APPLE PROCESSING BY-PRODUCTS AS A FEEDSTOCK FOR MANUFACTURE OF BIO-ETHANOL AND ORGANIC ACIDS

by

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ABSTRACT

Bio-conversion of agricultural wastes provides a viable solution to multiple environmental problems as well as production of natural products. Apple processing for manufacturing juice, pies and sauce results in significant volumes of under-utilized by-products. This study aims to optimize the method for producing fermentable sugars from apple processing by-products. The conditions required for pre-treatment, polyphenol removal and enzymatic hydrolysis were optimized. The optimized conditions for dilute sulfuric acid-based hydrothermal pre-treatment were acid concentration of 1.5% (w/v) at 91 ^{o}C for 16 min. The final yield of 12.7% fermentable sugars (glucose, fructose and galacturonic acid) was obtained after multi-step hydrolysis using commercial cellulase, pectinase and β-glucosidase at 9, 38 and 8 enzyme units/g FW, respectively. The other optimum conditions were temperature of 40 ^{o}C, pH at 4.0 and 24 h of reaction time. These fermentable sugars can further be converted into bio-ethanol and organic acids using specific yeast and bacteria.

LIST OF ABBREVIATIONS AND SYMBOLS USED

AAB	Acetic Acid Bacteria
ADF	Acid Detergent Fibre
AFEX	Ammonia Fibre Explosion
AIR	Alcohol Insoluble Residue
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
CRD	Completely Randomized Design
D	Dextrorotatory
DP	Degree of Polymerization
DW	Dry Weight
FW	Fresh Weight
FDW	Freeze Dried Weight
EU	Enzyme Unit
GAE	Gallic Acid Equivalents
GC	Gas Chromatography
HMF	Hydroxy methyl furfural
HPLC	High Performance Liquid Chromatography
ISO	International Organization for Standardization
min	Minutes
mL	Millilitre
MS	Mass Spectrometry
NDF	Neutral Detergent Fibre
Q3Gal	Quercitin-3-Galactoside
Q3Glu	Quercitin-3-Glucoside
Q3Rha	Quercitin-3-Rhamnoside
TDF	Total Dietary Fibre
ºC	Degree Celsius
ppm	Parts Per Million
SEM	Standard Error of the Mean
α	alpha
β	beta

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CHAPTER 1: INTRODUCTION

The world's total apple production in the year 2008-2009 has been reported to be more than 69.5 million tonnes, of which Canada contributed 455,361 tonnes (FAO, 2008; Statistic Canada, 2010). Apple processing is carried out to manufacture numerous food products such as juice, pie, sauce, jams and fresh cuts, which result in significant volumes of waste by-products (Rupasinghe and Kean, 2008). These processes utilize approximately 75% of fresh weight of fruit while 25% is left as waste (Sargent et al., 1984; Shah et al., 1994). Every year apple processing industries produce more than 5500 tonnes of apple processing by-products in Nova Scotia (Rupasinghe, 2003). Apple processing by-products mainly constitute pomace (containing peel, core, calyx, stem and seed left after juice processing), cores, peels and rejects from processing industry. These under-utilized biomasses represent a major disposal problem for the industry concerned because of their uncontrollable fermentation and high chemical oxygen demand (250-300 g/kg; Masoodi, 1998). However, these by-products, especially apple pomace, are also rich in both soluble and insoluble carbohydrates and appear to be excellent substrates for bio-processes (Vendruscolo et al., 2008). Fructose, glucose and sucrose form the major soluble sugars present in apple processing biomass. The major polysaccharides present in apple processing by-products include cellulose, hemicelluloses, pectin and lignin.

Like all other lignocellulosic biomasses, the complex polysaccharide structure of

apple processing by-products limits their efficient utilization. Lignocellulosic biomass has been marked with close physical and chemical associations between lignin, cellulose and hemicelluloses. High lignin/cellulose ratio in apple pomace has been considered as the reason for its low digestibility (Villas-Bôas et al., 2003). In addition to this, the cell wall structure of apples has been found to contain pectin matrix, which forms a shield around cellulose and hemicelluloses, thereby decreasing its enzymatic digestibility (Carpita and Gibeaut, 1993). Pectin-cellulose interactions have been suggested by several studies (Oechslin et al., 2003; Selvendran and Ryden, 1990).

Bio-conversion of apple processing by-products consists of at least four steps: size reduction, pre-treatment to enhance biomass digestibility, enzyme hydrolysis of cellulose to sugar monomers such as glucose and fermentation of sugars to bio-ethanol (Zheng et al., 2009). Size reduction accounts for increase in surface area of biomass to undergo rapid hydrolysis, thereby yielding high amount of fermentable sugars. Pre-treatment is aimed at disintegrate the lignin-cellulose complex, thereby increasing biomass digestibility for enzymes. Enzyme hydrolysis can be performed to produce fermentable sugar monomers at a high rate as compared to chemical conversion. For pectin rich residues such as apple pomace, use of pectinases along with cellulases could be an advantageous approach for efficient conversion of polysaccharides into monosaccharides. Finally, the fermentable sugars produced by enzyme hydrolysis can be fermented using *Saccharomyces cerevisiae* to yield bio-ethanol. This bio-ethanol can further be converted into organic acids such as acetic acid using specific strains of *Acetobacter.*

In addition to the release of sugars such as glucose, pre-treatment of apple processing by-products could lead to the formation of sugar degradation products such as furfurals, depending on the severity of the conditions applied. Also, apple waste is known to contain high amounts of specific polyphenols such as chlorogenic acid, epicatechin and phlorodzin (Rupasinghe and Kean, 2008). The by-products of pre-treatment, as well as phenolic compounds present in the waste have been demonstrated to inhibit the cellulolytic enzymes and fermenting micro-organisms (Palmqvist et al., 2000; Ximenes et al., 2010). Therefore, an additional step for the detoxification of these inhibitors could be advantageous in enhancing fermentable sugar yields.

Thus a chemical and enzymatic approach to enhance the release of fermentable sugars in apple processing by-products could be advantageous to yield high amounts of bio-ethanol and organic acids. In addition to this, detoxification of potential inhibitors such as phenolic compounds in apple processing by-products could improve the overall bio-conversion process.

CHAPTER 2: OBJECTIVES

The under-utilized processing waste from the apple industry is critical to the environment. Alternatively, bio-conversion of polysaccharides present in waste could be used to produce value-added products. Some studies have been carried out in the past to produce fermentable sugars from apple pomace. However, there is lack of information on the effect of pre-treatment and polyphenol removal on the enzyme hydrolysis of apple processing by-products. It is likely that the presence of pectin and lignin-like substances could impede enzymatic hydrolysis in apple processing by-products. It is also expected that the high glucose concentration generated from enzyme hydrolysis could cause feedback inhibition due to elevated levels of end product. Therefore, it is important to determine the hydrolytic efficiency at different operating conditions and investigate the impact of these factors when commercial cellulase enzyme is used. The overall objective of this study is to optimize the production of fermentable sugars from apple processing by-products, which can be used for bio-ethanol and organic acid production. The study would provide insight regarding the interaction of different factors such as pre-treatment, polyphenol oxidation, enzyme type, dosage, temperature, time and pH on the release of fermentable sugars in the hydrolyzates of processing by-products from the apple juice industry. The specific objectives were to:

1) determine the chemical composition of apple processing by-products;
2) optimize dilute acid-based hydrothermal pre-treatment of apple processing by-products for maximum glucose yield;

3) investigate the effect of commercial laccase on the oxidation of polyphenols present in apple processing by-products prior to enzyme addition; and
4) standardize the enzyme hydrolysis of apple processing by-products for the production of fermentable sugars by assessing the effects of different factors such as enzyme type, dosage, time, temperature and pH.

The overall research approach is given in **Figure 2.1**.

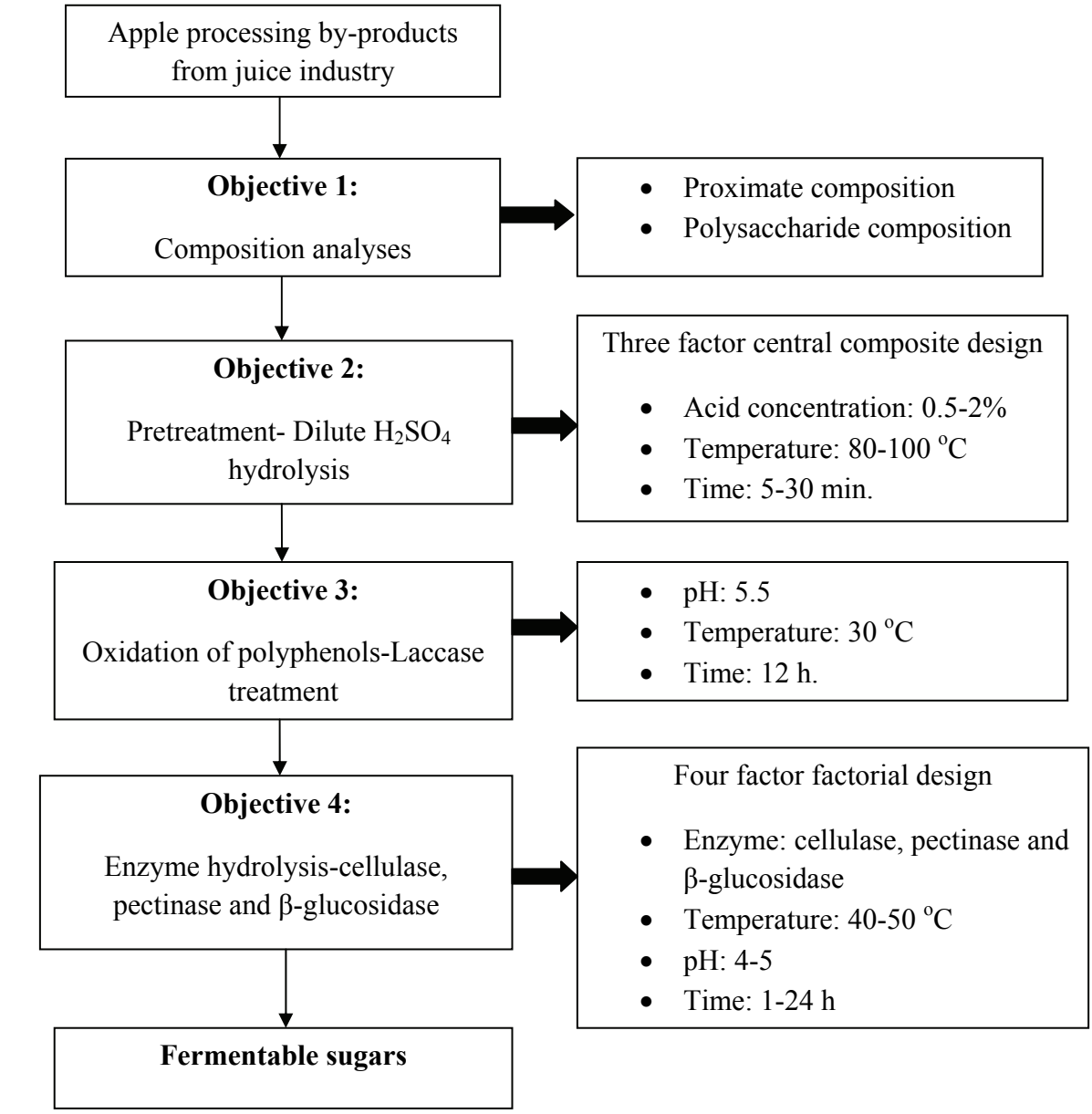


Figure 2.1 Overall research approach.

