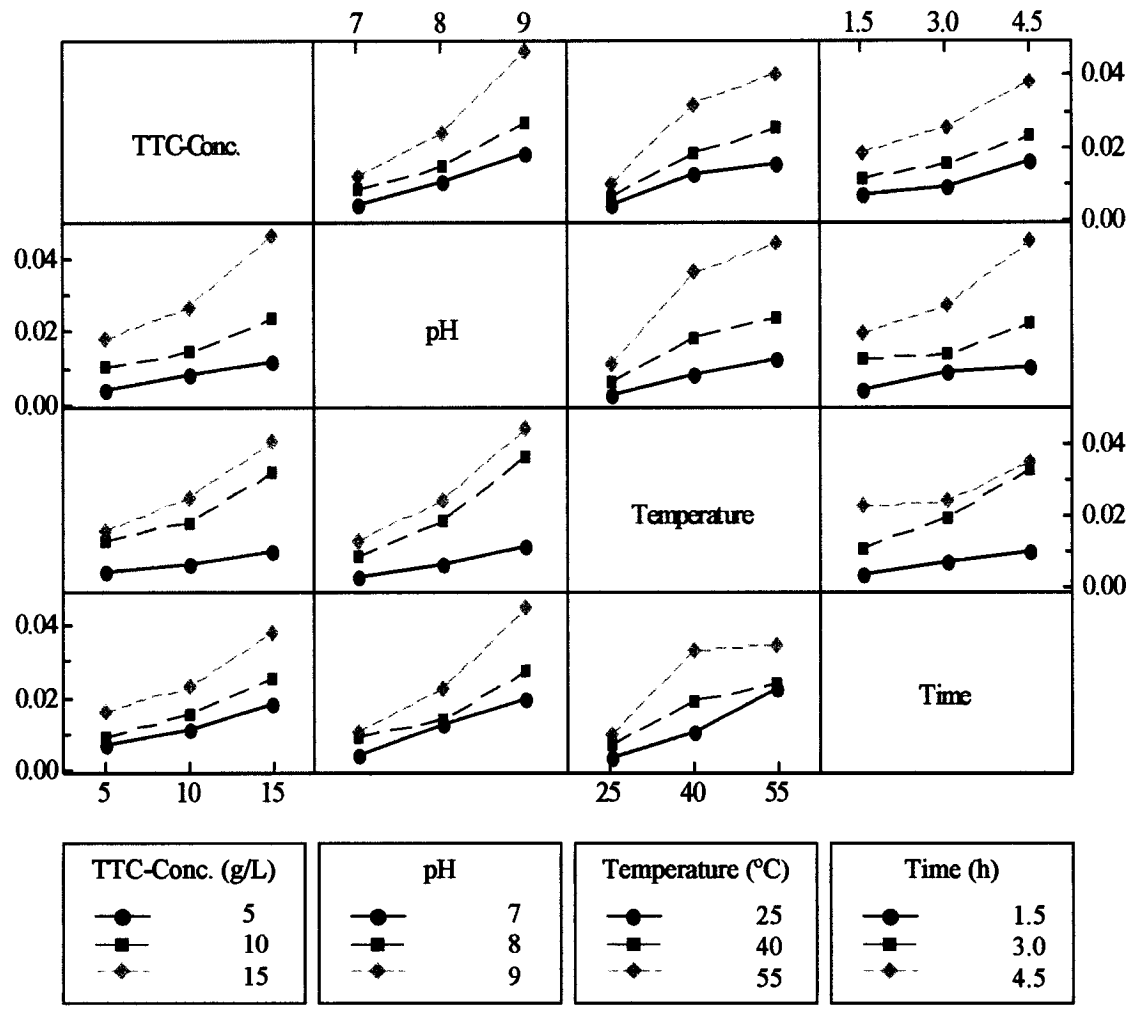


Figure 6.15. Interaction plots for the TTC concentration, pH, temperature and incubation time.

6.8.2.1.1 Effect of TTC Concentration: The results obtained from a preliminary experiment showed a very low TF yield when a TTC concentration of 5 g/L was used. Increasing the TTC concentration up to 15 g/L significantly enhanced the TF yield. The amount of increase in the TF yield with every 2.5 units (g/L) increase in TTC concentration was 27.53 %. The direct relationship between TTC concentration and TF yield (Figure 6.16a) can be described by the following exponential equation ($R^2 = 0.99$).

$$TF = 0.0802e^{0.0973C_{TTC}} \qquad (5 \leq C_{TTC} \leq 15 \text{ mg/L}) \qquad (6.11)$$

Table 6.11. Base values and range of each parameter used for the sensitivity analysis.

Variable	Unit	Level	Base	Range
TTC Concentration	g/L	5	15	5.0
				7.5
				10.0
				12.5
				15.0
pH	-	5	9	7.0
				7.5
				8.0
				8.5
				9.0
Temperature	°C	7	55	25
				30
				35
				40
				45
				50
				55
Incubation time	h	7	3	1.5
				2.0
				2.5
				3.0
				3.5
				4.0
				4.5

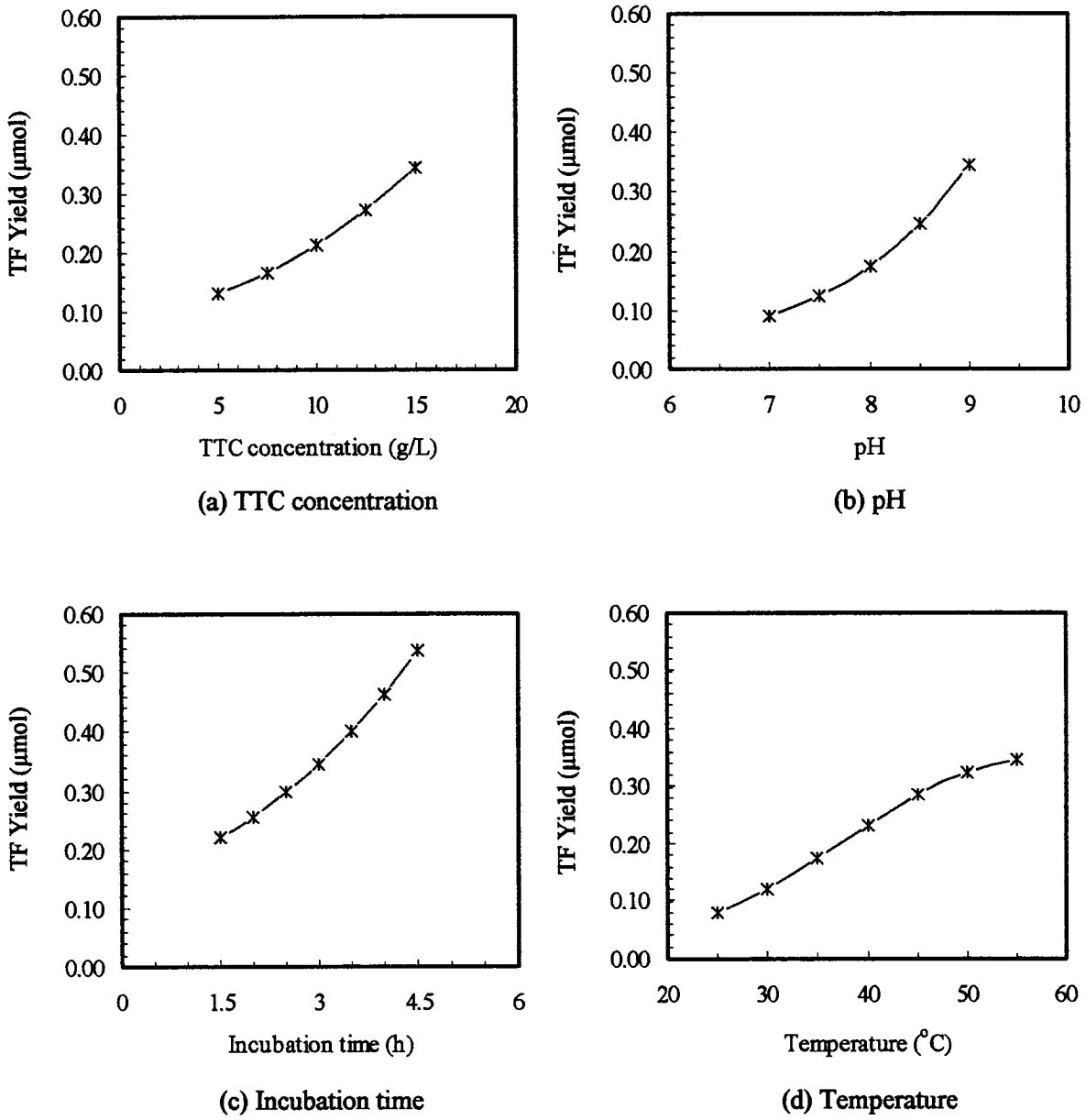


Figure 6.16. Effects of TTC concentration, pH, temperature and incubation time on TF yield.

A very small percentage (0.05-2.70 %) of the added TTC was reduced to triphenyl formazan (Table 6.8). Tetrazolium salts vary in their ability to penetrate through intact cell plasma membrane (Altman 1976; and Bernas and Dobrucki, 2000). Altman (1976) reported that intact cells and subcellular membranes can constitute barriers to the entry of tetrazolium salts. A low concentration of the salt at the enzymatic reaction site may,

therefore, be a rate limiting factor even if the concentration in the medium is sufficient. Bernas and Dobrucki (2000) provided evidence suggesting that MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) can readily penetrate intact plasma membranes while CTC (5-cyano-2,3-ditolyl tetrazolium chloride) remains extracellular and requires membrane permeable electron carrier to be reduced efficiently. Altman (1976) stated that MTT is able to penetrate mitochondrial membrane more readily than NT (3,3'-(4,4'-biphenylylene)-bis-(2,5-diphenyl-2H-tetrazolium chloride)) because it has a smaller molecular weight (414 vs. 668). Based on this explanation, it appears that the ability of TTC to penetrate cell membranes would be relatively higher since it has the smallest molecular weight (335) among the common tetrazolium salt (Table 6.1). On the contrary, however, Altman (1969) and Altman (1976) rated TTC as having the lowest reducibility among tetrazolium salts and found the activity of TTC to be affected by the presence of O_2. According to Bernas and Dobrucki (2000) molecular structure of tetrazolium salts as well as the presence of electron carrier may strongly influence the rates and the ability to penetrate through cellular membranes.

6.8.2.1.2 Effect of pH: In a preliminary experiment, no reduction of TTC took place under acidic conditions (< 6.5) and chemical reduction of TTC starts to take place at pH of 9.5 (Mahmoud and Ghaly, 2004). The results obtained from these experiments showed that the TF yield increased with increases in pH within the studied range (7-9 g/L). The amount of increase in the TF yield with every unit increase in pH was 40.42%. The direct relationship between pH and TF yield (Figure 6.16b) can be described by the following exponential equation ($R^2 = 0.99$).

$$TF = 0.0008e^{0.679pH} \qquad (7 \leq pH \leq 9) \qquad (6.12)$$

Enzymatic activities can only take place over a certain pH range. Ghaly and Ben-Hassan (1993) reported an optimum pH range of 7.0 ± 0.2 for measuring the dehydrogenase activity of *Kluyveromyces fragilis* and *Candida pseudotropicalis* using TTC. They stated that pH change (+/-) of 3 units was able to hinder the enzymatic activity. Shuler and Kargi (1992) reported that pH may affect enzymes in three ways: (a) changes in the

medium pH result in changes in the ionic form of the active sites of the enzymes, which in turn affect the activity of the enzyme and hence the reaction rate, (b) variations in pH may alter the three-dimensional shape of the enzyme and (c) sometimes the substrate contain ionic groups and hence the affinity of the substrate to the enzyme is affected by changes in the pH. Although, the enzymatic reduction of TTC took place with the pH range of 7-9 in this study, a pH of 9 appeared to be the optimum pH for measuring the dehydrogenase activity of *A. niger*.

6.8.2.1.3 Effect of Incubation Time: The results obtained from these experiments showed that the TF yield increased with the increase in incubation time within the studied range (1.5 – 4.5 h). The amount of increase in the TF yield with every one unit (h) increase in incubation time was 32.00 %. The direct relationship between incubation time and TF yield (Figure 6.16c) can be described by the following exponential equation (R^2 = 0.99).

$$TF = 0.1416e^{0.2968t_I} \qquad (5 \leq t_I \leq 15 \text{ h}) \qquad (6.13)$$

Ghaly and Ben-Hassan (1993) reported an optimum incubation time of 80 min for measuring the dehydrogenase activity of *Kluyveromyces fragilis* and *Candida pseudotropicalis* using TTC of 5 g/L concentration at a pH of 7 and a temperature of 20 °C. Ross (1971) reported a gradual decrease in formazan formation after an incubation time of 1 h. Griebe et al. (1997) reported a decrease in the rate of formazan formation after 2 h incubation time when CTC was incubated with activated sludge and stated that no significant increase was realized in formazan formation after 4 h incubation time. Although, the TF yield increased exponentially with time in this study, an incubation time greater that 4 h would be considered very lengthy.

6.8.2.1.4 Effect of Temperature: The results obtained from these experiments showed that the TF yield increased with the increase in temperature within the studied range (25-55 °C). However, the amount of increase in TF yield for every 10 °C increase in the temperature declined gradually as shown in Figure 6.17. The relationship between TF

yield and incubation temperature in the temperature range from 25 to 55 °C can be described by the following Arrhenius equation ($R^2 = 0.93$).

$$TF = 98 \times 10^4 e^{-40{,}098/RT} \qquad (25\ ^\circ C \leq T \leq 55\ ^\circ C) \qquad (6.14)$$

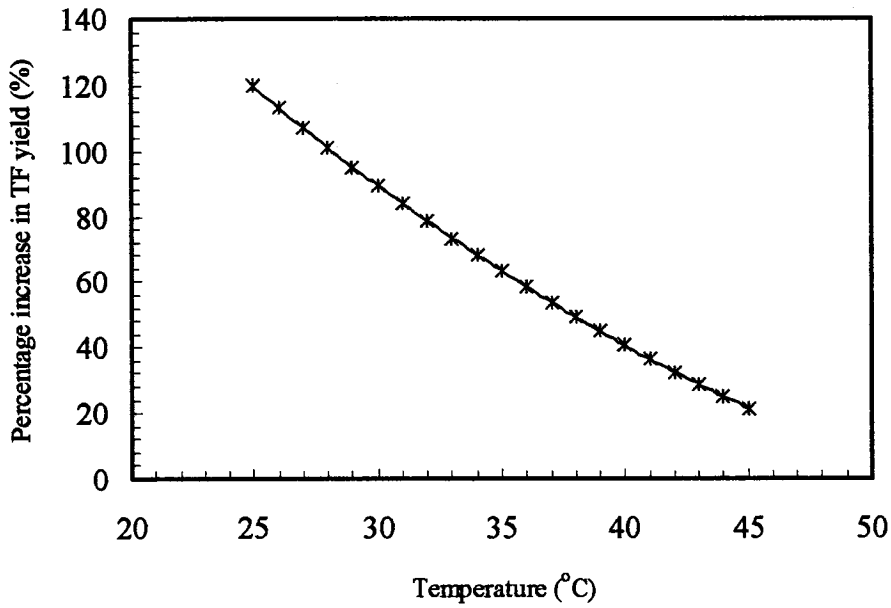


Figure 6.17. Percent increase in the amount of TF produced for every 10 °C increase in temperature.

Trevors (1984a) observed significant increase in dehydrogenase activity with increases in the incubation temperature up to 50 °C. Trevors (1984b) observed a high positive linear correlation between incubation temperature (in the range of 5 to 70 °C) and color development when measuring dehydrogenase activity in soils using INT. Wiseman (1985) stated that chemical reaction rates increase with rises in temperature by a factor of between 0 and 1 with each 10 °C rise in temperature and the relationship is usually described by Arrhenius equation (equation 6.8).

Doran (1995) reported that for enzyme reactions, a 10 °C rise in temperature between 20 and 30 °C increases the rate of reaction by a factor of 2-3. He also stated that the kinetics

of most enzymatic reactions can be sufficiently represented by the following Michaelis-Menten equation.

$$\upsilon = \frac{\upsilon_{max} C}{K_m + C} \tag{6.15}$$

Where:

- υ is the volumetric rate of reaction (μmol/mL.h).
- C is the concentration of the substrate (μmol/mL).
- υ_{max} is the maximum reaction rate at infinite substrate concentration and is proportional to the amount of active enzyme present (μmol/mL.h).
- K_m is the Michaelis-Menten constant and is equal to the substrate concentration at half the maximum reaction rate (μmol/mL).

The temperature affects the conformation of enzymes. Very rapid changes in the activities can take place over a small temperature range as variations in temperature affects both υ_{max} and K_m (Bu'lock and Kristiansen, 1987; and Shuler and Kargi, 1992). However, the rate of an enzymatic reaction increases with the increase in temperature up to a certain limit above which enzyme activity decreases as a result of thermal deactivation (or enzyme denaturation) as shown in Figure 6.18a (Wiseman, 1985; Shuler and Kargi, 1992; and Doran, 1995). The ascending part of the curve in Figure 6.18a describes the activation effect of temperature on enzyme while the descending part describes the deactivation effect of temperature on enzyme, which is much faster than enzyme activation. The rate of enzyme activation and deactivation can be described by the following Arrhenius equations (Bu'lock and Kristiansen, 1987; and Shuler and Kargi, 1992).

$$k_a = A_a e^{-\frac{E_a}{RT}} \tag{6.16}$$

$$k_d = A_d e^{-\frac{E_d}{RT}} \tag{6.17}$$

Where:

k_a is the activation rate constant (h^{-1})

E_a is the activation Energy (J/mol)

k_d is the deactivation rate constant (h^{-1})

E_d is the deactivation Energy (J/mol)

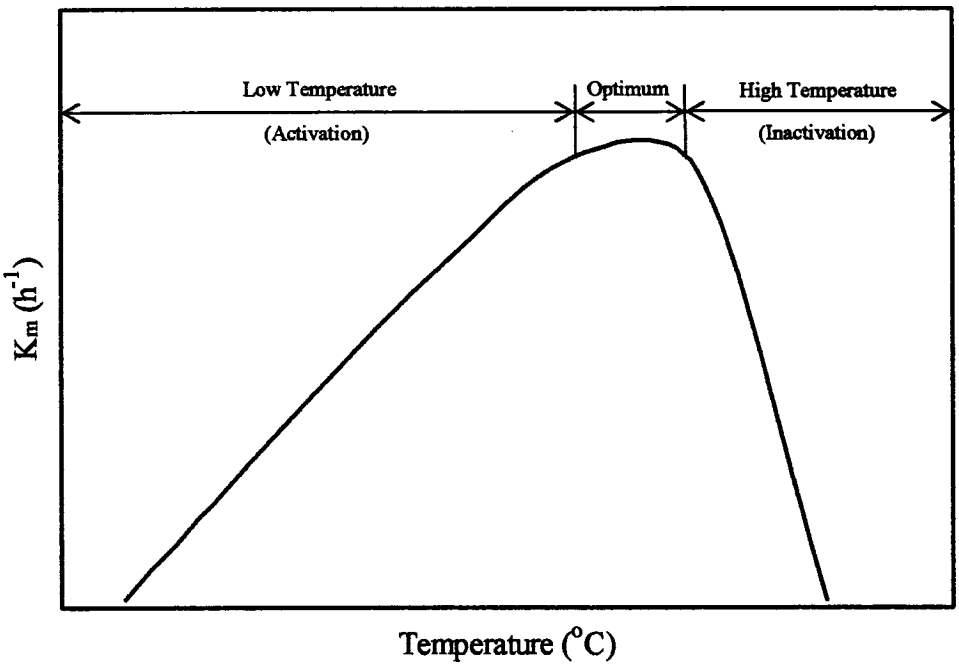
The overall maximum rate of reaction υ_{max} can, therefore, be expressed as follows (Bu'lock and Kristiansen, 1987).

$$\upsilon_{max} = A_a e^{-E_a/RT} - A_d e^{-E_d/RT} \qquad (6.18)$$

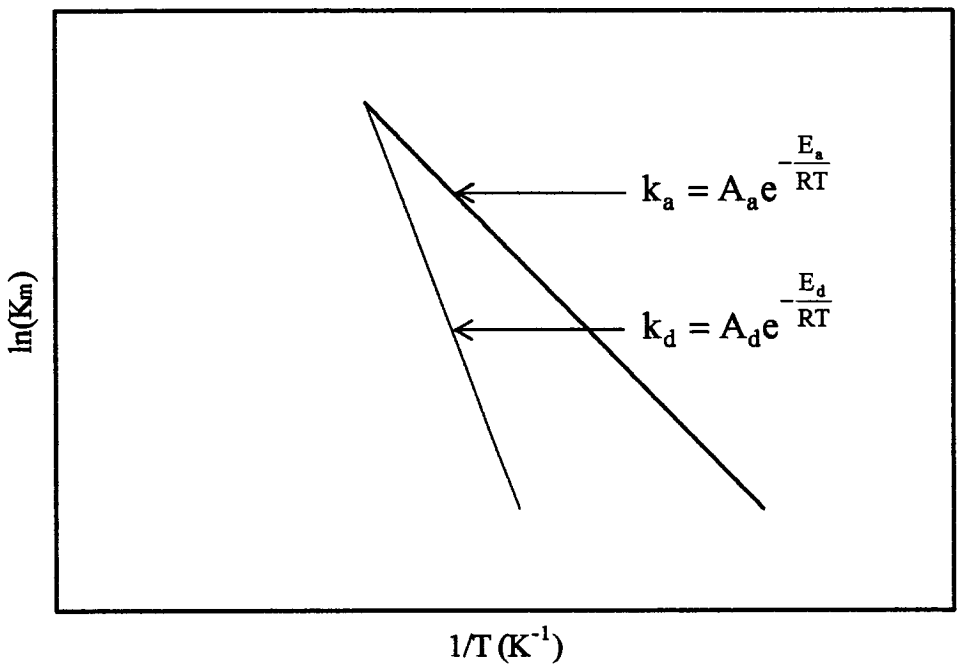
In equation (6.17), the first term accounts for the general increase in the reaction rate with temperature while the second term, which has much higher activation energy than the first term, is associated with the rapid reduction in the activity of the enzyme as the temperature increases beyond the optimum. There is a linear relationship between the logarithm of the reaction rate constant and the inverse of the absolute temperature as shown in Figure 6.18b (Bu'lock and Kristiansen, 1987).

The activation energy for the TTC biochemical chemical reduction for *A. niger* vegetative cells was 40,098 J/mol in the temperature range from 25 to 55 °C. The activation energy obtained in this study was within the range of 16,736-83,680 J/mol reported by Wiseman (1985) and Shuler and Kargi, (1992) and of 40,000-80,000 J/mol reported by Doran (1995) for enzyme catalyzed reactions.

Doran (1995) reported that the deactivation energy for many enzymes is of the order 170,000 - 400,000 J/mol, which means for 10 °C rise in temperature between 30 to 40 °C, the rate or enzymatic deactivation increases by a factor between 10 and 150. Shuler and Kargi, (1992) reported that a rise in temperature from 30 to 40 °C results in a 41 fold increase in enzyme denaturation. Hecht et al. (1990) stated that, under certain conditions, enzyme denaturation only occurs at temperatures higher than 55 °C. In this study, although the enzyme activity decreased gradually with the increase in temperature,



(a) reaction rate constant



(b) Activation and deactivation rate constants

Figure 6.18. Effect of temperature on enzyme activity.

enzyme denaturation was not realized within the studied temperature range (25-55 °C).

6.8.2.2 Effects of TTC Concentration and Cell Age. The results obtained from the previous experiments indicated that increasing the TTC concentration up to 15 g/L increased the TF yield exponentially. Since the rates of enzymatic reactions are affected by the substrate concentration (Shuler and Kargi, 1992; Doran, 1995; and Blanch and Clark, 1997) and inhibition can take place at higher concentrations (Andrews, 1968; Edwards, 1970; Han and Levenspiel, 1988; Shuler and Kargi, 1992; and Blanch and Clark, 1997), the toxicity effect of TTC concentrations beyond 15 g/L (up to 40 g/L) on *A. niger* cells of different ages was evaluated. The results obtained from these experiments showed that increasing the TTC concentration initially increased the triphenyl formazan yield to a maximum value and then decreased it. The optimum TTC concentration (that resulted in the maximum TF yield) was 30 g/L (44.8 μmol) for young cells (4 days old) and 20 g/L (29.9 μmol) for old cells (12 days old). Further increases in TTC concentration above these tolerance levels reduced the triphenyl formazan yield. This might be due to: (a) cell toxicity, which differed with age as younger cells appeared to have more toxicity tolerance and (b) cell viability as the amount of dehydrogenase enzymes present inside the fungal cells could be a function of the cell age.

According to Tengerdy et al. (1967) and Ohara and Saito (1995), tetrazolium salts inhibit the growth of microorganisms to some extent but using certain test conditions may fairly reduce the negative effect of the salts on the microbial growth and hence improve the quantitative determination of microbial growth. Griebe et al. (1997) detected a significant reduction in formazan formation at CTC concentration of 10 mM and pointed out that this reduction indicates physiological inhibition. Shuler and Kargi (1992) stated that the presence of toxic compounds in the medium results in the inactivation of cells or cell death and consequently the enzymatic activity is negatively affected. The authors indicated that the intracellular composition of cells varies depending on the type and age of cells and the composition of the medium.

The relationship between the reaction rate and substrate concentration for enzymatic reactions is usually described by Michaelis-Menten kinetics (a combination of zero order and first order kinetics) as shown in equation 5.13 (Doran, 1995). At low substrate concentrations, the reaction follows first order kinetics in which the reaction rate increases with the increase in substrate concentration. At high substrate concentration, the reaction rate follows zero order kinetics and approaches a constant maximum value which is independent of substrate concentration (i.e. $\upsilon = \upsilon_{max}$ regardless of increases in the substrate concentration). Figure 6.19 presents a typical curve of Michaelis-Menten plot. The values of υ_{max} and K_m can be obtained graphically by converting Michaelis-Menten equation into the following linear forms:

$$\frac{1}{\upsilon} = \frac{K_m}{\upsilon_{max} C} + \frac{1}{\upsilon_{max}} \qquad \text{(Lineweaver-Burk plot)} \qquad (6.19)$$

$$\frac{\upsilon}{C} = \frac{\upsilon_{max}}{K_m} + \frac{\upsilon}{K_m} \qquad \text{(Eadie-Hofstee plot)} \qquad (6.20)$$

$$\frac{C}{\upsilon} = \frac{K_m}{\upsilon_{max}} + \frac{C}{\upsilon_{max}} \qquad \text{(Langmuir plot)} \qquad (6.21)$$

The plots are shown in Figure 6.20 and the values of K_m and υ_{max} are presented in Table 6.12. The K_m values varied from 88.55 to 103.14 μmol and from 177.41 to 187.84 μmol for the 4 and 12 days old cultures, respectively. The υ_{max} values also varied from 0.95 to 1.07 μmol/h and from 1.12 to 1.18 μmol/h for the 4 and 12 days old cultures, respectively. The Lineweaver-Burk plot of $1/\upsilon$ versus $1/C$ gave a straight line with slope K_m/υ_{max} and intercept $1/\upsilon_{max}$ whereas the Eadie-Hofstee plot of υ/C versus C gave a straight line with slope $1/K_m$ and intercept υ_{max}/K_m. According to Doran (1995), the linearization process of both plots distorts the experimental errors in the data which results in reduced accuracy. The Langmuir plot of C/υ versus C gave a straight line with

slope $1/\upsilon_{max}$ and intercept K_m/υ_{max}. The linearization of data using the Langmuir plot minimizes distortions in experimental error.

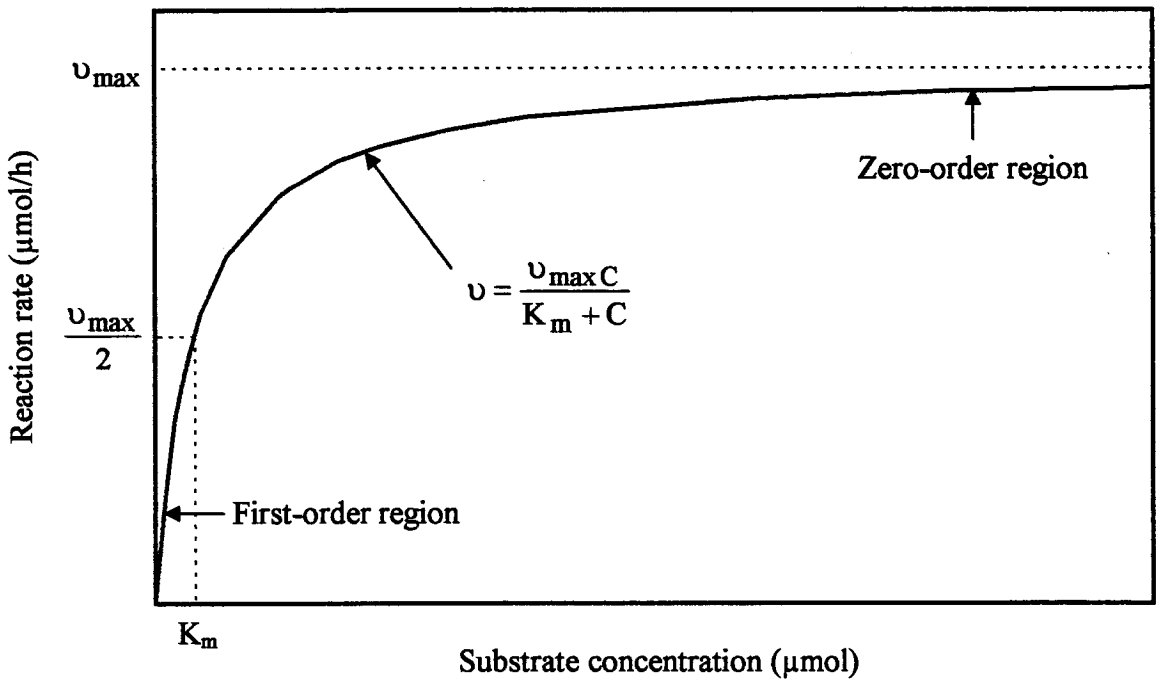
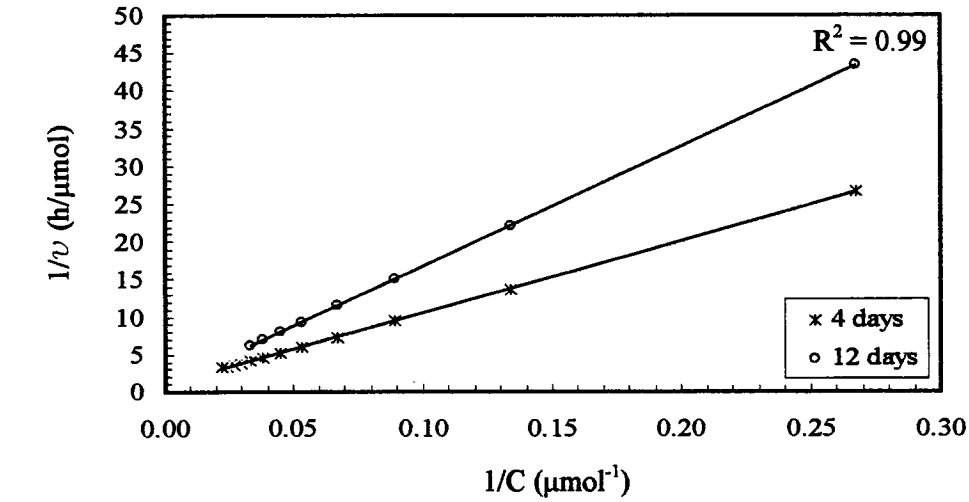


Figure 6.19. Michaelis-Menten plot.

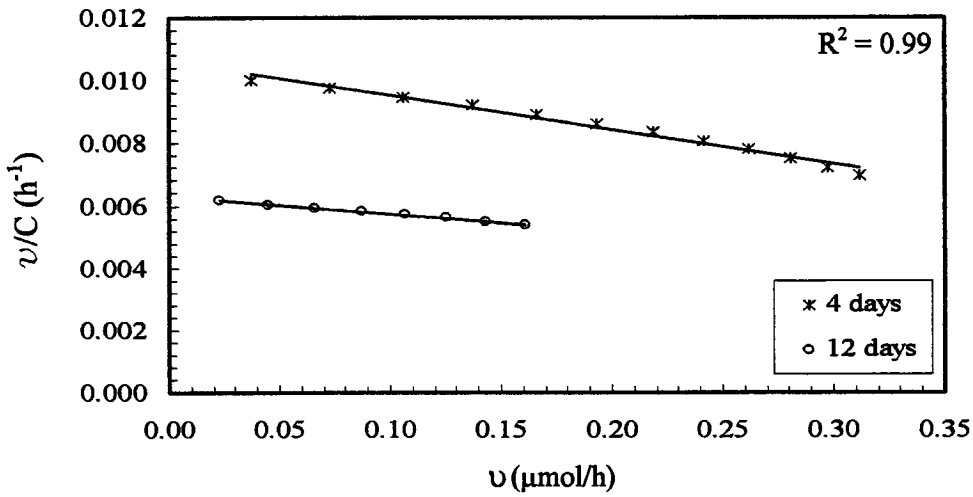
In this study, a linear relationship between C/υ and C was obtained and the maximum dehydrogenase activity at infinite TTC concentration (υ_{max}) and the Michaelis-Menten constant (K_m) were determined. Accordingly, the Michaelis-Menten kinetics for the young (4 days) and old (12 days) groups of cells can be written as follows:

$$\upsilon_4 = \frac{0.95C}{88.55 + C} \tag{6.22}$$

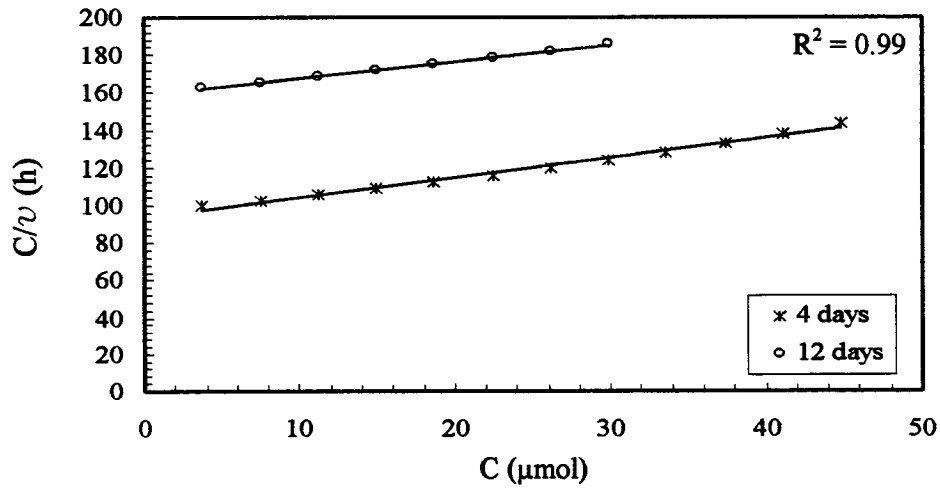
$$\upsilon_{12} = \frac{1.12C}{177.41 + C} \tag{6.23}$$



(a) Lineweaver-Burk Plot



(b) Eadie-Hofstee Plot



(c) Langmuir Plot

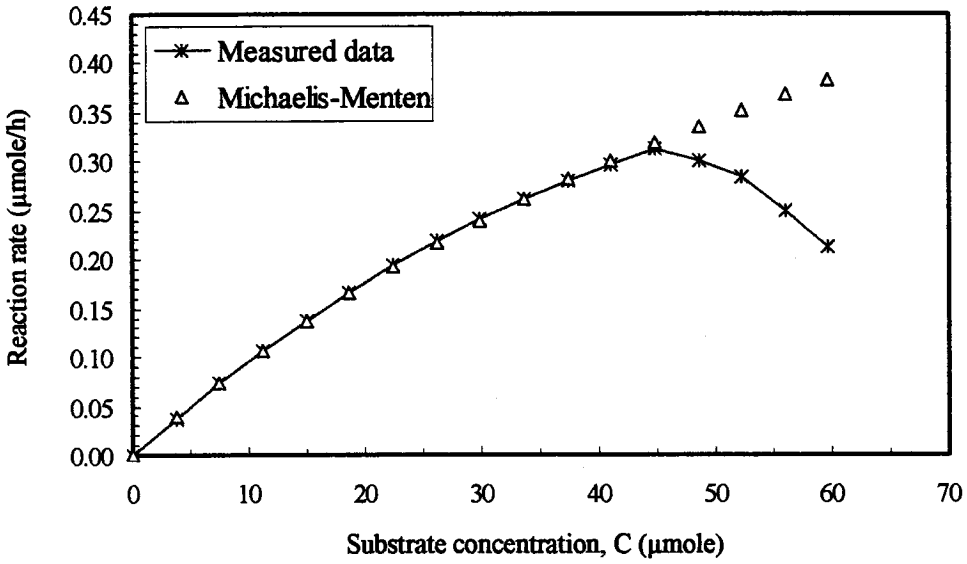
Figure 6.20. Graphical determination of K_m and υ_{max}.

Table 6.12. Enzyme kinetic constants K_m and υ_{max}.

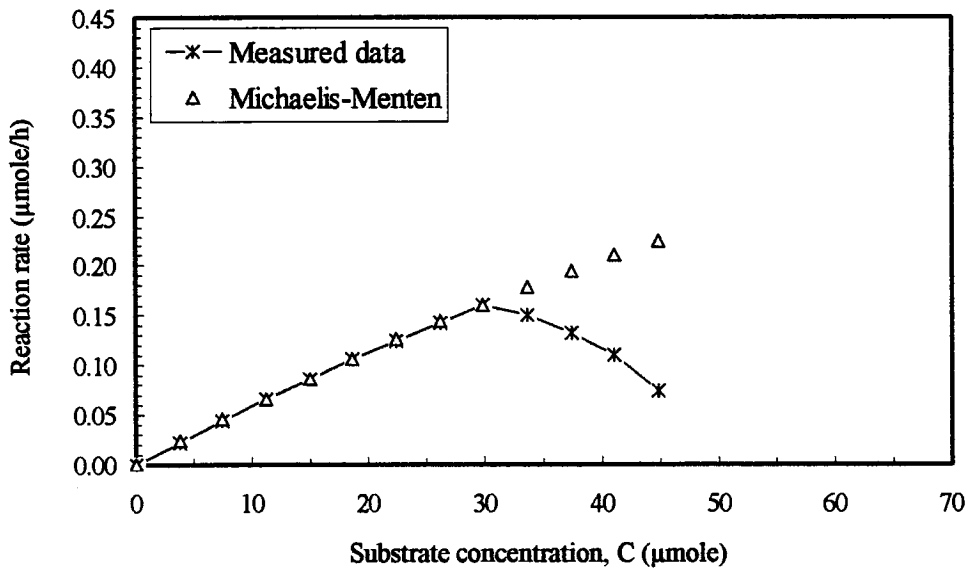
Plot	K_m (µmol)		υ_{max} (µmol/h)	
	4 days	12 days	4 days	12 days
Lineweaver-Burk Plot	103.14	187.84	1.07	1.18
Eadie-Hofstee Plot	90.91	178.57	0.96	1.13
Langmuir Plot	88.55	177.41	0.95	1.12

In another study by Ross (1971), a nonlinear relation between the reciprocals of dehydrogenase activities and of TTC concentrations in a Lineweaver-Burk plot was obtained. The author indicated that the determination of maximum dehydrogenase activities at infinite TTC concentration would be impossible. Furthermore, the above equations do not account for the substrate inhibition as shown in Figure 6.21. In this study, substrate inhibition concentration (C_{in}) was 44.8 and 29.9 µmol for young (4 days) and old cells (12 days), respectively. Shuler and Kargi (1992) and Blanch and Clark (1997) reported that some compounds (known as enzyme inhibitors) may bind to the enzymes causing reduction in their activity. In some cases, substrate may act as an inhibitor as substrate may bind to an alternative site to the active site of the enzyme and the resulting enzyme-substrate complex may be unreactive. The non productive binding is known as substrate inhibition and has been described by several models (Table 6.14). At low substrate concentrations the change in the reaction rate follows Michaelis-Menten kinetics (i.e. υ_{max} increases with increases in substrate concentration); while at high substrate concentrations the reaction rate becomes inversely dependent on substrate concentration as shown in Figure 6.22.

The values of kinetic constants (υ_{max} and K_m) obtained from the Langmuir plot and the inhibition concentration (K_i) obtained experimentally for the two cultures were used to test the validity of the models presented in Table 6.13. The υ_{max}, K_m and K_i values for the 4 days old culture were 0.95 µmol/h, 88.55 µmol and 44.8 µmol and for the 12 days old culture were 1.12 µmol/h, 177.41 µmol and 29.9 µmol, respectively. Figure 6.23



(a) 4 days old culture



(b) 12 days old culture

Figure 6.21. Measured and predicted data using Michaelis-Menten model.

shows comparison between the measured and the predicted data using the models. The model (No. 2) by Luong (1985) and Luong (1987) deviated significantly from the measured data and hence was excluded from the graph. However, none of the other models could describe the experimental data obtained from the current study. Therefore, in order to better account for the substrate inhibition, the following model was developed:

$$\upsilon = \frac{\upsilon_{max} C}{K_m + C} \qquad (C \leq C_{in})$$

$$\upsilon = \frac{\upsilon_{max} C}{K_m + C} - K_{in}(C - C_{in}) \qquad (C > C_{in}) \qquad (6.24)$$

Where:

C_{in} is the substrate concentration above which the reaction rate start to decrease (μmol)

K_{in} is the inhibition constant (h^{-1})

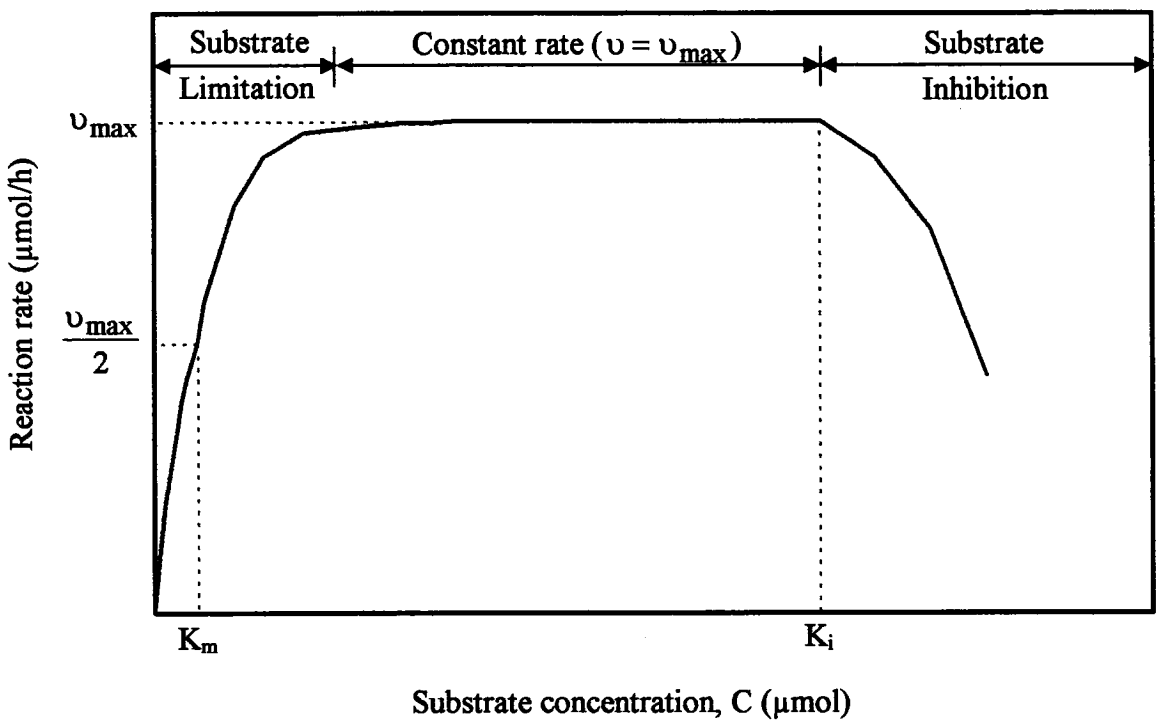
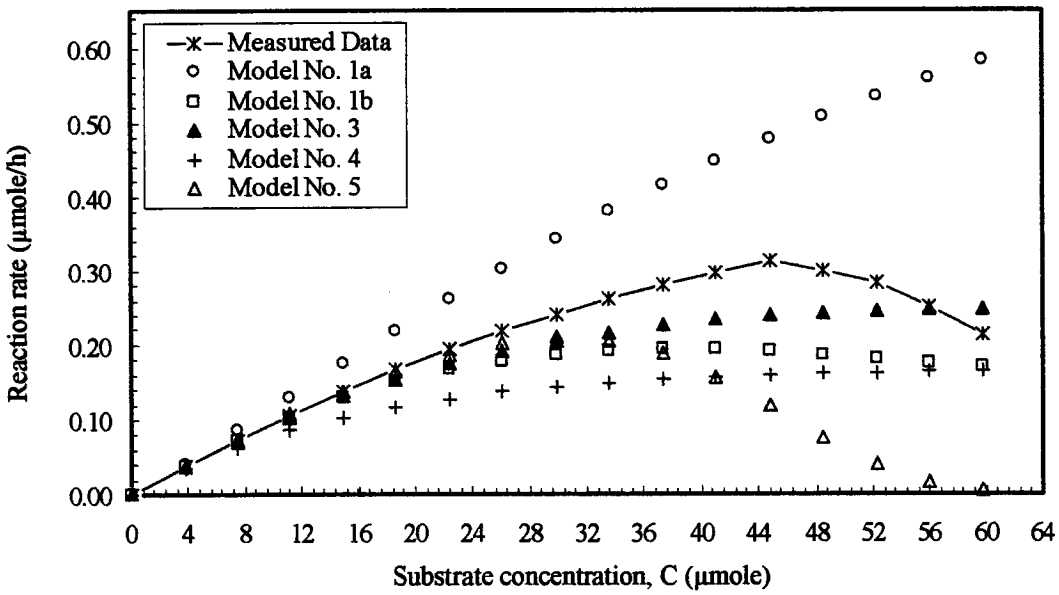


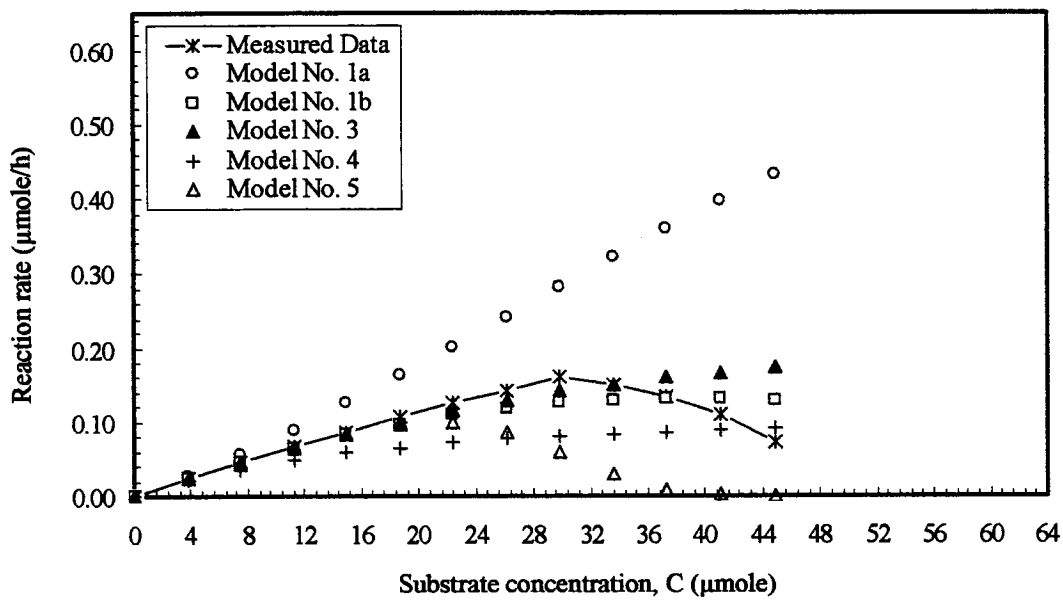
Figure 6.22. Effect of substrate concentration on reaction rate.

Table 6.13. Substrate inhibition models.

