ENZYMATIC EXTRACTION OF PROTEINS AND AMINO ACIDS FROM WHOLE FISH AND FISH WASTE

by

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ABSTRACT

Fish and fish waste can be used to produce various value added by products such as proteins, oil, omega-3 fatty acids, biodiesel, amino acids, peptides, collagen, gelatin and silage, each of which has various applications in the food industry, renewable energy and medicinal purposes. Fish protein contains amino acids and many bioactive peptides . Fish proteins are found in the flesh, head, frames, fin, tail, skin and guts in varying quantities. After removing the flesh, all other parts are considered waste which is not properly utilized. The aim of this study was to evaluate the enzymatic extraction of amino acids from fish protein for use as substrates in the microbial production of jadomycin, an antimicrobial agent and potential anti-cancer drug. In this study, enzymatic extraction of proteins was carried out using Alcalase enzyme at three enzyme concentrations (0.5, 1 or 2%) and four time intervals (1, 2, 3 and 4 h). The fish protein hydrolysate was dried using spray dryer to obtain protein powder. The highest protein yield (76.30% from whole fish and 74.53% from the frame) was obtained using 2.0% enzyme concentration after 4 h of hydrolysis. The enzymatic extraction of amino acids were carried out using the enzymes Alcalase and Neutrase (individually and in combination) and the effect of reaction time (24 and 48 h) on the hydrolysis of proteins was studied. The profiling of amino acids was carried out using gas chromatography. Fourteen amino acids were extracted from fish proteins of which twelve amino acids have been used by researchers for the production of jadomycins. These are: alanine (7.59%), glycine (5.82%), histidine (3.59%), isoleucine (5.30%), leucine (9%), lysine (7.34%) methionine (2.2%), phenylalanine (4.2%), serine (4.3%), threonine (5.40%), tyrosine (3.17%) and valine (7.2%). Tryptophan which is suitable of producing jadomycin was not present in the fish protein. No reports were found in the literature for jadomycin production from glutamic acid. Therefore, glutamic acid (9.85%), and proline (0.98%) which are present in the fish protein should be investigated for possible production of jadomycins.

LIST OF ABBREVIATIONS AND SYMBOLS USED

ACE:	Angiotensin I- Converting Enzyme
ADP:	Adenosine Diphosphate
AMP:	Adenosine Monophosphate
ATP:	Adenosine Triphosphate
BSA:	Bovine Serum Albumin
BSE:	Bovine Spongiform Encephalopathy
CSTR:	Continuous Stirred Reactor
DHA:	Docosahexaenoic Acid
EC	Enzyme Concentration
EMR:	Enzyme Membrane Reactor
EPA:	Eicosapentaenoic Acid
F	Frame
FTSG:	Fin Tail Skin and Gut
GC:	Gas Chromatography
gly-trp:	Glycine-Trypsin
H	Head
HCl:	Hydrochloric acid
Hx:	Hypoxanthine
ile-ile:	Isoleucine-Isoleucine
ile-trp:	Isoleucine-Trypsin
IMP:	Inosine Monophosphate
Ino:	Inosine
L-orn acetate:	L-ornithine Acetate
L-orn.HCl:	L-ornithine Hydrochloride
MSA:	Methanesulfonic Acid
NADH:	Nicotinamide adenine dinucleotide
NADPH:	Nicotinamide adenine dinucleotide phosphate
PAF:	Platelet Activating Factor
PEG:	Polyethylene Glycol

PFA:	Polytetrafluoroethylene
PPM:	Parts per Million
RT	Reaction Time
TLC:	Thin Layer Chromatography
val-ile:	Valine-Isoleucine
val-trp:	Valine-Trypsin
val-val:	Valine-Valine
WF	Whole Fish

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CHAPTER 1. INTRODUCTION

The fish processing industry in Canada is one of world's major exporters of seafood and marine products. Canada exports 75 % of its fish products to more than 80 countries. In the year 2011, exports from Canada amounted to 609,334,945 kg of fish worth $4.09 billion (FOC, 2011). Canada has the world's longest coastline (244,000 km) which makes 25% of the entire world's coastline. Atlantic Canada represents 40,000 km of coastline which comprises four major provinces. It exports high quality harvested groundfish, shellfish and pelagic fish accounting for 85% of the total landings (ACOA, 2009). The Pacific fishery accounted for 14% of the total landings whereas the freshwater fishery accounted for 1% of the total landings. Canadian aquaculture production include salmon, trout, steelhead, clams, oysters, mussels and scallops. The aquaculture production in Canada for the year 2010 reached 161,326 tonnes worth $926,504 (FOC, 2010; AMEC, 2003).

Most of the plants in the Atlantic Region use mechanized equipment for processing fish into fillets. The fish is first washed in large wash tanks and the skin is removed from the fillet by hand or by machines. The solid wastes from filleting, skinning and candling operations are rendered for pet food or as animal meal. The amount of the waste collected from each province is shown in the Table 1.1 (AMEC, 2003).

Marine capture fisheries contribute more than 50% of the total world fish production. About 70% of fish is processed resulting in a significant amount of fish waste (20-80% depending on the level of processing and type of fish) (AMEC, 2003). Each year a significant amount of the total catch from fish farming is discarded. Also, fish processing requires large volumes of potable water which results in a significant amount of waste water (FOC, 2005). The majority of fish wastes are disposed off in the ocean. The aerobic bacteria present in the water breakdown the organic matter in the presence of oxygen leading to a considerable reduction in the oxygen in water. There is also an overload of nitrogen, phosphorous, ammonia, which leads to pH variation, increased turbidity of the water and growth of algae. The decomposition process causes a reduction in the oxygen content, creating an anaerobic condition that leads to the release of foul gases such as

Table 1.1. Fish waste amount by province in 2001 (AMEC, 2003).

Province	Landing		Product		Waste	
	(Tonnes)	(%)	(Tonnes)	(%)	(Tonnes)	(%)
New Brunswick	113588	13.95	89012	78.36	24576	21.63
New Foundland and Labrador	267959	32.92	120999	45.15	146960	54.84
Nova Scotia	366381	45.01	146708	40.04	219673	59.95
Prince Edward Island	66046	8.11	39000	59.04	27046	40.95
Total	813974	100.00	395719	48.61	418255	51.38

hydrogen sulfide and ammonia, organic acids and green house gases such as carbon dioxide and methane (Tchoukanova et al., 2012).

The discards from the processing plants amount to 20 million tonnes which is equivalent to 25% of the world's total production from marine capture fisheries (AMEC, 2003). These waste can be used to produce fish protein concentrate, fish oils and enzymes such as pepsin and chymotrypsin. The fish oil is used for products such as margarine, omega-3 fatty acids and biodiesel. The fish protein concentrate is used as animal feed. Fish protein is also rich in amino acids which are highly suited for human consumption (Murray et al., 2001).

Amino acids from fish proteins can be utilized for the production of jadomycin which is an antimicrobial agent that has shown great potential as an anti-cancer drug. Jadomycins are produced as secondary metabolite by a soil microbe *Streptomyces venezuelae ISP5230* (Burdock et al., 2008). The biosynthetic pathway for the production of jadomycin involves the integration of amino acids into the oxazolone ring structure in the presence of a suitable nutritional environment and with applied stress caused by either ethanol, heat or phage shock. Different types of jadomycin can be produced using different amino acids in the production medium (Doull et al., 1994; Jakeman et al., 2006). The amino acids that are capable of producing jadomycins are all present in fish protein. Use of fish and fish waste would potentially improve the economics of jadomycin production and facilitate drug development. The aim of this study was to evaluate the enzymatic extraction of amino acids from fish protein for use as substrate in the production medium for jadomycin.

CHAPTER 2. OBJECTIVES

The main aim of this research was to evaluate the enzymatic extraction of proteins and amino acids from whole fish and fish waste for use as a substrate for production of jadomycin. The specific objectives were:

1. To study the effect of three different concentrations (0.5, 1 and 2%) of the enzyme Alcalase on the extraction of proteins from different parts of fish (whole fish, head, fin, tail, skin and gut, and frames).
2. To study the effect of four times (1, 2, 3 and 4 h) on the extraction of proteins from different parts of fish (whole fish, head, fin, tail, skin and gut, and frames).
3. To extract amino acids from the proteins extracted from whole fish and fish waste using the enzymes Alcalase and Neutrase and to:
    (a) evaluate the effectiveness of the enzymes Alcalase and Neutrase individually and in combination on the extraction of amino acids from fish proteins, and
    (b) study the effect of time (24 and 48 h) on the enzymatic hydrolysis of protein.
4. To determine the profile of amino acids extracted from fish proteins using thin layer chromatograph (TLC).
5. To characterize the amino acids extracted from fish proteins using gas chromatography-flame ionization detector (GC-FID).

CHAPTER 3. LITERATURE REVIEW

3.1. Fish Processing

Most fish processing plants process fish using the following steps: stunning of fish, grading, removal of slime, scaling, washing, deheading, gutting, cutting of fins, slicing into steaks, filleting, meat-bone separation, packaging, labeling and distribution.

3.1.1. Stunning of Fish

The stunning of fish is the first and most critical step in the processing of fresh water and farmed fish because prolonged agony experienced by the fish causes the production of undesired substances in the tissue. The oxygen deficiency in the blood and muscle causes accumulation of lactic acid and leads to paralysis of the neural system. Stunning of the fish produces movements enough to break vertebrae and rupture blood vessels. Red spots appear on the surface of the skin and in the muscle tissue near the backbone (Bykowski et al., 1996). Borderias et al. (2011) stated that electrical stunning is recommended for killing salmon and grass carp over carbon dioxide because it causes earlier onset of rigor mortis and faster adenosine triphosphate (ATP) depletion and increased shelf life. Electrical stunning carried out on turbot (*Scopthalmus maximus*) resulted in a rapid drop in the pH and potential increase in fillet gaping. Erikson et al. (2012) stated that the efficiency of electrical stunning depended on whether the fish is stunned in the head or through the whole body. Morzel et al. (2002) reported that during stunning, the initial pH was low and rapid onset of rigor mortis occurred and the flesh was softer, redder and darker when compared to fish cut by percussion or bleeding. Roth et al. (2002) reported that when Atlantic salmon were electrically stunned in water for 1.5 s, there was no accelerated rigor development and no injuries were observed in the fish.

Erikson et al. (2012) stated that some of the wild fish are subjected to asphyxiation on board after capture until they die. In some cases of farmed fish, the fish is directly plunged into iced water in which the temperature is kept close to 0°C. It is critical that the temperature is kept low because the fish would not die due to temperature shock but by asphyxia which affects the quality and texture of the fish (Borderias et al., 2011).

3.1.2. Grading of Fish

The second step in fish processing is fish grading by species and size. Grading of fish can be done manually or by using mechanical equipment. The mechanical grading equipment is more precise for fish before or after rigor mortis than for fish in a state of rigor mortis. The automated grading instruments are 6-10 times more efficient than manual grading (Tave et al., 1994; Lovshin et al., 1994). The basic benefits of the automated system are: low production costs and increased quality of fish products at the end of the processing chain (Borderias et al., 2011; Jensen, 1990).

3.1.3. Slime Removal

Fish secretes slime on its surface as a protection mechanism against harmful conditions. The slime secretion stops before rigor mortis. Pseudomonas species are one of the potent spoilers, always present in the sea water, and fish slime provides them a perfect environment to grow (Chai et al., 1975; Doyle., 1995). Anaerobic bacteria present during processing can produce hydrogen sulfide by taking up sulfur compounds from the slime, skin and flesh (Granata et al., 2012; Chen et al., 1982). Therefore, slime should be removed by continuous washing. Slime present in some of the species such as eel, trout and other fresh water species should be soaked in a solution of 2% baking soda and then washed in a cylindrical rotating washer (Borderias et al., 2011; Doyle., 1995).

3.1.4. Scaling of Fish

The process of scaling is one of the toughest and extremely labour intensive step in fish processing. The scales may also harbor bacterial pathogens and removing them will keep the fish fresh refrigerated or frozen (Ringo et al., 2010; Trust, 1986). The scaling can be done manually with a hard brush or scaling blades. Fish such as perch, pike-perch, carp and bream are difficult to remove the scales from and so these fish are blanched in boiling water for 3-6 seconds and then scaled using mechanized hand-held scalers in motion perpendicular to the long body axis. The electrical scalers are more efficient (complete elimination of scales) than the manual tools and save lot of time (Borderias et al., 2011).

3.1.5. Washing

The primary goal of washing is to clean and remove accumulated bacteria on the fish. The effective washing of fish depends upon the ratio of fish : water, the quality of water and kinetic energy of the water stream. The recommended washing ratio of fish : water is 1:1, but during processing the amount of water used increases by two fold. Use of potable water is recommended during freshwater fish processing (Borderias et al., 2011). Washing is carried out using vertical drum, horizontal drum and combination washer conveyor belt washers. The washing time is about 1-2 min and these mechanized washers can be used to process whole fish, deheaded and gutted fish as well as fish fillets. Washing action does not cause any physical damage to the product (Hossain et al., 2004). The washing is always continuous and is accomplished by spraying pressurized water and the dirty water is collected in the waste basins (Bechtel, 2003).The amount of wastewater produced during each step in fish processing is shown in Table 3.2 (Arvanitoyannis et al., 2008)**.**

3.1.6. Deheading

The fish head constitutes around 10-20% of its weight (Table 3.1) and it is considered as an inedible part (Waterman, 2001). The fish can be deheaded by three different ways: round cut, straight cut and contoured cut. It can be performed manually and mechanically. In most fish plants, manual deheading is performed because it causes minimal flesh loss. A cut around the operculum is a called round cut and it results in lowest meat loss. The contour cut is the one which runs perpendicular to the fish backbone and then at an angle of 45°. This cut is mainly used when the final product is a boneless and skinless fillet (Borderias et al., 2011). Manual cutting is easier for small fresh water fish, but larger fish ranging from 20-40 cm can be deheaded using mechanical devices. Machines with a guillotine cutter are suitable for larger fish under-going round or contour cuts. Machines with a manually-operated circular saw are suitable for larger fish undergoing straight cut (Bechtel., 2003; Jonatansson et al., 1986). The amount of deheaded waste produced from fish processing is shown in Table 3.2 (Arvanitoyannis et al., 2008).

Table 3.1. Physical composition of fish (Waterman, 2001)

Component	Average Weight (%)
Head	21
Gut	7
Liver	5
Roe	4
Backbone	14
Fins and lugs	10
Skin	3
Fillet, skinned	36

3.1.7. Gutting

Gutting of the fish is the removal of internal organs and optionally cleaning the body cavity of the peritoneum, kidney tissue and blood. In the gutting process, the fish is cut longitudinally to remove the internal organs on a table made of special material which is easy to wash and does not absorb fluids. The table should be rinsed and periodically disinfected. There are some mechanical gutting machines used for trout, eel and other fish, but their use increases the processing cost of the fish (Jonatansson et al., 1986). The internal organs constitutes around 5-8% of the fish weight, as shown in Table 3.1 (Waterman, 2001). The amount of waste in the gutting processing is shown in Table 3.2 (Arvanitoyannis et al., 2008).

3.1.8. Cutting of Fins

Fins constitute around 1-2% of the fish weight as shown in Table 3.1 (Waterman, 2001). The amount of fin waste after fish processing is shown in Table 3.2 (Arvanitoyannis et al., 2008). Fins are cut manually either by a knife or by mechanized rotating disc knives (Morkore et al., 2001). This process is mostly carried out after deheading and gutting. This process is difficult for cutting larger fish. So the mechanical knives are provided with a slit opening in which the fins are cut when the fish are passed through it manually (Borderias et al., 2011; Ewing, 1988).

3.1.9. Steaks and Fillets

Deheaded whole fish are sliced into steaks by cutting perpendicular to the backbone. Small and medium-sized fish are cut manually in a concave basin with evenly-spaced slots to facilitate slicing. The average thickness of the fish pieces are 2.5-4.5 cm. Large fish such as cyprinids are sliced mechanically because of their solid and massive backbone. These pieces are more popular in the retail market and the canning industry (Borderias et al., 2011; Hanson et al., 2001).

Fillets are pieces of meat containing only the dorsal and abdominal muscles. The fillets are processed manually and mechanically. Manual filleting is carried out in small

Table 3.2. Inputs and outputs of various fish processes (Arvanitoyannis et al., 2008).

Process	Inputs		Outputs					
	Fish (kg)	Energy (kW h)	Wastewater (m^3)	BOD (kg)	COD_5 (kg)	Nitrogen (kg N)	Phosphorous (kg P)	Solid waste (kg)
White fish filleting	1000	Ice: 10-12 Freezing: 50-70 Filleting: 5	5-11	35	50	-	-	Skin: 40-50 Heads: 210-250 Bones: 240-340
Oily fish filleting	1000	Ice: 10-12 Freezing: 50-70 Filleting: 2-5	5-8	50	85	2.5	0.1-0.3	400-450
Canning	1000	150-190	15	52	116	3	0.1-0.4	Head: 250 Bones: 100-150
Fish meal and fish oil	1000	Electricity: 32	-	-	-	-	-	-
Frozen Fish thawing	1000	-	5	-	1-7	-	-	-
De-icing and washing	1000	0.8-1.2	1	-	0.7-4.9	-	-	0-20
Grinding	1000	0.1-0.3	0.3-0.4	-	0.4-1.7	-	-	0-20
Scaling of white fish	1000	0.1-0.3	10-15	-	-	-	-	Scales: 20-40
Deheading of white fish	1000	0.3-0.8	1	-	2-4	-	-	Head and debris: 270-320
Filleting of deheaded white fish	1000	1.8	1-3	-	4-12	-	-	Frames and off cuts: 200-300
Filleting of ungutted oily fish	1000	0.7-2.2	1-2	-	7-15	-	-	Entrails, tails, heads and frames: 400
Skinning white fish	1000	0.4-0.9	0.2-0.6	-	1.7-5	-	-	Skin: 40
Skinning oily fish	1000	0.2-0.4	0.2-0.9	-	3-5	-	-	Skin: 40
Trimming and cutting of white fish	1000	0.3-3	0.1	-	-	-	-	-
Packaging of fillets	1000	5-7.5	-	-	-	-	-	-
Freezing and storage	1000	10-14	-	-	-	-	-	-
Unloading fish for	1000	3	2-5	-	27-34	-	-	-

Process	Inputs		Outputs					
	Fish (kg)	Energy (kW h)	Wastewater (m^3)	BOD (kg)	COD_5 (kg)	Nitrogen (kg N)	Phosphorous (kg P)	Solid waste (kg)
canning								
Grading of fish	1000	0.15	0.2	-	0.35-1.7	-	-	0.30
Precooking of fish to be canned	1000	0.3-11	0.07-0.27	-	-	-	-	Inedible parts: 150
Nobbing and packing in cans	1000	0.4-1.5	0.2-0.9	-	7-15	-	-	Head and entrails: 150 Bones and meat: 100-150
Draining of cans containing precooked fish	1000	0.3	0.1-0.2	-	3-10	-	-	-
Sauce filling	1000	-	-	-	-	-	-	Spillage of sauce and oil: varies
Can sealing	1000	5-6	-	-	-	-	-	-
Washing of cans	1000	7	0.04	-	-	-	-	-
Sterilization of cans	1000	230	3-7	-	-	-	-	-
Handling and storage of fish	1000	10-12	-	-	130-140	-	-	-
Unloading of fish	1000	3	2-5	-	27-34	-	-	-
Cooking of fish	1000	90	-	-	-	-	-	-
Pressing the cooked fish	1000	-	750kg water 150kg oil	-	-	-	-	Press cake: 100 dry matter
Drying of press cake	1000	340.0	-	-	-	-	-	-
Fish oil polishing	1000	Hot water	0.05-0.1	-	5	-	-	-
Stick water evaporation	1000	475	-	-	-	-	-	Concentrated stick water: 250 Dry matter: 50

freshwater fish industries and mechanical filleting is used for processing marine fish. Once the fillet leaves the filleting stations, three products remain: napes, block, and trimmed fillet. Napes are the thinnest part of a fillet that covered the guts before the fish was gutted. Blocks are parts which are removed from fillets for aesthetic purposes. Trimmed fillet is the final product in which the napes and pin bone attached to some fillets will be removed (Jonatansson et al., 1986).

3.1.10. Meat Bone Separation

Around 30-50% of the meat is usually left along the ribs and backbone during filleting. In smaller fish, the loss of meat is high and so minced meat is gaining more attention (Eide et al., 1982). Minced meat can also be produced from less valuable species after deheading and carefully removing their internal organs. In this process, the meat is removed from skin, scales and bones through automated devices called separators. The fish travels along a conveyor belt which runs closely to a cylinder which has holes in it. The meat is squeezed through the holes due to the pressure applied from the conveyer belt and the bones are scraped away (Borderias et al., 2011). The minced meat stability is much less than that of intact fish muscle and so it is frozen immediately. It is used to produce fish burgers, fish sticks, canned fish, vegetable mixes and fish dumplings (Venugopal et al., 1995).

3.2. Changes During Fish Production Process

During the processing of fish, certain changes take place in the fish tissue such as slime secretion on the surface of fish, rigor mortis, autolysis as enzymatic decomposition of tissues and microbiological spoilage (Bykowski et al., 1996).

3.2.1. Slime Secretion

Slime or mucus is a secretion product that occurs in almost all animals including fish and is involved in numerous life processes. In soft-bodied animals, mucus forms a protective covering, attenuating the effects of the environment. It lubricates the body

surface and facilitates locomotion and prevention against sharp edges (Jakowska, 1963). In fish, slime is formed in the skin and it is very active just after its death. The quantity of slime varies between different fish species, reaching up to 2-3% of the fish mass and can create problems during processing (Bykowski et al., 1996). Certain fish species such as hagfish secrete two types of exudates which includes normal epidermal mucus and extruded slime. The epidermal mucus provides a physical and biological barrier between fish and its aquatic environment. However, extruded slime is produced during feeding or when hagfish is stressed or provoked. The extruded slime provides protection against predators and other scavengers (Shephard, 1993).

3.2.2. Rigor Mortis

Rigor mortis occurs as a result of biochemical reactions which cause the muscle fibers to shorten and tighten, becoming stiff (Berkel et al., 2004). When the bones are removed prior to rigor mortis the length of the fillet shortens by 30% but the size of fillet becomes wider and thicker (Ghaly et al, 2010). However, its volume does not change (Bykowski et al., 1996). The permanence, resolution and intensity of the rigor mortis are important factors for fish processing. The delay in the occurrence of rigor mortis increases fish conservation. There are three types of rigor (pre rigor, full rigor and post rigor) based on parameters such as visual/tactile estimation of body rigidity and strength of deformation. Rigor mortis can be tracked by changes in the viscosity of a high ionic strength muscle extract (Erikson et al., 1997). Fish body temperature also plays a major role in the formation of rigor mortis, the higher the temperature the faster it begins and sooner it ends. This causes greater changes in the proteins and greater losses in the tissue juices. If the rigor mortis starts later, the shelf life of the fish is longer (Trucco et al., 1982). During the on-set of rigor mortis, the ATP content of the muscle drops below a critical level and this very often causes the connective tissue of the individual myomeres to break and leads to muscle separation that reduces the quality of fish (Berg et al., 1997; Park et al., 1990).

3.2.3.Autolysis

After the death of the fish, a complex biochemical process starts which leads to the

enzymatic decomposition of basic compounds such as tissues, proteins, lipids and carbohydrates. This decomposition breaks down proteins into amino acids which play an important role in the sensory features of fish as it makes great changes in the structure of muscle (becomes softer). The tissues have substantial liquid loss making them stiff and providing ideal condition for the spoilage microorganisms. Microorganisms also decompose other compounds containing nitrogen and lipids to peroxides, aldehydes, ketones and lower aliphatic acids that form ammonia, hydrogen sulfide, mercaptans, indole, and skatole, all of which releases unpleasant odours and results in an unpalatable product (Kristinsson et al., 2000; Bykowski et al., 1996; Gram et al., 1996). The accumulation of catabolites leads to the enzymatic breakdown of adenosine tri-phosphate ATP to adenosine diphosphate (ADP), then to adenosine monophosphate (AMP), then to inosine monophosphate (IMP) to inosine (Ino) and finally to hypoxanthine (Hx) (Gill et al., 1987).

3.3. Composition of Fish Waste

Most fish species mainly comprise of 80% water. However, the water content in some species can vary between 30 to 90% (Murray et al., 2001). The Composition of the fish varies according to the type of species, sex, age, nutritional status, time of year and health. Most of the fish contains 15-30% of protein, 0-25% of fat and 50-80% of moisture (Ghaedian et al., 1998). Suvanich et al. (2006) reported that the variation in the composition of catfish, cod, flounder, mackerel and salmon varied according to the species. Mackerel had the highest fat content (11.7%) and cod had the lowest (0.1%). Salmon had the highest protein content (23.5%) and flounder had the lowest (14%). The moisture content of the five fishes varied between 69 and 84.6% and the ash content of all the species were similar, as shown in Table 3.3.

The byproducts of the fish processing industry can be a great source of value added products such as protein amino acids, collagen, gelatin and oil (Disney et al., 1977). Solid fish waste consists of head, tails, skin, gut, fins and frames. It proves to be a great source of proteins (58%), ether extract or fat (19%) and minerals. Also, monosaturated acids, palmitic acid and oleic acid are abundant in fish waste (22%) as shown in Table 3.4

Table 3.3. Composition of the fish fillets determined by standard methods (Suvanich et al.,2006).

Fish Type	Fat (%)	Ash (%)	Protein (%)	Moisture (%)
Catfish	7.7	0.9	15.4	76.3
Cod	0.1	1.1	18.2	80.8
Flounder	0.7	1.3	14.0	84.6
Mackerel	11.7	1.1	18.8	69.0
Salmon	1.6	1.1	23.5	74.3

Table 3.4. Nutritional and mineral composition of fish waste (Esteban et al., 2007).

Nutrient	Fish waste
Crude protein (%)	57.92 ± 5.26
Ether extract (%)	19.10 ± 6.06
Crude fiber (%)	1.19 ± 1.21
Ash (%)	21.79 ± 3.52
Calcium (%)	5.80 ± 1.35
Phosphorous (%)	2.04 ± 0.64
Potassium (%)	0.68 ± 0.11
Sodium (%)	0.61 ± 0.08
Magnesium (%)	0.17 ± 0.04
Iron (ppm)	100 ± 42
Zinc (ppm)	62 ± 12
Manganese (ppm)	6 ± 7
Copper (ppm)	1 ± 1

Values in % or mg/kg (ppm) on a dry matter basis.

(Esteban et al., 2006).

3.3.1.Proteins

Fish frames contain significant amounts of muscle proteins. These muscle proteins are highly nutritious with a well-balanced amino acid composition and are easily digestible Therefore, proteins from this part of the fish waste can be extracted by enzymatic hydrolysis rather than being discarded as waste (Venugopal et al., 1996). Proteins derived from fish are nutritionally superior when compared to that of plant sources. They have a better balance of the dietary essential amino acids compared to all other animal sources (Friedman, 1996; Yanez et al., 1976). Fish muscles proteins are more heat sensitive than mammalian muscles proteins (Dunajski, 1979). Fish muscle proteins from the cold water species are more susceptible to denaturation by heat when compared to that of tropical water fishes. The T-50 values (the temperature required for 50% denaturation of the fish muscles) are influenced by the pH and were reported to be in the range of 29-350°C at a pH of 7.0 and in the range of 11-270°C at a pH of 5.5 (Kristinsson et al., 2000).

Fish muscle consists of two types: light and dark. The proportion of dark muscle is low in white fish such as cod and haddock where there is a small strip of dark or red muscle just under the skin on both sides of the body. In fatty fish such as herring and mackerel, the percentage of dark muscles is high and the muscles contain more vitamins and fats as shown in Figure 3.1(Murray et al., 2001). Light muscle is more abundant and contains about 18-23% proteins.

About 70-80% of the fish muscles are made up of structural proteins and the remaining 20-30% are composed of sarcoplasmic proteins with about 2-3% insoluble connective tissue proteins. Myofibrillar proteins are the primary food proteins and they make up about 66-77% of the total protein content in the fish meat. These myofibrillar proteins comprise of myosin (50-60%) and actin (15-30%) (Spinelli et al., 1982). The myosin fibers can be cleaved by proteases trypsin and chymotrypsin on one end and on the other end with papain. During this cleavage, the myosin fibers are divided into heavy meromyosin and light meromyosin with different functional properties. Actin occurs in

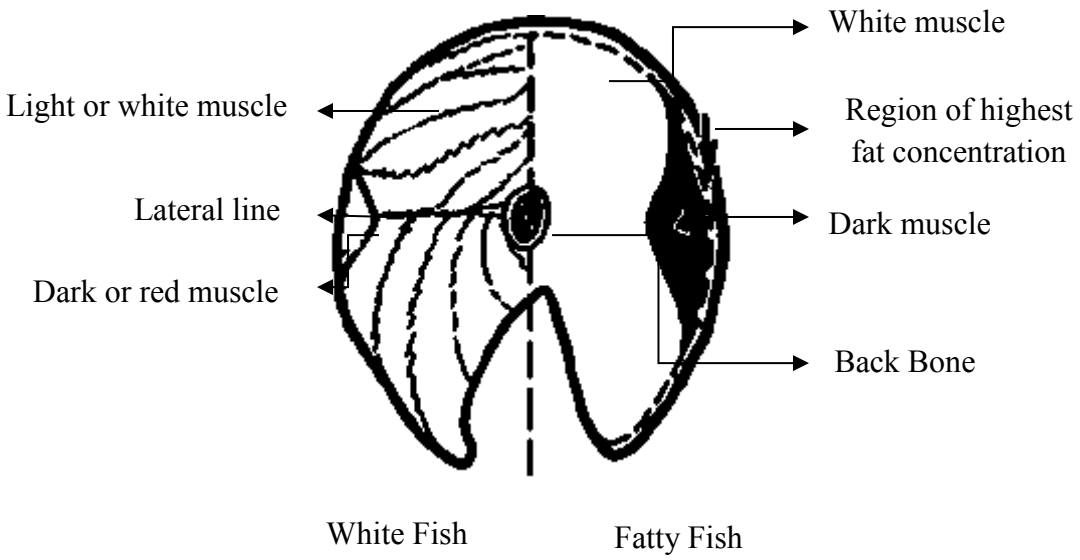


Figure 3.1. Types of muscles in white fish and fatty fish (Murray et al., 2001).

two forms, G-actin, a spherical monomer and F-actin, a large polymer which connects to myosin (Kristinsson et al., 2000).

3.3.2.Bioactive Peptides

Proteins extracted from the fish muscle also contain a number of peptides which have many bioactivities such as antihypertensive, antithrombotic, immune modulatory and antioxidative properties (Kim et al., 2000). The bioactive peptides obtained from the fish muscle also have anticoagulant and antiplatelet properties, which is the main reason behind the capability of peptides obtained from the fish to inhibit coagulation factors in the intrinsic pathway of coagulation (Je et al., 2005). The protein obtained by the enzymatic hydrolysis of the fish muscle has several nutritional and functional properties from which many biologically active peptides can be obtained (Benkajul et al., 1997).

3.3.3. Collagen and Gelatin

The fish skin waste is a good source for collagen and gelatin which are currently used in food, cosmetic and biomedical industries. Collagen and gelatin are two different forms of same macromolecule in which gelatin is a partially hydrolysed form of collagen. The collagen and gelatin are two unique and more significant forms of proteins in comparison to that of fish muscle proteins. The significance lies upon the amino acid content, more than 80% are non-polar amino acids such as glycine, alanine, valine and proline (Byun et al., 2001). Heat denaturation of collagen easily converts it into gelatin. The collagen and gealtin extracted from bovine sources pose the risk of mad cow disease or bovine spongiform encephalopathy (BSE), whereas the collagen and gelatin extracted from the fish skin eliminates the risk of BSE. The gelatin extracted enzymatically from fish skin has better biological activities as antioxidants and antihypertensive agents. The gelatin has a unique repeating sequence of glycine-proline-alanine in their structure compared to the peptides derived from fish muscle protein and it is the main reason behind the antioxidative property of gelatin (Kim et al., 2005; Byun et al., 2005).

3.3.4. Fish Oil

Fish processing byproducts contain fish oil. The amount generally depends upon the fat content of the specific fish species. Generally, fish contains 2-30% fat. Almost 50% of the body weight generated as waste during the fish processing would be a great potential source for good quality fish oil which can be used for human consumption. The fish oil consists of two main fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These two fatty acids are polyunsaturated fatty acids which are classified as omega-3 fatty acids. They are mainly found in the marine animals which have high polyunsaturated fatty acid content (Zuta et al., 2003).

3.3.5. Calcium

Fish bones are normally separated after removal of muscle proteins from the frames. The fish bone mainly accounts for 30% of the collagen and therefore it is an additional source of collagen along with fish skin. Fish bone also contains 60-70% of inorganic content such as calcium, phosphorous and hydroxyapatite (Kim et al., 2005). Generally calcium is deficient in most of the regular diets and to improve calcium intake, consumption of small whole fish can be nutritionally valuable. The fish bone obtained from the fish processing waste can the used to provide calcium. In order for it to be a fortified food, it should be converted into edible form by softening its structure with hot water treatment, hot acetic acid solutions or by super heated steam cooking (Ishikawa et al., 1990). Fish bone is a very good source of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) which can be used as a bone graft material in medical and dental applications. Previously autografts, allografts and xenografts were used to solve bone fractures and damages but they were found to be ineffective due to their mechanical instability and incompatability. The important properties of hydroxyapatite are: it does not break under physiological conditions, it is thermodynamically stable at physiological pH and it plays an active role in bone binding (Larsen et al., 2000).

3.3.6. Enzymes

The internal organs of the fish are a rich source of enzymes, many of which exhibit

high catalytic activity at relatively low concentrations. The enzymes which are available in fish include: pepsin, trysin, chymotrypsin and collagenases. These enzymes are commercially extracted from the fish viscera in a large scale. These proteinases extracted from the fish possess better catalytic properties, good efficiency at lower temperatures, lower sensitivity to substrate concentrations and greater stability in a wide range of pH (Zhou et al., 2011; Byun et al., 2005; Kim et al., 2005).

3.4. Utilization of spoiled Fish and Fish Waste

The fish processing industry is growing and it is estimated that around 145.1 million tonnes of fish was produced in the year 2009, out of which 27.3 million tonnes was used for the non-food products such as fish meal, fish oil and fish silage as shown in Table 3.5 and Table 3.6 (FAO, 2010).