CHAPTER 3: LITERATURE REVIEW

3.1 BIOMASS STRUCTURE

The major polysaccharides present in apple processing by-products are cellulose, hemicellulose, pectin and lignin (Villas-Boâs et al., 2002; Gullon et al 2007). Cellulose, a linear condensation homopolysaccharide, is composed of β-1→4 linked D-glucose units with a degree of polymerization (DP) ranging from 100 to 10,000, depending on plant species (Lynd et al., 2002; **Figure 3.1**). Cellobiose, a dimer of β-1→4 linked D-glucose, is the repeating unit of cellulose. The adjacent chains of cellulose are coupled by hydrogen bonds and van der Waal's forces, which result in a parallel alignment and a crystalline structure. The cellulose structure has straight and stable supra-molecular 6 fibres of great tensile strength. This is due to extensive hydrogen bonding of interchain and intrachain hydroxyl groups, thereby making it less accessible to enzymes and difficult to break down (Zhang and Lynd, 2004). Cellulose present in the cell wall of most lignocellulosic biomass is crystalline in structure (Mosier et al., 2005). However, a most recent study by (Chargot et al., 2011) has stated that cellulose present in apple cell wall is mostly amorphous in nature.

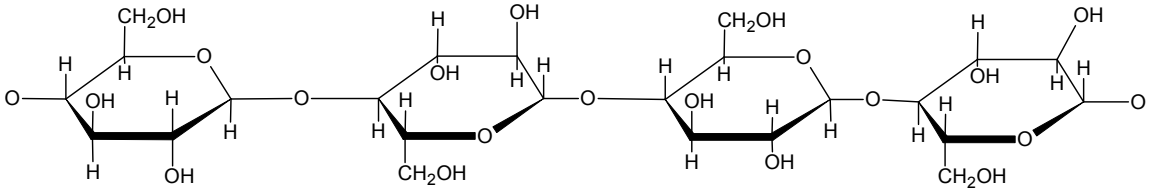


Figure 3.1 Chemical structure of cellulose

Hemicelluloses are heterogeneous polymers composed of pentoses (β-D-xylose, α-L-arabinose), hexoses (β-D-mannose, β-D-glucose, α-D-galactose) and or uronic acids (α-D-glucuronic, α-D-4-*O*-methylgalacturonic and α-D-galacturonic acids) which are linked together by glycosidic bonds (Kumar et al., 2008). Hemicelluloses can be categorized into xylans, mannans, arabinans and galactans based on the the main sugar components in their backbones. Xylan (D-xylopyranosyl units bound by β-1→4 glycosidic bonds) is the most common hemicellulose present in plant cell wall (**Figure 3.2**). Besides, minor amounts of L-fucose and L-rhamnose may also be present and hydroxyl groups of sugars can be substituted with acetyl groups (Gírio et al., 2010). The form and structure of hemicelluloses depend on their source such as wood or fruits (Dey and Brinson, 1984). In general, hemicelluloses are relatively small molecules, containing 70 to 200 residues of monossacharides. Hemicelluloses are bonded to cellulose by hydrogen bonds, and to lignin and pectin, by covalent bonds (Freudenberg, 1965).

Pectin can be described as a methylated ester of polygalacturonic acid, which contains chains of 300-1000 galacturonic acid units joined with α-1→4 linkages (Jayani et al., 2005). Pectin present in apple has two regions: homogalacturonan (smooth) (**Figure 3.3a**) and rhamnogalacturonan (hairy) (**Figure 3.3b**). Homogalacturonan contains α-1→4 linked partly methylesterified D-galacturonan residues, while rhamnogalacturonan constitutes highly branched arabinogalactan side chains, α-1→2 linked D-galacturonic acid units and α-1→4 linked L-rhamnose regions. In the latter, side chains of neutral sugars consist of mainly L-arabinose, D-galactose and D-xylose, which are covalently attached to the rhamnosyl residues of the backbone (De Vries et al.,

1986). According to De Vries et al. (1981), the distribution of the neutral sugars in apple pectin is discontinuous.

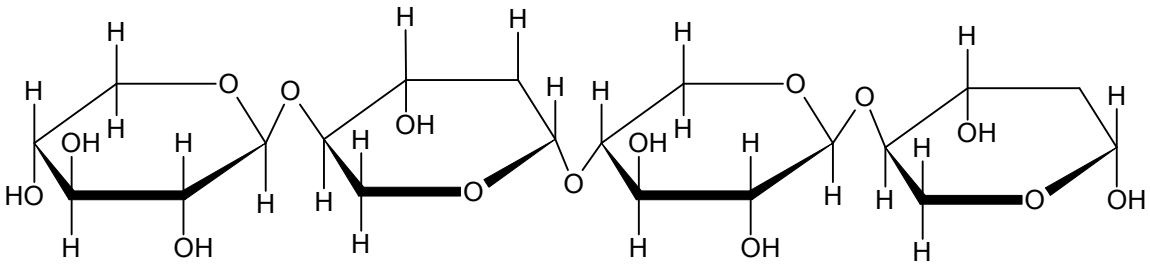


Figure 3.2. Chemical structure of xylan backbone of hemicellulose.

Lignin is an aberrant hydrophobic distribution of phenylpropanoid units, resulting from the oxidative polymerization of hydroxycinnamyl alcohol precursors (Higuchi, 1985). These alcohols, 4-hydroxycinnamyl alcohol, coniferyl alcohol, and sinapyl alcohol, lead to the formation of *p*-hydroxyphenyl, guaiacyl, and syringyl lignins, respectively (**Figure 3.4**). Lignin has the ability to withstand chemical or enzymatic degradation to protect cellulose from degradation. Lignin degradation is regarded as the rate controlling step in biomass degradation (Lee, 1997).

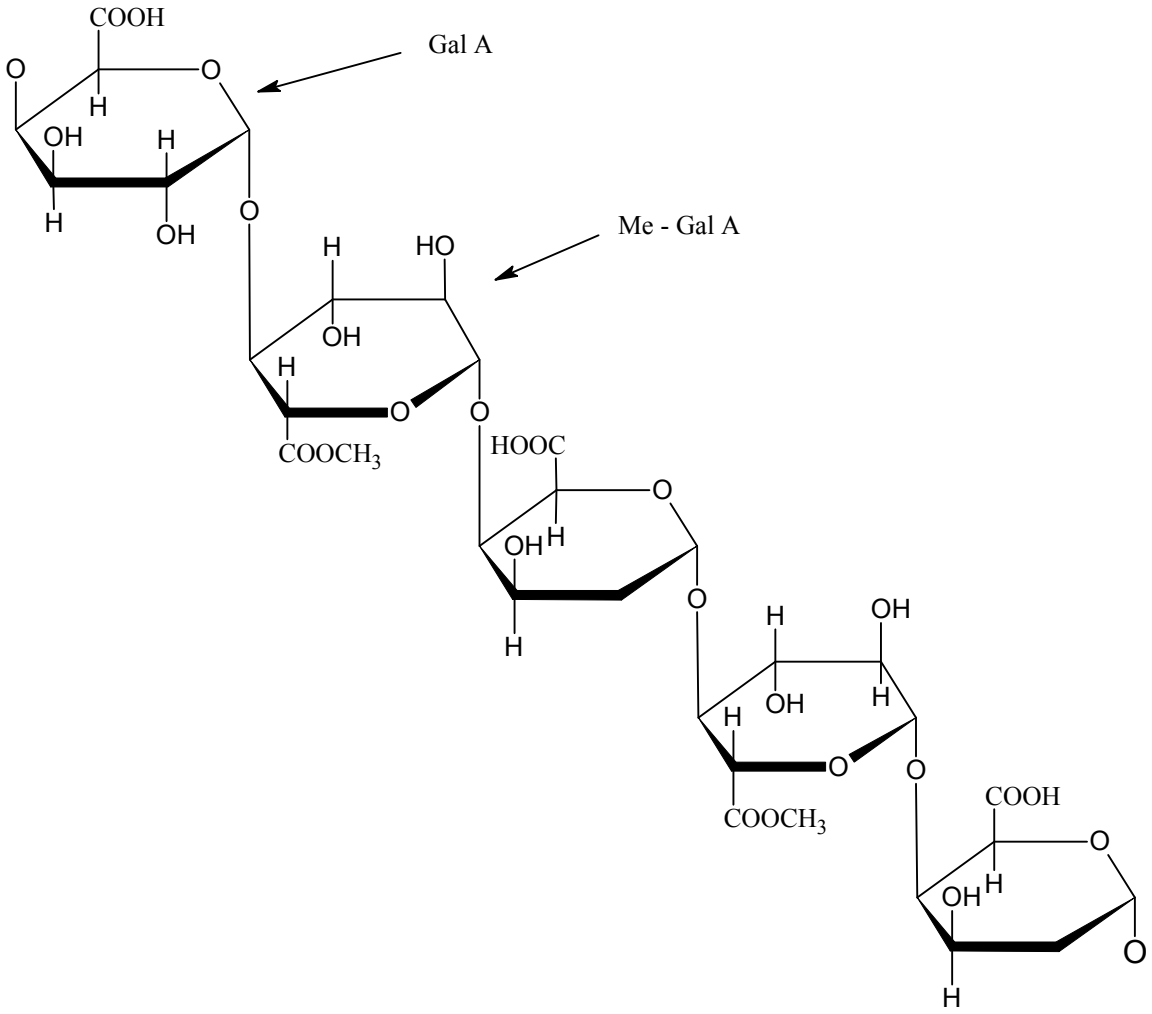


Figure 3.3a Chemical structure of homogalacturonan region of pectin (Gal A-Galacturonic acid and Me-Gal A-methyl esters of Gal A).