Model No.	Model		Reference
1a	$\upsilon = \frac{\upsilon_{max} \cdot C\left[1+\frac{C}{K_i}\right]}{K_m + C + \frac{C^2}{K_i}}$		Edwards (1970)
1b	$\upsilon = \frac{\upsilon_{max} \cdot C}{K_m + C + \frac{C^2}{K_i}\left[1+\frac{C}{K_i}\right]}$		
2	$\upsilon = \frac{\upsilon_{max} \cdot C}{K_m + C} - \left[1-\left(\frac{C}{K_i}\right)^n\right]$		Luong (1985) and Luong (1987)
3	$\upsilon = \frac{\upsilon_{max} C}{C \cdot \left(1+\frac{C}{K_i}\right) + K_m}$		Shuler and Kargi (1992) and Blanch and Clark (1997)
4	$\upsilon = \left[\frac{\upsilon_{max} \cdot C}{K_m + C}\right]\left[\frac{K_i}{K_i + C}\right]$		Ghaly and Al-Taweel (1994) and Ghaly and Ben-Hassan (1995)
5	$\upsilon = \frac{\upsilon_{max} \cdot C}{K_m + C} \cdot e^{-\left(\frac{C}{K_i}\right)^n}$		Tango and Ghaly (1999)
6a	$\upsilon = \frac{\upsilon_{max} \cdot C}{K_m + C}$	$(C \le C_{in})$	Current study
6b	$\upsilon = \frac{\upsilon_{max} \cdot C}{K_m + C} - K_{in}(C - C_{in})$	$(C > C_{in})$	



(a) 4 days old culture



(b) 12 days old culture

Figure 6.23. Measured and predicted data using various models.

The values of the substrate concentration above which the reaction rate start to decline (C_{in}) were obtained experimentally. In order to determine the inhibition constant (K_{in}), the values of ($\upsilon_{MM} - \upsilon_M$), which is calculated by subtracting the values of the measured reaction rate from the values of the calculated reaction rate obtained from Michaelis-Menten kinetics Equation, were plotted versus ($C-C_{in}$) values. A straight line with slope of K_{in} and intercept of zero was obtained (Figure 6.24). Therefore, the relationship between reaction rate and TTC concentration in this study for the young (4 days) and old (12 days) groups of cells can be described by the following equations (Figure 6.25) with coefficient of determination of 0.99 and 0.98, respectively.

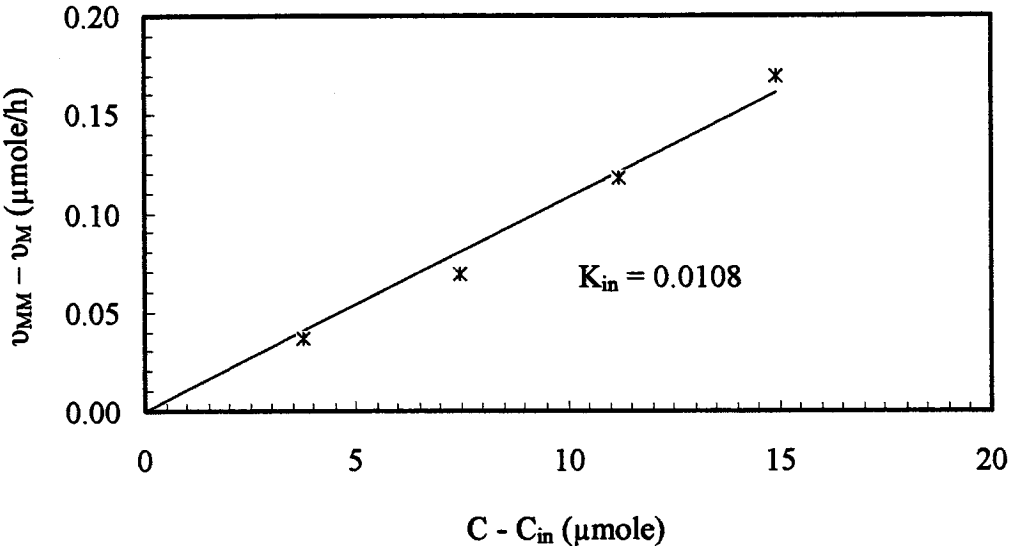
$$\upsilon_4 = \frac{0.95C}{88.55+C} \qquad (C \le C_{in})$$

$$\upsilon_4 = \frac{0.95C}{88.55+C} - 0.0108(C - C_{in}) \qquad (C > C_{in}) \qquad (6.25)$$

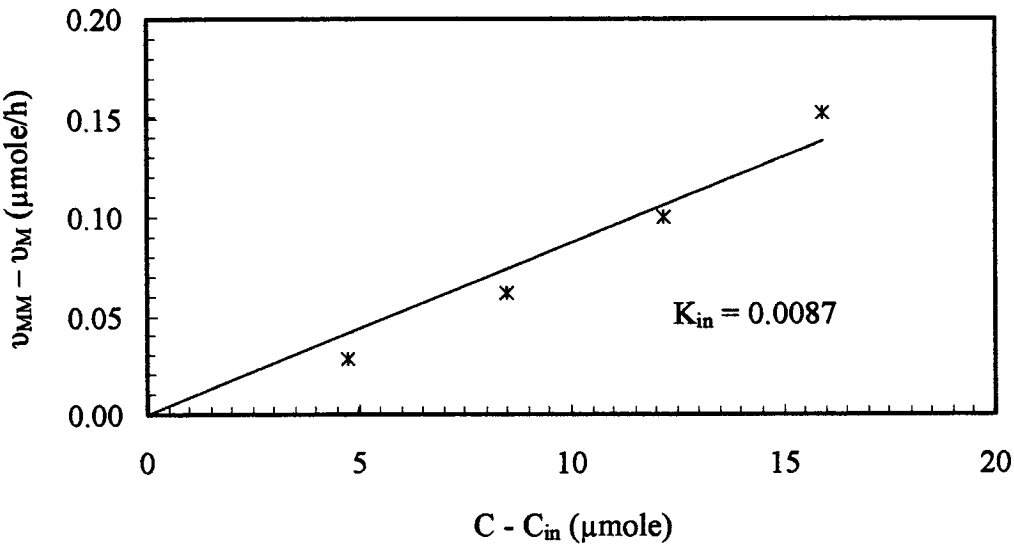
$$\upsilon_{12} = \frac{1.12C}{177.41+C} \qquad (C \le C_{in})$$

$$\upsilon_{12} = \frac{1.12C}{177.41+C} - 0.0087(C - C_{in}) \qquad (C > C_{in}) \qquad (6.26)$$

6.8.2.3 <u>Reaction Rate</u>. The results obtained from the previous experiments indicated that increasing the incubation time from 1.5 h to 4.5 h (90 – 270 minutes) increased the TF yield. In this study, the effect of incubation time (from 15 to 360 minutes) on the TF yield was investigated in order to calculate the reaction rate under both aerobic and anaerobic conditions. The TF yield increased significantly with the increase in incubation time up to 3 h. However, a further increase in the incubation time beyond 3 h provided substantively small amount of additional TF; increasing the incubation period by another 3 h (from 3 to 6 h) resulted in an additional TF of only 8.89 and 8.92 % for aerobic and anaerobic conditions, respectively. The relationship between TF yield and incubation time in the range of 0 to 3 h under aerobic and anaerobic conditions (Figure 6.26) can, therefore, be described by the following polynomial expressions ($R^2 = 0.99$):

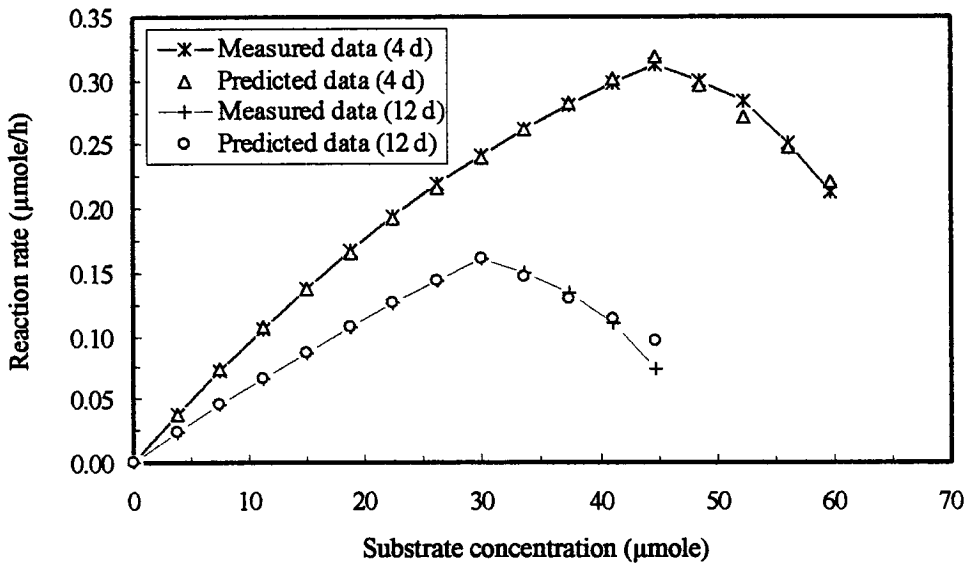


(a) 4 days old culture

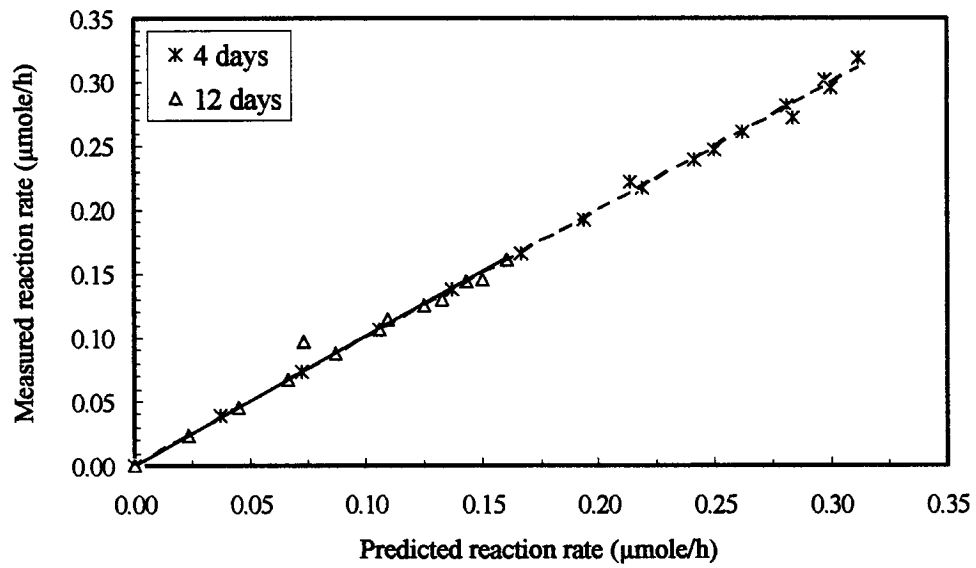


(b) 12 days old culture

Figure 6.24. Graphical determination of the inhibition constant (K_{in}).



(a) Substrate concentration



(b) Reaction rate

Figure 6.25. Proposed Model.

$$TF_a = 0.00691\ t_I - 0.000016\ t_I^2 \qquad (t_I \leq 3.0\ h) \qquad (6.27)$$

$$TF_{an} = 0.0106\ t_I - 0.000033\ t_I^2 \qquad (t_I \leq 3.0\ h) \qquad (6.28)$$

Where:

TF_a is the triphenyl formazan yield under aerobic conditions (μmol)

TF_{an} is the triphenyl formazan yield under anaerobic conditions (μmol)

t_I is the incubation time (min.)

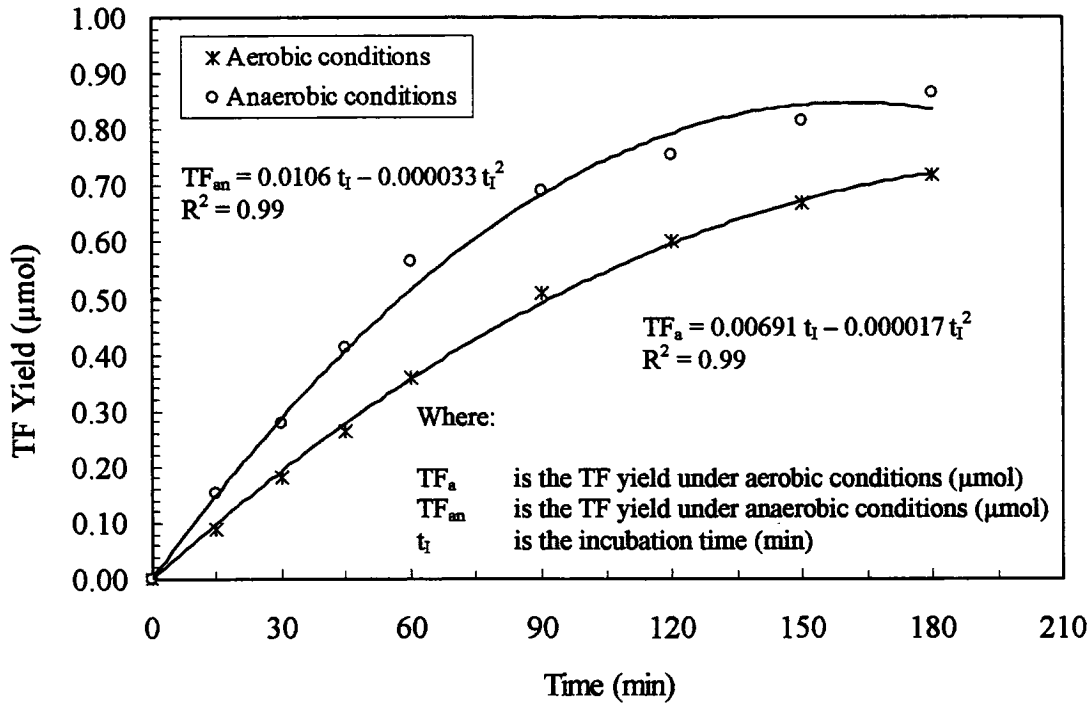


Figure 6.26. Correlation between TF yield and incubation time during the first three hours of incubation under optimum conditions of TTC concentration (20 g/L), pH (9) and temperature (55 °C).

The rate of TF production was estimated from the raw data of TF yield versus time presented in Figure 6.7. The reaction rate first increased with time during the first 45 minutes and then gradually decreased (from 45 to 360 minutes) as shown in Figure 6.27. The reason for the initial increase in the reaction rate might be due to the time required to

warm up the samples to the optimum incubation temperature (55 °C). While measuring the metabolic activity of soil using TTC as an electron acceptor, Casida (1977) reported that formazan formation did not remain linear during 24 h incubation period and the rate of formation started to decrease at about 9 to 12 h incubation time. Ross (1971) reported that the rate of formazan formation under anaerobic conditions and in the presence of buffer system was greatest after an incubation period of 1 h and then decreased gradually with further increase in incubation period. Griebe et al. (1997) reported a decrease in the rate of formazan formation after 2 h incubation time when CTC was incubated with activated sludge.

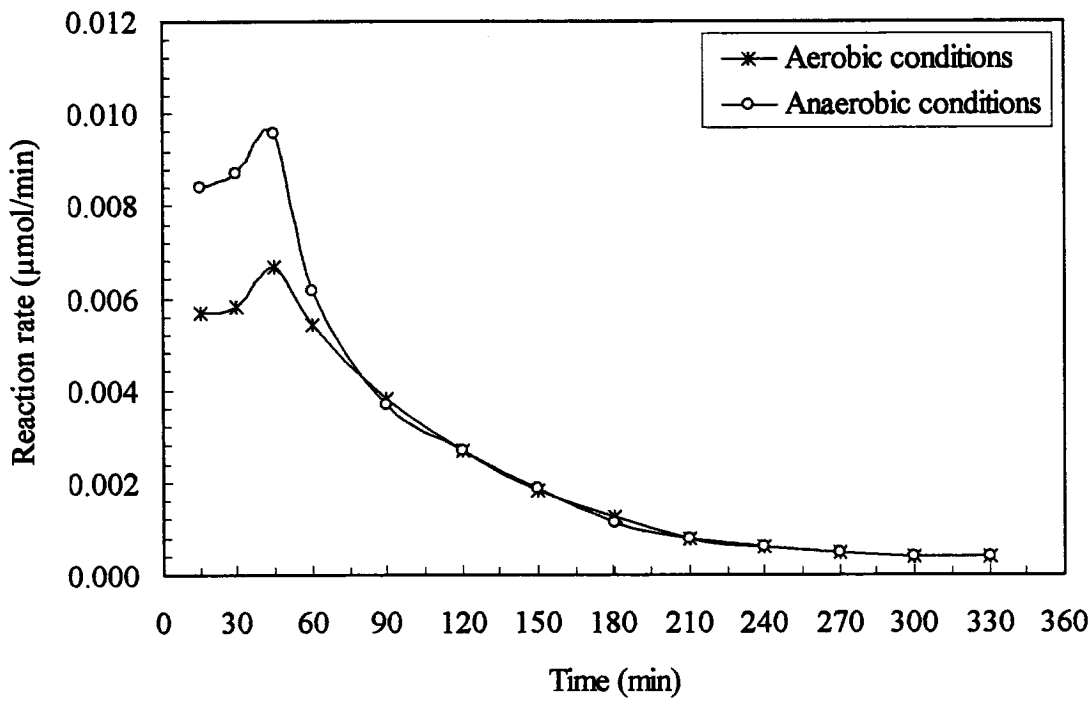


Figure 6.27. TTC reaction rate during the dehydrogenase activity measurement of *A. niger* under optimum conditions of TTC concentration (20 g/L), pH (9) and temperature (55 °C).

Generally, TF yield under anaerobic conditions was higher than that under aerobic conditions. Tetrazolium salts act as electrons acceptors from the oxidized substrate or coenzyme. In the absence of tetrazolium salts, these electrons would normally pass via

series of carriers to a physiological acceptor, which in most cases is oxygen. Ross (1971) stated that water-saturated samples to some extent create anaerobic conditions. However, Ross (1971) and Trevors (1984b) reported that TTC-test gives significantly higher formazan formation in systems incubated anaerobically than in systems incubated without complete anaerobiosis. Altman (1976) proposed that one electron reduction of TTC result in TTC free radical, which under anaerobic conditions is reduced further by the addition of another electron to triphenyl formazan. Under aerobic conditions the TTC radical reduces molecular oxygen to the superoxide radical $O^{\cdot}_2$ and itself becomes oxidised back to TTC. In this study about 18% of the TF produced under aerobic conditions was converted back to TTC.

Figure 6.28 shows the net TF conversion to TTC due to the presence of oxygen. The net TF conversion was defined as the difference between TF yield under anaerobic conditions and TF yield under aerobic conditions. During the incubation period in the presence of oxygen, two processes are taking place: (a) TF production as a result of dehydrogenase activity and (b) TF conversion to TTC as a result of the presence of O_2. The results showed that the net TF conversion increased with the increase in incubation time up to 1 h and then stared to decrease until it reached almost a constant value after 3 h incubation time.

The increase in the net TF conversion during the first 60 min might be due to the effect of temperature on TF oxidation reaction since the samples during this period were warming up causing an increase in the reaction rate. The net TF conversion decreased from 60 min to 120 min incubation time as a result of oxygen depletion. TF production increased with time during this period (60-120 min) while the TF oxidation rate decreased as a result of oxygen depletion and thus the net TF conversion declined. Beyond 180 min incubation time no significant increase in the TF production was realized resulting in stabilization of the net TF conversion.

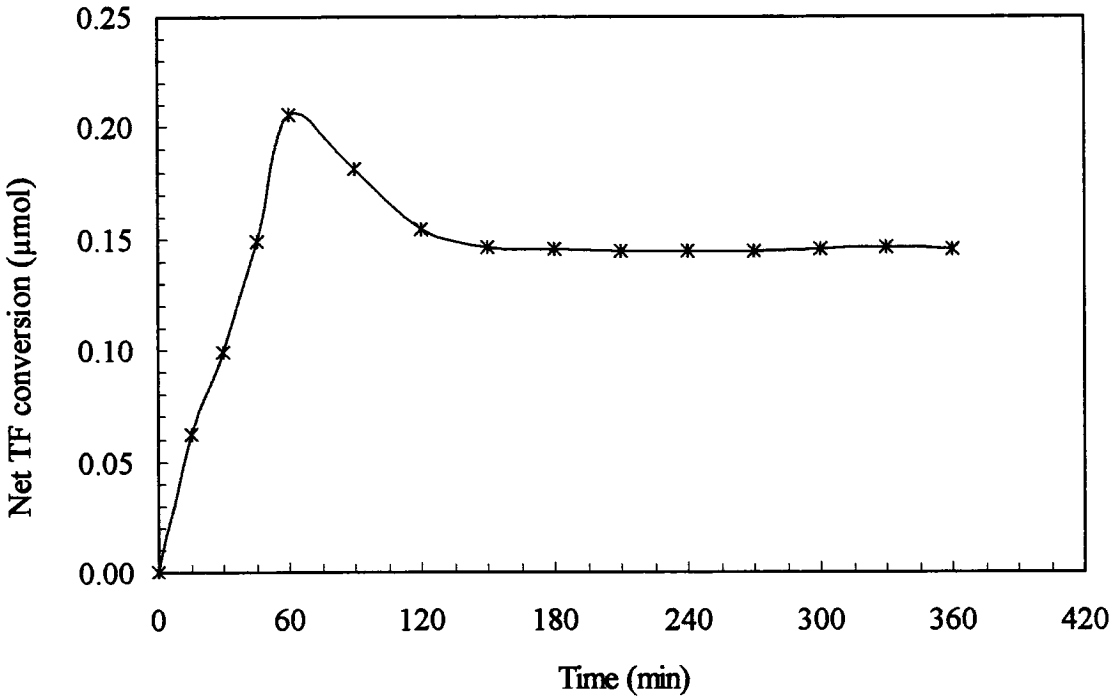


Figure 6.28. Net TF conversion with time for *A. niger* under optimum conditions of TTC concentration (20 g/L), pH (9) and temperature (55 °C).

5.8.2.4 *A. niger* Standard Curve in the Presence and Absence of O_2. The relationship between the dehydrogenase activity of *A. niger* cells as measured by the TF yield under optimum test conditions (a pH of 9, a temperature of 55 °C, a TTC concentration of 20 g/L and an incubation period of 3 h) and cell weight for both aerobic and anaerobic conditions was linear (Figure 6.8) and can be described by the following equations (R^2 = 0.99).

$$TF_a = 3.29\ W_c \quad (6.29)$$

$$TF_{an} = 4.01\ W_c \quad (6.30)$$

Where:

W_c is *A. niger* dry cell weight (g)

Tengerdy et al. (1967) reported that the reducing activity of actively growing bacteria is proportional with the number of cells assuming that all bacteria are in a similar metabolic state. The authors obtained a significant correlation between the reducing activity (represented by color intensity of reduced tetrazolium salt) of nutrient broth culture of *Escherichia coli* and *Staphylococcus aureus* and their viable plate count. Ghaly and Ben-Hassan (1993) obtained a linear relationship between triphenyl formazan yield (μmol) and concentration of active cells (number of cells/ mL) for two microorganisms: *Kluyveromyces fragilis* and *Candida pseudotropicalis*. In this study, TF yield under anaerobic conditions (4.01 μmol TF/ g cells or 1.2 mg TF/ g cells) was higher than that under aerobic conditions (3.29 μmol TF/ g cells or 0.96 mg TF/ g cells).

6.8.3 Measuring Dehydrogenase Activity of *A. niger* in the Presence of Shrimp Shells Using TTC

6.8.3.1 Effect of Filtration. During measuring dehydrogenase activity of *A. niger* in the presence of shrimp shells using TTC-test under the optimum test conditions (TTC concentration of 20 g/L, pH of 9, temperature of 55 °C, incubation time of 3 h and the absence of O_2), it was realized that the supernatant (the extracted triphenyl formazan solution) had dirty red color and high turbidity. The lack of purity of the red color interfered with the proper absorbance readings and consequently the TF yield determination. It was assumed that this observation might be due to the small solid particles present in the shrimp shell waste, which were difficult to separate by centrifugation, and/or fat and pigments in the shrimp shell waste, which are known to dissolve in the solvent (ethyl alcohol) used for extraction.

Lenhard et al. (1964), Casida (1977) and Chander and Brookes (1991) filtered their formazan solution before absorbance measurements. Therefore, the supernatant was filtered and the absorbance was measured after filtration. Filtration of the supernatant through a membrane micro-filter (0.45 μm pore size) enhanced the properties of the supernatant and consequently the absorbance readings by 37.9 – 84.2%. However, two main problems still existed: (a) the amount of alcohol used for extraction (2 washes of 2.5 mL each) was not enough since the shrimp shells solid matrix absorbed most of the added

alcohol and (b) the color extracted in the presence of shrimp shells was much higher than that extracted in the absence of shrimp shells indicating that the shrimp shells might have contributed to the reduction of the TTC.

6.8.3.2 Effect of Amount of Alcohol per Wash. The results obtained from this set of experiments showed that increasing the volume of alcohol per wash from 2.5 mL to 5 mL increased the TF yield by a factor of 4.87 - 6.56 as shown in Figure 6.11. Further increase in the number of washes and/or the alcohol volume per wash beyond two washes and/or 5 mL per wash, respectively, did not have any significant effect on the TF yield. Therefore, 2 washes with 5 mL instead of 2.5 mL alcohol per wash were recommended when dealing with solid samples such as shrimp shells. Several studies used different TF extraction techniques. Klein et al. (1971) used single wash with 10 mL methanol for extraction of TF from soil samples. Lenhard et al. (1964) extracted the formed formazan from activated sludge samples using a single wash of ethyl alcohol and bringing the extract to a final volume of 50 mL. Ghaly and Ben-Hassan (1993) extracted TF from cheese whey samples using two washes of 10 mL ethyl alcohol each and made up the extracted solution to 50 mL. Chander and Brookes (1991) extracted TF from soil samples using 7-8 washes of 10 mL each and diluted the extracted formazan solution to 100mL. Casida (1977) extracted TF from soil samples by placing the sample in a funnel containing filter paper and then washing with methanol until 50 mL of methanol was collected.

6.8.3.3 Contribution of Shrimp Shells to TTC Reduction. The results obtained from this set of experiments showed that the amount of formazan extracted from 0.1 g *A. niger* cells mixed with 1 g shells (0.828 µmol) was higher than double the amount of formazan extracted from 0.1 g *A. niger* cells (0.289 µmol), thus giving TF contribution of 0.539 µmol by the shrimp shells. However, the TF yield for 1 g shrimp shells alone was 0.617 µmol. The range of variation in the degree of extracted color between the replicates of the samples containing shrimp shells was high compared to the variation in color between the replicates of the samples containing cell mass only. The high variation in the particle size of the shrimp shells may have caused variations in the shells surface contact area

with the alcohol and as a result contributed to the high variation in the degree of color extracted from the shells. The contribution of the shrimp shells to the formazan yield could be the result of: (a) the dehydrogenase activity of intrinsic microbial population in the shrimp shells and/or (b) chemical reactions between the TTC and some of the shrimp shells constituents.

6.8.3.4 Effect of Autoclaving. The plate count of autoclaved *A. niger* cells and shrimp shells proved that the autoclaving technique was effective in killing the intrinsic microbial population in the shrimp shells as well as the *A. niger* vegetative cells since no colonies were realized on the plates after incubation for 3 days at room temperature (~24 °C). However, the TF results of this set of experiment showed that autoclaving did not completely suppress the dehydrogenase activity. The residual dehydrogenase activities after autoclaving of 0.1 g cells, 1 g shrimp shells and 0.1 g cells mixed with 1 g shrimp shells were 10.84, 42.74 and 37.78% of the dehydrogenase activities of unautoclaved samples, respectively.

Several studies reported on the formation of colored formazans with control spore samples, which were killed either by autoclaving or alcohol treatment (Nelson and Olsen, 1967; An and Hendrix, 1988; Miller et al., 1993; and Meier and Charvat, 1993). Altman (1974) stated that based on the chemistry of the formation of colored formazans, the existence of any color indicates a positive reaction. Nelson and Olsen (1967) reported the existence of a contaminant organism, *Aspergillus fumigatus*, which produced an extracellular nonenzymatic agent that reduced TTC in their control spores tested at 40 °C. Ziętara and Skorkowski (1995) reported that when cells are exposed to temperatures few degrees higher than their growth temperature, heat shock proteins, which are responsible for immediate stress protection or conduct cellular repair processes, start to be synthesized. The authors stated that the presence of heat shock protein DnaK protects the activity of lactate dehydrogenase LDH-A_4 isoenzyme against mild thermal inactivation. Acosta-Martínez and Tabatabai (2000) reported that enzyme activity of field-moist soils heated to 110 to 120 °C was completely destroyed while that of air-dried soils contained residual activity after heating at 120 °C for two hours. The authors mentioned that the

accuracy of detecting residual or low enzyme activity depends on the assay method as well as the conditions under which the assay is conducted.

The TF yield obtained from autoclaved *A. niger* cells indicated that dehydrogenase enzymes were not completely deactivated by autoclaving although the autoclaved cells were incapable of growing. The high TF yield obtained from autoclaved shrimp shells indicated that a chemical reaction between TTC and one or more of the shrimp shells constituents took place. Shrimp shells are composed of chitin, protein, lipids, pigments and minerals. Hansen and Illanes (1994) and Guillou et al. (1995) reported that crustaceans contains carotenoid pigments mainly astaxanthin and astaxanthin ester. Simpson (1978) stated that very low levels of pH or alkaline conditions would rapidly destroy astaxanthin. Bustos et al. (2003) reported that astaxanthin is highly unsaturated and are greatly susceptible to oxidative degradation. Altman (1976) reported that reduction of TTC might occur in the presence of some amino acids such as ascorbic acid, cysteine or glutathione since they are considered effective reductants at pH values above 9.

6.8.3.5 *A. niger* Standard Curve. The standard curve results (Figure 6.14) for *A. niger* in the presence of autoclaved and unautoclaved shrimp shells under the optimum TTC-test conditions (at a pH of 9, a temperature of 55 °C, a TTC concentration of 20 g/L, 3 h incubation period and in the absence of O_2) showed lower inconsistency compared to that for *A. niger* in liquid media under the same conditions. The coefficients of variation among replicates were 4.6-23.6 % and 21.5-40.0 % and the correlation coefficients (R^2) were 0.94 and 0.44 for autoclaved and unautoclaved samples, respectively. Although, autoclaving of shrimp shells decreased the contribution of the shrimp shells to TF yield, it was considered impractical and costly.

Figure 6.29 shows *A. niger* standard curves obtained in liquid media and in the presence of unautoclaved shrimp shells. The dash dotted line shows the standard curve obtained for *A. niger* in liquid media under anaerobic conditions while the dashed line presents the standard curve obtained for *A. niger* in the presence of unautoclaved shrimp shells under

anaerobic conditions. The solid line was constructed from the *A. niger* standard curve in liquid media under anaerobic conditions plus the average contribution of shrimp shells to the TF yield. The standard curve obtained in the presence of unautoclaved shrimp shells deviated from the constructed line of the TF yield data obtained in the absence of shrimp shells plus the average contribution of shrimp shells. In addition, the coefficient of variation for the data used to fit the standard curve with shrimp shells was high (21.5-40.0 %) and the correlation coefficient was low (0.44). The standard curve developed in the presence of shrimp shells can be represented by the following linear expression:

$$TF = 0.8873 + 2.4256\, W_{cw} \tag{31}$$

Where:

W_{cw} is *A. niger* wet cell weight (g)

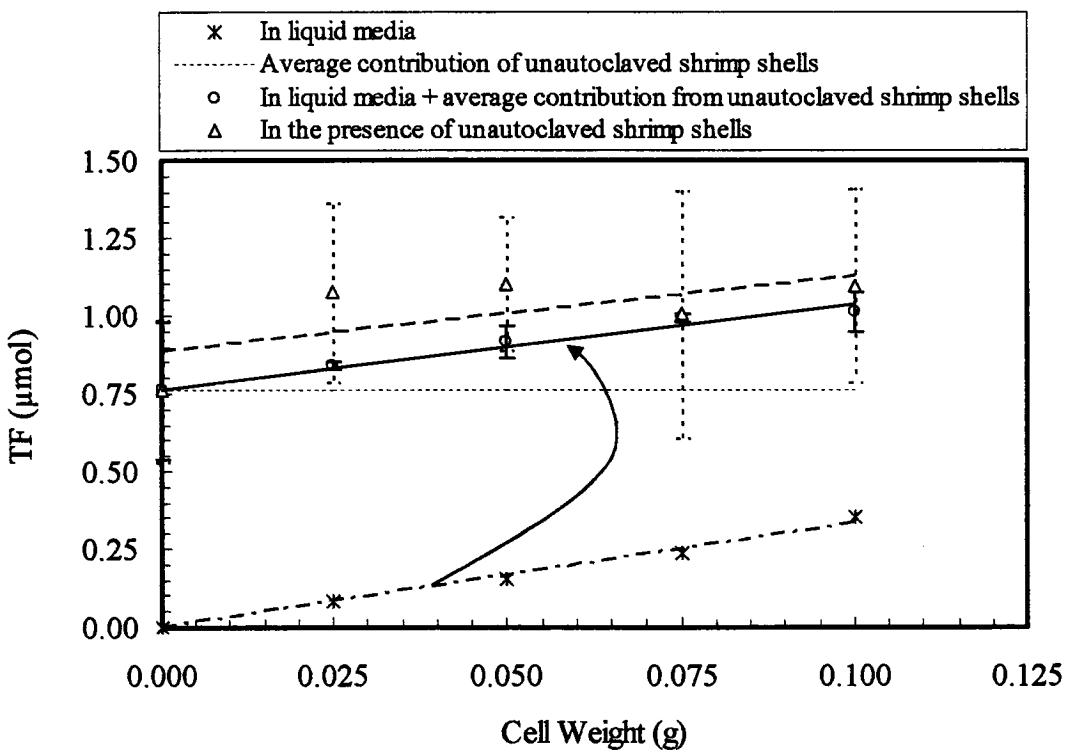


Figure 6.29. *A. niger* standard curve in the presence of shrimp shells under optimum conditions of TTC concentration (20 g/L), pH (9), temperature (55 °C), incubation time (3 h) and absence of oxygen.

The developed TTC-test can be used for *A. niger* quantification during solid-state fermentation of shrimp shells for chitin purification, however, several factors should be taken into consideration in order to enhance the accuracy of the test and the inconsistency of the results: (a) some of the shrimp shells constituents react chemically with the TTC causing TTC reduction and TF formation, (b) there is inconsistency in the shrimp shell waste composition between different batches, (c) during solid-state fermentation of shrimp shells the characteristics of the solid matrix as well as the constituents of the shells differ over time and consequently the chemical reaction of shrimp shells with the TTC also changes over time, (d) shrimp shells contain some intrinsic microorganisms that might contribute to TTC reduction and TF formation, (d) autoclaving of shrimp shells before fermentation is costly and impractical and (e) residual dehydrogenase activity was realized even after autoclaving.

Therefore, the average contribution of the shrimp shells to TTC reduction and TF formation should be taken into account while generating the standard curve for *A. niger* quantification during solid-state fermentation of shrimp shells using TTC. A standard curve should be constructed each time from the shrimp shells batch to be used in the fermentation in order to overcome the inconsistency in the shrimp shells composition. In this study, the control, which was used to zero the spectrophotometer, was prepared by incubating sample with sterilized distilled water instead of TTC/glucose solution. However, it is recommended to prepare controls by autoclaving samples and then incubating them with TTC/glucose solution since it was found that residual dehydrogenase activity remained even after autoclaving.

6.9 Conclusions

The following conclusions were made from this study:

1. The colorless TTC was sensitive to both pH and temperature and was reduced chemically (non-enzymatically) to the red dye TF at high pH (> 9.5) and/or high temperatures (> 70 °C).

2. TTC chemical reduction was more sensitive to changes in pH than changes in temperature.
3. The TF yield was affected by the sample composition and moisture content.
   (a) Applying the TTC-test to samples that contain heavy metals, in particular copper, may give misleading readings due to the formation of formazan copper complex, which reduces the red color intensity.
   (b) Samples with low moisture content gives TF yield lower than samples with high moisture content.
4. TF yield from *A. niger* vegetative cells was affected by TTC concentration, pH, temperature, incubation time, presence of oxygen and cell age.
   (a) TF yield increased significantly with the increase in pH (from 7 to 9), temperature (from 25 to 55 °C) and incubation time (from 1.5 to 3 h).
   (b) The biochemical reduction of TTC by dehydrogenase enzymes of *A. niger* was more sensitive to changes in temperature followed by pH, TTC concentration and incubation time.
   (c) Part of the TF produced in the presence of oxygen (aerobic conditions) was converted back to TTC. Therefore, conducting the TTC-test in the absence of O_2 (anaerobic conditions) increased the TF yield.
   (d) The tolerance level of TTC was affected by the cell age; the older the cells the lower the tolerance level of TTC.
5. TTC-test can effectively be used for dehydrogenase activity measurements of *A. niger* vegetative cells in liquid media. The optimum test conditions are a TTC concentration of 20 g/L, a pH of 9, a temperature of 55 °C, and incubation time of 3 h and anaerobic conditions. However, cell age should be taken into consideration.
6. Filtration and the amount of alcohol per wash are important parameters when performing the TTC-test on solid samples.

(a) Small solid particles affected the purity of the extracted red color and interfered with the proper absorbance readings and consequently the TF yield determination. Filtration of the supernatant enhanced the absorbance readings by 37.9 – 84.2%

(b) Increasing the volume of alcohol per wash from 2.5 mL to 5 mL increased the TF yield by a factor of 4.87 - 6.56. 5 mL alcohol per wash was recommended when dealing with shrimp shell samples.

7. TTC-test can be used for *A. niger* quantification during solid-state fermentation of shrimp shells if the following factors are taken into consideration:

(a) Some of the shrimp shell constituents react chemically with the TTC causing TTC reduction and TF formation.

(b) There is inconsistency in the shrimp shell waste composition between different batches.

(c) Shrimp shell characteristics as well as composition change over time during the fermentation process and so do the chemical reactions between the shells and the TTC.

(d) Shrimp shells contain some intrinsic microorganisms that might contribute to TTC reduction and TF formation.

(e) Residual dehydrogenase activity was realized even after autoclaving.

8. In order to develop an accurate standard curve for *A. niger* quantification during solid-state fermentation of shrimp shells:

(a) The average contribution of the shrimp shells to TTC reduction and TF formation should be taken into account.

(b) A standard curve should be constructed each time from the shrimp shells batch to be used

(c) Controls should be prepared by autoclaving samples and incubating them with TTC/glucose solution.

6.10 Recommendations

1. Measuring the dehydrogenase activity using tetrazolium salts is simple, fast, inexpensive and can be applied for biomass quantification in liquid media. However, test should be carried out in the absence of O_2 and the test conditions should be optimised for each microorganism/substrate system. No single optimum condition could be applied for all cases. The most important parameters are tetrazolium salt concentration, pH, temperature and incubation time.
2. It was quite obvious in this study that shrimp shells contributed to the TTC reduction. Therefore, further studies need to be conducted in order to determine which constituent(s) in the shrimp shells is (are) responsible for the addition of color. The reaction of shrimp shell constituents such as amino acids and pigments with the TTC should be studied separately in order to define which constituent is responsible for the TTC chemical reduction.
3. Many tetrazolium salts are available and could be used for dehydrogenase activity measurements. Although TTC is one of the tetrazolium salts most commonly used for this purpose and despite the low molecular weight and the cheap price of the salt, the sensitivity of the TTC to O_2 might affect the popularity of its usage. Further studies of the suitability of more tetrazolium salts are recommended. TV and MTT are worth studying because of their low molecular weights, which is a very important characteristic that affects the penetration ability of the salts.

7. CHITIN PURIFICATION FROM SHRIMP SHELLS

7.1 Introduction

Each year, the seafood processing industry generates large amounts of solid wastes, which have become a major environmental concern to the main producing countries due to the high perishability and the bulky nature of the waste material (Hall and Silva, 1992; Hall et al., 1994; and Zakaria et al., 1998). Environmental and economic pressures are also forcing shellfish producers to reduce the waste from the processing plants (Hall and Reid, 1995). Shellfish waste is a rich source of many valuable products including: chitin, protein, pigments (e.g. astaxanthin), and flavor compounds. However, chitin is the most economically valuable component of the waste material (Zakaria et al., 1998). Chitin and its derivatives are versatile environmentally friendly modern materials that have been used in virtually every significant segment of the economy. They have a wide range of properties. Over the years a wide range of applications for chitin and its derivatives have been developed in areas such as water treatment, pulp and paper, biomedical devices and therapies, cosmetics, biotechnology, agriculture, food science, and membrane technology (Dunn et al., 1997). The number, importance and variety of industrial uses of these materials are growing rapidly each year.

Chitin present in the crustacean waste is associated with proteins, inorganic material (mainly calcium carbonate), and lipids including pigments. The traditional method of chitin extraction from crustacean waste involves the use of strong acid (HCl) and alkali (NaOH) for demineralization and deproteinization, respectively (Kong, 1975; Gagné and Simpson, 1993; Bustos and Healy, 1994; Hall et al., 1994; and Zakaria et al., 1998). This method has many disadvantages: (a) it yields an end product with variable and inconsistent physiochemical properties, (b) it results in an effluent wastewater that creates disposal problems, (c) it wastes the other valuable components present in the waste material and (d) the process costs are fairly high. Therefore, a less expensive environmentally friendly method for chitin extraction from crustacean waste is needed (Kim and Lee, 1995; and Sophanodora and Hutadilok, 1995).

Several studies proposed the use of biotechnological approaches such as lactic acid fermentation for demineralization and to some extent deproteinization (Hall and De Silva, 1992; Hall et al., 1994; Hall and Reid, 1995; and Beaney et al., 2005) and proteolytic enzymes for deproteinization either by using proteolytic microorganisms, which secret extracellular proteases (Shimahara et al., 1982; Cano-Lopez et al., 1987; Shimahara et al., 1988; Gagné and Simpson, 1993; Bustos and Healy, 1994a; Bustos and Healy, 1994b; Wang and Chio, 1998; and Teng et al., 2001), or by adding purified proteolytic enzymes (Takeda and Abe, 1962; Takeda and Katsuura, 1964; and Wang and Chio, 1998) for the extraction of the chitin from shrimp shells waste. Deproteinization by purified enzymes is not as effective as living microbes because the presence of microorganisms provides a constant and gradual increase of protease throughout the fermentation period and microorganisms can grow in the interstices of shell waste structure and allow for closer contact between the enzyme producing site and the protein substrate (Michael, 1994; Bustos and Healy, 1994; and Teng et al., 2001). Chitin extraction using microorganisms appears to be a good alternative to the harsh chemical treatment since: (a) it is considered less expensive compared to the chemical method, (b) it results in an effluent that is less harmful to the environment and (c) it preserves the natural state of the biopolymer.

Aspergillus niger is an important microorganism that has been used in different industrial applications including production of organic acids, such as citric and gluconic acids, and extracellular enzymes, such as pectinase, amylase and protease, (Berka et al., 1992; Ray, 1996; and Naidu and Panda, 2003). Pandey (1992) reported that *A. niger* can produce more than 19 types of enzymes including alpha amylase, beta amylase, maltase, lactase, catalase, proteinase, trehalase, tannase, dipetalase, polypetalase, lipase, cellulose, amidase, glucose oxidase, glucose dihydrogenase, urease, insulase, melibase and zymase (trace). *A. niger* is also considered a source of chitin as it contains up to 42.0% chitin of the dry weight of the fungal cell wall; cell wall weight is 20-40% of the dry cell weight (Stagg and Feather, 1973; and Kong, 1975). Therefore, *A. niger* has a great potential for deproteinization of crustacean waste. The proteolytic enzymes released from the fungi can deproteinize the shells and the chitin in the cell wall of the fungi can be considered an additional source of chitin (Teng et al., 2001). Hesseltine (1977) stated that *A. niger*

species are often most suited to solid-state fermentation because their growth conditions are similar to those of the natural habitats. Supplementation of additional carbon source, particle size, heat treatment and pH are important parameters in solid-state fermentation.

Using organic acids such as lactic and/or acetic acids for the demineralization process is a promising idea since organic acids: (a) are less harmful to the environment, (b) can preserve the characteristics of the purified chitin and (c) can be produced from low cost biomass such as cheese whey. In addition, the resulting organic salts from the demineralization process can be used as an environmentally friendly de-icing/anti-icing agent.

7.2 Objectives

The aim of this study was to develop a bio-process for chitin purification from shrimp shells. The proposed bio-process involved two steps: (a) the use of a chitin containing fungus (*Aspergillus niger*), which is known to produce proteolytic enzymes, in order to deproteinize the shrimp shells and (b) the use of a cheese whey fermentation process effluent, in which organic acids (lactic acid and/or acetic acid) were produced, to demineralize the deproteinized shells obtained from the first step. The deproteinization step employed solid-state fermentation to break down the protein in the shrimp shell waste, thus enabling a certain degree of chitin purification while producing protein liquor, which could be used as animal feed. The lactic and/or acetic acids produced from the cheese whey fermentation will dissolve calcium carbonate and other minerals, thus allowing the production of high quality chitin and organic salts, which could be used as an environmentally friendly de-icing/anti-icing agents.

The specific objectives were to:

1. Evaluate the ability of the fungus species (*Aspergillus niger*) to carry out the deproteinization process.

(a) Study the effect of addition of external carbon source (galactose) at different initial concentrations (10, 20 and 30 % w/w) on the performance characteristics of the deproteinization process.

(b) Study the effect of shrimp shell particle size (intact shrimp shell waste versus ground shrimp shell waste) on the performance characteristics of the deproteinization process.

(c) Study the effect of initial shrimp shell pH (7.0 and 8.64) on the performance characteristics of the deproteinization process.

(d) Study the effect of autoclaving of shrimp shells on the performance characteristics of the deproteinization process.

2. Evaluate the effectiveness of organic acid to demineralize deproteinized shells.

(a) Investigate the effects of acid type and demineralization procedure on the performance of the demineralization process.

(b) Study the effect of retention time on the performance of the demineralization process.

(c) Study the effect of shells to acid ratio on the performance of the demineralization process.

7.3 Experimental Apparatus

The experimental setup (Figure 7.1) consisted of a main frame, three drum bioreactors, an aeration system and a data acquisition system. It was used for studying the effects of various parameters on shrimp shell deproteinization using *A. niger*. The small reactor size allowed the use of small amount of shrimp shell waste for each run. Figure 7.2 shows front and back views of the drum bioreactors system along with the aeration system and the humidifier.