3.4.1. Fish Silage

Fish silage is an excellent protein source having high biological properties for animal feeding. Fish silage is a liquid product made from whole fish or parts of fish that are liquefied by the action of enzymes in the fish in the presence of added acid. The enzymes present in the acidic medium break down fish proteins into smaller soluble units while the acid helps to speed up their activity and prevent bacterial spoilage. Fish silage can be made from spoiled fish, sub-utilized species, by-products from marine fishing, commercial fish waste and industrial residues. (Disney et al., 1977; Vidotti et al., 2003).

The proteins present in the fish silage can also be hydrolysed to free amino acids, making the silage the most available source of amino acids for protein biosynthesis. The composition of amino acids in the various states of fish silage is shown in the Table 3.7 (Vidotti et al., 2003). During fish silage preparation the raw material is chopped into small pieces and a 3% by weight solution of 98% formic acid is added and mixed well and stored for 48 days. The pH of the mixture should be less than 4 to prevent bacterial action (Espe et al., 1999; Tatterson et al., 1974). Fish silage can also be prepared by a fermentation method in which fish is chopped, minced and mixed with 5% (w/w) sugar beet molasses. A culture of *Lactobacillus plantarum* is inoculated into molasses and

Table 3.5. World fisheries and aquaculture production in million tonnes (FAO, 2010).

Production	2004	2005	2006	2007	2008	2009
INLAND						
Capture	8.6	9.4	9.8	10.0	10.2	10.1
Aquaculture	25.2	26.8	28.7	30.7	32.9	35.0
Total inland	33.8	36.2	38.5	40.6	43.1	45.1
MARINE						
Capture	83.8	82.7	80.0	79.9	79.5	79.9
Aquaculture	16.7	17.5	18.6	19.2	19.7	20.1
Total marine	100.5	100.1	98.6	99.2	99.2	100.0
TOTAL						
Total capture	92.4	92.1	89.7	89.9	89.7	90.0
Total aquaculture	41.9	44.3	47.4	49.9	52.5	55.1
Total world fisheries	134.3	136.4	137.1	139.8	142.3	145.1

Table 3.6. World fisheries and aquaculture utilization in million tonnes (FAO, 2010).

Production	2004	2005	2006	2007	2008	2009
Human consumption	104.4	107.3	110.7	112.7	115.1	117.8
Non-food uses	29.8	29.1	26.3	27.1	27.2	27.3
Population (billions)	6.4	6.5	6.6	6.7	6.8	6.8
Per capita food fish supply (kg)	16.2	16.5	16.8	16.9	17.1	17.2

Table 3.7. Amino acid composition (g/100g CP) and protein content (Vidotti et al., 2003).

Amino acids	SW	FSW	ASW	FW	FFW	AFW	TR	FTR	ATR
Tryptophan	0.79	0.65	0.66	0.97	0.87	1.34	0.52	0.61	0.43
Lysine	10.12	9.16	7.90	7.48	9.92	9.09	9.75	5.94	6.77
Histidine	5.24	5.85	5.70	2.65	3.08	2.75	2.02	2.52	2.20
Arginine	3.03	2.19	6.11	3.62	1.80	7.72	2.46	2.49	7.27
Aspartic acid	9.05	10.79	7.83	10.17	9.62	6.20	10.16	11.79	8.98
Threonine	2.85	4.97	4.58	3.18	5.12	5.28	2.76	4.68	4.72
Serine	2.71	3.23	4.49	3.39	3.52	5.53	2.04	3.72	5.11
Glutamic acid	13.57	14.45	14.04	16.18	13.83	9.26	13.88	14.76	13.10
Proline	3.19	3.66	5.74	4.37	5.57	7.78	7.75	7.22	5.94
Glycine	6.49	5.87	8.17	6.20	6.32	11.55	7.50	9.22	12.32
Alanine	8.60	7.41	7.39	9.27	8.12	6.00	8.81	8.92	7.63
½ Cystine	0.81	0.69	1.54	0.97	1.03	0.63	1.40	0.86	1.34
Valine	6.42	5.77	4.16	5.95	5.83	3.92	6.62	5.06	4.31
Methionine	6.88	6.03	3.75	3.19	4.97	5.31	2.80	5.54	5.37
Isoleucine	5.31	5.05	3.10	5.38	5.00	3.10	6.24	4.63	2.51
Leucine	9.16	8.00	7.33	9.61	9.31	7.57	10.32	6.72	6.23
Tyrosine	1.78	1.90	3.45	2.40	2.02	2.73	1.22	1.70	2.43
Phenylalanine	3.99	4.32	4.08	5.02	4.07	4.26	3.76	3.63	3.35
CP (g/kg)	776.7	596.1	699.1	496.2	420.9	443.8	429.9	358.4	395.9

SW- Commercial saltwater fish waste;
FSW- fermented saltwater fish silage;
ASW-acid salt water fish silage;
FW- commercial freshwater fish waste;
FFW- fermented freshwater fish silage;
AFW- acid freshwater fish silage;
TR- tilapia filleting residue;
FTR- fermented tilapia residue silage;
ATR- acid tilapia residue silage;
CP- crude protein (dry matter)

incubated until a population of 10^7 bacteria per g of molasses and this culture is added in the ratio of 2 ml/kg to the minced fish. The inoculum is incubated at 30°C for 7 days inside sealed plastic buckets. The autolysis is later stopped by heating the silage at 90°C for 30 min (Zahar et al., 2002; Fagbenro et al., 1995).

According to Gildberg (1992) many bioactive products including peptone, oil, and pepsin can be obtained from fish silage. Fish like Atlantic cod and salmon have high amounts of pepsin in the stomach. The optimal storage conditions for the recovery of pepsin are pH of 3 and 25°C for 3 days. By ultra-filtration and spray drying, the stomach silage can provide crude pepsin corresponding to 0.5-1 g pure pepsin per kg. The purity of the crude pepsin extracted ranges from 2-10%. The cod stomach and viscera silage provides 100 g low molecular weight peptone per kg of the raw material.

According to Goddard et al. (2005) fish silage extracted can be mixed with wheat bran and oven dried at 105°C. Co-drying fish silage with cereals reduces the drying times of the silage and improves the nutritional content of the silage. To prevent spoilage of the dried silage it should contain little bit of moisture content for the microorganisms to grow. Water levels greater than 120 g/kg can support bacterial, mold and yeast growth.

3.4.2. Fish Sauce

Fish sauce is made from small pelagic fish or by-products using salt fermentation. Fish are mixed with salt in the ratio of 3:1 at 30°C for six months and an amber protein solution is drained from the bottom of the tank. It can be used as a condiment on vegetable dishes and is very nutritious due to the presence of essential amino acids (Gildberg, 2004). Fermented fish sauce has various biological activities including angiotensin I-converting enzyme (ACE) inhibitory activity and insulin secretion-stimulating activity. Various studies reported ACE inhibitory activity in the fermented fish sauce from salmon, sardine and anchovy. Three ACE peptides (gly-trp, ile-trp and val-trp) were found in fermented fish sauce (Ishimura et al., 2003; Okamoto et al., 1995 a & b)

3.4.3. Fish Oil

Fish oils are readily available sources for long chain polyunsaturated fatty acids which consist of omega-3 fatty acids mainly composed of cis-5,8,11,14,17-eicosapentaenoic acid (EPA) and cis-4,7,10,13,16,19-docosahexaenoic acid (DHA) (Khoddami et al., 2009). The omega-3 fatty acids have many beneficial bioactivities including prevention of atherosclerosis, protection against arrhythmias, reduced blood pressure, benefit to diabetic patients, protection against manic-depressive illness, reduced symptoms in asthma patients, protection against chronic obstructive pulmonary diseases, alleviate symptoms of cystic fibrosis, improving survival of cancer patients, reduction in cardiovascular disease and improved learning ability (Tawfik et al., 2009; Kim et al., 2005; Kim et al., 2006). The American Heart Association has recommended at least two servings of fish every week to reduce the effect of cardiovascular diseases (Kris-Etherton et al., 2002).

Fish oil can be extracted from fish waste and fish by various methods such as the goldfisch method, chloroform/methanol/ water extraction method, and acid digestion method (Shahidi, 2001).

In the Goldfisch method, 10 g of the predried samples is placed in a ceramic extraction thimble. To the sample, 40 ml of hexane or petroleum ether is added and kept for 4 to 7 h. The sample is cooled and the solvent is evaporated at 95°C for 30 min. The sample is cooled and the weight of the lipid is calculated as follows (Xiao, 2010; Shahidi, 2001; Dobush et al., 1985).

Weight of lipid = (weight of container + extracted lipid) - (weight of container) (3.1)

$$\text{Lipid content (\%)} = \frac{\boldsymbol{Mass\ of\ lipid\ extracted\ (g)}}{\boldsymbol{weight\ of\ the\ original\ sample\ (g)}} * \mathbf{100} \quad (3.2)$$

In the chloroform/methanol method, 50 g of fish cut into small pieces is homogenized in 100 ml of methanol. Then, 50 ml of chloroform is added and homogenized for 30 s. An additional 50 ml of chloroform is added and homogenized for 30 s. Then, 50 ml of water is added and homogenized for 30 s. The content is centrifuged

at 3300 g at 5°C and the supernatant is filtered through Whatman No.1 filter paper. This procedure is repeated three times and all the supernatants are combined and passed through 2.5 cm thick layer of anhydrous sodium sulfate using Whatman No. 1 filter paper. The solvent is evaporated under reduced pressure at 40°C in rotary evaporator and the weight and concentration of lipid are calculated as shown in equations (3.1) and (3.2) (Shahidi, 2001; Lee et al, 1996; Folch et al., 1957)

In the acid digestion method, 5 g of ground sample is hydrolysed using 6 N HCl at 80°C for 1 h or at 110°C for 4-24 h until complete dissolution. The lipids are extracted using 1:1 (v/v) chloroform/methanol, retaining the organic layers each time. The organic solvent is removed at 40°C under reduced pressure using rotary evaporator. Then, the weight and content of lipid are calculated as shown in equation (3.1) and (3.2) (Shahidi, 2001).

3.5. Fish Protein Production

Fish proteins are extracted from fish using chemically and enzymatic methods. Protein hydrolysates obtained from these process are used in the food industry including, milk replacers, protein supplements, stabilizers in beverages and flavor enhancers.

3.5.1. Chemical Extraction

The most common extraction method used for the fish proteins is the solvent extraction method. The standard protocol for the solvent extraction of proteins reported by Sikorski et al. (1981) is shown in Figure 3.2. The whole fish is first ground and the protein is extracted using isopropanol. After grinding, the supernatant is collected and extracted three times. The first extraction is carried out at 20-30°C for 50 min in isopropanol. The second extraction is carried out at 75°C for 90 min with isopropanol. The third extraction is carried out at 75°C for 70 min with azeotropic isopropanol. The final supernatant fraction is collected, dried, milled and screened to separate out bone particles. Hermansson et al. (1971) reported that the fish protein concentrate can also be produced at a higher temperature of 50°C but it will have lower emulsifying properties and poor solubility. The disadvantages of this method are poor functionality, off-flavors,.

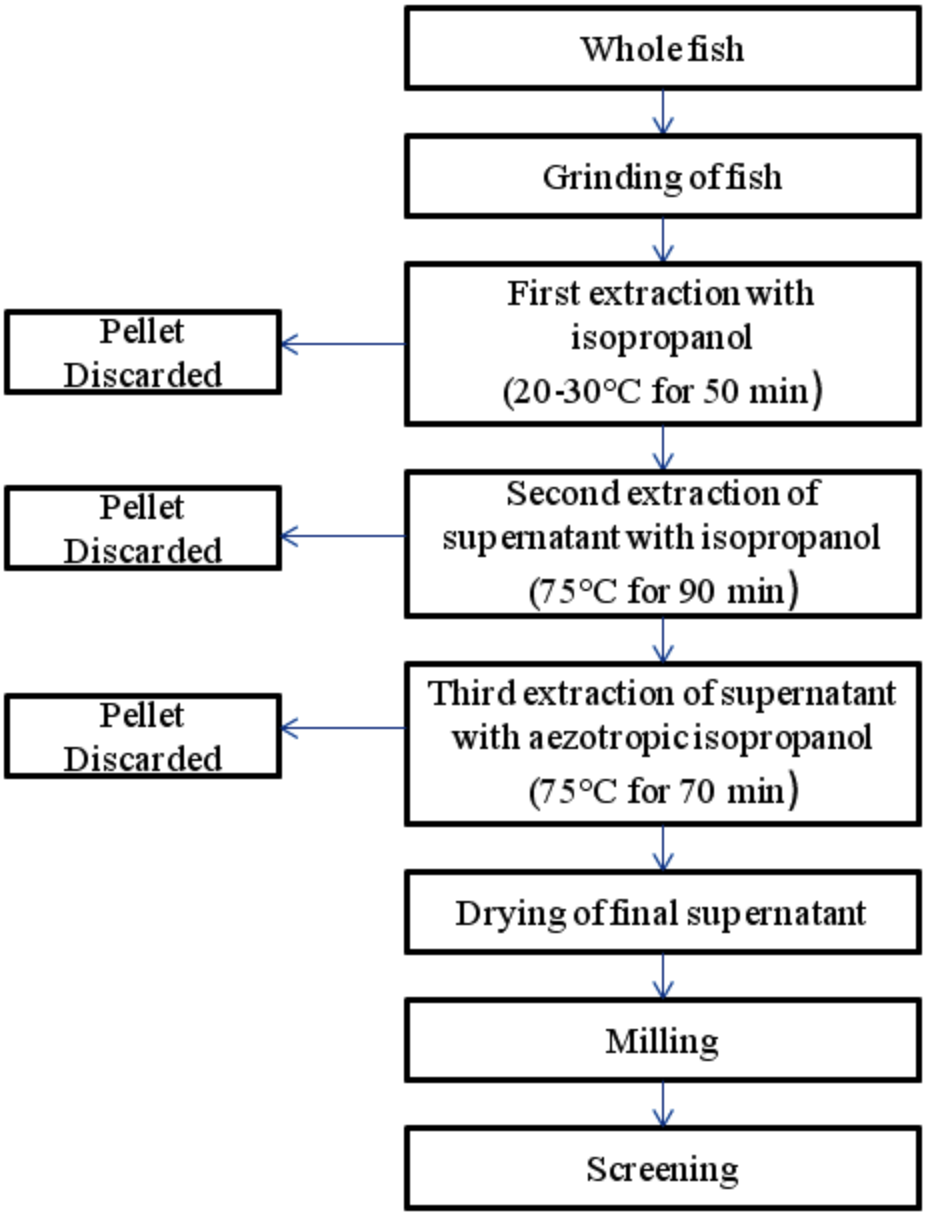


Figure 3.2. Extraction of fish protein using solvent (Sikorski et al., 1981).

high cost of production and traces of the solvent in the final product, making it commercially unsuccessful.

Another chemical method for the production of fish protein concentrate and gelatin was reported by Arnesen et al. (2006) as shown in Figure 3.3. 2000g of the Atlantic cod was added to 2000 ml of water and the pH was adjusted to 11 with 3 M NaOH (62 ml). The first extraction was carried out for 15 min and the sample was centrifuged at 4000 g for 15 min at 4°C. The pellet was suspended in 2000 ml and the pH was adjusted to 11 with 3 M NaOH (15 ml). The second extraction was carried out for 60 min and the sample was then centrifuged. The pellet was again suspended in 2000 ml of water and the pH was adjusted to 2 with 3M HCl (145 ml). The third extraction was carried out for 15 min and centrifuged The supernatants from the three extracts were pooled together and the pH was adjusted to 7 with 3 M NaOH. The samples were allowed to precipitate for 15 min at room temperature and the soluble protein was separated by centrifugation at 4°C for 60 min at 5000 g Altogether 47.5% of the total protein was recovered from the pooled extract from muscle and soft tissues. The solids remaining after the third extraction included bone, skin and residual muscle tissues.

Batista (1999) reported on the extraction of proteins from hake and monkfish waste using a chemical method. The minced fish waste was mixed with water in ratios of 20:1 - 20:5 for a time ranging from 5-120 min. The pH was in the range of 1-12 and the temperature range was in the range of 22-55°C. The extraction was carried out with HCl in the acid phase and with NaOH and $Ca(OH)_2$ in the alkaline phase. After the extraction is completed, the protein extracts were centrifuged for 15 min at 5000 rpm and the supernatant obtained was filtered through glass wool. The results indicated that the minimum solubility for hake waste proteins was seen at a pH in the range of 5-6 for hake fish waste and at pH of 5 for monkfish waste. The amount of proteins solubilized at optimum pH was 17% for hake fish waste and 9% for monkfish waste. The extraction time influenced the amount of protein solubilized in both hake and monk fish wastes. Higher yield was obtained at 45°C when NaOH was used for extraction and at 50°C when $Ca(OH)_2$ was used for extraction. The ratio of 10:1 (fish:water) was found to be more convenient giving better yield.

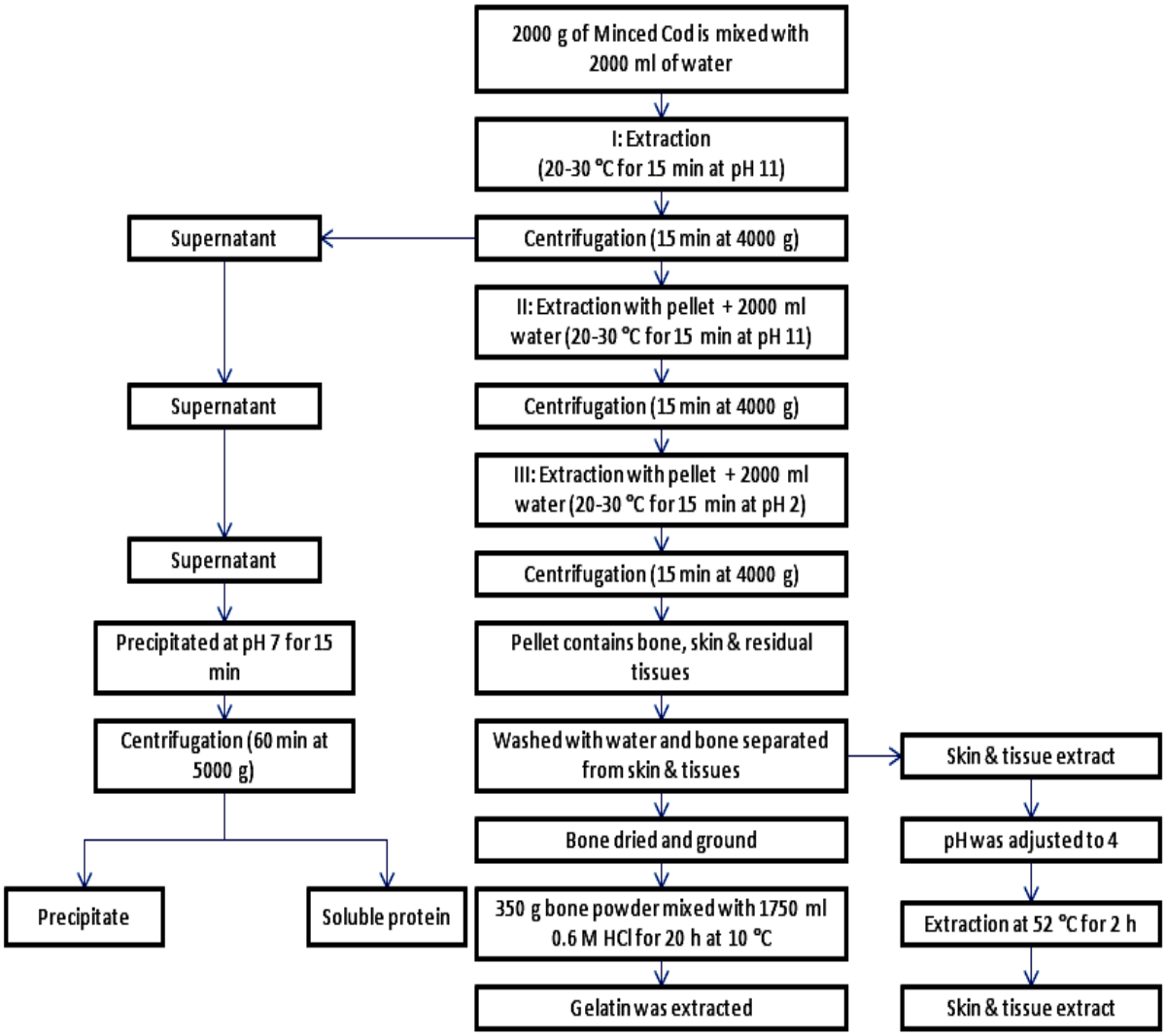


Figure 3.3. Extraction of muscle proteins and gelatin from Atlantic cod (Arnesen et al., 2006).

Undeland et al. (2002) extracted proteins with acid and alkali from ground whole herring fish (120-300 g) in a blender using 9 volumes of ice cold distilled water. The proteins present in the homogenate were solubilized by adding 2N HCl or 2N NaOH in drops until a pH 2.8 or 10.8 is reached. The protein suspension was centrifuged for 15 min at 18000 g which gives four layers of a floating emulsion layer, a clear supernatant, soft gel like sediment and harder bottom sediment. The supernatant was separated from emulsion layer by filtration through double layer cheese cloth. The solubilized proteins were precipitated by adjusting the pH in the range of 4.8-7. The precipitated proteins were centrifuged at 100000 g for 20 min. The results indicated that the extract solubilized by acid had 92.1±3.4% proteins compared to 88.6±3.8% solubilized by alkali.

Kelleher et al. (1991) used lithium chloride for the extraction of protein from fish muscle. 40 g of ground muscle were mixed with 760 ml (1:20) of 4.2% LiCl and 0.02 M Li_2CO_3 at a pH of 7.2 and a temperature of 25°C. The contents were homogenized in a blender for 2 min and centrifuged at 2600 g for 20 min. The supernatant was collected and analyzed for protein using the Biuret method. The same procedure was used but LiCl was replaced by 5% NaCl with 0.02 M $NaHCO_3$ and 6% KCl with 0.02M $KHCO_3$. The results indicated that lithium chloride was much better than sodium or potassium chloride with respect to blending time. Also, lithium chloride had a stable and consistent protein yield over a wide range of concentrations compared to the other two salts.

Nurdiyana et al. (2008) optimized the extraction of proteins from freeze dried fish waste using response surface methodology. The fish waste obtained was minced in a blender and pretreated with petroleum ether to remove the fat. Then defatted fish waste was freeze dried for 24 hrs. The freeze dried fish waste was mixed with distilled water in the ratio of 1:10 before adding NaOH. The results from the response surface optimization methodology indicated that the optimum ratio of NaOH : sample was 1.54:1, the optimum speed of rotation was 105, and the optimum extraction time was 49 min at a pH of 10.5. The predicted protein yield under these conditions was 85.02 mg/ml compared to an experimental protein yield of 83.51 mg/ml.

3.5.2. Enzymatic Hydrolysis

The enzymatic hydrolysis of various biopolymers in foodstuffs, such as polysaccharides, proteins and pectins, is an important process which is used to improve the physical, chemical and organoleptic properties of the original food in relation to the nutritive value and the intestinal absorption characteristics. The enzymatic hydrolysis of protein is carried out under acid or alkaline controlled conditions without degrading their nutritional qualities for the acceptance in the food industry and broad spectrum of products can be produced for a wide range of applications (Shahidi et al., 1995). Many protein hydrolysates are subjected to enzymatic hydrolysis to produce special diets for babies and sick adults. This is possible only when the hydrolysates are low in bitterness, osmotically balanced, hypoallergic and have good flavor. Most of these diets are composed of peptides and are rich in amino acids (Tello et al., 1994).

Although enzymatic hydrolysis has been widely applied to various livestock and poultry meat and milk, there are few studies on the production of fish protein hydrolysate using enzymes. Fish processing waste has also been underutilized for use as a feed or a fertilizer (Kristinsson et al, 2000). Most of the research studies conducted on the enzymatic hydrolysis of fish protein seems to be laboratory or small scale oriented and have their limitations when scaled up to industrial scale (Gildberg., 1993). However, the large scale production of fish protein hydrolysates are carried out in various countries including France, Japan and other countries in Southeast Asia. The process has several disadvantages including low yields, initial high cost of enzymes, inactivation of enzymes after hydrolysis either by heat or by pH and the inability to reuse enzymes (Deesle et al., 1988).

The enzymatic processing of fish waste could be helpful in producing a broad spectrum of food ingredients and industrial products for a wide range of applications (Kim et al., 2006). The enzymes used in the food industry for the preparation of fish protein hydrolysate are mostly carbohydrases, proteases and lipases.

Proteases are one of the most highly used enzymes in the food industry (Godfrey et al., 1983). Proteases are derived from animal, plant and microbial sources. Some of

enzymes extracted from plant sources include papain, bromelain and keratinases. Enzymes extracted from animals include trypsin, chymotrypsin, pepsin and renin. Because of the inability of plant and animal proteases to meet current demand in the market, there is an increase in the demand for microbial proteases (Rao et al., 1998; Kristinsson et al., 2000). Bacterial proteases are often used in the production of protein hydrolysate. They are mainly neutral or alkaline and are produced by the genus Bacillus. The neutral proteases are active in the pH range of 5-8 and have low temperature tolerance. The alkaline proteases are active in the pH range of 7-10 and have broad specificity (Rao et al., 1998).

Alcalase is an alkaline enzyme produced from *Bacillus licheniformis* which is developed by Novo Nordisk (Bagsvaerd, Denmark) for the detergent industry. This enzyme has been proven to be one of the best enzymes used to prepare fish protein hydrolysate (Sugiyama et al., 1991; Benjakul et al., 1997). Shahidi et al. (1995) stated that fish protein hydrolysate produced by Alcalase had better functional properties, a high protein content with an excellent nitrogen yield, an amino acid composition comparable to that of muscle and a higher nutritional value than those from other enzymes such as Neutrase.

Liaset et al. (2000) reported on the extraction of protein hydrolysate from fish frames from Atlantic cod and Atlantic salmon using four different enzymes Neutrase, Alcalase, pepsin and kojizyme. The study revealed that after 120 min of hydrolysis, salmon treated with Alcalase and cod treated with pepsin yielded higher protein recoveries of 67.6% and 64% respectively.

Shahidi et al. (1995) reported on the extraction of protein hydrolysate from capelin (*Mallotus villosus*) using Alcalase, Neutrase and papain. The samples were also subjected to autolytic hydrolysis. The results revealed that protein recoveries with commercial enzymes reached 51.6-70% in comparison with the autolytic hydrolysis yield of 22.9%. Alcalase hydrolysis had the highest protein recovery compared to other enzymes.

The effects of initial inactivation of endogenous enzymes, water and different enzymes on the yield of proteins and oil from cod (*Gadus morhua*) were studied by

Slizyte et al. (2005a). The enzymes used in the hydrolysis were Alcalase and Lecitase ultra. The results revealed that initial heating of raw material changed its composition and inactivated the endogenous enzymes. The yield of fish protein hydrolysate had higher amount of lipids such as phospholipids and other polar lipids. Alcalase with the addition of water produced good quality fish protein and oil.

Guerard et al. (2001) extracted protein from yellowfin tuna (*Thunnus albacores*) wastes using Alcalase. The freeze dried protein hydrolysate was used as nitrogen substrate for microbial cultures such *E. coli*, *L. casei*, *S. Cerevisiae*, *S. odorus*, *P. roqueforti* and *A. niger*.

Enzymatic hydrolysis was performed on the Atlantic spiny dogfish (*Squalus acanthias*), which is of low commercial value but potential source for high quality protein by Diniz et al. (1997). The optimized variables for protein extraction from dogfish waste were a temperature of 35°C, reaction time of 23.8 h, rotation speed of 171 rpm, and an enzyme: substrate ratio of 1.5. The yield in the extraction process was 80.75g/L (Nurdiyana et al., 2008).

Beak et al. (1995) reported on the extraction of proteins from Crayfish processing byproducts using alkaline protease optimase. The optimal conditions for the enzymatic hydrolysis were a temperature of 65°C, pH of 8-9, reaction time of 2.5 h, and an enzyme concentration of 0.3%. The maximum protein yield was 75%.

3.6. Amino Acids Production

Amino acids are building blocks of proteins. They have wide nutritional value, taste, medicinal action and chemical properties. All amino acids are sold in different quantities each year as shown in Table 3.8. They are used as food additives, in pharmaceutical applications, feed and food supplements. The largest consumer of amino acids is the food flavoring industry which uses monosodium glutamate, alanine, aspartate and arginine to improve the flavor of food. The second largest consumer of amino acids is the animal feed industry which uses lysine, methionine, threonine, tryptophan and others to improve the nutritional quality of animal feed. The total amino acid market in 1996 was estimated

Table 3.8. Global production of amino acids in 1996 (Ikeda, 2003).

Amino acid	Amount (ton/year)	Process	Uses
L- Glutamate	1000000	Fermentation	Flavour enhancer
D,L-Methionine	350000	Chemical	Food, Feed supplement and pharmaceutical
L- Lysine HCL	250000	Fermentation	Feed supplement
Glycine	22000	Chemical	Pharmaceutical, soy sauce
L-Phenylalanine	8000	Fermentation, synthesis	Aspartame
L- Aspartic acid	7000	Enzymatic	Aspartame, Pharmaceutical
L- Threonine	4000	Fermentation	Feed supplement
L- Cysteine	1500	Extraction, Enzymatic	Pharmaceutical
D, L- Alanine	1500	Chemical	Flavor, sweetener
L- Glutamine	1300	Fermentation	Pharmaceuticals
L- Arginine	1200	Fermentation	Flavor, Pharmaceuticals
L- Tryptophan	500	Fermentation, Enzymatic	Feed supplement, Pharmaceuticals
L- Valine	500	Fermentation	Pharmaceuticals
L- Leucine	500	Fermentation, extraction	Pharmaceuticals
L- Alanine	500	Enzymatic	Pharmaceuticals
L- Isoleucine	400	Fermentation	Pharmaceuticals
L- Histidine	400	Fermentation	Pharmaceuticals
L- Proline	350	Fermentation	Pharmaceuticals
L- Serine	200	Fermentation	Pharmaceuticals
L- Tyrosine	120	Extraction	Pharmaceuticals

to be $4.5 billion. The market value of amino acids has drastically increased since 1996 (Ikeda, 2003). Fermentation products in 2004 were estimated to be $14.1 billion and $17.8 billion in 2009 and amino acids were the second most important category after antibiotics (Leuchtenberger et al., 2005). The production of amino acids is divided into four types: microbial fermentation, enzymatic synthesis, chemical synthesis, extraction and protein hydrolysis (Kusumoto, 2001). The common methods practiced for the production of individual amino acids, which are used as flavouring agents, feed additives and in infusion solutions is shown in Table 3.9 (Leuchtenberger et al., 1984).

3.6.1. Fermentation Method

The fermentation method is used for the production of L- amino acids. The cost of production depends upon the carbon source, fermentation yield, purification and the yield of the overall process. The fermentation process is made more advantageous with the use of continuous processes for the production of amino acids, due to the higher yield of amino acids achieved than that from batch processes (Ikeda., 2003). Advances in the fermentation technology and improvements in strain producing amino acids have enabled industrial scale production of L- lysine as well as glutamate (de Graaf et al., 2001).

The demand for the L- lysine in 2005 was estimated to be 850,000 tons. The fermentation scheme of L- lysine HCl is shown in Figure 3.4 (Leuchtenberger et al., 2005). Different amino acids are produced from different microorganisms as shown in Table 3.10. Microbial strains for the production of amino acids are classified into three types: (a) wild type strains that are capable of producing specific amino acids under controlled culture conditions, (b) auxotropic mutants that can bypass feedback regulations by partially starving them of their nutrients and (c) strains genetically modified to improve the biosynthetic capacity of the cells to produce specific amino acids by amplifying the gene codes for rate limiting enzymes (Ikeda., 2003).

Amino acids such as L- Threonine and L- Tryptophan can be produced using fermentation of recombinant strains of *Escherichia coli* (Debabov, 2003; Ikeda et al., 1999). The extraction method is being also employed for a few kinds of amino acids such as L-cysteine, L- leucine and L-tyrosine. The manufacturing of amino acids by

Table 3.9. Production of L-amino acids (Leuchtenberger et al., 1984)

Amino Acid	Chemical Synthesis	Extraction	Fermentation	Enzymatic Catalysis
L-Alanine		+		+
L-Arginine		+	+	
L-Aspartic acid		+		+
L-Cystine		+		
L-Cysteine	+			
L-Glutamic acid (Na)		(+)	+	
L-Histidine		+	+	
L-Isoleucine		+	+	
L-Leucine		+		
L-Lysine (HCl)			+	+
L-Methionine				+
L-Phenylalanine	(+)	(+)	(+)	+
L-Proline		+	(+)	
L-Serine		+	+	
L-Threonine		+	+	
L-Tryptophan			+	
L-Tyrosine		+		
L-Valine		+	(+)	+

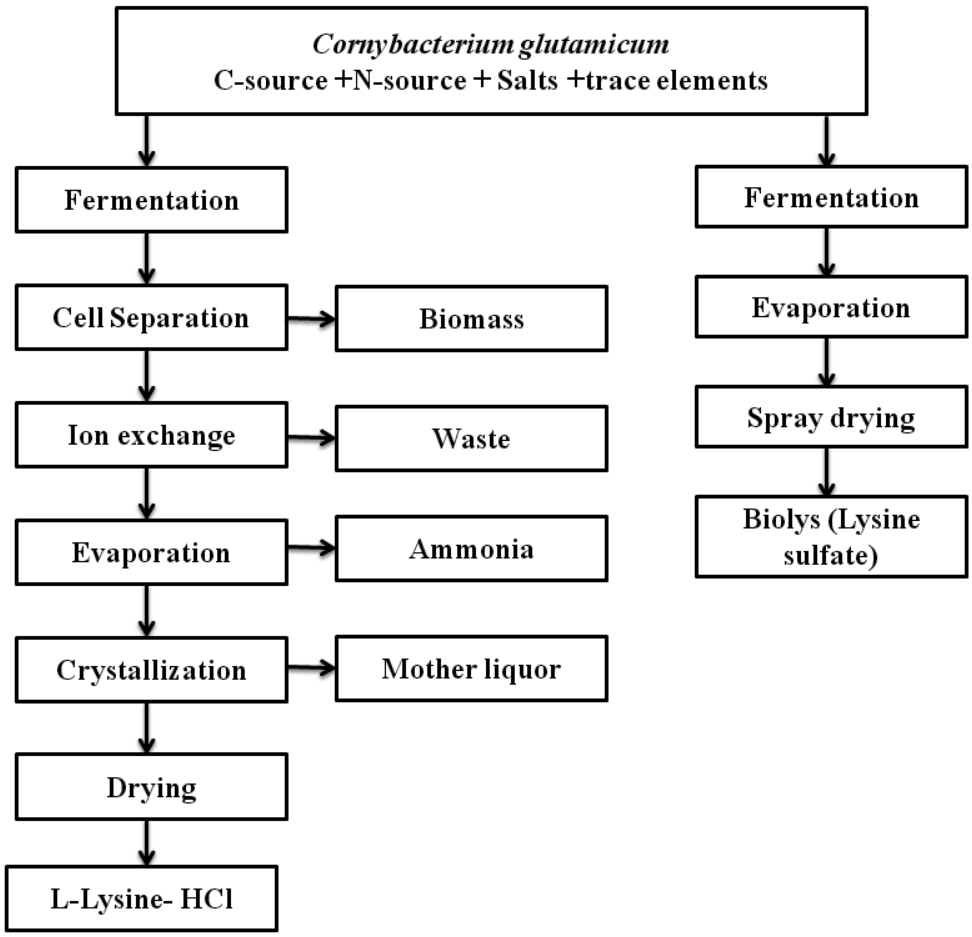


Figure 3.4. Fermentation of L-lysine-HCl in comparison with lysine sulfate (Leuchtenberger et al., 2005).