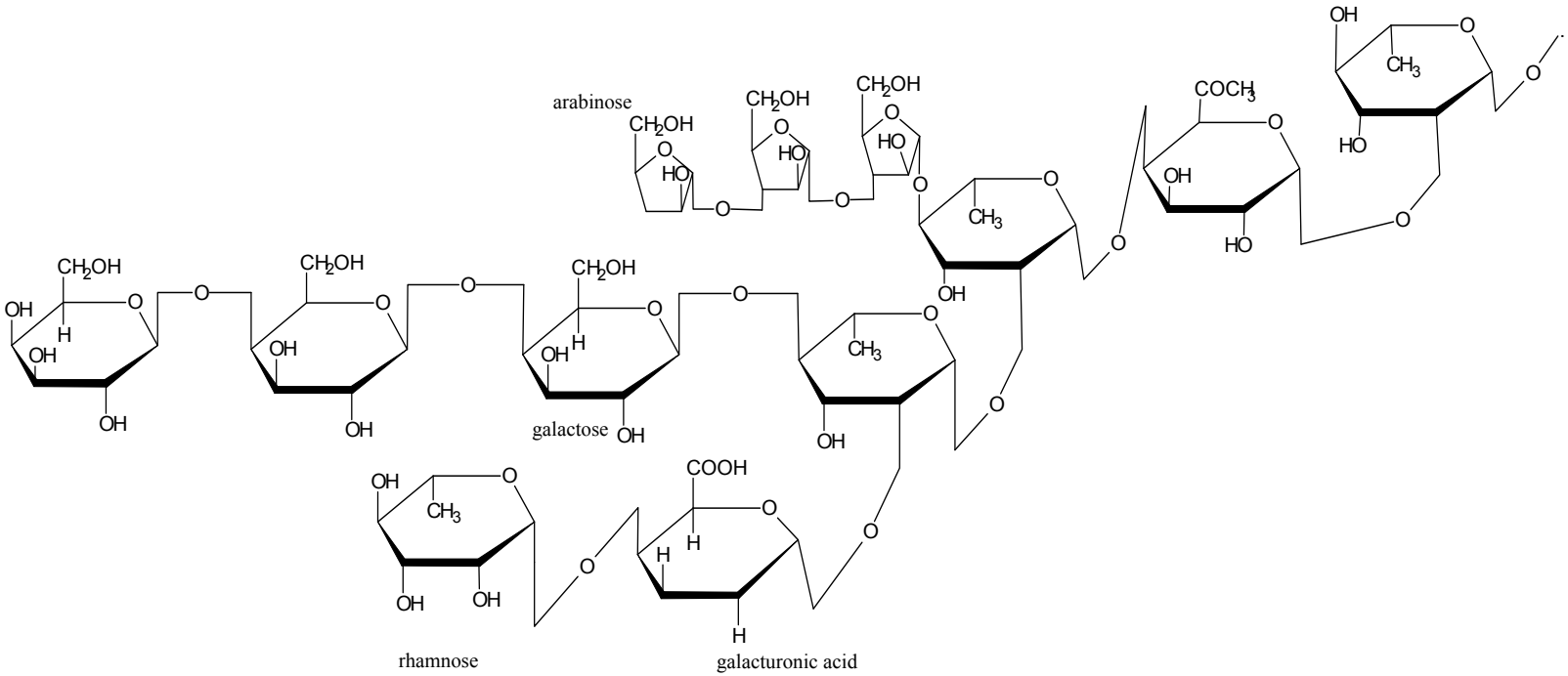


Figure 3.3b Chemical structure of rhamnogalacturonan region of pectin

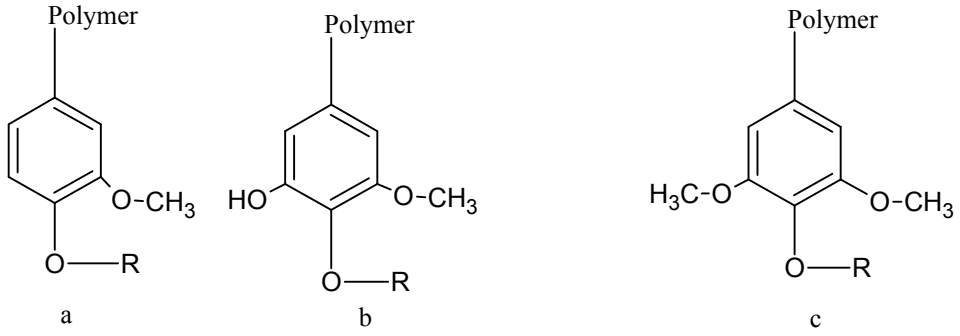


Figure 3.4 Monomers of lignin: a: *p*-hydroxyphenyl, b: guaiacyl and c: syringyl units

3.2 COMPOSITION OF APPLE POMACE

The composition of apple pomace is summarized in **Table 3.1.** The given variability in the values is due to different growing conditions, method of extraction and cultivars used. Fructose, glucose and sucrose are the major monosaccharides present in apple pomace. In addition to these, minor amounts of arabinose, rhamnose, xylose, galactose and mannose have also been reported (Gullón et al., 2007). Arabinose and galactose form the monomeric units of hemicelluloses and pectin present in apple processing waste (Bittner et al., 1982; Downing, 1989). Starch content of apple pomace is relatively low as it gets converted to reducing sugars in the ripening process.

According to Sudha et al. (2007), the total dietary fibre content of apple pomace is 51.1% on dry basis. The concentration and type of fibre varies from tissue to tissue with carpels being rich in glucans and xyloglucans, while flesh contains a high proportion of pectic components (Massiot et al., 1994). Both epidermis and carpels of apples contain non-polysaccharide insoluble material such as waxes and lignin (Colin-Henrion et al., 2009).

Table 3.1 Composition of apple pomace

Component	Value (DW)	References
Proximate composition		
Dry matter	25-30%	Bhushan et al., 2008
Crude protein	2.8-4.1%	Gullón et al., 2007
Crude fat	3.01 to 4.70	Cho and Hwang, 2000
Ash	2.11 to 3.50	Cho and Hwang, 2000
Monosaccharides		
Fructose	13.6-35%	Ngadi and Correia, 1992; Voget et al., 1985; Waugh, 1981
Glucose	6.1-13.3%	Wang and Thomas, 1989; Waugh, 1981
Sucrose	1.4-5.2%	Hours et al., 1988; Voget et al., 1985
Polysaccharides		
Cellulose	12.0-23.2%	Givens and Barber, 1987
Pectin	5.0-16.0%	Marcon et al., 2005
Hemicellulose	5.0-6.1%	Wolter et al., 1980
Lignin	15.8%	Gullón et al., 2007
Organic acids		
Malic acid	1.0–1.3%	Gullón et al., 2007
Minerals		
Sodium	0.02-0.04%	Alibes et al., 1984;
Calcium	0.1-0.3%	Givens and Barber, 1987)

According to a study by Renard et al. (1995), Klason lignin (acid soluble lignin) content of depectinized apple pomace was found to be almost 20% (186 mg/g DW). It was thought that this Klason lignin content was due to the polymerization of phenolic compounds, which precipitate on the cell wall matrix, thereby preventing hydrolysis of other polysaccharides present.

Malic acid forms the major organic acid present in apples, thus minor amounts (1.0–1.3% on dry weight basis) are found in apple pomace as well (Gullón et al., 2007).

On a dry weight basis, apple pomace has been found to contain 0.02-0.04% sodium, 0.1-0.3% calcium, along with very minor contents of magnesium, manganese and phosphorous (Alibes et al., 1984; Givens and Barber, 1987). In addition to these, apple pomace is a promising source of specific polyphenols such as epicatechin, chlorogenic acid, phloridzin, a number of quercitin glycosides and so on (Rupasinghe and Kean, 2008; Schieber et al., 2003). The phenolic composition of pomace varies according to the apple variety and the type of processing used for juice extraction (Paganini et al., 2005).

3.3 UTILIZATION OF APPLE PROCESSING BY-PRODUCTS

Pectin production has been viewed as the most reasonable and economical method of utilizing apple pomace so far (Endress, 2000). However, a mild brown hue of apple pectin caused by enzymatic browning could hamper its use in very light-colored foods (Schieber et al., 2003). Efforts to bleach apple pomace by alkaline peroxide treatment resulted in pectin degradation and the loss of the polyphenols (Renard et al., 1996). It has been demonstrated that enzymatic liquefaction of apple pomace with pectinases and cellulases lead to enhanced release of phenolics, which represents an alternative approach to utilizing apple pomace Will et al., 2000). Efforts have been made to utilize apple pomace by converting it into organic acids (Gullón et al., 2008), liquid bio-fuel (Jewell and Cummings, 1984), animal feeds (Vendruscolo et al., 2007), edible fibres (Grigelmo-Miguel and Martín-Belloso, 1999) and many other products in order to reduce the associated waste disposal problems. According to Kennedy et al. (1999a), the ideal use for apple pomace is yet to be discovered.

3.4 APPLE PROCESSING BY-PRODUCTS AS SUBSTRATES FOR BIO-FUEL

The interest in fuel based on renewable sources has been increasing since the oil crisis in 1973. On an industrial scale, first generation bio-ethanol has been produced mostly from corn starch and cane sugar. However, certain challenges like food security have led the researchers to look for alternative feed stock for bio-ethanol which are more competitive and inexpensive. To date, most common lignocellulosic biomass sources are pine, poplar, corn stover, sugarcane bagasse, agricultural residues, switch grass and other perennial grasses. In comparison to non-renewable gasoline, bio-ethanol is cleaner in burning as it is more oxygenated (Wheals et al., 1999). Bio-ethanol has the potential to replace aromatic and sulfur containing compounds used in gasoline. Also it may decrease urban smog by reducing nitric oxide (NO) emissions to improve air quality. In addition to these, bio-ethanol, due to its high oxygen content, could reduce the generation of known hazardous volatile organic compounds and carbon monoxide in vehicle exhaust (Putsche and Sandor, 1996; Yoon et al., 2009).

Owing to their high polysaccharide content and low-cost renewable source, apple processing by-products have the potential to be converted into bio-ethanol. This can provide alternative energy supply, at the same time, can reduce their disposal related problems. Furthermore, bio-conversion of apple processing biomass into bio-ethanol seems to be an attractive option to reduce greenhouse gas emissions from fossil fuel based transportation sector.

3.5 APPLE PROCESSING BY-PRODUCTS AS SUBSTRATE FOR FERMENTATION TO PRODUCE ORGANIC ACIDS

Organic acids can be produced by fermentation of enzymatic hydrolyzates from apple processing by-products. Cost reduction in the process can be achieved, for example, by using cheaper raw materials, optimizing the process parameters such as temperature, pH, enzyme activity, microbial loadings and reducing the nutrient supplementation of fermentation media. Apple processing by-products show several benefits as a raw material for organic acid production, including: (i) high content of fermentable sugars such as glucose and fructose, which are good carbon sources for acetic acid manufacture; (ii) high content of polysaccharides (pectin, cellulose and hemicelluloses) which can be enzymatically hydrolyzed to give monosaccharides; (iii) presence of minerals (e.g. phosphorous, magnesium, manganese etc.) which are required by micro-organisms to grow. Apple pomace has been mostly studied as a source for conversion into organic acids such as citric acid and lactic acid (Dhillon et al., 2011; Gullón et al., 2008).

3.6 APPROACH FOR THE BIO-CONVERSION OF APPLE PROCESSING BY-PRODUCTS

Conventional technologies for bio-conversion of cellulosic waste consist of feedstock size reduction, pre-treatment, hydrolysis and fermentation (Zheng et al., 2009). Size reduction of the biomass effectively increases the accessible surface area during hydrolysis. Pre-treatment is targeted to disrupt the barrier formed by lignin and hemicelluloses on cellulose structure and expose it to enzymatic hydrolysis. Enzymatic hydrolysis of biomass is carried out to yield simple sugars at a high rate as compared to chemical treatments. This is due to higher production yields by enzymes at relatively moderate temperature and pH conditions. The simple sugars produced during enzymatic

hydrolysis can be simultaneously fermented into bio-ethanol and organic acids using specific yeast or bacterial strains.