7.3.1 Main Frame

The main frame (Figure 7.3) was made of two polyvinyl chloride (PVC) rectangular sheets (13 mm thick) and two irregular hexagon stainless steel sheets (3 mm thick). One of the PVC sheets (560 × 460 mm) was used as a base and the other one (560 × 380 mm)

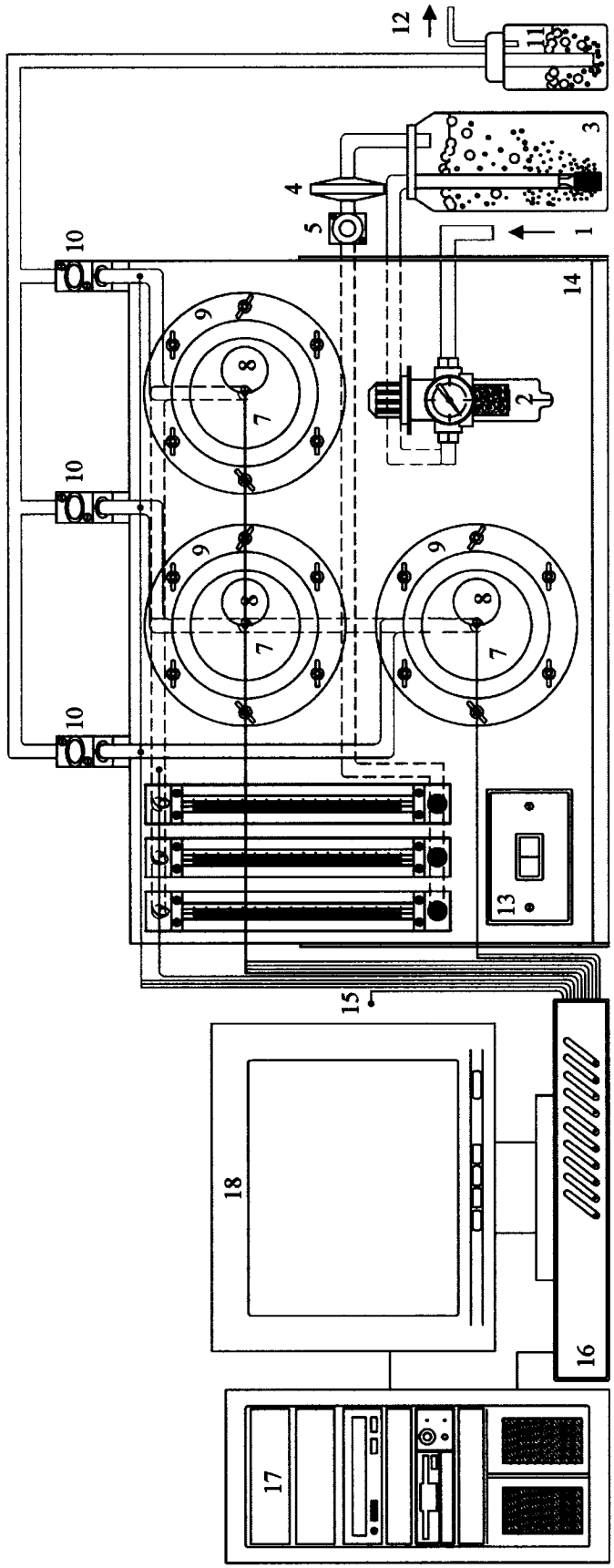
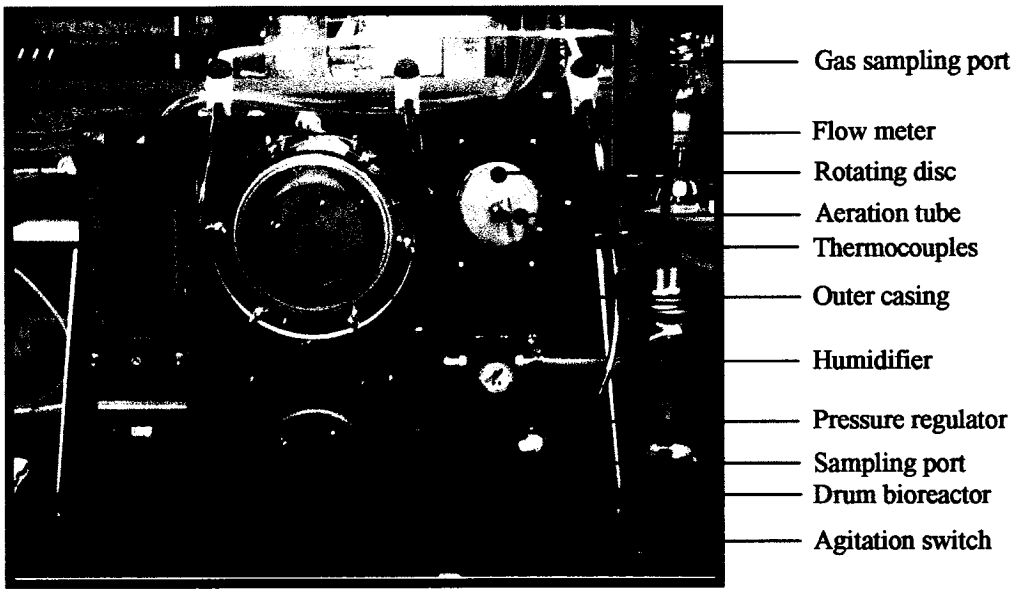
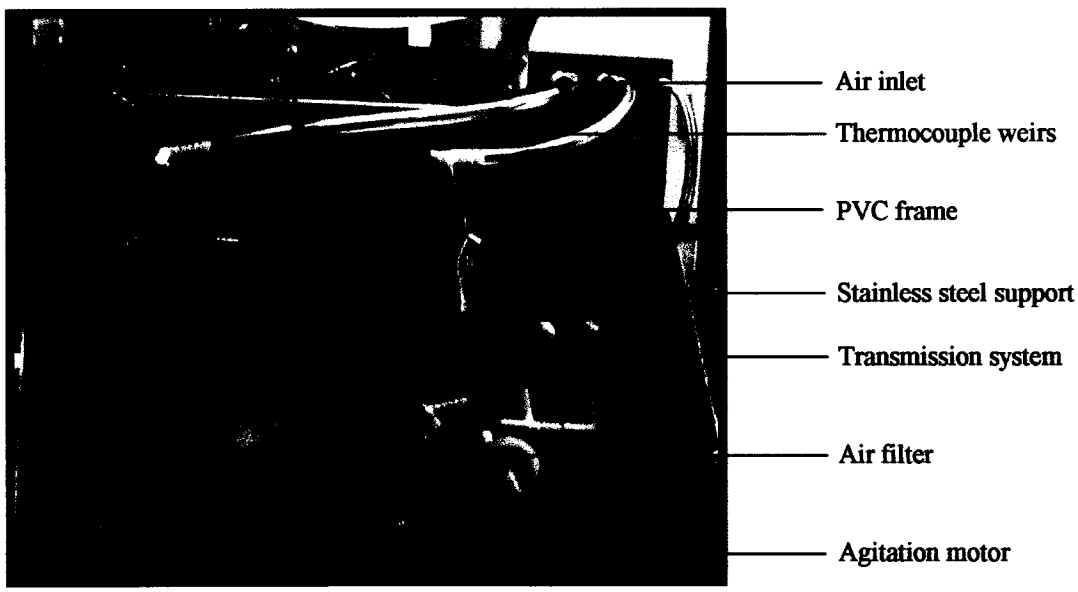


Figure 7.1. Experimental setup.



(a) Front view



(b) Back view

Figure 7.2. Front and back views of the small experimental setup.

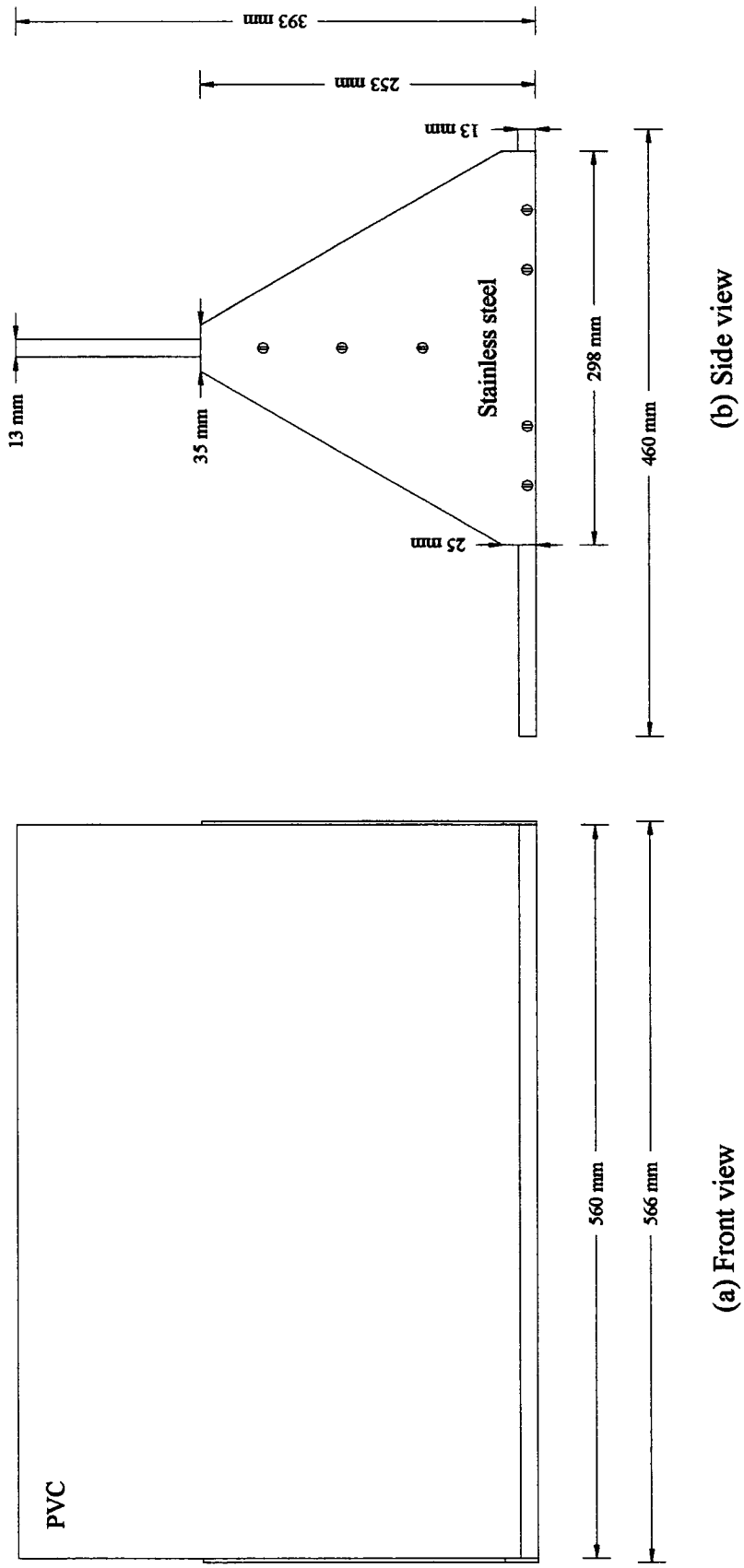


Figure 7.3. The main frame.

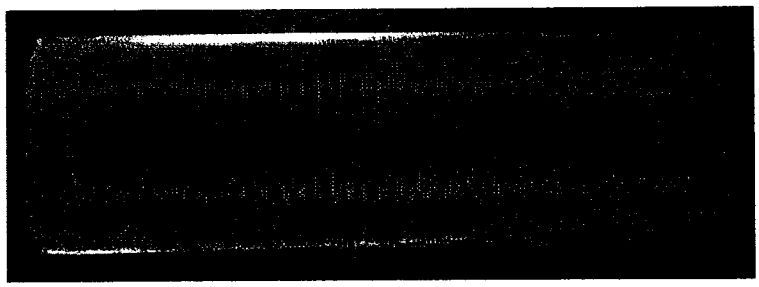
was fixed and glued vertically on the base. The two irregular hexagon stainless steel sheets were used to support the main frame and hold the two PVC sheets together. Each irregular hexagon stainless steel sheet was fixed to the two PVC sheets by means of six stainless steel screws. The main frame held the drum bioreactors, the pressure regulator, the flow meters, the inlet air and exhaust gas manifolds, tubing and sampling ports, the thermocouple wires, the mixing motor along with the transmission system and the switch.

7.3.2 Bioreactor

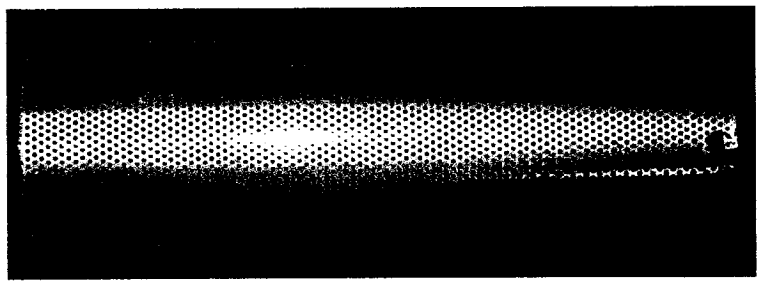
Three 1.8 L drum type bioreactors with a mixing motor and transmission system were used. Each drum bioreactor (Figure 7.4) consisted of a removable inner stainless steel mesh (aperture of 1.5 mm) which was used as a lining for an outer stainless steel horizontally rotating basket of 88 mm diameter and 292 mm length. One stainless steel plate, with a drilled hole for sampling, was used to close one end of the rotating basket. The other end was left opened for charging and cleaning purposes and was designed so that it can be recessed and secured into a rotating disc after charging the reactor. An outer casing made from a Plexiglas cylinder of 12.5 mm diameter was installed for each bioreactor. The Plexiglas cylinder was recessed and secured into the main frame from one end by means of six stainless steel screws. The other end of the Plexiglas cylinder was covered by a removable circular Plexiglas plate and was recessed and secured by means of six stainless steel screws and fastened by wing nuts. A rubber gasket lining (O-ring, 2.5 mm thick) was used at both ends of the Plexiglas cylinder in order to provide an air tight seal. A hole was drilled through the cylinder wall for the release of the exhaust gas. The rotating discs were connected to a motor (Synchronous motor, 20-34245G-24007, xerox127P1292/B, Sigma Instruments Inc., Braintree, Mass., USA) through a transmission system.

7.3.3 Aeration System

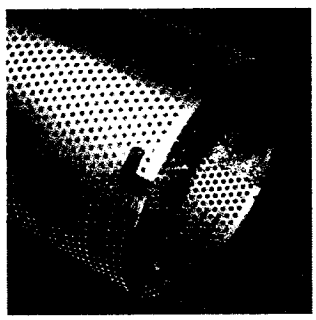
Air was supplied continuously at the required flow rate inside each drum bioreactor from the laboratory air supply. The air passed first through a pressure regulator (ARO, Model no. 129125-510, Bryan, Ohio) in order to regulate the air pressure around 5 kPa and then through a 1-L humidifier which contained 0.75 L sterilized distilled water kept at room

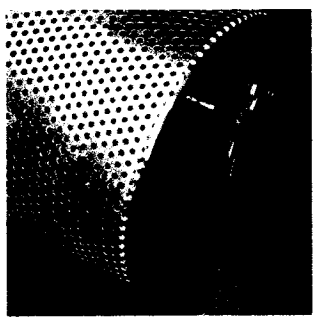


(a) Stainless steel mesh

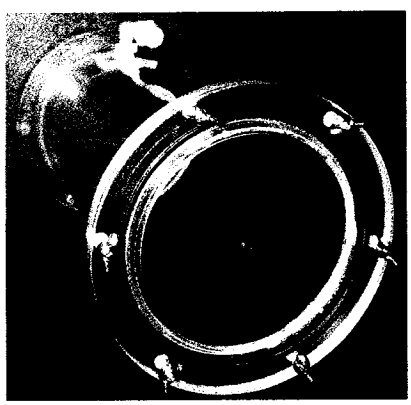


(b) Stainless steel rotating basket

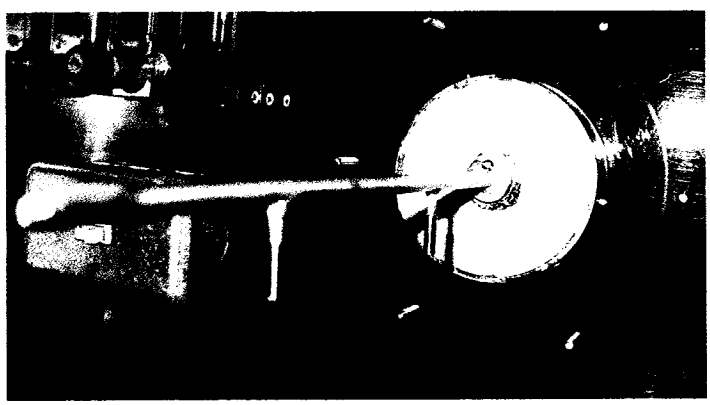




(c) Both ends of the rotating basket



(d) Plexiglas cylinder



(e) rotating disc, aeration tube and thermocouples

Figure 7.4. The drum bioreactor.

temperature. The humidified air was passed through a bacterial filter and then through a flow meter (No. 60648, Cole-Parmer Instrument Co., Illinois, USA) and finally introduced into the reactor through a small perforated stainless steel tube that ran along the center of the basket. The aeration tube was fixed through the center of the rotating disc and remains stationary while the basket is rotating. The air inlet sampling port was placed right after the bacterial filter whereas the three exhaust gas sampling ports were located on the exhaust tubes; each was made of a rubber septum. The three exhaust gas tubes were connected to a manifold and the exhaust gas was bubbled through a small container of water in order to create a slight gas pressure in the bioreactors.

7.3.4 Data Acquisition System

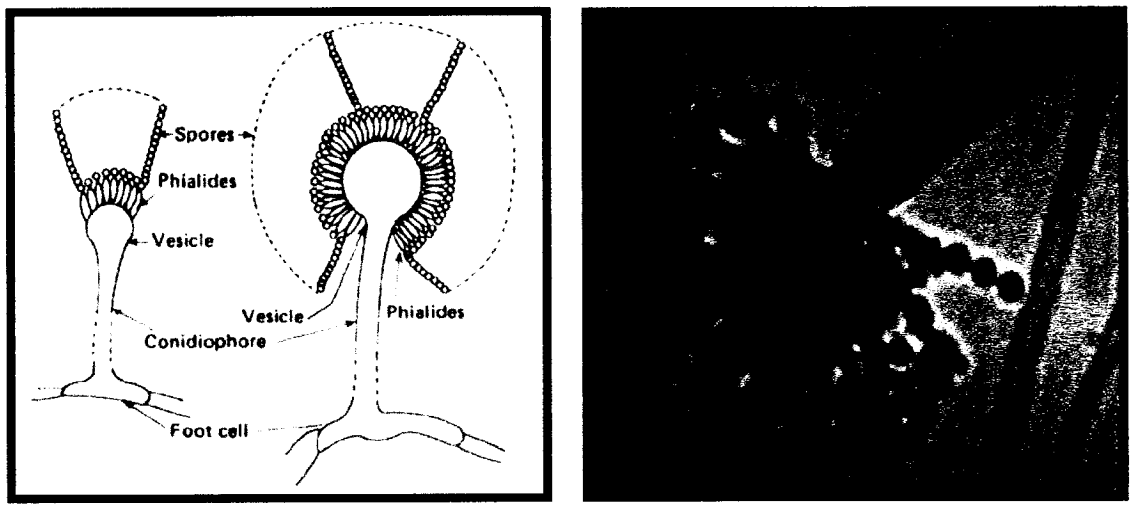
Eleven T-type thermocouples (Thermo Electric Ltd., Brampton, Ontario) were used to measure the temperature during the course of the fermentation process. Two thermocouples were threaded through the aeration tube of each bioreactor and used to measure the temperature of the material inside the bioreactor. The other five thermocouples were used to measure the ambient temperature (one), inlet air temperature (one) and exhaust gas temperature of each bioreactor (three). The temperature data were monitored and stored using a data acquisition system which consisted of a master unit (Multiscan 1200, Omega, Stamford, CT), a thermocouple/volt scanning card (MTC/24, Omega, Stamford, CT), a software (Tempview, Omega, Stamford, CT) and a personal computer (IBM, Pentium III).

7.4 Experimental Material

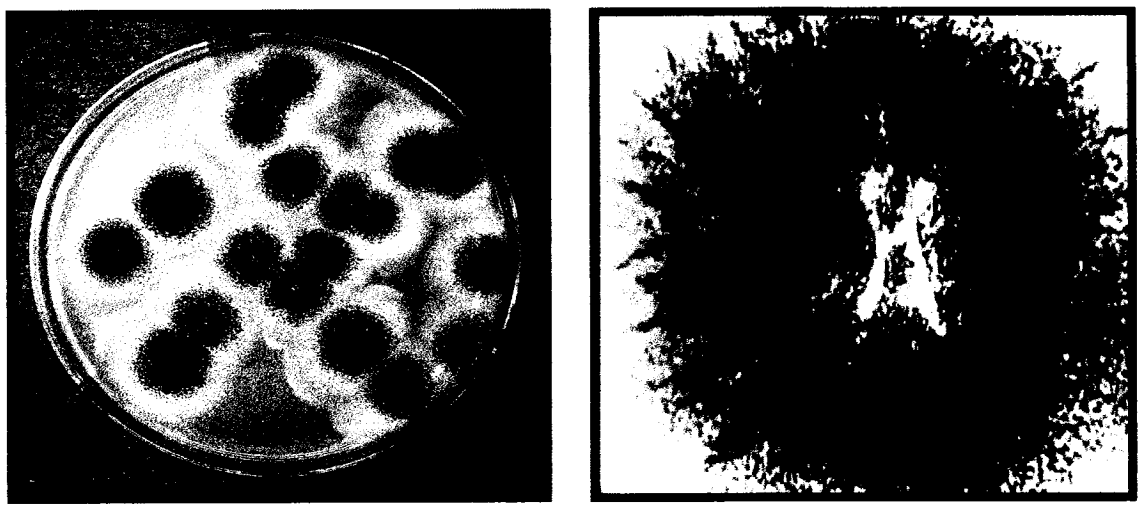
7.4.1 Microorganisms

The fungus *Aspergillus niger* (Figure 7.5) was chosen for this study for two reasons: (a) it is well known to produce acid protease and (b) it has chitin in its cell wall which is considered an additional source of chitin. Generally, the genus *Aspergillus* is characterized by a well developed foot cell at the base of the conidiophore. The colony consists of colorless mycelium from which conidiophora (Figure 7.5a) arise. The spores develop the color and the powdery appearance of the colony surface. *Aspergillus niger*

has round head and black color and its colonies grow rapidly (Figure 7.5b). It is present in soil and on dust, cereals and fruits (Collins and Lyne, 1984).



(a) conidiophore bearing black conidia



(b) culture and colonial morphology

Figure 7.5. *Aspergillus niger* colonies.

Aspergillus niger (ATCC 16513) was obtained from the American Type Culture Collection (Rockville, Maryland) as freeze dried cells. Figure 7.6 shows the procedure for reviving the freeze dried culture. The freeze dried culture was revived in 6 mL of 0.1% sterilized peptone solution, which was prepared by dissolving 1 g bacto-peptone (Difco, Detroit, Michigan, USA) in 1 L deionized-distilled water and then sterilized in an autoclave (Model No. STM-E, Market Forge Sterilmatic, New York) at 121 °C and 103.4

kPa for 30 minutes. The rehydrated culture was kept in the peptone solution in a capped test tube for 24 hours at room temperature (24 °C). One mL of the rehydrated *A. niger* was transferred to each of three test tubes containing 9 mL potato dextrose broth (PDB), which contained Infusion from 200 g potatoes (4 g/L) and 20 g/L Bacto dextrose. The test tubes were kept tightly capped for 48 h at room temperature (24 °C) and then stored in the fridge at 4 °C and subcultured every two months.

A spore stock suspension was obtained by growing the fungus on Czapek's agar, which contained 30.00 g/L saccharose, 2.00 g/L sodium nitrate, 1.00 g/L dipotassium phosphate, 0.50 g/L magnesium sulfate, 0.50 g/L potassium chloride, 0.01 g/L ferrous sulfate and 15.00 g/L agar at room temperature (25 °C) for 4 days. The conidia were harvested from the surface by adding sterilized deionized distilled water containing 0.01% (v/v) Tween 80 (prepared by dissolving 0.1 mL Tween 80 in 1 L distilled deionized water and then autoclaving at 121° C and 103.4 kPa for 30 minutes) and gently scraping the surface with a sterile spatula. The spore concentration was determined using direct standard plate count method according to the procedures described in the Standard Method for the Examination of Dairy Products (Messer et al., 1985). The prepared suspension was stored in the refrigerator at about 4 °C until needed. A fresh suspension was prepared on a weekly basis when needed.

7.4.2 Shrimp Shells

Northern Pink Shrimp (*Pandalus borealis)* shell waste was used in this study. *Pandalus borealis* is commonly fished in the North Atlantic both on the East Coast of Canada and the West Coast of Norway. This species has a mean length of 22-25 mm at maturity (Hansen and Aschan, 2000). Figure 7.7 shows the morphology and external anatomy of the shrimp species. The processing discards of these shrimp may account for up to 80% of the original weight of the material (Shahidi and Synowiecki, 1992).

The shrimp shells were obtained from Ocean Nutrition Ltd. of Bedford, Nova Scotia. The material came from a shell processing plant in Mulgrave, Nova Scotia. These shrimp were caught in the fall of 2001, as part of the offshore Northern Shrimp Fishery by a

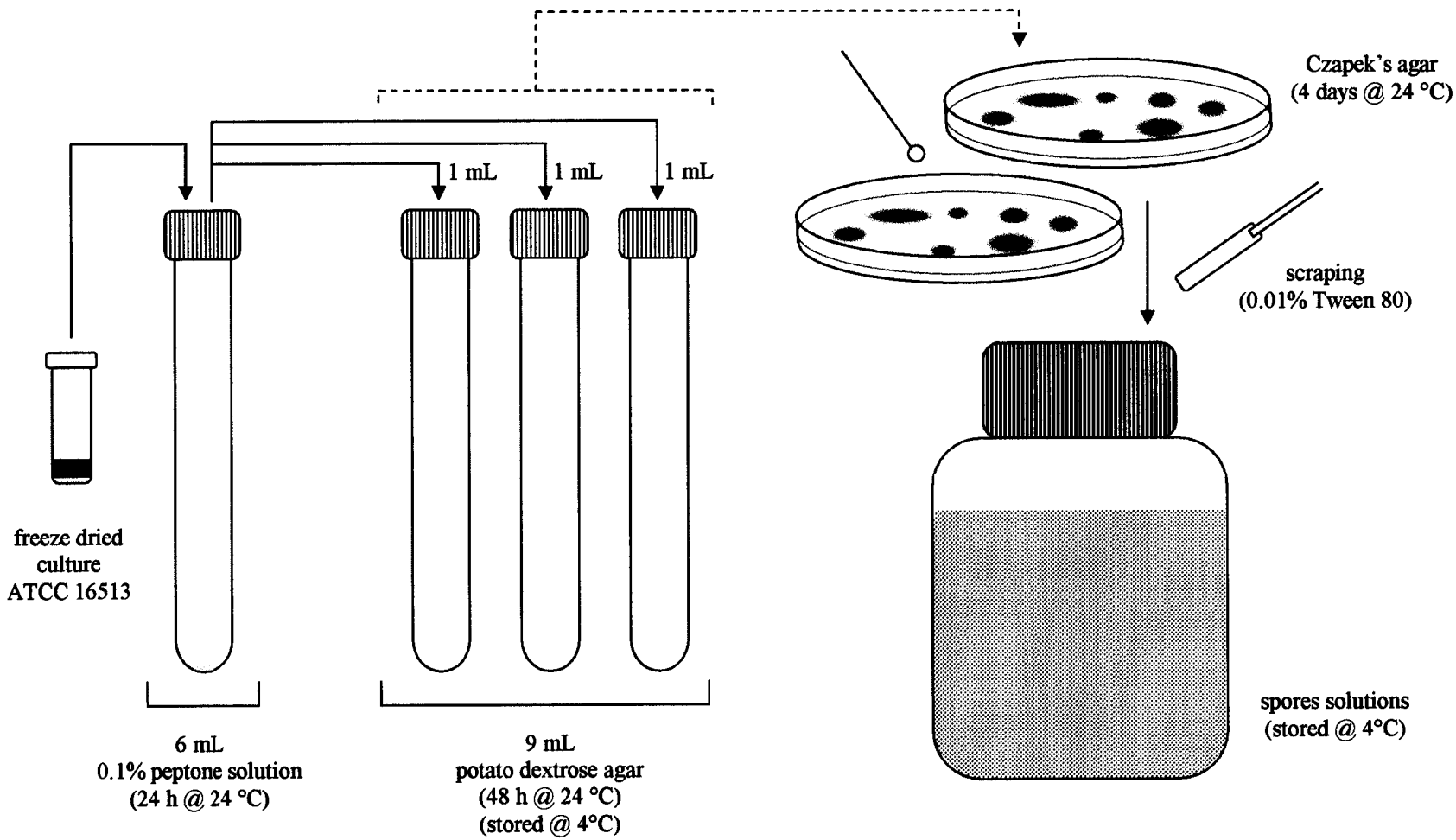


Figure 7.6. Reviving freeze dried culture and spores solution preparation.

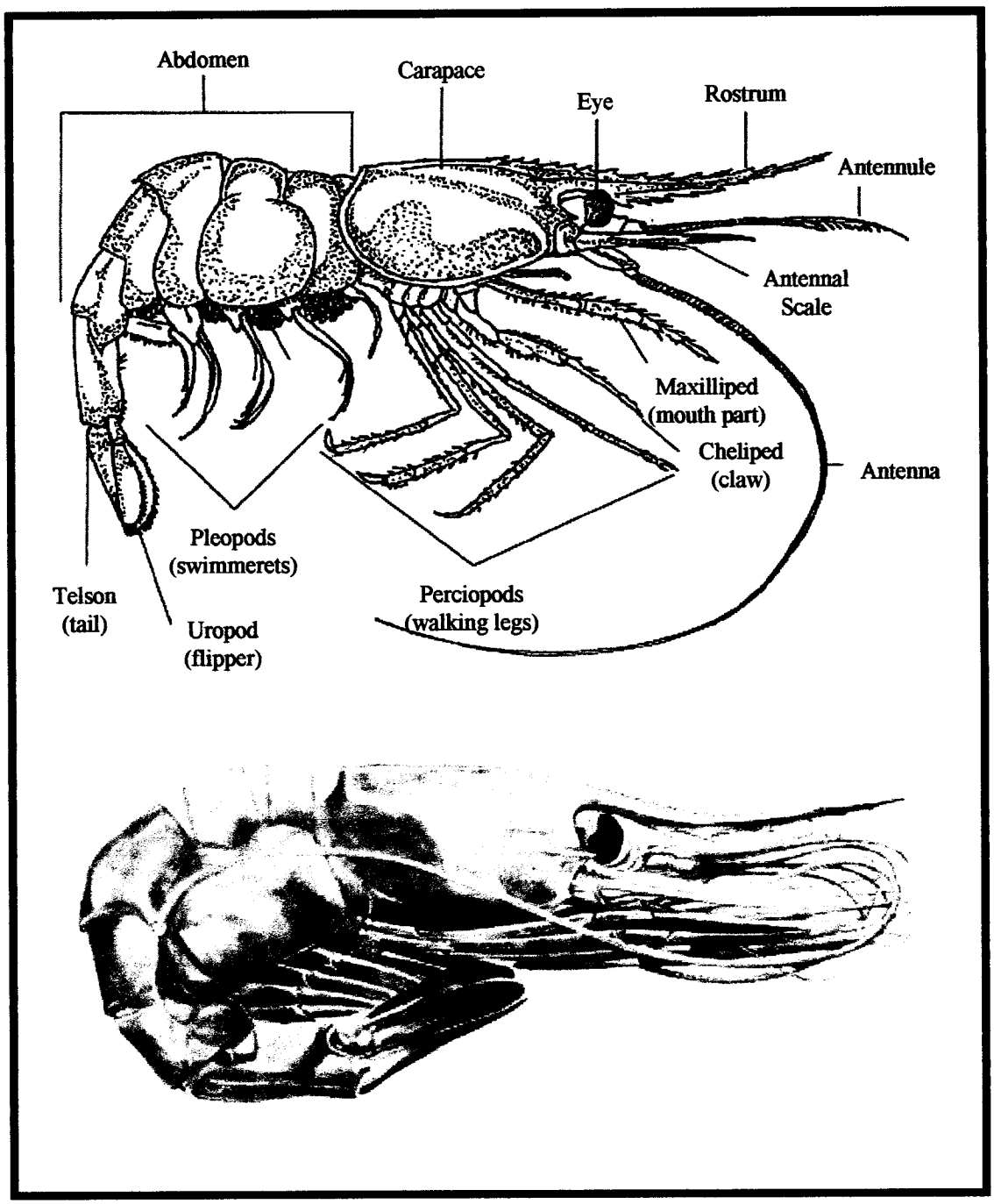


Figure 7.7. Morphology and external anatomy of Northern Pink Shrimp *Pandalus borealis* (Marine Resources Aquarium, 1993 and Superhighway, 2002).

vessel owned by Clearwater Fine Foods Inc., and then individually quick-frozen on board the vessel. They were stored frozen in this manner until arrival at a cooking/peeling plant

operated by St. Anthony Seafoods Ltd., St. Anthony, Newfoundland where they were cooked in boiling salt water for 10 minutes. Following cooking, the shrimp were sent to automated peeling machines where the shell and meat portions were separated. The shell material was collected and dried in large kiln dryers in Mulgrave before shipping for any further processing. The obtained shrimp shells were stored at about -25 °C in the storage facility (Associated Freezers of Canada, Dartmouth, Nova Scotia) till needed.

Table 6.4 shows some of the characteristics of the shrimp shells used in the study. Table 7.1 and Figure 7.8 show the particle size distribution of intact and ground shrimp shell waste used in this study. For the experiments in which ground shrimp shells was used, the shrimp shell waste was ground using a conventional food processor (General Electric Company, Wal-Mart Canada Corp., Mississauga, Ontario). For the experiments with autoclaved shrimp shells, the shrimp shells waste was autoclaved in an autoclave (Model No. STM-E, Market Forge Sterilmatic, New York) at 121 °C and 103.4 kPa for 45 minutes.

7.5 Experimental Protocol

Seven sets of experiments were carried out. The first four sets were used to investigate the deproteinization of shrimp shells using *A. niger* (Table 7.2). The first set of experiments was used to study the ability of *A. niger* to deproteinize shrimp shells and the effect of initial galactose concentration on the performance of the deproteinization process. The second set was used to study the effect of the particle size of shrimp shells on the performance of the deproteinization process. The third set was used to study the effect of initial pH on the performance of the deproteinization process. The fourth set was used to study the effect of autoclaving of shrimp shell waste on the performance of the deproteinization process. The fifth, six and seven sets of experiments were carried out using 1 L beakers. The fifth set was used to study the effects of acid type as well as demineralization condition on efficiency of the demineralization process. The sixth and seventh sets were used to study the effects of retention time between lactic acid solution and crude chitin and shells to acid ratio on the efficiency of the demineralization process.

Table 7.1. Particle size distribution of the shrimp shells used in the study.

Size (mm)	Under (%)		In range (%)	
	Intact	Ground	Intact	Ground
6.300	98.11	100.00	1.89	0.00
4.000	88.97	100.00	9.14	0.00
2.000	59.33	98.20	29.64	1.80
0.850	35.74	70.25	23.59	27.95
0.300	11.25	34.92	24.49	35.33
0.180	3.96	25.14	7.29	9.78
0.075	0.92	11.97	3.04	13.17
< 0.075			0.92	11.97

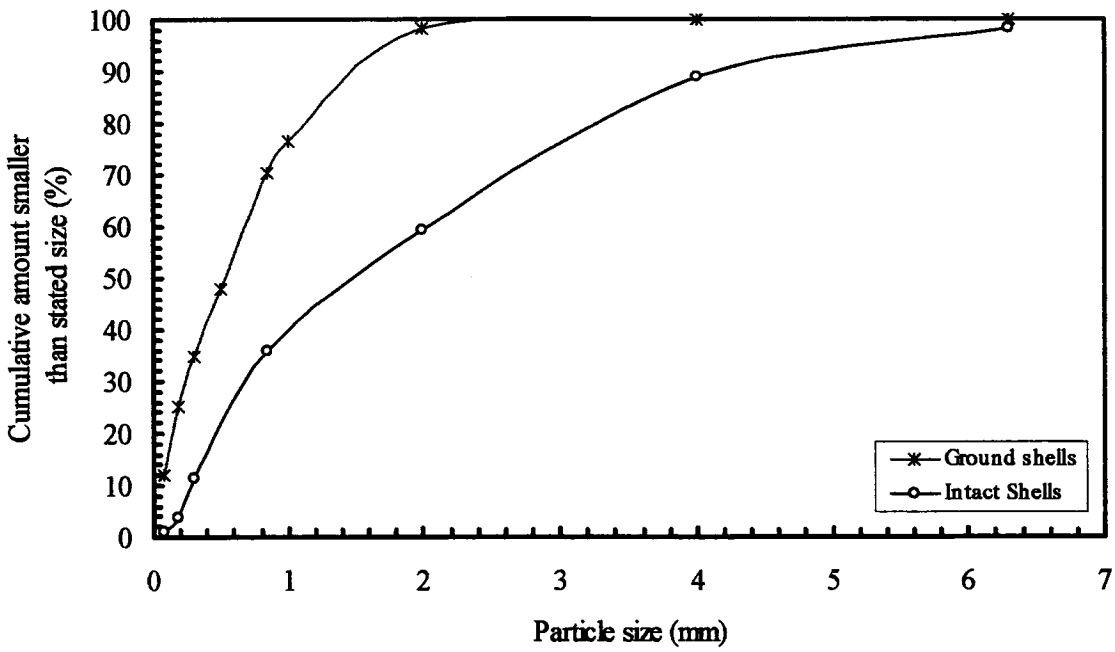


Figure 7.8. Particle size distribution curve for intact and ground shrimp shells.

Table 7.2. Experimental design for the shrimp shells deproteinization process.

Parameters	Experiments			
	First Set	Second Set	Third Set	Fourth Set
Studied parameter	Initial galactose Concentration	Particle size	Initial pH	Autoclaving
Load	75% of the bioreactor volume	75% of the bioreactor volume	75% of the bioreactor volume	75% of the bioreactor volume
Initial moisture content	60%	60%	60%	60%
Inoculum size	1×10^7 spores per 1g	1×10^7 spores per 1g	1×10^7 spores per 1g	1×10^7 spores per 1g
Aeration	5 VMM	5 VMM	5 VMM	5 VMM
Agitation	Intermittent: 15 min every 6 h	Intermittent: 15 min every 6 h	Intermittent: 15 min every 6 h	Intermittent: 15 min every 6 h
Particle size	Intact shrimp shell waste	Intact and ground shrimp shell waste	Ground shrimp shell waste	Ground shrimp shell waste
Autoclaving	Autoclaved shells	Autoclaved shells	Autoclaved shells	Autoclaved and unautoclaved shells
Initial galactose concentration	10, 20 and 30% w/w	20% w/w	20% w/w	20% w/w
Initial pH	8.64	8.64	8.64 and 7.00	8.64

7.5.1 Deproteinization Process

7.5.1.1 Effect of Initial Galactose Concentration. Three reactors were used in this set of experiments. The effect of three initial galactose concentrations (10, 20 and 30% w/w) on deprotenization of shrimp shells was studied. The shrimp shell waste was autoclaved (Model No. STM-E, Market Forge Sterilmatic, New York, USA) at 121°C and 103.4 kPa for 45 min. The sugar solution was prepared by dissolving the required galactose weight in the appropriate volume of deionized distilled water and then autoclaving at 121°C and 103.4 kPa for 30 min. Each reactor was loaded up to 75% of its capacity (200 g shells based on dry weight). The initial moisture content of the shrimp shells was adjusted to 60% with the addition of sugar and spores solutions and the material was mixed thoroughly. An inoculum concentration of 1×10^7 spores per 1g shrimp shell waste was used. Air was introduced inside each reactor at a flow rate of 5 VMM (volume air in mL per shell waste mass in grams per minute). The experiment ran for 6 days. At the start of the experiment, the reactors were rotated (1 rpm) continuously for 30 min and then intermittently for 15 min every 6 h.

7.5.1.2 Effect of Particle Size. Two reactors were used in this set of experiments. Each reactor was loaded up to 75% of its capacity. The first reactor was loaded with 200 g intact shrimp shell waste while the second reactor was loaded with 400 g ground shrimp shell waste. The particle size distribution of the intact shrimp shell waste used in this study is shown in Figure (7.8). The shrimp shell waste was autoclaved (Model No. STM-E, Market Forge Sterilmatic, New York, USA) at 121°C and 103.4 kPa for 45 min. The initial galactose concentration was adjusted to 20% w/w, which was proven to be the optimum initial galactose concentration from the first set of experiments. The sugar solution was prepared by dissolving the required galactose weight in the appropriate volume of deionized distilled water and then autoclaving at 121°C and 103.4 kPa for 30 min. The initial moisture content of the shrimp shells was adjusted to 60% with the addition of galactose and spores solutions and mixing thoroughly. An inoculum concentration of 1×10^7 spores per 1g shrimp shell waste was used. Air was introduced inside each reactor at a flow rate of 5 VMM. The experiment ran for 5 days. At the start

of the experiment, the reactors were rotated (1 rpm) continuously for 30 min and then intermittently for 15 min every 6 h.

7.5.1.3 Effect of Initial pH. Two reactors were used in this set of experiments. No initial pH adjustment was made in the first reactor while the initial pH of the fermentation material in the second reactor was lowered to 7.0 by the addition of lactic acid solution. The initial sugar concentration was adjusted to 20% w/w. The sugar solution was prepared by dissolving the required galactose weight in the appropriate volume of lactic acid solution (75.6 g/L) and then autoclaving at 121°C and 103.4 kPa for 30 min. The initial moisture content of the shrimp shells was adjusted to 60% with the addition of galactose and spores solutions and the material was mixed thoroughly. An inoculum concentration of 1×10^7 spores per 1g shrimp shell waste was used. Air was introduced inside each reactor at a flow rate of 5 VMM. The experiment ran for 5 days. At the start of the experiment, the reactors were rotated (1 rpm) continuously for 30 min and then intermittently for 15 min every 6 h.

7.5.1.4 Effect of Autoclaving. Two reactors were used in this set of experiments. The first reactor was loaded with 400 g unautoclaved ground shrimp shells while the second reactor was loaded with 400 g autoclaved ground shrimp shells. No initial pH adjustment was made. The initial galactose concentration was adjusted to 20% w/w. The sugar solution was prepared by dissolving the required galactose weight in the appropriate volume of deionized distilled water and then autoclaving at 121°C and 103.4 kPa for 30 min. The initial moisture content of the shrimp shells was adjusted to 60% with the addition of galactose and spores solutions and the material was mixed thoroughly. An inoculum concentration of 1×10^7 spores per 1g shrimp shell waste was used. Air was introduced inside each reactor at a flow rate of 5 VMM. The experiment ran for 5 days. At the start of the experiment, the reactors were rotated (1 rpm) continuously for 30 min and then intermittently for 15 min every 6 h.

7.5.2 Demineralization Process

7.5.2.1 Effect of Acid Type and Demineralization Conditions. In this set of experiments, two organic acids (lactic and acetic acids) and one mineral acid (hydrochloric acid) were used to demineralize microbially deproteinized shells under two demineralization conditions (Table 7.3).

Table 7.3. Studied demineralization conditions.

Parameter	Demineralization condition	
	A[a]	B[b]
Concentration[c]	1 N	1.7 N
Temperature	100 °C	Room temperature
Retention time	1 h	6 h
Shells to acid ratio	1:50	1:10

[a] According to Zakaria (1997)
[b] According to Ghanem et al. (2003)
[c] In case of using HCl otherwise 75.6 g/L lactic acid and 75.0 g/L acetic acid were used.

Tango and Ghaly (2002) attained a high lactic acid concentration of 75.6 g/L from cheese whey fermentation using an immobilized packed bed of *Lactobacillus helveticus* in continuous mode at 36 h retention time and initial lactose concentration of 100 g/L. Based on this study, a lactic acid solution of 75.6 g/L concentration was synthesized and used for the demineralization study. The lactic acid solution was prepared by the addition of 73.54 mL 85% w/w DL-lactic acid (Sigma-Aldrich Canada Ltd., Oakville, Ontario) to de-ionized distilled water and making up the solution to 1 L.

Yang et al. (1999) attained a high acetate concentration of 75.0 g/L from cheese whey fermentation using recycle fed-batch immobilized coculture of *Lactococcus lactis* and *Clostridium formicoaceticum*. Based on this study, an acetic acid solution of 75.0 g/L concentration was synthesized and used for the demineralization study. The acetic acid solution was prepared by the addition of 71.57 mL glacial acetic acid (99.8%) (Fisher Scientific, Montreal, Quebec, Canada) to de-ionized distilled water and making up the solution to 1 L.

Ground autoclaved shrimp shell material collected after 120 h of deproteinization was used. The deproteinized shells were washed thoroughly several times with deionized distilled water until the wash water was clear and dried in an oven (Isotemp Oven, Model 655F, Fisher Scientific, Montreal, Quebec, Canada) at 60 °C before use in this set of experiments.

The first set of experiments was carried out according to the demineralization procedure described by Zakaria (1997). In this procedure (A), 1 g of microbially deproteinized shells (MDS) was mixed with 50 mL acid (36.46 g/L HCl, 75.60 g/L lactic acid and 75.00 g/L acetic acid) and placed in a water bath at 100 °C for 1 h. The second set of experiments was carried out according to the procedure described by Ghanem et al. (2003). In this procedure (B), 1 g of microbially deproteinized shells was mixed with 10 mL acid (61.98 g/L HCl, 75.60 g/L lactic acid and 75.00 g/L acetic acid) and placed on a stir plate (Thermix® Stirrer Model 120MR, Fisher Scientific, Montreal, Quebec, Canada) at room temperature (24 °C) for 6 h.

Samples were then filtered under suction using coarse porosity filter paper (reeve angel grade 202, Whatman Inc., Clifton, NJ, USA) and washed few times with deionized distilled water. The filter paper and collected material were removed and dried at 105 °C in an oven. The purified chitin was then collected from the filter paper and analyzed for mineral content.

7.5.2.2 Effect of Retention Time. From the previous sets of experiments it was found that demineralization conditions did not have a significant effect on the minerals removal efficiency. Therefore, in this set of experiments demineralization condition B was used since it saves energy and acid (it is performed at room temperature and uses less amount of acid). The effect of reducing the retention time from 6 to 2 h on the minerals removal efficiency of the microbially deproteinized shells using demineralization condition B was studied. Lactic acid and acetic acid solutions (75.6 and 75.0 g/L concentration, respectively) were used. The crude chitin along with either the lactic acid or the acetic acid solution were placed in a beaker and mixed continuously using a stir plate

(Thermix® Stirrer Model 120MR, Fisher Scientific, Montreal, Quebec, Canada) for the required retention time at room temperature (24 °C). Samples were then filtered under suction using coarse porosity filter paper (reeve angel grade 202, Whatman Inc., Clifton, NJ, USA) and washed few times with deionized distilled water. The filter paper and collected material were removed and dried at 105 °C in an oven. The purified chitin was then collected from the filter paper and analyzed for its mineral content.

7.5.2.3 Effect of Crude Chitin to Acid Ratio. From the previous set of experiments, it was found that reducing the retention time from 6 to 2 h caused a slight decrease in the minerals removal efficiency. Therefore, in this set of experiments, the effect of crude chitin to acid solution ratio (1:10, 1:20 and 1:30 g crude chitin: mL acid solution) on the minerals removal efficiency of microbially deproteinized shells at 2 h retention time was studied. Ground autoclaved shrimp shell material collected after 120 h of deproteinization was used. Lactic acid and acetic acid solutions (75.6 and 75.0 g/L concentration, respectively) were used. The deproteinized shells were washed thoroughly several times with deionized distilled water until the wash water was clear and dried in an oven (Isotemp Oven, Model 655F, Fisher Scientific, Montreal, Quebec, Canada) at 60 °C before use in this set of experiments. The crude chitin along with the acid solution was placed in a beaker and mixed continuously using a stir plate (Thermix® Stirrer Model 120MR, Fisher Scientific, Montreal, Quebec, Canada) for 2 h at room temperature (24 °C). Samples were then filtered under suction using coarse porosity filter paper (reeve angel grade 202, Whatman Inc., Clifton, NJ, USA) and washed few times with deionized distilled water. The filter paper and collected material were removed and dried at 105 °C in an oven. The purified chitin was then collected from the filter paper and analyzed for mineral content.

7.6 Experimental Analyses

Particle size distribution of the intact shrimp shell waste was first determined. Shrimp shell samples of 10 g each were collected every 12 h during the course of the fermentation period (5-6 days) and analyzed for moisture content, pH, galactose

concentration, protease activity, ammonium nitrogen, total Kjeldahl nitrogen, protein and chitin. Exhaust gas samples were also taken every 12 h and analyzed for carbon dioxide concentration. The bulk temperature was monitored and recorded every 10 minutes. The dried purified chitin samples were analyzed for their minerals content.

7.6.1 Determination of Particle Size Distribution

A known weight of intact and ground shrimp shells were sieved for 30 min using a sieve shaker (Model RX-86, Fisher Scientific, Montreal, Quebec, Canada) with 7 different sieve sizes (6.300, 4.000, 2.000, 0.850, 0.300, 0.180, 0.075 mm aperture size). Each particle size fraction obtained was weighed and the percentage from the total weight was calculated.

7.6.2 Moisture Contents

A known weight of shrimp shell sample was placed in a preweighed aluminum dish. The dish and contents were then placed in an oven (Isotemp Oven, Model 655F, Fisher Scientific, Montreal, Quebec, Canada) at 105 °C for 24 hours. The aluminum dish along with the dried sample were first placed in a desiccator to cool down, the weight was then recorded. The moisture content was determined as follows:

$$MC = \frac{W_{ws} - W_{ds}}{W_{ws}} \times 100 \tag{7.1}$$

Where

MC is the moisture content (%)

W_{ws} is the weight of the wet sample (g)

W_{ds} is the weight of the dry sample (g)

7.6.3 pH

20 mL of deionized distilled water was added to one gram of shrimp shell sample and kept at room temperature (24 °C) for 30 min with frequent stirring using a stir plate (Thermix® Stirrer Model 120MR, Fisher Scientific, Montreal, Quebec, Canada). The

sample was let stand for two minutes and the pH was then measured using a pH meter (Model 805MP, Fisher Scientific, Montreal, Quebec, Canada).

7.6.4 Galactose Concentration

20 mL of deionized distilled water was added to one gram of fermented shrimp shell sample and kept at room temperature (24 °C) for 30 min with frequent stirring using a stir plate (Thermix® Stirrer Model 120MR, Fisher Scientific, Montreal, Quebec, Canada). The extract was then filtered under suction using coarse porosity filter paper (reeve angel grade 202, Whatman Inc., Clifton, NJ, USA) and the supernatant was used for galactose concentration measurements. Galactose concentration was measured using the phenol-sulfuric acid method. The method is based on that simple sugars, oligosaccharides, polysaccharides and their derivatives give a stable orange-yellow color when reacted with phenol and concentrated sulfuric acid (Dubois et al., 1956). The intensity of the color is proportional to the amount of galactose present in the sample and can be measured at 492 nm.