Table 3.10. Amino acid producing micro organisms (Ikeda., 2003).

Amino acid	Strain/mutant	Culture conditions	Estimated yield (g/100 g sucrose)
L-Alanine	*Arthrobactor oxylans* DAN75	Glucose 15%, 30°C, 120 h	45-55
L-Arginine	*Brevibacterium flavum* AJ12429	Molasses 89% (as glucose), 30°C, 72 h	30-40
L-Histidine	*Cornybacterium glutamicum* F81/pCH99	Molasses 12% (as glucose), 30°C, 116 h	15-20
L-Isoleucine	*Escherichia coli* H-8461	Glucose, 30°C, 45 h	20-30
L-Lysine HCl	*Cornybacterium glutamicum* B-6	Molasses, 32°C, 48 h	40-50
L-Phenylalanine	*Escherichia coli* MWPWJ304/PMW16	Glucose 18.5%, 37°C, 48 h	20-25
L-Serine	*Methylobacterium sp. MN43*	Methanol 10.4%, Glycine 59%, 28°C, 5 days	30-35
L-Threonine	*Escherichia coli* KY 10935	Glucose 22%, 30°C, 77 h	40-50
L-Tryptophan	*Cornybacterium glutamicum* KY9218/Pik9960	Sucrose 25%, 30°C, 80 h	20-25
L-Tryptophan	*Escherichia coli*	Glucose 19.8%, 30°C, 52 h	20-25
L-Valine	*Cornybacterium glutamicum*	Glucose 23%, 30°C, 70-90 h	30-40

fermentation comprises of fermentation, crude isolation and purification processes. L-glutamine was successfully isolated purified from *Cornybacterium glutamicum* as shown in Figure 3.5 (Ajinomoto, 2013). To start the process, stocks of *C. glutamicum* are used in shake flask cultures and the resulting cells are transferred to a large tank. The pH is maintained at 7.8 during the process. After 14 h of growth, the temperature is increased from 32-33°C to 38°C. During the process, 160 g/L glucose is fed to a reactor after 36 h. After the fermentation process is completed, the crude isolate is obtained and most of impurities contained in the fermentation broth are removed to achieve required quality for the intended use (Kusumoto, 2001; Pfefferle et al., 2003).

3.6.2. Enzymatic Method

Enzymatic methods are used in small scale industries for the production of synthetic D- and L-amino acids (Kamphuis et al., 1990). Leuchtenberger et al. (2005) reported that the enzymatic method is used for producing optically active and pure D- and L- amino acids in higher concentrations with fewer by-products which in turn makes the downstream processing simpler. Soluble enzymes have been used for the production of enantiomerically pure amino acids but led to the loss of biocatalyst after each batch. Immobilization methods were, therefore, used for the production of amino acids. An enzyme membrane reactor (EMR) was introduced which made it possible to retain the enzyme as most of enzymes have molecular weights in the range of 10-150 kDa and a molecular weight cut-off of 10 kDa was used in the membranes (Woltinger et al., 2005). The amino acids which are produced using enzyme membrane reactor are shown in Table 3.11 (Leuchtenberger et al., 1984).

The set up for membrane reactors used for production of amino acids includes dead-end membrane, recycle membrane, diffusion membrane and multiphase membrane. In a dead-end reactor, the solution containing enzymes is pushed towards the membrane, the product is obtained on the other side and the enzymes are retained (Figure 3.6 a). In a recycle membrane reactor, the substrate containing enzymes is continuously recycled from and to the reactor through the filtration membrane (Figure 3.6 b). If the soluble enzymes are used, the reaction takes place in both vessel and membrane module but only

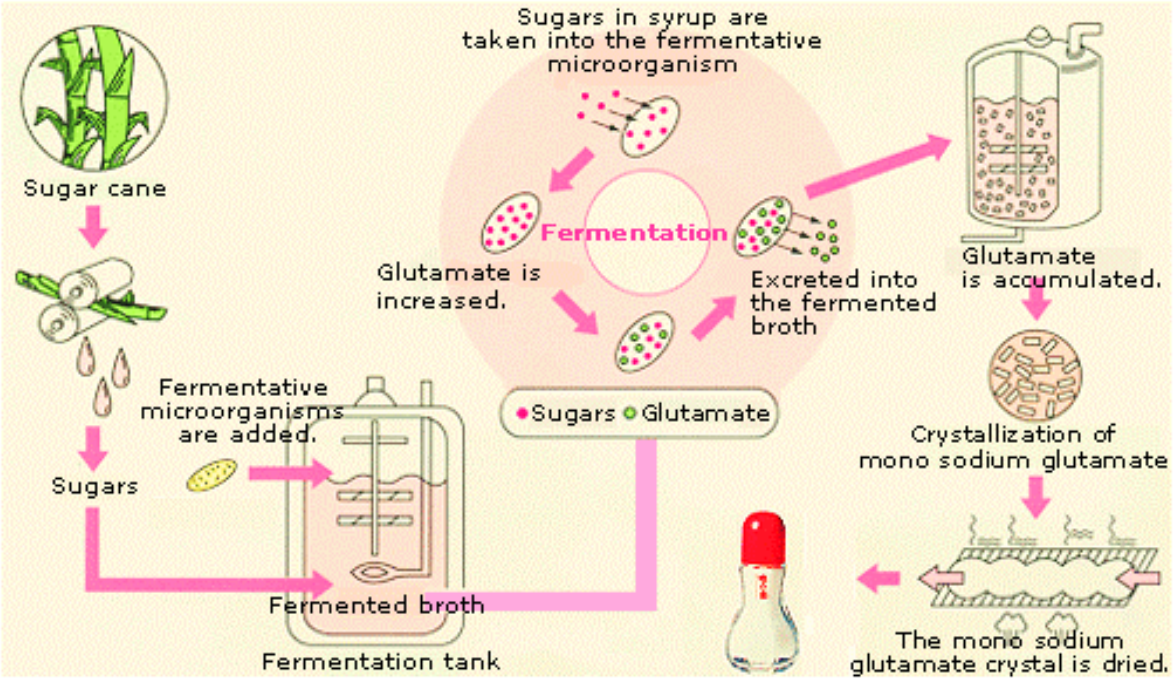
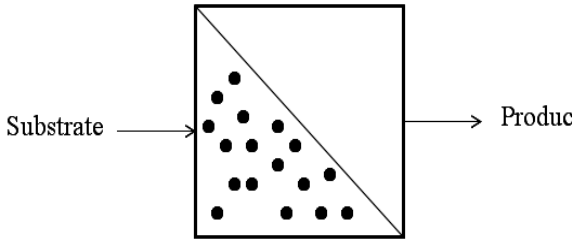


Figure 3.5. Production of mono sodium glutamate by fermentation (Ajinomoto, 2013).

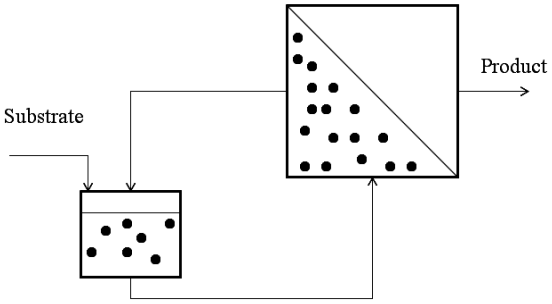
Table 3.11. Amino acids produced using enzyme membrane reactor (Leuchtenberger et al., 1984).

Substrate	Enzyme	Product
N-acetyl-DL-alanine	Acylase (a)	L-Alanine
N-acetyl-DL-methionine	Acylase (a & b)	L-Methionine
N-acetyl-DL-valine	Acylase (a & b)	L-Valine
N-acetyl-DL-phenylalanine	Acylase (b)	L-Phenylalanine
N-acetyl-DL-trytophan	Acylase (b)	L-Tryptophan
Fumaric acid	Fumarase	L-Malic acid
Fumaric acid	Aspartase	L-Aspartic acid
Pyruvic acid	L-Alanine dehydrogenase	L-Alanine
α - Keto isocaproic acid	L-Leucine dehydrogenase	L-Leucine
Phenyl-pyruvic acid	L-Lactate dehydrogenase	L-Phenyl lactic acid

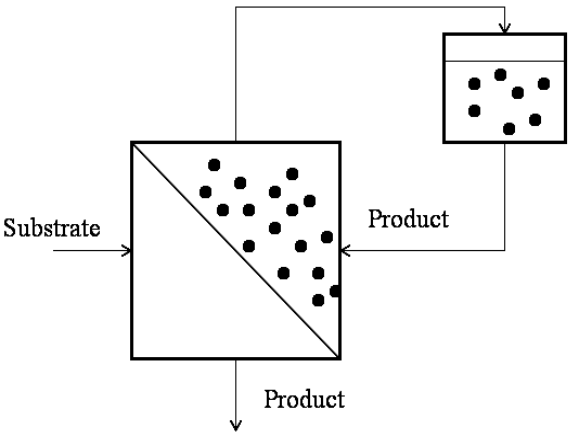
* a - Acylase from pork kidney; b- Acylase from *Aspergillus oryzae*



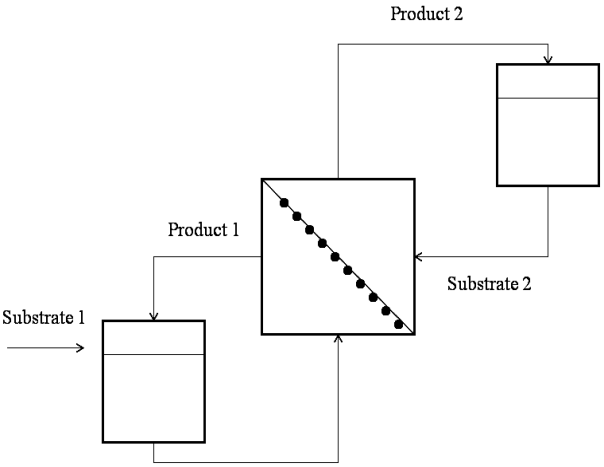
(a) Dead-end membrane module



(b) Recycle membrane module



(c) Diffusion membrane module



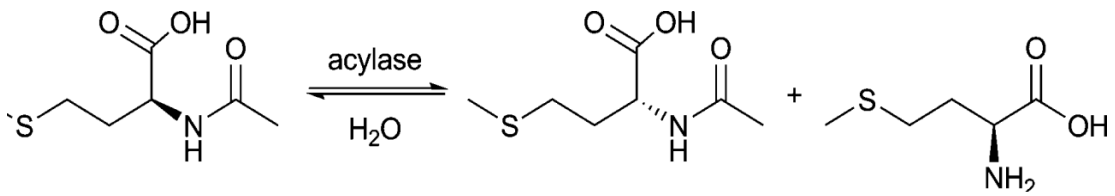
(d) Multiphase membrane module

Figure 3.6. Membrane Reactors (Prazeres et al., 2001).

occurs in the module if immobilized enzyme are used (Woltinger et al., 2005; Prazeres et al., 2001). The diffusion membrane reactor allows passive diffusion of substrate molecules through the membrane to the adjacent compartment containing enzymes (Figure 3.6 c). These reactors are used only for low molecular weight substrates (as the substrate is diffused back after catalysis) and are not usually used for industrial production. The multi-phase reactors are capable of creating interfacial contact between enzyme and substrate at the membrane matrix through diffusion (Figure 3.6 d). In this type of reactor the membrane acts as a support between two liquid phases and in some cases positive pressure can be applied in order to prevent the phases from mixing (Prazeres et al., 2001).

The main advantages of using enzyme membrane reactors (EMR) include: (a) development of continuous processes for the production of amino acids, (b) higher productivity, (c) controllable environment, shift in the chemical equilibrium, (d) improved rate of reaction in product inhibitions, (e) concentration of process streams (f) possibility to conduct multiphase process reactions, (g) no enzyme fixation costs, (h) interchangeability of substrate/enzyme systems, (i) use of multienzyme system, (j) sterilizability of the plant and (k) no diffusion limitaion. The main drawbacks of EMR are: (a) a specific substrate is used to produce corresponding amino acids, (b) lacks operational stability, the enzymes are resistant towards product inactivation, (c) high substrate and salt concentrations, (d) it cannot withstand high temperature and organic solvents, (e) it is not stable at low or high pH values, (f) poisoning of enzyme, (g) deactivation of enzymes, (h) concentration polarization, (i) fouling and (j) enzyme leakage. (Prazeres et al., 2001; Kamphuis et al., 1990).

The enzyme Acylase has been used widely for the production of amino acids. Wandrey (1977) reported on the production of L-Methionine from N-acetyl-L-methionine catalyzed by Acylase as shown in Equation 3.3.

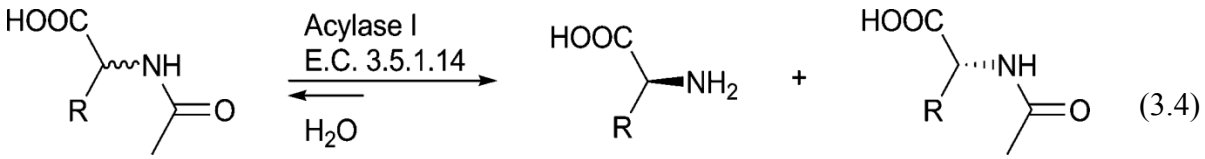


(3.3)

N-acetyl-L-methionine

L-Methionine

Janssen et al. (2011) reported on the industrial application of acylase process by Evonik Degussa using an enzyme membrane reactor for the production of several amino acids as shown in Equation 3.4



(3.4)

L-amino acid + acetic acid N-acetyl-D-amino acid

The dehydrogenase technology with integrated cofactor regeneration has been used to produce enantiomerically pure amino acids from prochiral compounds such as α-ketoacids in a single step with high yield. L-amino acids (such as L-tert-leucine and L-methionine) were obtained by reductive amination or by reduction by optically pure alcohols (Woltinger et al., 2005). The enzymes used in the process were leucine dehydrogenase (from *Bacillus stereothermophilus*) and formate dehydrogenase (from *Canadida boidinii*). Since the molecular weight of NAD^+ is less than 600 Da, it could not be retained by the ultrafiltration membrane and so NADH was covalently bound to the water soluble polyethylene glycol (PEG-NADH) and was used as a catalyst in a reactor. The NAD^+ present in the system was formed by the reductive amination of α-ketoacid by leucine dehydrogenase which is reduced back to NADH by formate dehydrogenase. This process is very useful for the production of non-natural amino acids or chiral alcohols which cannot be produced by fermentation and has been applied for the production of L-*tert*-lecuine and L-*neopentyl*-glycine (Janssen et al., 2011; Leuchtenberger et al., 1984). Ohshima et al. (1985) also used the dehydrogenase process for the production of L-leucine and the results indicated that the enzymes used in the process were from thermophilic bacteria and so they exhibited higher activity and stability at high temperatures.

The industrial production of L-tert-leucine is shown in Figure 3.7. In this process, reductive amination of trimethylpyruvate is carried out using L-leucine dehydrogenase to form L-tert-leucine. The main advantages of this process are: (a) desired optically active compounds are obtained directly from the prochiral precursors (α-ketoacid) eliminating

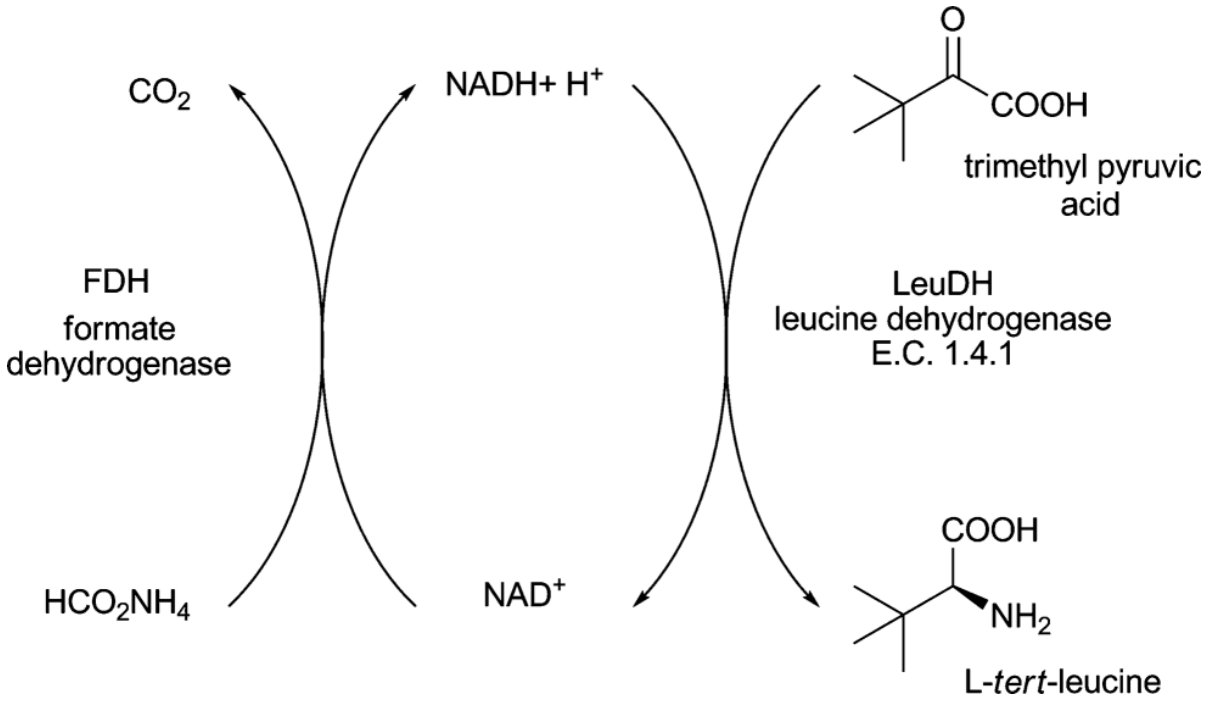
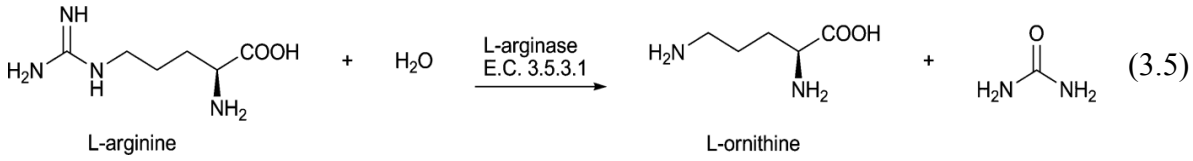


Figure 3.7. Synthesis of L-*tert*-leucine by reductive amination of trimethylpyruvate (Findrik et al., 2009).

the step for optical resolution, derivatization and racemization of the product, (b) its low cost, and (c) ability to shift the reaction equilibrium and drive it to completion which helps in simplifying product recovery and prevents the accumulation of inhibitory co-factor by-products (Findrik et al., 2009).

L-ornithine plays an important role in the pharmaceutical industry as a parenteral nutrition product in the form of L-ornithine hydrochloride (L-orn.HCl) and L-ornithine acetate (L-orn acetate) for the treatment of hepatic diseases. The enzymatic production of L- ornithine using enzymes is an alternative to fermentation process. The activity of L-arginase enzyme depends upon the form of enzyme used in the process. L-arginase extracted from the calf or bovine liver is mainly used instead of immobilized arginase. The soluble form of arginase is always preferred in enzyme membrane reactors due to the high solubilities of reactants and products (Janssen et al., 2011). Woltinger et al. (2005) stated that the shear stress due to the mechanical agitation leads to early deactivation of enzyme and therefore the production of L-ornithine is carried in a special membrane in which the substrates and enzymes are transported by hydraulic transport. Certain stabilizing agents such as manganese ions and ascorbic acid further reduce the early deactivation of enzymes. The process conducted on pilot scale showed that the enzyme consumption level was less than 400 units per kg and conversion yield was 83-88%. The conversion process is shown in Equation 3.5.



(3.5)

3.6.3.Chemical Method

The chemical synthesis method can only produce D, L- forms of amino acids and needs a costly optical resolution step. (Calmes et al., 1999). The main advantage of using chemical synthesis to produce amino acids is that it can be carried out on a very large scale and in a continuous manner. The main disadvantage of this process is that the amino acids produced will be in a racemic mixture of both L and D amino acids. The products

must then be resolved to obtain separate D and L amino acids followed by recovery and recycling. Amino acids such as glutamic acid, methionine and lysine are produced by chemical synthesis. Glutamic acid was earlier produced by the Steffen process, which is used for the isolation of sugar from sugar beets. Glutamic acid occurs in the form of glutamine in sugar beets which cyclizes to form pyroglutamic acid. Hydrolysis is carried out at a pH range of 10.5-11.5 and 85°C for 2 h. After hydrolysis, the pH is adjusted to 3.5 and glutamic acid is precipitated. The amount of glutamine present in the sugar beet is half the amount present in wheat gluten, corn gluten and de-fatted soy beans (Ault, 2004).

In 1950's glutamic acid was produced using acrylonitrile as the starting material which was converted to β-cyanopropionaldehyde by an oxo reaction. The aldehyde present in β-cyanopropionaldehyde was converted to an amino analog of cyanohydrin, which was then hydrolyzed to glutamic acid using the Strecker's process as shown in Figure 3.8 (Sano, 2009; Ault, 2004). The production of glutamic acid by the Strecker's process was continuous and was operated at high temperature and pressure. There was no carbon containing by-product and two equivalent amounts of ammonia were produced which were recycled for the synthesis of ammonium cyanide (Ault, 2004). A racemic mixture of glutamic acid was fed to L- and D- glutamic acid seed crystals. These seed crystals enabled the crystallization of only its optical isomer and each isomer could be grown and centrifuged separately (Sano, 2009).

3.6.4.Extraction Method

The extraction method is dependent on the starting material which can include: hair, keratin, feather, blood meal and soybeans. The standard procedure comprises of the hydrolysis with aqueous acid, in which the amino acids are captured when passing the hydrolysate over a strongly acidic ion exchange resin. The resin is later washed with water and eluted with aqueous ammonia which frees the amino acids. This is one of the most economical processes for the production of tyrosine and Cysteine. The main drawback of this process is that the raw materials cannot keep up with the increasing demand for amino acids (Ault, 2004; Ikeda, 2003).

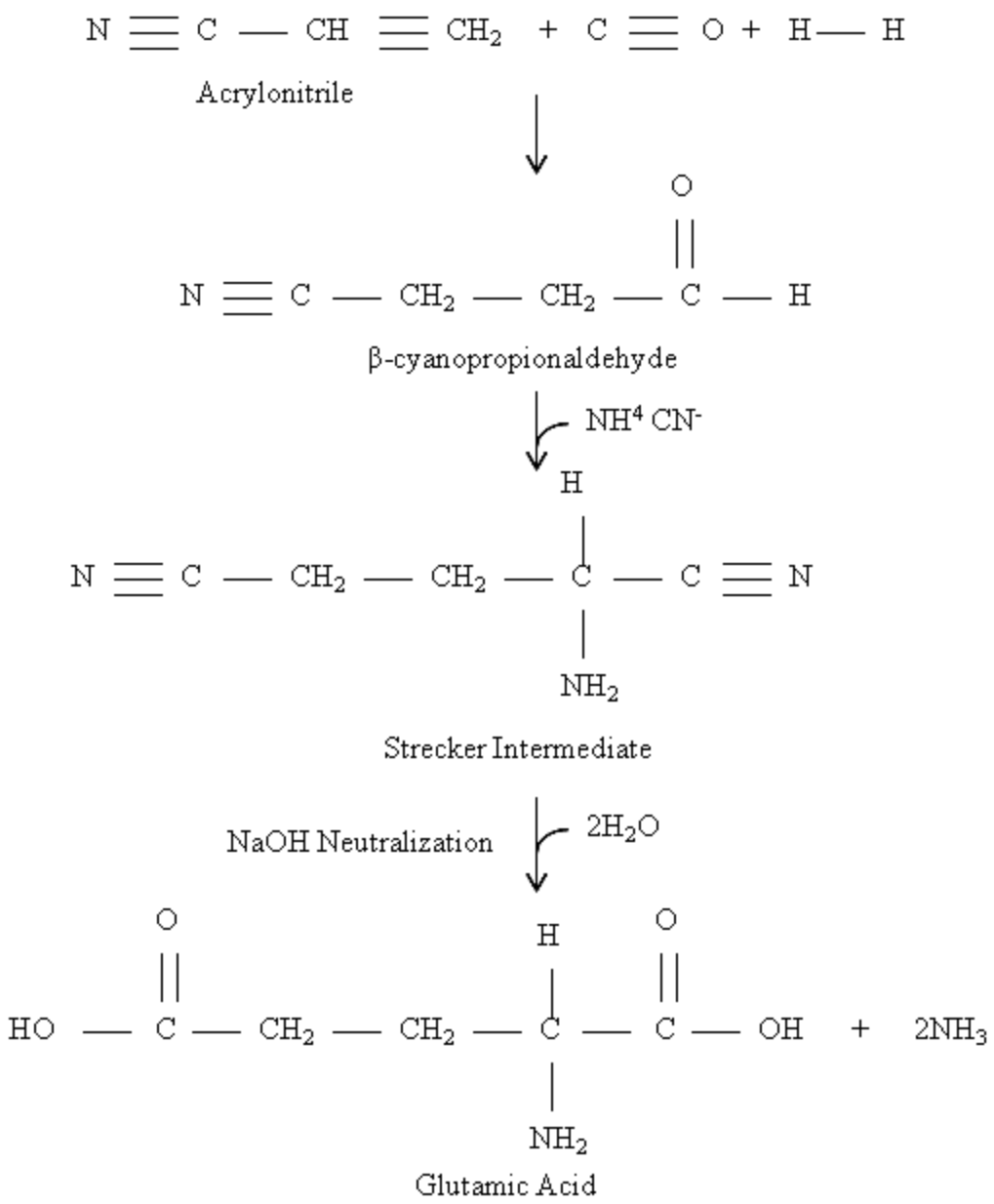


Figure 3.8. Chemical synthesis of glutamic acid (Ault, 2004).

Sano (2009) reported on the extraction of amino acids from wheat gluten which was identified as the major source of glutamate. The process consists of three steps: extraction,isolation and purification. In the extraction process, gluten was first separated from the wheat flour by washing the starch from the dough. The crude gluten was transferred to pottery vessels and mixed with hydrochloric acid and heated for 20 h. After 20 h, the hydrolysates were filtered to remove the black residue resulting from the reaction of amino acids with carbohydrates. The filtrate was then transferred to the same vessel and concentrated for 24 h and then transferred to another vessel to crystallize for 1 month. After 1 month, the crystals of L-glutamic acid hydrochloride were isolated from the liquid through filtration and redissolved in water. The pH was adjusted to 3.2 and stored for 1 week for L-glutamic acid crystallization. The crystals had two polymorphs granular α-form and stable, thin β-form. The α-form contained only glutamate crystals with improved purity. The separated L-glutamic acid α-form crystals were dissolved in water and the pH was adjusted to 7 and filtered and decolorized using activated charcoal. The filtered solution was concentrated by heating and cooled to form monosodium L-glutamate.

3.7. Protein Hydrolysis

Proteins can be broken down into peptides of varying sizes. The agents used in the acidic hydrolysis of proteins are shown in the Table 3.12. Chemical and biological methods are most commonly used for the hydrolysis of proteins (Kristinsson et al., 2000). The aim of the hydrolysis process is to liberate amino acids and recover them without degrading their properties. The factors affecting the hydrolysis of proteins are temperature, time, hydrolysis agent and additives. The combination of these factors affects the quality and yield. (Fountoulakis et al., 1998). Protein hydrolysis can be carried out with the use of chemicals or enzymes.

3.7.1.Chemical Hydrolysis

Chemical hydrolysis can be carried out using acids, alkalis or microwave.

Table 3.12. Hydrolysis agents of protein hydrolysis (Fountoulakis et al., 1998)

Hydrolysis agent	Hydrolysis Conditions	Additives	Specific Determination
6 M HCl	110°C, 24 h	0.02% Phenol	All residues except of Cys, Trp
6 M HCl or 4 M MSA	110°C, 24 h	0.2% Sodium azide	Cys
6 M HCl	110°C, 18 h	5% Thioglycolic acid, 0.1% phenol, 3,3'-dithiodipropionic acid	Cys
6 M HCl		3-Bromopropylamine	Cys
6 M HCl	145°C, 4 h	Samples previously oxidized with performic acid	Cys, Met, Lys
4 M MSA		3-(2-aminoethyl)indole	Trp, Methionine sulfoxide
4 M MSA	115°C, 22 h	Samples previously alkylated, tryptamine	All residues
4 M MSA	160°C, 45 min		All residues
4 M MSA or 5.7 M HCl	150°C, 90 min	Oxidation with performic acid 50°C, 10 min	All residues
3 M *p-Toluenesulfonic* acid			Methionine sulfoxide
12 M HCl–propionic acid (1:1)	150°C, 90 min		Resin-bound peptides
12 M HCl–propionic acid (1:1) *p-Toluenesulfonic acid*	840 W, 1–7 min microwave 15 min, microwave		Resin-bound peptides
DCl	medium power, 30 min microwave		Sensitive residues
2.5MMercaptoethanesulfonic acid	176°C, 12.5 min	S-Pyridylethylated samples	Cys, Trp
6 M HCl–TFA (6:3)	120°C, 16 h	Dithiodiglycolic acid, 1% phenol	Cys
HCl		Thioglycolic acid	Trp
HCl	110°C, 24 h	0.4% β-Mercaptoethanol	Trp
HCl	166°C, 25 min or 145°C, 4 h	3% Phenol	Trp
HCl	145°C, 4 h	Tryptamine	Trp
6 M HCl	145°C, 4 h gas phase	Tryptamine[3-(2-aminoethyl)]indole	Trp
TFA–HCl (1:2)	166°C, 25-50 min	5% Thioglycolic acid	Trp, Met
7 M HCl, 10% TFA		10% Thioglycolic acid, indole	Trp

3.7.1.1.Acid Hydrolysis: Acid hydrolysis is the most commonly used process for the hydrolysis of proteins. The process itself is very harsh and hard to control, but is still the preferred method for hydrolyzing vegetable proteins. Acid hydrolysis is normally carried out using hydrochloric acid and in some cases with sulfuric acid (Blendford., 1994). The conventional acidic hydrolysis of fish proteins is carried out using 6 M HCl for 20-24 h at 110°C under vacuum (Shahidi et al., 1995). Under these conditions of hydrolysis, aspargine and glutamine are completely hydrolyzed to aspartic acid and glutamic acid, respectively. Tryptophan is completely destroyed and cysteine cannot be directly determined from the acid hydrolysed samples. Tyrosine, serine and threonine are partially hydrolysed. There is usually5-10% loss in the recovery (Fountoulakis et al., 1998).

The conventional method of acid hydrolysis was modified by adding 50% acetic acid in order to reduce the hydrolysis time. The study revealed that during conventional hydrolysis the recoveries of amino acids are very low and therefore in the presence of organic acid it is possible to reach the hydrophobic regions of proteins (Westall et al., 1972). Tsugita et al. (1982) used formic acid, acetic acid, trifluoroacetic acid and propionic acid. Trifluoroacetic acid was found to be a strong acid with a pKa of 0.23, high vapour pressure and low boiling point (72.5°C).The dipeptide consisting of valine and isoleucine (Val-Val, Val-Ile, Ile-Val and Ile-Ile) in the proteins were hydrolyzed at 160°C for 25 min with various combinations of mixtures of hydrochloric acid and organic acid. The results indicated that combination of trifluoroacetic acid and HCl in the ratio of 1:1-1:2 showed a recovery of 100% when compared to other organic acids as shown in Table 3.13. To check the stability of amino acids, the amino acid mixtures were heated to 130°Cand 210°C for 25 min and 50 min.

Fountoulakis et al. (1998) reported that the time and temperature are always important variables to consider in conventional acid hydrolysis processes. Hydrolysis with 6 M HCl at 145°C for 4 h gives comparable recoveries and quantification in comparison to conventional hydrolysis with 6M HCl at 110°C for 24 h. The recoveries of threonine and serine were reduced by 50% after 4h, whereas the valine and isoleucine recoveries increased by 100%. In comparison, shortened hydrolysis at elevated

Table 3.13. Recovery of amino acids from *valyl*- glutamic acid (Tsugita et al., 1982).

Acid Mixture	Composition (v/v)	Recovery (%)
Formic acid: HCl	1:1	85
	1:2	95
Acetic acid: HCl	1:1	97
	1:2	100
Trifluoroacetic acid: HCl	2:1	85
	1:1	100
	1:2	100
Propionic acid: HCl	1:1	90
	1:2	97

temperatures gave similar or superior results to those of conventional hydrolysis at 110°C for 24 h.

Csapo et al. (2008) stated that in addition to the recovery, the duration also affects the degree of racemization of the hydrolyzate. When using conventional protein hydrolysis, racemization is 1.2-1.6 times higher compared to the hydrolysis carried out at elevated temperatures of 160°C-180°C. At higher temperatures, proteins are hydrolyzed rapidly into free amino acids and racemization of free amino acids is always slower than that of amino acid bound to polypeptides. During conventional acid hydrolysis the proteins are hydrolyzed at a much slower rate, during which the amino acids bound to polypeptide bonds are exposed to heat for a longer time causing racemization. According to Ozols (1990), there are certain peptide bonds that are very tough to cleave, including Ile-Val, Val-Val, Ile-Val, resulting in only 50-70% at 110°C in 24 h and therefore hydrolysis must be carried out for 92 h or even 120 h.