3.6.1 PRE-TREATMENT

During lignocellulosic bio-conversion, cellulose should be made accessible to cellulases by pretreating the substrate (Ballesteros et al., 2004). Hydrothermal pre-treatments such as steam explosion and dilute acid hydrolysis are commonly used for lignocellulosic biomass where the presence of lignin hinders the enzymatic hydrolysis. Besides, other pre-treatments using ammonia fibre explosion, sulfur dioxide (SO_2)-steam explosion, concentrated acids and lime [$Ca(OH)_2$] are also used but high costs are involved with their application. A comparative study carried out by Wyman et al. (2005) stated the necessity of an acid or a base to loosen up the lignocellulosic structure at a reasonable cost before enzymatic hydrolysis.

During processing of biomass, pre-treatment is considered to be one of the most crucial and expensive steps due to high inputs such as heat, acid/base and reaction time (Mosier et al., 2005). Based on the application and type of substrate used, pre-treatment can be classified into three different categories: physical, chemical and biological. Some bio-processes apply a combination of different pre-treatments to increase the product yield. Careful selection of pre-treatment can enhance the efficiency of the process andcan simultaneously reduce the cost.

3.6.1.1 PHYSICAL PRE-TREATMENT

Physical pre-treatments include size reduction of biomass by mechanical size reduction (comminution), steam explosion (also called auto hydrolysis) and liquid hot water treatment (McMillan, 1994). Except for mechanical communition, the critical parameters for the other two treatments are temperature, application time, particle size of

biomass, and moisture content. During physical pre-treatment, biomass is heated at high pressure in liquid water/saturated steam for a certain time to assist in delignification and release of sugars trapped in the lignin-hemicellulose network. In addition to these, some by-products of sugars may also be produced due to acidic properties of water used (Cantarella et al., 2004). Both steam explosion and liquid hot water pre-treatment have been reported to be effective for pentose recovery, sugar extraction and improving cellulose accesibility to enzymes (Grous et al., 1986; Van Walsum, 1996).

3.6.1.2 CHEMICAL PRE-TREATMENT

Chemical pre-treatments have received numerous interests and mainly comprise of acid hydrolysis, alkaline hydrolysis, ammonia fibre/freeze explosion, sulfur dioxide (SO_2) catalyzed steam explosion and ionic liquid pre-treatment.

3.6.1.2.1 ACID PRE-TREATMENT

Dilute acid hydrolysis is the most common pre-treatment method applied for almost all types of biomass. The most commonly used acids for dilute acid pre-treatment are sulfuric acid (Martin et al., 2007), nitric acid (Brink, 1994) and hydrochloric acid (Hererra et al., 2003). Dilute sulfuric acid pre-treatment at concentration usually 4.0% (w/v), has been most explored due to its high reaction rate and effectiveness in removal of lignin (Esteghlalian et al., 1997; Kumar et al., 2009). In addition to this, high temperature in the dilute-acid treatment has been found to favor xylan and cellulose hydrolysis (Mcmillan, 1994). A study by Lu et al. (2007) demonstrated dilute sulfuric acid pre-treatment of corn stover at acid concentrations of 2, 4, and 6% and temperatures of 80, 100 and 120 °C. The study determined that a sulfuric acid concentration of 2.0 % and a reaction time of 43 min. at 120 °C were optimum for corn stover in terms of yield of total reducing sugars. Another study by Taherzadeh et al. (1997a) demonstrated that

sulfuric acid pre-treatment (0.5% w/v) of wood for 7 minutes hydrolyzed more than 80% of the hemicellulose at temperatures below 200 ^{o}C. However, the maximum overall yield of glucose was found at hydrolysis temperatures above 220^{o}C, suggesting the recalcitrance of crystalline cellulose. Acid hydrolysis has been found to release oligomers and monosaccharides by a reaction in which acid catalyzes breakdown of cellulose to glucose followed by breakdown of the glucose released to form HMF and other degradation compounds (Mosier et al., 2005).

Initial hydrolysis using concentrated acids also has been studied (Goldstein et al., 1983; Vedernikov et al., 1991). Concentrated acids were found to yield higher amounts of sugars and consequently higher ethanol yield (Taherzadeh and Karimi, 2007). However, the high acid concentration (30-70%), dilution and heating during hydrolysis process makes it corrosive. In order to overcome these shortcomings, the process demands for expensive alloys or specialized non-metallic constructions such as ceramic or carbon-brick lining. The used acid is recovered in a complex system of absorption towers, a stripper, a falling film evaporator and several heat exchangers with an aim to recirculate it (Sivers and Zacchi, 1994). In addition, the neutralization step prior to enzymatic hydrolysis produces substantial amounts of gypsum. Therefore, the economic feasibility of this process is challenged due to high investments involved in acids, detoxification of inhibitors and an additional step required for neutralization before enzymatic hydrolysis.

3.6.1.2.2 ALKALINE PRE-TREATMENT

Besides acid pre-treatment, delignification and hemicelluloses solublization can also be achieved by alkaline hydrolysis. The intermolecular ester bonds which are cross

linked to xylan hemicelluloses and lignin get saponified using pre-treatment with bases such as sodium hydroxide (Carrillo et al., 2005), calcium hydroxide (Chang et al., 2001), potassium hydroxide (Chang and Holtzapple, 2000) and ammonia hydroxide (Prior and Day, 2008). Research has found that biomass from hardwood, herbaceous crops, and agricultural residues are more suitable for alkaline pre-treatment (Bjerre et al., 1996). However, alkaline pre-treatment incorporates considerable amount of salts into the hydrolyzates, whose removal increase the process costs.

3.6.1.2.3 AMMONIA FIBRE EXPLOSION (AFEX)

Ammonia fibre explosion is a chemical pre-treatment that produces negligible inhibitors and requires no particle size reduction. It works on the same principle as steam explosion except for the fact that it uses hot liquid ammonia under high pressure for a specific holding time. A slow release of pressure after holding time leads to increased accessibility of cellulose to enzymes due to disruption of lignin and solublization of hemicelluloses (Mes-Hartree et al., 1988; Mosier et al., 2005;). AFEX process has been studied for various lignocellulosic materials and has been reported to be less effective in high lignin containing biomass such as newspapers and aspen chips (mainly from *Populus* species; McMillan, 1994). A study by Teymouro et al. (2004) evaluated AFEX pre-treatment on corn stover (approximately 17% lignin) for several parameters including ammonia loading, moisture content of biomass, reaction temperature and residence time. Based on approximately 98% theoretical yield of glucose obtained, the optimum conditions for pre-treatment were determined to be temperature of 90 °C, an ammonia/dry corn stover mass ratio of 1:1, a moisture content of corn stover of 60% (dry weight basis), and a residence time (holding at target temperature) of 5 min. The

study also showed that AFEX treated sample yielded 2.2 times more ethanol than untreated sample.

3.6.1.2.4 CATALYZED STEAM EXPLOSION

One of the most economically feasible chemical pre-treatment is steam explosion with some acidic chemicals such as sulfur dioxide and sulfuric acid (Tengborg et al., 1998). Carbon dioxide has also been used because of its ability to form carbonic acid when dissolved in water (Zheng et al., 1998). Catalyzed steam explosion seems to be more promising than uncatalyzed steam explosion process due to its ability to completely solublize hemicelluloses and increased digestibility of the substrate. However, degradation of carbohydrates due to high heat involved in the reaction conditions has been marked as a disadvantage of the process. This can be overcome by careful selection of scraped heat exchangers, which do not allow the substrate to stick to the walls of the reactor.

3.6.1.2.5 IONIC LIQUID PRE-TREATMENT

Recently, several research interests are engaged in finding a cheaper and better way for biomass pre-treatment and fractionation (Zheng et al., 2009). New pre-treatment technologies have given attention to the use of ionic liquids (ILs) as cellulose solvents. Due to their low melting point and a variety in their component ions, it is possible to adjust their physiochemical properties. A range of alkylimidazolium salts containing 1-N-butyl-3-methylimidazolium chloride (BMIMCl), 1-ethyl-3-methylimidazolium chloride (EMIMCl), 1-N-butyl-3-methylimidazolium acetate (BMIMAc) and some others containing alkylsulfate, alkylsulfonate, and alkylphosphate as anion derivatives have been referred to as green solvents for cellulose (Fukaya et al., 2008; Kosan et al.,

2008). Ionic liquids have potential to dissolve high concentrations of cellulose at low energy requirements, when compared to almost all other pre-treatment conditions (Fukaya et al., 2008; Kosan et al., 2008). The studies have reported 100% recovery of ionic liquid solvents after their application. These non-volatile solvents are found to be environmental friendly, thermo-stable and less viscous (Feng et al., 2008). However, more research is required to address some important issues like their action mechanism on lignin-hemicellulose complex, toxicological information, and regeneration before commercializing these compounds for biomass pre-treatment (Zheng et al., 2009).

3.6.1.3 BIOLOGICAL PRE-TREATMENT

Biological treatment uses micro-organisms or enzymes in pretreatment of lignocellulosic biomass. Biomass can be biologically modified using white/brown/soft rot fungi because of their ability to undergo oxidation (Lee et al., 2007; Singh et al., 2008). Common extracellular phenoloxidases, namely, lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac), due to their low substrate specificity and strong oxidative activity are responsible for causing the disruption of lignin (Kirk and Ferrell, 1987). White rot fungi have attracted research interests with respect to lignin degradation followed by brown and soft rot fungi, which demonstrated more efficiency in cellulose degradation (Ander and Eriksson, 1978; Schurz, 1978). Besides, various ancillary enzymes that attack hemicellulose, such as xylanase, glucuronidase, acetylesterase, feruloylesterase, β-xylosidase and glucomannanase have been studied for a variety of biomass (Aranda et al., 2004; Georgieva et al., 2008; Mtui and Nakamura, 2005, Roman et al., 2006).

3.6.2 POTENTIAL INHIBITORS TO ENZYMATIC HYDROLYSIS AND FERMENTATION

The degradation of sugars during pre-treatment step leads to the formation of by-products which can have an inhibitory action to further steps in bio-conversion of lignocellulosic biomass (Lu et al., 2009; **Figure 3.5**). The amount and type of inhibitors formed during pre-treatment step depend on type of pre-treatment used, composition of substrate and conditions at which it is carried out (Cantarella et al., 2004; Ximenes et al., 2010). Acid pre-treatment usually involves high temperature under acidic conditions, which produces or releases a range of compounds such as furfural, hydroxymethylfurfural (HMF), phenolic compounds and weak acids (e.g. acetic acid) (Lu et al., 2009; Palmqvist and Hägerdal., 2000). **Table 3.2** shows the concentration of common inhibitors as a result of various pre-treatments of lignocellulosic biomass.