The chemicals used in performing the galactose analyses included 5% aqueous solution of phenol and concentrated sulfuric acid (95-98%). 2 mL of each sample were transferred to a test tube. 1 mL of phenol solution and 5 mL of concentrated sulfuric acid were added to each tube. Tubes were then tight capped and the contents were mixed using a vortex mixer (Sybron Maxi Mix model M-16715, Thermolyne Corporation, Dubuque Iowa, USA). The tubes were allowed to stand for 10 minutes at room temperature and then the contents were mixed again using a vortex mixer. Tubes were placed in a water bath at 30 °C for 15 minutes after which the contents were mixed again using a vortex mixer. The tubes were allowed to stand for 30 minutes and 200µL of each tube were carefully loaded into duplicate wells in a microtiter plate. The absorbance was measured using a microplate reader ((µQuant, Bio-Tek Instruments, Inc., Winooski, Vermont, USA).

In order to determine the galactose concentration using phenol-sulfuric acid method, a standard curve was developed from solutions of galactose and deionized distilled water with different concentrations. First, a standard solution of 100 µg/mL galactose was

prepared by dissolving 10 mg galactose in 100 mL deionized distilled water. Then, a set of 6 solutions with galactose concentration of 5, 10, 15, 20, 25, and 30 μg/mL) were prepared. Finally, the test procedure was performed on the prepared solutions. The measured absorbances were plotted against the known galactose concentrations (μg/mL) as shown in Figure 7.9. A blank sample of pure deionized distilled water was used. The following linear relationship between the galactose concentration and the absorbance was obtained, with a coefficient of determination (R^2) value of 0.98:

$$C_{ga} = 416.67\ (\bar{A}_{492}) \tag{7.2}$$

Where

C_{ga} is the galactose concentration (μg/mL)

$\bar{A}_{492}$ is the absorbance measured at 492 nm

Before measuring the galactose concentration, samples were first diluted in order to fit the galactose concentration range (0 to 30 μg/mL) of the standard curve.

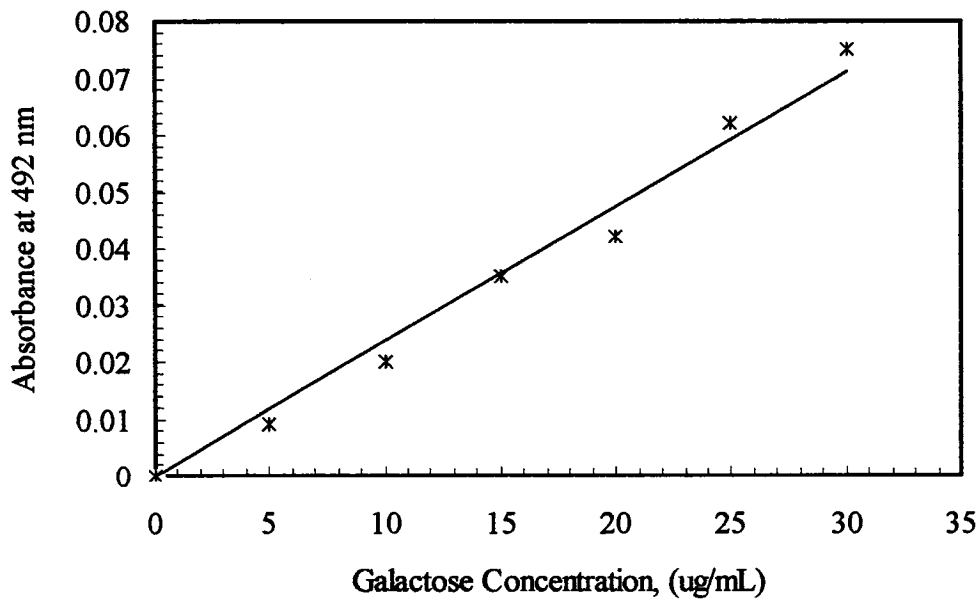


Figure 7.9. Galactose concentration standard curve.

7.6.5 Protease Activity

Protease produced by *A. niger* was first extracted from the samples (1 g each) using 20 mL deionized distilled water and kept at room temperature for 30 min with continuous stirring using a stir plate (Thermix® Stirrer Model 120MR, Fisher Scientific, Montreal, Quebec, Canada). The extract was then filtered under suction using coarse porosity filter paper (reeve angel grade 202, Whatman Inc., Clifton, NJ, USA) and the supernatant was used for the assay of enzyme. Protease activity was measured using Protease Colorimetric Detection kit (Product Code PC0100, Sigma, Saint Louis, Missouri, USA). Description of the assay is given in the data sheet provided with the kit (Appendix A). The assay was based on using a casein substrate, which is cleaved by the protease to trichloroacetic acid soluble peptides. The formed peptides contain tyrosine and tryptophan residues, which react with the Folin and Ciocalteu's reagent causing color change, which can be estimated calorimetrically at 660 nm. The color change was determined using a microplate reader (μQuant, Bio-Tek Instruments, Inc., Winooski, Vermont, USA).

7.6.6 Ammonium Nitrogen

Samples were washed thoroughly several times with deionized distilled water until the wash water was clear, dried in an oven (Isotemp Oven, Model 655F, Fisher Scientific, Montreal, Quebec, Canada) at 60 °C till constant weight (24 h) and ground using a small conventional grinder in case of using intact shrimp shell waste. Ammonium nitrogen (NH_4-N) of dry ground samples was determined directly using KJELTEC AUTO 1030 Analyzer (Fisher Scientific, Montreal, Quebec, Canada).

7.6.7 Total Kjeldahl Nitrogen

Samples were washed thoroughly several times with deionized distilled water until the wash water was clear, dried in an oven (Isotemp Oven, Model 655F, Fisher Scientific, Montreal, Quebec, Canada) at 60 °C till constant weight (24 h) and ground using a small conventional grinder in case of using intact shrimp shell waste. Total Kjeldahl nitrogen (TKN) of dry ground samples was determined after digestion in concentrated sulfuric acid in order to convert the organic nitrogen to ammonium nitrogen, which was then

measured in the digest using KJELTEC AUTO 1030 Analyzer (Fisher Scientific, Montreal, Quebec, Canada).

The digestion was basically done by heating the sample with concentrated sulfuric acid containing kjeltabs, which contained 3.5 g K_2SO_4 and 0.0035 Se. The K_2SO_4 promotes the oxidation of organic matter and conversion of organic nitrogen to ammonium nitrogen by increasing the temperature of the digest. Se is a catalyst which increases the rate of oxidation of organic matter by sulfuric acid. It was realized that the amount of sulfuric acid used for digestion is very critical since nitrogen losses might take place (Appendix B). For the analyses of nitrogen of shrimp shells, 5 mL sulfuric acid with 1 kjeltab per 0.2 g dry weight sample and 45 minutes digestion time at 420 °C was used in this study.

7.6.8 Chitin

Organic nitrogen was calculated by subtracting the ammonium nitrogen from the total Kjeldahl nitrogen. Chitin content was determined based on the fact that chitin contains about 6.89% organic nitrogen (Zakaria, 1997). In order to determine the chitin nitrogen, samples were first deproteinized and demineralized.

The deproteinization process was performed using 5% (w/v) NaOH solution. One gram of ground shrimp shell sample (dry weight) along with 100 mL of sodium hydroxide solution was placed in a 250 mL wide-mouth flask. The flask with the contents was covered with a piece of tin foil and sealed with a rubber band in order to ensure retention of all reacted materials and was then placed in a boiling water bath for 1 h. The sample was filtered under suction through a buchner funnel with coarse porosity filter paper (reeve angel grade 202, Whatman Inc., Clifton, NJ, USA) and washed thoroughly with deionized distilled water. The deproteinized sample was dried in an oven (Isotemp Oven, Model 655F, Fisher Scientific, Montreal, Quebec, Canada) at 60 °C till constant weight (24 h). The weight of the recovered dry deproteinized sample was determined.

The deproteinized dried sample was then demineralized using 50 mL of 1.0 M HCl. The deproteinized sample along with the hydrochloric acid was placed in a 250 mL wide-mouth flask. The flask with the contents was covered with a piece of tin foil and sealed with a rubber band in order to ensure retention of all reacted materials and was then placed in a boiling water bath for 1 h. The demineralized sample was then filtered under suction through a Buchner funnel with coarse porosity filter paper (reeve angel grade 202, Whatman Inc., Clifton, NJ, USA) and washed thoroughly with deionized distilled water. The deproteinized-demineralized sample was then dried in an oven (Isotemp Oven, Model 655F, Fisher Scientific, Montreal, Quebec, Canada) at 60 °C till constant weight (24 h). The weight of the recovered dry deproteinized-demineralized sample was determined. Chitin yield, mineral composition of shrimp shells and extracted chitin and mineral removal efficiency resulted from this chemical deproteinization/demineralization process are illustrated in Appendix (C).

Ammonium nitrogen and total Kjeldahl nitrogen of the dried deproteinized-demineralized sample were then determined. The chitin content was then calculated as described in the following equations.

$$(\text{Org.-N})_c = [(\text{TKN}_c - (\text{NH}_4\text{-N})_c] \times W_c/W_s \quad (7.3)$$

$$\text{CH}_c = (\text{Org.-N})_c \times 14.51 \quad (7.4)$$

Where

CH_c	is chitin content (mg/kg)
$(\text{Org.-N})_c$	is organic nitrogen of the recovered chitin based on the sample weight (mg/kg)
TKN_c	is total Kjeldahl nitrogen of the recovered chitin (mg/kg)
$(\text{NH}_4\text{-N})_c$	is ammonium nitrogen of the recovered chitin (mg/kg)
W_c	is weight of recovered chitin (g)
W_s	is weight of sample (g)

7.6.9 Protein

The protein content of the samples was determined based on the fact that the protein contains about 16% nitrogen. The protein content was then calculated:

$$(\text{Org.-N})_s = \text{TKN}_s - (\text{NH}_4\text{-N})_s \tag{7.5}$$

$$\text{PR}_c = [(\text{Org.-N})_s - (\text{Org.-N})_c] \times 6.25 \tag{7.6}$$

Where

PR_c	is protein content (mg/kg)
$(\text{Org.-N})_s$	is organic nitrogen of the sample (mg/kg)
TKN_s	is total Kjeldahl nitrogen of the sample (mg/kg)
$(\text{NH}_4\text{-N})_s$	is ammonium nitrogen of the sample (mg/kg)

7.6.10 Ash Content

Deproteinized shells as well as purified chitin was filtered under suction through a Buchner funnel with coarse porosity filter paper (reeve angel grade 202, Whatman Inc., Clifton, NJ, USA). The recovered solids were washed thoroughly several times using deionized distilled water and dried in an oven (Isotemp Oven, Model 655F, Fisher Scientific, Montreal, Quebec, Canada) at 60 °C for 24 h. The dried deproteinized samples and the purified chitin samples were analyzed for their ash content. Samples were placed in a muffle furnace (Isotemp® Muffle Furnace model 186A, Fisher Scientific, Montreal, Quebec, Canada) at 700 °C for 2 hours. The sample was taken from the muffle furnace and placed in a desiccator to cool down, thereafter the weight was recorded. The ash content was determined as follows:

$$\text{AC} = \frac{W_{ds} - W_a}{W_{ds}} \times 100 \tag{7.7}$$

Where

AC	is the ash content (%)

W_a is the weight of the ash (g)

7.6.11 Minerals

Purified chitin was filtered under suction through a Buchner funnel with coarse porosity filter paper (reeve angel grade 202, Whatman Inc., Clifton, NJ, USA). The recovered solids were washed thoroughly several times using deionized distilled water and dried in an oven (Isotemp Oven, Model 655F, Fisher Scientific, Montreal, Quebec, Canada) at 60 °C for 24 h. The dried purified chitin samples were analyzed for their minerals content. Quantitative trace element analyses (magnesium, calcium, manganese, potassium, sodium, iron, silicon, aluminum, titanium and copper) were done using an Atomic Absorption Spectrophotometer (SpectrAA 55B, Varion, Mulgrave, Victoria, Australia) in the Minerals Engineering Center, Dalhousie University, Halifax, Nova Scotia. For magnesium, calcium, manganese, potassium, sodium, iron and copper analyses, the samples were first digested with hydrochloric, nitric, hydrofluoric and perchloric acids (30, 10, 10 and 5 mL/g sample, respectively) in a closed vessel at a temperature of 100 °C and then the elements were determined by flame atomic absorption with detection limit of 1 ppm. For silicon, aluminum and titanium analyses, 1 g of the sample was fused with a flux of lithium metaborate and lithium tetraborate and leached with 1:9 nitric acid. Sulfur was determined with Leco Sulfur analyzer along with Leco Induction Furnace (Leco Corporation ST. Joseph, HI, USA). Phosphorus was determined as P_2O_5 by a colorimetric method using spectrophotometer with micro flow-thru system (Spectoronic 100, Bausch & Lomb Incorporation, Rochester, New York, USA) at 430 nm.

7.6.12 Visualization of Shrimp Shells

The shrimp shells were visually inspected at the end of the deproteinization with the naked eye as well as under an incident light stereomicroscope (Carl Zeiss Stemi SV8, Carl Zeiss Canada Ltd., Ontario) at a magnification of 60X. The stereoscope was equipped with a cold light source (SCOHTT KL 1500, SCHOTT North America Inc., NY) and a single chip CCD color video camera (Sony DXC-101, Sony of Canada Ltd.).

7.7 Results

7.7.1 Deproteinization Process

7.7.1.1 **Effect of Initial Galactose Concentration.** The effect of initial galactose concentration on various parameters (temperature, pH, moisture content, galactose utilization, carbon dioxide evolution, protease activity, residual protein and chitin concentration) during the deproteinization process was investigated.

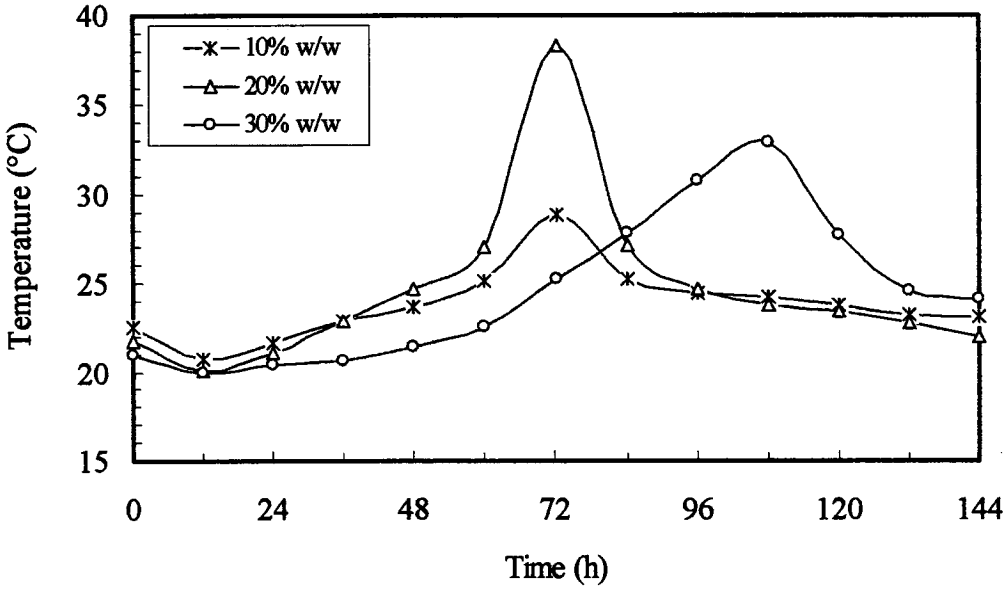
7.7.1.1.1 Temperature: Figure 7.10 shows the change in the temperature of the shrimp shells and the exhaust gas during the course of deproteinization as affected by initial galactose concentrations (10, 20, 30% w/w). The values are the average of two replicates. The coefficient of variation ranged from 0.87 to 6.03%.

7.7.1.1.2 pH: Figure 7.11 shows the change in pH during the course of deproteinization as affected by the initial galactose concentrations (10, 20, 30% w/w). The values are the average of two replicates. The coefficient of variation ranged from 0.92 to 5.16%.

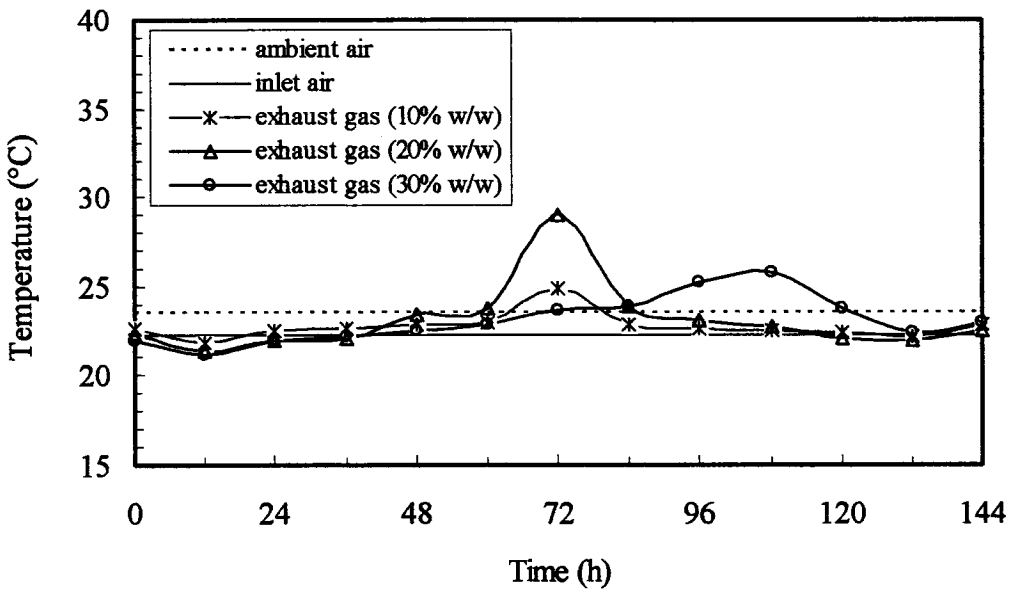
7.7.1.1.3 Moisture Content: Figure 7.12 shows the change in moisture content of the shrimp shells during the course of deproteinization as affected by initial galactose concentrations (10, 20 and 30% w/w). The values are the average of two replicates. The coefficient of variation ranged from 0.04 to 8.97%.

7.7.1.1.4 Galactose Utilization: Figure 7.13 shows the effect of initial galactose concentrations (10, 20 and 30% w/w) on the residual galactose concentration in the shrimp shells during the course of deproteinization. The values are the average of two replicates. The coefficient of variation ranged from 3.77 to 15.71%.

7.7.1.1.5 Carbon Dioxide Evolution: Figure 7.14 shows the effect of initial galactose concentration on carbon dioxide concentration in the exhaust gas. The values are the average of two replicates. The coefficient of variation ranged from 1.29 to 13.56%.



(a) shrimp shell



(b) exhaust gas

Figure7.10. Effect of initial galactose concentration on the shrimp shell and exhaust gas temperatures during the deproteinization process.

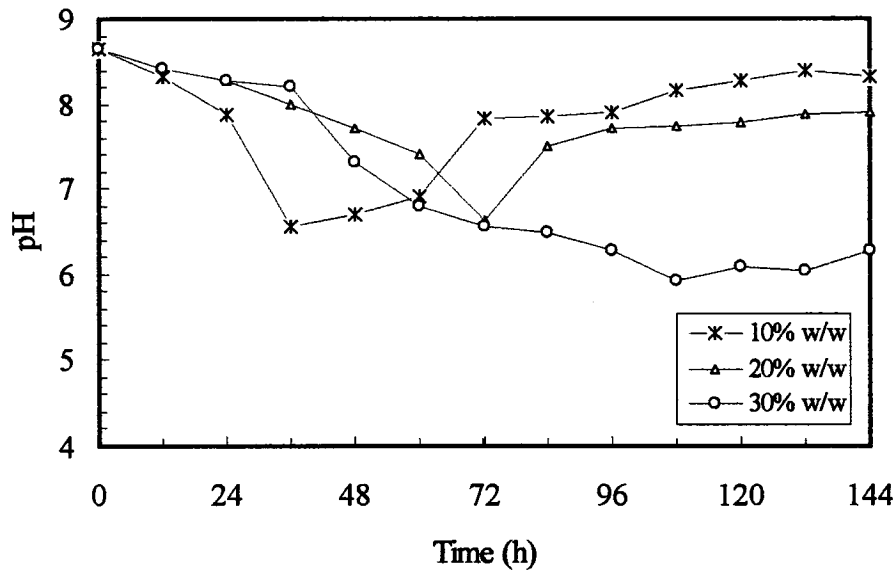


Figure 7.11. Effect of initial galactose concentration on pH during the deproteinization process.

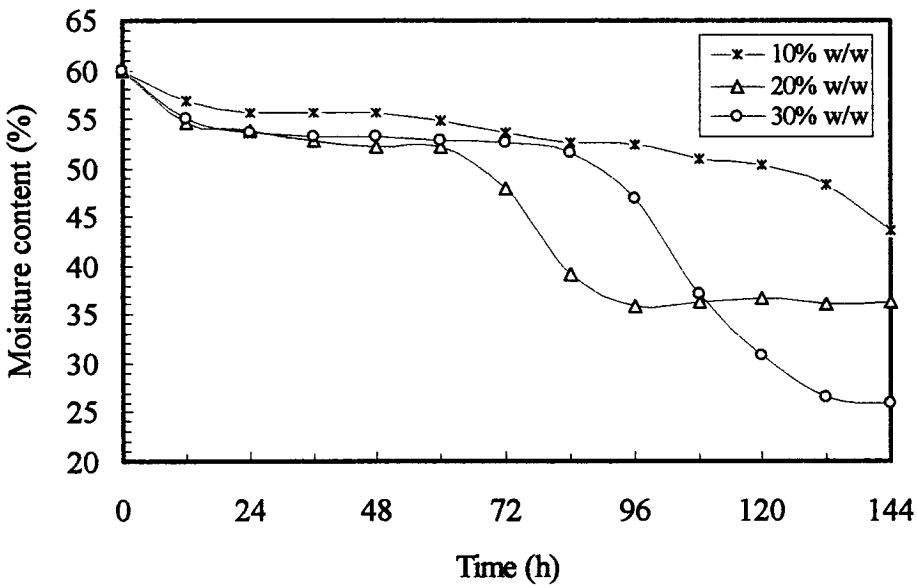


Figure 7.12. Effect of initial galactose concentration on moisture content during the deproteinization process.

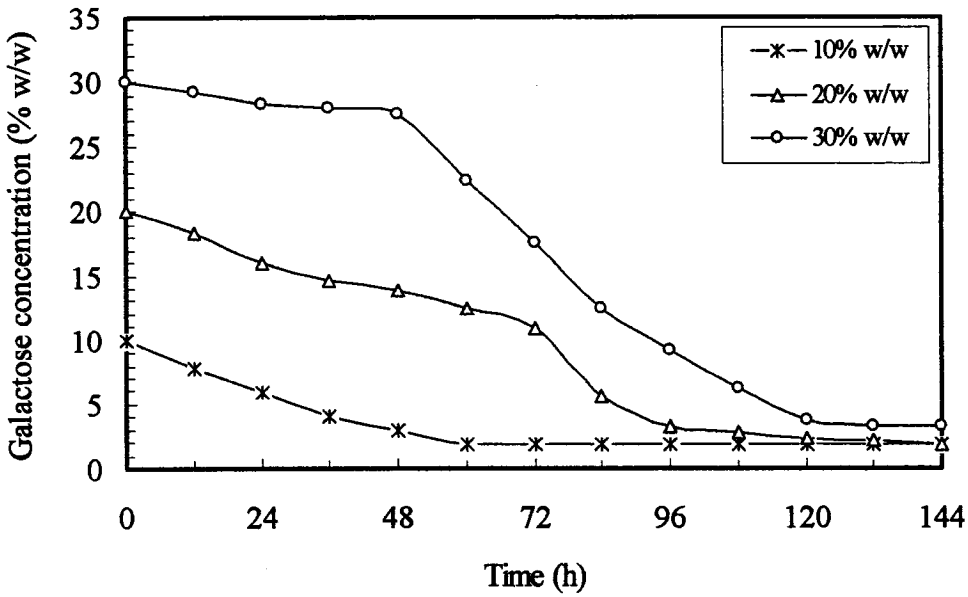


Figure 7.13. Effect of initial galactose concentration on residual galactose during the deproteinization process.

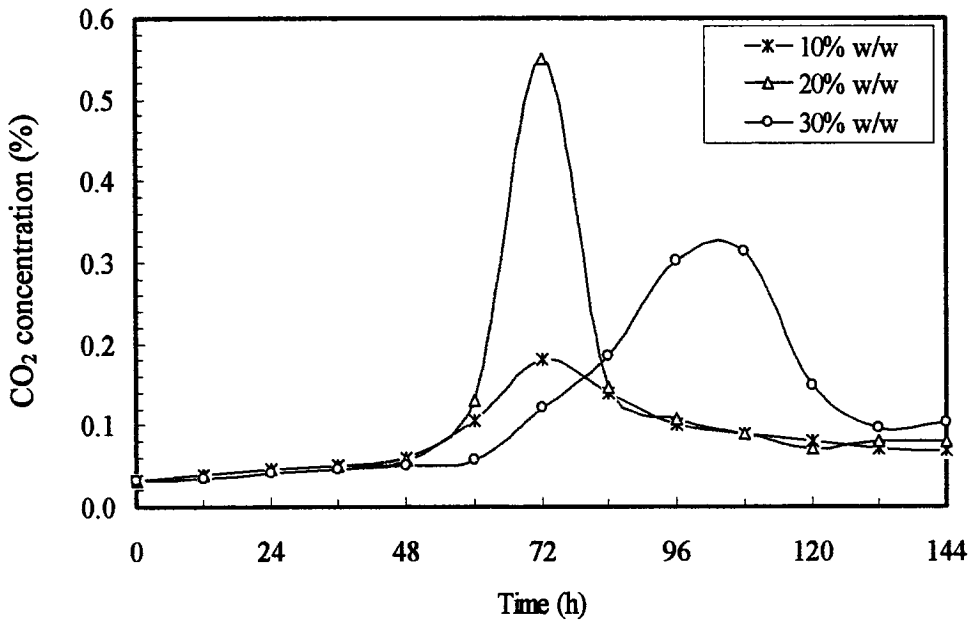


Figure 7.14. Effect of initial galactose concentration on carbon dioxide concentration in the exhaust gas during the deproteinization process.

7.7.1.1.6 Protease Activity: Figure 7.15 shows the effect of initial galactose concentration on protease activity during the course of deproteinization. The values are presented in units per gram dry shrimp shells. One unit of protease activity was defined as the amount of enzyme which can hydrolyze casein to produce color equivalent to 1.0 μmole (181 μg) of tyrosine per minute at pH 7.5 at 37 °C (color by Folin & Ciocalteu's Reagent).

7.7.1.1.7 Protein Concentration: Figure 7.16 shows the effect of initial galactose concentration on residual protein in the shrimp shells during the deproteinization process. The values are presented based on the dry weight of the samples.

7.7.1.1.8 Chitin Concentration: Figure 7.17 shows the effect of initial galactose concentration on chitin content of the shrimp shells during deproteinization process. The values are presented based on the dry weight of the samples.

7.7.1.1.9 Visualization of Shrimp Shells: Figure 7.18 shows the effect of initial galactose concentration on the appearance of the shrimp shells at the end of the deproteinization process. Figure 7.19 shows the appearance of the shrimp shells under the microscope (60X magnification) at the end of the deproteinization process as affected by the initial galactose concentration.

7.7.1.2 **Effect of Particle Size.** The effect of particle size on various parameters (temperature, pH, moisture content, galactose utilization, carbon dioxide evolution, protease activity, residual protein and chitin concentration) during the deproteinization process was investigated.

7.7.1.2.1 Temperature: Figure 7.20 shows the change in the temperature of the shrimp shells and the exhaust gas during the course of deproteinization as affected by the shrimp shell particle size. The values are the average of two replicates. The coefficient of variation ranged from 0.59 to 5.29%.

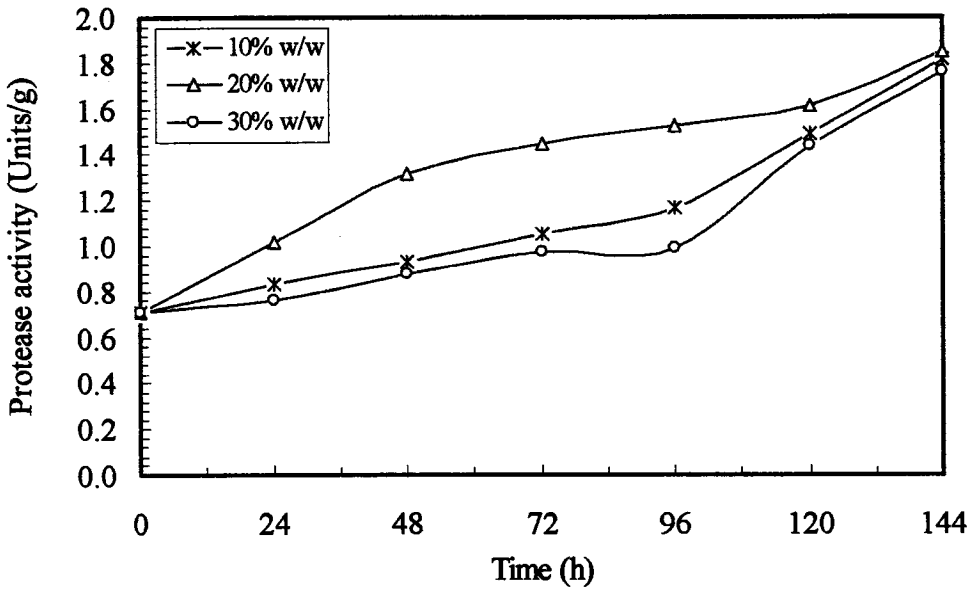


Figure 7.15. Effect of initial galactose concentration on protease activity during the deproteinization process.

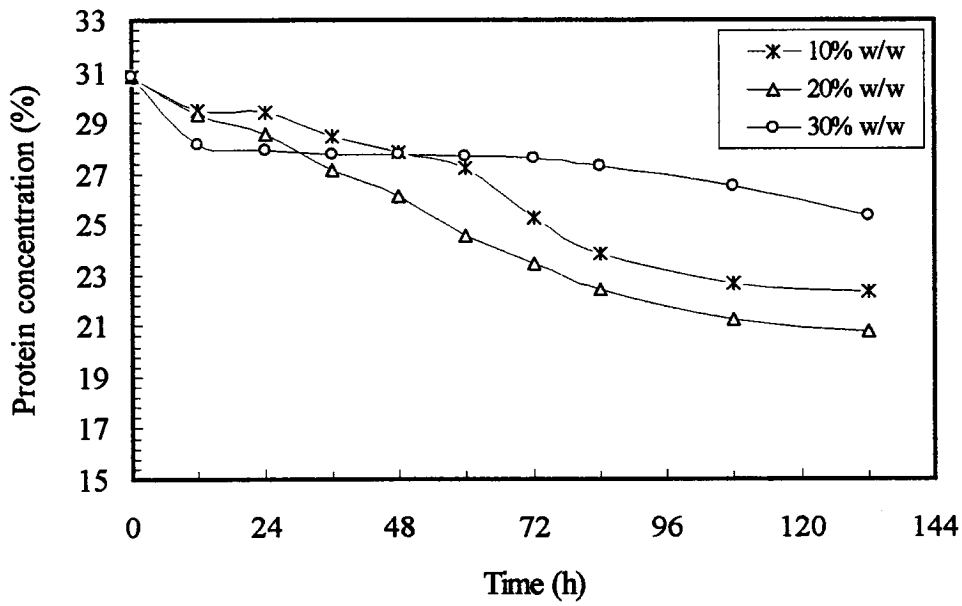


Figure 7.16. Effect of initial galactose concentration on residual protein during the deproteinization process.

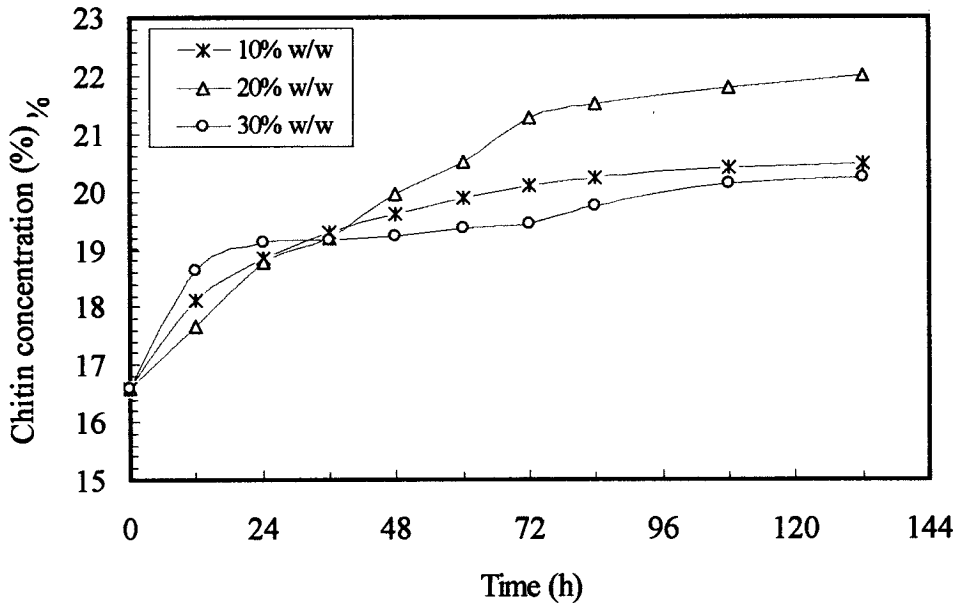


Figure 7.17. Effect of initial galactose on chitin concentration during the deproteinization process.

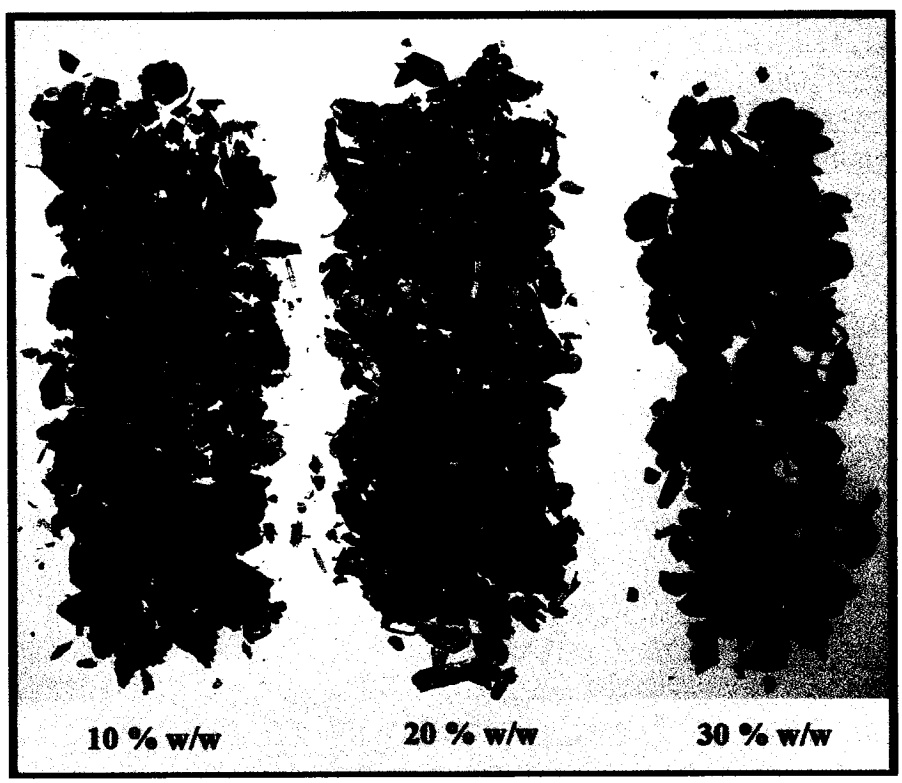
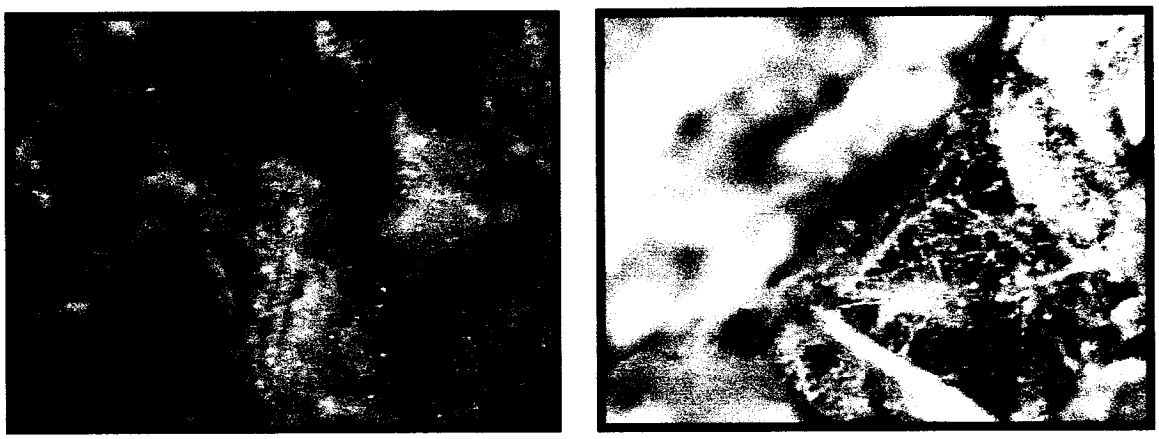
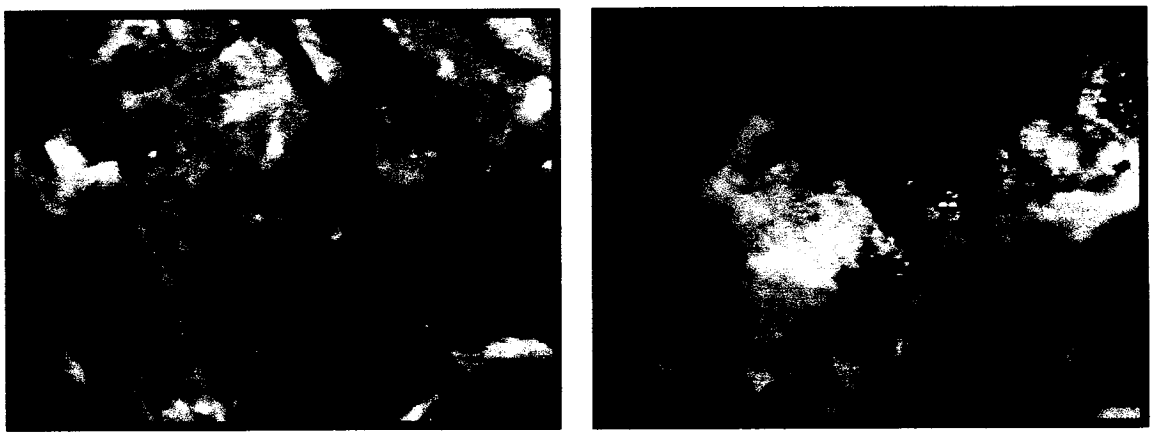


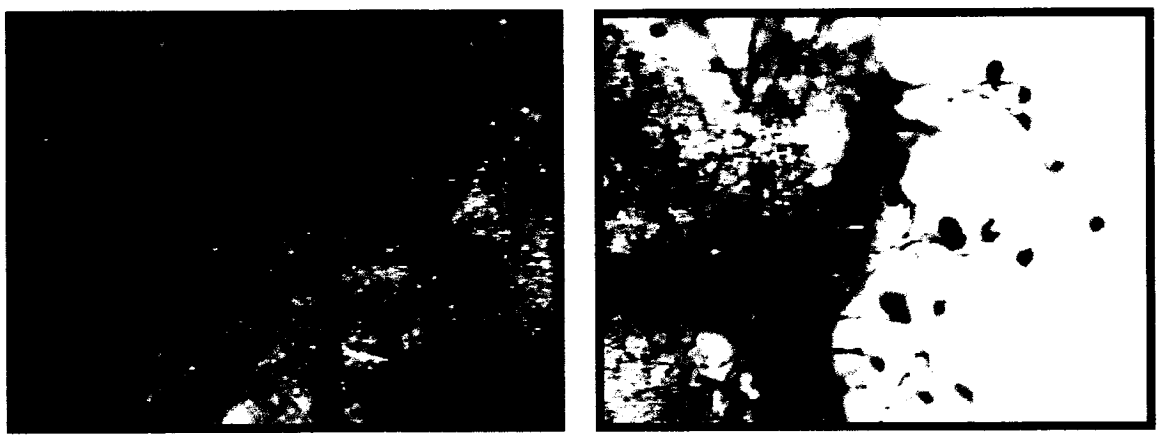
Figure 7.18 Shrimp shells after 144 h of deproteinization as affected by the initial galactose concentrations.



(a) 10 % w/w galactose

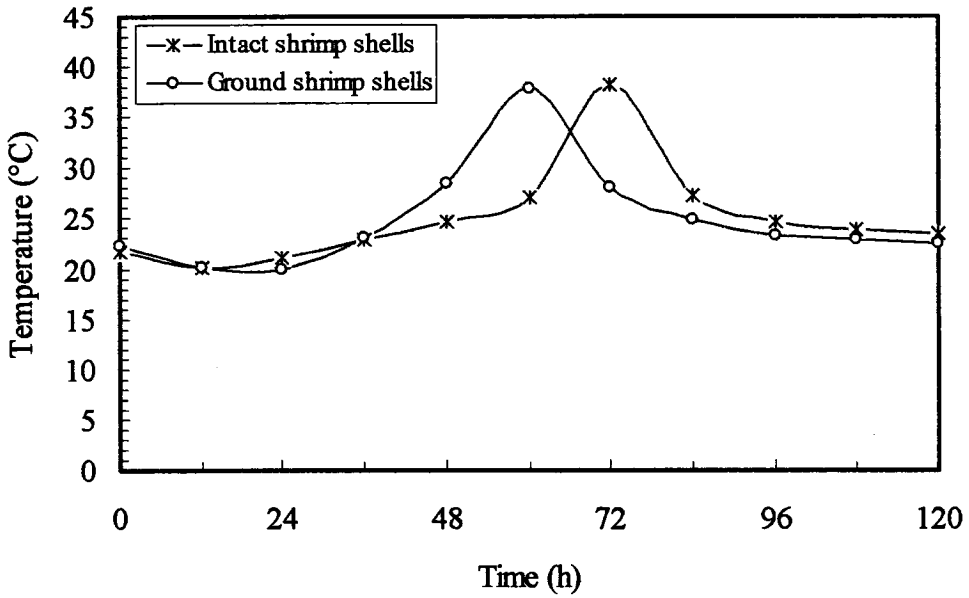


(b) 20 % w/w galactose

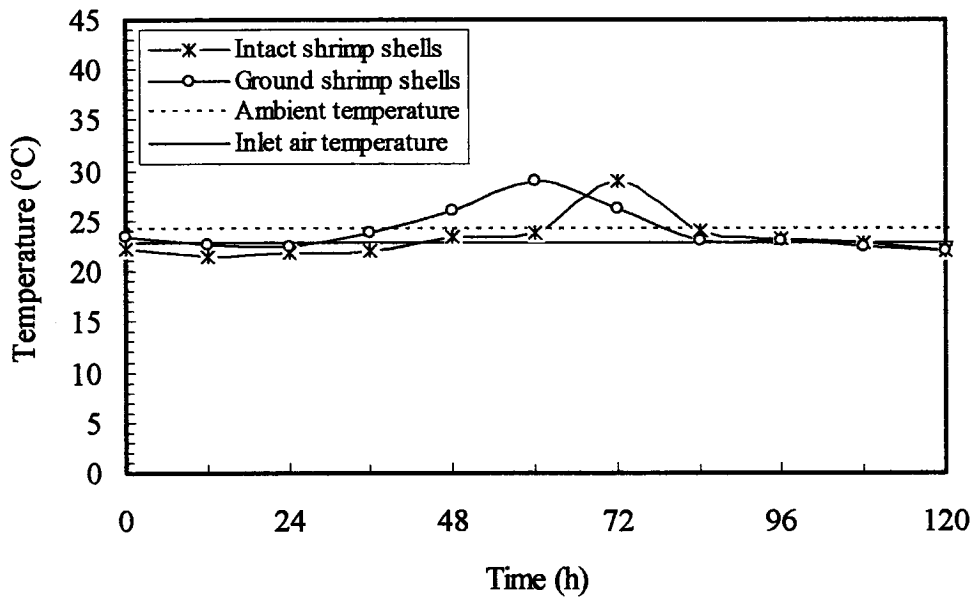


(c) 30 % w/w galactose

Figure 7.19. Visual images (60 X) of the shrimp shells after 144 h of deproteinization as affected by the initial galactose concentrations.



(a) shrimp shells



(b) exhaust gas

Figure 7.20. Effect particle size on the shrimp shell and exhaust gas temperatures during the deproteinization process.

7.7.1.2.2 pH: Figure 7.21 shows the change in pH during the course of deproteinization as affected by the shrimp shell particle size. The values are the average of two replicates. The coefficient of variation ranged from 0.81 to 4.21%.

7.7.1.2.3 Moisture Content: Figure 7.22 shows the change in moisture content of the shrimp shell during the course of deproteinization as affected by the shrimp shell particle size. The values are the average of two replicates. The coefficient of variation ranged from 0.07 to 6.21%.

7.7.1.2.4 Galactose Utilization: Figure 7.23 shows the residual galactose concentration in the shrimp shells during the course of deproteinization as affected by the shrimp shell particle size. The values are the average of two replicates. The coefficient of variation ranged from 1.47 to 12.92%.

7.7.1.2.5 Carbon Dioxide Evolution: Figure 7.24 shows the effect of shrimp shell particle size on carbon dioxide concentration in the exhaust gas. The values are the average of two replicates. The coefficient of variation ranged from 1.41 to 12.58%.

7.7.1.2.6 Protease Activity: Figure 7.25 shows the effect of shrimp shell particle size on protease activity during the course of deproteinization. The values are presented in units per gram dry shrimp shell waste.

7.7.1.2.7 Protein Concentration: Figure 7.26 shows the effect of shrimp shell particle size on the residual protein in the shrimp shells during the deproteinization process. The values are presented based on the dry weight of the samples.

7.7.1.2.8 Chitin Concentration: Figure 7.27 shows the effect of shrimp shell particle size on the chitin content of the shrimp shells during the deproteinization process. The values are presented based on the dry weight of the samples.

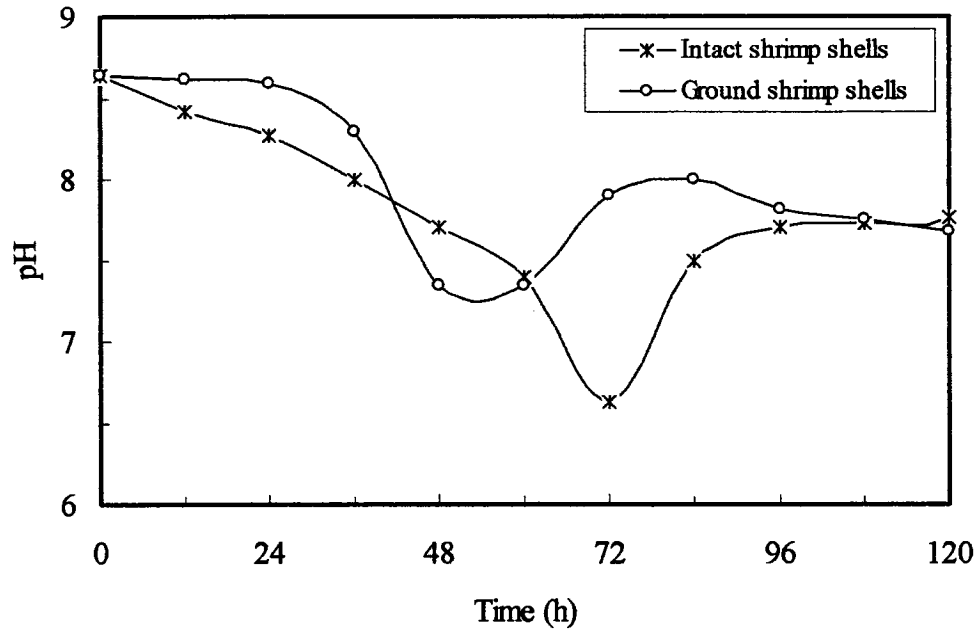


Figure 7.21. Effect of shrimp shell particle size on the pH during the deproteinization process.