Blackburn. (1978) used Methanesulfonic acid (MSA) in the hydrolysis in the presence of 3-(2-aminoethyl)indole. The advantage of using methanesulfonic acid in comparison to HCl is that tryptophan is not destroyed and methionine is determined as methionine sulfoxide. To reduce the losses incurred by the acid hydrolysis certain protective agents such as phenol, thioglycolic acid, mercaptoethanol, indole or tryptamine are added to the sample Adebiyi et al. (2005) used a single step hydrolysis process with 4 M methanesulfonic acid at 115°C for 22 h in the presence of 0.02% tryptamine, in which even tryptophan and cysteine were able to be determined.

Chiou et al. (1988) hydrolyzed proteins using methanesulfonic acid, in which 0.5 mg of protein samples were added with 0.5 ml 4 M methanesulfonic acid containing 0.2 % 3-(2-aminoethyl)indole. The hydrolysis tubes were flushed with nitrogen gas for 1 min and closed tightly. The tubes were heated at 160°C for 45 min. At the end of hydrolysis, the samples were partially neutralized with 8 M sodium hydroxide to pH 2. The samples were using amino acid analyzer and the results were compared with conventional HCl hydrolysis. The results indicated that by hydrolyzing the proteins at a higher temperature and shorter time, accurate results were produced for all amino acids, including tryptophan and half-cystine which are normally degraded in a conventional HCl acid hydrolysis

process. An additional advantage of this process is that there is no degradation of serine, threonine and tyrosine as in the case of prolonged hydrolysis. The recoveries of amino acids range between 97-102% at 160°C for 45 min. Methanesulfonic is also non-volatile in nature and cannot be evaporated after hydrolysis and therefore it is often neutralized to pH 2 before analysis.

Liu et al. (1971) hydrolyzed proteins using 3 N p-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl)indole at 110°C for 22, 48 and 72 h. At the end of hydrolysis, 2 ml of NaOH was added and transferred to 5 ml volumetric flask, in which the total volume was made up to 5 ml before analysis. The results indicated that p-toluenesulfonic acid hydrolysis can be carried out to identify and quantify tryptophan but cannot be used for the analysis of proteins which are contaminated with carbohydrates such as those in animal feed.

3.7.1.2. Alkaline Hydrolysis: Hydrolysis of proteins can be carried out using sodium hydroxide, potassium hydroxide or with barium hydroxide. The alkaline treatment is specifically used for the determination of tryptophan. It is also applied to the samples which have a higher percentage of carbohydrates as in the case of foods and formulation of pharmaceutical solutions which have higher percentage of monosaccharides. The major disadvantage of this method is that serine, threonine, arginine and cysteine are destroyed and all other amino acids are racemized (Gupta et al., 1997).

Linder et al. (1995) stated that many deleterious reactions occur in alkaline solutions during hydrolysis. These reactions are initiated by hydrogen abstraction from the alpha carbon of an amino acid which includes racemization of L- amino acids to produce D-amino acids. Lahl et al. (1994) reported that D- amino acids are not absorbed by humans and that alkaline hydrolysis also splits disulfide bonds with loss of cysteine, serine and threonine via β-elimination reactions and formations of lysinoalanine, orinithinoalanine, lanthionine and β-amino alanine. These eliminations can also lead to production of toxic substances such as lysinoalanine and are undesirable in foods.

The chemicals that are produced during the alkaline hydrolysis of protein are shown in the Figure 3.9 (Kristinsson et al., 2000). Protein phosphorylation plays a major role in

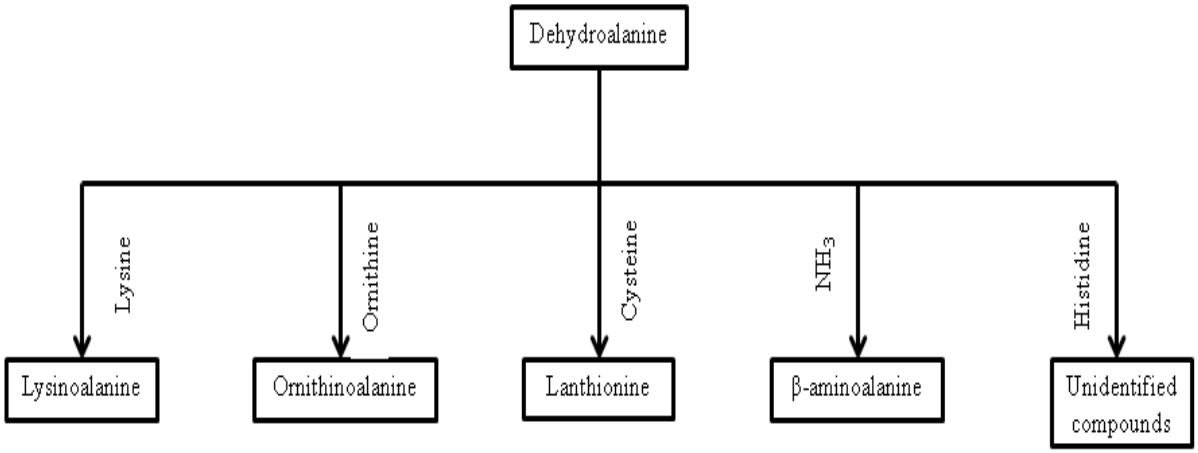


Figure 3.9. Chemicals formed after alkaline hydrolysis (Kristinsson et al., 2000).

cell biology and biomedical sciences. Addition of phosphate to the amino acid side chain by esterification causes conformational changes to the protein in terms of activity and stability. The typical acceptors of phosphate in their ring structure are the hydrophobic amino acids such as tyrosine, serine, tryptophan, histidine, aspartic acid, glutamic acid, lysine, arginine and cysteine.

Yan et al. (1988) reported the partial hydrolysis of amide bonds using acid, alkali or enzyme can release phosphoamino acids. Fountoulakis et al, (1998) reported that potassium hydroxide hydrolysis was applied for the quantification of phosphorylated and sulfated tyrosine and for analysis of phosphohistidine. Proteolytic hydrolysis of protein samples, followed by alkali hydrolysis results in superior yields of phopshoamino acids (Yan et al., 1988).

3.7.1.3. Microwave Induced Hydrolysis of Proteins: In the microwave induced hydrolysis of proteins, substrate can be hydrolysed in either liquid or gas phase mode with HCl or other reagents. In this method the instant uptake of the radiation energy results in the reduction of the overall hydrolysis time from many hours to few minutes: 1-30 min for the liquid phase hydrolysis and 20-45 min for gas phase hydrolysis.

Kaiser et al. (2005) studied microwave induced hydrolysis of proteins. Amino acids were hydrolysed with a CEM Mars 5000 microwave equipped with a protein hydrolysis accessory kit which included four Teflon vessels for the samples. The major advantages of the process were (a) it allows processing 40 samples in 3 h, (b) only 100 µl was needed for analysis, (c) the samples contain only hydrochloric acid and moisture with less impurities and 4) more feasible for smaller sized samples.

Margolis et al. (1991) studied the hydrolysis of proteins by microwave energy using bovine serum albumin that was hydrolyzed with 10 mol/L HCl in a clean acid leached Teflon (PFA) pressure vessel which was free of the metal impurities and kept in the microwave system at 125°C for 2-4 h. The results showed that most of amino acids were completely hydrolyzed within 2 h except valine, isoleucine and leucine which were resistant to hydrolysis. Tryptophan was not stable and could not measured. When the

time was increased to 4 h, threonine, serine and tyrosine were stable compared to the conventional hydrolysis process and the results were accurate.

Wu et al. (1992) used microwave energy to study peptide cleavage. In this study, peptides (1 mg/ml) were dissolved in dilute hydrochloric acid (0.006, 0.015, 0.03, 0.06 M) and water in 0.3 ml teflon vials. The vials were flushed with nitrogen gas for about 1 min and placed in microwave oven for time intervals ranging from 1 to 7 min. The results indicate that the peptides in 0.06 M HCl were completely hydrolyzed in 3 min and peptides in 0.03 M HCl were hydrolyzed in 4 min. The peptide content in the neutral solution was less than 15% after 4 min. The microwave energy that is emitted is absorbed into the liquid media by two mechanisms, ionic conduction and dipole rotation. Therefore, input power during microwave hydrolysis plays an important role and 572 W or 650 W power is very suitable for microwave hydrolysis. This process was developed to control the cleavage sites especially for the peptide bonds connected to aspartic acid residues and it is very useful in obtaining defined acid-cleaved peptide fragments.

Joergensen et al. (1995) performed microwave assisted hydrolysis on protein samples. The results suggested that a hydrolysis time of 10-30 min is more than enough to cleave all the peptide linkages with no loss of serine or threonine and methionine was found to be stable with the addition of thioglycolic acid during the hydrolysis process. As in the case of conventional hydrolysis process, cysteine and tryptophan could not be quantified. To quantify cysteine, the sample was subjected to pre-hydrolysis oxidation, in which 20-50 mg of the protein samples were added to 5 ml ice cold performic acid (0.5 ml 30% H_2O_2 and 4.5 ml formic acid) and 250 µl of 200 mM norleucine was also added along with 250 µl of 10% phenol at 0°C for 18 h. After 18 h, the reagents were dried under vacuum using a freeze dryer and 5 ml water and 10 ml 30% HCl were added to the dried samples to perform microwave hydrolysis.

Weiss et al. (1998) performed liquid phase and gas phase hydrolysis on protein solutions using a microwave technique. In liquid phase microwave hydrolysis, 500 µl of protein solution was added to 6M HCl containing 0.02% phenol or to 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole. The hydrolysis vial was placed in the microwave oven inside a beaker containing 200 ml water for equal

absorption of energy and hydrolysis was carried out at 155°C (900 W) for 4 min. In the gas phase microwave hydrolysis, 300 µl of protein solution was evaporated to dryness, 6 M HCl was added and hydrolysis was performed at 1000 W for 5 min and 500 W for 15 min after flushing the vials with argon gas. The results indicated that using this microwave technique, the hydrolysis was completed within 4 min and gave results comparable to that of conventional hydrolysis with higher losses of serine and threonine and a higher percentage of racemization of amino acids.

3.7.2. Enzymatic Hydrolysis

The main advantage of this process is that it allows quantification of aspargine and glutamine and other sensitive residues, which are normally destroyed by acid and alkali hydrolysis, and does not cause any racemization during digestion. However, the widespread application of this type of hydrolysis is hampered by the relative specificity of the proteases for certain residues. For complete hydrolysis of the protein, a combination of different proteases is necessary with a longer incubation time. Therefore this method is not applied for serial analysis (Fountoulakis et al., 1998). Linder et al, (1995) stated that due to the presence of several peptide bonds and their specific accessibility to enzymatic reaction, the enzymatic hydrolysis is a complex process. The specificity of the enzymes is not the only factor affecting the hydrolysis but some environmental factors such as temperature and pH play an important role on the hydrolysis of the proteins.

Systems of immobilized proteases have been used to obtain complete hydrolysis of proteins, in which the hydrolysis was carried out for 18-24 h and resulted in recoveries of 92-103% (Church et al., 1984; Fountoulakis et al., 1998).

Ge et al. (1996) investigated the complete hydrolysis of casein using immobilized endopeptidase and exopeptidase in packed jacketed columns. The proteases used were trypsin, chymotrypsin, papain, pepsin and *Aspergillus oryzae* protease. The casein was solubilized with 2 mol/L sodium hydroxide at a pH of 8 and filtered to remove any impurities. Then, the casein was passed through a heat exchanger for pasteurization. The casein solution was passed through an endopeptidase column which was packed with

immobilized endopeptidase. Then, the solution was passed through the second column which was packed with immobilized porcine pancreatic exopeptidase. Finally, the solution was passed through the third column which was packed with porcine kidney exopeptidase. After three stages of hydrolysis the product was collected in a storage tank. Each glass column was water-jacketed and connected to a circulating water bath at 50°C. The columns were 50 cm in length and 3.5 cm inner diameter with 50 g of immobilized enzymes. The results from this study revealed that attempts to hydrolyze soy bean protein and egg white protein by this process failed because of their relatively high molecular weight compared to casein. These proteins can only be hydrolyzed after preliminary hydrolysis of soluble protease. The results also suggested that the combined use of enzymes with different peptide specificity was important to improve the degree of hydrolysis and free amino acid content from the casein hydrolysates. Kristinsson et al. (2000) stated that regardless of the enzyme concentration, having a sample containing more than 8% protein seemed to have an inhibiting effect on the hydrolysis of the proteins.

Bai et al. (1999) reported on the combined use of soluble and immobilized protease. Soy bean hydrolysates were treated with 2000 ml 6% protein solutions and the soy bean protein solutions were treated first with 3 g of *Aspergillus niger* acidic protease at pH 2 for 4 h. The resulting product was adjusted to pH 7, treated with 3 g of *Aspergillus oryzae* protease for 4h, heated to 100°C for 10 min, passed consecutively through an immobilized *Aspergillus oryzae* protease reactor at a flow rate of 5 ml/min and consecutively passed through an immobilized kidney exopeptidase reactor at a flow rate of 5 ml/min. Egg white proteins (5% w/v) were first treated with 2 g papain and 2 g of *Aspergillus oryzae*. The resulting product was heated at 100°C for 10 min and passed through an immobilized *Aspergillus oryzae* protease reactor and then through an immobilized pancreatic exopeptidase at a flow rate of 5ml/min. The results revealed that the hydrolysis of proteins with soluble protease was effective in reducing the molecular size and further improved when subjected to more soluble and immobilized proteases. The immobilized proteases in the system improved the degree of hydrolysis and the free amino acid ratio. The process had many advantages such as moderate operating conditions, low salt residues, high retention of acid and alkaline sensitive amino acid and

semi-continuous production of protein hydrolyzate with a high content of free amino acids. In addition, the cost of operation was lower than the traditional processes.

3.8. Jadomycin

3.8.1. Biosynthesis and Structure of Jadomycin

The soil microbe *Streptomyces venezuelae* ISP5230 produces jadomycin B as a secondary metabolite under specific nutrient and stress conditions such heat, alcohol and phage shocking. The jadomycin is a glycosylated natural product which contains five-membered oxazolone ring in its structure. The oxazolone ring formation is due to the reaction with the amino acid present in the culture medium with a biosynthetic aldehyde precursor. This pathway generates reactive aldimines which undergoes series of transformations to form a cyclized product (Jakeman et al., 2009).

Doull et al. (1994) reported that there is an unusual nitrogen atom in the oxazolone ring structure as a result of the direct incorporation of isoleucine molecule for jadomycin B as shown in Figure 3.10. Borissow et al. (2007) and Rix et al. (2004) reported that different jadomycins can be produced by replacing isoleucine with other amino acids in the production medium and twelve different types of jadomycins have been identified as shown in Table 3.14.

3.8.2.Growth and Shocking Process

The jadomycin production process takes place in three steps: growth, shocking and production. During the growth phase, a culture of *Streptomyces venezuelae* ISP5230 is grown on nutrient rich MYM agar at 30°C for 48 h. The colonies are later scraped into the nutrient rich MYM broth medium and the bacteria is allowed to grow at 30°C and 250 rpm for 24 h. The composition of the growth medium is shown in Table 3.15 (Burdock et al., 2008).

During the production phase, the grown innoculum is transferred to nutrient deprived production medium containing amino acids. The shocking of the bacteria is carried out in the production medium. Doull et al. (1994) performed ethanol shocking of bacteria at 6%

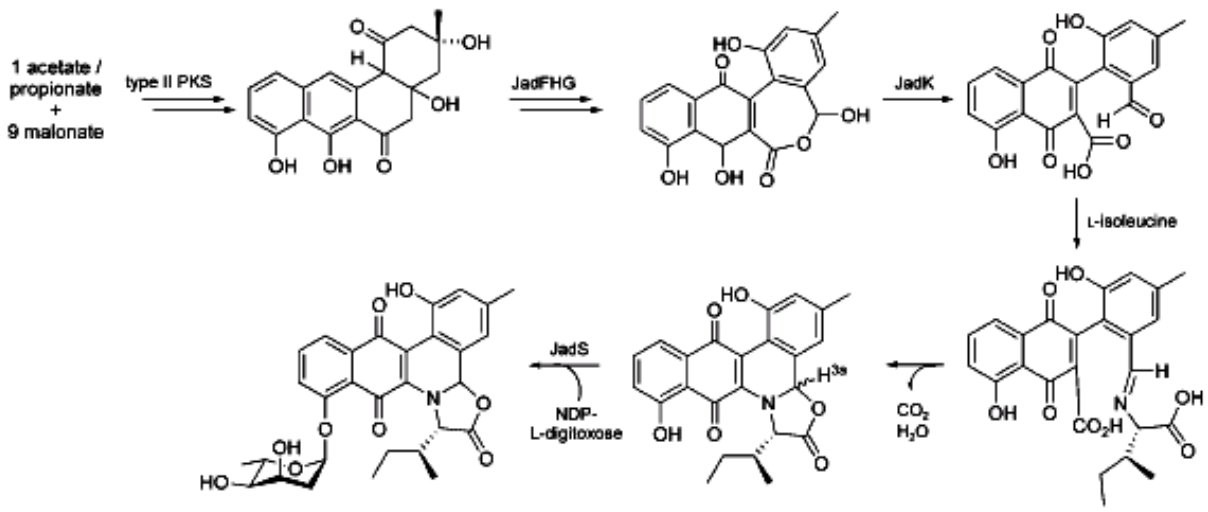


Figure 3.10. Jadomycin B biosynthesis with isoleucine (Borissow et al., 2007)

Table 3.14. Jadomycins produced by *S.venezuelae* ISP5230 by ethanol shock (Borissow et al., 2007).

Amino acid	Jadomycin Type
Alanine	Jadomycin Ala
Glycine	Jadomycin G
Histidine	Jadomycin H
Isoleucine	Jadomycin B
Leucine	Jadomycin L
Methionine	Jadomycin M
Phenylalanine	Jadomycin F
Serine	Jadomycin S
Threonine	Jadomycin T
Tryptophan	Jadomycin W
Tyrosine	Jadomycin Y
Valine	Jadomycin V

Table 3.15. Media composition for jadomycin prodcution (Burdock et al., 2008).

Media Component	Chemical Formula	Amount per liter
Growth Medium		
Maltose	$C_{12}H_{22}O_{11}$	4.0 g
Yeast Extract		4.0 g
Malt Extract		10.0 g
Agar		15.0 g
MOPS	$C_7H_{15}NO_4S$	1.9 g
Production Medium		
MSM Solution		
Magnesium Sulfate	$MgSO_4$	0.4 g
MOPS	$C_7H_{15}NO_4S$	1.9 g
Salt Solution		9.0 mL
Sodium Chloride (1% w/v salt solution)	NaCl	
Calcium Chloride (1% w/v salt solution)	$CaCl_2$	
Ferrous Sulfate (0.2% w/v salt solution)	$FeSO_4.\ 7H_2O$	4.5 mL
Trace Mineral Solution		4.0 mL
Zinc Sulfate (88% w/v trace mineral solution)	$ZnSO_4.\ 7H_2O$	
Cupric Sulfate (3.9% w/v trace mineral solution)	$CuSO_4.\ 7H_2O$	
Manganese Sulfate (0.61% w/v trace mineral solution)	$MnSO_4.\ 7H_2O$	
Boric Acid (0.57% trace mineral solution)	H_3BO_3	
Ammonium Molybdate (0.37% trace mineral solution)	$(NH_4)_6.\ 4H_2O$	
GM Solution		
Glucose (dextrose)	$C_6H_{12}O_6$	5.4 g
Phosphate Stock Solution (9 mM)		
Potassium Phosphate	K_2HPO_4	10.5 g
Potassium Phosphate Monobasic	KH_2PO_4	4.5 g
Amino Acid		
Glycine	$C_2H_5NO_2$	4.5 g

(v/v), in which the highest jadomycin yield was obtained with the cultures treated with ethanol after 6-13 h and the jadomycin yield reduced after 48 h. The report also suggested that different amino acids produced different colored pigments with different jadomycins possibly representing the biosynthesis of jadomycin B analogs. Similarly, Jakeman et al. (2006) and Burdock et al. (2008) performed ethanol shocking at 3% v/v to produce jadomycin. Burdock et al. (2008) reported that jadomycin yield increased rapidly for the first 15 h after the ethanol at 6 h. The report indicated that a small amount of jadomycin is produced during the first 6 h when the innoculum was transferred from growth media to the nutrient deprived production medium, but significant amount of jadomycin was produced only after shocking the bacteria with ethanol.

Doull et al. (1994) performed heat shocking on the bacteria at 42°C for 1 h and shifted back to 27°C to produce jadomycin. The report indicated that heat shock after 9 h post inoculation was effective for significant jadomycin production. The report also suggested the use of phage shocking, in which the phage SV1 was added to the production medium 2 h after the inoculation of bacteria and the production was carried out for 48 h at 30°C. The phage infection is known to induce various heat shock genes in the bacterial cells and the association of *Streptomyces venezuelae* with phage SV1 has induced heat shock response in it to produce jadomycin, but this process is seldom used to produce jadomycin.

CHAPTER 4. MATERIALS AND METHODS

4.1. Experimental Materials

4.1.1. Glassware

The glassware used in this research included test tubes, beakers, reagent bottles, Pyrex bottles and pipettes. All the glassware was washed using soap and hot water and rinsed with distilled water before use.

4.1.2. Chemicals and Enzymes

The chemicals and enzymes used include: Alcalase, Neutrase, potassium phosphate monobasic, potassium phosphate dibasic, concentrated sulfuric acid, concentrated hydrochloric acid, Bovine serum albumin, copper sulfate, sodium carbonate, sodium tartrate, 2N Folin and Ciocalteu's Phenol Reagent, trichloroacetic acid and acetone. All the chemicals were obtained from Sigma-Aldrich, Oakville, Ontario, Canada. The standard amino acids were obtained from Bioshop, Ontario, Canada.

4.1.3. Reagents

The reagents included: 1N hydrochloric acid, 6N hydrochloric acid, 1N sodium hydroxide, 20% trichloroacetic acid, 1M potassium phosphate monobasic and 1M potassium phosphate dibasic. 1M pH 8 phosphate buffer was prepared by adding 94.7 ml of 1M potassium phosphate mono-basic and 5.3 ml of 1M potassium phosphate dibasic with 100 ml of distilled water.

4.1.4. Equipment

The equipment used in the experiments were: Precision 2870 Series water bath shaker (Thermo Scientific, Ohio, USA), Precision 280 Series water bath (Thermo Scientific, Ohio, USA), Yamato RE540 rotary evaporator (Yamato Scientific America, USA), Mini Pulvis Spray GS-310 spray dryer (Yamato Scientific America, USA), Thermo Scientific UV-Vis spectrophotometer, Isotemp 655 F oven (Fisher Scientific,

Ohio, USA), Sorvall RT1 Centri-fuge (Thermo Scientific, Ohio, USA), Branson 2510 sonicator, Metler AE 200 and PM 4600 balance (Mettler-Toledo International Inc., Mississauga, Canada), .Orion 5 Star pH meter (Thermo Scientific, Massachusetts, USA), Hach DRB200 Reactor Block (Hach Company, Colorado, USA), Hewlett-Packard HP5890 Series II gas chromatograph, coupled with Flame Ionization Detector (FID) (Agilent Technologies, Mississauga, Ontario, Canada), Non-Packed Silica Capillary Column (Alltech Associates, Bannockburn, Illinois, USA), Silica G TLC plates (Sigma-Aldrich, Oakville, Ontario, Canada) and and Genesys 10 S UV-VIS spectrophometer (Thermo Scientific, Ohio, USA).

4.1.5. Fish Samples

Whole Mackerel fish was obtained from Sea Crest Fisheries, Nova Scotia, Canada. The fish was collected in sealed plastic bags and transported to the Biological Engineering Laboratory and stored in a freezer at -20°C.

4.2. Experimental Design

The experimental work is divided into two parts: protein extraction and amino acid extraction. The parameters studied in each are shown in Tables 4.1 and 4.2.

In the first part (Figure 4.1), proteins were extracted enzymatically from the whole fish and fish waste using the enzyme Alcalase. The fish waste consisted of the head, frames, gut, skin, fin and tail. During the enzymatic hydrolysis of proteins from different parts of fish, three enzyme concentrations (0.5, 1 and 2%) and four reaction times (1, 2, 3 and 4 h) were tested. The temperature, pH and raw material: buffer ratio were kept at 55°C, 7.5 and 1:1, respectively.

The second of part of the experimental work was devoted to the enzymatic extraction of amino acids (Figure 4.2). The enzymes used were: Alcalase, Neutrase and a combination of Alcalase and Neutrase. The hydrolysis was carried out using two different retention times (24 and 48 h) and a set ratio of raw material: amount of enzyme (20 µl/g). The temperature and pH were kept at 55°C and 7.5 for the Alcalase and 45°C and 6.5 for

Table 4.1. Protein extraction parameters.

Factors	Parameters
Enzyme	Alcalase
Enzyme concentration	0.5, 1.0 and 2.0 %
Reaction time	1, 2, 3 and 4 h
pH	7.5
Temperature	55°C

No. of replicates = 3
No. of runs = 36

Table 4.2. Amino acids extraction parameters.

Factors	Parameters
Enzyme	Alcalase, Neutrase, and Alcalase + Neutrase
Enzyme: Substrate Ratio	20µl/g
pH	7.5 and 6.5
Temperature	55°C and 45°C
Reaction time	24 and 48 h

No. of replicates = 3
No. of runs = 18

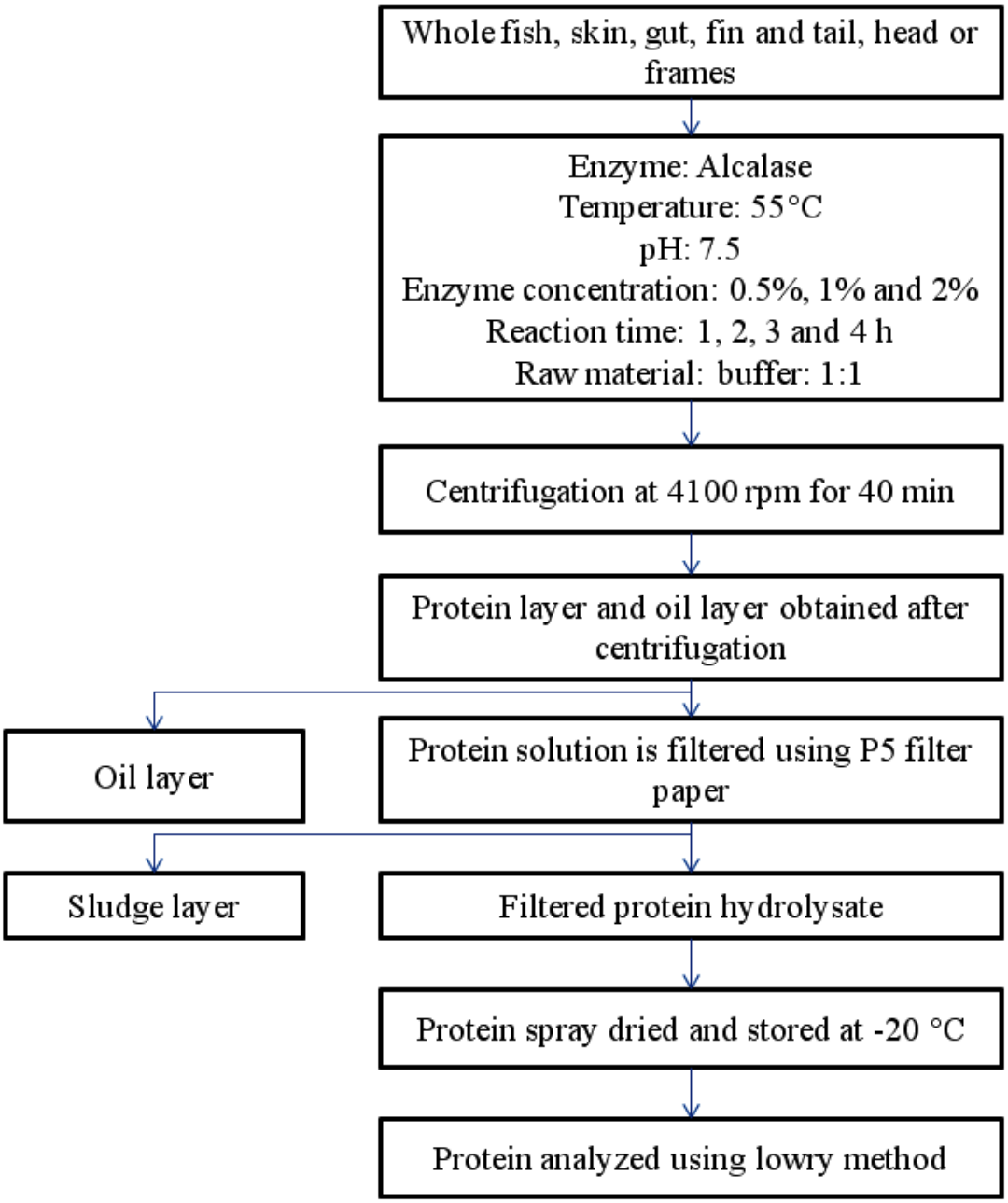


Figure 4.1. Enzymatic extraction of proteins from whole fish and fish waste.

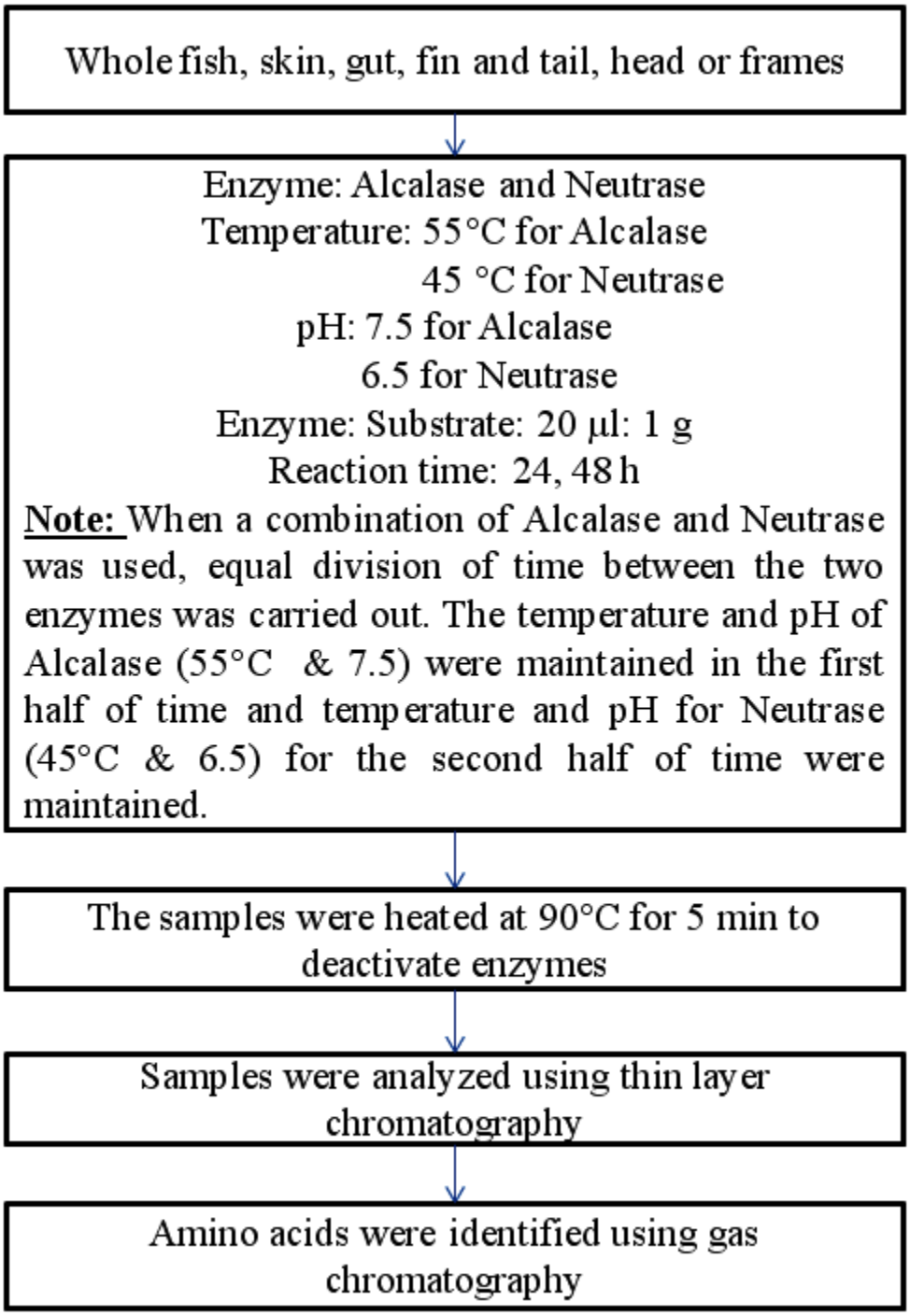


Figure 4.2. Enzymatic extraction of amino acids from protein.

Neutrase, respectively. When using a combination of enzymes, the total time was divided equally (12+12 h and 24+24 h) between the two enzymes and the optimum temperature and pH for Alcalase (55°C and 7.5) were used during the first half of the experiment and the optimum temperature and pH for Neutrase (45°C and 6.5) were used during the second half. The amino acids obtained from the extraction were identified using thin layer chromatography and the amino acid profiling was determined using gas chromatography.

4.3. Experimental Procedure

4.3.1. Enzymatic Extraction of Proteins

The enzymatic extraction of protein was carried out according to the procedure described in Figure 4.3. The whole mackerel fish was minced in a homogenizer (Model No.4532s/s, Hobart Manufacturing Co. Ltd, Ontario, Canada) without adding any water. The minced fish (50 g) was first placed in a 500 ml glass bottle and heated in a water bath (Precision 280 Series, Thermo Scientific, Ohio, USA) at 90°C for 10 min before the extraction to deactivate the endogenous enzymes. Then, 50 ml of 1M potassium phosphate buffer (pH 7.5) in the ratio of 1:1 (fish : buffer) was added to the fish and mixed well using a magnetic stirrer. The total volume was found to be 100 ml and the pH of the mixture was measured using a pH meter (Orion 5 Star pH meter, Thermo Scientific, Massachusetts, USA). The pH was adjusted to 7.5 with 1N NaOH. The glass bottle was then placed in a water bath shaker (Precision 2870 Series, Thermo Scientific, Ohio, USA) at 140 rpm and 55°C and kept for 30 min. Then, the temperature was measured using a thermometer. The enzymatic hydrolysis was started by adding 0.5, 1 or 2% (by weight of raw material) Alcalase. After hydrolysis for 1, 2, 3 and 4 h, the mixture was taken and placed in another water bath (Precision 280 Series, Thermo Scientific, Ohio, USA) at 90°C for 5-10 min to inactivate the enzymes. The mixture was then allowed to cool and centrifuged (Sorvall RT1 Centrifuge, Thermo Scientific, Ohio, USA) at 4100 rpm for 40 min. Four layers (Figure 4.4) were formed in the centrifuge tubes: upper oil layer, light-lipid layer, soluble clear protein layer and bottom sludge layer (un-hydrolyzed fish tissue), respectively. The upper oil layer was removed and stored at.