3.6.2.1 PHENOLIC COMPOUNDS

Phenolic acids are released as a result of disruption of lignin and degradation of carbohydrates during hydrolysis of lignocellulosic substrates (Ximenes et al., 2010). Enzymatic hydrolysis leads to depolymerization of polyphenolic compounds. A considerable inhibitory effect of phenolic compounds toward enzymatic hydrolysis and fermentation of lignocellulosic biomass has been reported (Ximenes et al., 2010). The activities of cellulases, hemicellulases and β-glucosidases are affected by the presence of phenolic compounds in the hydrolyzate (Sineiro et al., 1997). Bio-conversion can be hampered due to the presence of these inhibitors, thereby decreasing the final product yield. Removal of such inhibitors is necessary for increased recovery of the final product. The low molecular weight polyphenols have been reported to be most toxic to fermentation (Buchert et al., 1989; Clark and Mackie, 1984). Complex interactions between phenolic compounds and mixtures of enzymes (cellulase and β-glucosidase)

have been suggested as the cause of inhibition (Ximenes et al., 2010). Among phenolic acids, *p*-hydroxybenzoic acid, syringic acid, hydroxybenzoic acids (Ando et al., 1986), cinnamic acid, vanillic acid (Nishikawa et al., 1988) and catechol (Jönsson et al., 1998) have been found to inhibit enzymatic hydrolysis of different lignocellulosic materials. Since several phenolic acids and derivatives are found in apple pomace, it may be necessary to remove them for improved yield of sugars and bio-ethanol.

3.6.2.2 FURFURAL AND HMF

HMF is the by-product of hexose degradation and furfural is produced as a result of xylose degradation (Ulbricht et al., 1984). These furan derivatives are reported to decrease the ethanol yield by restricting the yeast growth or giving rise to a longer lag phase during growth (Almeida et al., 2007). In order to investigate the toxicity of the furfural, Navarro (1994) carried out a series of fermentation runs using 0.2, 0.5, and 0.9 mg/kg (DW) of *Saccharomyces cerevisiae* inoculum and 0.1, 0.3, and 0.5 mg/kg of furfural. The results showed that high initial yeast concentration is required to overcome the inhibory effect of furfural at an early stage of fermentation. Also, at furfural concentration of 0.5 mg/g, the rate of glucose consumption was greatly reduced. Another study by Taherzadeh et al (1999) showed that addition of furfural (0.4 mg/g) to an exponentially growing batch culture of *Sacchromyces cerevisiae* led to a decrease of specific growth rate and ethanol productivity by 93% and 68%, respectively. Microbial cell death has been reported to occur due to high furfural concentrations in fermentation media (Palmqvist et al., 1999).

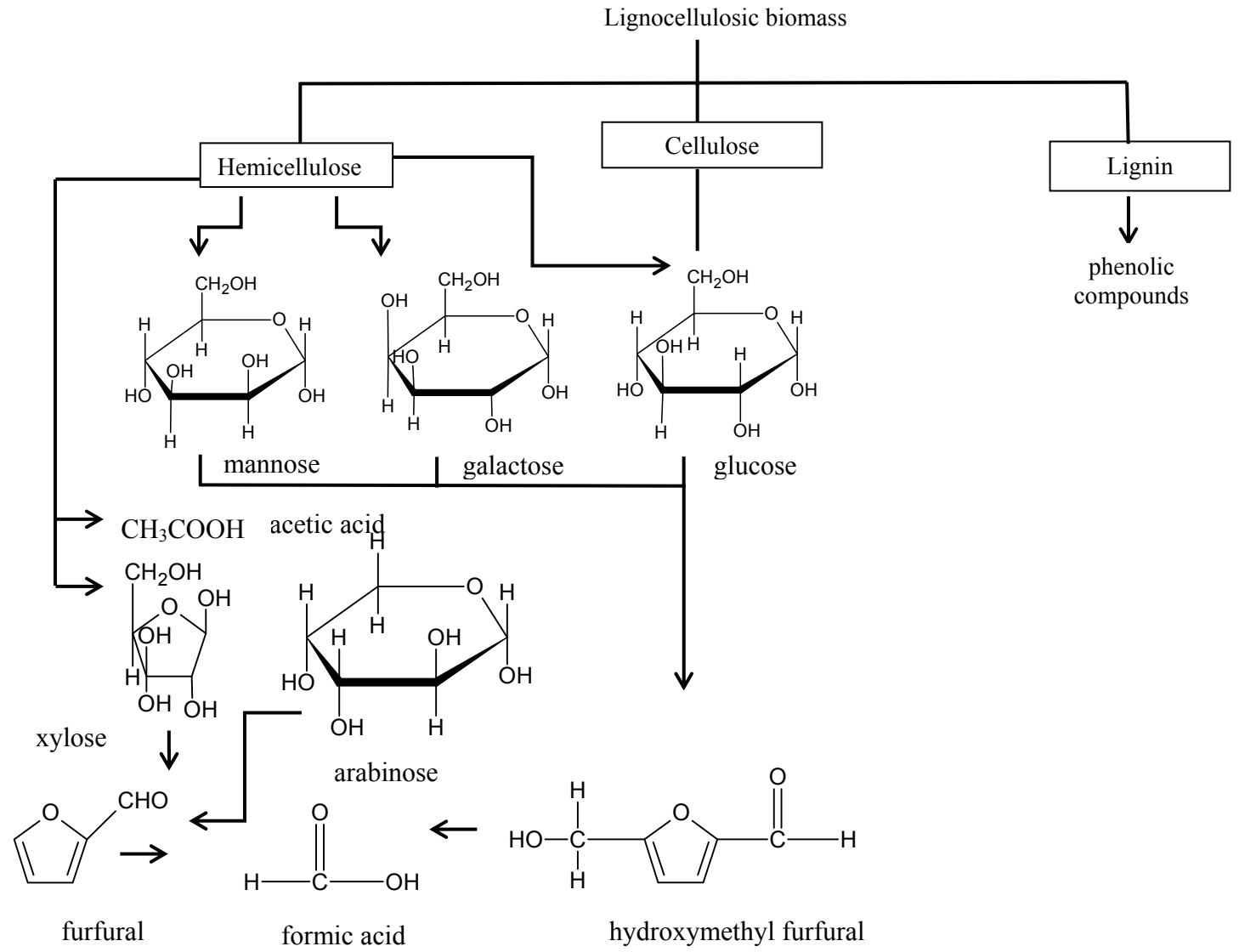


Figure 3.5 Reactions occurring during hydrolysis of lignocellulosic biomass (The furan derivatives and phenolic compounds will react further to form different products; Adapted from Palmqvist et al., 2000).

Table 3.2 Concentrations of common inhibitory compounds present in pre-treated lignocellulosic biomass.

Pre-treatment	Substrate	Inhibitor concentration (mg/g)					Reference
		Acetic acid	Furfural	HMF	Formic acid	Phenols	
Hydrothermal pre-treatment (0.5% H_2O_2 w/v; 190-200^oC; 3-6 min.)	Wheat straw	0.2-0.3	0-0.1	0-0.03	0.1-0.2	0-0.02	Thomsen et al., 2009
Dilute acid pre-treatment (0.5-4.4% w/v H_2SO_4; 150-240^oC; 1-30 min)	softwood	0.1-0.6	0-0.4	0.01-0.6	0-1.0	-	Larsson et al., 1999
Alkaline wet oxidation (6.5 g/L Na_2CO_3; 195^oC; 12 bar O_2)	Wheat straw	0.6	0	0	0.7	0.3	Klinke et al., 2001
Dilute acid pre-treatment (5% w/v H_2SO_4; 188-234 ^oC; 7 min.)	wood	0.2-1	0.03-0.3	0.02-0.3	-	-	Taherzadeh et al., 1997a
Wet oxidation (2 g/L Na_2CO_3; 195^oC; 15 min.; 12 bar O_2)	sugarcane	0.4	0.06	0.2	0.1	0.07	Martin et al., 2001
Steam explosion with SO_2	Corn stover	0.2	0.01	1.1	-	0.5	Öhgren et al., 2006a

The study also demonstrated that growth of micro-organisms is more sensitive to furfural than is to ethanol production. Studies have suggested that furfural and HMF inhibit key enzymes in glycolysis such as hexokinase and triosephosphate dehydrogenase (Banerjee et al., 1981). In addition, enzymes associated with citric acid cycle and ethanol formation, such as alcohol dehydrogenase and aldehyde dehydrogenase, have also been suggested (Taherzadeh et al., 2000). Both furfural and HMF are metabolized by *Saccharomyces cerevisiae* into furfuryl alcohol and 5-hydroxymethyl furfuryl alcohol, respectively (Taherzadeh et al., 1997b; Weigert et al., 1988). Synergistic action of furfural and HMF on fermentation has also been demonstrated by Taherzadeh et al. (2000). The study showed that addition of furfural (0.2 mg/g) and furfural (0.2 mg/g) together to batch cultivations of *S. cerevisiae* caused the CO_2 evolution rate to decrease by 62%.

3.6.2.3 WEAK ACIDS: ACETIC ACID AND FORMIC ACID

Acetic acid is formed as a result of hemicellulose degradation, while formic acid results from breakdown of furfural and HMF (Ulbricht et al., 1984). Enzymatic hydrolysis and fermentation of biomass depends crucially on pH. The dissociation constants (pK_a) of acetic acid and formic acid at 25 ^oC are 4.75 and 3.75, respectively. Due to partial dissociation and lipo-soluble nature of acetic and formic acid, they can diffuse across the plasma membrane of cells. The mechanism of inhibition by weak acids is based on the inflow of undissociated acid into the cytosol of micro-organisms (Axe and Bailey, 1995). The neutral intracellular pH causes the acid to dissociate, thus dropping down the cytosolic pH. However, a study by Larson et al. (1998) demonstrated that low amounts of weak acids (<0.6 mg/g) in hydrolyzate enhanced ethanol yield at pH 5.5 and vice versa.

3.6.3 REMOVAL OF INHIBITORS

Several biological, physical and chemical methods have been studied for the removal of inhibitors from the substrate prior to fermentation. The removal methods need to be selected based on the type of inhibitor, substrate and micro-organism used. Physical methods require steam treatment of substrate, chemical methods are based on treatment with strong alkali and sulphite and biological methods make use of enzymes such as peroxidases and laccase (**Table 3.3**).

Among biological methods, treatments with peroxidases, laccase and *Trichoderma reesei* are widely used for inhibitor removal prior to enzymatic hydrolysis and fermentation. Laccase (*p*-diphenol oxidase; E.C. 1.10.3.2) is a polyphenoloxidase which catalyzes the oxidation of number of phenolic compounds (Chiacchierini et al., 2004; Dur´an and Esposito, 2000). Phenolic compounds released as a result of lignin disruption during pre-treatment can be polymerized by oxidation using laccase. The catalysis occurs due to reduction of one oxygen molecule to water and oxidation of electrons from aromatic compounds. Laccase is an extracellular secretion of several fungi, white rot-fungi being the most studied ones (Shraddha et al., 2011). Laccase from *Trametes versicolor* has been studied for fruit juice and wine stabilization (Artik et al., 2004; Minussi, 2007). Also, this enzyme finds application in phenolic removal in lignocellulosic bio-conversion (Jönsson et al., 1998). A study by Palmqvist et al. (1997) demonstrated that soft-rot fungus, *Trichoderma reesei*, degraded inhibitors in a hemicellulose hydrolysate obtained after steam pretreatment of willow, thereby resulting in four times increase in ethanol yield. While laccase has been reported to be effective against phenolic compounds, *T. reesei* was found to degrade acetic acid, furfural and benzoic acid derivatives in pre-treated hydrolyzate (Palmqvist et al., 1997).