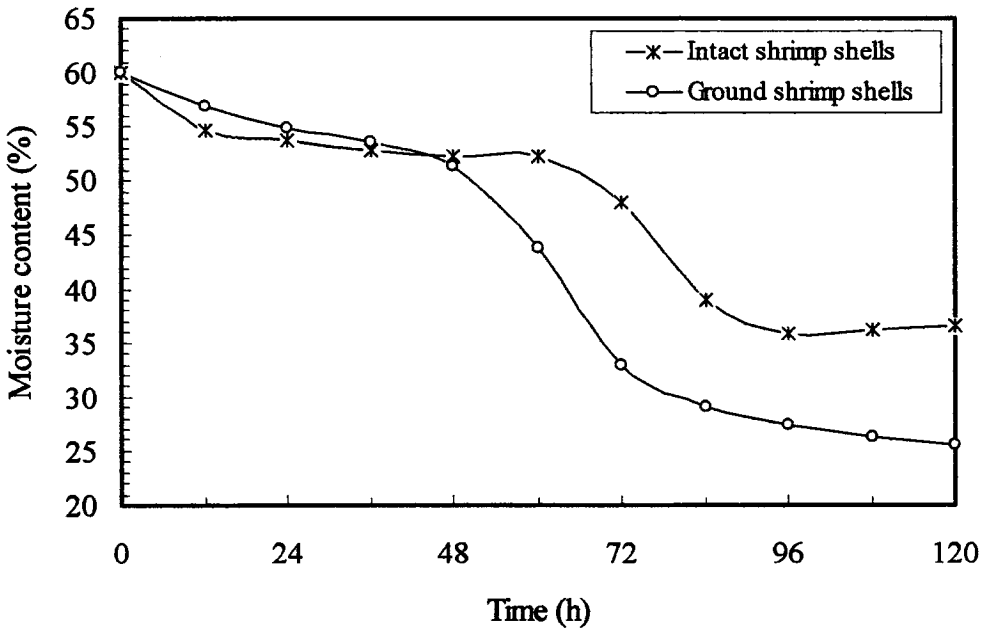


Figure 7.22. Effect of shrimp shell particle size on moisture content measurements during the deproteinization process.

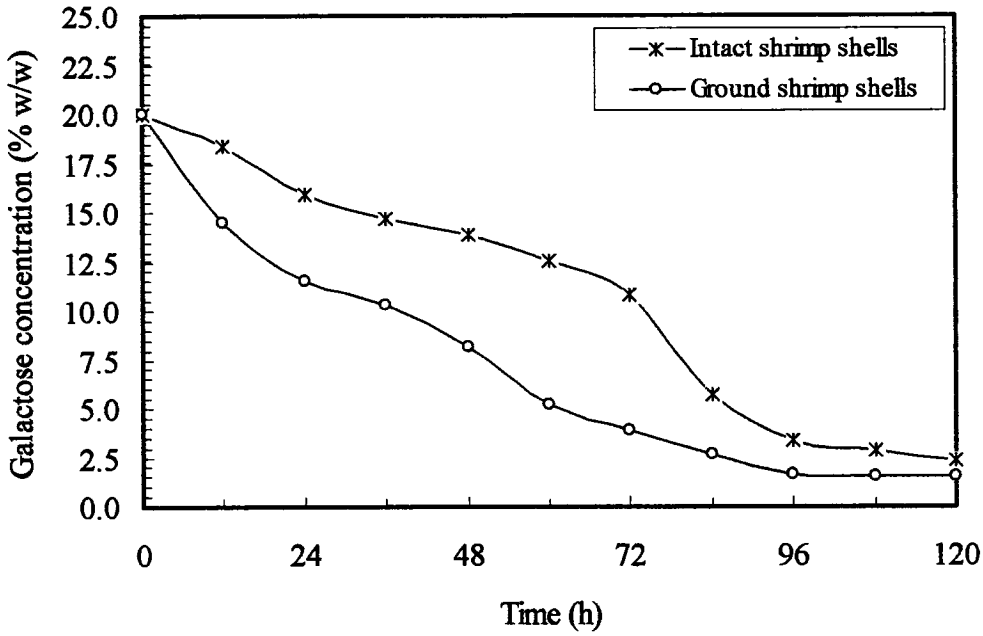


Figure 7.23. Effect of shrimp shell particle size on residual galactose during the deproteinization process.

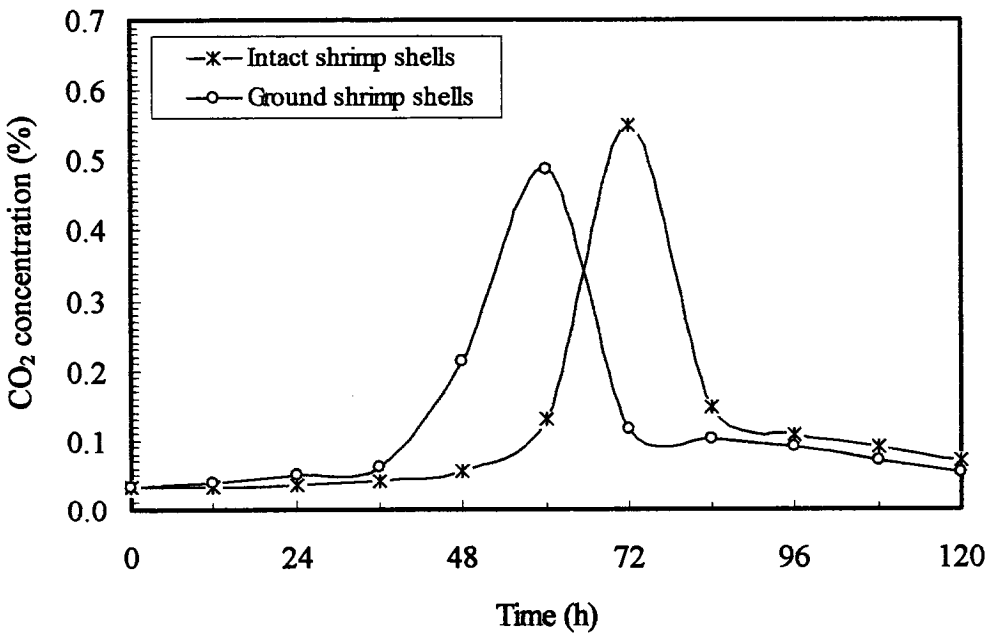


Figure 7.24. Effect of shrimp shell particle size on carbon dioxide concentration in the exhaust gas during the deproteinization process.

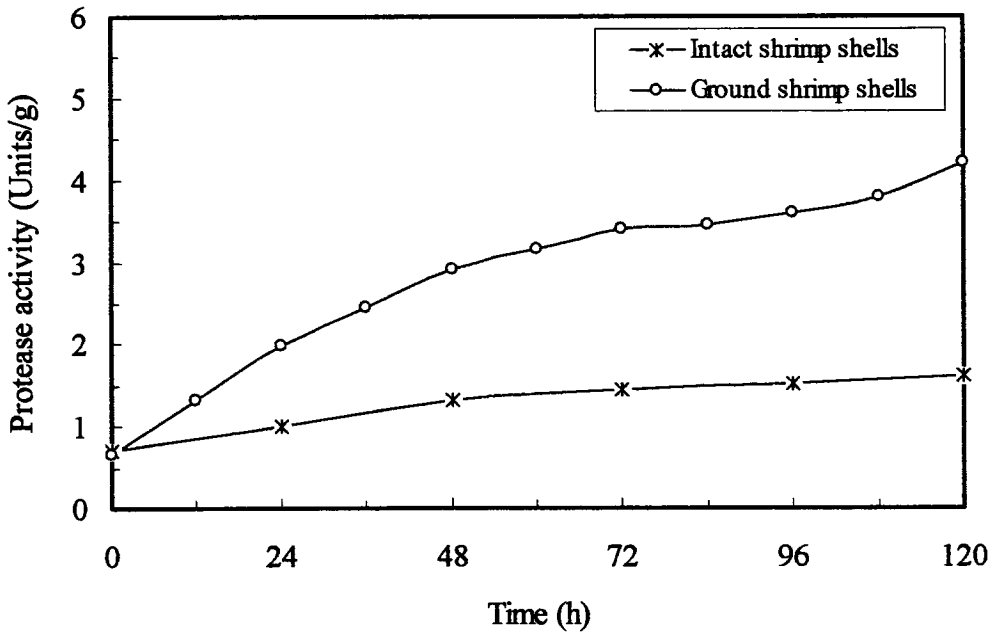


Figure 7.25. Effect of shrimp shell particle size on protease activity during the deproteinization process.

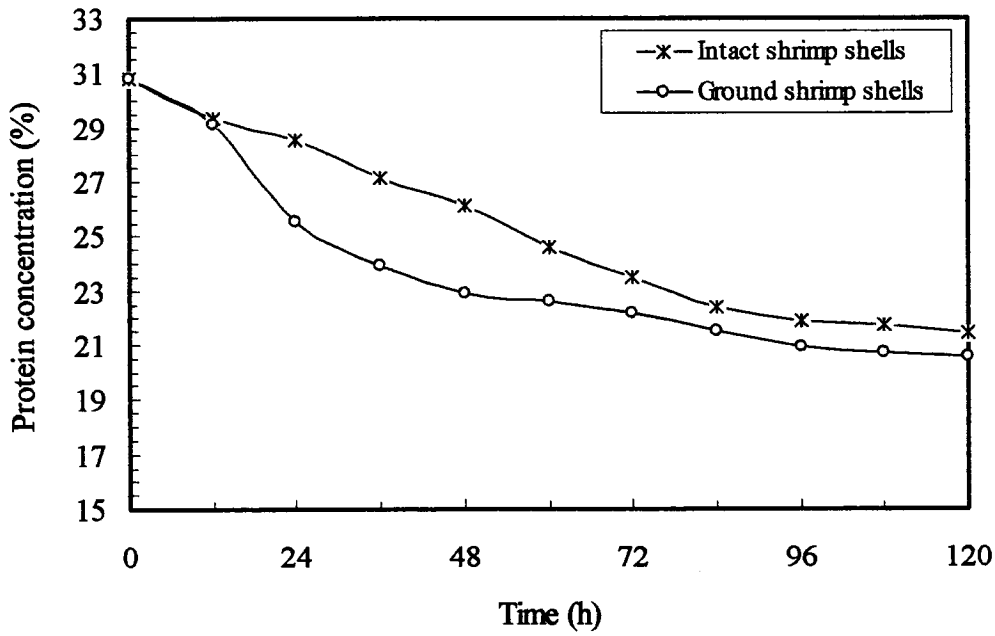


Figure 7.26. Effect of shrimp shell particle size on residual protein during the deproteinization process.

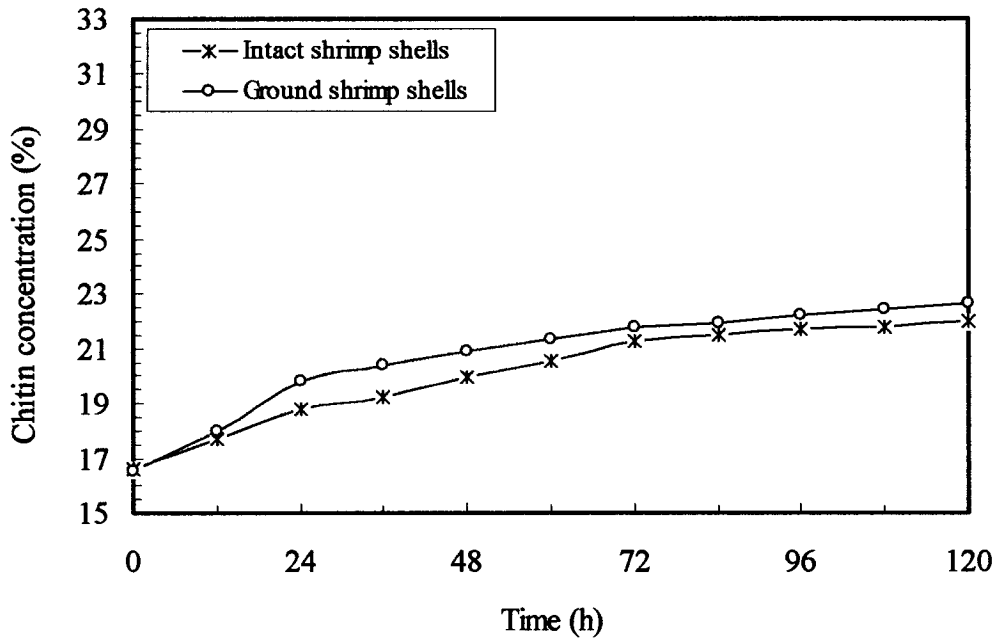
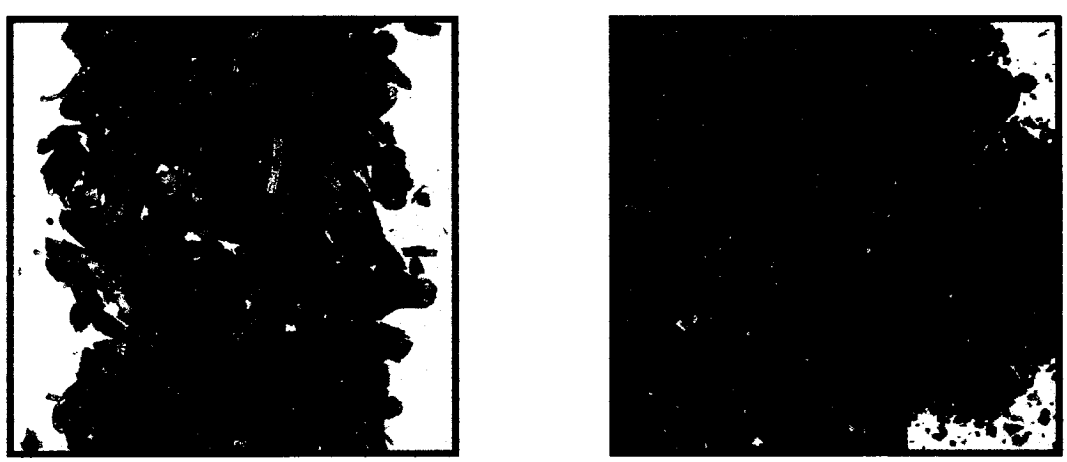


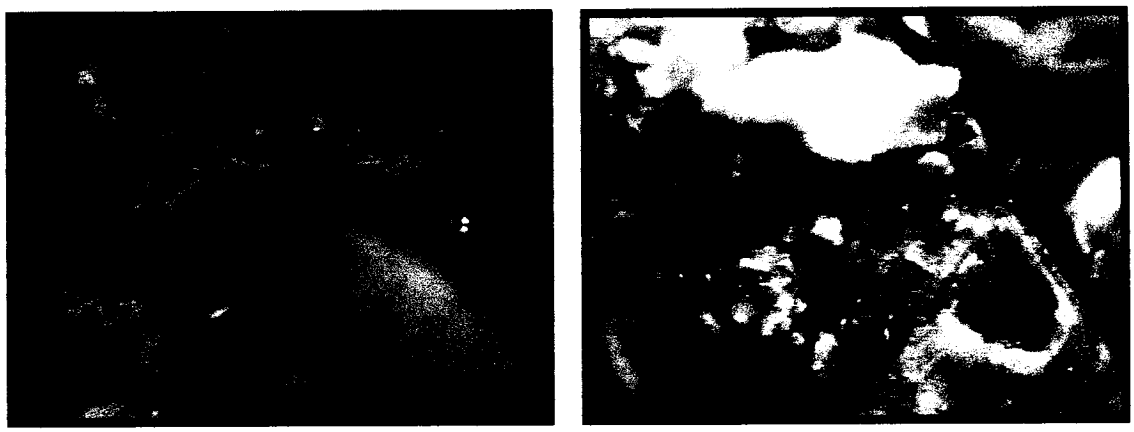
Figure 7.27. Effect of shrimp shell particle size on chitin concentration during the deproteinization process.

7.7.1.2.9 Visualization of Shrimp Shells: Figure 7.28 shows the effect of particle size on the appearance of the shrimp shells at the end of the deproteinization process. Figure 7.29 shows the appearance of the intact and ground shrimp shells under the microscope (60X magnification) at the end of the deproteinization process.



(a) Intact shrimp shells (b) Ground shrimp shells

Figure 7.28. Appearance of shrimp shells after 120 h of deproteinization.



(a) Intact shrimp shells (b) Ground shrimp shells

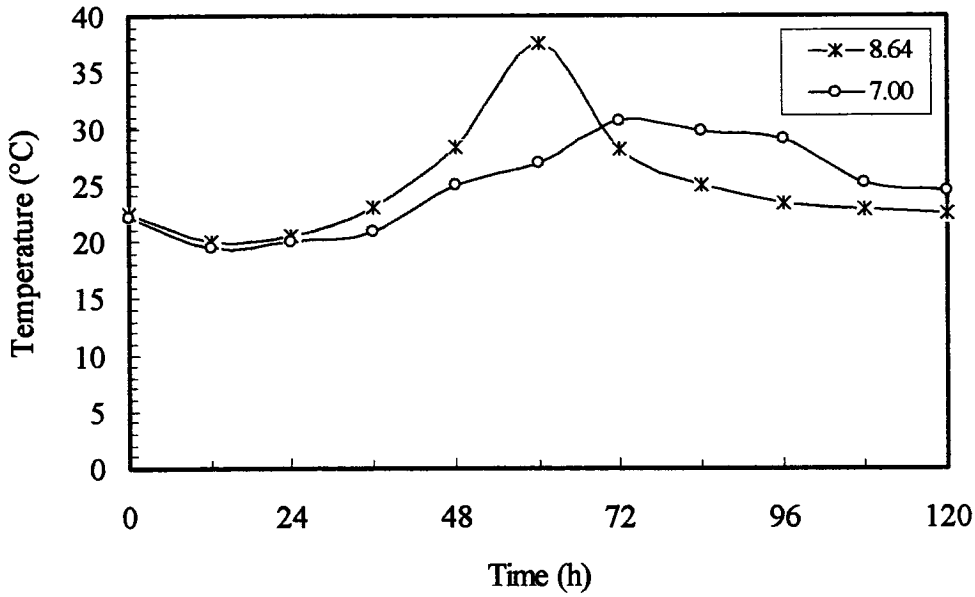
7.29. Visual images (60 X) of the shrimp shells after 120 h of deproteinization.

7.7.1.3 Effect of Initial pH. The effect of initial pH on various parameters (temperature, pH, moisture content, galactose utilization, carbon dioxide evolution, protease activity, residual protein and chitin concentration) during the deproteinization process was investigated.

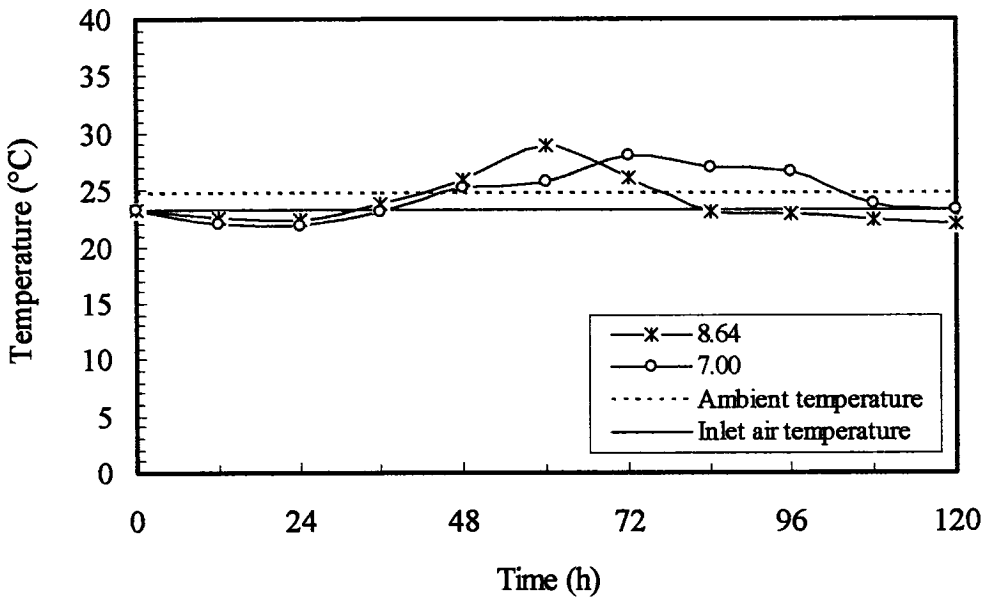
7.7.1.3.1 Temperature: Figure 7.30 shows the change in the temperature of the shrimp shell bed and the exhaust gas during the course of deproteinization as affected by the initial pH. The values are the average of two replicates. The coefficient of variation ranged from 0.29 to 4.97.

7.7.1.3.2 pH: Figure 7.31 shows pH change during the course of deproteinization as affected by the initial pH. The values are the average of two replicates. The coefficient of variation ranged from 0.29 to 3.62%.

7.7.1.3.3 Moisture Content: Figure 7.32 shows the change in moisture content of the shrimp shell during the course of deproteinization as affected by the initial pH. The values are the average of two replicates. The coefficient of variation ranged from 0.24 to 4.53%.



(a) shrimp shells



(b) exhaust gas

Figure 7.30. Effect of initial pH on the shrimp shell and exhaust gas temperatures during the deproteinization process.

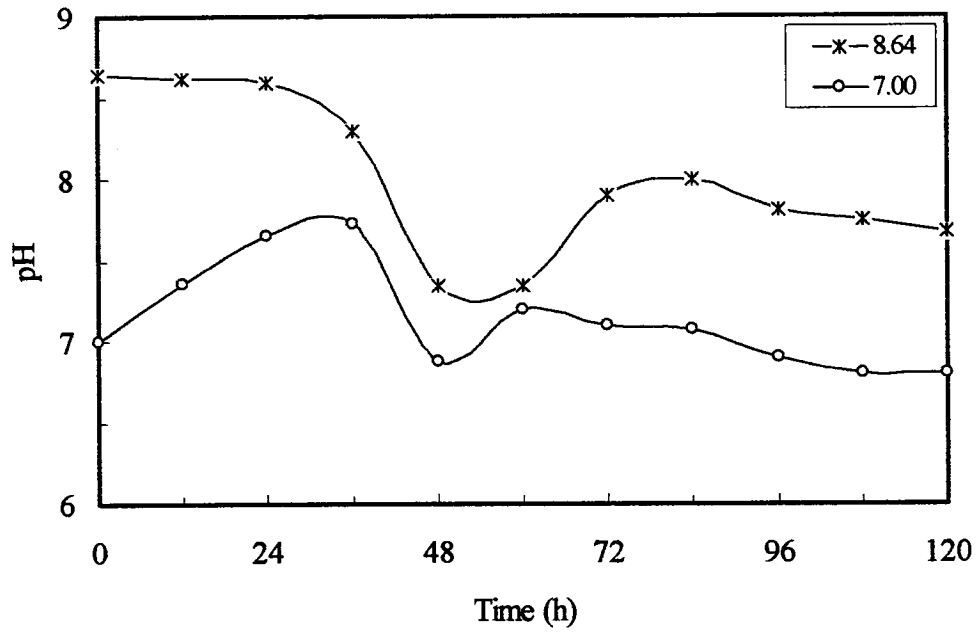


Figure 7.31. Effect of the initial pH on the pH during the deproteinization process.

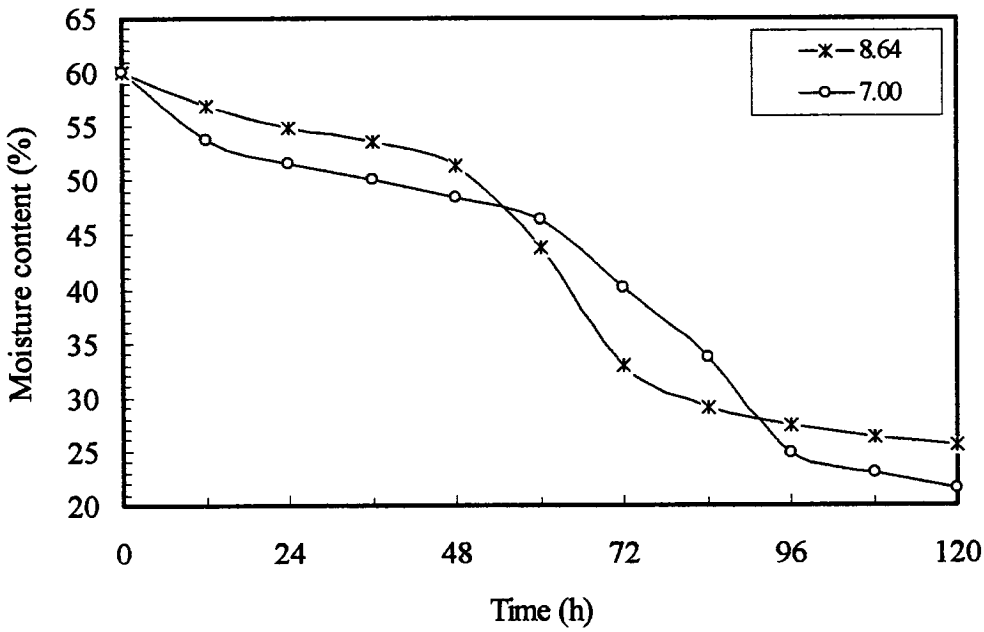


Figure 7.32. Effect of the initial pH on moisture content during the deproteinization process.

7.7.1.3.4 Galactose Utilization: Figure 7.33 shows the residual galactose concentration in the shrimp shells during the course of deproteinization as affected by the initial pH. The values are the average of two replicates. The coefficient of variation ranged from 2.91 to 12.39%.

7.7.1.3.5 Carbon Dioxide Evolution: Figure 7.34 shows the effect of initial pH on carbon dioxide concentration in the exhaust gas. The values are the average of two replicates. The coefficient of variation ranged from 1.99 to 15.34%.

7.7.1.3.6 Protease Activity: Figure 7.35 shows the effect of initial pH on protease activity during the course of deproteinization. The values are presented in units per gram dry shrimp shell waste.

7.7.1.3.7 Protein Concentration: Figure 7.36 shows the effect of initial pH on residual protein in the shrimp shells during the deproteinization process. The values are presented based on the dry weight of the samples.

7.7.1.3.8 Chitin Concentration: Figure 7.37 shows the effect of initial pH on chitin content of the shrimp shells during the deproteinization process. The values are presented based on the dry weight of the samples.

7.7.1.3.9 Visualization of Shrimp Shells: Figure 7.38 shows the effect of initial pH on the appearance of the shell waste at the end of the deproteinization process. Figure 7.39 shows the appearance of the shrimp shells under the microscope (60X magnification) at the end of the deproteinization process.

7.7.1.4 **Effect of Autoclaving.** The effect of Autoclaving on various parameters (temperature, pH, moisture content, galactose utilization, carbon dioxide evolution, protease activity, residual protein and chitin concentration) during the deproteinization process was investigated.

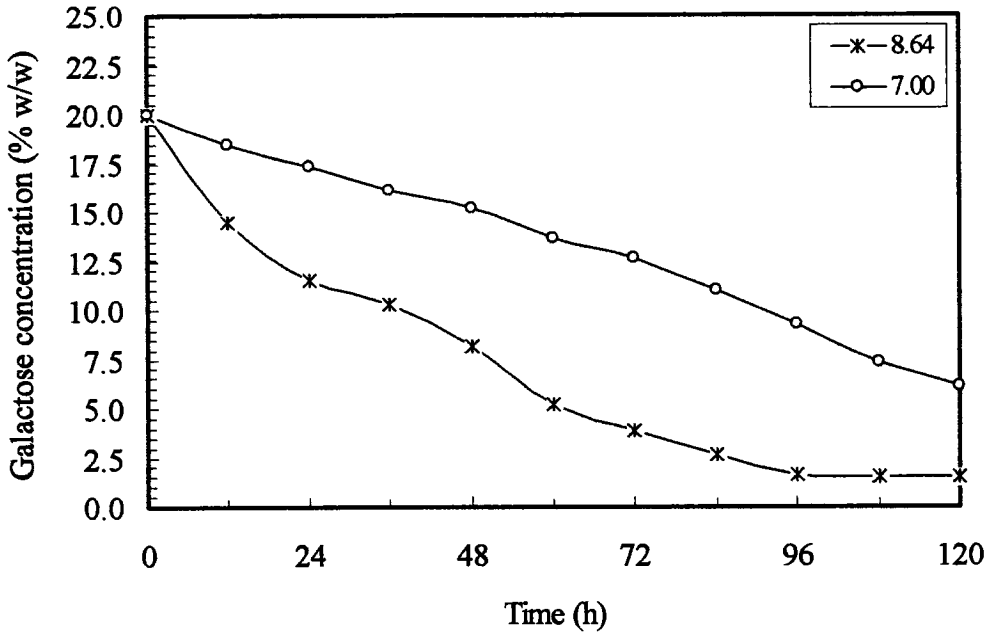


Figure 7.33. Effect of the initial pH on residual galactose during the deproteinization process.

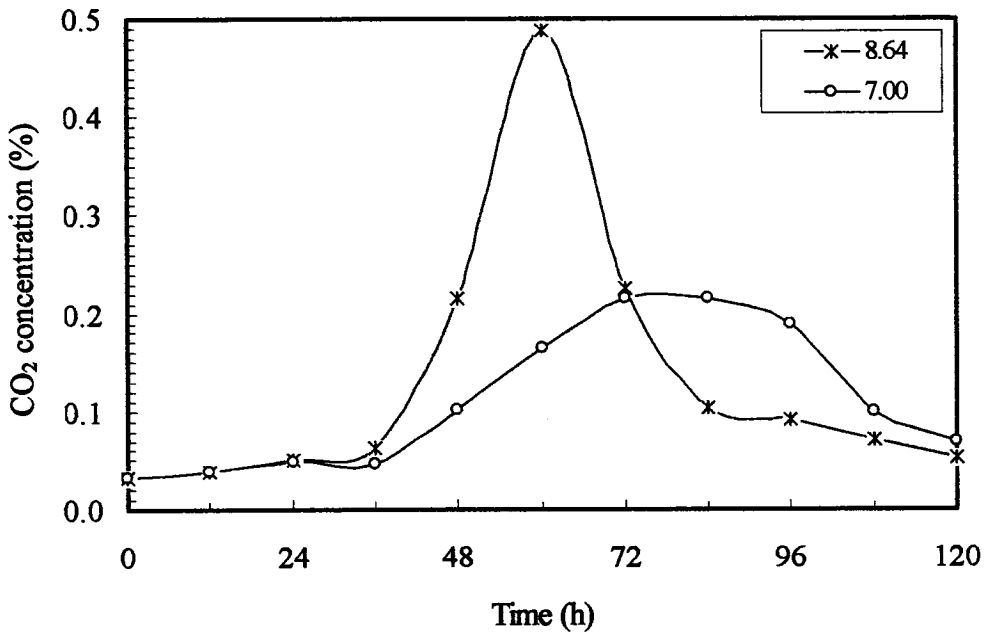


Figure 7.34. Effect of the initial pH on carbon dioxide concentration in the exhaust gas during the deproteinization process.

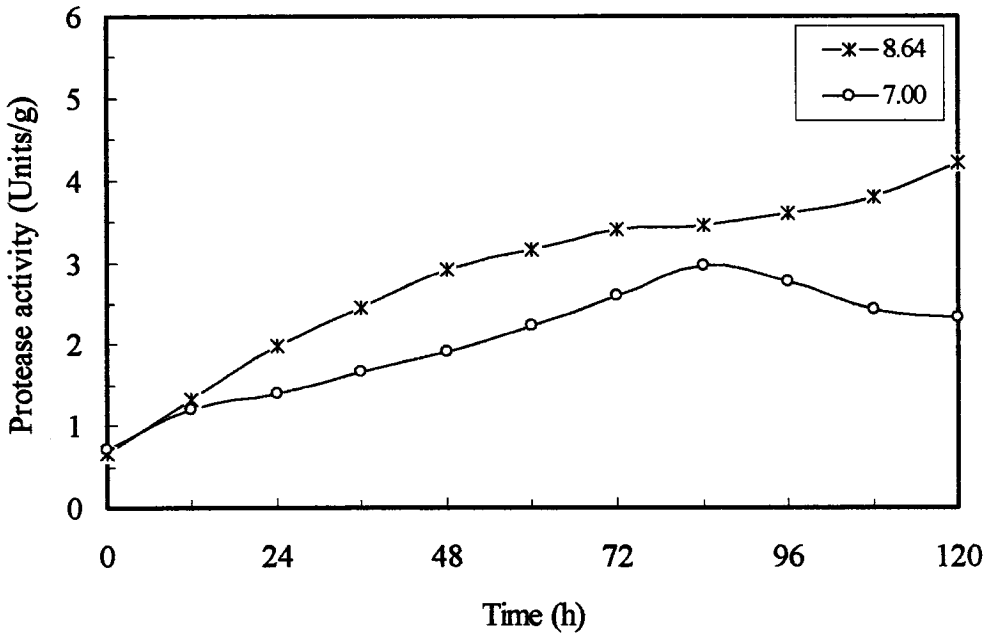


Figure 7.35. Effect of the initial pH on protease activity during the deproteinization process.

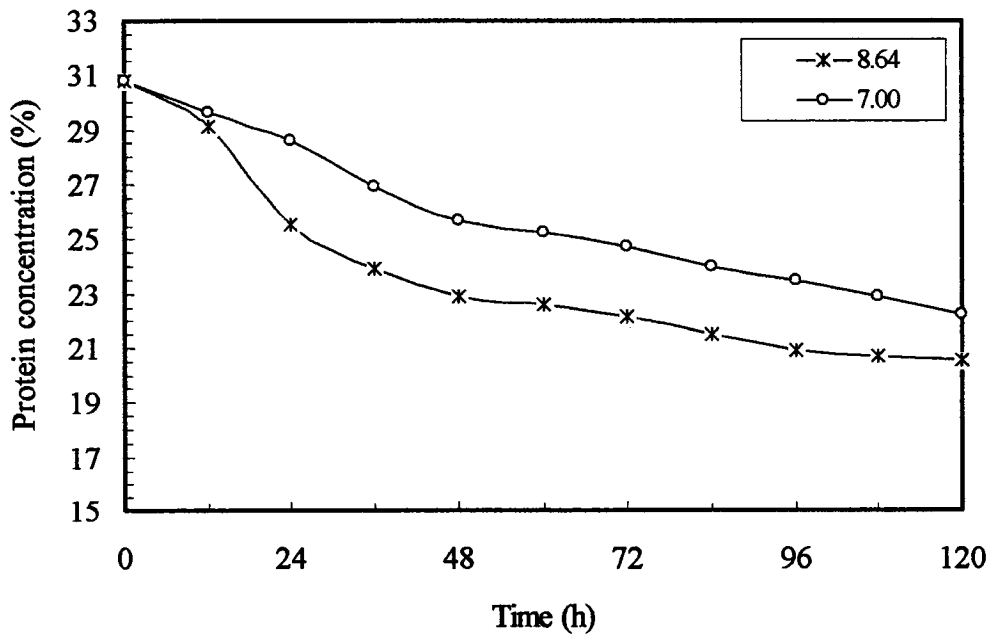


Figure 7.36. Effect of the initial pH on residual protein during the deproteinization process.

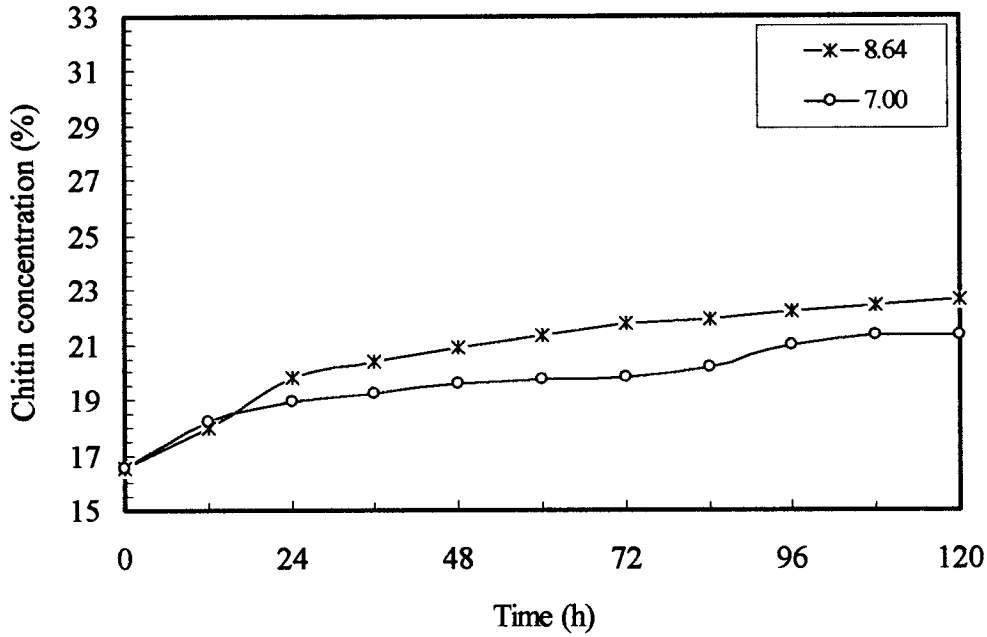
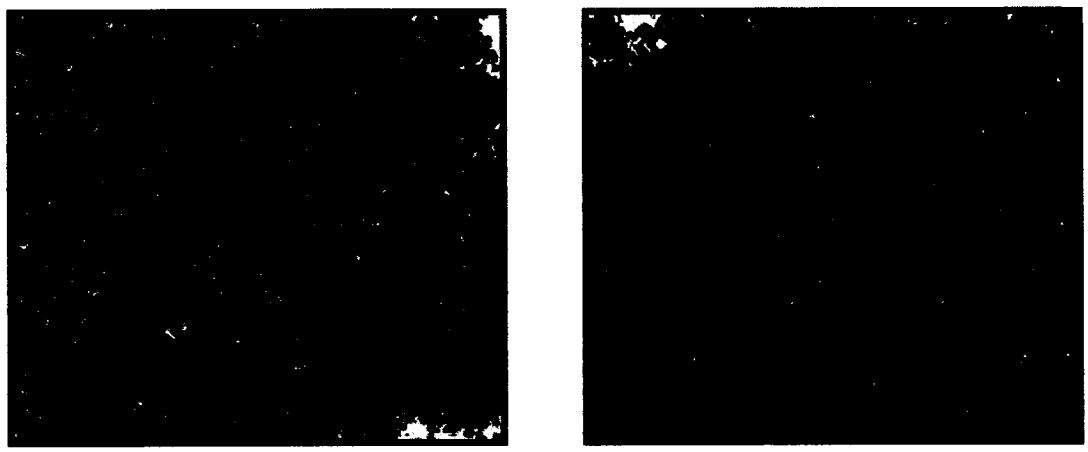
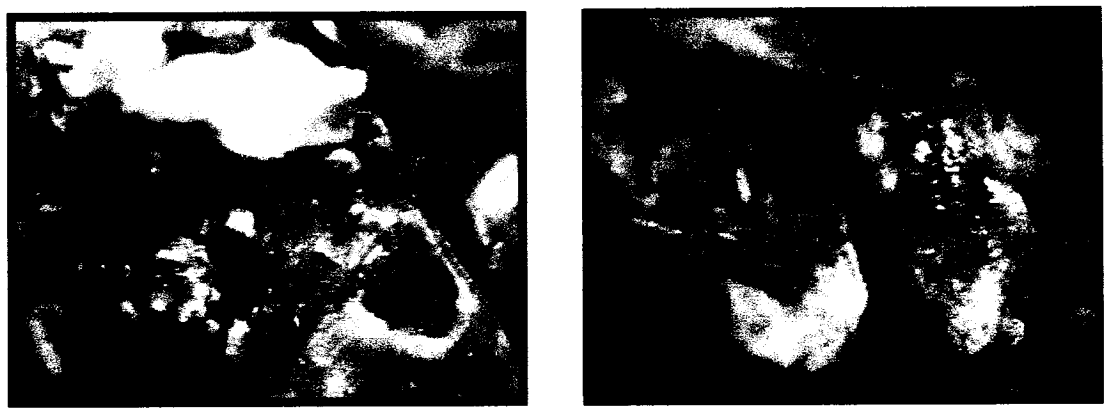


Figure 7.37. Effect of the initial pH on chitin concentration during the deproteinization process.



(a) pH of 8.64 (b) pH of 7.00

Figure 7.38. Appearance of shrimp shells after 120 h deproteinization as affected by the initial pH.



(a) pH of 8.64 (b) pH of 7.00

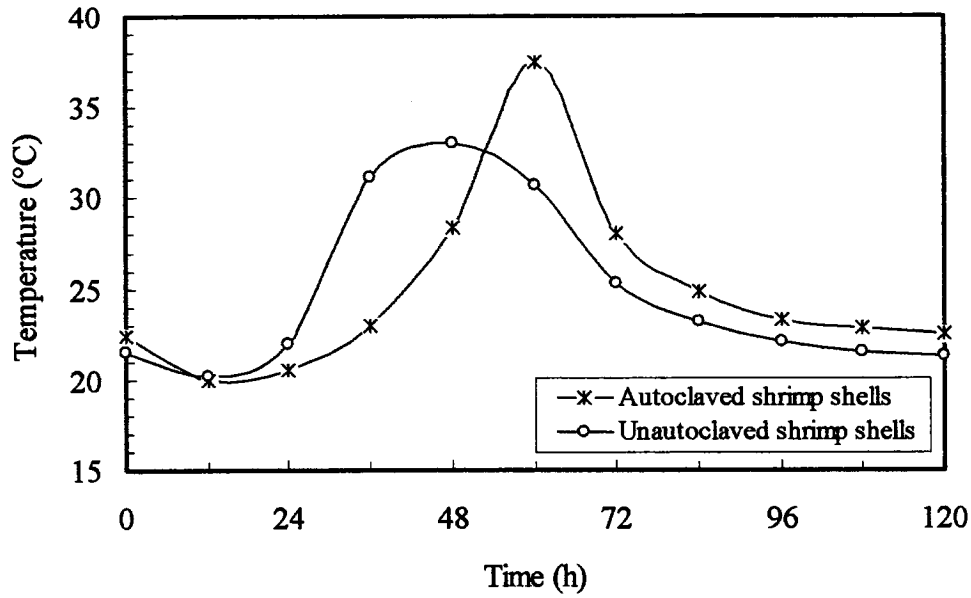
7.39. Visual images (60 X) of shrimp shells after 120 h of deproteinization as affected by the initial pH.

7.7.1.4.1 Temperature: Figure 7.40 shows the change in the temperature of the shrimp shells and the exhaust gas during the course of deproteinization as affected by autoclaving. The values are the average of two replicates. The coefficient of variation ranged from 0.40 to 4.32.

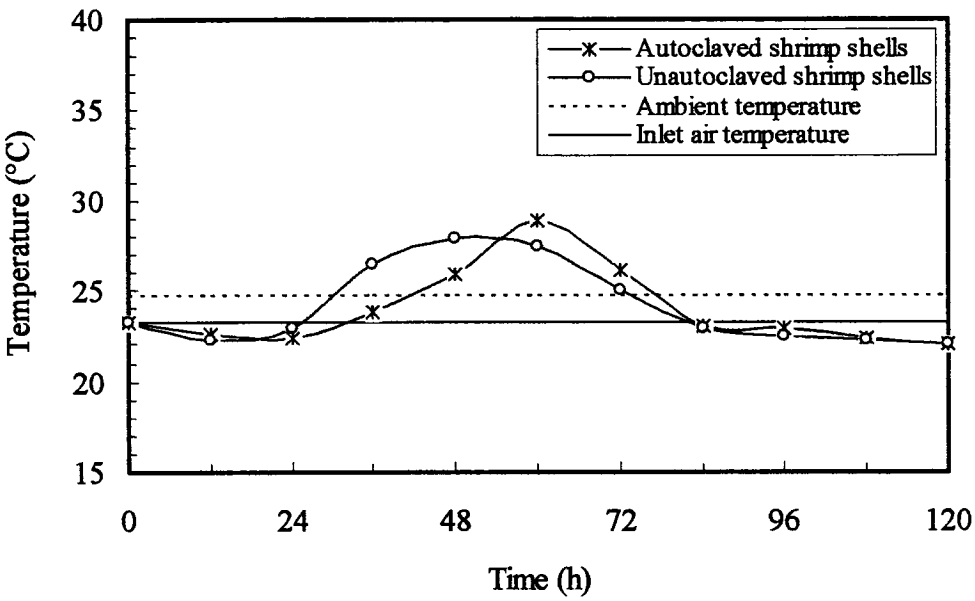
7.7.1.4.2 pH: Figure 7.41 shows pH change during the course of deproteinization as affected by autoclaving of the shrimp shells. The values are the average of two replicates. The coefficient of variation ranged from 0.46 to 3.51%.

7.7.1.4.3 Moisture Content: Figure 7.42 shows the change in moisture content of the shrimp shell during the course of deproteinization as affected by autoclaving of the shrimp shells. The values are the average of two replicates. The coefficient of variation ranged from 0.06 to 8.22%.

7.7.1.4.4 Galactose Utilization: Figure 7.43 shows the residual galactose concentration in the shrimp shells during the course of deproteinization as affected by autoclaving of the shrimp shells. The values are the average of two replicates. The coefficient of variation ranged from 1.92 to 14.51%.



(a) shrimp shells



(b) exhaust gas

Figure 7.40. Effect of autoclaving on the shrimp shell and exhaust gas temperatures during the deproteinization process.

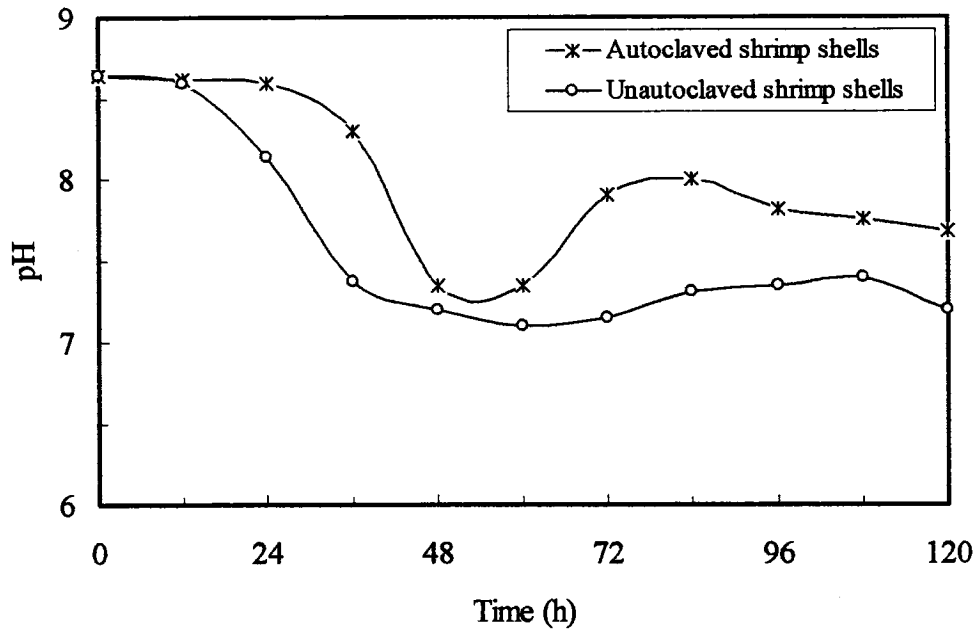


Figure 7.41. Effect of autoclaving on the pH during the deproteinization process.

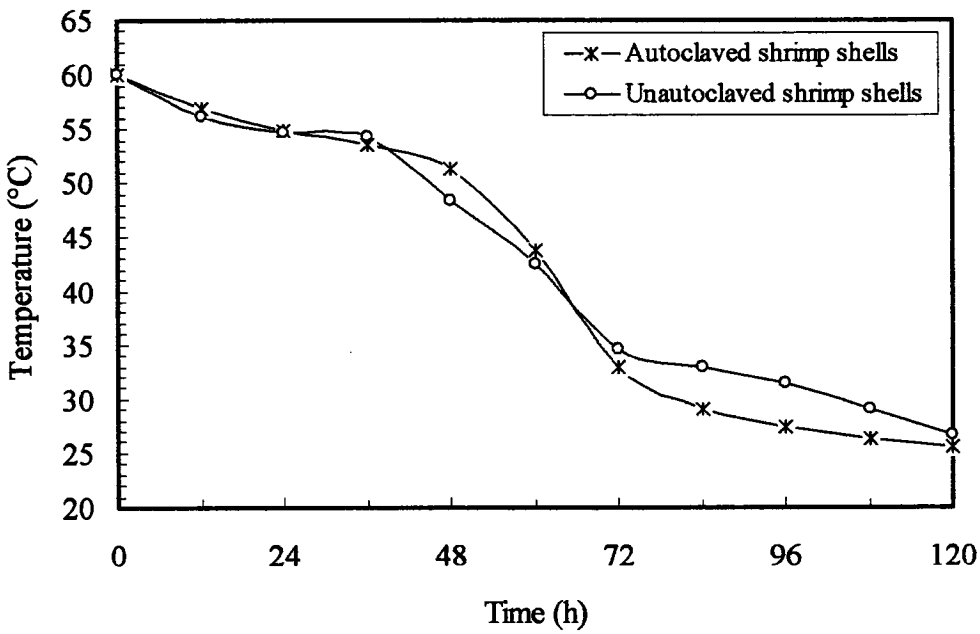


Figure 7.42. Effect of autoclaving on moisture content during the deproteinization process.