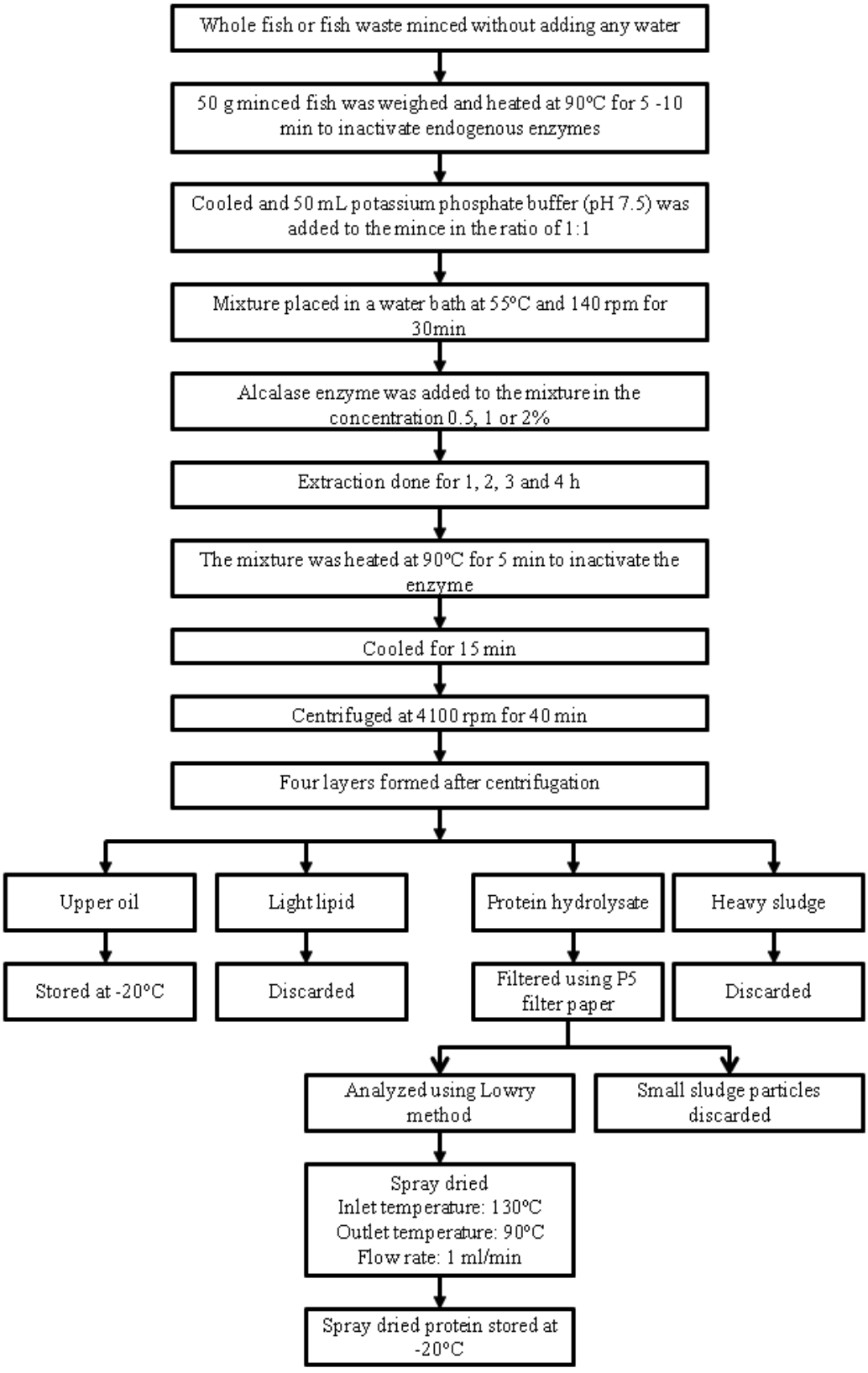


Figure 4.3. Enzymatic extraction of protein from whole fish.

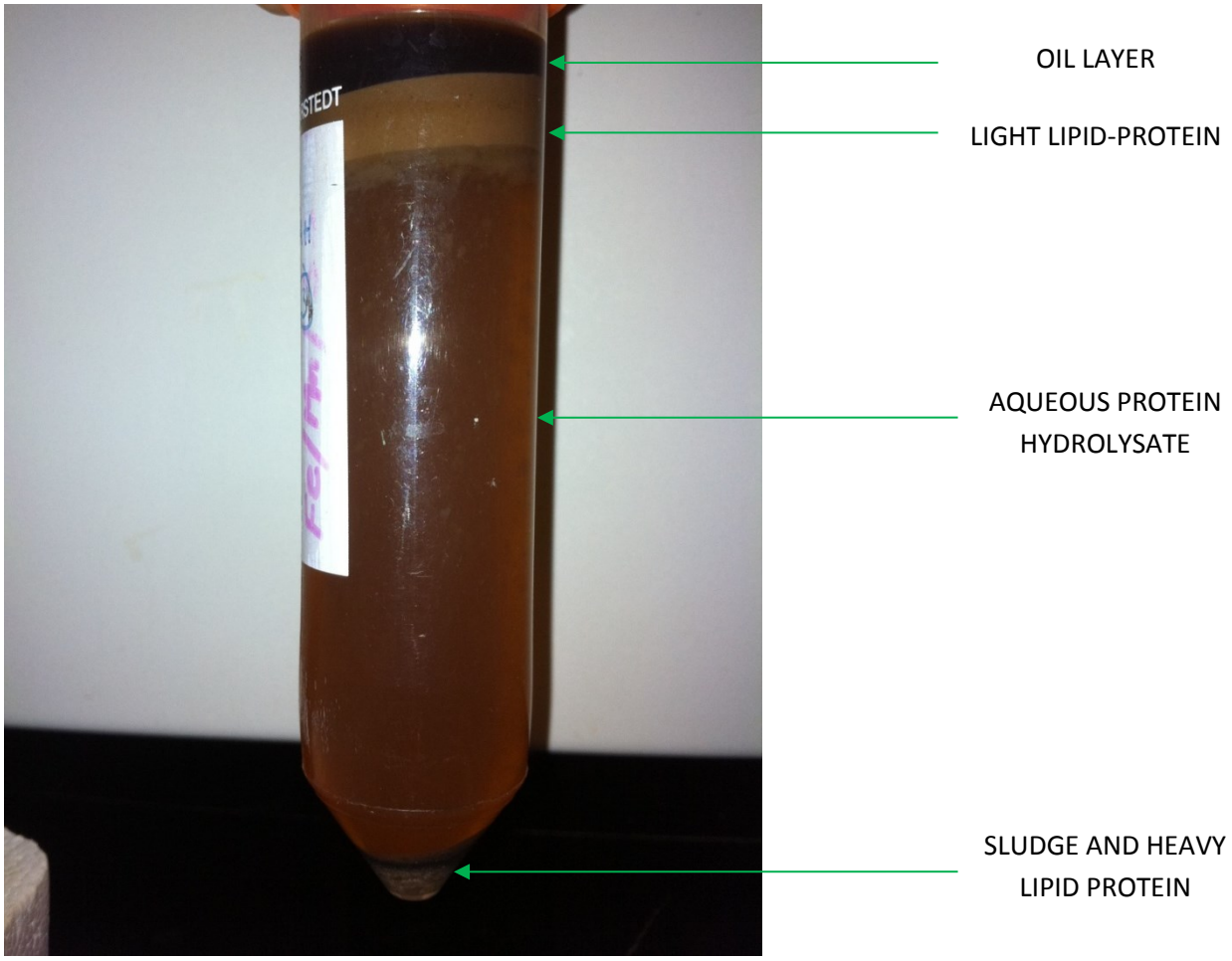


Figure 4.4. Four layers when recovering soluble fish protein hydrolyzate.

-20°C. The soluble protein layer was removed by tilting the centrifuge tubes without disturbing the lipid layer. The lipid layer and sludge layer was discarded. The protein hydrolysate obtained was then filtered using Fisher P5 filter paper. The filtered protein hydrolysate was then spray dried using spray dryer (Mini Pulvis Spray GS-310, Yamato Scientific America, USA) at an inlet temperature of 130°C and outlet temperature of 90°C and flow rate of 1-2 ml/min. The spray dried protein was stored at -20°C until further use. The protein hydrolysate was also analyzed using the Lowry method. The enzymatic hydrolysis of proteins from the various parts of mackerel fish (head, frames and fins, tails, gut and skin together) was carried out using the same procedure.

4.3.2. Hydrolysis of Proteins

The hydrolysis of proteins was done in order to extract amino acids from the proteins derived from the whole fish as shown in Figure 4.5. 4 g of dried protein samples were weighed using a digital scale (Metler AE 200, Mettler-Toledo International Inc., Mississauga, Canada) and added to 100 ml of distilled water making a 4% solution. Then the solutions were placed in a water bath shaker (Precision 2870 Series water bath shaker, Thermo Scientific, Ohio, USA) which was preheated at 55ºC and kept in the water bath for 30 min. Then, Alcalase enzyme was added to the samples at the rate of 20 µL/g of protein. The hydrolysis was continued for either 24 h or 48 h at 140 rpm. The samples were heated in a water bath (Precision 280 Series, Thermo Scientific, Ohio, USA) for 5 min at 90ºC to deactivate enzyme. The samples were then cooled and analyzed using thin layer chromatography (TLC (Sigma-Aldrich, Oakville, Ontario, Canada)), samples were spray dried (Mini Pulvis Spray GS-310, Yamato Scientific America, USA) at an inlet temperature of 130°C and outlet temperature of 90°C and flow rate of 1-2 ml/min. The spray dried amino acids were analyzed using gas chromatography (Hewlett-Packard HP5890 Series II gas chromatograph, coupled with Flame Ionization Detector (Agilent Technologies, Mississauga, Ontario, Canada).

Hydrolysis of proteins with Neutrase was carried out using the same procedure but the temperature and pH were adjusted to 45 ºC and 6.5, respectively. For hydrolysis of proteins using the two enzymes in combination, the pH was adjusted to 7.5 and the

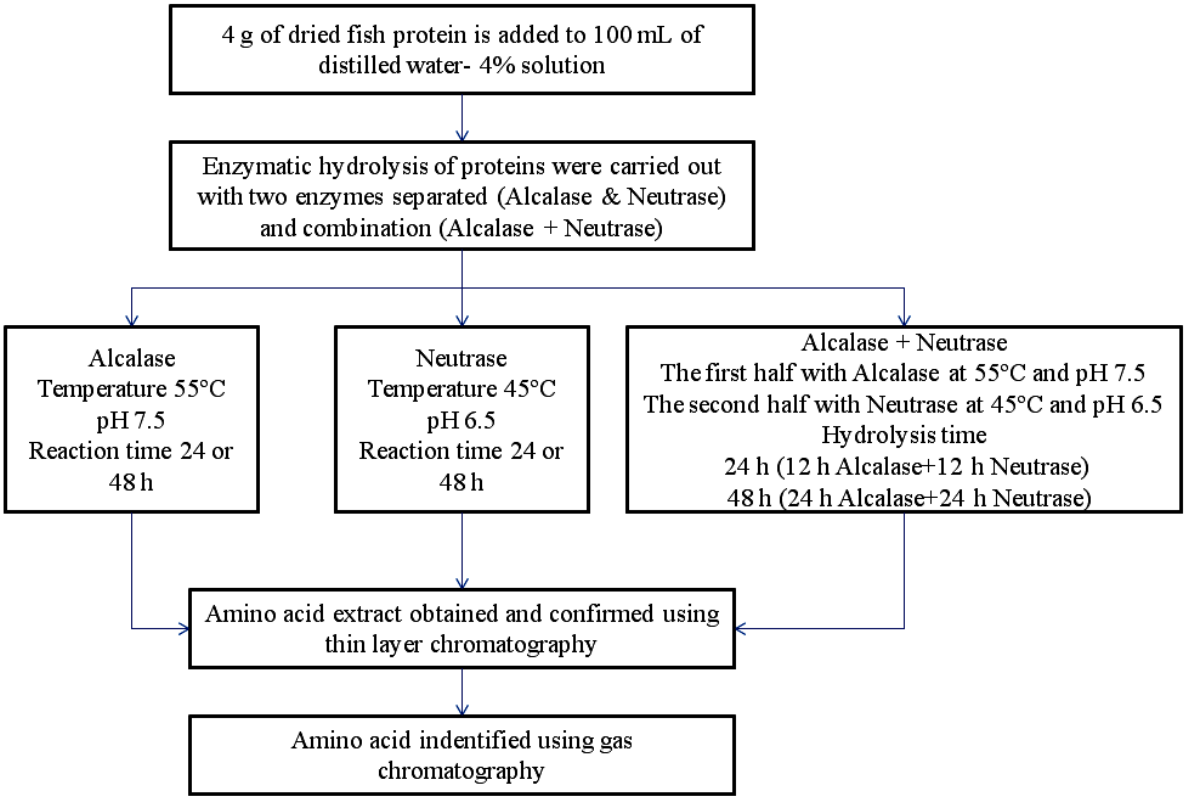


Figure 4.5. Enzymatic hydrolysis of proteins to extract amino acids.

sample was placed in a water bath preheated at 55ºC and kept in the water bath for 30 min before adding Alcalase enzyme (20 µL/g of protein). The hydrolysis was continued for 12 h and the pH was reduced from 7.5 to 6.5 using 1N HCl and the temperature of the water bath was reduced to 45ºC. The Neutrase enzyme was added and the hydrolysis was continued for another 12 h. The same procedure was repeated but allowing 24 h for each enzyme.

4.4. Experimental Analyses

4.4.1. Chemical Analysis

The moisture content, fat content, protein content, ash content and carbohydrate content were performed on the fish samples. These analyses were performed by Nova West Laboratory Ltd., Nova Scotia, Canada.

4.4.2. Lowry Analysis

The protein concentration was determined by the Lowry method (Lowry et al., 1951; Gerhardt et al., 1994). In this procedure, the proteins were first pretreated with copper ion in alkali solution. Then, the aromatic amino acids in the treated sample reduce the phosphomolybdatephosphotungstic acid present in the Folin reagent. As the endpoint of the reaction has a blue color, the amount of protein in the sample can be estimated by reading the absorbance (Genesys 10 S UV-VIS spectrophometer, Thermo Scientific, Ohio, USA) at 750 nm.

Solution A was an alkaline solution consisting of 2.8598 g NaOH and 14.3084 g Na_2CO_3 mixed in 500 ml volumetric flask. Solution B was made by adding 1.4232 g $CuSO_4.5H_2O$ to 100 ml water in a volumetric flask. Solution C was prepared by adding 2.85299 g sodium tartarate to 100 ml water in a volumetric flask. Lowry solution was prepared fresh daily by combining solution A + B + C together in the ratio 100:1:1. Folin Reagent was prepared fresh, by adding 5 ml of 2N Folin and Ciocalteu's Phenol Reagent to 6 ml of distilled water. Bovine serum albumin (BSA) was used as the standard protein solution.

Several concentrations were prepared for the development of standard curve. 0.05 g of BSA was added to a 500 ml volumetric flask containing distilled water. The final concentration of the BSA mixture was 100 mg/l. Dilutions ranging from 0 to 100 mg/L were prepared (Table 4.3). The results of the absorbance values of the standard BSA concentrations are shown in the Table 4.4. A linear plot was drawn from the absorbance values and the linear relationship between absorbance and protein concentration is shown in the Figure 4.6.

4.4.3.Thin Layer Chromatography

Thin layer chromatography is the process of separating compounds based upon their differential affinity in which the stationary phase is the thin layer of silica gel on a solid plate and the mobile phase is the phase in which a small amount of sample spotted on the plate is carried through the stationary phase via capillary action. The procedures described by Srivastava et al. (1972) were undertaken for the proper movement of the samples. A silica gel TLC plate (Sigma-Aldrich, Oakville, Ontario, Canada) was obtained and a thin line was drawn 2-3 cm from the bottom and also from the top of the plate. On the bottom line, 21 evenly spaced markings were made for spotting the amino acid standards. All the amino acid standards were prepared in 10 ml test tubes in the concentration of 1mg/ml distilled water. The prepared amino acids standard were spotted on the markings using capillary tubes. The spotting of amino acids was carefully done in such a way that the spot were small (bigger spots might lead to an imprecise result). After spotting, the plate was allowed to air dry. In the TLC tank, the solvent system consisting of butanol, glacial acetic acid and water in the ratio of 4:1:1 (the mobile phase) was prepared. The solvent system in the tank was exactly 1 cm from bottom of the tank. The TLC plate was then placed in the tank and was closed tightly. The solvent was allowed to run up to the top marked line on the plate. After that the TLC plate was carefully taken out, the line on the top of the sheet was the solvent front. The plate was allowed to completely dry and then taken to the fume hood to spray 2% ninhydrin in ethanol solution on it. It was then kept in a hot air oven (Isotemp 655 F oven, Fisher Scientific, Ohio, USA) to dry at 100ºC for 5 min. After drying, the spots were visible and they were immediately circled and spotted in the center with a pencil. The Retardation factor (Rf)

Table 4.3. Dilutions from the BSA stock solution (100 mg/L) for the standard curve

Distilled water (ml)	BSA stock solution (ml)	Final concentration (mg/L)
10	0	0
9	1	10
8	2	20
7	3	30
6	4	40
5	5	50
4	6	60
3	7	70
2	8	80
1	9	90
0	10	100

Table 4.4. Absorbance values of the standard BSA measured at 750 nm

Protein Concentration (mg/L)	Absorbance at 750 nm
0	0.000
10	0.064
20	0.131
30	0.168
40	0.211
50	0.256
60	0.308
70	0.361
80	0.412
90	0.492
100	0.580

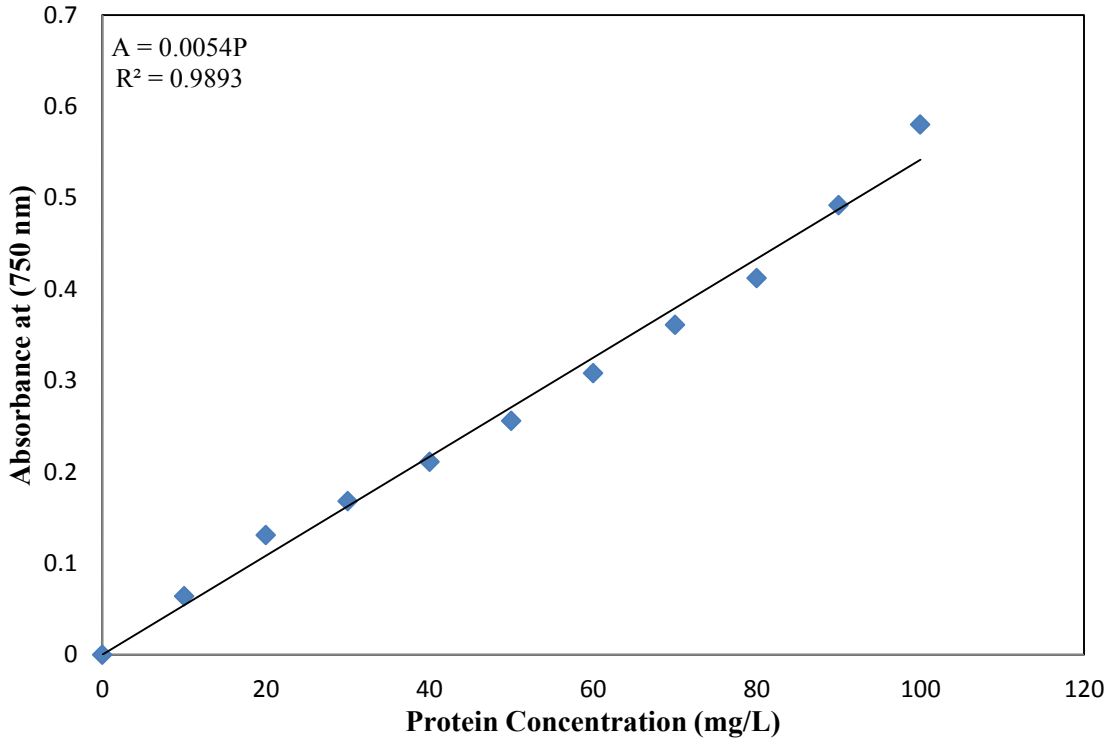


Figure 4.6.Standard curve for protein concentration

value of each amino acid was calculated using the following formula

Rf =(Distance travelled by the spot)/(Distance travelled by the solvent) (4.1)

In a test tube, 100 µl from each amino acid standard solution was mixed together. To identify the amino acids present in a hydrolysed sample, the solution with the combined amino acids and hydrolysed sample solution were spotted together on a same chromatogram plate and compared.

4.4.4.Gas Chromatograph of Amino acids

A gas chromatograph (Hewlett-Packard HP5890 Series II gas chromatograph, Agilent Technologies, Mississauga, Ontario, Canada) was used to identify and quantify each amino acid in the samples. Non-packed silica capillary column (Alltech Associates, Bannockburn, Illinois, USA) with intermediate polarity, a length of 25 m and diameter of 0.54 mm was used. Helium was used as the carrier gas and the stationary phase in the column was amino acids. The initial pressure was 34.47 kPa, the flow rate was 4 m/min with a velocity of 1cm/sec. A Flame Ionization Detector was used for the detection of amino acids. The rate of temperature for heating was set at 10ºC/min to reach a final temperature of 210ºC. The total time for each run was 60 min. The sample size injected into the chromatogram was 10µl. An amino acid derivatization kit (Alltech Chemicals Ltd.), in which 40 mg of the amino acids were placed in a small reaction vial, was used. 3 ml of 0.2 M HCl was added and the solution was heated to 110ºC for 5 minutes. Then, the vials were dried under a stream of dry nitrogen. Acetyl chloride was slowly added to isopropanol and this mixture was added to the dried sample and heated to 100ºC for 45 min. The vial was later uncapped and heated to approximately 115ºC under a stream of dry nitrogen to remove excess reagent. The vial was later cooled in an ice bath. After cooling, 3 ml of methylene chloride and one ampoule of pentafluoropropionic anhydride (PFPA) was added to the vial and it was reheated to 100ºC for 15 min. The vial was again cooled to ambient temperature and dried under a stream of dry nitrogen and dissolved in suitable solvent for analysis using gas chromatograph.

4.4.5. Fish Protein Hydrolysate Yield

The amount of fish protein hydrolysate was calculated by the volume of supernatant obtained to the total volume of raw material used during the extraction process. It was calculated as follows

$$\textbf{Fish Protein Hydrolysate yield}\ (\%) = \frac{\textbf{Volume of supernatant (ml)}}{\textbf{Total volume of raw material (ml)}} * \mathbf{100} \quad (4.2)$$

4.4.6. Recovered Protein

The percentage recovered protein from the fish is the ratio of the amount of dried protein extracted to the total weight of the raw material used in the extraction process. It was

calculated as follows

$$\textbf{Recovered Protein}\ (\%) = \frac{\textbf{Weight of dried protein (g)}}{\textbf{Weight of raw material (g)}} * \mathbf{100} \quad (4.3)$$

4.4.7. Protein Yield

The protein yield from the fish is the ratio of protein yield obtained during the extraction process to the amount of estimated Kjeldhal protein. It was calculated as follows:

$$\textbf{Protein yield}\ (\%) = \frac{\textbf{Recovered protein}\ (\%)}{\textbf{Kjeldhal protein}\ (\%)} * \mathbf{100} \quad (4.4)$$

4.4.8. Recovered Oil

The percentage recovered oil is the ratio of the amount of oil extracted to the total weight of raw material used in the extraction process. It was calculated as follows:

$$\textbf{Recovered Oil}\ (\%) = \frac{\textbf{Weight of oil extracted (g)}}{\textbf{Weight of raw material (g)}} * \mathbf{100} \quad (4.5)$$

4.4.9. Oil Yield

The oil yield is the ratio of the oil yield of oil to the percentage estimated fat content from the raw material. It is calculated as follows

$$\boldsymbol{Oil\ yield}\ (\%) = \frac{\boldsymbol{Recovered\ oil}\ (\%)}{\boldsymbol{Yield\ of\ estimated\ fat}\ (\%)} * \mathbf{100} \tag{4.6}$$

4.4.10. Amino acid Yield

The individual amino acid yield is the ratio of peak area of the corresponding amino acid to the total area count of the chromatograph. It is calculated as follows

$$\boldsymbol{Amino\ acid\ yield}\ (\%) = \frac{\boldsymbol{Peak\ area\ A} * \mathbf{100}}{\sum(\boldsymbol{Peak\ area\ A} + \boldsymbol{Peak\ area\ B} + \cdots + \boldsymbol{Peak\ area\ N})} \tag{4.7}$$

4.4.11. Statistical Analysis

Statistical analysis of the data was conducted using Minitab statistics software (Ver 16.2.2 , Minitab Inc., Canada). Both analysis of variance (ANOVA) and Tukey grouping were performed on the data..

CHAPTER 5. RESULTS

5.1. Weight Distribution and Nutritional Composition

The average weight of a whole fish was 487.11 g. The weight distribution of the different parts of the fish is shown in Table 5.1. The flesh, head, frame, fins and tails, skin and gut make up 286.91g (58.90%), 75.87g (15.58%), 37.12g (7.62%), 5.71g (1.17%), 34.74g (7.13%), and 36.69g (7.53%), respectively. The nutritional composition (moisture, protein, fat, carbohydrate and ash contents) of whole fish and fish parts are shown in Table 5.2. The average protein, fat, carbohydrate and ash contents were 15.57, 16.52, 0.65 and 1.68% for the whole fish, 12.30, 17.16, 1.17 and 3.74% for the head, 14.16, 10.43, 0.31 and 3.48% for the frame and 12.18, 20.84, 0.00 and 1.36% for the fins, tails, skin and gut, respectively. The whole fish had the highest protein content (15.57%) while the fins, tails, skin and gut had the highest fat content (20.84%) and head had the highest carbohydrate content (1.17%).

5.2. Protein Extraction

Fish protein was extracted from the whole fish (WF), head (H), frames (F) and fin, tail, skin and gut (FTSG). The extraction was carried out using 50 g of material, with 50 ml of 1M phosphate buffer (pH of 7.5), at three different enzyme concentrations (0.5, 1.0, or 2.0%) and four different reaction times (1, 2, 3 and 4 h). After extraction, fish protein hydrolysate was obtained by centrifugation. The amount of fish protein hydrolysate extracted was calculated using Equation 4.2 and spray dried to obtain dried protein powder. The protein powder obtained was weighed and the amount of protein yield from each sample was calculated. The results are shown in Tables 5.3-5.6.

Analysis of variance (ANOVA) was performed on the protein yield data as shown in Table 5.7. The effects of fish parts, enzyme concentration and time were significant at the 0.001 level. There were significant interactions among the various parameters at the 0.001 level.

The results obtained from the Tukey's grouping is shown in Table 5.8. The difference

Table 5.1. Weight distribution of mackerel fish.

Sample	Whole (g)	Flesh (g)	Head (g)	Frames (g)	Fins & Tails (g)	Skin (g)	Gut (g)
1	470.03	256.41	82.23	41.87	7.62	31.51	38.53
2	403.18	213.2	66.34	19.48	3.67	41.96	32.73
3	514.74	302.63	79.41	38.71	9.73	32.43	32.18
4	492.58	301.65	81.7	26.58	5.32	38.08	39.25
5	366.48	187.79	63.57	36.66	4.77	30.73	31.17
6	580.41	340.69	85.73	53.06	6.07	30.3	53.12
7	438.25	246.08	65.76	30.79	3.42	38.26	32.3
8	557.25	343.86	80.94	34.05	6.23	30.95	32.3
9	529.35	313.98	73.57	52.96	4.79	38.17	37.85
10	518.85	302.85	79.49	38.96	5.5	35.01	37.52
Average	487.11	286.91	75.87	37.12	5.71	34.74	36.69

Table 5.2. Nutritional composition of mackerel fish and fish waste.

Sample	Moisture (%)	Protein (%)	Fat (%)	Carbohydrate (%)	Ash (%)
Head	65.63	12.30	17.16	1.17	3.74
Frames	71.62	14.16	10.43	0.31	3.48
Fins, Tails, Skin and Gut	65.62	12.18	20.84	0.00	1.36
Whole Fish	65.58	15.57	16.52	0.65	1.68

Table 5.3. Protein recovery and yield from whole fish.

Enzyme Concentration (%)	Reaction Time (h)	Recovered Protein (g)	Protein Yield (%)
0.5	1	3.53±0.15	45.34
	2	3.86±0.12	49.58
	3	4.53±0.11	59.22
	4	5.21±0.01	66.92
1	1	3.67±0.03	47.18
	2	4.17±0.10	53.52
	3	5.14±0.12	65.98
	4	5.54±0.01	71.16
2	1	4.24±0.11	54.46
	2	4.86±0.05	62.43
	3	5.44±0.02	69.88
	4	5.94±0.02	76.30

Estimated protein: 15.57%
Fish Weight: 50g

Table 5.4. Protein recovery and yield from head.

Enzyme Concentration (%)	Reaction Time (h)	Recovered Protein (g)	Protein Yield (%)
0.5	1	3.28±0.03	53.39
	2	3.55±0.02	57.78
	3	3.75±0.04	60.98
	4	3.82±0.02	62.17
1	1	3.22±0.02	52.41
	2	3.53±0.02	57.45
	3	3.75±0.02	61.03
	4	3.92±0.03	63.79
2	1	3.39±0.02	55.18
	2	4.00±0.09	65.04
	3	4.22±0.02	68.67
	4	4.36±0.03	70.84

Estimated protein: 12.30%
Fish Weight: 50g

Table 5.5. Protein recovery and yield from frame.

Enzyme Concentration (%)	Reaction Time (h)	Recovered Protein (g)	Protein Yield (%)
0.5	1	3.52±0.11	49.67
	2	3.97±0.01	56.07
	3	4.19±0.02	59.18
	4	4.39±0.03	61.96
1	1	3.81±0.04	53.86
	2	4.25±0.02	59.98
	3	4.46±0.02	62.99
	4	4.68±0.02	66.05
2	1	3.63±0.02	51.22
	2	4.71±0.03	66.57
	3	4.97±0.05	70.20
	4	5.28±0.02	74.53

Estimated protein: 14.16%
Fish Weight: 50g

Table 5.6. Protein recovery and yield from fin, tail, skin and gut.

Enzyme Concentration (%)	Reaction Time (h)	Recovered Protein (g)	Protein Yield (%)
0.5	1	2.48±0.06	40.78
	2	2.75±0.04	45.16
	3	2.95±0.03	48.39
	4	3.28±0.02	53.80
1	1	2.96±0.02	48.6
	2	3.37±0.05	55.34
	3	3.58±0.04	58.84
	4	3.78±0.02	62.01
2	1	3.45±0.04	56.70
	2	3.95±0.03	64.81
	3	4.14±0.02	67.98
	4	4.26±0.02	69.95

Estimated protein: 12.18%
Fish Weight: 50g

Table 5.7. Analysis of variance for protein yield.

Source	DF	SS	MS	F	P
Total	143	9836.37			
Model					
Parts	3	587.33	195.78	395.34	0.001
EC	2	2888.51	1444.26	2916.43	0.001
RT	3	5083.29	1694.43	3421.61	0.001
Parts*EC	6	486.24	81.04	163.65	0.001
Parts*RT	9	486.34	54.04	109.12	0.001
EC*RT	6	128.11	21.35	43.12	0.001
Parts*EC*RT	18	129.00	7.17	14.47	0.001
Error	96	47.54	0.50		

DF: Degree of freedom
SS: Sum of square
MS: Mean of square
EC: Enzyme concentration
RT: Reaction time
R^2: 99.52%

Table 5.8. Tukey's grouping on protein yield.

Factors	Level	N	Mean Yield (%)	Tukey Grouping
Parts	WF	36	60.17	A
	H	36	60.73	B
	F	36	61.02	B
	FTSG	36	56.03	C
Enzyme Concentration (%)	0.5	48	54.40	A
	1.0	48	58.76	B
	2.0	48	65.30	C
Reaction time (h)	1	36	50.73	A
	2	36	57.81	B
	3	36	62.78	C
	4	36	66.63	D

Groups with the same letter are not significantly different from each other at the 0.05 level.
WF: Whole Fish
FTSG: Fin, tail, skin and gut
F: Frames
H: Head

between the head and frame was not significant from each other at the 0.05 level. They were, however, significantly different from the whole fish (WF) and fin, tail, skin and guts (FTSG) at 0.05 level. The results indicated that the highest protein yield was obtained from the frame (F) and head (H) with a mean protein yield of 61.02 and 60.73%, respectively. The three enzyme concentrations (0.5, 1.0 and 2.0%) were significantly different from each other at the 0.05 level. The highest protein yield of 65.30% was achieved at the 2% enzyme concentration. The four reaction times (1, 2, 3 and 4 h) were significantly different from each other at 0.05 level. The results indicate that the highest mean protein yield of 66.63% was achieved after 4 h.

5.2.1. Effect of Enzyme Concentration on Protein Yield

The effect of enzyme concentrations (0.5, 1 and 2%) at different time intervals (1, 2, 3 and 4 h) on the protein yield from different parts are shown in Figure 5.1. The results indicated that the protein yield at the 0.5% enzyme concentration was the lowest for all fish parts and the protein yield at the 2% enzyme concentration was the highest for all fish parts.

For the 0.5% enzyme concentration, the protein yield after 1 h was 45.34, 53.39, 49.67 and 40.78% for the whole fish (WF), head (H), frame (F) and fin, tail, skin and guts (FTSG), respectively. When the enzyme concentration was increased from 0.5 to 1%, the protein yield increased from 45.34 to 47.18% (4.06%), from 40.78 to 48.60% (19.19%) and from 49.67 to 53.86% (8.43%) for the whole fish (WF), fin, tail, skin and guts (FTSG) and frame (F), respectively and decreased from 53.39 to 52.41% (1.83%) for head (H). However, when the enzyme concentration was further increased from 1 to 2%, the protein yield decreased from 53.86 to 51.22% (4.89%) for frame (F), and increased from 47.18 to 54.46% (15.42%), from 48.60 to 56.70% (16.66%), from 52.41 to 55.18% (3.29%), from whole fish (WF), fin, tail, skin and guts (FTSG) and head (H), respectively. Similar trends were observed with all other reaction times (2, 3 and 4 h), increasing the enzyme concentration from 0.5 to 2.0% increased the protein yield for all fish parts.