One of the physical removal method of inhibitors include rotovaporation followed by suspension. A study by Wilson et al. (1989) showed that the effect of fermentation of an acid hydrolysate of aspen with *Pichia stipitis* increased ethanol yield from 0 to 13% of a reference fermentation. The fermentation medium contained reduced inhibitors after roto-evaporation almost to dryness and subsequent resuspension of the residue in fermentation medium. The increase in ethanol yield was ascribed to a decrease in the concentration of acetic acid, furfural and vanillin by 54%, 100% and 29%, respectively, compared with the concentrations in the hydrolyzate. However, the economic feasibility is challenged due to high costs involved in the process.

Chemical methods of inhibitor removal include treatments with calcium hydroxide, sodium hydroxide and sulphite (Olsson et al., 1995; Palmqvist and Hahn-Hägerdal, 2000; Palmqvist et al., 1998). According to a study by van Zyl et al. (1988), alkali treatment (increasing pH to 9-10) with calcium hydroxide ($Ca(OH)_2$) and readjustment to 5.5 with sulfuric acid has been reported to result in higher fermentability than reference fermentation. The mechanism of action is suggested as precipitation of inhibitors (van Zyl et al., 1988). Also it could be due to the instability of some inhibitors at high pH. Furthermore, a study by Larsson et al. (1999) described that treatment of dilute-acid hydrolysates of spruce with sodium sulphite led to decrease in the concentrations of furfural and HMF. Synergistic effect of sulphite and calcium hydroxide has been shown to be more efficient in degrading inhibitors in hemicellulose hydrolysate prior to fermentation by recombinant *Escherichia coli* (Olsson et al., 1995). However, this detoxification of inhibitors was at the expense of increased process cost and sugar loss. Furthermore, $Ca(OH)_2$ based method seems less feasible for a full-scale

ethanol plant because of risk of calcium salt precipitation, that can foul distillation columns, evaporators and heat-exchanger surfaces.

3.6.4 ENZYMES FOR HYDROLYSIS OF APPLE PROCESSING BY-PRODUCTS

This is the second step of bio-conversion technology after pre-treatment. Apple processing by-products consisting of polysaccharides such as pectin, cellulose and hemicellulose can be converted into monosaccharides, disaccharides and oligosaccharides by the action of pectinolytic, cellulolytic and hemicellulolytic enzymes respectively. Enzymatic hydrolysis is preferred over chemical conversion strategies because of higher product yields, negligible by-product formation, low energy requirements and environmentally friendly processing (Saha, 2000; Wingren et al., 2005).

3.6.4.1 ENZYMES FOR DEPOLYMERIZATION OF PECTIN

3.6.4.1.1 PROTOPECTINASES

Protopectinase (PPase) is used to convert insoluble protopectin (water insoluble) into soluble pectin (Jayani et al., 2005). Further, PPases can be divided into two categories depending upon the region they act on. A type protopectinase acts on the polygalacturonic acid region of protopectin, while B type acts on the polysaccharide chain connecting polygalacturonic acid and cell wall constituents (Jayani et al., 2005).

Table 3.3 Summary of methods used for removal of inhibitors arising from pre-treatment of lignocellulosic biomass.

Method	Treatment	Mode of action	Inhibitors removed	References
Biological	Lignin peroxidase, manganese peroxidase, Laccase (*Trametes versicolor*), glyoxal oxidase	Oxidative polymerization	Low molecular weight phenolic compounds	Jönsson et al., 1998; Cullen, 1997;
	Fungus (*Trichederma reesei*)	Change in content or structure of phenolic compounds	Acetic acid, furfural and benzoic acid derivatives	Palmqvist et al., 1997
Physical	Rotoevaporation of hydrolyzate followed by resuspension	Evaporation of unwanted compounds	Acetic acid, furfural and some phenolic compounds	Wilson et al., 1989
Chemical	Sodium hydroxide, Lime [$Ca(OH)_2$]	Precipitation of toxic compounds at high pH	Furfural, HMF	Palmqvist., 1998
	Sulphite	Subduction of unfavorable reduction potential	Furfural, HMF	Larsson et al., 1999; Palmqvist and Hahn-Hägerdal., 2000
	Lime and sulphite combined	Precipitation of toxic compounds	Furfural, HMF, Hibbert's ketones and aldehydes	Olsson et al., 1995; Van Zyl et al., 1988

3.6.4.1.2 PECTINESTERASES

Pectinesterase (PE; E.C. 3.1.1.11; **Figure 3.6**) is responsible for hydrolysis of pectin by removal of methoxyl esters, resulting in acid pectins and methanol (Whitaker, 1984). Sources of PE can be plants, bacteria and fungi (Hasunuma et al., 2003). According to Jayani et al. (2005), PEs are stable at a temperature range of 40-50 ^{o}C and have a pH range between 4.0-8.0 with fungal PE having a lower pH optima than bacterial PE.

3.6.4.1.3 POLYGALACTURONASES

Polygalacturonases (PGs) hydrolyze the polygalacturonic acid chain. Endo PGs (E.C. 3.2.1.15) are found in bacteria, yeast and fungi (Sakai et al., 1993 while exo-PGs (E.C. 3.2.1.67) can be of fungal origin (with monogalacturonic acid as the end product) as well as of bacterial origin (with digalactouronic acid as the end product; Luh and Phaff, 1951). PGs perform best within a pH range of 3.5-5.5 and temperature range of 30-50 ^{o}C (Jayani et al., 2005).

3.6.4.1.4 PECTATE LYASES

Pectate lyases (PLs) depolymerize pectic substances by a trans-eliminative split at C-4 and simultaneous removal of H atom at C-5 (Sakai et al., 1993). They can be endo PL (E.C. 4.2.2.2), exo PL (E.C. 4.2.2.9), endopolymethylgalacturonate lyase (E.C. 4.2.2.2) and exopolymethylgalactouronate lyase (E.C. 4.2.2.9; Jayani et al., 2005). Commercial PLs can be of both fungal and bacterial origins have pH optima of 7.5-10.0 and temperature optima of 40-50 ^{o}C.

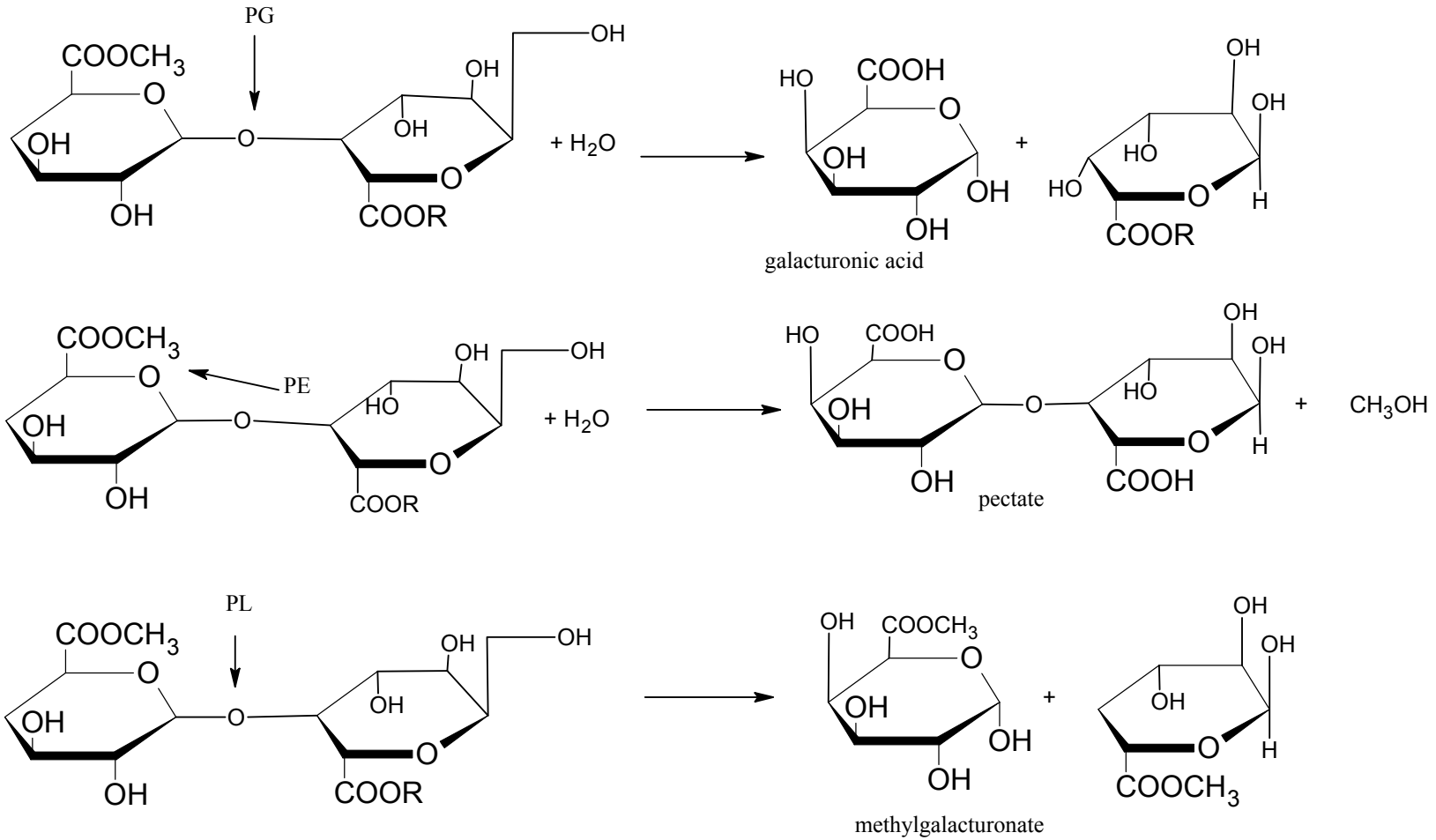


Figure 3.6 Mode of action of pectinases.

3.6.4.2 ENZYMES FOR CELLULOSE HYDROLYSIS

Cellulose can be converted into glucose via a hydrolysis process involving three types of enzymes called cellulases. Endoglucanases (E.C. 3.2.1.4) are the class of cellulases that are responsible for the cleavage of internal β-1→4 D-glycosidic bonds at random positions in amorphous cellulose polysaccharide chains, yielding oligosaccharides of varying chain lengths and generating new chain ends (**Figure 3.7;** Lynd et al., 2002). Exoglucanases, including cellodextrinases (E.C. 3.2.1.74) and cellobiohydrolases (E.C.3.2.1.91) cleave β-1→4 D-glycosidic bonds from reducing or non-reducing ends of cellulose chains (microcrystalline structure), and produce either glucose or cellobiose units. Cellobiose is the repeating unit of cellulose with two glucose molecules linked through β-1→4 glycosidic bonds and can be further converted into glucose by β-glucosidase (E.C. 3.2.1.21; **Figure 3.8;** van Rooyen et al., 2005). β-glucosidase also hydrolyzes soluble cellodextrins to glucose (Lynd et al., 2002). These three steps occur simultaneously to degrade cellulose (Lynd et al., 2002). During enzymatic hydrolysis, the accumulation of excess cellobiose in the product inhibits the further hydrolysis by cellulases (Howell et al., 1975).