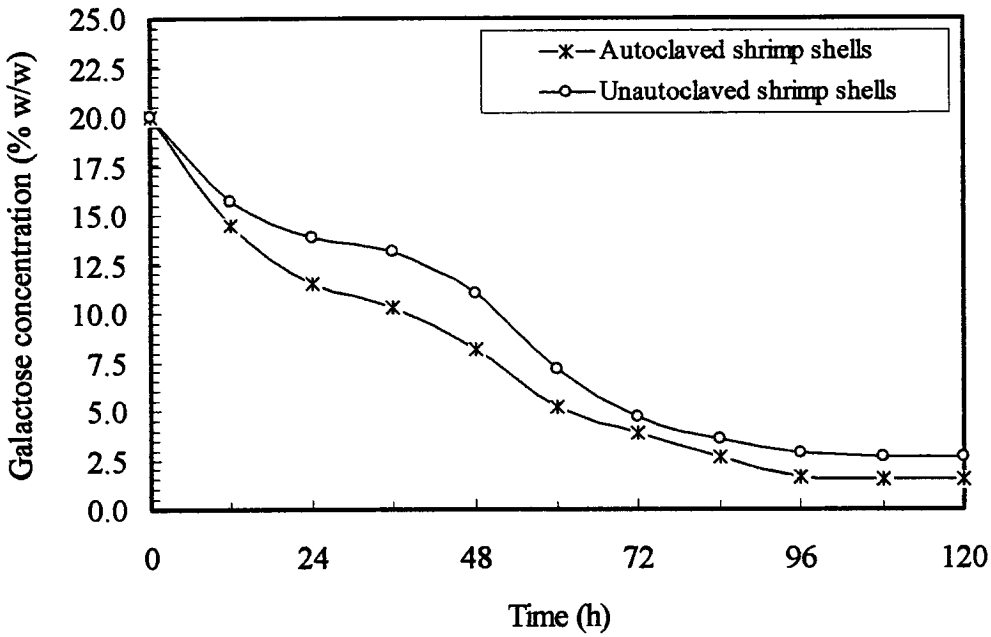


Figure 7.43. Effect of autoclaving on residual galactose during the deproteinization process.

7.7.1.4.5 Carbon Dioxide Evolution: Figure 7.44 shows the effect of autoclaving of shrimp shells on carbon dioxide concentration in the exhaust gas. The values are the average of two replicates. The coefficient of variation ranged from 2.01 to 10.98%.

7.7.1.4.6 Protease Activity: Figure 7.45 shows the effect of autoclaving of shrimp shells on protease activity during the course of deproteinization. The values are presented in units per gram dry shrimp shell waste.

7.7.1.4.7 Protein Concentration: Figure 7.46 shows the effect of autoclaving of shrimp shells on chitin content of the shrimp shells during the course of deproteinization. The values are presented based on the dry weight of the samples.

7.7.1.4.8 Chitin Concentration: Figure 7.47 shows the effect of autoclaving of shrimp shells on residual protein in the shrimp shells during the course of deproteinization. The values are presented based on the dry weight of the samples.

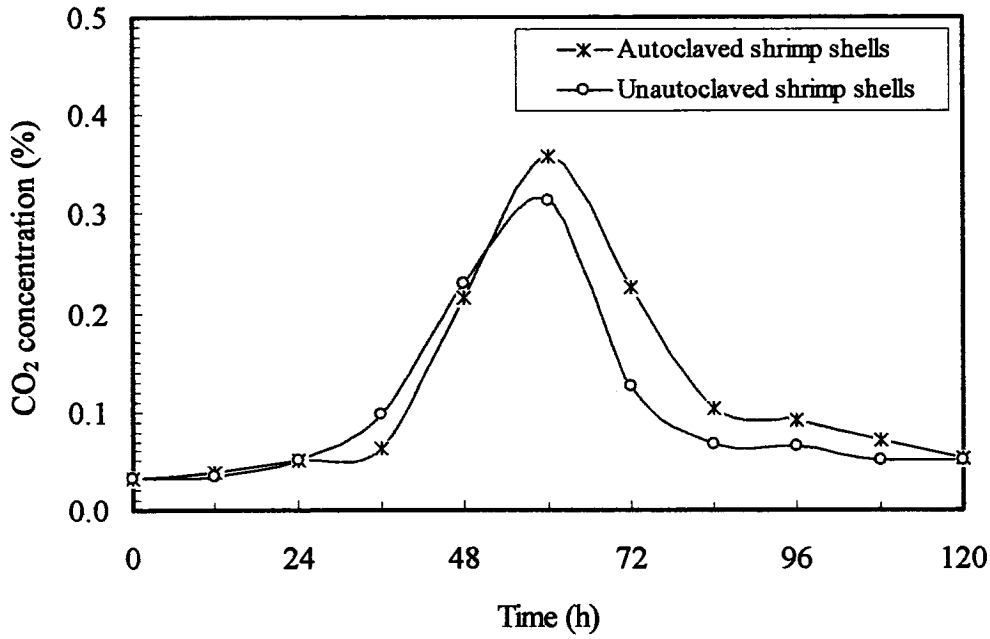


Figure 7.44. Effect of autoclaving on carbon dioxide concentration in the exhaust gas during the deproteinization process.

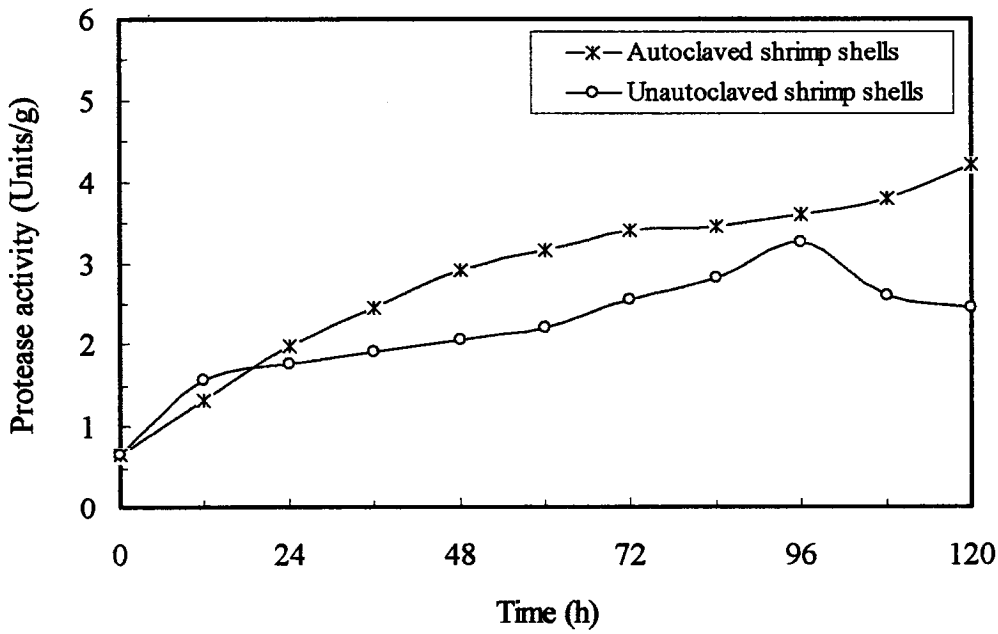


Figure 7.45. Effect of autoclaving on protease activity during the deproteinization process.

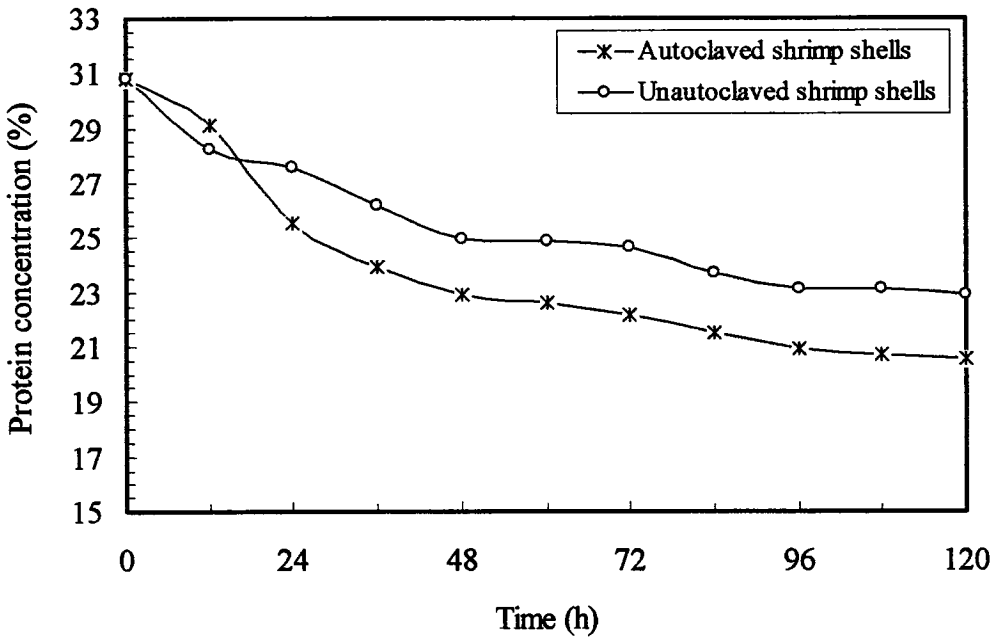


Figure 7.46. Effect of autoclaving on residual protein during the deproteinization process.

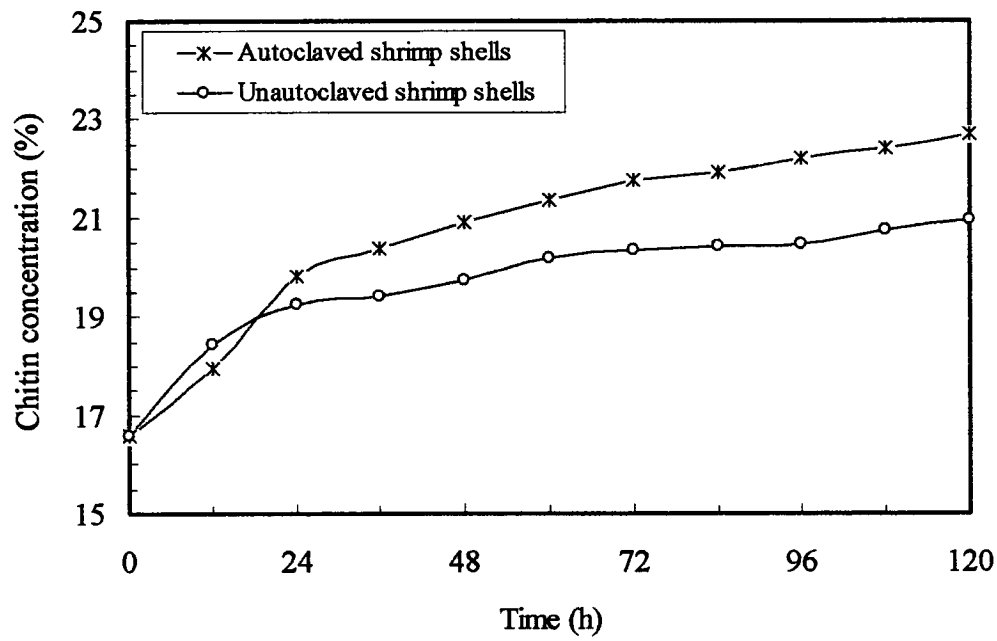
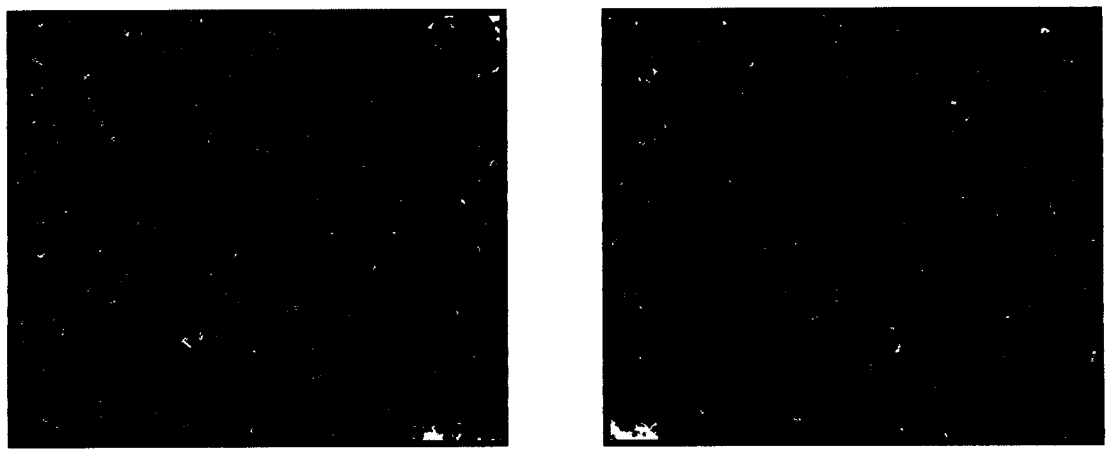


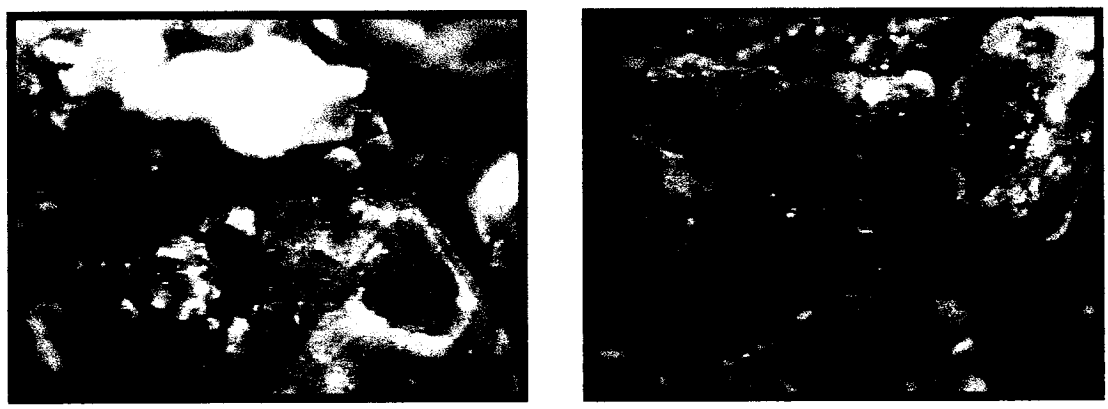
Figure 7.47. Effect of autoclaving on chitin concentration during the deproteinization process.

7.7.1.4.9 Visualization of Shrimp Shells: Figure 7.48 shows the appearance of autoclaved and unautoclaved shrimp shells at the end of the deproteinization process (120 h). Figure 7.49 shows the appearance of autoclaved and unautoclaved shrimp shells under the microscope (60X magnification) at the end of the deproteinization process. Figure 7.50 shows the effect of autoclaving on the microbial population after 24 and 72 h fermentation time.



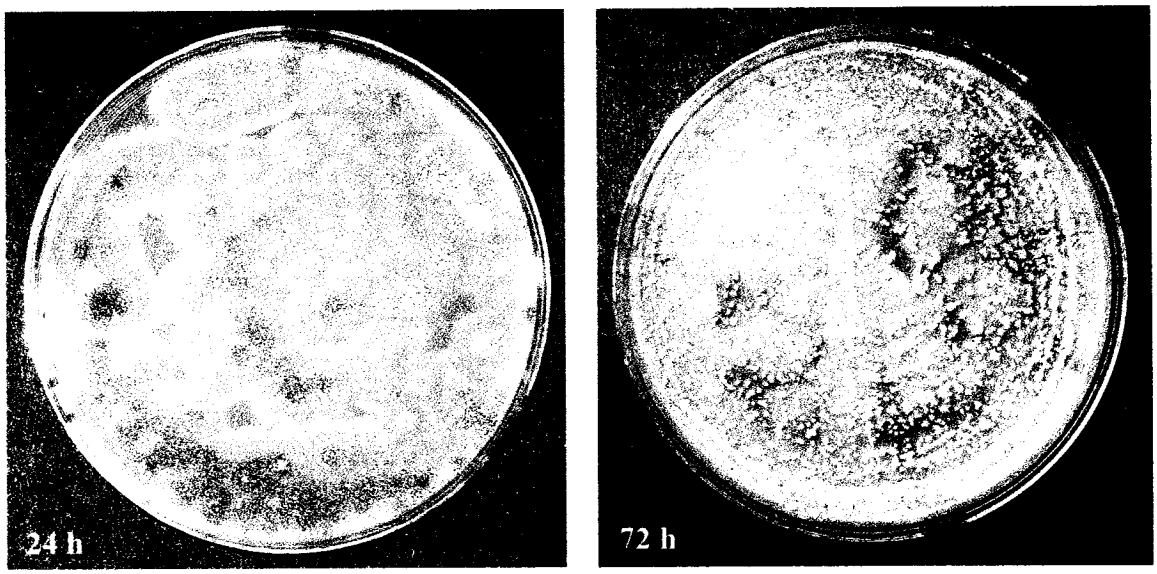
(a) Autoclaved shrimp shells (b) unautoclaved shrimp shells

Figure 7.48. Appearance of autoclaved and unautoclaved shrimp shells after 120 h deproteinization.

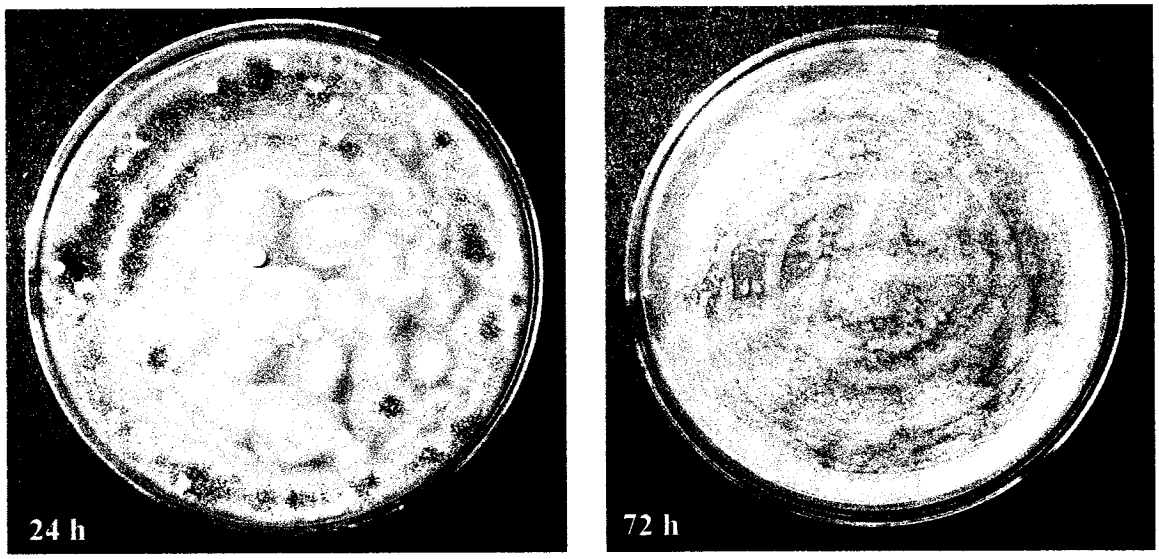


(a) Autoclaved (b) Unautoclaved

7.49. Visual images (60 X) of autoclaved and unautoclaved shrimp shells after 120 h deproteinization.



(a) Autoclaved shells



(b) Unautoclaved shells

Figure 7.50. Plated autoclaved and unautoclaved shrimp shell samples after 24 and 72 h of deproteinization.

7.7.2 Demineralization Process

7.7.2.1 Effect of Acid Type and Demineralization Conditions. Table 7.4 and Figure 5.51 show the minerals composition of microbially deproteinized shells (MDS) and demineralized shells (DS) using hydrochloric, lactic and acetic acids under two

Table 7.4. Effects of acid type and demineralization condition on minerals composition of demineralized shells.

Element	MDS	Concentration (%)					
		HCl		LA		AC	
		A	B	A	B	A	B
Total	30.650	3.150	3.260	3.690	4.600	8.529	9.100
Al	0.078	0.321	0.442	0.123	0.340	0.347	0.050
Ca	13.439	0.115	0.132	0.784	0.702	3.216	4.030
Cu	0.020	0.010	0.005	0.005	0.009	0.006	0.007
Fe	0.056	0.172	0.059	0.053	0.209	0.111	0.051
K	0.016	0.034	0.016	0.016	0.024	0.017	0.010
Mg	0.555	0.047	0.035	0.030	0.072	0.049	0.030
Mn	0.009	0.005	0.010	0.001	0.006	0.010	0.007
Na	0.041	0.045	0.023	0.022	0.073	0.022	0.010
P	2.860	0.046	0.083	0.534	0.410	1.685	1.630
S	0.199	0.235	0.265	0.268	0.293	0.257	0.258
Si	0.460	0.417	0.755	0.192	0.730	0.598	0.180
Others	12.917	1.703	1.435	1.662	1.732	2.213	2.837

A Temperature of 100 °C, shells to acid ratio of 1:50 and retention time of 1 h (Zakaria, 1997)
B Temperature of 24 °C, shells to acid ratio of 1:10 and retention time of 6 h (Ghanem et al., 2003)

Table 7.5. Effects of acid type and demineralization condition on minerals removal efficiency.

Element	Removal efficiency (%)					
	(A)			(B)		
	HCl	LA	AC	HCl	LA	AC
Total Minerals	96.71	94.07	85.56	95.02	92.12	82.55
Calcium	99.73	97.13	87.59	99.54	97.26	82.38

A Temperature of 100 °C, shells to acid ratio of 1:50 and retention time of 1 h (Zakaria, 1997)
B Temperature of 24 °C, shells to acid ratio of 1:10 and retention time of 6 h (Ghanem et al., 2003)

demineralization conditions described by Zakaria (1997) and Ghanem et al. (2003). Table 7.5 and Figure 5.52 show the removal efficiencies of the total minerals and calcium for the demineralized shells.

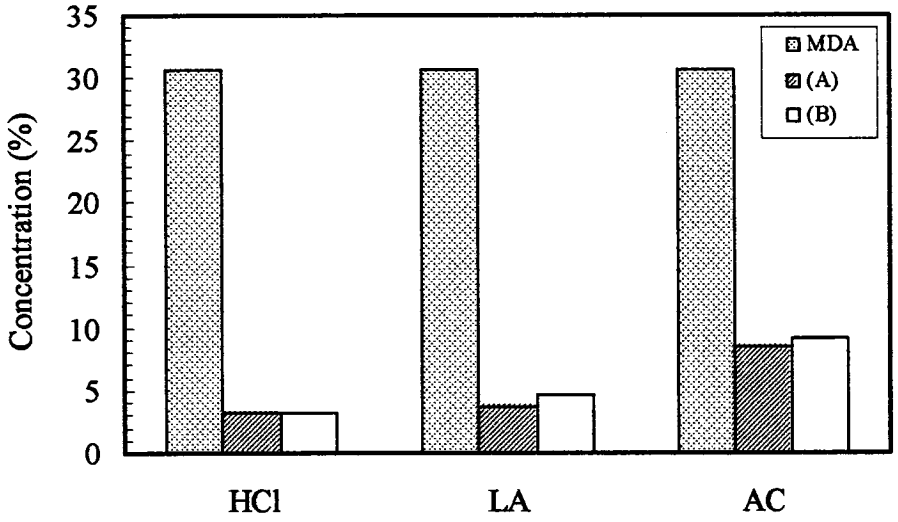
7.7.2.2 Effect of Retention Time. Table 7.6 and Figure 5.53 show the minerals composition of microbially deproteinized shells (MDS) and Demineralized shells (DS) using lactic and acetic acids using demineralization procedure B (1:10 shells to acid ration at room temperature) for 2 and 6 h retention times. Table 7.7 and Figure 5.54 show the removal efficiencies of the total minerals and calcium for the demineralized shells.

7.7.2.3 Effect of Crude Chitin to Acid Ratio. Table 7.8 and Figure 5.55 show the minerals composition of microbially deproteinized shells (MDS) and demineralized shells (DS) using organic acids (lactic and acetic) using the modified demineralization procedure B (at room temperature) at 2 h retention time for 1:10, 1:20 and 1:30 shells to acid ratio. Table 7.9 and Figure 5.56 show the removal efficiencies of the total minerals and calcium for the demineralized shells.

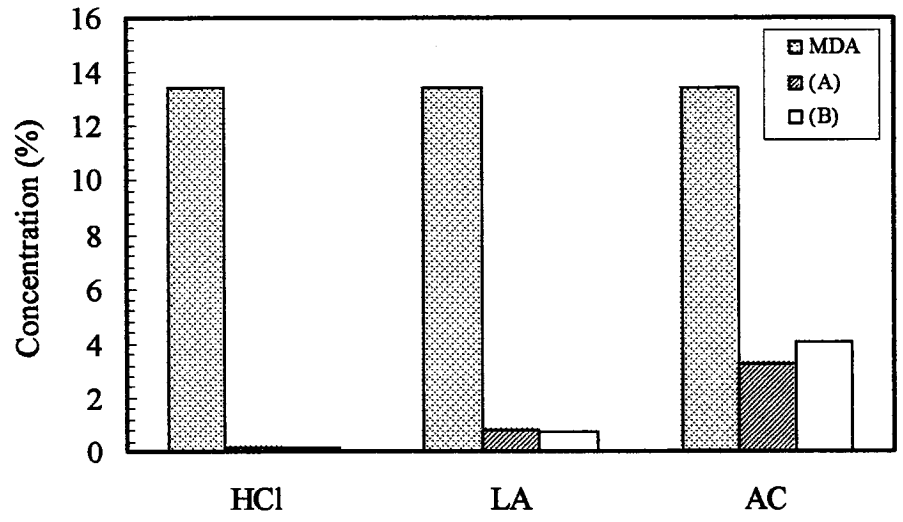
7.8 Discussion

7.8.1 Deproteinization Process

7.8.1.1 Effect of Initial Galactose Concentration. Andrade et al. (2002) reported that although a protein source must be presented for protease production, lack of carbon source was also noticed to cause a significant decrease in protease production. Teng et al. (2001) reported that shrimp shells can serve as nitrogen and mineral sources for the fungus *A. niger* but the medium should be supplemented with a suitable carbon source. El-Samragy et al. (1996) and Chen et al. (1990) used cheese whey permeate as a carbon source for the production of citric acid using a strain of *A. niger* and reported that raw cheese whey can serve as a potential source of carbon and energy for the production of biomass. However, Hossain and Brooks (1983) stated that not all *A. niger* strains are able to readily utilize lactose as a carbon source. Chakraborty et al. (1995) studied the effect of five carbon sources (D-glucose, Sucrose, maltose, lactose and fructose) on the

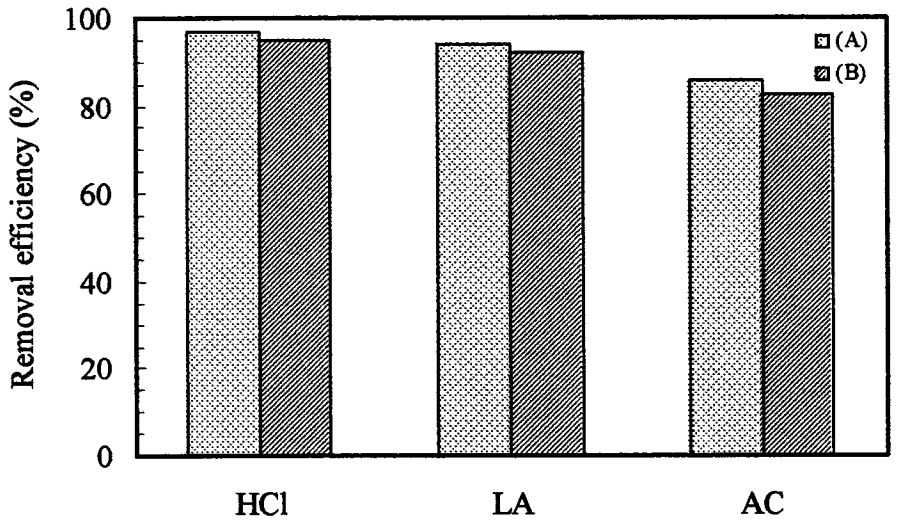


(a) Total minerals

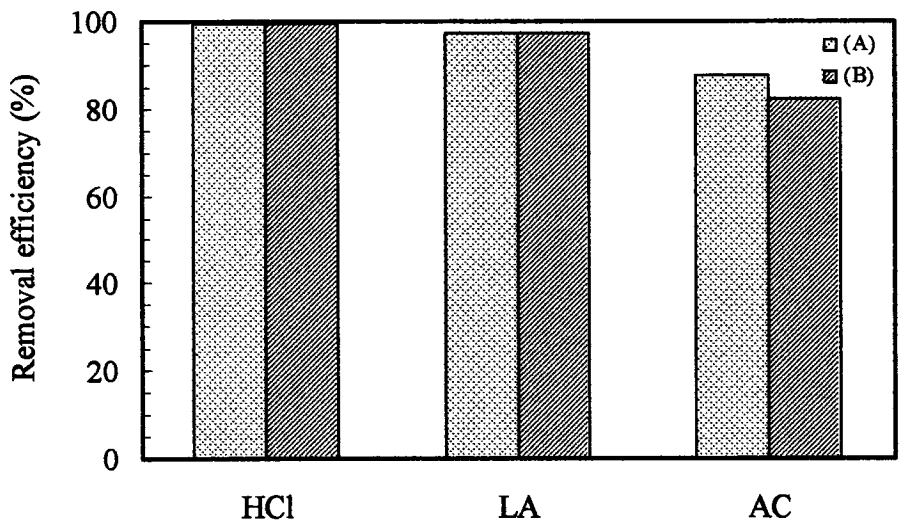


(b) Calcium

Figure 5.51. Concentrations of minerals in microbially deproteinized shells (MDS) and demineralized shells using hydrochloric acid (HCl), lactic acid (LA) and acetic acid (AC) under two demineralization conditions (A: Temperature of 100 °C, shells to acid ratio of 1:50 and retention time of 1 h; B: Temperature of 24 °C, shells to acid ratio of 1:10 and retention time of 6 h).



(a) Total minerals



(b) Calcium

Figure 5.52. Minerals removal efficiency for demineralized shells using hydrochloric acid (HCl), lactic acid (LA) and acetic acid (AC) under two demineralization conditions (A: Temperature of 100 °C, shells to acid ratio of 1:50 and retention time of 1 h; B: Temperature of 24 °C, shells to acid ratio of 1:10 and retention time of 6 h).

Table 7.6. Effect of retention time on minerals composition of demineralized shells using different acids at room temperature.

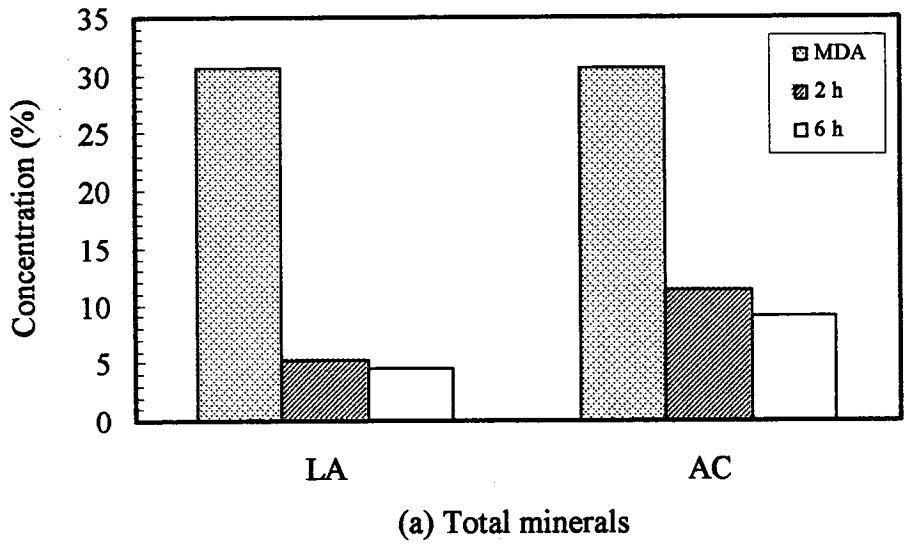
Element	MDS	Concentration (%)			
		LA		AC	
		6 h	2 h	6 h	2 h
Total	30.650	4.600	5.279	9.100	11.388
Al	0.078	0.340	0.356	0.050	0.332
Ca	13.439	0.702	1.563	4.030	4.682
Cu	0.020	0.009	0.014	0.007	0.012
Fe	0.056	0.209	0.201	0.051	0.103
K	0.016	0.024	0.012	0.010	0.033
Mg	0.555	0.072	0.080	0.030	0.089
Mn	0.009	0.006	0.004	0.007	0.008
Na	0.041	0.073	0.117	0.010	0.034
P	2.860	0.410	0.500	1.630	2.385
S	0.199	0.293	0.243	0.258	0.252
Si	0.460	0.730	0.817	0.180	0.506
Others	12.917	1.732	1.372	2.837	2.952

MDS Microbially deproteinized shells
AC Acetic acid
LA Lactic acid

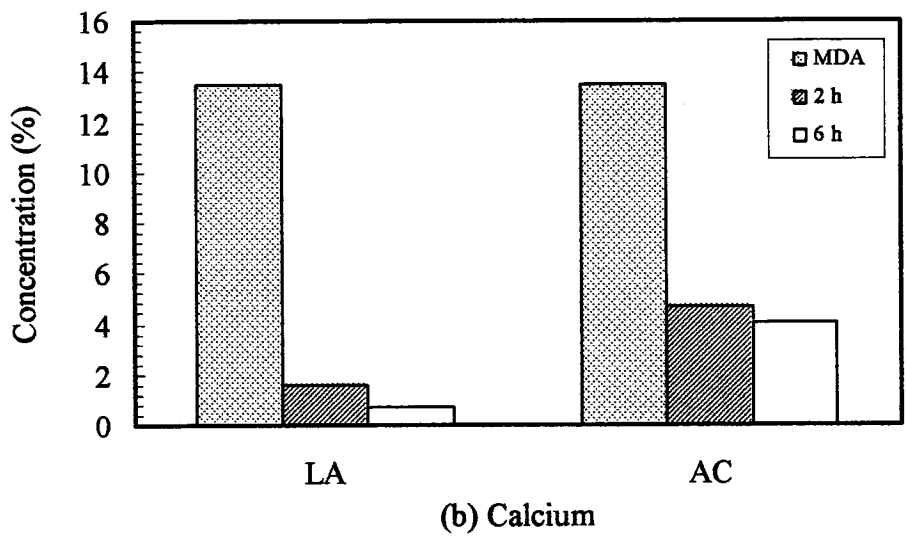
Table 7.7. Minerals removal efficiency as affected by retention time and acid type at room temperature.

Element	Removal efficiency (%)			
	LA		AC	
	2 h	6 h	2 h	6 h
Total Minerals	90.29	92.12	79.56	82.55
Calcium	93.44	97.26	80.84	82.38

LA Lactic acid
AC Acetic acid

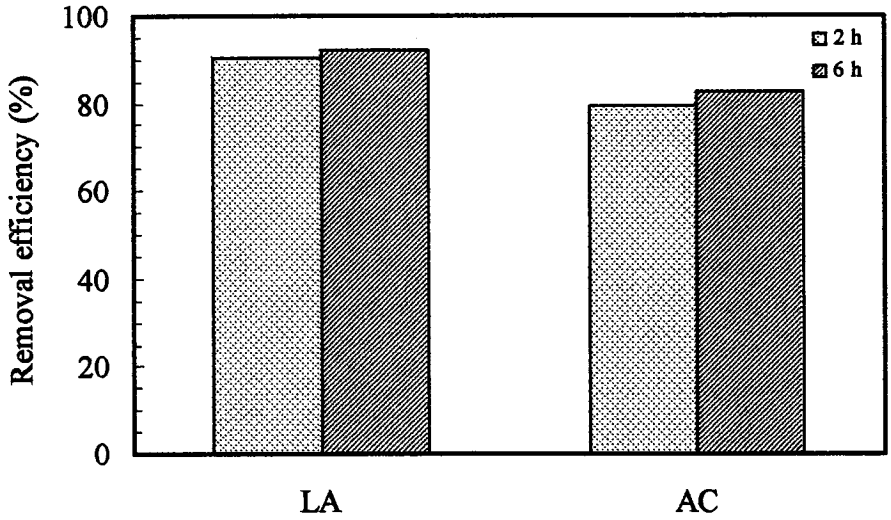


(a) Total minerals

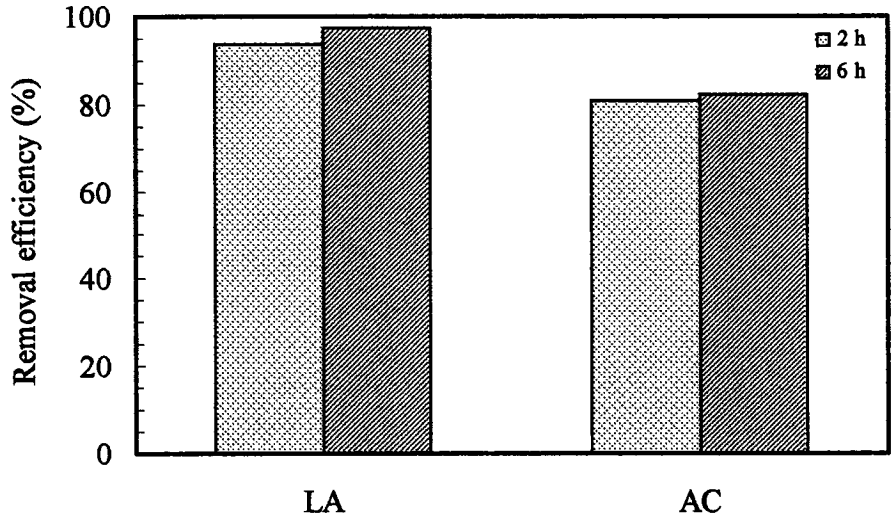


(b) Calcium

Figure 5.53. Concentrations of minerals in microbially deproteinized shells (MDS) and demineralized shells using lactic (LA) and acetic (AC) acids at room temperature and different retention times.



(a) Total minerals



(b) Calcium

Figure 5.54. Minerals removal efficiency for demineralized shells using lactic acid (LA) and acetic acid (AC) at room temperature at different retention times.

Table 7.8. Effect of shells to acid ratio on minerals composition of demineralized shells.

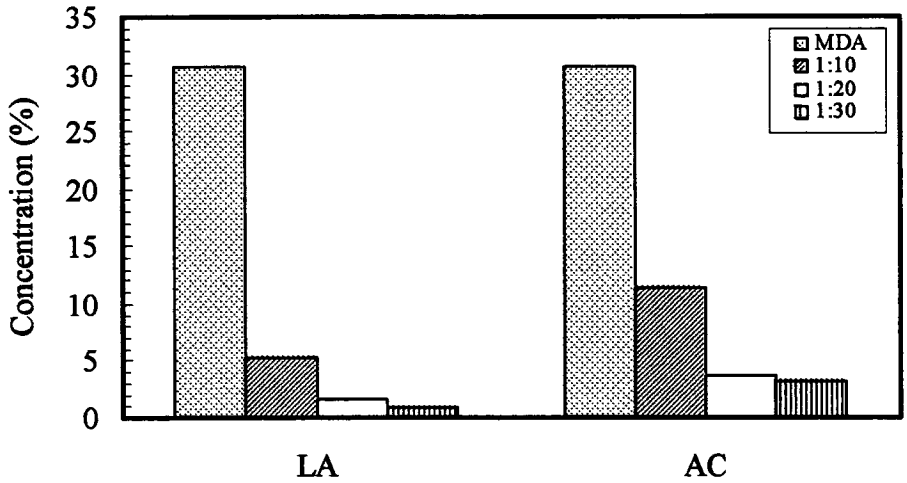
Element	MDS	Concentration (%)					
		LA			AC		
		1:10	1:20	1:30	1:10	1:20	1:30
Total	30.650	5.279	1.509	0.862	11.388	3.710	3.070
Al	0.078	0.356	0.065	0.040	0.332	0.150	0.163
Ca	13.439	1.563	0.481	0.095	4.682	1.750	1.140
Cu	0.020	0.014	0.003	0.003	0.012	0.003	0.003
Fe	0.056	0.201	0.035	0.035	0.103	0.049	0.062
K	0.016	0.012	0.011	0.015	0.033	0.010	0.010
Mg	0.555	0.080	0.046	0.024	0.089	0.020	0.020
Mn	0.009	0.004	0.002	0.001	0.008	0.007	0.006
Na	0.041	0.117	0.014	0.015	0.034	0.020	0.010
P	2.860	0.500	0.240	0.170	2.385	0.910	0.670
S	0.199	0.243	0.244	0.246	0.252	0.280	0.256
Si	0.460	0.817	0.252	0.164	0.506	0.310	0.590
Others	12.917	1.372	0.115	0.054	2.952	0.201	0.140

MDS Microbially deproteinized shells
AC Acetic acid
LA Lactic acid
Retention time = 2 h
Temperature = 24 °C

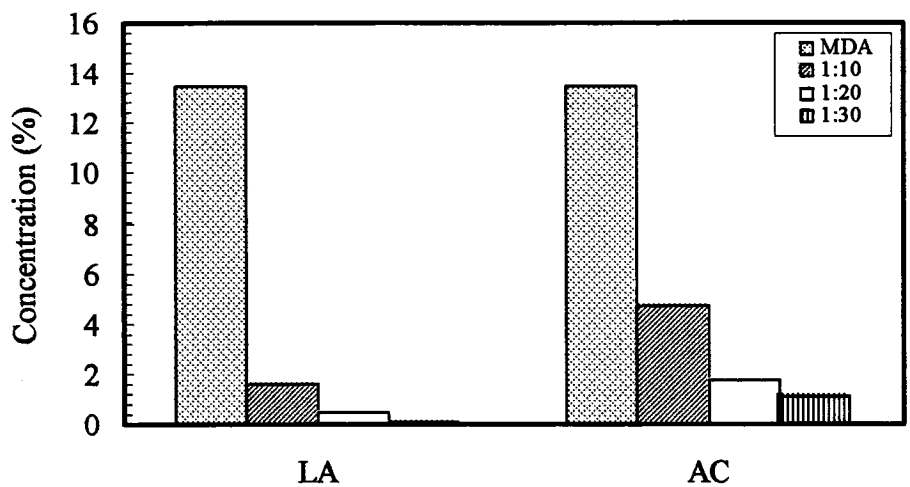
Table 7.9. Minerals removal efficiency as affected by shells to acid ratio.

Element	Removal Efficiency (%)					
	LA			AC		
	1:10	1:20	1:30	1:10	1:20	1:30
Total minerals	90.29	97.40	98.53	79.56	86.36	94.83
Calcium	93.44	99.11	99.63	80.84	85.33	95.62

AC Acetic acid
LA Lactic acid

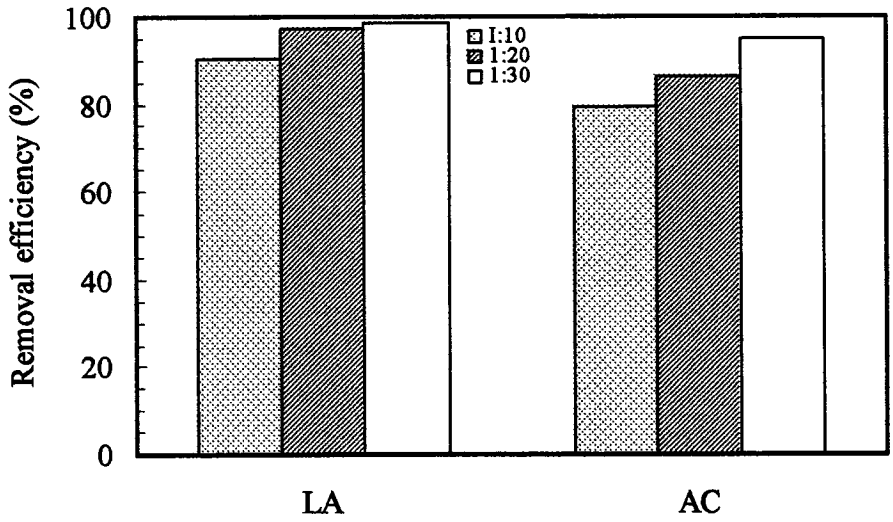


(a) Total minerals

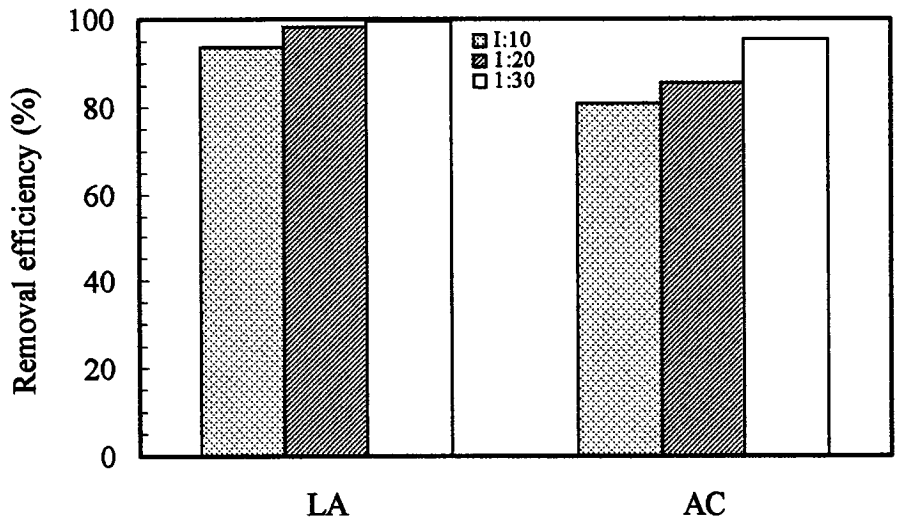


(b) Calcium

Figure 5.55. Concentrations of minerals in microbially deproteinized shells (MDS) and demineralized shells using lactic (LA) and acetic (AC) acids at different shells to acid ratio.



(a) Total minerals



(b) Calcium

Figure 5.56. Minerals removal efficiency for demineralized shells using hydrochloric acid (HCl), lactic acid (LA) and acetic acid (AC) at different retention times.

production of protease from *A. niger* grown in wheat bran. The authors reported that all the sugars tested inhibited the protease production, which indicated catabolic repression. Singh et al. (1975) and Taragano et al. (1997) reported that although glucose enhanced the mycelial growth of *A. niger* it caused inhibition for acid protease synthesis. Ashour et al. (1996) tested the effect of incorporation of six carbon sources (glucose, galactose, fructose, sucrose, lactose, starch and cellulose) into cheese whey on *A. niger* protease production and reported that among the tested carbon sources only galactose (doubled the enzyme yield) and fructose (caused a slight increase in the enzyme yield) had positive effects on the enzyme production. Based on the latter study, galactose was used as an additional carbon source and three initial galactose concentrations (10, 20, 30% w/w) were tested.

7.8.1.1 Temperature: Temperature is one of the most significant parameters in solid-state fermentation since it may affect the growth and activities of microorganisms (Knapp and Howell, 1980; Mudgett, 1986; and Zakaria, 1997). The average ambient air temperature was 23.49 ± 0.56 °C. The inlet air was supplied from the laboratory air supply and had a temperature lower than the ambient temperature (22.25 ± 0.56 °C). Generally, the temperature of the shrimp shell increased as a result of heat production in the bioreactor by metabolic activity (Figure 7.10a). The heat stored in the bioreactor is the net of metabolic heat production minus the heat losses (by conduction through the shrimp shells and the bioreactor walls, by convection with the exhaust gas and latent heat through water evaporation from the shrimp shells). Microorganisms utilize organic matter for synthesis of new microbial cells (growth) and product formation as well as respiration (energy). Grajek (1988), Saucedo-Castañeda et al. (1990), Pandey (1990a), Pandey (1990b), Pandey (1992) and Ghildyal et al. (1993) stated that large amount of heat is generated during solid-state fermentations as a consequence of microbial activity (respiration or oxidation of sugars), which causes heat accumulation and thus temperature increase of the fermentation medium. Aidoo et al. (1982) reported that about 2511.48 kJ/kg solids of heat generation have been reported in several solid-state fermentations for the production of enzymes, biomass and acids.