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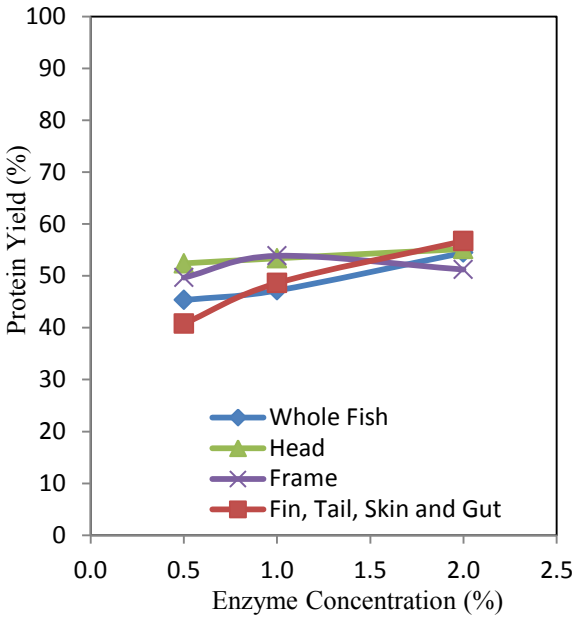
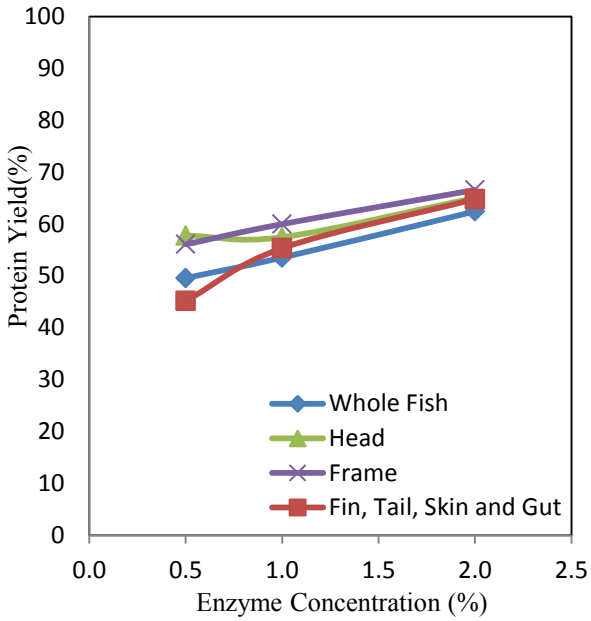
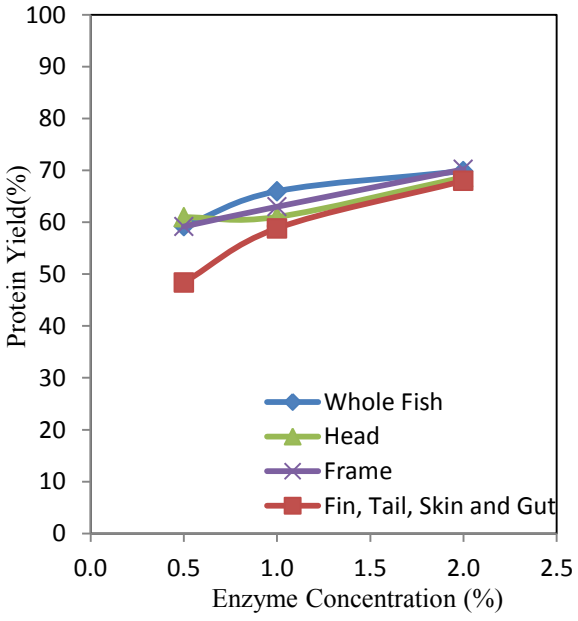


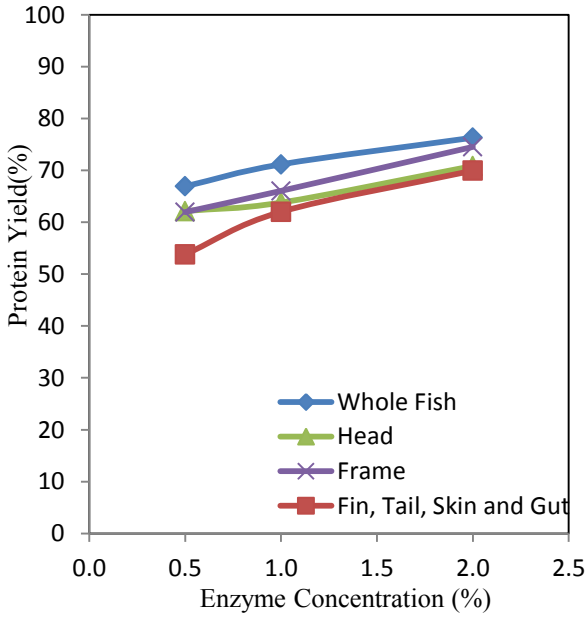
(a) 1 h



(b) 2 h



(c) 3 h



(d) 4 h

Figure 5.1. Effect of enzyme concentrations on protein yield from different fish parts at different reaction times.

5.2.2.Effect of Reaction Time on Protein Yield

The effect of reaction times (1, 2, 3 and 4 h) on the protein yield at different enzyme concentrations (0.5, 1 and 2%) is shown in Figure 5.2. The results indicated that the protein yield from the different parts was the lowest after 1 h and the highest after 4 h

For the 0.5% enzyme concentration, increase in the reaction times from 1 to 4 h, increased the protein yield from 45.34 to 66.92% (47.59%), from 40.78 to 53.80% (31.94%), from 53.39 to 62.17% (16.44%) and from 49.67 to 61.96% (24.73%) for the whole fish (WF), fin, tail, skin and gut (FTSG), head (H) and frame (F) increased, respectively. Similar trends were observed at the other enzyme concentrations. Increases of 50.82, 27.59, 21.71 and 22.64% and 40.09, 23.36, 28.39 and 45.50% for the 1% and 2% enzyme concentrations for whole fish (WF), fin, tail, skin and gut (FTSG), head (H) and frame (F), respectively.

5.3. Amino Acids Extraction

The hydrolysis of fish proteins was performed to extract amino acids by enzymatic breakdown of the peptide bonds in the proteins. The amino acids were identified by Thin Layer Chromatography and quantified by Gas Chromatography.

5.3.1. Thin Layer Chromatography

Thin layer chromatography (TLC) was performed on Silica G TLC plates. The standard amino acids were first subjected to TLC and the distance travelled by each amino acid was measured to calculate the retardation factor of each amino acid using Equation 4.1 as shown in Table 5.9. The standard amino acid chromatogram is shown in Figure 5.3. The enzymatic hydrolysis of proteins was carried out using Alcalase, Neutrase and combination of Alcalase and Neutrase for 24 and 48 h. After the hydrolysis process, the samples were first heated at 90ºC for 5 min to deactivate the enzymes and then allowed to cool down. The TLC plates were spotted with standard amino acids and samples for identification. After completing the run, the plates were dried and sprayed with ninhydrin to identify the presence of amino acids after hydrolysis Figure 5.4

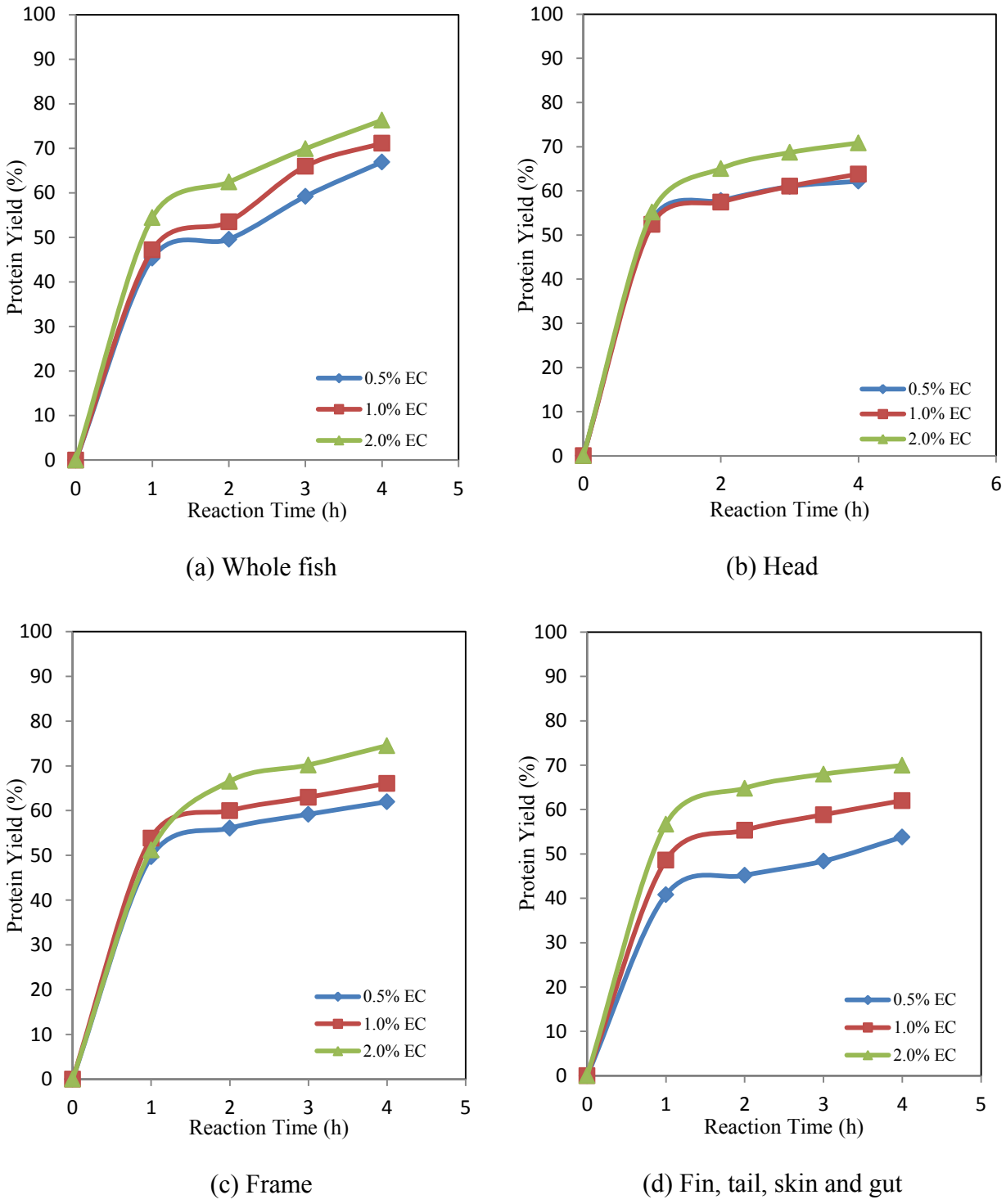


Figure 5.2. Effect of reaction time on protein yield from different fish parts at different enzyme concentrations

Table 5.9. Thin layer chromatograph of standard amino acids.

Amino acid	Distance travelled		Retardation factor
	By Spot (cm)	By Solvent (cm)	
L-Alanine	3.9	12.5	0.312
L-Arginine-Hcl	1.5	12.5	0.120
L-Aspargine	2.5	12.5	0.200
L-Aspartic acid	2.8	12.5	0.224
L-Cysteine-Hcl	2.9	12.5	0.232
L-Cystine	1.1	12.5	0.088
L-Glutamic acid	3.9	12.5	0.312
L-Glutamine	2.6	12.5	0.208
L-Glycine	3.1	12.5	0.248
L-Histidine	1.7	12.5	0.136
L-Hydroxyproline	2.9	12.5	0.232
L-Isoleucine	6.9	12.5	0.552
L-Leucine	7.2	12.5	0.576
L-Lysine	1.0	12.5	0.080
L-Methionine	5.9	12.5	0.472
L-Phenylalanine	7.0	12.5	0.560
L-Proline	2.5	12.5	0.200
L-Serine	3.2	12.5	0.256
L-Threonine	3.6	12.5	0.288
L-Tryptophan	7.0	12.5	0.560
L-Tyrosine	6.7	12.5	0.536
L-Valine	5.5	12.5	0.440

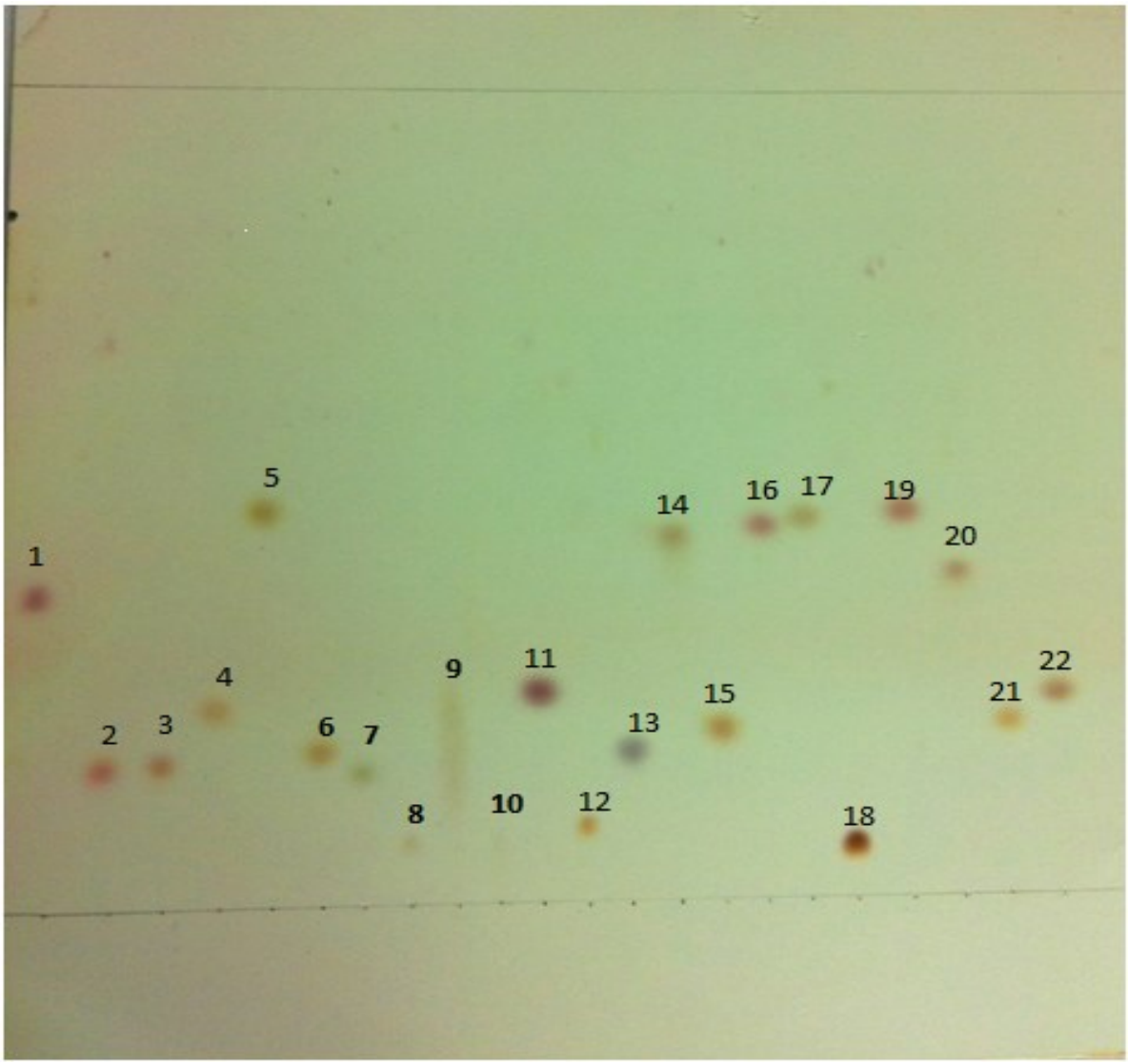


Figure 5.3. Thin layer chromatograph of standard amino acids.

1: L-Valine; 2: L-Proline; 3: L-Glutamine; 4: L-Threonine; 5: L-Tryptophan; 6: L-Hydroxy-proline; 7: L-Aspargine; 8: L-Histidine; 9: L-Cysteine-Hcl; 10: L-Cystine; 11: L-Alanine; 12: L-Arginine-Hcl; 13: L-Aspartic acid; 14: L-Tyrosine; 15: L-Glycine; 16: L-Isoleucine; 17: L-Phenylalanine; 18: L- Lysine; 19: L-Leucine; 20: L-Methionine; 21: L-Serine; 22: L-Glutamic acid

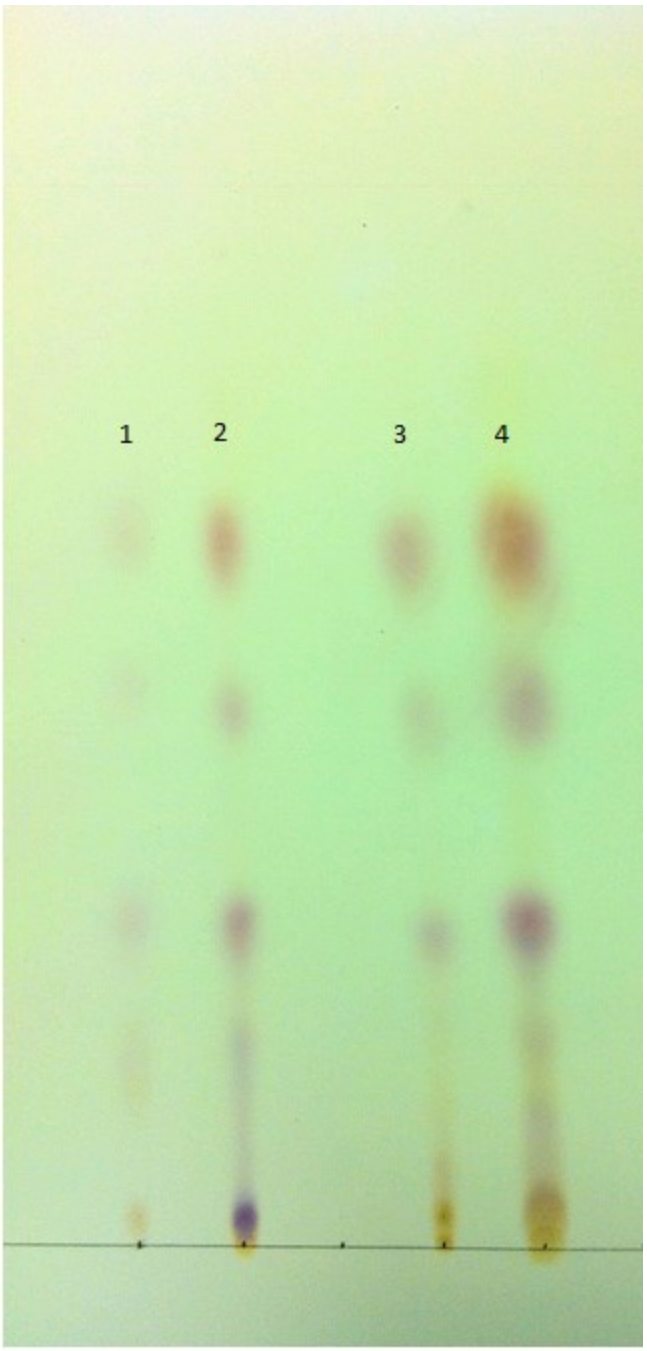


Figure 5.4. Enzymatic hydrolysis of proteins using Alcalase.

1: Standard amino acids; 2: Standard amino acids; 3: Alcalase extract after 24 h;

4: Alcalase extract 48 h.

represents a chromatogram containing standard amino acids in the first and second spots, the third spot is the sample hydrolyzed with Alcalase for 24 h and the fourth spot is the sample hydrolyzed with Alcalase for 48 h. Figures 5.5 and 5.6 represent the chromatogram for identification of amino acids after hydrolysis for 24 or 48 h with Neutrase and combination of Alcalase and Neutrase, respectively. The differentiation in the spots were not clearly seen due to the streaking and spreading of spots and single dimensional chromatography was not helpful in separation of amino acids.

5.3.2. Gas Chromatography

The amino acids were quantified using gas chromatography. The proteolytic digestion of fish proteins was conducted using Alcalase, Neutrase and combination of Alcalase and Neutrase for 24 and 48 h. At the end of hydrolysis, the samples were spray dried to obtain amino acid powder and the amino acids were subjected to derivatization for gas chromatograph analysis. The results are shown in Table 5.10. The results indicated that fourteen amino acids were quantified for the samples hydrolysed for 48 h and only ten amino acids could be quantified for the samples hydrolysed using combination of enzymes for 24 h.

Twelve amino acids were quantified individually using Alcalase for 48 h, whereas only three amino acids were quantified for the samples hydrolysed for 24 h. When the amino acids were hydrolysed using Neutrase for 48 h, ten amino acids were quantified and only three amino acids were quantified when the samples were hydrolysed for 24 h. The yield of amino acids was the highest for the samples hydrolysed using a combination of enzymes for 48 h.

5.4. Oil Yield

Oil was produced from the enzymatic extraction of fish protein as a secondary product in this study. The quantity of oil obtained after the enzymatic hydrolysis of fish parts was calculated using Equation 4.5. The amount of fat present in each part of fish was determined. The results are shown in Table 5.2 The oil yield from each part at

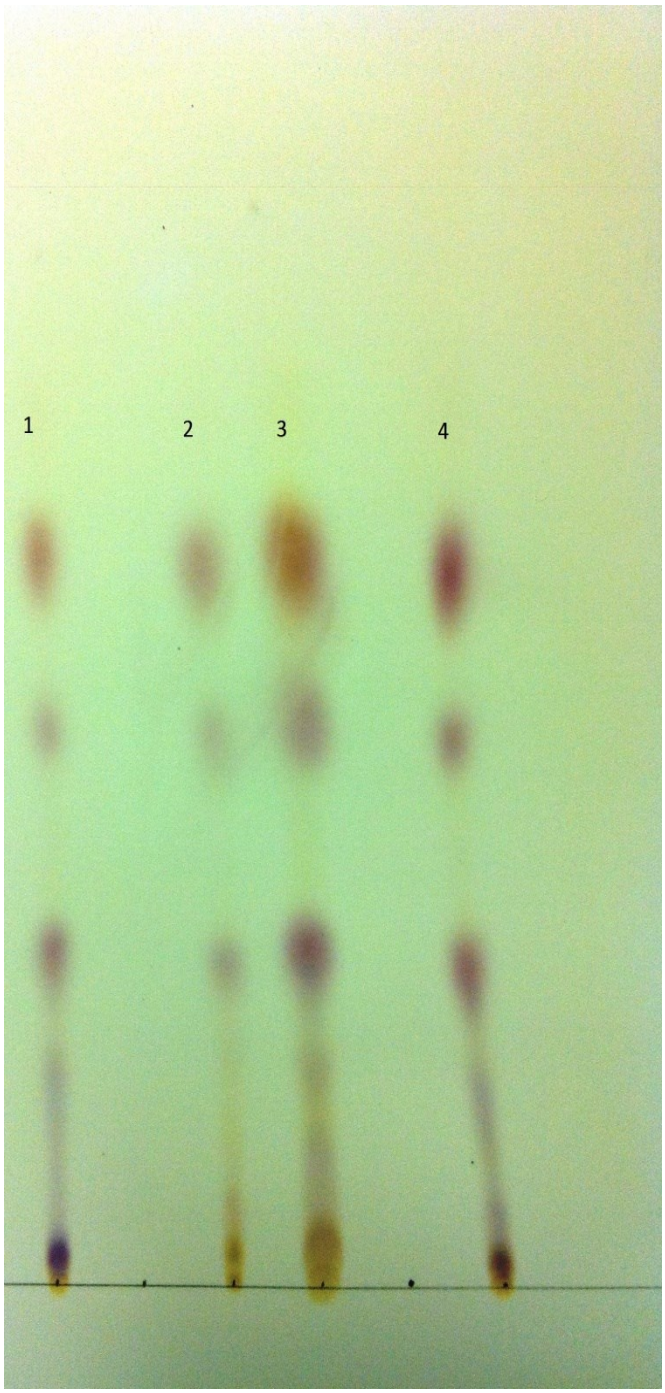


Figure 5.5. Enzymatic hydrolysis of proteins using Neutrase.

1: Standard amino acids; 2: Standard amino acids; 3: Neutrase extract after 48 h;

4: Neutrase extract 24 h.

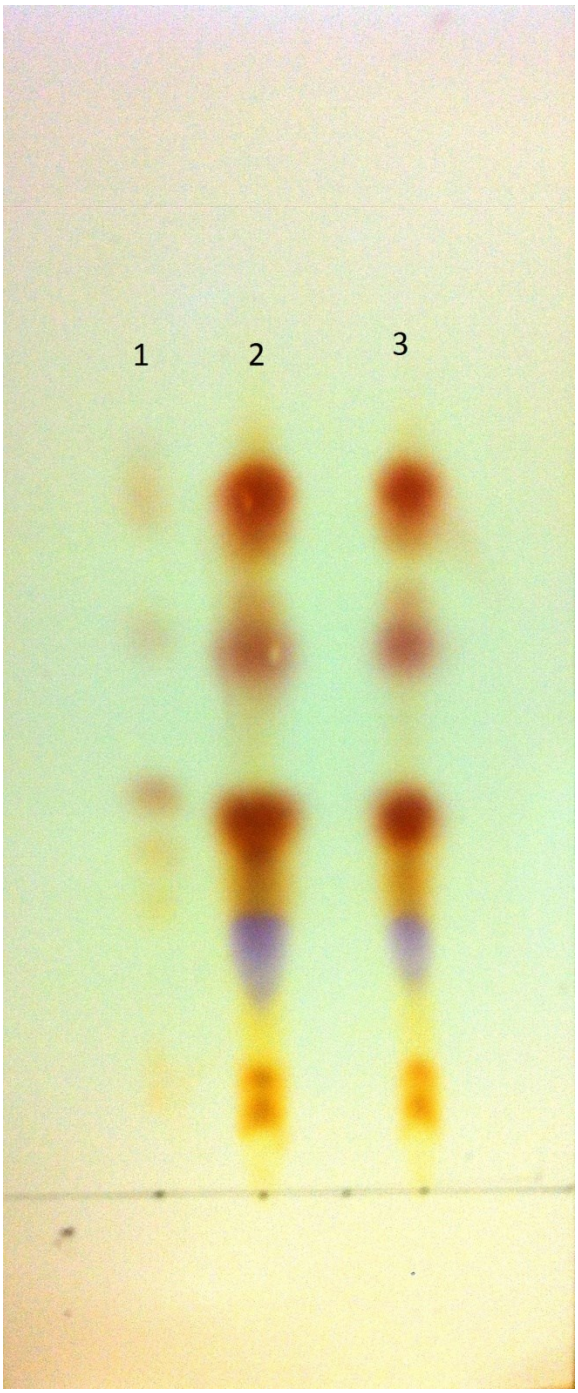


Figure 5.6. Enzymatic hydrolysis of proteins using Combination of Neutrase and Alcalase after 24 hrs

1: Standard amino acids; 2: Alcalase + Neutrase - 24 h; 3: Alcalase + Neutrase - 48 h

Table 5.10. Amino acid composition from enzymatic hydrolysis using gas chromatography.

Name	Weight Percent					
	Alcalase		Neutrase		Combination	
	24 h	48 h	24 h	48 h	24 h	48 h
Alanine	1.63±0.311	5.06±0.28	1.29±0.07	4.73±0.24	4.62±0.28	7.59±0.24
Glycine	0.00±0.00	2.99±0.48	0.00±0.00	2.35±0.29	0.00±0.00	5.82±0.37
Valine	1.74±0.24	4.04±0.33	1.43±0.29	3.67±0.38	3.10±0.30	7.20±0.30
Threonine	0.00±0.00	1.09±0.21	0.00±0.00	1.03±0.32	1.08±0.14	5.40±0.51
Serine	0.89±0.47	2.71±0.07	0.80±0.25	1.99±0.22	0.86±0.08	4.30±0.48
Leucine	0.00±0.00	4.49±0.24	0.00±0.00	3.32±0.33	2.49±0.28	9.00±0.04
Isoleucine	0.00±0.00	1.74±0.41	0.00±0.00	1.30±0.48	3.12±0.20	5.30±0.16
Proline	0.00±0.00	0.56±0.11	0.00±0.00	0.51±0.24	0.00±0.00	0.98±0.17
Phenylalanine	0.00±0.00	2.49±0.39	0.00±0.00	2.23±0.31	2.12±0.23	4.20±0.31
Methionine	0.00±0.00	1.79±0.22	0.00±0.00	0.00±0.00	1.92±0.31	2.20±0.31
Aspartic acid	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Glutamic acid	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	2.34±.0.13	9.85±0.10
Arginine	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Histidine	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	3.59±0.38
Lysine	0.00±0.00	4.36±0.28	0.00±0.00	0.00±0.00	2.32±1.41	7.34±0.23
Tyrosine	0.00±0.00	1.54±0.29	0.00±0.00	0.74±0.28	0.00±0.00	3.17±0.11

different enzyme concentrations (0.5, 1.0 or 2%) and different time intervals were calculated using Equation 4.6. The results are shown in Tables 5.11-5.14.

Analysis of variance (ANOVA) was performed on the oil yield data as shown in Table 5.15. The effects of fish parts, enzyme concentration and time were significant at the 0.001 level. The interactions among the various parameters were also significant at the 0.001 level.

The results of the Tukey's grouping are shown in Table 5.16. The various fish parts were significantly different from each other and from the whole fish except for the fin, tail, skin and gut (FTSG) which was not significantly different from the whole fish (WF) at the 0.05 level. The highest oil yield of 66.82% was obtained from the head (H). All enzyme concentrations were significantly different from each other at the 0.05 level. The highest oil yield of 65.81% was achieved with 2.0% enzyme concentration. All the reaction times were significantly different from each other at the 0.05 level. The highest oil yield of 70.79% was achieved after 4 h.

5.4.1. Effect of Enzyme Concentration on Oil Yield

The effect of enzyme concentrations on oil yield at different reaction times (1, 2, 3 and 4 h) is shown in Figure 5.7. The results showed that the highest oil yield was obtained with 2% enzyme concentration and the lowest oil yield was obtained at the 0.5% enzyme concentration.

For the 0.5% enzyme concentration, the oil yield after 1 h was 44.23, 40.12, 55.82 and 34.81% for the whole fish (WF), fin, tail, skin and guts (FTSG), frame (F) and head (H), , respectively. When the enzyme concentration was increased from 0.5% to 1%, the oil yield increased from 44.23 to 46.83% (5.89%), from 40.12 to 50.25% (25.25%), from 55.82 to 56.96% (2.04%) and from 34.81 to 37.07% (6.48%) for the whole fish (WF), fin, tail, skin and guts (FTSG), head (H) and frame (F), respectively. When enzyme concentration was further increased from 1 to 2%, the oil yield increased from 46.83 to 48.13% (2.78%), from 50.25 to 57.46% (14.36%), and from 37.07 to 56.99% (53.73%) for the whole fish (WF), fin, tail, skin and guts (FTSG) and frame (H), respectively but

Table 5.11. Oil recovery and yield from whole fish.

Enzyme Concentration (%)	Reaction Time (h)	Recovered Oil (g)	Oil Yield (%)
0.5	1	3.63±0.07	44.23
	2	4.86±0.04	58.66
	3	5.24±0.03	63.30
	4	5.52±0.05	66.59
1	1	3.87±0.05	46.83
	2	5.18±0.05	62.63
	3	5.66±0.04	68.47
	4	6.16±0.02	74.60
2	1	3.98±0.04	48.13
	2	5.04±0.07	61.22
	3	5.80±0.02	70.31
	4	6.26±0.02	75.71

Estimated Fat: 16.52%
Fish Weight: 50g

Table 5.12. Oil recovery and yield from head.

Enzyme Concentration (%)	Reaction Time (h)	Recovered Oil (g)	Oil Yield (%)
0.5	1	4.83±0.18	55.82
	2	5.39±0.15	60.81
	3	5.88±0.02	67.07
	4	6.20±0.02	70.68
1	1	4.99±0.03	56.96
	2	5.92±0.03	67.45
	3	6.26±0.02	71.46
	4	6.38±0.02	72.76
2	1	4.90±0.01	55.83
	2	6.11±0.09	70.00
	3	6.51±0.03	74.36
	4	6.68±0.02	76.26

Estimated Fat: 17.16%
Fish Weight: 50g

Table 5.13. Oil recovery and yield from frame.

Enzyme Concentration (%)	Reaction Time (h)	Recovered Oil (g)	Oil Yield (%)
0.5	1	1.82±0.03	34.81
	2	2.95±0.03	56.29
	3	3.16±0.03	60.72
	4	3.26±0.03	62.60
1	1	1.94±0.02	37.07
	2	2.96±0.03	56.97
	3	3.21±0.02	61.40
	4	3.51±0.02	67.22
2	1	2.98±0.02	56.99
	2	3.11±0.02	59.55
	3	3.38±0.02	64.94
	4	3.89±0.02	74.74

Estimated Fat: 10.43%
Fish Weight: 50g

Table 5.14. Oil recovery and yield from fin, tail, skin and guts.

Enzyme Concentration (%)	Reaction Time (h)	Recovered Oil (g)	Oil Yield (%)
0.5	1	4.20±0.07	40.12
	2	5.94±0.02	57.01
	3	6.64±0.02	63.79
	4	7.02±0.06	67.15
1	1	5.24±0.03	50.25
	2	6.64±0.02	63.72
	3	6.92±0.02	66.49
	4	7.12±0.02	68.40
2	1	5.99±0.02	57.46
	2	6.79±0.11	65.54
	3	7.21±0.01	69.19
	4	7.58±0.02	72.77

Estimated Fat: 20.84%
Fish Weight: 50g

Table 5.15. Analysis of variance for oil yield.