Cellulases can be produced by various bacteria (*Streptomyces lividans* and *Cellulomonas fimi*) and fungi (*Trichoderma reesei* and *Humicola grisea*), out of which *Trichoderma reesei* is found to be very efficient (Sandgren et al., 2005). A common characteristic of most fungal cellulases is a modular structure which contains both catalytic and carbohydrate-binding modules (CBMs), joined together by a flexible linker peptide (Beguin, 1994; van Tilbeurgh, 1986). The presence of CBMs is particularly important for the function of exoglucanases, since they act on the micro-crystalline

structure of insoluble cellulose (Teeri, 1997). The CBM is assumed to be responsible for binding to the cellulose surface to facilitate cellulose hydrolysis by bringing the catalytic domain in close proximity to the insoluble cellulose.

Cellulase enzyme systems exhibit a synergistic effect on the substrate, which means that higher collective activity than the sum of the activities of individual enzymes. Four forms of synergism have been reported: (i) endo-exo synergy between endoglucanases and exoglucanases, (ii) exo-exo synergy between exoglucanases processing from the reducing and non-reducing ends of cellulose chains, (iii) synergy between exoglucanases and β-glucosidases that remove cellobiose as end products of the first two enzymes, and (iv) intramolecular synergy between catalytic domains and CBMs (Lynd et al., 2002).

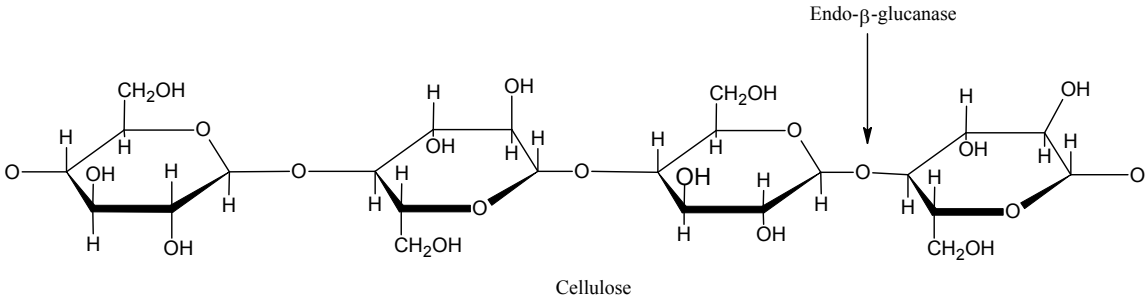


Figure 3.7 Mode of action of cellulose

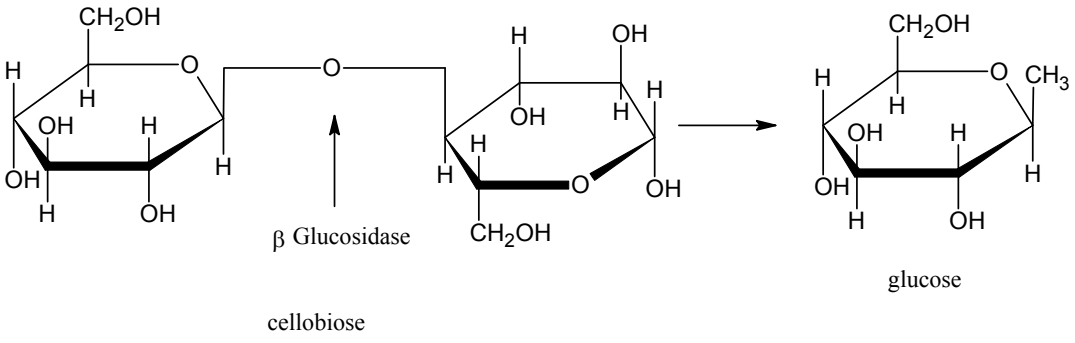


Figure 3.8 Mode of action of β-glucosidase on cellobiose

3.6.4.3 ENZYMES FOR HEMICELLULOSE HYDROLYSIS

Besides pectinases and cellulases, xylanases are also found to assist lignocellulosic hydrolysis process by depolymerizing hemicelluloses (Shallom and Shoham, 2003). Since hemicellulose is a complex mixture of pentose and hexose sugars embedded in cellulose matrix, hydrolyzing micro-organism undergoes several intrinsic challenges. For efficient hydrolysis of hemicellose, an array of glycoside hydrolases and carbohydrate esterases such as endoxylanase (E.C. 3.2.1.8), β-xylosidase (E.C. 3.2.1.37), α-L-arabinofuranosidase (E.C. 3.2.1.55), α-glucuronidase (E.C. 3.2.1.139), α-galactosidase (E.C. 3.2.1.22) and acetylxylan esterase (E.C. 3.1.1.72) are required (Shallom and Shoham, 2003). During hemicelluloses hydrolysis, endo-xylanase randomly attacks the main chains of xylans, and β-xylosidase hydrolyzes xylooligosacharides to xylose. The α-L-arabinfuranosidase, α-glucuronidase and α-galactosidase release arabinose, 4-*O*-methyl glucoronic acid ad galactose substituents, respectively, from the xylan backbone (Saha, 2000).

The enzymatic transformation of a substrate into product by a glycoside hydrolase proceeds through a series of intermediates and transition states, and in many cases, is mediated via distortion/relaxation cycles of a ground-state low-energy $^4C_1$ chair conformation of the glycosyl residue. The mechanism for cleavage of C-O-C bond in cellulose proceeds through protonation of glucosides. The mechanism of hydrolysis in a hydrolase enzyme occurs using a proton donor and nucleophile or base. The reaction passes through a series of intermediates and transition states (White and Rose 1997). An intermediate complex with the oxygen and proton is thought to be formed. This is followed by addition of a water molecule to the anomeric (1) carbon of a glucose unit in the cellulose, which causes breaking of the chain due to removal of bridge oxygen with

the other (4) carbons. This process occurs in exo-fashion, separating one or two glucose molecules at a time.

3.6.5 MICRO-ORGANISMS FOR BIO-ETHANOL PRODUCTION

Baker's yeast, *S. cerevisiae*, is one of the oldest and best characterized micro-organism available to date for ethanol production. The specific yeast has high ethanol productivity and is capable of better tolerating fermentation inhibitors compared to other known microorganisms (Clark and Mackie, 1984; Larsson et al., 2001; Martin et al., 2003). According to studies carried out by Hahn-Hagerdal et al. (2006) and Palmqvist et al. (2001) *S. cerevisiae* is capable of tolerating process derived fermentation inhibitors (such as acetic acid, furfural and HMF) below a cretain concentration. This specific concentration varies with the type of strain used and fermentation conditions. However, lower ethanol yields below theoretical levels have been observed when the concentration level of fermentation inhibitors goes beyond threshold (Robinson, 2003). Theoretical yield of ethanol from apple pomace has been reported to be 78% using 0.1 g/L of yeast (Jarsoz, 1988). Genetically engineered yeast strain of *S. cerevisiae* has been used for increased yield of ethanol from apple pomace (Knor and Sinskey, 1985; **Table 3.4**).

Other than yeast, some bacteria have been widely used for fermentation of six carbon sugars present in apple processing waste. Galacturonic acid in apple pomace cannot be fermented by yeast. Hence, *Esherichia coli* K011, a recombinant strain, was developed which carries the *pet* operon on its chromosome (Ohta et al., 1991; **Table 3.5**). Briefly, pyruvate decarboxylase and alcohol dehydrogenase II genes from *Zymomonas mobilis* were incorporated into the chromosome of *E. coli* to produce *E. coli* KO11, which can ferment galacturonic acid and other sugars present in apple pomace, though at a smaller rate than by yeast (Grohmann 1995a; Grohmann and Baldwin, 1992).

There is still a need for a recombinant organism that produces high ethanol, has high tolerance to fermentation inhibitors and can utilize a broader range of sugar substrates.

3.6.6 MICRO-ORGANISMS FOR VINEGAR PRODUCTION

The origin of vinegar making process can be dated back to spoilage of alcoholic beverages. Traditionally, vinegar has been produced by alcoholic fermentation followed by fermentation using acetic acid bacteria (AAB). The acetic acid bacteria are a group of gram-negative bacteria having motile rods that work under aerobic conditions to carry out incomplete oxidation of alcohol and sugars, leading to the accumulation of organic acids as end products (Madigan et al., 2008). AAB are heterogeneous assemblage organisms, most of which are mesophilic with a temperature range of 25-40 ^{o}C (Madigan et al., 2008; Sokollek et al., 1998). Among several genera of AAB, *Acetobacter* and *Gluconobacter* species are more discussed as they are used mostly for industrial purposes. *Acetobacter* is a gram negative, obligate aerobe coccus or rod shaped bacterium with the size of 0.6-0.8 X 1.0-4.0 μm, (motile or non-motile). Some strains are capable for converting acetic acid to carbon dioxide and water. *Acetobacter* uses ethanol as carbon source preferably and flourishes during wine fermentation (Drydale and Fleet, 1985; Du-Toit and Lambrechts, 2002; Joyeux et al., 1984; Kadere et al., 2008). The strains of *Acetobacter* have been isolated from several natural sources such as grape, date and palm resources and coconut (Kadere et al., 2008). The growth and survival of AAB depends on various factors such as ethanol concentration, acetic acid concentration, oxygen, temperature and nutrient availability. A study by Nanda et al. (2001) showed that acetic acid concentration below 10 g/L resulted in significant increase in the growth rate of AAB (particularly at low ethanol concentration).

Table 3.4 Performance of some hexose and pentose fermenting microbial strains under laboratory conditions.

Microbial strain	Substrate	Ethanol yield (g/g initial sugar)	Fermentation time (h)	Reference
S. cerevisiae 424A (LNF-ST)	Corn stover	0.41-0.45	24	Sedlak et al., 2004
S. cerevisiae TMB3006	Spruce	0.37	96	Hahn-Hägerdal and Pamment, 2004
S. cerevisiae TMB3400	Spruce, corn stover	0.30-0.33	96	Öhgren et al., 2006b
Escherichia coli K-O11	Sugarcane baggase, corn fiber	0.49	N/A	Hahn-Hägerdal and Pamment, 2004

N/A, not available

However, acetic acid concentration above 20 g/L hampered the growth of AAB severely, with virtual inhibition at 50 g/L acetic acid irrespective of the amount of ethanol present. Among the most important acetic acid bacteria, the strains of genus *Acetobacter* are mainly involved in vinegar production (Kadere et al., 2008; Sokollek et al., 1998).

3.7 COMMERCIAL PRODUCTION OF VINEGAR

Household vinegar is about 5 g/L acetic acid. There are several methods to prepare vinegar on commercial basis including submerged state fermentation (developed by Hromatka and Ebner, 1951a, b), fed batch fermentation, liquid state fermentation and semi-continuous mode of fermentation (De Ory et al., 2004). Several technical modifications in terms of aeration, stirring, heating etc. have been done to regulate the

fermentation conditions. Generally, acetic acid fermentation of fruit-based ethanol is carried out at a temperature range of 25-35 ^{o}C for about 7-10 days using *Acetobacter aceti* as the fermentation micro-organism. Multiplication of bacteria and acidification should occur simultaneously. The rate of multiplication of *Acetobacter* depends on the acetic acid concentration in the fermentation media. In general, the multiplication rate slows down at 13-15 g/L acetic acid and above 15 g/L it stops. Berroud (2000) showed the possibility of making highly concentrated vinegar (170-180 g acetic acid/L) using acetic acid bacteria beyond their viability. The study showed 120 g/L value of acetic acid concentration to be the limiting factor in reaching high acetic acid concentration. A study by Park et al. (1989) showed that the number of viable acetic acid bacteria stops increasing above 40 g acetic acid/L. They also showed that the bacterial population was inhibited by ethanol (the substrate) at about 40 g/L. In order to resolve this problem, fed batch system was launched to maintain the substrate concentration by selective feeding. Vinegar can be prepared by a freeze concentrating process in which vinegar with lower percentage acidity is subjected to freezing. The ice formed is separated out by centrifugation and thus the vinegar left behind contains higher percentage acetic acid. This process is economically not feasible because of high input involved in terms of energy.