The initial galactose concentration affected the shrimp shell and exhaust gas temperature profiles. Generally, the temperature of the medium decreased in the first 12 hours regardless of the initial galactose concentrations. At the beginning of the fermentation process, heat losses from the bioreactor due to evaporation (latent heat) and the cooling effect of the inlet air was higher than heat generation since microorganisms usually experience a lag period during which the microorganisms adapt themselves to the new environment. A typical batch growth curve (Figure 7.57) includes: (a) lag phase, during which microbial population remains unchanged and adaptation to the surrounding environment takes place, (b) logarithmic or exponential growth phase, during which the specific growth rate is constant (c) deceleration growth phase, during which growth decelerates either because of the depletion of an essential nutrient or the accumulation of an inhibitory product, (d) stationary phase, during which the net growth rate is zero as a result of the balancing of reproduction rate by an equivalent death rate and (e) death phase, during which cells die faster than new cells are produced.

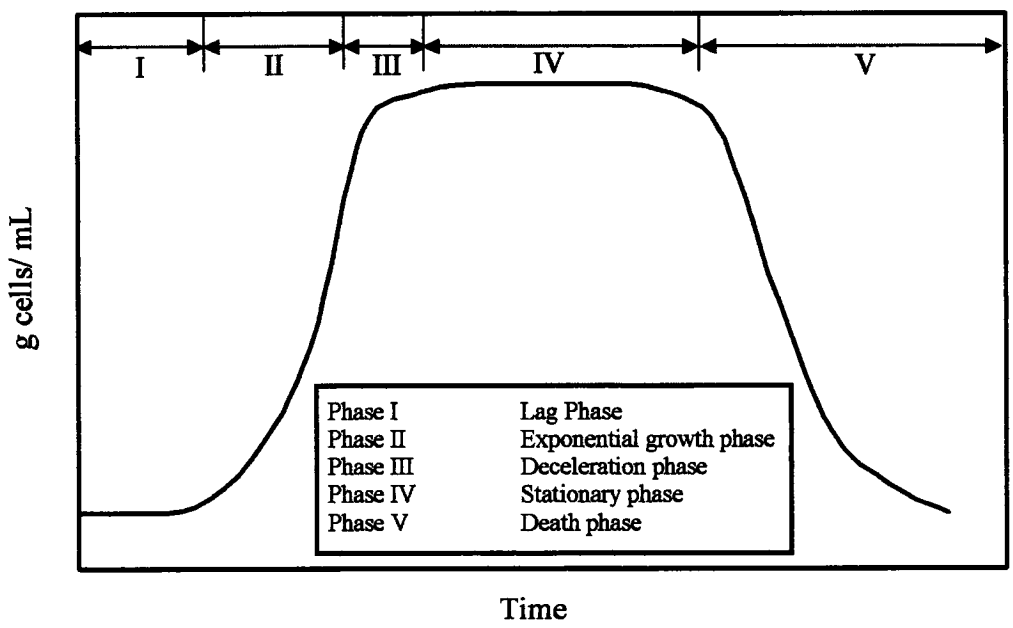


Figure 7.57. Typical batch growth curve.

Several studies (Ghaly et al., 1992a; Ghaly et al., 1992b; Ben-Hassan et al., 1993; Ghaly and El-Taweel, 1995; and Ghaly et al., 2003) showed that the microbial growth and the temperature curves are similar in shape and the temperature curve can be used to determine microbial kinetics. Table 7.10 shows some of the growth kinetic parameters, calculated based on shrimp shell temperature measurements obtained in this study. The lag phase and the specific growth rate were determined graphically according to the procedure described by Ghaly et al. (1989).

Table 7.10. Growth kinetic parameters[a] at various initial galactose concentrations.

Parameter	Initial galactose concentration (% w/w)		
	10	20	30
Lag phase (h)	12	12	12
Exponential phase (h)	60	60	96
Specific growth rate (h^{-1})	0.0042	0.0089	0.0058
Stationary phase (h)	2	5	11
Initial temperature (°C)	21.8	21.8	21.8
Maximum temperature (°C)	28.9	38.3	32.9
Time to reach maximum temperature (h)	72	72	108

[a] values were calculated from the temperature curve

No change in the lag period (12 h) was observed when the galactose concentration increased from 10 to 30% w/w. Also, no change in the length of the exponential growth phase (48 h) was obtained when the galactose concentration was increased from 10 to 20% w/w. A further increase in the galactose concentration (30% w/w) increased the length of the exponential growth phase to 96 h. The stationary phase increased from 2 to 5 h as initial galactose concentration increased from 10 to 20% w/w. A further increase in the initial galactose concentration (30% w/w) increased the stationary phase to 11h. The maximum temperatures attained in this study were 28.9, 38.3 and 32.9 °C after 72, 72 and 108 h for 10, 20 and 30% w/w initial galactose concentration, respectively.

The specific growth rates for *A. niger* were found to be 0.0042, 0.0089 and 0.0058 h^{-1} for the initial galactose concentrations of 10, 20 and 30%, respectively. These values were very low compared to those of 0.343 – 2.452 h^{-1} obtained by Haq et al. (2005) for *A. niger* grown at initial pH of 6.0 on molasses sugar supplemented with different concentrations of ammonium nitrate as a source of nitrogen. Esfahani et al. (2004) attained a maximum specific growth rate of 0.29 h^{-1} for *A. niger* grown on wheat bran in solid-state fermentation at an initial pH of 4.0, an initial moisture content of 55% and a temperature of 35 °C. Pedersen et al. (2000) reported a specific growth rate of about 0.2 h^{-1} for *A. niger* grown in batch culture for the production of glucoamylase.

The results showed that the maximum temperature attained at the initial galactose concentration of 20% w/w was much higher than that attained at initial galactose concentration of 10% w/w (38.3 °C vs. 28.9 °C) as shown in Figure 7.58. This indicated that galactose at 10% w/w concentration was a limiting factor for microbial growth as evidenced by the metabolic heat production. Further increase in the initial galactose to 30% w/w concentration caused inhibition; longer exponential growth phase (96 h) and lower maximum temperature (32.9 °C). The rise in the exhaust gas temperature was due to heat losses by convection and followed a similar trend to that of the shrimp shells temperature (Figure 7.10). The temperature of the exhaust gas reached maximum values of 24.9, 29.0 and 25.8 °C after 72, 72 and 108 h for initial galactose concentrations of 10, 20 and 30%, respectively.

Several authors reported on the temperature rise in bioreactors during solid-state fermentation. Gowthaman et al. (1993) obtained a temperature profile for the production of amyloglucosidase using wheat bran and *A. niger* in solid-state fermentation similar to the one obtained in this study. Pandey (1990a) reported a maximum substrate temperature of 41 °C during solid-state fermentation of wheat bran using *A. niger* in a 500-mL flask for the production of the enzyme glucoamylase. Rathbun and Shuler (1983) reported 3 °C/cm temperature gradient during active mold growth within a bed depth of 6.35 cm when fermenting soybeans using *Rhiopus oligosporus*. Saucedo-Castañeda et al. (1990) reported an axial temperature gradient of 0.17 °C/cm and a radial temperature gradient of

5 °C/cm in a packed-bed bioreactor during the fermentation of cassava using *A. niger*. Ghildyal et al. (1993), Ghildyal et al. (1994), and Pandey (2003) reported that temperatures in the middle of the bed can reach about 20 °C higher than the temperature of the inlet air. In the current study, the peak temperature of the shells material was about 7, 17 and 11 °C higher than the inlet air temperature for initial galactose concentration of 10, 20, and 30% w/w, respectively. There were no temperature gradients in the radial or axial direction due to mixing. Szewczyk and Myszka (1994) studied the effect of temperature on the growth of *A. niger* in solid-state fermentation and found that the net growth constant started to decrease at temperatures above 32 °C as a result of temperature deactivation. Yang and Chiu (1986) indicated that the mycelial growth had maximal value at 26 °C. Based on their results they recommended the cultivation of *A. niger* at 26-30 °C for 24 h for mycelium propagation and then adjust the temperature to 20-26 °C for maximal protease production.

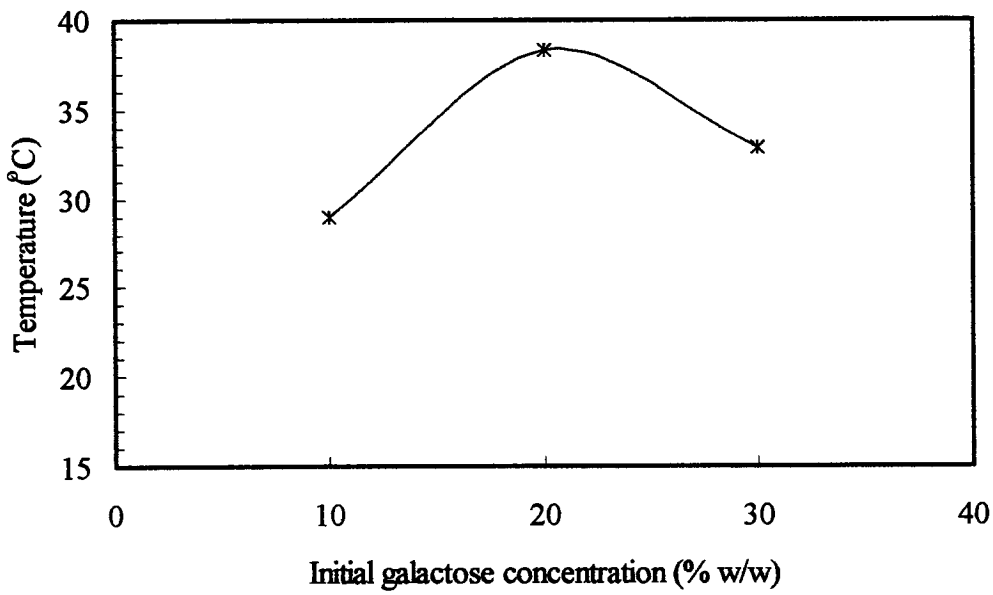


Figure 7.58. Maximum shrimp shells temperature as affected by initial galactose concentration.

7.8.1.1.2 pH: The results showed that the pH of the shrimp shells decreased with time up to a certain value and then increased again. The decrease in the pH of the shrimp shells

was due to acid protease production and deproteinization process while the increase in the pH of the shrimp shell was due to the buffering capacity of the calcium carbonate released from the shrimp shells (Yang and Ling, 1989; and Zakaria et al., 1998) as well as the production of ammonium nitrogen (Yang, 1988). The lowest pH was 6.55, 6.63 and 5.92 which was reached after 36, 72 and 108 h of deproteinization, after which the pH started to increase reaching 8.32, 7.9 and 6.28 by the end of the deproteinization process (144 h) for initial galactose concentrations of 10, 20 and 30% w/w, respectively.

Teng et al. (2001) reported a decrease in the medium pH (between 3 and 6) as a consequence of shrimp shells deproteinization using *A. niger*. Villegas et al. (1993) stated that changes in pH due to the growth of microorganisms are linked to the ionic balance established by substrate uptake and product formation. Andrade et al. (2002) reported that the medium pH decreased during the period of enzyme synthesis and then stayed constant up to the end of fermentation during protease production using *Mucor circinelloides* as a result of metabolites accumulation resulting from D-glucose degradation. Yang (1993) stated that microbial utilization of ammonium sulfate can cause pH decrease while nitrate and urea cause the pH to rise as a result of nitrate reduction to $R\text{-}NH_3$ or urea decomposition and suggested that pH control during solid-state fermentation can be achieved by using different ratio of ammonium salts and urea, ammonium nitrate or sodium nitrate, or a buffering agent such as $CaCO_3$. Matsushima et al. (1981) indicated that the drop in pH during acid protease production might be due to the accumulation of organic acid and the residue of sulfate ion in the utilization of $(NH_4)_2SO_4$. Zakaria et al. (1998) reported a drop in the pH (minimum value of 5) over the first 48 h of lactic acid fermentation of scampi waste after which the pH increased reaching a final value of 6.6 as a result of the buffering capacity of the solubilized calcium. Beaney et al. (2005) reported rapid decrease in pH (less than 3.5 over 7 days) during lactic acid fermentation of prawn shells as a result of metabolic lactic acid production.

7.8.1.1.3 Moisture Content: In this study, the initial moisture content was adjusted to 60%. The results showed that initial galactose concentration affected the moisture content of the shrimp shells. Generally, the moisture content declined with time during the

deproteinization process reaching 43.71, 36.20 and 25.91% by the end of the deproteinization process for the galactose concentrations of 10, 20 and 30% w/w, respectively. The net moisture content of the shrimp shell bed can be defined by the following equation:

$$MC_{net} = MC_i + MC_m - MC_e \quad (7.9)$$

Where:

MC_{net}	Is the net moisture content (%)
MC_i	Is the initial moisture content (%)
MC_m	Is the contribution of metabolic heat production to moisture content (%)
MC_e	Is the amount of moisture lost through evaporation with the exhaust gas (%)

The initial slight decrease in the moisture content indicated a period of microbial activity where metabolic water was produced (Yang and Ling 1989; Yang, 1993; and Asagbra et al., 2005) while the steep decline in the moisture content observed with the 20 and 30% w/w galactose concentrations indicated that moisture lost through evaporation overweighed the metabolic water production. Yang and Ling (1989) reported on the importance of moisture content in solid-state fermentation and stated that low moisture content caused the substrate to be too dry for microbial growth and product formation and high moisture content caused packing of the substrate and prevention of gas exchange. Yang and Chiu (1986) reported optimum initial moisture content between 50-58% for the production of protease by solid-state fermentation of sweet potato residue supplemented with rice bran and minerals using *A. niger*. The authors stated that an initial moisture content of 71% caused the substrate to stick together preventing gas exchange and causing low protease production, whereas an initial moisture content of 41% made the substrate too dry for mycelial growth causing very poor protease production. Diaz et al. (1993) reported that microbial activity became inhibited as moisture content decreases below 40% and it completely ceased when moisture content reached 8 to 12%. In this

study, the initial moisture content of the shrimp shells was adjusted to 60%, which fell below 40% (36.20 and 25.91%) during the entire deproteinization process for the initial galactose concentrations of 20 and 30% w/w, respectively.

7.8.1.1.4 Residual Galactose Concentration: The results showed that galactose concentration decreased with time for all initial galactose concentrations. For the initial galactose concentration of 10% w/w, about 70% of the added galactose was depleted after 48 h of deproteinization whereas for the initial galactose concentration of 20%, the galactose concentration decreased gradually over the first 72 h reaching 10.8% w/w then rapidly thereafter reaching 3.3% w/w after 96 h of deproteinization. For initial galactose concentration of 30% w/w, the galactose concentration decreased slightly reaching 27.5% w/w over the first 48 h of deproteinization and then decreased at a higher rate reaching 3.8% w/w after 120 h of deproteinization. About 91.00, 90.85 and 83.35% of the initial galactose of 10, 20 and 30% w/w were utilized, respectively. Residual galactose concentration of 1.80, 1.83 and 3.33% w/w remained unutilized by the end of the deproteinization process. Andrade et al. (2002) studied the effect of initial D-glucose concentrations (40, 60 and 80 g/L) on the production of extracellular proteases by *Mucor circinelloides* and stated that at high D-glucose concentrations (60 and 80 g/L), the substrate was not totally consumed while at low D-glucose concentration (40 g/L) no residual glucose was detected by the end of cultivation (96 h). Haq et al. (2005) reported 58.67% sucrose utilization after 144 h incubation during citric acid production using *A. niger*.

7.8.1.1.5 CO_2 Concentration: The results showed that concentration of carbon dioxide in the exhaust gas with time followed the same trend as the temperature of the shrimp shells. A strong correlation between the concentration of carbon dioxide in the exhaust gas and the temperature of the shrimp shells (Figure 7.59) was obtained. Gowthaman et al. (1993) also obtained a CO_2 profile similar to the temperature profile during solid-state fermentation of wheat bran using *A. niger* for the production of amyloglucosidase. Temperature and carbon dioxide evolution are considered strong indicators of microbial activity. Metabolic heat as well as CO_2 evolution have been used as indirect methods for

biomass quantification during solid-state fermentation (Sugama and Okazaki, 1979; Okazaki et al., 1980; Carrizalez et al., 1981; Rathbun and Shuler, 1983; Lonsane et al., 1985; and Bellon-Maurel et al., 2003). Carrizalez et al. (1981) used carbon dioxide measurement data for the determination of microbial growth constant and reported a specific growth rate of 0.31 h^{-1} for *A. niger* culture on cassava flour as a soul carbon source. Rathbun and Shuler (1983) stated that the relationship of CO_2 evolution to growth is accurate since carbon dioxide can evolve from endogenous metabolism and also possibly from anaerobic metabolism of sugars. The authors used the rate of CO_2 evolution (gas flow times mole fraction CO_2) as a measure of the rate of microbial growth.

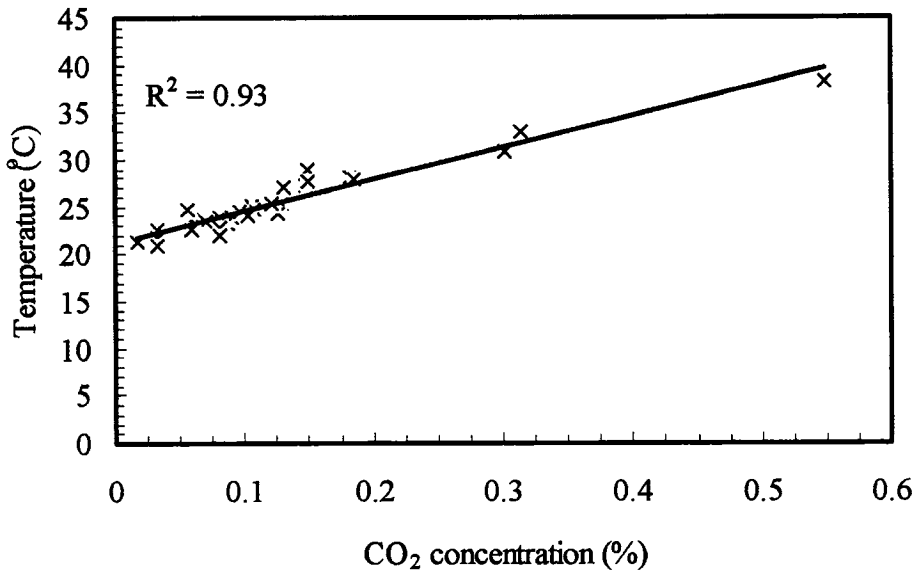


Figure 7.59. Correlation between carbon dioxide concentration in the exhaust gas and temperature of the shrimp shells.

7.8.1.1.6 Protease Activity: The results showed that initial galactose concentration affected the protease activity. The protease activity increased gradually with time from an initial value of 0.71 units per gram dry weight reaching 1.82, 1.85 and 1.77 units per gram dry weight for the 10, 20 and 30% w/w initial galactose concentrations, respectively. During the course of the deproteinization process, the protease activities for

initial galactose concentration of 20% w/w were significantly higher than those for initial galactose concentrations of 10 and 30% w/w.

Zielińska and Kączkowski (1971) stated that acid proteases are produced by many black *Aspergilli* such as *A. avamori*, *A. saitoi* and *A. niger* and that the production of these enzymes is stimulated by the presence of certain concentration of organic nitrogen especially proteins. However, the protein source also affects the proteolytic activity since the amino acid composition is a limiting factor in the synthesis of acid protease. Teng et al. (2001) screened 34 fungal strains for their proteolytic activity and concluded that most of the tested *A. niger* strains were found to give higher protease activity compared to the other strains. Ashour et al. (1996) reported that protease yield from the fungus *A. niger* during cheese whey fermentation increased with incubation period and reached a maximum value after 6 days. Ray et al. (1992) reported that protease production was greater during the exponential growth phase of the yeast *Candida humicola*. Bulthuis et al. (1991) reported a predominant protease production during the logarithmic and early stationary phase and low but steady production during the stationary phase of *Bacillus licheniformis*. Andrade et al. (2002) reported a decrease in the yield factor for protease produced on glucose consumed and an increase in the yield factor for biomass on glucose with increasing the initial D-glucose concentration from 40 to 80 g/L indicating a catabolic repression. Teng et al. (2001) reported protease activities in the range of 0.8 ± 0.3 to 6.8 ± 0.4 units (one unit activity was defined as 1 μM of tyrosine produced in 1 min) for 17 *A. niger* strains after 5 days of incubation at 25 °C and 200 rpm of the spores in a medium (pH 7.4) containing 1.0% casein, 0.4% D-glucose, 0.4% yeast extract, 1.0 g/L $K_2HPO_4.3H_2O$, 0.5 g/L $MgSO_4.7H_2O$and 0.1 mL/L Vogel trace element solution. The results obtained in this study are within that range.

7.8.1.1.7 ***Protein Concentration:*** The results showed that protein concentration of the shrimp shells decreased with deproteinization time for the three initial galactose concentrations as a result of protein break down caused by the proteolytic enzymes produced by *A. niger*. The protein concentration decreased from an initial value of

30.84% to final values of 22.34, 20.77, and 25.30 % over 132 h of deproteinization for the 10, 20 and 30% w/w initial galactose concentrations, respectively.

The highest protein removal efficiency (about 33.33%) was achieved with initial galactose concentration of 20% w/w. Several authors reported protein removal efficiencies in the range of 55-95% from shellfish waste using either purified enzymes or microorganisms that produce proteolytic enzymes (Shimahara et al., 1982; Cano-Lopez et al., 1987; Shimahara et al., 1988; Bustos and Healy, 1994; and Wang and Chio, 1998). The deproteinization time in these studies ranged from as short as 24 h to as long as 16 days. Zakaria et al. (1998) used lactic acid fermentation for chitin recovery from scampi waste and reported that about 77.5% of the original scampi waste protein was solubilized during the fermentation process (5 days). Beaney et al. (2005) reported about 50% decrease of the original protein concentration in prawn shell waste using lactic acid fermentation and stated that complete deproteinization through a purely biotechnological process seems hard to achieve. The low deproteinization efficiency observed in this study could be due to: (a) the large particle size of the shrimp shells, (b) the high pH of the shrimp shells that might have interfered with protease synthesis and/or activity and (c) protein denaturation that might have happen during shrimp shells autoclaving.

7.8.1.1.8 Chitin Concentration: The results showed that chitin concentration in the shrimp shells increased with deproteinization time as a result of protein removal. The chitin concentration increased from an initial value of 16.59% to final values of 20.48, 21.99, and 20.24% over 132 h of deproteinization for the 10, 20 and 30% w/w initial galactose concentrations, respectively. The values of chitin concentration were determined in the deproteinized samples without demineralization. The shrimp shells used in this study contained 31.73% minerals. Zakaria et al. (1998) reported an increase in the concentration of chitin (from 12.05 to 17.48%) as a result of lactic acid fermentation of scampi waste. Cira et al. (2002) reported increase in chitin concentration (from 11.4-13.1% to 20.3-23.2%) as a result of lactic acid fermentation of shrimp waste. Beaney et al. (2005) reported 67.9-72.3% chitin after lactic acid fermentation of prawn shells.

7.8.1.1.9 Visualization of Shrimp Shells: The appearances of the shrimp shells were compared at the end of the deproteinization process (144 h) as affected by the initial galactose concentrations. The appearance of the spent shrimp shells obtained from the experiment that received initial galactose concentration of 10% w/w almost did not change and had a pale pink-orange color with some tan patches. A fair amount of moisture remained in the deproteinized shells. For the initial galactose concentration of 20% w/w, the spent shells had a pale pink-orange color with some tan patches. The spent shrimp shells obtained from this run were not as wet as the spent shrimp shells obtained from the run that received 10% w/w initial galactose concentration. The existence of the pink-orange color was an indication of the presence of pigments, which were not utilized during the fermentation process. The spent shrimp shells obtained from the run that received an initial galactose concentration of 30% w/w appeared to be very dry and had a gray-black color due to the presence of *A. niger* spores. High galactose concentration (30% w/w) inhibited the growth of *A. niger* and enhanced the non productive sporulation. Beaney et al. (2005) compared chitin purified by lactic acid fermentation and chitin extracted by a chemical method and reported that the color of extracted chitin using lactic acid fermentation ranged from pale pink-orange to off-white with tan tones due to the presence of impurities such as pigments and proteins in the extracted chitin. However, they reported a light tan color for chitin extracted by the chemical method as a result of the removal or degradation of most of the pigments.

The spent shells were also inspected under the microscope at 60X magnification (Figure 7.19). Some white precipitants were noticed on the shells obtained from the runs that received initial galactose concentrations of 10 and 20% w/w (Figure 7.19a and b). The white precipitants were more in case of initial galactose concentration 20% w/w and were believed to be the substances that were broken down from the shell matrix during the deproteinization process. For initial galactose concentration of 30% w/w (Figure 7.19c), the white precipitants were not noticed and the shells were covered with a large amount of spores.

7.8.1.2 Effect of Particle Size.

7.8.1.2.1 Temperature: The results showed that the temperature profiles for intact and ground shrimp shells were similar. The growth kinetics was determined graphically from the temperature data according to the procedures described by Ghaly et al. (1989). Table 7.11 shows some of the growth kinetic parameters for intact and ground shrimp shells. Lag periods of 12 and 24 h were observed for the intact and the ground shells, respectively. The exponential growth phase was 60 h for intact shells and 36 h for ground shells. The maximum temperatures attained were 38.3 and 37.8 °C after 72 and 60 h for intact and ground shells, respectively. Size reduction of the shrimp shells increased the surface area to volume ration, thus increasing the fraction of substrate (galactose and protein) initially available for the microorganisms. As more substrate was available, the culture organism experienced longer lag period and shorter exponential growth phase. The specific growth rate for *A. niger* was 0.0089 and 0.0221 h^{-1} for intact and ground shells, respectively.

Table 7.11. Growth kinetic parameters[a] for intact and ground shrimp shells.

Parameter	Particle size	
	Intact	Ground
Lag phase (h)	12	24
Exponential phase (h)	60	36
Specific growth rate (h^{-1})	0.0089	0.0221
Stationary phase (h)	5	4
Initial temperature (°C)	21.8	21.8
Maximum temperature (°C)	38.3	37.8
Time of maximum temperature (h)	72	60

[a] values were calculated from the temperature curve

The average inlet air temperature was 22.8 ± 0.59 °C. The temperature of the exhaust gas increased as a result of heat losses from the shrimp shells by convection. The exhaust gas temperature profile was similar to the shrimp shell temperature profile. The temperature of the exhaust gas reached maximum values of 29.0 and 28.9 °C after 72 and 60 h for

intact and ground shells, respectively. Mudgett (1986) reported that smaller particle size results in smaller packing densities of the substrate and less void space between the substrate particles, which might reduce the area for heat transfer and gas exchange with the surroundings. However, this was not an issue in this study since agitation was provided allowing for more heat transfer and gas exchange.

7.8.1.2.2 pH: The results showed that the pH of the shrimp shells decreased from an initial value of 8.64 to final values of 6.63 and 7.34 after 72 and 48 h of deproteinization for intact and ground shells, respectively. Then, the pH increased reaching 7.77 and 7.68 by the end of the deproteinization process (120 h) for the intact and ground shells, respectively.

7.8.1.2.3 Moisture Content: The moisture content decreased with time slightly during the first 48 h of deproteinization, then sharply thereafter. The results showed that the shrimp shells particle size affected the moisture content during the deproteinization process. The moisture content declined from an initial value of 60.00 to 52.20 and 51.44% during the first 60 and 48 h of deproteinization for intact and ground shrimp shells, respectively. The moisture content started to decline at a higher rate reaching 36.63 and 25.54 % by the end of the deproteinization process (120 h) for intact and ground shrimp shells, respectively. The surface area to volume ration was higher in case of ground shells allowing for more moisture to be evaporated from the shells. Mudgett (1986) reported that smaller particle sizes cause the moisture to be distributed in thinner films on the external surfaces of the substrate that are exposed to the gas environment.

7.8.1.2.4 Residual Galactose Concentration: The galactose concentration decreased with time. The results showed that the rate of galactose utilization was significantly higher in case of ground shrimp shells. Size reduction increased the surface area to volume ration, thus increasing the fraction of glucose accessible to the microorganisms. Residual galactose of 2.33 and 1.48% remained unutilized by the end of the deproteinization process (120 h) for intact and ground shells, respectively. Mudgett (1986) reported that size reduction results in higher surface nutrient concentrations and shorter pathways for

nutrients diffusion either on the surface or in the pores of the substrate. Molony et al. (1984) reported that substrates with finer particles showed improved degradation as a result of the increase in the surface area. Pandey et al. (1988) reported that substrates with finer particles resulted in high degree of solubilization. However, Pandey (1991b) reported that with small particle substrates part of the released sugars during fermentation of wheat bran using *A. niger* remained unutilized while with substrates containing large particles (larger than 500 μm) and with substrates of mixed particle sizes insignificant amount of sugar was left unutilized.

7.8.1.2.5 CO_2 Concentration: The results showed that concentration of carbon dioxide in the exhaust gas with deproteinization time followed the same trend as the temperature of the shrimp shells. A strong correlation between the concentration of carbon dioxide in the exhaust gas and the temperature of the shrimp shells (Figure 7.60) was obtained. Maximum carbon dioxide concentrations of 0.55 and 0.4877% were obtained after 72 and

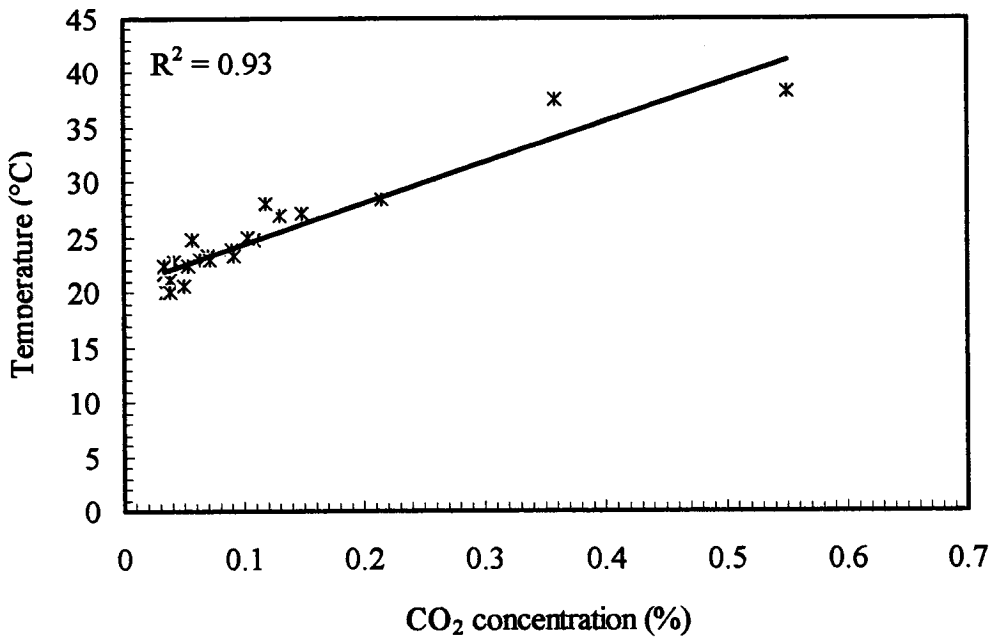


Figure 7.60. Correlation between carbon dioxide concentration in the exhaust gas and temperature of the shrimp shells.

60 h for intact and ground shrimp shells, respectively. No studies reported on the direct effect of particle size on carbon dioxide evolution. However, particle size of the substrate affects the growth of microorganisms and consequently CO_2 evolution as a result of microbial metabolic activity.

7.8.1.2.6 Protease Activity: The results showed that the protease activity increased significantly in case of ground shrimp shells. Size reduction of the shrimp shells increased the fraction of shrimp shell protein accessible by the microorganisms. Wang and Chio (1998) and Andrade et al. (2002) stated that a protein source must be presented for stimulating proteases production. For intact shrimp shells, the protease activity increased from 0.71 to 1.62 units per gram dry shrimp shell weight over the course of the deproteinization process whereas for ground shrimp shells, the protease activity increased from 0.67 to 4.21 units per gram dry shrimp shell weight over the course of the deproteinization process. Thus, grinding increased the production of proteases by 2.6 folds.

Pandey (1991b) studied the effect of wheat bran particle size on the production of the enzyme glucoamylase by *A. niger* and found that substrates of particle size 425-500 μm and 500-600 μm gave the highest productivity and that a mixed particle sizes containing particles smaller than 180 μm to larger than 1.4 mm resulted in comparable results. Kumar and Lonsane (1987) reported increase in Gibberellic acid production in coarse wheat bran probably because of larger void fraction and better oxygen transfer and CO_2 removal. Krishna and Chandrasekaran (1996) reported an optimum particle size of 400 μm for the production of α-amylase from banana waste using *Bacillus subtilis* in solid-state fermentation. However, Chakraborty et al. (1995) reported no difference in acid protease production for different wheat bran sizes. Zakaria (1997) studied the effect of particle size on lactic acid solid-state fermentation for chitin purification from shellfish waste and reported that mincing shellfish waste (6 mm average diameter) promotes good fermentation resulting in substantial level of chitin purification and that large particle sizes promotes the growth of spoilage organisms.

7.8.1.2.7 ***Protein Concentration:*** The results showed that the decrease in the protein concentration was faster in case of ground shrimp shells. The protein concentration decreased from an initial value of 30.84 to final values of 26.09 and 22.90% after 48 h of deproteinization for intact and ground shells, respectively. However, a small difference between the protein concentration for intact and ground shells was noticed at the end of the deproteinization process (120 h). Over the course of the deproteinization process (120 h), the protein concentration decreased from an initial value of 30.84 to final values of 21.45 and 20.59% for intact and ground shells, respectively. Initial galactose concentration had a more pronounced effect on the final protein concentration than particle size.

7.8.1.2.8 ***Chitin Concentration:*** The results showed small differences between chitin concentration in the intact and ground shrimp shells over the course of the deproteinization process. The chitin concentration increased from an initial value of 16.59 to final values of 21.99 and 22.68% at the end of the deproteinization process for intact and ground shells, respectively.

Initial galactose concentration had a more pronounced effect on the final chitin concentration than particle size. Shrimp shells size reduction caused 0.69% increase in chitin concentration at the end of the deproteinization process while 1.51% increase in the chitin concentration was obtained when increasing initial galactose concentration from 10 to 20% w/w.

7.8.1.2.9 ***Visualization of Shrimp Shells:*** The appearances of the intact and ground shells at the end of the deproteinizatoin process (120 h) were compared. The color of the shells (pale pink-orange with some tan patches) was almost the same for intact and ground shells. Some of the ground shrimp shells agglomerated and formed small balls. This problem was not encountered with the intact shells. Lonsane et al. (1985) reported that agglomeration of substrate particles into balls is a common problem when using drum type bioreactors as a result of the tumbling effect. When the shells were inspected under the microscope at 60X magnification, it appeared that the ground shells had more white

precipitants, which were believed to be as a result of break down of substances from the shell matrix.

7.8.1.3 Effect of Initial pH.

7.8.1.3.1 Temperature: The results showed that the temperature profiles were different for the runs with initial pH of 8.64 and 7:00. No distinguished temperature peak value was noticed with the run that had an initial pH of 7.00 but a longer stationary phase was noticed. A temperature peak of 37.5 °C was noticed after 60 h of deproteinization with the run that had an initial pH of 8.64. The growth kinetics were determined graphically from the temperature data according to the procedures described by Ghaly et al. (1989). Table 7.12 shows some of the growth kinetic parameters for the two runs. The length of the lag period was 24 and 36 h for the initial pH of 8.64 and 7:00, respectively. The exponential growth phase (36 h) was the same for both pHs. The stationary phase significantly increased from 4 h for initial pH of 8.64 to 36 h for initial pH of 7:00. Maximum temperatures of 37.5 and 30.6 °C were attained after 60 and 72 h for the initial pH of 8.64 and 7.00, respectively. Decreasing the initial pH from 8.64 to 7.00 decreased the specific growth rate for *A. niger* from 0.0221 h^{-1} to 0.0081 h^{-1}. The significant reduction in the specific growth phase for initial pH of 7.00 can be explained as a result of the inhibitory effect of the lactic acid used to lower the initial pH of the shrimp shells.

Table 7.12. Growth kinetic parameters[a] for initial pH of 8.64 and 7.00.

Parameter	pH	
	8.64	7
Lag phase (h)	24	36
Exponential phase (h)	36	36
Specific growth rate (h^{-1})	0.0221	0.0081
Stationary phase (h)	4	36
Initial temperature (°C)	21.8	21.8
Maximum temperature (°C)	37.5	30.6
Time of maximum temperature (h)	60	72

[a] values were calculated from the temperature curve

It was noticed that the initial pH had a similar effect (in magnitude) on the specific growth rate to that of the particle size of the shells. Reducing the particle size of the shrimp shells resulted in an increase in the specific growth rate by a factor of 2.5 and increasing the initial pH of the shells from 7:00 to 8.64 increased the specific growth rate by a factor of 2.7. High galactose concentration had less inhibitory effect than lactic acid. Increasing the initial galactose concentration from 20 to 30% w/w decreased the specific growth rate by a factor of 1.5 while lactic acid addition for lowering the initial pH from 8.64 to 7:00 decreased the specific growth rate by a factor of 2.7.

7.8.1.3.2 pH: For the run with initial pH of 8.64, the pH of the shrimp shells decreased from 8.64 to 7.34 during the first 60 h of deproteinization and then increased reaching 8.06 by the end of the deproteinization process (120 h). For the run with an initial pH of 7.00, the pH of the shrimp shells increased from 7.00 to 7.73 during the first 36 h of deproteinization (which corresponded to the lag period) as a result of the buffering capacity of the calcium carbonate in the shells and then decreased reaching 6.88 at 48 h of deproteinization and remained almost constant until the end of the deproteinization process (120 h).

7.8.1.3.3 Moisture Content: The moisture content of the shrimp shells did not change significantly as a result of the changes in the initial pH value. The moisture content of the shrimp shells declined from an initial value of 60% to final values of 25.5 and 21.5% over the course of the deproteinization process for the runs with initial pH of 8.64 and 7.00, respectively.

7.8.1.3.4 Residual Galactose Concentration: The results showed that the rate of galactose utilization was significantly higher for initial pH of 8.64. For the run with initial pH of 8.64, the galactose concentration decreased gradually from 20 to 1.48% over the course of the deproteinization process (120 h) whereas for the run with initial pH of 7.00, the galactose concentration decreased almost linearly from 20 to 6.17% over the course of the deproteinization process (120 h). The high residual galactose concentration experienced by the end of the deproteinization process with initial pH of 7:00 resulted

from the inhibitory effect of lactic acid which was used to adjust the initial pH of the shrimp shells.

7.8.1.3.5 CO_2 Concentration: The results showed that concentration of carbon dioxide in the exhaust gas followed the same trend as the temperature of the shrimp shells. A strong correlation between the concentration of carbon dioxide in the exhaust gas and the temperature of the shrimp shells was obtained (Figure 7.61). Maximum carbon dioxide concentrations of 0.49 and 0.22% were obtained at 60 and 84 h for the runs with initial pH of 8.64 and 7.00, respectively. The lactic acid used to adjust the initial pH of the shrimp shells inhibited the microbial growth and as a result the carbon dioxide concentration in the exhaust gas was reduced.

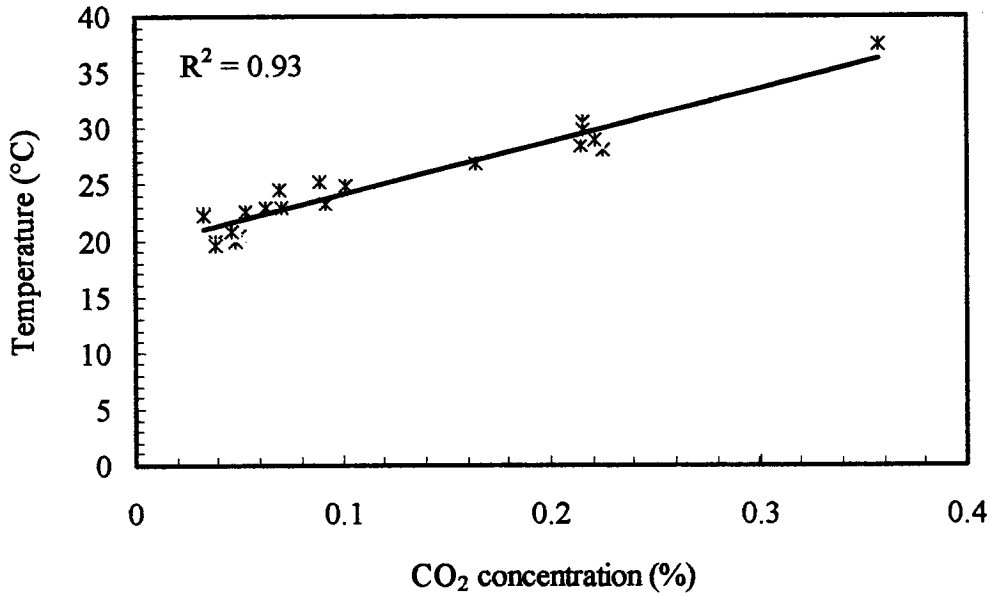


Figure 7.61. Correlation between carbon dioxide concentration in the exhaust gas and temperature of the shrimp shells.

7.8.1.3.6 Protease Activity: The results showed that the protease activity increased significantly in case of initial pH of 8.64. Lactic acid was inhibitory for the growth of *A. niger* as well as acid protease synthesis. The protease activity increased from 0.67 to 4.21 and from 0.71 to 2.34 units per gram dry shrimp shell weight over the course of the

deproteinization process (120 h) for initial pH of 8.64 and 7.00, respectively. The absence of lactic acid resulted in 1.8 folds increase in the protease production.

A high galactose concentration had less inhibitory effect than lactic acid. Increasing the initial galactose concentration from 20 to 30% w/w resulted in 4.6% decrease in the protease activity while addition of lactic acid resulted in 44.5% decrease in the protease activity.

*7.8.1.3.7 **Protein Concentration:*** The results showed that the decrease in the protein concentration in the shrimp shells for initial pH of 8.64 was higher than that for initial pH of 7.00. Over the course of the fermentation process (120 h), the protein concentration decreased from an initial value of 30.84 to final values of 20.59 and 22.22 for the initial pH of 8.64 and 7.00, respectively.

The effects of initial pH and particle size of the shrimp shells on protein concentration were almost similar. A high initial galactose concentration had more effect on the final protein concentration than lactic acid. Increasing the initial galactose concentration from 20 to 30% and the presence of lactic acid resulted in 21.76 and 7.89% increase in the protein concentration of the shells at the end of the deproteinization process, respectively.

*7.8.1.3.8 **Chitin Concentration:*** The results showed an insignificant difference between chitin concentration in the shrimp shells for initial pH of 8.64 and 7.00. The chitin concentration for initial pH of 8.64 was slightly higher that that for initial pH of 7.00. It increased from an initial value of 16.56 to final values of 22.68 and 21.35 for the initial pH of 8.64 and 7.00, respectively. The effects of initial pH, particle size of the shrimp shells and initial galactose concentration on chitin concentration were similar.

*7.8.1.3.9 **Visualization of Shrimp Shells:*** In case of initial pH of 7.00, the extent of shrimp shell agglomeration was higher than in case of initial pH of 8.64. More of the white precipitant was accumulated on the surface of the shrimp shells for initial pH of 8.64 compared to initial pH of 7.00 as noticed under the microscope (60X).

7.8.1.4 Effect of Autoclaving.

7.8.1.4.1 Temperature: The results showed that the temperature profiles for autoclaved and unautoclaved shells were similar. The growth kinetics were determined graphically from the temperature data according to the procedures described by Ghaly et al. (1989). Table 7.13 shows some of the growth kinetic parameters for autoclaved and unautoclaved shrimp shells. Lag periods of 12 and 24 h were observed for the unautoclaved and autoclaved shells, respectively. The exponential growth phase was 36 h for autoclaved shells and unautoclaved shells. The stationary phase was 4 h for autoclaved shells and 3 h for unautoclaved shells. The maximum temperatures attained were 37.5 and 33.0 °C after 60 and 48 h for autoclaved and unautoclaved shells, respectively. The specific growth rate for *A. niger* was 0.0221 h^{-1} for autoclaved shells and 0.0187 h^{-1} for unautoclaved shells.

Table 7.13. Growth kinetic parameters[a] for autoclaved and unautoclaved shrimp shells.

Parameter	Autoclaving	
	Autoclaved	Unautoclaved
Lag phase (h)	24	12
Exponential phase (h)	36	36
Specific growth rate (h^{-1})	0.0221	0.0187
Stationary phase (h)	4	3
Initial temperature (°C)	21.8	21.8
Maximum temperature (°C)	37.5	33.0
Time of maximum temperature (h)	60	48

[a] values were calculated from the temperature curve

The average inlet air temperature was 23.3 ± 0.54 °C. The temperature of the exhaust gas increased as a result of heat losses from the shrimp shells by convection. The exhaust gas temperature profile was similar to the shrimp shell temperature profile. The temperature of the exhaust gas reached maximum values of 28.9 and 27.9 °C after 60 and 48 h for the autoclaved and unautoclaved shells, respectively.

7.8.1.4.2 pH: The results showed that for the autoclaved shrimp shells, the pH decreased from 8.64 to 7.34 after 48 h of deproteinization and then increased reaching 7.68 at the end of the deprotienization process (120 h). For the unautoclaved shells, the pH decreased from 8.64 to 7.10 after 60 h of deproteinization and then remained constant until the end of the deproteinization process.

7.8.1.4.3 Moisture Content: The results showed that the moisture contents of the shrimp shells were similar for both the autoclaved and unautoclaved shells. The moisture content decreased from an initial value of 60.00% to final values of 25.54 and 26.72% at the end of the deproteinization process for the autoclaved and unautoclaved shrimp shells, respectively. The effects of the initial galactose concentration and the particle size on the moisture content of the shrimp shells were more pronounced than the effects of initial pH and autoclaving.

7.8.1.4.4 Residual Galactose Concentration: The results showed that the rate of galactose utilization was slightly higher for autoclaved shrimp shells compared to that of unautoclaved shrimp shells. The galactose concentration decreased gradually over the course of the deproteinization process. About 1.48 and 2.67% residual galactose remained unutilized by the end of the deproteinization process (120 h) for the autoclaved and unautoclaved shells, respectively. The effect of autoclaving on residual galactose concentration was similar to the effects of initial galactose concentration and particle size while the effect of pH was more pronounced.

7.8.1.4.5 CO_2 Concentration: The results showed that carbon dioxide concentration in the exhaust gas measured during the deproteinization process for both the autoclaved and unautoclaved shells had similar profiles. However, higher concentrations of CO_2 were observed with the autoclaved shrimp shells. The CO_2 concentration also followed the same trend as the temperature of the shrimp shells. A strong correlation between the concentration of carbon dioxide in the exhaust gas and the temperature of the shrimp shells was obtained (Figure 7.62).

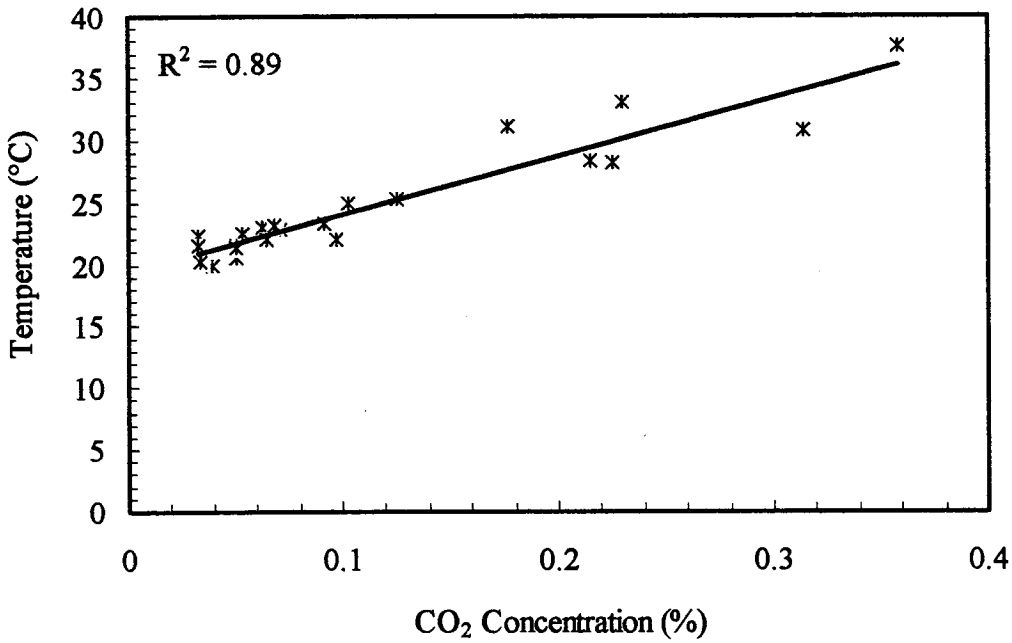


Figure 7.62. Correlation between carbon dioxide concentration in the exhaust gas and temperature of the shrimp shells.