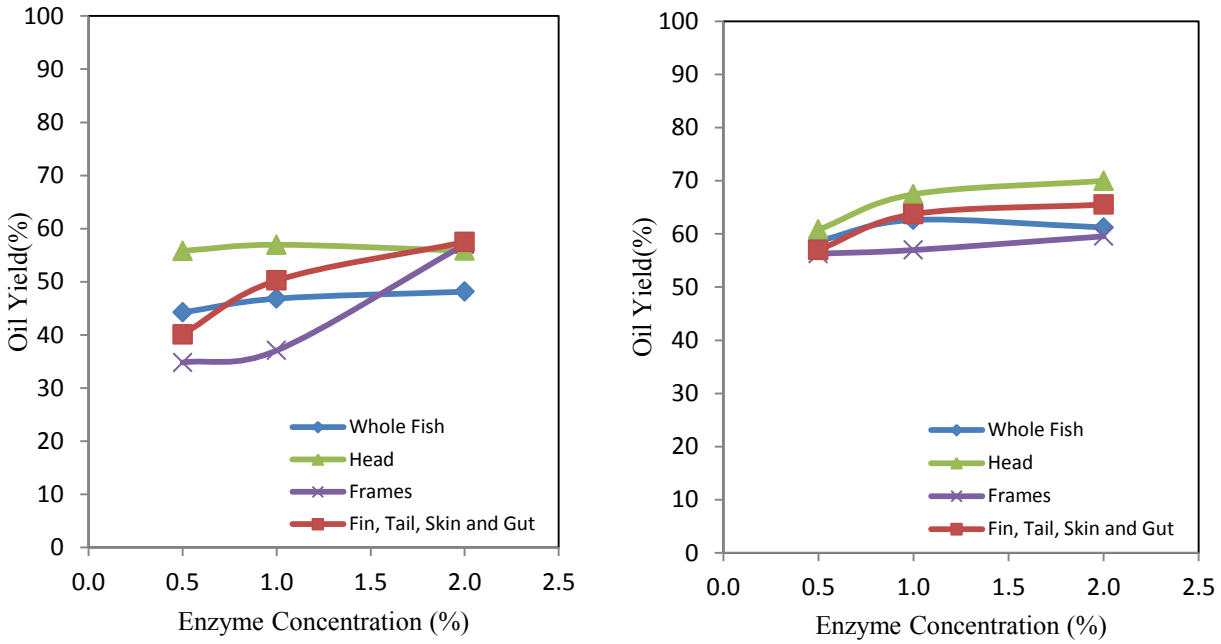
Source	DF	SS	MS	F	P
Total	143	14039.81			
Model					
Parts	3	1415.13	471.71	4397.85	0.001
EC	2	1426.68	713.34	6650.60	0.001
RT	3	9971.54	3323.85	30988.81	0.001
Parts*EC	6	236.85	39.48	368.04	0.001
Parts*RT	9	260.45	28.94	269.80	0.001
EC*RT	6	133.94	22.32	208.13	0.001
Parts*EC*RT	18	584.92	32.50	302.96	0.001
Error	96	10.30	0.11		

DF: Degree of freedom
SS: Sum of square
MS: Mean of square
EC: Enzyme concentration
RT: Reaction time
R^2: 99.89%

Table 5.16. Tukey grouping on oil yield.

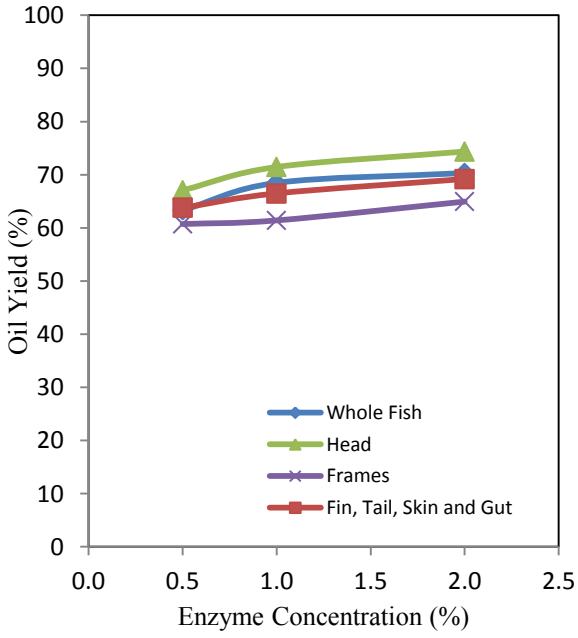
Factors	Level	N	Mean Yield (%)	Tukey Grouping
Parts	WF	36	61.72	A
	H	36	66.62	B
	F	36	57.78	C
	FTSG	36	61.82	A
Enzyme concentration (%)	0.5	48	58.10	A
	1.0	48	62.04	B
	2.0	48	65.81	C
Reaction time(h)	1	36	48.71	A
	2	36	61.65	B
	3	36	66.79	C
	4	36	70.79	D

Groups with the same letter are not significantly different from each other at the 0.05 level.
WF: Whole Fish
FTSG: Fin, tail, skin and gut
F: Frames
H: Head

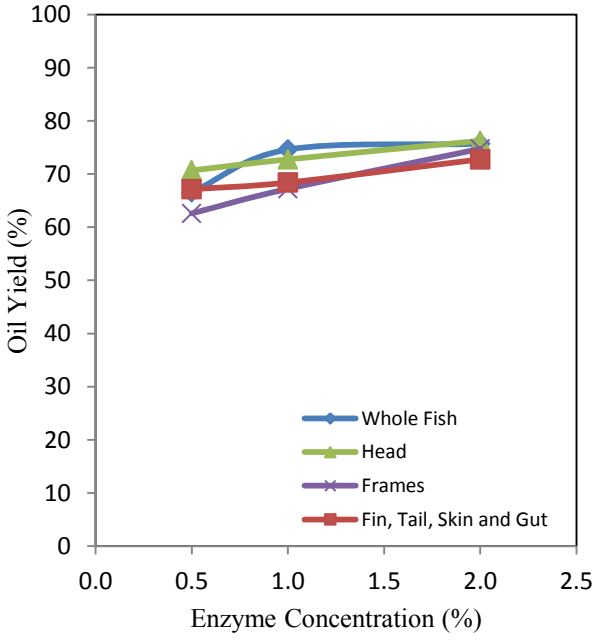


(a) 1 h

(b) 2 h



(c) 3 h



(d) 4 h

Figure 5.7. Effect of enzyme concentrations on oil yield from different fish parts at different reaction times.

oil yield of head (H) decreased from 56.96 to 55.83% (1.97%). Similar trends were observed with the other reaction times (2, 3 and 4 h) for all the fish parts.

5.4.2.Effect of Reaction Time on Oil Yield

The effect of time at different enzyme concentrations (0.5, 1 and 2%) on the oil yield from the whole fish and three different fish parts are shown in Figure 5.8. For the 0.5% enzyme concentration, when the time was increased from 1 to 4 h, the oil yield from whole fish (WF), fin, tail, skin and gut (FTSG), head (H) and frame (F) increased from 44.23 to 66.59% (50.54%), 40.12 to 67.15% (67.38%), 55.82 to 70.68 (26.62%), 34.81 to 62.60% (79.80%), respectively. There were increases of 59.32, 36.14, 27.73 and 81.32% and 57.29, 26.62, 36.57 and 31.14% for the 1 and 2% enzyme concentrations when time was increased from 1 to 4 h for whole fish (WF), fin, tail, skin and gut (FTSG), head (H) and frame (F), respectively.

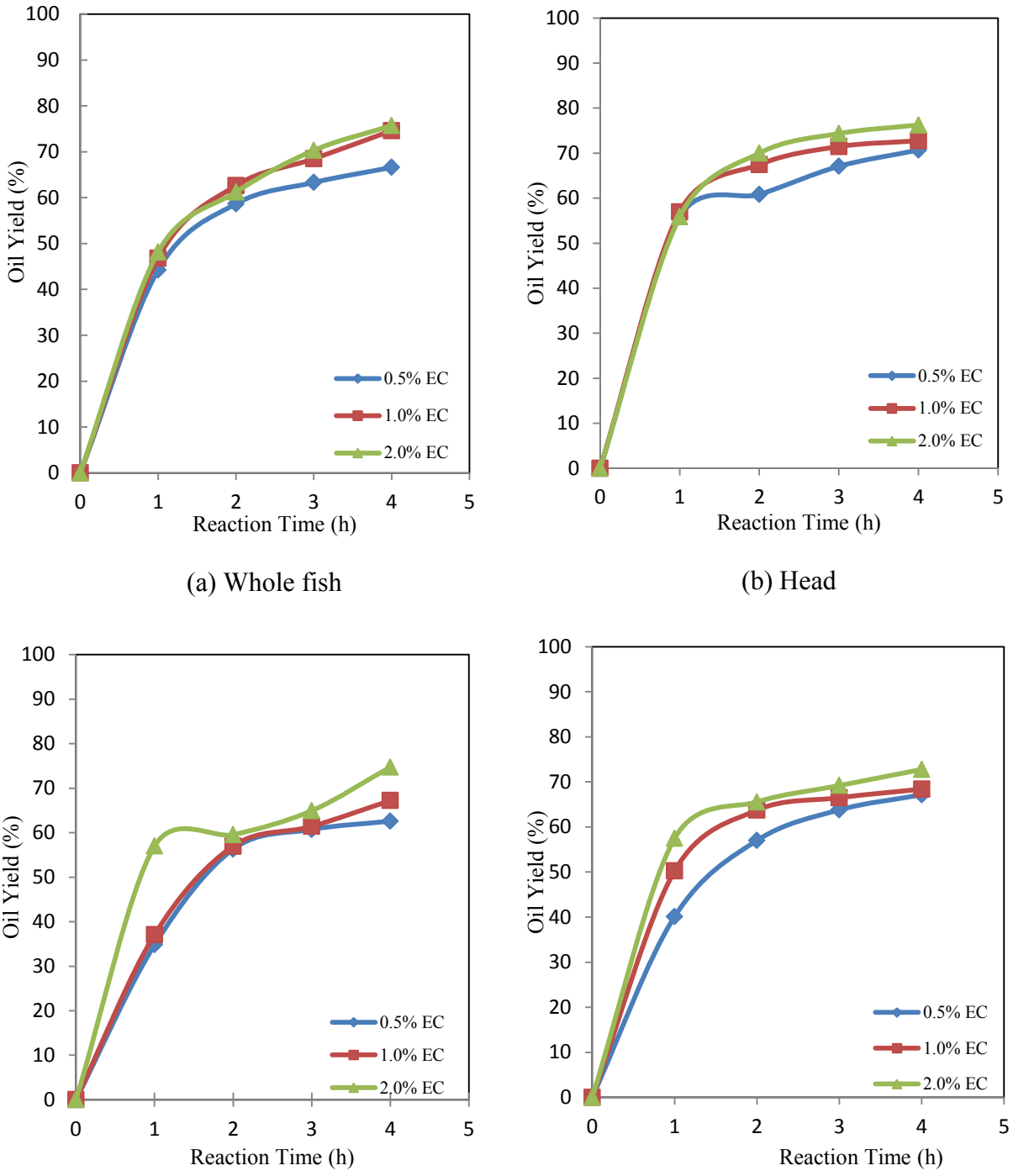


Figure 5.8. Effect of reaction time on oil yield from different fish parts at different enzyme concentrations.

CHAPTER 6. DISCUSSION

6.1. Protein Extraction

The selection of enzyme plays an important role in the extraction of proteins from fish and fish waste. Several researchers (Hoyle et al., 1994, Shahidi et al., 1995, Kristinsson et al., 2000, Guerard et al., 2001, and Gbogouri et al., 2004) reported that Alcalase was the best enzyme to be used for the extraction of proteins from fish and fish waste. Kristinsson et al. (2000) stated that Alcalase is prominently used in the hydrolysis of proteins from fish due to its high degree of hydrolysis in a relatively short time. In this study, the enzymatic hydrolysis of the whole fish and fish waste (head, frame, fin, tail, skin and gut) was carried out to obtain protein for use as a substrate for production of amino acids that can be further used for production of jadomycin. The enzyme Alcalase was used and the extraction of protein was carried out at 55°C which is the optimum temperature for Alcalase as reported by several authors (Diniz. et al., 1996, Shahidi et al., 1995, Guerard et al., 2001, Guerard et al., 2002 and Ovissipour et al., 2009). The effects of enzyme concentration (0.5, 1 or 2%) and reaction time (1, 2, 3 and 4 h) were investigated in order to determine the protein yield from whole fish and various fish parts. The highest protein yield from whole fish (WF), head (H), frame (F) and fin, tail, skin and gut (FTSG) after 4 h of hydrolysis at the 2% enzyme concentration was 76.30, 70.84, 74.53 and 69.95%, respectively. The enzyme concentration and the reaction time had significant effects on the protein yield.

6.1.1. Enzyme Concentration

When the enzyme concentration was increased from 0.5 to 1% at 1 h, the increases in protein yield were 4.06, 1.82, 8.43 and 19.19% and when the enzyme concentration was further increased from 1 to 2% at 1 h, the increases in protein yields were 15.42, 5.27, 4.89 and 16.16% for whole fish (WF), head (H), frame (F) and fin, tail, skin and gut (FTSG). Similar trends were seen for all the time intervals with all fish parts. Benjakul et al. (1997) obtained similar results and indicated that the increase in the Alcalase enzyme concentration increased the overall proteolysis rate and the solubilization of protein. Tello et al. (1994), Marquez et al. (1999) and Moreno et al. (1993) obtained similar results

from kinetic studies involving the hydrolysis of whey-proteins, bovine hemoglobin and vegetable proteins, respectively.

Guerard et al. (2002) reported that protein proteolysis can happen sequentially, releasing one peptide at a time (one by one mechanism) or by forming intermediates that are further hydrolyzed as the time progresses (zipper mechanism). In this study, during the hydrolysis of fish, there was rapid burst phase of reaction followed by a steady phase till the end of the experiment indicating that the enzymatic reaction followed the zipper mechanism. Guerard et al. (2001) reported that as long as there was plenty of substrate, the initial rate of reaction increased linearly when the enzyme concentration was increased up to 3%, after which the reaction followed the zipper mechanism as it depends upon the reaction time during extraction process. In this study, the enzyme concentration was kept below 2%. The increase in enzyme concentration (from 0.5 to 2%) increased the protein yield because more enzyme molecules became associated with fish particles thus releasing more protein molecules into the system (Kristinsson et al., 2000 ; Shahidi et al., 1995).

Srividhya et al. (2006) reported that in the zipper mechanism, the enzyme binds to the substrate and remains as a complex until all the protein is completely digested. The zipper mechanism is described as follows,

$$E + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} C_1 + X_1 \xrightarrow{k_2} C_2 + X_2 \xrightarrow{k_3} \ldots C_{n-1} + X_{n-1} \xrightarrow{k_n} X_n + E \quad (6.1)$$

Where:

E = Enzyme

S = Substrate

K_1, K_{-1}, K_2, $K_{3....}$ K_n = Rate constant

C_1, C_2...C_{n-1} = Intermediate complex

X_1, X_2...X_n = Small peptide fragment

The enzyme (E) binds with the substrate (S) to form a complex (C_1) and produces a low molecular weight fragment (X_1). In the first step of the cleavage, the enzyme is irreversibly bound, forming another complex (C_2) and small peptide fragment (X_2). The process continue until all the peptides are released and the final fragment (X_n) is produced. The rate of reactions describing the zipper mechanism are as follows,

$$\frac{dS}{dt} = -k_1 SE + k_{-1} C_1 \tag{6.2}$$

$$\frac{dC_1}{dt} = k_1 SE - k_{-1} C_1 - k_2 C_1 \tag{6.3}$$

$$\frac{dC_i}{dt} = k_i C_{i-1} - k_{i+1} C_i \qquad for\ i = 2, 3 \ldots\ldots, n \tag{6.4}$$

$$\frac{dE}{dt} = -k_1 SE + k_{-1} C_1 + k_n C_{n-1} \tag{6.5}$$

Where:

$C_1 \ldots C_i$ = Intermediate complexes for i = 2, 3...n

dS/dt = Rate of substrate utilization (g/L/min)

dC/dt = Rate of intermediate complex production (g/L/min)

dE/dt = Rate of enzyme activity (1 katal or 1 mol/s)

SE = Substrate enzyme complex

K_1, K_{-1}, K_2, $K_{3 \ldots}$ K_n = Kinetic rate constants (s^{-1})

If the reaction is carried out using ($S_0/E_0 > 1$), then the reaction must be following the zipper mechanism and it does not follow first order kinetics. The zipper mechanism follows first order kinetics only if the substrate concentration to enzyme concentration is very low ($S_0/E_0 < 1$) and the number of intermediates (n) formed also follows first order kinetics. If the substrate present in the system gets depleted following the first order kinetics it would be difficult to differentiate between the one-by-one step mechanism and zipper mechanism and the number of intermediates cannot be studied with the substrate depletion.

. Choisnard et al. (2002) reported that during pepsin hydrolysis of protein, one molecule of pepsin molecule cleaved the protein substrate once and then dissociates following one-by-one mechanism. In another case, they found one pepsin molecule cleaves the protein substrate multiple times without dissociating from it, thereby generating intermediate peptide products following zipper mechanism. They concluded that the pepsin hydrolysis can be between two phases of reaction and it is not clear whether the rate of decay of the starting substrate follows first order kinetics for both mechanisms.

Shahidi et al. (1995) reported that during the initial phase of hydrolysis, bulk soluble proteins were released and no increase in the release of soluble hydrolysates were seen when additional enzyme was added to the system during the stationary phase of the hydrolysis. This may be due to the product inhibition present during the hydrolysis or due to total cleavage of all the susceptible peptide bonds. The authors also suggested that removal of products during the hydrolysis can improve the rate and the recovery of proteins.

Gildberg (1993) reported that an increase in the enzyme concentration increased the rate of reaction. The author suggested that fish tissue is a very complex substrate and contains a large amount of proteinase inhibitors which make it difficult to explain the hydrolysis process. Kristinsson et al. (2000) stated that kinetics of the fish protein hydrolysis process is complicated due to the presence of various types of peptide bonds present and their specificity for the attack by enzymes during the process. Diniz et al. (1996) reported that once all the substrate present in the system gets attached to the active sites of enzyme, there will be free enzyme which may inhibit the hydrolysis process and may even hydrolyze itself. Therefore, increasing the enzyme concentration above 4% is not recommended and it is not cost effective.

The results showed that increasing the enzyme concentration by 400% (from 0.5 to 2%) increased the protein yield by of 3.13- 43.52% depending upon the fish part and reaction time used as shown in Table 6.1. Increasing the enzyme concentration for a small increase in protein yield may appear unjustified. Therefore, the concentration of

0.5% should be used for the protein extraction unless the enzyme is reused or an immobilized reactor is used in order to reduce the cost associated with the enzyme.

6.1.2.Reaction Time

Increasing the reaction time also increased protein yield. All the hydrolytic curves obtained at different enzyme concentrations (0.5, 1 and 2%) and different times (1, 2, 3 and 4 h) tend to have an initial rapid phase during the first 1.5 h and a phase of slow increase after the core proteins are hydrolyzed (amount of substrate in the system is decreased). Even though the protein yield slowly increased till 4 h, the percentage increases in protein yield from 3 to 4 h started to decrease from 19.43 to 13.01%, from 23.48 to 7.85% and from 11.93 to 9.19% for 0.5, 1 and 2% enzyme concentrations during whole fish (WF) hydrolysis. This trend of enzymatic hydrolysis of protein was observed with fish parts. Shahidi et al. (1995), Guerard et al. (2002) and Gbogouri et al. (2004) reported on hydrolysis studies using reaction times ranging between 1 and 5 h. Guerard et al. (2001) reported that increasing the time above 5.5 h did not cause any insignificant increase in the protein yield and stopped the hydrolysis process at 4 h.

Liaset et al. (2000) reported that during the enzymatic hydrolysis of cod using pepsin, Alcalase and Neutrase, the reaction mechanism followed two first order kinetics processes, in which the first process involves an initial fast reaction in which loosely bound polypeptide chains were cleaved to form an insoluble protein particle and in the second process the compact proteins were digested. The author also suggested that this mechanism of slow reaction at the end may be due to decrease in enzyme activity, substrate saturation or product inhibition.

Gbogouri et al. (2004) observed same phenomenon when using Alcalase to extract proteins from salmon byproducts. Guerard et al. (2002) compared umamizyme with Alcalase during hydrolysis of tuna waste and observed the regular decrease of the hydrolysis curves suggesting an enzyme deactivation or enzyme inhibition and lack of peptides bond for hydrolysis.

The results showed that increasing the reaction time by 400% (from 1 to 4 h)

Table 6.1. The increase in protein yield as a result of increase in enzyme concentration from 0.5 to 2%.

Parts	Time (h)	Increase in Protein Yield (%)
Whole fish	1	20.11
	2	25.91
	3	18.00
	4	14.01
Head	1	3.35
	2	12.57
	3	12.62
	4	13.95
Frame	1	3.13
	2	18.72
	3	18.62
	4	20.29
Fins, Tail, Skin and Gut	1	39.04
	2	43.52
	3	40.50
	4	30.01

increased the protein yield by 16.45 - 50.82% depending upon the fish part and enzyme concentration used as shown in Table 6.2. Increasing the time for a small increase in protein yield will increase the capital and operating costs of production. A shorter reaction time will allow more throughput and/or reduce the volume of the reactor thereby reducing the cost of protein extraction. Therefore, a 1 h reaction time for protein extraction is recommended.

6.2.Amino acid extraction

Enzymatic hydrolysis of protein was carried out to extract amino acids for the production of jadomycin. Jadomycin is an antibiotic produced from *Streptomyces venezuelae* in three steps: growth, shocking and production. In the growth step, the *Streptomyces venezuelae* is grown in nutrient enriched maltose-yeast extract medium (MYM). In the shocking step, the grown inoculum is transferred to a nutrient deprived amino acid rich production media and shocked with 3% (v/v) ethanol. In the production step, the production of jadomycin is induced after shocking and amino acids are incorporated within the oxazolone ring structure. The presence of amino acids is an important ingredient for jadomycin production. Twelve types of jadomycin can be produced in the presence of different amino acids including alanine, glycine, histidine, isoleucine, leucine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine and valine

In this study, fourteen amino acids were identified and quantified after hydrolyzing the protein samples using a combination of Alcalase and Neutrase enzymes for 48 h. High values of all the amino acids were obtained. Alcalase (endopeptidase and serine protease) and Neutrase (metallo endoprotease) was able to attack the protein backbone and cleave the peptide bonds to release amino acids. However, these enzyme were not able to cleave some amino acids such as arginine and aspartic acid. The yield of proline was also low which is similar to those reported by Church et al. (1984) and Hill et al. (1962).

Hill et al. (1962) hydrolyzed proteins using papain, carboxypeptidase, enolase,

Table 6.2. The increase in protein yield as a result of increase in reaction time from 1 to 4h.

Parts	Enzyme concentration (%)	Increase in Protein Yield (%)
Whole Fish	0.5	47.59
	1	50.82
	2	40.09
Head	0.5	16.45
	1	21.72
	2	28.39
Frame	0.5	22.64
	1	24.74
	2	45.50
Fins, Tail, Skin and Gut	0.5	31.95
	1	27.59
	2	23.36

oxidized ribonuclease, leucine aminopeptidase, prolidase and α-corticotropin. Their results indicated that proteolytic hydrolysis of proteins depended upon the properties and type of enzyme used in the process. Papain being an endopeptidase degraded most of the proteins to yield amino acids. Leucine aminopeptidase is one enzyme which can degrade proteins that do not contain proline and prolidase is capable of completely hydrolyzing proline. However, when carboxypeptidase was used together with aminopeptidase and prolidase it did not have any significant effect. In this study, the results obtained using a combination of these enzymes were closely comparable to the yields from acid hydrolysis.

D'Aniello et al. (1993) hydrolysed proteins using 6 M HCl at 37°C for 24 h or at 90°C for 15 min followed by hydrolysis using pronase enzyme at 50°C for 24 h. The results suggested that during partial chemical hydrolysis step only 4-8% of proteins were hydrolysed into peptones, peptides and amino acids. The main advantage of carrying out the procedure at 37°C was that no racemization of amino acids occurs and when the temperature was ramped up to 50°C for enzymatic hydrolysis using pronase the rate of enzymatic hydrolysis was increased and the enzyme did not get deactivated as 90% hydrolysis of proteins was achieved. In the present study, the enzymes were not able to cleave two amino acids (cystine and aspartic acid) due to the initial acid hydrolysis process, after which the enzyme (pronase) was able to act upon the loose peptide linkages to release amino acids into the system in a shorter period of time.

Church et al. (1984) hydrolysed proteins using immobilized pronase, proteinase K, carboxypeptidase A and B, aminopeptidase M, intestinal mucosa exopeptidase and prolidase for 18-24 h to achieve complete hydrolysis of proteins. The results suggested that use of enzymatic hydrolysis provided an alternative method to hydrolyze proteins without addition of acid or alkali and it does not cause racemization of amino acids. Some disadvantages of enzymatic hydrolysis includes multiple pH and temperature changes if hydrolysis is extended more than 24 h. The results indicated that the rate of hydrolysis of pronase individually from 0-4 h were similar to the rate of combined exopeptidases, endopeptidase and neutral enzymes at the same time interval, but when time was increased from 4-24 h the rate of hydrolysis drastically increased for the

combination of enzymes with 90% hydrolysis of proteins. The report also indicated that cysteine, cystine, arginine, proline, glutamine and aspargine bonds were most resistant towards proteolytic cleavage. In the present study, the enzymes were unable to cleave cystine and arginine and the yield of proline was low.

Bujard et al. (1966), Bieleski et al. (1966), Jones et al. (1966), Ali et al. (1982) and Matysik et al. (1986) stated that thin layer chromatography is a simple and rapid analytical method for the identification of amino acids. It is a qualitative analysis of amino acids in which individual amino acids cannot be well differentiated due to streaking and spreading of spots and the Rf values are not reproducible. In this study, the amino acids extracted from fish protein using two enzymes Alcalase and Neutrase (individually and together in combination) were identified using single dimensional thin layer chromatography. The results suggested that changing the solvent system and carrying out two dimensional thin layer chromatography can help in defining the individual amino acids present in the sample.

Stroud (1974) reported that the bond cut by the serine proteases is the one that joins amino acids together to form proteins. Each amino acid is composed of an amino group (NH_2) and a carboxyl group (COOH) attached to a single alpha carbon atom. The carbon atom is also attached to a hydrogen atom and one of the twenty side chains, by which the amino acids are identified. During synthesis of proteins, the carboxyl group of one amino acid is connected to the next amino group by the extraction of water molecule to form a linkage -CO-NH-.(peptide bond). During the hydrolysis of proteins, a water molecule consisting of a hydrogen atom and a hydroxyl group is added for each peptide bond broken, thereby restoring the amino and carboxyl group at the site of cleavage to their free amino acid form. In this study, Alcalase being a serine protease belonging to subtilisin family and Neutrase being a metallo endoprotease, contains three residues critical for catalysis: a serine, a histidine and an aspartic acid (Mansfeld, 2007 and Rawlings et al., 2007). These three residues are important to break peptide bond in the protein backbone to release amino acid in the system. The specific mechanism for Alcalase and Neutrase breaking the peptide bond has not been reported in the literature. However, Bachovchin et al. (1978) and Brandt. (2011) reported a mechanism for

chymotrypsin which is also a serine protease containing three residues including: a serine, a histidine and an aspartic acid for the catalysis of peptide bond. Therefore, both Alcalase and Neutrase are suggested to follow the same mechanism.

Marquez et al. (1999) and Sousa et al. (2004) reported that there are four steps involved during the general proteolytic cleavage of proteins which include substrate binding, opening of the peptide bond, proton exchange and titration of amino group. During the hydrolysis of the proteins, the serine 195 pivots approach and attack the carbonyl carbon of the substrate (-CO-) group and at the same time the hydrogen ion or proton of the hydroxyl group is transferred to the nearby histidine 57, and the serine hydroxyl oxygen forms a covalent bond with the carbonyl carbon. As a result, the double bond connecting the carbon and oxygen in the substrate carbonyl group is transformed into a single bond. During protonation, the proton is delivered to histidine 57 and remains there for a short time and then gets transferred to the -NH- group in the substrate on the other side of the peptide bond that is being broken. In the final step, water attacks the ester bond between the peptide and the serine 195 oxygen and forms the second product peptide with normal carboxyl group and regenerated the serine hydroxyl group. The second peptide is then dissociated from the enzyme to allow another catalytic cycle to begin. The whole hydrolytic process is shown Figure 6.1.

6.3.Oil Extraction

During the enzymatic extraction of protein from the whole fish and fish waste (head, frame, fin, tail, skin and gut), oil was obtained as the by-product. The oil obtained was dark in color due to presence of products released from hemoglobin degradation as reported by Batista et al. (2009). The oil obtained can be utilized for the production of biodiesel in a two step process in which the oil is pretreated with 1% sulfuric acid followed by transesterification using methanol and potassium hydroxide as a catalyst (El-Mashad et al., 2008). Shimada et al. (2002) reported on the extraction of biodiesel by two and three step ethanolysis processes. The report indicated that the conversion yield of biodiesel from the oxidized fatty acid compounds is lower than that of unoxidised oil. Lin et al. (2010) reported that the biodiesel produced from crude fish oil had a lower

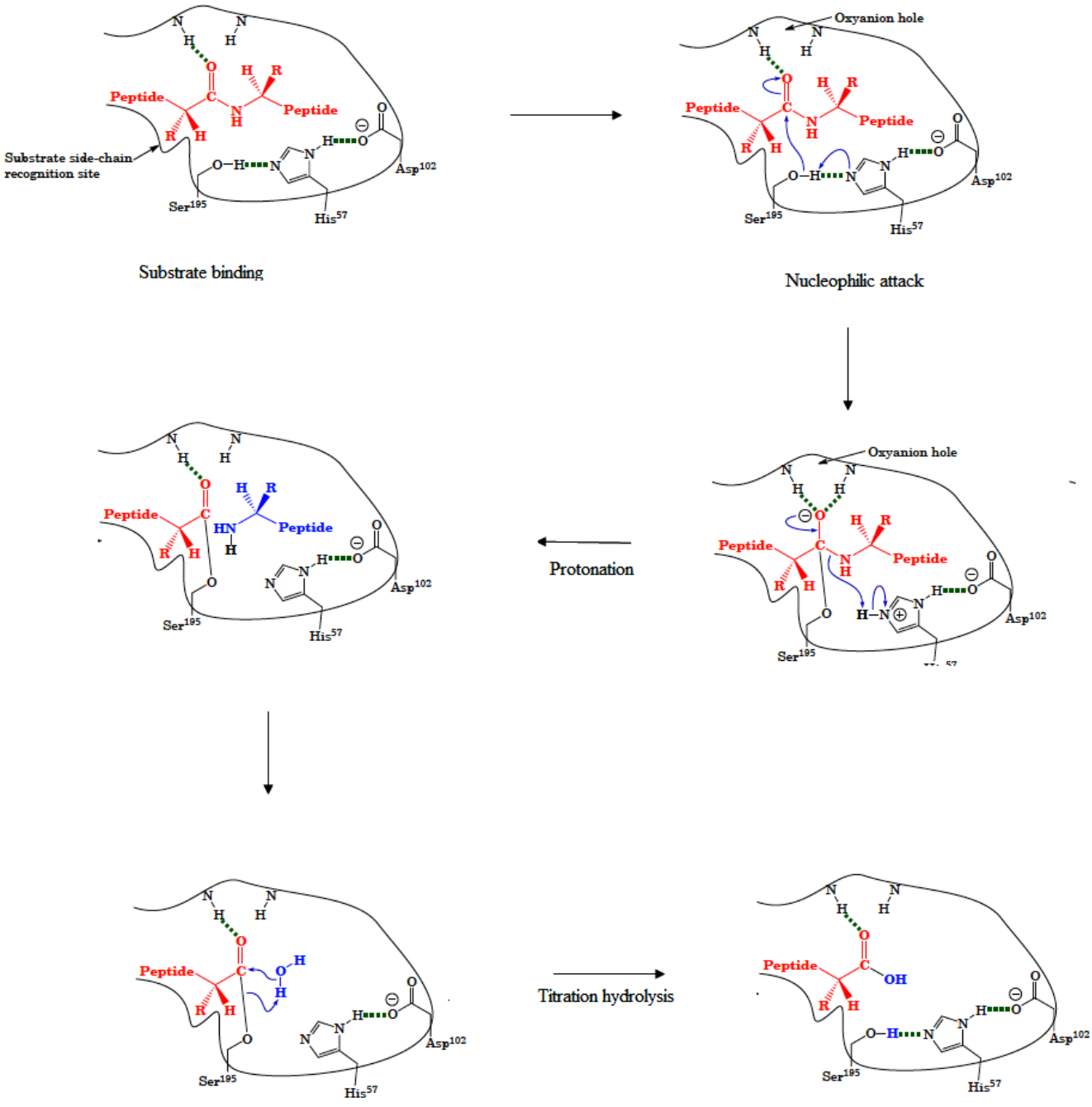


Figure 6.1. Catalytic mechanism of serine proteases (Bachovchin et al., 1978; Brandt, 2011).

oxidative stability and the addition of a combination of antioxidants including 0.1% butylated hydroxytoluene (BHT) and 0.1% butylated hydroxyanisole (BHA) to the oil increased the oxidative stability of biodiesel, its kinematic viscosity and carbon residue at the beginning of the storage period.

Fish oil can be extracted by various means such as hexane extraction, supercritical fluid extraction and enzymatic extraction. The enzymatic extraction of oil uses commercially available proteases (Linder et al., 2005). The type of enzyme, enzyme concentration and reaction time play important roles in the quality and recovery of oil from the fish (Mbatia et al., 2010; Hathwar et al., 2011). In this study, the fish oil was extracted from mackerel whole fish (WF), head (H), frame (F) and fin, tail, skin and guts (FTSG) using different concentrations of Alcalase enzyme (0.5, 1 and 2%) at 55°C and different reaction times (1, 2, 3 and 4 h). The highest oil yield was achieved after 4 h of hydrolysis and 2% enzyme concentration from whole fish (WF), fin, tail, skin and gut (FTSG), head (H) and frame (F) were 75.71, 72.77, 76.26 and 74.74%, respectively. The enzyme concentration and the reaction time had significant effects on the oil yield.

6.3.1.Enzyme Concentration

Increasing in the enzyme concentration from 0.5 to 2% increased the oil yield. Mbatia et al. (2010) stated that increasing enzyme concentration increases the rate of hydrolysis but may not significantly increase the oil yield due to the limitation of substrate availability for the enzyme to bind. In this study, the highest oil yield was obtained at 2% enzyme concentration. Previous studies (Mbatia et al., 2010; Linder et al., 2005; Slizyte et al., 2005a; Slizyte et al., 2005b; Dauskas et al., 2005) have used enzyme concentrations ranging between 0.05 and 2% and indicated that increasing the enzyme concentration more than 1% was insignificant for the oil yield and therefore the enzyme concentration is not increased more than 2%.

Mbatia et al. (2010) used 0.5% bromelain and 0.5%protex to extract oil from nile perch and salmon heads at 55°C. The results indicated that a maximum oil yield of 11.6 g/100g and 15.7 g/100g was achieved for bromelain and protex, respectively . The oil recovered from the available total lipids in salmon heads and nile perch using bromelain

and protex were 65% and 88% and 81% and 81%, respectively. The study also suggested that increasing the enzyme concentration increased the hydrolysis rate but did not increase the oil yield from fish. Linder et al. (2005) used three different enzymes Neutrase, Alcalase and Flavourzyme at a concentration of 0.05% and three temperatures (45, 55 and 50°C) for 2 h to extract oil from salmon heads. The oil yield using Neutrase, Flavourzyme and Alcalase were 17.2, 17.0 and 17.4%, respectively. The oil yield from this current study was slightly less than the yield reported by Mbatia et al. (2010) and Linder et al. (2005). This is due to the addition of buffer during the hydrolysis which affected the oil yield.

6.3.2. Reaction Time

In this study, the highest oil yield was obtained after 4 h. Even though the oil yield slowly increased till 4 h, the percentage increases in oil yield from 3 to 4 h started to decrease from 7.91 to 5.18%, from 9.32 to 8.95% and from 14.85 to 7.67% for 0.5, 1 and 2% enzyme concentrations during whole fish (WF) hydrolysis. Mbatia et al. (2010) and Linder et al. (2005) obtained the highest oil yield at a hydrolysis time of 4 h and reported that increasing the time from 4 to 14 h did not improve the oil yield and changed the oil color to brown due to the formation of brown pigments from the reaction of carbonyls (aldehydes) produced from the oxidation of polyunsaturated fatty acids (PUFA) with amino acids and proteins. The tissue hydrolysis achieved between 1-4 h was sufficient to release lipids. The reports suggested that the decrease in oil yield after 2 h could have been due to interaction of more lipids with hydrolyzed proteins when higher amounts of water was added to the reaction. Slizyte et al. (2005a), Slizyte et al. (2005b), Linder et al. (2005) and Mbatia et al. (2010) reported that the optimum hydrolysis time for oil extraction was 2 h.

6.4. Extraction Variables

After hydrolysis and centrifugation, four layers were observed: upper oil layer, light-lipid layer, soluble clear protein layer and bottom sludge layer. Similar results were reported by Spinelli et al. (1982) and Gildberg (1992). The protein hydrolysate was spray dried to obtained protein powder to determine the protein yield as described by Hoyle et

al. (1994), Nilsang et al. (2004) and Bhaskar et al. (2008). The amount of protein recovered depended upon the amount of protein present in the raw material and the hydrolysis conditions (enzyme concentration and time). The protein and oil recovery from whole fish (WF), fin, tail, skin and gut (FTSG), head (H) and frame (F) increased with increases in the enzyme concentration (from 0.5% to 2.0%) and the reaction time (from 1 to 4 h). The results indicated that the highest protein yield was obtained from whole fish (WF). The amount of protein obtained from fish waste (frames (F), head (H) and fin, tail, skin and gut (FTSG)) was lower than the whole fish (WF). However, fish wastes (together) can be utilized for the extraction of proteins without any segregation of fish waste parts during fish processing. The recommended extraction parameters for the protein extraction from mackerel fish waste are shown in Table 6.3.

Response surface methodology is a collection of statistical and mathematical techniques used for developing, improving and optimizing process. Diniz et al. (1996) used response surface methodology to describe the optimum conditions for the protein extraction from dog fish (*Squalus acanthias*) using Alcalase. The results indicated that pH range of 8-8.5, a temperature range of 50-60°C and enzyme concentration of 3.6% were optimal for Alcalase-assisted hydrolysis. Gbogouri et al. (2004) hydrolyzed salmon and reported a protein recovery of 71.0% at a 5.5% enzyme concentration, a pH of 8 and a temperature of 58°C after 2 h, which is lesser than the protein yield (76.30%) achieved at 2% enzyme concentration in this study. This agrees with the findings of other researchers (Guerard et al., 2001; Diniz et al., 1996). Guerard et al. (2001) extracted proteins from yellowfin tuna (*Thunnus albacares*) using Alcalase (0.2-3% w/w) at a pH of 8.0 and a temperature of 55°C for 6 h and obtained the highest protein recovery at 3% enzyme concentration. However, increasing the enzyme concentration beyond 1% slightly increased the protein yield.

Beaulieu et al. (2009) extracted proteins from Atlantic mackerel (*Scomber scombrus*) using protamex under optimum conditions (0.001% enzyme concentration, a pH of 8 and a temperature of 40°C for 120 min) and achieved 77.8% recovery, which is similar to the protein recovery (76.30%) achieved from whole fish in this study.

Table 6.3. Effective parameters for protein extraction from fish and fish waste.

Factors	Parameters
Enzyme	Alcalase
Enzyme concentration	0.5 %
Reaction Time	1 h
pH	7.5
Temperature	55°C

Shahidi et al. (1995) extracted proteins from capelin (*Mallotus villosus*) fish using four different enzymes including Alcalase (1.05% (w/w)), Neutrase (1.05% (w/w)), papain (0.14% (w/w)) and endogenous enzymes. The results indicated that the capelin treated with Alcalase gave a superior protein recovery of 70.6% after 120 min of hydrolysis compared to those of 51.6, 57.1 and 22.9% from Neutrase, papain and endogenous enzymes, respectively and is less than the protein yield (76.30%) achieved at 2% enzyme concentration but it is similar to the protein yield (71.16%) achieved at 1% enzyme concentration from whole fish in this study.

Vieira et al. (1995) reported a protein recovery yield of 61.9, 44.9 and 70.1% using papain, pepsin and fungal protease at 0.5% from Brazilian lobster heads after 5 h of hydrolysis. Bhaskar et al. (2008) extracted proteins from visceral waste of catla (*Catla catla*) using Alcalase under optimal condition (enzyme concentration of 1.5%, a pH of 8.5,a temperature of 50°C for 135 min) and achieved a protein yield of 71.54%. Bhaskar et al. (2008b) extracted proteins from visceral waste of catla (*Catla catla*) using multifect-neural under optimal condition (enzyme concentration of 1.25%, temperature of 55°C for 165 min) and achieved a protein yield of 70.54%. Holanda et al. (2006) recovered protein from shrimp processing waste using Alcalase and pancreatin (enzyme concentration of 3%, a temperature of 60°C for 30 min) and achieved a protein yield of 59.50 and 50.55%, respectively. Alcalase also recovered 18% more proteins than pancreatin. The protein yields from these studies are similar to the protein yield achieved at 2% enzyme concentration in this study.

Ovissipour et al. (2009) reported that the time and temperature plays an important role in determining the protein yield using Alcalase from Persian sturgeon. The results indicated that when the time was increased from 30 to 205 min, the protein recovery increased by 9.91% and the yield was 38.38% at 35°C. When the temperature increased from 35 to 55°C, the protein recovery increased by 61.43% and the protein recovery was 61.96% at 205 min, indicating the highest protein yield was obtained at 55°C and 205 min which is lesser than the protein recovery (76.30%) achieved at 2% enzyme concentration from whole fish in this study.

The maximum protein recovery was obtained at 4 h and 55°C for whole fish (WF), fin, tail, skin and guts (FTSG), head (H), and frames (F) are similar to those reported by Guerard et al. (2001) and Kristinsson et al. (2000). The highest protein yield (76.30%) from whole fish (WF) at 2% enzyme concentration after 4 h of hydrolysis was superior to those reported by Gbogouri et al. (2004), Ovissipour et al. (2009) and Shahidi et al. (1995). The protein yield at 2% enzyme concentration and 4 h hydrolysis from fin, tail, skin and guts (FTSG), head (H) and frame (F) were similar to those reported by Vieira et al. (1995), Bhaskar et al. (2008), Bhaskar et al. (2008b) and superior to those report by Holanda et al. (2006).

Dauskas et al. (2005) reported maximum oil recovery of 82.8% from cod viscera without digestive tract using flavourzyme enzyme. The lowest oil recovery of 36.4% was achieved by using Neutrase enzyme on viscera with backbone. The authors suggested that at the end of hydrolysis lipids were formed in three forms: free oil, emulsion and sludge. The formation of emulsion is not desirable and increase in the amount of emulsion decreases the amount of free oil produced. The study suggested that addition of water during hydrolysis increased the formation of emulsion and decreased the production of free oil. In this study the highest oil yield (76.26%) was achieved from head and it was less than the oil yield reported by the author which is due to the addition of buffer during the hydrolysis process.

Slizyte et al. (2005a) used flavourzyme and Neutrase to extract oil from cod and reported that the decrease in the amount of free oil fraction can be attributed to the presence of large amounts of proteins in the raw material (digestive tracts, flesh and backbones) which together with the oil present in the liver forms various complexes when heated during thermal deactivation of endogenous enzymes. During heat inactivation, the proteins in the raw material were denatured and precipitated. Only a small portion of denatured proteins can be solubilised and the remaining forms a lipid-protein complex which eventually reduce the release of lipids into the oil fraction. The report also suggested that the minimum amount of lipids in the raw material should be more than 8.5 g/100 g to form an emulsion and to decrease the formation of emulsion the amount of protein must be higher than 16.5 g/100 g.

Slizyte et al. (2005b) used Alcalase and lecitase ultra to extract lipids from cod by-products and found that heating to inactivate the endogenous enzyme affected the oil yield. The type of treatment, initial heating of raw material and addition of water to the raw material played a significant role in determining the amount of oil and emulsion. The results indicate that the highest amount of oil was obtained from hydrolysis using Alcalase (after initial heating and without addition of water) lowered the emulsifying properties of fish protein. The report also suggested that Alcalase was the best enzyme for oil extraction.

In this study, buffer was added during the extraction process which played an important role for both protein and oil yield. Fish and fish waste contains 60-70% of water and further dilution with addition of buffer attributed to a decrease in the oil yield as reported by Mbatia et al. (2010). That addition of water increased the recovery of soluble proteins and it was difficult to obtain maximum soluble proteins and lipids under same hydrolysis conditions. The effects of addition of water and initial heating from the previous studies of Dauskas et al. (2005), Slizyte et al. (2005a) and Slizyte et al. (2005b) also suggest the same phenomenon on the effects of addition of water and initial heating.

6.5. Jadomycin Production

The aim of this study was to extract amino acids for use in jadomycin production. Twelve jadomycins can be produced from amino acids as shown in Table 6.4. Burdock et al. (2008) reported that a concentration of 4.5 g/L (0.45%) of individual amino acid is required for the production of jadomycin. Out of fourteen amino acids produced, twelve amino acids were present in relatively reasonable concentrations suitable of producing jadomycin. These were alanine (7.59%), glycine (5.82%), histidine (3.59%), isoleucine (5.30%), leucine (9%), lysine (7.34%), methionine (2.2%), phenylalanine (4.2%), serine(4.3%), threonine (5.40%), tyrosine (3.17%) and valine (7.2%). In this study, tryptophan which is suitable for producing a jadomycin was not present in the fish protein. Leu et al. (1981) extracted sixteen amino acids from the Atlantic Mackerel (*Scomber scombrus*) and reported that the amino acid tryptophan was not present in the fish. Doull et al. (1994) reported that when glutamic acid was used in the production

Table 6.4. Jadomycin produced by *S.venezuelae* ISP5230 by ethanol shock (Borissow et al., 2007; Fan et al., 2012).

Suitable Amino acids for Jadomycin Production	Jadomycin	Extracted Amino Acids	
		(wt %)	Suitability
Alanine	Jadomycin Ala	7.59	✓
Glycine	Jadomycin G	5.82	✓
Histidine	Jadomycin H	3.59	✓
Isoleucine	Jadomycin B	5.30	✓
Leucine	Jadomycin L	9.00	✓
Lysine	Jadomycin K	7.34	✓
Methionine	Jadomycin M	2.20	✓
Phenylalanine	Jadomycin F	4.20	✓
Serine	Jadomycin S	4.30	✓
Threonine	Jadomycin T	5.40	✓
Tryptophan	Jadomycin W	0.00	×
Tyrosine	Jadomycin Y	3.17	✓
Valine	Jadomycin V	7.20	✓

* A minimum concentration of 0.45% was considered for economic reasons.

medium, the color of the medium was orange and lysine indicating the possibility of jadomycin production. No other reports were found in the literature on jadomycin production from glutamic acid. Therefore, the possibility of producing jadomycins from glutamic acid (9.85%), and proline (0.98%) which were present in the fish protein should be investigated.

Doull et al. (1994), Jakeman et al. (2006), Borissow et al. (2007) and Burdock et al. (2008) produced several jadomycins using *Streptomyces venezuelae* ISP5230 with individual amino acids in the production medium. No reports were found in the literature to suggest the use of more than one amino acid in the same production medium. Therefore, the amino acids extracted from fish protein in this study have to be separated and purified before use for the production of individual jadomycins.

CHAPTER 7. CONCLUSIONS

The effect of enzyme concentration (0.5, 1 and 2%) and time (1, 2, 3 and 4 h) on the extraction of proteins from the whole fish (WF) and fish waste (head (H), frame (F), fin, tail, skin and gut (FTSG)) were studied. Oil was produced as a byproduct. The effect of two types of enzymes (Alcalase and Neutrase) individually and together in combination and time (24 and 48 h) on the extraction of amino acids from fish protein were studied. The following are the conclusions obtained from the study.

1. The protein yield increased with increases in enzyme concentration from (0.5 to 2%) for the whole fish and fish waste parts because the enzyme molecules become associated with the fish particles, thereby releasing more protein molecules into the system.
   (a) The effect of enzyme concentration on protein yield was significant at the 0.05 level and there were significant interactions between the enzyme concentration and parts.
   (b) The differences in protein yield among the fish parts (whole fish, head, frames, fin, tail, skin and gut) was significant at the 0.05 level.
   (c) The highest protein yield was obtained from whole fish (WF).
   (d) Among the fish waste the highest protein yield was obtained from the frame (F) and the lowest protein yield was obtained from the fin, tail, skin and gut (FTSG).

2. The protein yield increased with increases in reaction time (1, 2, 3 and 4 h) for the whole fish and fish waste parts.
   (a) The effects of reaction time on protein yield was significant at the 0.05 level and there was significant interaction between the time and parts.
   (b) The protein yield increased rapidly in the first 1.5 h and then increased slowly until the hydrolysis was stopped at 4 h.
   (c) The highest protein yield was obtained at 4 h from whole fish and fish waste.

3. During protein hydrolysis, the enzyme Alcalase (being an serine endopeptidase) and the enzyme Neutrase (being a neutral metallo endoprotease) act upon the peptide bonds to release amino acids into the system.
   (a) Highest amount of amino acids were obtained from the samples hydrolyzed using combination of enzymes (Alcalase+Neutrase) for 48 h.
   (b) Two amino acids arginine and aspartic acid could not be quantified as the enzymes were not able to cleave them.

4. All the amino acids suitable for the jadomycin production can be obtained from enzymatic hydrolysis of fish proteins.
   (a) Due to their high yield, alanine, glycine, isoleucine, leucine, lysine, phenylalanine, serine, threonine, valine, histidine, methionine and tyrosine were recommended for the use as substrate for jadomycin production.
   (b) All the amino acids were above the minimum cut off concentration of 0.4% for the jadomycin production.
   (c) Tryptophan which is suitable of producing jadomycin was not present in the fish protein
   (d) The feasibility of jadomycin production from glutamic acid and proline should be investigated.

5. The oil was obtained as the by-product of the protein extraction and the oil yield also increased with increase in the enzyme concentration (0.5, 1 and 2%) for the whole fish and fish waste parts.
   (a) The effects of enzyme concentration on oil yield was significant at the 0.05 level and there was a significant interaction between the enzyme concentration and parts.
   (b) The differences in oil yield among the fish parts (whole fish, head, frames, fin, tail, skin and gut) were significant at the 0.05 level.
   (c) The highest oil yield was obtained from the head and the lowest oil yield was obtained from the fin, skin, gut and tail.

(d) The oil obtained after enzymatic hydrolysis was dark in color due to the formation of brown pigments from reaction of carbonyls produced from oxidation of polyunsaturated fatty acids with amino acids and proteins.

6. The oil yield increased with increases in time for the whole fish and fish waste parts.
   (a) The effect of time on the oil yield was significant at the 0.05 level.
   (b) The highest oil yield was obtained at 4 h from whole fish and fish waste parts.
   (c) The oil yield increased rapidly in the first 1.5 h and then increased until the reaction was stopped at 4 h.

7. The addition of buffer during the hydrolysis played an important role as it increased the formation of emulsion, decreased the production of free oil and increased the recovery of soluble proteins.

8. The initial heating of raw material played an important role as it helped to increase the oil yield but it would have been more effective if no water or buffer was added to the system.

CHAPTER 8. CONCLUSIONS AND FUTURE WORK

1. Fish waste (fish parts) contained less amount of protein than whole fish but can be utilized for the production of protein without any segregation. However, a preservation method should be investigated to avoid protein hydrolysis.
2. Immobilized enzymes should be evaluated for the production of amino acids from fish proteins for better yield and reduced reaction time.
3. The reusability of enzymes should be investigated
4. Enzyme membrane reactor (EMR) can be used at the pilot scale for production of amino acids and their effectiveness should be evaluated
5. The amino acids produced from protein hydrolysis should be separated by column chromatography and synthetic ion-exchange chromatography. All their economies for jadomycin production should be evaluated.

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APPENDICES

APPENDIX A

PROTEIN EXTRACTION

WF: Whole Fish

H: Head

F: Frame

FTSG: Fin, Tail, Skin and Gut

EC: Enzyme concentration

Sample weight: 50 g

Sample Calculation

1. $Recovered\ Protein\ (\%) = \frac{Weight\ of\ dried\ protein\ (g)}{Weight\ of\ raw\ material\ (g)} * 100$

   Whole fish (WF) at 0.5% and 1 h, the protein recovered was 3.53 g. Therefore the percentage of recovered protein is as follows,

   $Recovered\ Protein\ (\%) = \frac{3.53\ (g)}{50\ (g)} * 100 = 7.06\%$

2. $Protein\ yield\ (\%) = \frac{Recovered\ protein\ (\%)}{Kjeldhal\ protein\ (\%)} * 100$

Whole fish (WF) at 0.5% and 1 h, the percentage protein recovered was 7.06% and the estimated protein content of whole fish was 15.57%. Therefore the percentage of protein yield is as follows,

$Protein\ yield\ (\%) = \frac{\mathbf{7.06}\ (\%)}{\mathbf{15.57}\ (\%)} * 100 = 45.34\%$

Table A1. Extraction of protein from whole fish and fish waste

PART	EC (%)	Time (h)	Recovered Protein (g)	Recovered Protein (g)	Recovered Protein (g)	Average (g)	Recovered Protein (%)	Recovered Protein (%)	Recovered Protein (%)	Average (%)	Protein Yield (%)	Protein Yield (%)	Protein Yield (%)	Average (%)
WF	0.5	1	3.4	3.5	3.69	3.53	6.8	7	7.38	7.06	43.67	44.96	47.40	45.34
WF	0.5	2	3.78	3.8	4	3.86	7.56	7.6	8	7.72	48.55	48.81	51.38	49.58
WF	0.5	3	4.4	4.58	4.61	4.53	9.28	9.16	9.22	9.22	59.60	58.83	59.22	59.22
WF	0.5	4	5.2	5.21	5.22	5.21	10.4	10.42	10.44	10.42	66.80	66.92	67.05	66.92
WF	1	1	3.65	3.66	3.71	3.67	7.3	7.32	7.42	7.35	46.89	47.01	47.66	47.18
WF	1	2	4.12	4.28	4.1	4.17	8.24	8.56	8.2	8.33	52.92	54.98	52.67	53.52
WF	1	3	5	5.21	5.2	5.14	10	10.42	10.4	10.27	64.23	66.92	66.80	65.98
WF	1	4	5.55	5.54	5.53	5.54	11.1	11.08	11.06	11.08	71.29	71.16	71.03	71.16
WF	2	1	4.32	4.28	4.12	4.24	8.64	8.56	8.24	8.48	55.49	54.98	52.92	54.46
WF	2	2	4.85	4.91	4.82	4.86	9.7	9.82	9.64	9.72	62.30	63.07	61.91	62.43
WF	2	3	5.46	5.44	5.42	5.44	10.92	10.88	10.84	10.88	70.13	69.88	69.62	69.88
WF	2	4	5.92	5.94	5.96	5.94	11.84	11.88	11.92	11.88	76.04	76.30	76.56	76.30
H	0.5	1	3.25	3.31	3.29	3.28	6.5	6.62	6.58	6.57	52.85	53.82	53.50	53.39
H	0.5	2	3.57	3.55	3.54	3.55	7.14	7.1	7.08	7.11	58.05	57.72	57.56	57.78
H	0.5	3	3.71	3.78	3.76	3.75	7.42	7.56	7.52	7.50	60.33	61.46	61.14	60.98
H	0.5	4	3.82	3.81	3.84	3.82	7.64	7.62	7.68	7.65	62.11	61.95	62.44	62.17
H	1	1	3.24	3.22	3.21	3.22	6.48	6.44	6.42	6.45	52.68	52.36	52.20	52.41
H	1	2	3.51	3.55	3.54	3.53	7.02	7.1	7.08	7.07	57.07	57.72	57.56	57.45
H	1	3	3.75	3.74	3.77	3.75	7.5	7.48	7.54	7.51	60.98	60.81	61.30	61.03
H	1	4	3.91	3.9	3.96	3.92	7.82	7.8	7.92	7.85	63.58	63.41	64.39	63.79
H	2	1	3.38	3.39	3.41	3.39	6.76	6.78	6.82	6.79	54.96	55.12	55.45	55.18
H	2	2	3.96	3.94	4.1	4.00	7.92	7.88	8.2	8.00	64.39	64.07	66.67	65.04
H	2	3	4.22	4.21	4.24	4.22	8.44	8.42	8.48	8.45	68.62	68.46	68.94	68.67
H	2	4	4.33	4.36	4.38	4.36	8.66	8.72	8.76	8.71	70.41	70.89	71.22	70.84
F	0.5	1	3.64	3.46	3.45	3.52	7.28	6.92	6.9	7.03	51.41	48.87	48.73	49.67
F	0.5	2	3.97	3.96	3.98	3.97	7.94	7.92	7.96	7.94	56.07	55.93	56.21	56.07

PART	EC (%)	Time (h)	Recovered Protein (g)	Recovered Protein (g)	Recovered Protein (g)	Average (g)	Recovered Protein (%)	Recovered Protein (%)	Recovered Protein (%)	Average (%)	Protein Yield (%)	Protein Yield (%)	Protein Yield (%)	Average (%)
F	0.5	3	4.17	4.19	4.21	4.19	8.34	8.38	8.42	8.38	58.90	59.18	59.46	59.18
F	0.5	4	4.36	4.42	4.38	4.39	8.72	8.84	8.76	8.77	61.58	62.43	61.86	61.96
F	1	1	3.84	3.81	3.79	3.81	7.68	7.62	7.58	7.63	54.24	53.81	53.53	53.86
F	1	2	4.21	4.25	4.28	4.25	8.42	8.5	8.56	8.49	59.46	60.03	60.45	59.98
F	1	3	4.44	4.46	4.48	4.46	8.88	8.92	8.96	8.92	62.71	62.99	63.28	62.99
F	1	4	4.68	4.66	4.69	4.68	9.36	9.32	9.38	9.35	66.10	65.82	66.24	66.05
F	2	1	3.63	3.64	3.61	3.63	7.26	7.28	7.22	7.25	51.27	51.41	50.99	51.22
F	2	2	4.69	4.71	4.74	4.71	9.38	9.42	9.48	9.43	66.24	66.53	66.95	66.57
F	2	3	4.92	5.01	4.98	4.97	9.84	10.02	9.96	9.94	69.49	70.76	70.34	70.20
F	2	4	5.26	5.28	5.29	5.28	10.52	10.56	10.58	10.55	74.29	74.58	74.72	74.53
FTSG	0.5	1	2.42	2.49	2.54	2.48	4.84	4.98	5.08	4.97	39.74	40.89	41.71	40.78
FTSG	0.5	2	2.71	2.78	2.76	2.75	5.42	5.56	5.52	5.50	44.50	45.65	45.32	45.16
FTSG	0.5	3	2.94	2.92	2.98	2.95	5.88	5.84	5.96	5.89	48.28	47.95	48.93	48.39
FTSG	0.5	4	3.28	3.26	3.29	3.28	6.56	6.52	6.58	6.55	53.86	53.53	54.02	53.80
FTSG	1	1	2.96	2.98	2.94	2.96	5.92	5.96	5.88	5.92	48.60	48.93	48.28	48.60
FTSG	1	2	3.31	3.41	3.39	3.37	6.62	6.82	6.78	6.74	54.35	55.99	55.67	55.34
FTSG	1	3	3.59	3.62	3.54	3.58	7.18	7.24	7.08	7.17	58.95	59.44	58.13	58.84
FTSG	1	4	3.76	3.78	3.79	3.78	7.52	7.56	7.58	7.55	61.74	62.07	62.23	62.01
FTSG	2	1	3.49	3.46	3.41	3.45	6.98	6.92	6.82	6.91	57.31	56.81	55.99	56.70
FTSG	2	2	3.92	3.94	3.98	3.95	7.84	7.88	7.96	7.89	64.37	64.70	65.35	64.81
FTSG	2	3	4.14	4.16	4.12	4.14	8.28	8.32	8.24	8.28	67.98	68.31	67.65	67.98
FTSG	2	4	4.26	4.28	4.24	4.26	8.52	8.56	8.48	8.52	69.95	70.28	69.62	69.95

APPENDIX B

OIL EXTRACTION

WF: Whole Fish

H: Head

F: Frame

FTSG: Fin, Tail, Skin and Gut

EC: Enzyme concentration

Sample weight: 50 g

Sample Calculation

1. $Recovered\ Oil\ \ (\%) = \frac{Weight\ of\ oil\ (g)}{Weight\ of\ raw\ material\ (g)} * \ 100$

   Whole fish (WF) at 0.5% and 1 h, the protein recovered was 3.63 g. Therefore the percentage of recovered protein is as follows,

   $Recovered\ Oil\ \ (\%) = \frac{3.63\ (g)}{50\ (g)} * \ 100 = 7.12\%$

2. $Oil\ yield\ (\%) = \frac{Recovered\ Oil\ (\%)}{Yield\ of\ estimated\ fat\ (\%)} * \ 100$

Whole fish (WF) at 0.5% and 1 h, the percentage protein recovered was 7.06% and the estimated protein content of whole fish was 15.57%. Therefore the percentage of protein yield is as follows,

$Oil\ yield\ (\%) = \frac{7.06\ (\%)}{16.52\ (\%)} * \ 100 = 44.23\%$

Table B1. Extraction of oil from whole fish and fish waste

PART	EC (%)	Time (h)	Recovered Oil (g)	Recovered Oil (g)	Recovered Oil (g)	Average (g)	Recovered Oil (%)	Recovered Oil (%)	Recovered Oil (%)	Average (%)	Oil Yield (%)	Oil Yield (%)	Oil Yield (%)	Average (%)
WF	0.5	1	3.64	3.69	3.56	3.63	7.28	7.38	7.12	7.26	44.07	44.67	43.95	44.23
WF	0.5	2	4.86	4.82	4.89	4.86	9.72	9.64	9.78	9.71	58.84	58.35	58.80	58.66
WF	0.5	3	5.24	5.21	5.26	5.24	10.48	10.42	10.52	10.47	63.44	63.08	63.40	63.30
WF	0.5	4	5.525	5.46	5.56	5.52	11.05	10.92	11.12	11.03	66.89	66.10	66.77	66.59
WF	1	1	3.82	3.91	3.89	3.87	7.64	7.82	7.78	7.75	46.25	47.34	46.89	46.83
WF	1	2	5.22	5.12	5.2	5.18	10.44	10.24	10.4	10.36	63.20	61.99	62.71	62.63
WF	1	3	5.62	5.69	5.66	5.66	11.24	11.38	11.32	11.31	68.04	68.89	68.48	68.47
WF	1	4	6.16	6.17	6.14	6.16	12.32	12.34	12.28	12.31	74.58	74.70	74.54	74.60
WF	2	1	4.01	3.94	3.98	3.98	8.02	7.88	7.96	7.95	48.55	47.70	48.14	48.13
WF	2	2	5.12	5.01	4.99	5.04	10.24	10.02	9.98	10.08	61.99	60.65	61.02	61.22
WF	2	3	5.82	5.8	5.79	5.80	11.64	11.6	11.58	11.61	70.46	70.22	70.26	70.31
WF	2	4	6.26	6.24	6.28	6.26	12.52	12.48	12.56	12.52	75.79	75.54	75.79	75.71
FTSG	0.5	1	4.12	4.22	4.26	4.20	8.24	8.44	8.52	8.40	39.54	40.50	40.31	40.12
FTSG	0.5	2	5.92	5.96	5.94	5.94	11.84	11.92	11.88	11.88	56.81	57.20	57.01	57.01
FTSG	0.5	3	6.66	6.64	6.62	6.64	13.32	13.28	13.24	13.28	63.92	63.72	63.72	63.79
FTSG	0.5	4	6.98	6.99	7.09	7.02	13.96	13.98	14.18	14.04	66.99	67.08	67.37	67.15
FTSG	1	1	5.21	5.26	5.24	5.24	10.42	10.52	10.48	10.47	50.00	50.48	50.26	50.25
FTSG	1	2	6.66	6.62	6.64	6.64	13.32	13.24	13.28	13.28	63.92	63.53	63.72	63.72
FTSG	1	3	6.94	6.92	6.91	6.92	13.88	13.84	13.82	13.85	66.60	66.41	66.44	66.49
FTSG	1	4	7.14	7.12	7.11	7.12	14.28	14.24	14.22	14.25	68.52	68.33	68.36	68.40
FTSG	2	1	5.98	5.99	6.01	5.99	11.96	11.98	12.02	11.99	57.39	57.49	57.52	57.46
FTSG	2	2	6.86	6.84	6.66	6.79	13.72	13.68	13.32	13.57	65.83	65.64	65.13	65.54
FTSG	2	3	7.2	7.22	7.21	7.21	14.4	14.44	14.42	14.42	69.10	69.29	69.19	69.19
SGFT	2	4	7.59	7.58	7.56	7.58	15.18	15.15	15.21	15.18	72.84	72.70	72.84	72.79
H	0.5	1	4.92	4.94	4.62	4.83	9.84	9.88	9.24	9.65	56.10	56.33	55.04	55.82
H	0.5	2	5.29	5.32	5.56	5.39	10.58	10.64	11.12	10.78	60.32	60.66	61.46	60.81

PART	EC (%)	Time (h)	Recovered Oil (g)	Recovered Oil (g)	Recovered Oil (g)	Average (g)	Recovered Oil (%)	Recovered Oil (%)	Recovered Oil (%)	Average (%)	Oil Yield (%)	Oil Yield (%)	Oil Yield (%)	Average (%)
H	0.5	3	5.89	5.88	5.86	5.88	11.78	11.76	11.72	11.75	67.16	67.05	67.01	67.07
H	0.5	4	6.18	6.22	6.19	6.20	12.36	12.44	12.38	12.39	70.47	70.92	70.66	70.68
H	1	1	5.01	4.99	4.96	4.99	10.02	9.98	9.92	9.97	57.13	56.90	56.86	56.96
H	1	2	5.89	5.94	5.92	5.92	11.78	11.88	11.84	11.83	67.16	67.73	67.46	67.45
H	1	3	6.28	6.26	6.24	6.26	12.56	12.52	12.48	12.52	71.61	71.38	71.38	71.46
H	1	4	6.36	6.4	6.39	6.38	12.72	12.8	12.78	12.77	72.52	72.98	72.79	72.76
H	2	1	4.89	4.9	4.91	4.90	9.78	9.8	9.82	9.80	55.76	55.87	55.87	55.83
H	2	2	6.19	6.12	6.01	6.11	12.38	12.24	12.02	12.21	70.58	69.78	69.63	70.00
H	2	3	6.51	6.54	6.49	6.51	13.02	13.08	12.98	13.03	74.23	74.57	74.27	74.36
H	2	4	6.68	6.7	6.67	6.68	13.36	13.4	13.34	13.37	76.17	76.40	76.21	76.26
F	0.5	1	1.84	1.79	1.82	1.82	3.68	3.58	3.64	3.63	35.28	34.32	34.84	34.81
F	0.5	2	2.92	2.94	2.98	2.95	5.84	5.88	5.96	5.89	55.99	56.38	56.50	56.29
F	0.5	3	3.19	3.15	3.14	3.16	6.38	6.3	6.28	6.32	61.17	60.40	60.59	60.72
F	0.5	4	3.24	3.29	3.26	3.26	6.48	6.58	6.52	6.53	62.13	63.09	62.58	62.60
F	1	1	1.92	1.94	1.96	1.94	3.84	3.88	3.92	3.88	36.82	37.20	37.20	37.07
F	1	2	2.99	2.96	2.94	2.96	5.98	5.92	5.88	5.93	57.33	56.76	56.82	56.97
F	1	3	3.19	3.21	3.22	3.21	6.38	6.42	6.44	6.41	61.17	61.55	61.49	61.40
F	1	4	3.49	3.52	3.51	3.51	6.98	7.04	7.02	7.01	66.92	67.50	67.24	67.22
F	2	1	2.96	2.98	2.99	2.98	5.92	5.96	5.98	5.95	56.76	57.14	57.08	56.99
F	2	2	3.09	3.12	3.11	3.11	6.18	6.24	6.22	6.21	59.25	59.83	59.57	59.55
F	2	3	3.4	3.38	3.36	3.38	6.8	6.76	6.72	6.76	65.20	64.81	64.81	64.94
F	2	4	3.91	3.89	3.88	3.89	7.82	7.78	7.76	7.79	74.98	74.59	74.66	74.74

APPENDIX C

AMINO ACID EXTRACTION

Sample Calculation

Table C1. Hydrolysis of proteins using combination of enzymes for 48 h

Amino acid	Peak Area	Yield (wt %)
Alanine	2799165.7	7.59
Glycine	2146395.9	5.82
Valine	2655335.1	7.20
Threonine	1991501.3	5.40
Serine	1585825.1	4.30
Leucine	3319168.92	9.00
Isoleucine	1954621.7	5.30
Proline	361420.6	0.98
Phenylalanine	1548945.5	4.20
Methionine	811352.4	2.20
Glutamic acid	3632645.9	9.85
Histidine	1323979.6	3.59
Lysine	2706966.6	7.34
Tyrosine	1169085.0	3.17
Unknown	8873244.7	24.06
Total	36879654.7	100

$$Amino\ acid\ yield\ (\%) = \frac{Peak\ area\ A}{\sum(Peak\ area\ A + Peak\ area\ B + \cdots + Peak\ area\ N)} * 100$$

Peak area of alanine: 2799165.79

Total area: 36879654.7

$$Alanine\ (wt\%) = \frac{2799165.79}{36879654.7} * 100 = 7.59\%$$