7.8.1.4.6 Protease Activity: The results showed that the protease activity was higher in case of autoclaved shrimp shells. The protease activity increased with time from an initial value of 0.67 to final values of 4.21 ad 2.44 units per gram dry shrimp shell weight for the autoclaved and unautoclaved shrimp shells, respectively.

7.8.1.4.7 Protein Concentration: The results showed that the protein concentration in the shrimp shells was lower in case of autoclaved shrimp shells. The protein concentration decreased from an initial value of 30.84 to final values of 20.59% and 22.94% over the course of the deproteinization process (120 h) for the autoclaved and unautoclaved shells, respectively.

7.8.1.4.8 Chitin Concentration: The results showed that the chitin concentration in the shells increased from an initial value of 16.56 to final values of 22.68% and 20.93% over the course of the deproteinization process for the autoclaved and unautoclaved shrimp shells, respectively.

7.8.1.4.9 Visualization of Shrimp Shells: The extent of shrimp shell agglomeration was much higher in case of unautoclaved shrimp shells than in case of autoclaved shrimp shells. More of the white precipitant accumulated on the surface of the autoclaved shrimp shells compared to that was accumulated on the surface of the unautoclaved shrimp shells as noticed under the microscope (60X). Samples of the autoclaved and unautoclaved shrimp shells were plated on potato dextrose agar after 24 and 72 h of deproteinization. The plates showed the existence of unidentified microorganisms for both autoclaved and unautoclaved shells. However, the population of the unidentified microorganisms was much higher in the case of unautoclaved shells.

7.8.1.5 Comparative Analysis. The results obtained from this study revealed the ability of *A. niger* to produce extracellular proteases in the presence of shrimp shells and with the supplementation of galactose as an additional carbon source. As a result of the presence of proteases, the shrimp shells were deproteinized to a certain degree by the end of the deproteinization process. Fish proteins are complex molecules, which consist of chains of simpler molecules (amino acids) linked together by peptide bonds. Proteases are proteins structured in such a way that allow them to act as catalysts in the breakage of peptide bonds through a process called hydrolysis. The following equation describes protein hydrolysis in the presence of proteases.

$$\text{Fish protein} \xrightarrow[\text{Proteases}]{\text{Hydrolysis}} \text{Polypepyides} + \text{Peptides} + \text{Amino acids} \qquad (7.10)$$

Most of the shorter amino acid chains broken off from the fish protein are water-soluble and therefore can be easily washed (Hale, 1974). Bustos and Healy (1994) stated that the degree to which a microorganism will hydrolyze a protein substrate depends on its capacity to produce proteases and the stability of such protease under the reaction conditions.

The initial galactose concentration affected the protease production as well as the degree of deproteinization. Based on the results of the temperature of the shrimp shells and the concentration of CO_2 in the exhaust gas, initial galactose concentration of 10% w/w did

not support enough growth for *A. niger* while initial galactose concentration of 30% w/w caused an inhibition. The problem of substrate inhibition is well documented. Pandey et al. (1999) reported that substrates that provide all the needed nutrients to the culture organisms are considered ideal substrates but in some cases some of the nutrients may be available in suboptimal quantities and/or even absent. Therefore, the substrate should be supplemented externally with nutrients in order to initiates biomass production, induces enzyme synthesis, provides balanced growth conditions, or in some cases prolongs secondary metabolite production Mudgett (1986).

However, a proper amount of nutrient is required to meet organism metabolic demand for optimum growth and/or product formation. King and Cheetham (1987) reported that at high concentrations of nutrient the organism usually suffers inhibition and the specific growth rate is reduced, resulting in a growth rate curve similar to the one shown in Figure 7.63. In some cases, inhibition is caused by the increase osmotic pressure within the cell, but high concentrations of substrate may also have toxic effects on the organism or may trigger the organism to use a different metabolic route (King and Cheetham, 1987).

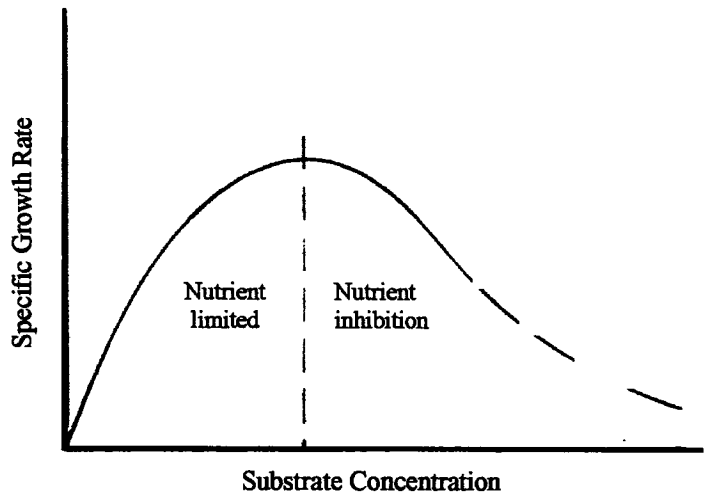


Figure 7.63. The effect of substrate inhibition on growth rate curve (King and Cheetham, 1987).

Based on the results of the protease activity and the protein concentration in the shrimp shells at the end of the deproteinization process, at the optimum initial galactose concentration of 20% w/w, protease production as well as protein removal efficiency were fairly low compared to those documented in the literature.

Particle size is of a great importance in solid-state fermentations. Size reduction of the substrate to a certain limit is known to greatly enhance the fermentation process. Table 7.1 and Figure 7.8 show the particle size distribution of the shrimp shells used in this study. The intact shells were of mixed particle size in the range of less than 6.3 to greater than 0.075 mm. After grinding, the shells were of mixed particle size in the range of less than 2 to greater than 0.075 mm. 59.33% of the intact shells and 98.20% of the ground shells were of a particle size less than 2 mm. The majority of the particles of the intact shells (77.72%) were in the range of less than 4.00 to greater than 0.30 mm while the majority of the particles of the ground shells (63.28%) were in the range of less than 2.00 to 0.30 mm.

The results of the temperature of the shrimp shells and the concentration of CO_2 in the exhaust gas showed that the particle size affected the growth kinetics. Size reduction resulted in a higher specific growth rate and a shorter exponential growth phase. The protease production was significantly enhanced (2.6 folds) in the case of the ground shells. However, the enhancement in the protein removal efficiency did not correspond to the increase in the protease reduction. A protein concentration of 21.45 and 20.59% were obtained for the intact and ground shells, respectively. This might be explained as a result of unsuitable conditions for the produced proteases to hydrolyze the shells proteins. The optimum pH for cell growth and proteases production is different from the optimum pH for maximum enzymatic activity and hydrolysis of proteins.

The data available in the literature on the effect of pH on the growth of *A. niger* and acid protease production are contradictory. Some authors reported on the significant effect of pH for the growth and acid protease production of *A. niger* while others found no significant effect of the pH. Singh et al. (1975) stated that initial pH adjustment to a

certain value was found to be necessary for maximum acid protease production from *Aspergillus niger* and obtained a maximum enzyme production when the initial medium pH was adjusted to 7.0 without pH control during the fermentation period. Zielińska and Kączkowski (1971) stated that the initiation of acid protease synthesis by *A. niger* might be bond to the lowering of pH value below 4.0. Yang and Chiu (1986) studied the effect of initial substrate pH on the mycelial growth and protease production of *A. niger* grown on sweet potato residue supplemented with rice bran as a nitrogen source and minerals with initial moisture content of 50-58 % and reported that pH lower than 1.9 or higher than 8.28 inhibited both the mycelial growth and the protease production. The authors also reported an optimum initial pH of between 3.10 and 7.12 for mycelial growth and 3.55 and 4.30 for protease production. However, Chakraborty et al. (1995) reported that initial pH in the range from 4 to 10 did not affect protease production by *A. niger* grown on wheat bran. Protease of *A. niger* is an acid resistant protease with optimum pH of 2.3 (Bailey and Ollis, 1986). Koaze et al., (1964) isolated two kinds of proteolytic enzymes from the crude enzyme powder obtained from the broth filtrate cultured with the same strain of *Aspergillus niger* used in this study. The first protease exhibited optimum activity on milk casein at pH of 2.6 and temperature of 55 °C when the incubation was done at pH 2.6 while the second protease exhibited optimum activity at pH of 2.0 and temperature of 70 °C or 60 °C when the incubation was done at pH 2.6 or 1.5, respectively.

Many studies reported the use of acids to lower the pH of the substrate in solid-state fermentations in order to create a selective environment for fungal growth. Steinkraus et al. (1960) soaked soyabeans in 0.85% lactic or acetic acids for tempeh fermentation. Wood (1982) used hydrochloric acid for lowering the pH for the production of soy sauce. The authors reported also the addition of acetate (0.2 to 0.8% by weight) in Koji processing. Nunokawa (1972) used lactic acid (0.5% by volume) in rice wine production. Underkofler (1954) acidified bran to a pH of 3.5 to 4.5 for amylolytic enzymes production using hydrochloric acid instead of water.

Lowering the initial pH of the shrimp shells was expected to enhance the deproteinization process by (a) achieving the optimum pH for fungal growth and protease production, (b) achieving the optimum pH for the proteolytic activity and (c) preventing the risk of the growth of undesirable microorganisms. However, the results obtained from this study showed that initial pH of 8.64 gave better results compared to initial pH of 7.00. The results showed also significant lower rate of galactose utilization and higher residual galactose concentration for pH of 7.00 due to lactic acid inhibition. Also, the specific growth rate was significantly decreased and the lag period was increased. The protease production for pH of 8.64 was 1.6 folds higher than that of pH of 7.00. The protein concentration at the end of the deproteinization process was 20.59 and 22.22% for the runs that has initial pH of 8.64 and 7.00, respectively.

Diniz and Martin (1997) stated that the extent of hydrolytic degradation of protein is highly dependent on both pH and temperature and that low molecular weight peptides were obtained at optimum pH, which indicates high proteolytic activity. The authors reported that yield as well as the composition and the molecular weight distribution of the peptides in the protein hydrolysates depends on factors such as: (a) concentration and specificity of the enzyme, (b) temperature, (c) pH, (d) extent of native protein denaturation, (e) presence of inhibitory substances and (f) degree of hydrolysis obtained.

Proteins are known to denature when exposed to high temperatures. Protein denaturation may alter the secondary, tertiary or quaternary structure of the molecules. Mudgett (1986) stated that denaturation of proteins make them more susceptible to enzymatic hydrolysis. However, the negative effects of high temperatures on protein hydrolysis are also documented in the literature. Wang and Chio (1998) reported that high cooking temperatures cause low protein removal efficiency during deproteinization of shrimp and crab shells with the protease of *Pseudomonas aeruginosa* K-187. They explained this as a result of the Maillard reaction associated with cooking at high temperatures that renders the proteins resistant to deproteinization. Hale (1974) reported on the effect of cooking on fish protein hydrolysis using proteolytic enzymes and stated that cooking resulted in a very poor yields and nutrition value of fish protein concentrates.

In this study, using unautoclaved shells negatively affected the deproteinization process. The protease activity of the autoclave shells was significantly higher than that of the unautoclaved shells (1.7 fold) at the end of the deproteinization process. The protein concentration at the end of the deproteinization process was 20.59 and 22.94 for autoclaved and unautoclaved shells, respectively. The low deproteinization attained in this study with unautoclaved shells might be due to the effect of heat as well as the growth of the indigenous microorganisms in the shrimp shells, which might in turn affect the growth of *A. niger*. Samples from the shrimp shells after 24 and 72 h of deproteinization were plated on potato dextrose agar (Figure 7.50) and some unidentified bacteria were noticed in the plates in a very high population in case of unautoclaved shells. Wang and Hesseltine (1979) reported that moisture content of 60% is ideal for bacterial growth while 45% moisture content is adequate for fungal but not bacterial growth. Hesseltine (1972) stated that in solid-state fermentations the addition of water in early fermentation stages may increase the risk of contamination. Mudgett (1986) reported that most bacteria grow at high moisture levels (above 40%), therefore, for solid-state fermentations at higher moisture contents mild thermal processing conditions such as steaming at 100 °C for a short time might be required. The author also referred to the effect of steaming on opening the structure of the solid substrate for mycelial penetration.

According to this study, the optimum conditions for the deproteinization of shrimp shells using *A. niger* were initial galactose concentration of 20% w/w, ground shells, pH of 8.64 (no initial pH adjustment) and autoclaved shells. However, at these conditions, the lowest protein concentration attained was 20.59%, which is still high and further enhancement to the process is still needed. The enhancement in acid protease production attained at the optimum conditions did not affect the deproteinization process significantly revealing that the conditions were suitable for production of proteases but not for their optimum activity.

7.8.2 Demineralization Process

7.8.2.1 Minerals Composition of Shrimp Shells and Microbially Deproteinized Shells. The total mineral content of the shrimp shells used in this study was 31.73%. The most abundant minerals in the shrimp shells were Ca, P, Mg, S and Na, which accounted for 44.75, 7.06, 1.94, 1.48 and 1.10% of the total shell mineral composition, respectively. The amount of calcium present in the shells was 6 and 23 times higher than the amounts of phosphorus and magnesium, respectively. Hansen and Illanes (1994) stated that the major mineral component of shellfish waste is calcium. Beaney et al. (2005) reported that the most abundant minerals in prawn shell were Ca, Mg, Na, Sr, K and Fe in that order and that calcium was by far the most abundant (about 17 times more calcium present than magnesium). In the microbially deproteinized shells (MDS), the total mineral composition was slightly reduced from 31.73 to 30.65%. The reduction in calcium, sodium, sulfur, potassium and magnesium were 0.76, 0.31, 0.27, 0.07 and 0.06%, respectively. After the deproteinization process shrimp shells were washed with distilled deionized water until the wash water was clear. About 1.08% of the total minerals in the shells were removed in the wash water.

7.8.2.2 Effect of Acid Type and Demineralization Conditions. The results showed insignificant difference in the demineralization efficiency between conditions A (Zakaria, 1997) and B (Ghanem et al., 2003) when using HCl. The total mineral content was reduced from the initial value of 30.65% in the MDS to final values of 3.15 and 3.26% in the demineralized shells (DS) for conditions A and B, respectively. The calcium (the most abundant mineral in the shells) was reduced from the initial value of 13.44% in the MDS to final values of 0.12 and 0.13% in the DS for conditions A and B, respectively. In case of lactic acid, an insignificant difference in calcium removal was noticed between the two demineralization conditions (calcium concentration was reduced from the initial value of 13.44% in the MDS to final values of 0.78 and 0.70% in the DS for conditions A and B, respectively). However, the reduction in the total mineral content differed slightly between the two demineralization conditions. The total mineral concentration was reduced from the initial value of 30.65% to final values of 3.69 and 4.60% for condition A and B, respectively.

The effectiveness of acetic acid in removing the minerals from the shells was lower than those of hydrochloric and lactic acids. The total minerals content was reduced from 30.65% in the MDS to 8.53 and 9.10 in the DS using acetic acid under conditions A and B, respectively. The calcium concentration was reduced from 13.44% in the MDS to 3.22 and 4.03% in the DS using acetic acids under conditions A and B, respectively.

The removal efficiencies of the total minerals and the calcium were calculated based on the microbially deproteinized sample weight and the recovered demineralized sample weight. The removal efficiencies of the total minerals and the calcium using demineralization condition A were 96.71and 99.73%, 94.07 and 97.13% and 85.56 and 87.59% for HCl, LA and AC, respectively. The removal efficiencies of the total minerals and the calcium using demineralization condition B were 95.02 and 99.54%, 92.12 and 97.26% and 82.55 and 82.38% for HCl, LA and AC, respectively. In general, insignificant difference between condition A and B was observed. The removal efficiencies using the three acids were acceptable although that of acetic acid was lower.

Zakaria et al. (1998) used lactic acid fermentation for chitin purification from Scampi waste and reported that approximately 61% of the calcium present in the scampi waste at the start of fermentation was solubilized and that the purified chitin contained about 19.3% calcium. Cira et al. (2002) achieved reduction in calcium concentration from 14.6-15.9% to 6.3-8.1% during lactic acid fermentation of shrimp waste. Ghanem et al. (2003) studied the effect of various shrimp processing procedures on the quality and quantity of purified chitin and achieved reduction in the ash concentrations, using 1.7 N HCl in a 1:10 shells to acid ratio at room temperature for 6 h retention time, from 20.08 and 37.13% in the shrimp shells to 8.93 and 0.83% in the purified chitin for shrimp shells obtained by different shrimp processing procedures. The calcium concentration was reduced from 3.56 to 2.38% and from 7.12 to 0.02% for the two shell types. Using lactic acid fermentation, Beaney et al. (2005) achieved reduction in the total inorganic matter and calcium concentrations from 61.8 to 19.6 and 14.2% and from 0.78 to 0.31 and 0.17%, respectively. The authors also used 1M HCl in a 1:15 shells to acid ratio for 2 h

retention time at room temperature and achieved reduction in the total inorganic matter concentration from 61.8 to 0.2%.

7.8.2.3 Effect of Retention Time. It was concluded that there was insignificant difference between the two tested conditions (A and B). Therefore, condition B was used for further investigations since it saves energy and acid. The results showed that reducing the retention time from 6 to 2 h caused a slight reduction in minerals removal. The total mineral concentrations for 6 and 2 h retention times were 4.60 and 5.28% and 9.10 and 11.39 for lactic and acetic acids, respectively. The calcium concentrations in the DS for 6 and 2 h retention times were 0.70 and 1.56% and 4.03 and 4.68 for lactic and acetic acids, respectively. The total minerals removal efficiencies for 2 and 6 h retention times were 90.29 and 92.12% and 79.56 and 82.55% for lactic and acetic acids, respectively. The calcium removal efficiencies for 2 and 6 h retention times were 93.44 and 97.26% and 80.84 and 82.38% for lactic and acetic acids, respectively. A slight decrease in the removal efficiencies was obtained when the retention time was reduced from 6 to 2 h. However, lactic acid achieved higher removal efficiency than acetic acid.

7.8.2.4 Effect of Shells to Acid Ratio. The effect of acid to shells ratio (1:10, 1:20 and 1:30), when using lactic and acetic acids at 2 h retention time, was investigated. The results showed a significant increase in minerals removal when the shells to acid ratio increased from 1:10 to 1:20. Further increase in the shells to acid ratio to 1:30 caused only a slight decrease in the mineral concentrations. In case of using lactic acid, the total minerals concentrations in the demineralized shells were 5.28, 1.51 and 0.86% for 1:10, 1:20 and 1:30 shells to acid ratio, respectively. The calcium concentrations in the DS were 1.56, 0.48 and 0.10% for 1:10, 1:20 and 1:30 shells to acid ratio, respectively. The total minerals and calcium removal efficiencies were 90.29 and 93.44, 97.40 and 99.11 and 98.53 and 99.63% for 1:10, 1:20 and 1:30 shells to acid ratio, respectively. In case of using acetic acid, the total minerals concentrations in the demineralized shells were 11.39, 3.71 and 3.07% for 1:10, 1:20 and 1:30 shells to acid ratio, respectively. The calcium concentrations in the DS were 4.68, 1.75 and 1.14% for 1:10, 1:20 and 1:30 shells to acid ratio, respectively. The total minerals and calcium removal efficiencies

were 79.56 and 80.84%, 86.36 and 85.33% and 94.83 and 95.62% for 1:10, 1:20 and 1:30 shells to acid ratio, respectively.

7.8.2.5 Deicing Agent Production. Alkaline salts of carboxylic acids had been found to have good de-icing ability (Ketcham et al., 1996; Bang and Johnston, 1998; and Jin et al., 2002). Using lactic and/or acetic acids for the demineralization of shrimp shells would result in a solution containing a mixture of lactate and/or acetate salts. The most abundant salt in the resultant solution would be calcium lactate and/or calcium acetate since the calcium comprises about 44.75% of the total minerals present in the shrimp shells used in the current study. The ratio of 1 g shells to 20 mL acid was found reasonable for the demineralization of the shells. The total amount of acid used per kilogram shells on molar basis was 16.79 moles for lactic acid (90.07 g/mol) and 24.98 moles for acetic acid (60.05 g/mol). Goycoolea et al. (2000) stated that the most important factor for the complete removal of minerals is to ensure that there is sufficient acid present (regardless of type), the minimum being at least stoichiometrically equal to the total amount of minerals present in the sample. Table 7.14 shows the minerals composition of the shrimp shells used in this study on a molar basis.

Although lactic acid was more effective for the demineralization process than acetic acid, there was no clear evidence found in the literature that proves the effectiveness of lactate salts for de-icing/anti-icing practices. In addition, the solubility of calcium lactate is significantly lower than the solubility of calcium acetate (3.1 and 37.4 g salt per 100 g of water at 0 °C for calcium lactate and calcium acetate, respectively). The use of acetic acid for the demineralization process is more justified. Numerous studies reported on the potentials of acetate salts especially calcium magnesium acetate (CMA) as a de-icing agent. In 1979, calcium magnesium acetate was identified as a non-corrosive environmentally-friendly alternative to chloride salts for road de-icing practices (Marynowski et al., 1985). The major disadvantage of CMA is its relatively high production cost, which is mainly attributed to the cost of acetic acid (Dionysiou et al., 2000). Yang et al. (1992) stated that acetic acid used to produce calcium magnesium acetate, as a road de-icer, does not need to be as pure as acetic acid used in the chemical

Table 7.14. Mineral composition of shrimp shells.

Element	Valance	Molecular weight	Concentration[a]		
			%[b]	g/kg	mol/kg
Al	+3	26.982	0.08	0.250	0.0093
Ca	+2	40.078	44.75	142.000	3.5431
Cu	+1, +2	63.546	0.01	0.024	0.0004
Fe	+2, +3	55.845	0.10	0.308	0.0055
K	+1	39.098	0.27	0.871	0.0223
Mg	+2	24.305	1.94	6.150	0.2530
Mn	+2, +3, +4, +7	54.938	0.04	0.116	0.0021
Na	+1	22.990	1.10	3.480	0.1514
P	+3, +5, -3	30.974	7.06	22.400	0.7232
S	-2, +4, +6	32.065	1.48	4.700	0.1466
Si	-4, +2, +4	28.086	0.25	0.794	0.0283
Others			42.92	136.21	

[a] total mineral concentration in the shrimp shells was 317.3 g minerals per kg shells
[b] percent of the total mineral content

industry. Producing acetic acid from cheese whey would reduce its production cost significantly. The use of acetic acid for the demineralization of shrimp shells in the ratio of 1 g shells: 20 mL acetic acid would result in 560.38 g calcium acetate per 1 kg shells. The process could be described by the following equations:

$$\text{Lactose} \xrightarrow{\textit{L. lactis}} \text{Lactic acid} \xrightarrow{\textit{C. formicoaceticum}} \text{Acetic acid} \qquad (7.11)$$

$$\underset{\text{Acetic acid}}{2CH_3COOH} + \underset{\text{Calcium carbonate}}{CaCO_3} \longrightarrow \underset{\text{Calcium Acetate}}{Ca(C_2H_3O_2)_2} + H_2O + CO_2 \qquad (7.12)$$

Dionysiou et al. (2000) stated that depending on different conditions the following reactions can occur:

$$\underset{\text{Acetate ion}}{Ca^{2+} + 2CH_3COO^{-1}} \longrightarrow \underset{\text{Calcium acetate}}{Ca(C_2H_3O_2)_2} \qquad (7.13)$$

$$Mg^{2+} + 2CH_3COO^{-1} \longrightarrow Mg(C_2H_3O_2)_2 \quad (7.14)$$

Acetate ion — Magnesium acetate

$$xCa^{2+} + yMg^{2+} + zCH_3COO^{-1} \longrightarrow Ca_xMg_y(C_2H_3O_2)_z \quad (7.15)$$

Acetate ion — Calcium magnesium acetate (CMA)

Marynowski et al. (1985) reported that liquid potassium acetate is being used now for airport runways de-icing practices. The use of acetic acid for the demineralization of shrimp shells in the ratio of 1 g shells: 20 mL acetic acid would result in 2.19 g potassium acetate per 1 kg shells.

$$K^{1+} + CH_3COO^{-1} \longrightarrow K(C_2H_3O_2) \quad (7.16)$$

Acetate ion — Potassium acetate

Bang and Johnston (1998) stated that a deicing agent is known to be more effective when it has a lower molecular weight with higher solubility. Table 7.15 shows the solubilities of sodium chloride, calcium lactate and some acetate salts. Sodium chloride is commonly used for ice control practices. However, concerns regarding corrosion and adverse environmental effects associated with the use of sodium chloride prompted the evaluation of organic salts. The solubility of calcium lactate is very low compared to sodium chloride. The solubilities of acetate salts are comparable to the solubility of sodium chloride.

7.9 Conclusions

7.9.1 Deproteinization Process

1. The fungus *A. niger* was able to produce proteases in the presence of shrimp shells and galactose as a carbon source, thus causing the deproteinization of the shrimp shells.
2. Initial galactose concentration affected the different parameters of the deproteinization process (temperature, pH, moisture content, galactose utilization, carbon dioxide evolution, protease activity, residual protein and chitin concentration).
   (a) Initial galactose concentration of 10% w/w did not support enough microbial growth and that of 30% w/w inhibited the growth of *A. niger* as was evident from

Table 7.15. Solubilities[a] of some salts in water at various temperatures (Dean, 1999).

Substance	Formula	Temperature (°C)								
		0	10	20	30	40	60	80	90	100
Sodium chloride	NaCl	35.7	35.8	35.9	36.1	36.4	37.1	38.0	38.5	39.2
Calcium lactate	$Ca(C_3H_5O_3)_2.5H_2O$	3.1		$5.4^{15°}$	7.9					
Calcium acetate	$Ca(OAc)_2.2H_2O$	37.4	36.0	34.7	33.8	33.2	32.7	33.5	31.1	29.7
Magnesium acetate	$Mg(C_2H_3O2)_2$	56.7	59.7	53.4	68.6	75.7	118			
Potassium acetate	$KC_2H_3O_2$	216	233	256	283	325	350	381	398	
Sodium acetate	$NaC_2H_3O_2$	36.2	40.8	46.4	54.6	65.6	139	153	161	170

[a] Solubilities are expressed as the number of grams of substance of stated molecular formula which when dissolve in 100 g of water make a saturated solution at the stated temperature.

the temperature and carbon dioxide evolution results.

(b) Although the initial galactose concentration of 20% w/w was found to be optimum, the protease activity was low (1.85 units per gram dry weight) and the protein concentration (20.77%) was high at the end of the deproteinization process (144 h).

3. Particle size had a significant effect on the deproteinization parameters.
   (a) Size reduction of the shrimp shells resulted in a 2.6 fold increase in the protease production
   (b) The reduction in the protein concentration at the end of the deproteinization process (120 h) did not correspond to the increase in the protease activity. The pH was far from being optimum for the enzymes produced during the deproteinization process
4. Lowering the initial pH from 8.64 to 7.00 using lactic acid had a negative effect on the deproteinization parameters. Lactic acid caused an inhibition to the growth of *A. niger* as well as the protease production.
5. Autoclaving enhanced the deproteinization process by making the proteins more susceptible to hydrolysis and preventing the risk of contamination.
6. The optimum conditions were found to be initial galactose concentration of 20% w/w, ground shells, autoclaved shells and without initial pH adjustment using lactic acid.
7. Under the optimum condition the residual protein was still high (20.59%,) and further enhancement to the deproteinization process is needed.

7.9.2 Demineralization Process

1. The effectiveness of organic acids (lactic and acetic) for the demineralization of shrimp shells was comparable to that of hydrochloric acid.
   (a) Under the same demineralization conditions (shells to acid ratio, temperature and retention time), the total minerals removal efficiencies ranged from 95.02 to 96.71%, from 92.12 to 94.07% and from 82.55 to 85.56% for hydrochloric, lactic and acetic acids, respectively.
   (b) Under the same demineralization conditions (shells to acid ratio, temperature and retention time), the calcium removal efficiencies ranged from 99.54 to 99.73%,

from 97.13 to 97.26% and from 82.38 to 87.59% for hydrochloric, lactic and acetic acids, respectively.

2. A slight difference between demineralization condition A (shells to acid ratio of 1:50, temperature of 100 °C and retention time of 1 h) and B (shells to acid ratio of 1:10, temperature of 24 °C and retention time of 6 h) was obtained with the three acids (hydrochloric, lactic and acetic).
   (a) The total minerals and calcium removal efficiencies using hydrochloric acid were 96.71 and 99.73% and 95.02 and 99.54% for condition A and B, respectively.
   (b) The total minerals and calcium removal efficiencies using lactic acid were 94.07 and 97.13% and 92.12 and 97.26% for condition A and B, respectively.
   (c) The total minerals and calcium removal efficiencies using acetic acid were 85.56 and 87.59% and 82.55 and 82.38% for condition A and B, respectively.
3. Retention time had an insignificant effect on the removal efficiency of minerals using organic acids
   (a) When lactic acid was used, a reduction in the retention time from 6 to 2 h resulted in reduction in the total minerals and calcium removal efficiencies from 92.12 to 90.29% and from 97.26 to 93.44%, respectively.
   (b) When acetic acid was used, a reduction in the retention time from 6 to 2 h resulted in reduction in the total minerals and calcium removal efficiencies from 82.55 to 79.56% and from 82.38 to 80.84%, respectively.
4. Increasing the shells to acid ratio from 1:10 to 1:20 increased the total minerals and calcium removal efficiencies significantly. Further increase in the shells to acid ratio to 1:30 had insignificant effect on total minerals and calcium removal efficiencies
5. Lactic acid was more efficient than acetic acid for the demineralization process of shrimp shells. However, if a de-icing salt is to be produced, the use of acetic acid is more justified since acetate salts have excellent de-icing capabilities.
6. Shells to acid ratio of 1:20, temperature of 24 °C (room temperature) and retention time of 2 h was found to be acceptable for the demineralization process of microbially deproteinized shells. Under these conditions the total minerals and calcium removal efficiencies were 97.4 and 99.11% and 86.36 and 85.33% for lactic and acetic acids, respectively.

7. Using acetic acid in the ratio of 1:20 g shells to mL acid for the demineralization of shrimp shells would result in the production of 560.38 g calcium acetate and 2.19 g potassium acetate for each 1 kg shells if all the calcium and potassium present in the shrimp shells will be removed.

7.10 Recommendations

7.10.1 Initial pH

The high pH of the shrimp shells is believed to be the main reason for the low efficiency of the deproteinization process achieved in this study. However, lowering the initial pH from 8.64 to 7.00 using lactic acid affected the deproteinization process negatively. This was believed to be as a result of the inhibition of the lactic acid to the microbial growth and protease production. It is well documented that *A. niger* can grow over a wide range of pH and that low pH favor the microbial growth and acid protease production. Low pH values also create a selective environment for fungal growth and prevent the growth of contaminant microorganisms. In addition the optimum pH of acid protease from *A. niger* lies in the range of 2.0 to 3.0. Moistening the shrimp shells with buffered or unbuffered medium at low pH is worth investigating. However, the medium should not have any toxic effect on *A. niger*.

It is also recommended that the demineralization process using unwashed microbially deproteinized shells be investigated. Most of the hydrolyzed proteins as well as proteases are removed with the wash water. Using unwashed shells for the demineralization process would allow acid proteases to hydrolyze the residual proteins under better pH conditions in the presence of the acid used for demineralization. Also the presence of water during the demineralization process would facilitate protein hydrolysis since hydrolysis is basically a cleavage of peptide bonds that occurs through the addition of water molecule. However, in this case a method for the separation of proteins from organic salts should be developed.

7.10.2 Temperature Control

The removal of metabolic heat during the deproteinization process might have a positive effect on the growth of *A. niger* and the production of acid protease. Singh et al. (1975) stated that the acid protease production by *A. niger* was shown to be temperature dependent. The authors reported no microbial growth at incubation temperature of 45 °C and maximum enzyme production at incubation temperature of 35°C. Yang and Chiu (1986) reported maximum protease activity at temperatures between 20-26 °C and significant decrease in protease production at temperatures higher than 30 °C for *A. niger* grown on sweet potato residue supplemented with rice bran as a nitrogen source and minerals. Introducing a cooling coil inside the bioreactor for metabolic heat removal is recommended.

7.10.3 Moisture Content

A significant reduction in the moisture content during the deproteinization process was noticed, although the air was humidified before introducing it into the bioreactor. The temperature of the inlet air increased as a result of the metabolic heat produced by *A. niger* resulting in an increase in the air water holding capacity. The recovery of the water lost from the bioreactor with the exhaust gas is recommended. The exhaust gas can be passed through a condensation tower and the recovered water can be pumped back into the bioreactor through the aeration tube.

In this study, the initial moisture content was 60%. The growth of unidentified microorganisms was noticed. Therefore, it is recommended to lower the initial moisture content in such a way that support the growth of *A. niger* and suppress the growth of contaminant microorganisms.

7.10.4 Fed-Batch Sugar Addition

High initial galactose concentration (30% w/w) inhibited *A. niger* growth and acid protease production. Fed-batch galactose addition would result in longer growth phase and more acid protease production and consequently more protein removal.

7.10.5 De-icing Agent Production

The effectiveness of using lactic acid and acetic acid fermentation broths rather than synthesized acid solutions for the demineralization of microbial deproteinized shells is worth studying. It is also recommended to test the de-icing characteristics of the resulting de-icing agents compared to those commercially available.

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9. APPENDICES

Appendix A

Data Sheet for the Protease Colorimetric Detection Kit

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ProductInformatio

Protease Colorimetric Detection Kit

Product Code **PC0100**
Storage Temperature 2–8 °C

TECHNICAL BULLETIN

Product Description
The Protease Colorimetric Detection Kit provides a complete set of reagents to detect and quantify protease activity. For convenience the kit utilizes a simple assay that can be performed in either cuvette or multiwell plate format. The assay method uses a casein substrate. As the casein is cleaved by the protease, trichloroacetic acid (TCA) soluble peptides are generated. These peptides contain tyrosine and tryptophan residues that react with the Folin & Ciocalteu's (F-C) Reagent to produce a color change. The F-C Reagent will also react with peptides containing cystine, cysteine, and histidine residues, but to a lesser extent. The amino acids reduce the tungstate and/or molybdate in the F-C Reagent, thereby generating one or more compounds with a characteristic blue color that can be colorimetrically quantitated at 660 nm.

This kit has been tested to detect a wide variety of proteases, including serine, cysteine, metallo, and aspartic proteases. Furthermore, this kit has been optimized to detect and quantify a diverse range of proteases found in physiological applications, and quantifies most non-specific proteases.

This kit will detect 0.1 unit/ml (approximately 2 µg) of trypsin with a 10 minute incubation time. This sensitivity may be increased by modifications, such as increased incubation time. If even higher sensitivity is required, the Protease Fluorescent Detection Kit (Product Code PF0100) is recommended.

Components
Sufficient reagents are provided to perform 200 one ml assays.

Protease Detection Substrate: Casein supplied as a powder (Product Code P 9996)	20 x 10 mg
2.0 N Folin & Ciocalteu's Phenol Reagent (Product Code F 9252)	10 ml
500 mM Sodium Carbonate Solution (Product Code S 5444)	125 ml
Enzyme Diluent 10 mM Tris acetate with 5 mM calcium acetate, pH 7.5 (Product Code E 9155)	125 ml
1.1 mM Tyrosine Standard Solution (Product Code T 2950)	1 ml
Protease from *Bacillus polymyxa* (Product Code P 6141)	1 g
0.6 N Trichloroacetic Acid (TCA) (Product Code T 0199)	5 ml

Equipment Required but Not Provided
- Pipettes
- Cuvettes or 96 well plates
- Microcentrifuge tubes
- Microcentrifuge
- Spectrophotometer suitable for measuring absorption at 660 nm

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions
Protease Detection Substrate - The substrate should be freshly prepared. Resuspend one 10 mg vial of casein powder (Product Code P 9996) in 1.5 ml of deionized water. This is sufficient for 10 one ml assays.

Folin & Ciocalteu's (F-C) Reagent - Mix 1 part of the 2.0 N Folin & Ciocalteu's Phenol Reagent (Product Code F 9252) with 3 parts of deionized water. For example, to prepare 4 ml, add 1 ml of the 2.0 N Folin & Ciocalteu's Phenol Reagent to 3 ml of deionized water.

TCA Working Solution - Prepare a 110 mM working solution by mixing 920 µl of the 0.6 N TCA Solution (Product Code T 0199) with 4.08 ml of deionized water, for a total volume of 5.0 ml.

Protease Control – Use the Protease from *Bacillus polymyxa* (Product Code P 6141) to prepare a solution containing 0.1–0.2 unit per ml in the Enzyme Diluent (Product Code E 9155). The activity of the Protease from *Bacillus polymyxa* is approximately 1 unit per mg of solid. The Protease Control can be used to confirm the assay is performing properly. For the assay of a different, specific protease, it is recommended to prepare a control solution containing the specific protease in the appropriate incubation buffer.

Protease Sample - In order to quantify the protease activity in the sample, prepare an enzyme solution containing 0.1–0.2 unit per ml in the provided Enzyme Diluent. If the activity is unknown, several dilutions may be required to determine the optimal concentration range.

Storage/Stability
The kit and its components are stable as supplied, for at least 2 years if stored at 2–8 °C.

When the kit is received, the 2.0 N Folin & Ciocalteu's Phenol Reagent (Product Code F 9252) can be stored at room temperature. The reagent should be bright yellow in color. Discard any reagent that has acquired a green tint.

It is recommended that working solutions be freshly prepared.

Procedure
This kit has been optimized to detect a diverse range of proteases found in physiological applications. It is suitable for detection of serine, cysteine, metallo, and aspartic proteases; however, modifications may be required to detect some specific proteases. Modifications to the procedure may include pH adjustments, the addition of metal ions, or a reformulation of the incubation buffer. The researcher must determine the optimal procedure conditions for the protease specific to their application. Note that this procedure can be scaled up or down according to the requirements of the instruments being used.

1. To a microcentrifuge tube, add 130 µl of Protease Detection Substrate and 25 µl of the Protease Sample or Control. For protease samples with high protease activity, a dilution may be required.
2. To prepare the Blank sample, add 130 µl of the Protease Detection Substrate and 25 µl of Enzyme Diluent to a microcentrifuge tube.
3. Mix and incubate at 37 °C for exactly 10 minutes.
4. After incubation, add 130 µl of the TCA Working Solution to each microcentrifuge tube.
5. Mix and incubate at 37 °C for 20 minutes.
6. Centrifuge the tubes for 5 minutes at 10,000 x *g*.

Absorbance Measurements
Cuvettes

7. Being careful not to disturb any pellet that may have formed, pipette 250 µl of the supernatant (step 6) into a separate fresh microcentrifuge tube or other suitable container.
8. Add 625 µl of the Sodium Carbonate Solution (Product Code S 5444) and 125 µl of the diluted F-C Reagent to each microcentrifuge tube.
9. Mix and incubate at 37 °C for 30 minutes.
10. Cool to room temperature.
11. Transfer 1 ml to a suitable cuvette.
12. Mix and record the absorbance at 660 nm.

Multiwell Plates

7. Pipette 50 µl of the supernatant (step 6), 125 µl of the Sodium Carbonate Solution (Product Code S 5444), and 25 µl of the diluted F-C Reagent into each well of a 96 well plate.
8. Seal or cover the plate to avoid evaporation.
9. Incubate at 37 °C for 30 minutes.
10. Remove the cover and record the absorbance at 660 nm.

Tyrosine Standard Curve
A standard curve is generated using the Tyrosine Standard Solution (Product Code T 2950), thereby facilitating quantitation of the protease activity.

1. Generate the standard curve by pipetting reagents (see Table 1) into microcentrifuge tubes or other suitable containers.

Table 1.
Reaction Scheme for Tyrosine Standard Curve

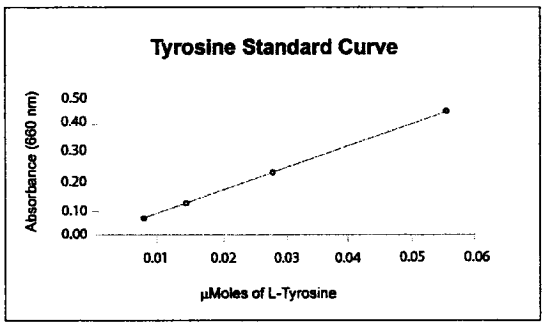
Reagent	Standard Blank	Standard 1	Standard 2	Standard 3	Standard 4
Tyrosine Standard	0.0 µl	5.0 µl	10.0 µl	25.0 µl	50.0 µl
Water	250 µl	245 µl	240 µl	225 µl	200 µl
Sodium Carbonate	625 µl	625 µl	625 µl	625 µl	625 µl
Diluted F-C Reagent	125 µl	125 µl	125 µl	125 µl	125 µl
µmoles of Tyrosine	0	0.0055	0.0110	0.0275	0.055

2. Mix and incubate at 37 °C for 30 minutes.
3. Transfer 1 ml to a suitable cuvette or 200 µl to each well of a 96 well plate.
4. Record the absorbance at 660 nm.
5. Subtract the absorbance of the Standard Blank from the absorbance of each Standard.

$$\Delta A_{660\ Standard} = A_{660\ Standard} - A_{660\ Standard\ Blank}$$

6. Plot the ΔA_{660} versus µmoles of tyrosine (see Figure 1).

Figure 1.
Typical Standard Curve generated using the Tyrosine Standard Solution.



Protease Activity Calculations
The protease activity is determined using the Tyrosine Standard Curve. A standard curve should be generated each time the assay is performed.

1. Subtract the absorbance of the Sample Blank from the absorbance of each Sample.

$$\Delta A_{660\ Sample} = A_{660\ Sample} - A_{660\ Sample\ Blank}$$

2. Determine the µmoles of tyrosine equivalents liberated using the equation derived for the Tyrosine Standard Curve.
3. Determine the units of protease activity per ml of protease sample using the following equation:

$$\text{Units/ml} = \frac{\text{µmole of Tyr x reaction volume}}{\text{sample vol x reaction time x vol assayed}}$$

µmole of Tyr = µmole of tyrosine equivalents released
reaction volume = total volume (in ml) of assay
sample vol = volume (in ml) of protease sample used
reaction time = time (in minutes) of reaction incubation
vol assayed = volume (in ml) used in colorimetric determination

Unit Definition: One unit will hydrolyze casein to produce color equivalent to 1.0 µmole (181 µg) of tyrosine per minute at pH 7.5 at 37 °C (color by Folin & Ciocalteu's Reagent).

For example, using the procedure for absorbance measurement with a cuvette and a tyrosine equivalent equal to 0.0275 µmoles, the units/ml would be determined as follows:

$$\text{Units/ml} = \frac{0.0275 \times 0.285}{0.025 \times 10 \times 0.25} = 0.125\ \text{µmoles ml}^{-1}\ \text{min}^{-1}$$

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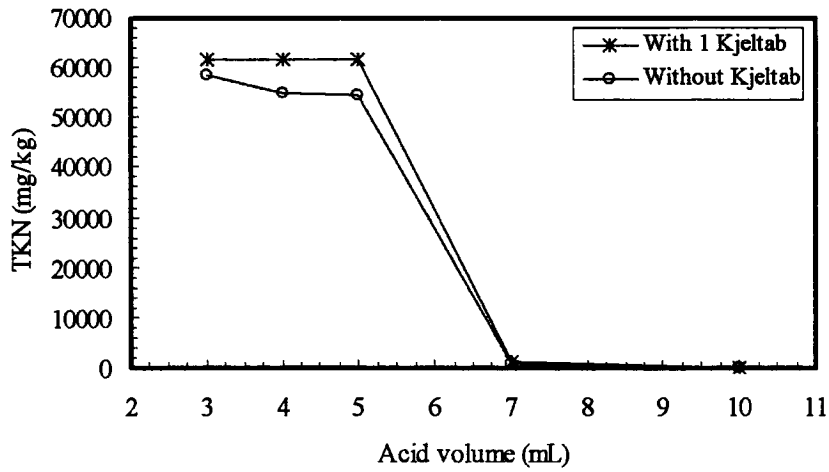
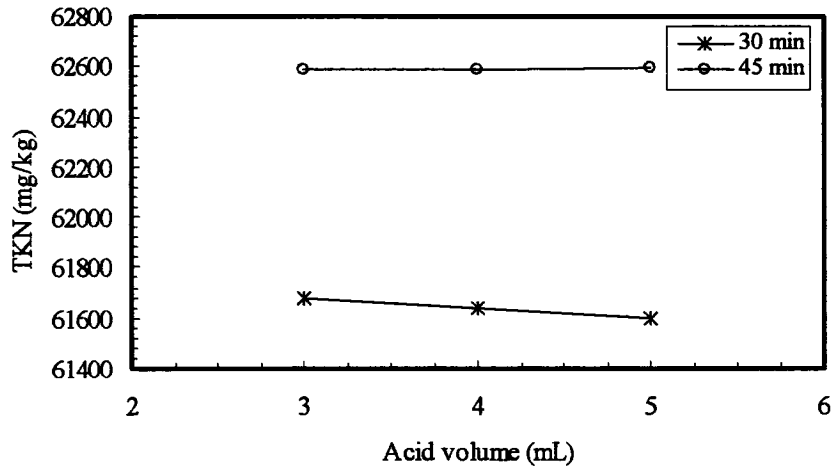
Appendix B

Effect of Sulfuric Acid Volume, Addition of Kjeltabs and Digestion Time on Total Kjeldhal Nitrogen Determination

The effects of sulfuric acid volume, addition of kjeltabs and digestion time on nitrogen recovery for the determination of total kjeldahl nitrogen was investigated in order to develop the optimum digestion parameters for TKN analysis of shrimp shells. The effects of sulfuric acid volume (3, 4, 5, 7 and 10), addition of Kjeltabs (1 Kjeltabs vs. no Kjeltabs) and digestion time (30 and 45 minutes) on the nitrogen recovery are shown in Figure B.1 shows the results. It was found that excess sulfuric acid caused significant nitrogen losses (up to 100%). The optimum digestion parameters were 5 mL sulfuric acid volume, addition of 1 Kjeltabs and digestion time of 45 min.



(a) effect of acid volume and addition of Kjeltab



(b) effect of acid volume and digestion time

Figure B.1. Total kjeldahl nitrogen of shrimp shells at different digestion conditions.

Appendix C

Analyses of Chitin Purified from Shrimp Shells Using Chemical Approach (Process Required for the Analyses of Chitin and Protein Contents)

Table C.1 shows the chitin yield from the procedure followed for chitin extraction from shrimp shell waste. The average chitin yield was about 21.35%. The mineral composition of both the shrimp shell waste and the extracted chitin is shown in Table C.2. Zakaria (1997) reported that final calcium content of about 0.1% is usually taken as an optimum value. In this study, the extracted chitin using chemical purification contained 0.1 – 0.06% calcium. The mineral removal efficiency is shown in Table C.3.

Table C.1. Chitin yield.

Replicate	Shrimp shells weight (g)	Extracted chitin weight (g)	Extracted chitin yield (%)
1	1.1276	0.2385	21.1511
2	1.0099	0.2143	21.2199
3	1.0165	0.2172	21.3674
4	1.0116	0.2199	21.7378
5	1.0284	0.2212	21.5091
6	1.0100	0.2135	21.1386
Average	1.0340 ± 0.0464	0.2208 ± 0.0092	21.3540 ± 0.2354[a]

[a] STD = 0.2354 and CV = 1.10%

Table C.2. Minerals composition of the shrimp shells and the extracted chitin.

Minerals	Minerals concentration (mg/kg)			
	Shrimp shells	Extracted chitin		
		(1)	(2)	Average
Mg	6150	724	784	754
Ca	142000	1000	621	810.5
Mn	116	80	43	61.5
K	871	108	116	112
Na	3480	444	570	507
Fe	308	70	98	84
P	22400	360	320	340
S	4700	1900	9900	5900
Si	794	1030	1360	1195
Al	250	222	185	203.5
Ti	<90	<90	<90	-

Table C.3. Process minerals removal efficiency.

Parameter	Shrimp shells		Extracted chitin		Minerals removal efficiency (%)		
	(1)	(2)	(1)	(2)	(1)	(2)	Average
Weight (g)	1.0116	1.0100	0.2199	0.2135	-	-	-
Minerals content (mg)[a]							
Mg	6221340	6211500	159208	167384	97.44	97.31	97.37
Ca	143647200	143420000	219900	132584	99.85	99.91	99.88
Mn	117346	117160	17592	9181	85.01	92.16	88.59
K	881104	879710	23749	24766	97.30	97.18	97.24
Na	3520368	3514800	97636	121695	97.23	96.54	96.88
Fe	311573	311080	15393	20923	95.06	93.27	94.17
P	22659840	22624000	79164	68320	99.65	99.70	99.67
S	4754520	4747000	417810	2113650	91.21	55.47	73.34
Si	803210	801940	226497	290360	71.80	63.79	67.80
Al	252900	252500	48818	39498	80.70	84.36	82.53
Ti	-	-	-	-	-	-	-

[a] Total minerals content calculated based on the total sample weight