CHAPTER 4: DETERMINATION OF CHEMICAL COMPOSITION OF APPLE PROCESSING BY-PRODUCTS AS FEEDSTOCK FOR BIO-CONVERSION

4.1 ABSTRACT

Processing of apple for numerous food products generates significant amounts of by-products which are underutilized and cause disposal problems. These by-products are very rich in carbohydrates and are potential feedstocks for enzymatic and microbial conversions to bio-fuels and other value-added products. They contain large amounts of soluble sugars and polysaccharides, especially cellulose, hemicellulose, lignin and pectin. Bio-conversion of lignocellulosic biomass is hindered by the structural and chemical complexity of biomass. This makes this lignocellulose biomass a challenge to be used as feedstock for value-added products. The aim of the present study was to determine the chemical composition of apple processing by-products. In addition to this, the cell wall isolates from freeze dried apple processing by-products were assessed for their polysaccharide and monosaccharide composition. The results showed the presence of high fibre, low protein and low lipid content. Freeze dried apple processing by-products revealed the presence of high amount of free glucose which has the potential to be utilized by micro-organisms. On a freeze dry basis, the polysaccharide composition of apple processing by-products was found to be 20% cellulose, 5% hemicellulose and 9% pectin. In addition, lignin content was about 19% of freeze dried apple processing by-products. The cell wall analysis showed that the dominance of cellulose over all other polysaccharides, representing about 45% of the isolated cell wall material. Hemicellulose and lignin accounted for 35.5% and 11% of cell wall isolate, respectively. The cell wall isolate contained high amount of xylose (101 mg/g cell wall isolate), followed by arabinose (37.9 mg/g cell wall isolate) and galactose (18.8 mg/g cell wall isolate). Besides, minor amounts of other neutral sugars such as mannose, fucose and rhamnose were also noted. All polysaccharides in these residues can be hydrolyzed to monomeric sugars by mixtures of cellulase, hemicellulase and pectinase enzymes. Microbial conversions of sugar rich hydrolyzates from these residues can be further performed by using specific yeast and bacteria that can utilize five carbon sugars along with six carbon sugars.

Keywords: Apple processing by-products, lignocellulosic biomass, cell wall isolates, polysaccharide, monosaccharide, cellulose, hemicellulose, lignin

4.2 INTRODUCTION

The growth of global horticulture industries generates large quantities of fruit waste (25%–30% of total fruit weight) (Sargent et al., 1984; Shah et al., 1994). In Nova Scotia itself, the apple processing industry produces significant amounts of by-products every year (Rupasinghe and Kean, 2008). These apple processing by-products are comprised of pomace, peels, cores, seeds, stems and rejected apples. Apple processing biomass contains a large amount of water and thus can easily ferment, leading to serious disposal-related issues. Over the past few decades, there has been a growing global orientation towards the efficient utilization of natural resources. Owing to the presence of high amounts of reducing sugars, polysaccharides and other functionally important bio-molecules such as polyphenols, vitamins and proteins, apple processing by-products appear as an attractive feedstock for further bio-conversion into different metabolites with higher commercial value (Vendruscolo et al., 2008).

Bio-conversion of lignocellulosic biomass is impeded by structural and chemical complexity (Zaldivar et al., 2001). This makes this lignocellulose biomass a challenge to be used as feedstock for value-added industrial products such as bio-ethanol and organic acids production. The effective use of lignocellulosic matter as a substrate for bio-ethanol production is largely dependent on the extent to which it is lignified. Cellulose and hemicellulose, when hydrolyzed into their component sugars, can be converted into ethanol and/or organic acids through fermentation. Therefore, it becomes important to characterize the components of polysaccharides before proceeding to further bio-conversion steps.

The composition of biomass depends on the cultivar, growing environment, origin of apples and stage of ripening (Sato et al., 2010). Any assessment of the potential of apple processing by-products for further processing should be based on reliable data concerning its average composition and its polysaccharide characterization. Therefore, the objective of the present study was to characterize apple processing by-products for their proximate composition, dietary fibre content and polysaccharide composition.

4.3 MATERIALS AND METHODS

4.3.1 APPLE PROCESSING BY-PRODUCTS

The apple processing by-products were collected from J. W. Mason and Sons Ltd., Windsor, NS, Canada and Apple Valley Foods Inc., Kentville, NS, Canada during the year 2008-2009. One primary batch of apple processing by-products was collected randomly from the two locations. The apple processing by-products were from 'Idared' and 'Northern Spy' cultivars of apples, which were combined for further use. The collected sample was further sub-sampled for experimental purposes. Apple pomace, residue from juice extraction contained peel, core, seeds and rice husks. Rice husks are added to facilitate pressing of juice and could be present upto 5-10% of the total weight. The apple processing by-products were from 'Idared' and 'Northern Spy' cultivars of apples (combined) and were stored at -20 ^{o}C until use.

4.3.2 MATERIALS AND CHEMICALS

Reagent grade sulfuric acid (72%) and sugar standard of D-glucose was acquired from Sigma Aldrich, Oakville, ON, Canada; whereas analytical grade ethanol was obtained from Fisher Scientific, Ottawa, ON, Canada. For reducing sugars analysis, anhydrous sodium carbonate, sodium bicarbonate, sodium sulphate and copper (II) sulphate were obtained from Sigma Aldrich, Oakville, ON, Canada. The potassium

sodium tartrate, ammonium molybdate and disodium arsenate were obtained from Fisher Scientific, Ottawa, ON, Canada.

4.3.3 PROXIMATE ANALYSIS

Proximate analysis partitions compounds present in a feedstock into six categories (moisture, ash, crude protein, crude lipid, crude fibre and digestible carbohydrates) based on its chemical properties. Apple processing by-products were analyzed for proximate composition using methods of the AOAC (2006): moisture (Method 965.08B), protein (Method 984.13 (A-D), crude fat (Method 920.39 (A)) and ash (Method 942.05). The raw material was also subjected to mineral analysis (calcium, magnesium, sodium, potassium, magnesium, manganese, copper zinc and phosphorous) according to the methods of AOAC (Method 968.08). All the determinations were carried out at Laboratory Services of Nova Scotia Department of Agriculture, Harlow Institute, Truro, NS.

4.3.4 FIBRE ANALYSIS

Soluble and insoluble fibre content of apple processing by-products were analyzed using the method (Method 991.43) of AOAC (2006) at Maxxam Analytics Inc**.** (Mississauga, ON, Canada), an ISO 17025 registered analytical laboratory. Acid detergent fibre *(*AOAC method 973.18 (A)) and neutral detergent fibre (Holst, 1973) were analyzed at Laboratory Services of Nova Scotia Department of Agriculture, Harlow Institute, Truro, NS. The amounts of pectin and hemicellulose were estimated by difference method according to following equations:

Neutral detergent fibre = cellulose + hemicellulose + lignin +ash

Acid detergent fibre = cellulose + lignin + ash

Hemicellulose = Neutral Detergent Fibre - Acid Detergent Fibre (Van Soest, 1991)

Total dietary fibre = Soluble fibre + Insouble fibre

Pectin = Total Dietary Fibre - Neutral Detergent Fibre

4.3.5 DETERMINATION OF REDUCING SUGARS

Total reducing sugar content of the raw material was determined using the Nelson Somogyi method (Nelson, 1944; Somogyi, 1952). This method consisted of extraction of 0.5 g (freeze dried and ground) sample with hot 80% ethanol (60^{o}C) twice (5 mL each time). The sample was centrifuged and collected supernatant was evaporated by keeping it on a water bath at 80 ^{o}C. The sugars were dissolved in 10 mL of deionized water. An aliquot of 0.2 mL was added to test tube and diluted with deionized water to 2 mL volume. This was followed by addition of alkaline copper tartrate reagent and boiling for 10 min. To the cooled test tubes, 1 mL of arsenomolybolic acid reagent was added and solution was allowed to react for 10 min. The absorbance of the solution was read at 620 nm using a Beckman DU-70 spectrophotometer (Fullerton, CA, USA). Solution of D-glucose (Sigma Aldrich, Oakville, ON, Canada) was used as standard to plot the calibration curves. The standard solution was prepared in deionized water and a range of 10-50 mg/L was used for analysis.

4.3.6 CELL WALL COMPOSITION

The monosaccharide composition of cell wall and lignin content were carried out at the Cell Wall Analytical Facility, Great Lakes Bioenergy Research Center, Michigan State University, East Lansing, MI, USA. The cell wall composition, crystalline cellulose content, lignin content and its composition in biomass cell wall were analyzed according to procedures standardized by Foster et al. (2010a, b). A brief overview of the method employed for the analysis is shown in **Figure 4.1**.

The analyses were performed on freeze-dried samples of apple processing by-products to avoid or reduce cell wall degradation due to oven drying. The determinations were carried out both on whole apple processing by-products (non-AIR), as well as on their alcohol insoluble residue (AIR). The non-AIR samples were simply hydrolyzed by trifloroacetic acid (TFA), without including any washing steps, whereas the AIR resulted from sequential treatments with solvents removing proteins, lipids and starch. Since alcohol insoluble residue involved multiple washing steps, it was suspected that it might have removed some of the wall material. The starch was removed from the residue with an amylase treatment, resulting in only cell wall material. It was assumed that any free sugar would have been removed during this process. Following the AIR treatment, the residue was weighed before and after the cell wall analysis.

Total cellulose content was determined after Updegraff treatment (acetic acid: nitric acid: water 8:1:2 v/v) and strong acid (72% sulfuric acid) hydrolysis of crystalline cellulose into glucose followed by a colorimetric assay. Total lignin content was measured according to the method reported by Fukushima et al. (1991). This method is called acetyl bromide soluble lignin method (ABSL). Lignin was extracted from cell wall using acidic dioxane and was analyzed spectophotometrically. The monomeric composition of lignin was carried out according to method developed by Robertson and Mansfield (2009). This method, called thioacidolysis, is based on lignin extraction using dioxane and solubilizing the lignin monomers, followed by derivitization and GC/MS analysis. The monosaccharide composition was determined using trifloroacetic acid treatment, followed by alditol acetate derivitization to make the compounds volatile for

GC/MS analysis. The monosaccharides were reduced using sodium borohydride to alditols and converted to their corresponding acetates after acetate anhydride treatment.

4.3.7 EXPERIMENTAL DESIGN AND STATISTICS

All analyses were carried out on three subsamples. Completely randomized designs (CRD) were used for the experiments. The data were presented as mean and standard deviation. For cell wall matrix composition, data were represented as mg/g of cell wall material.

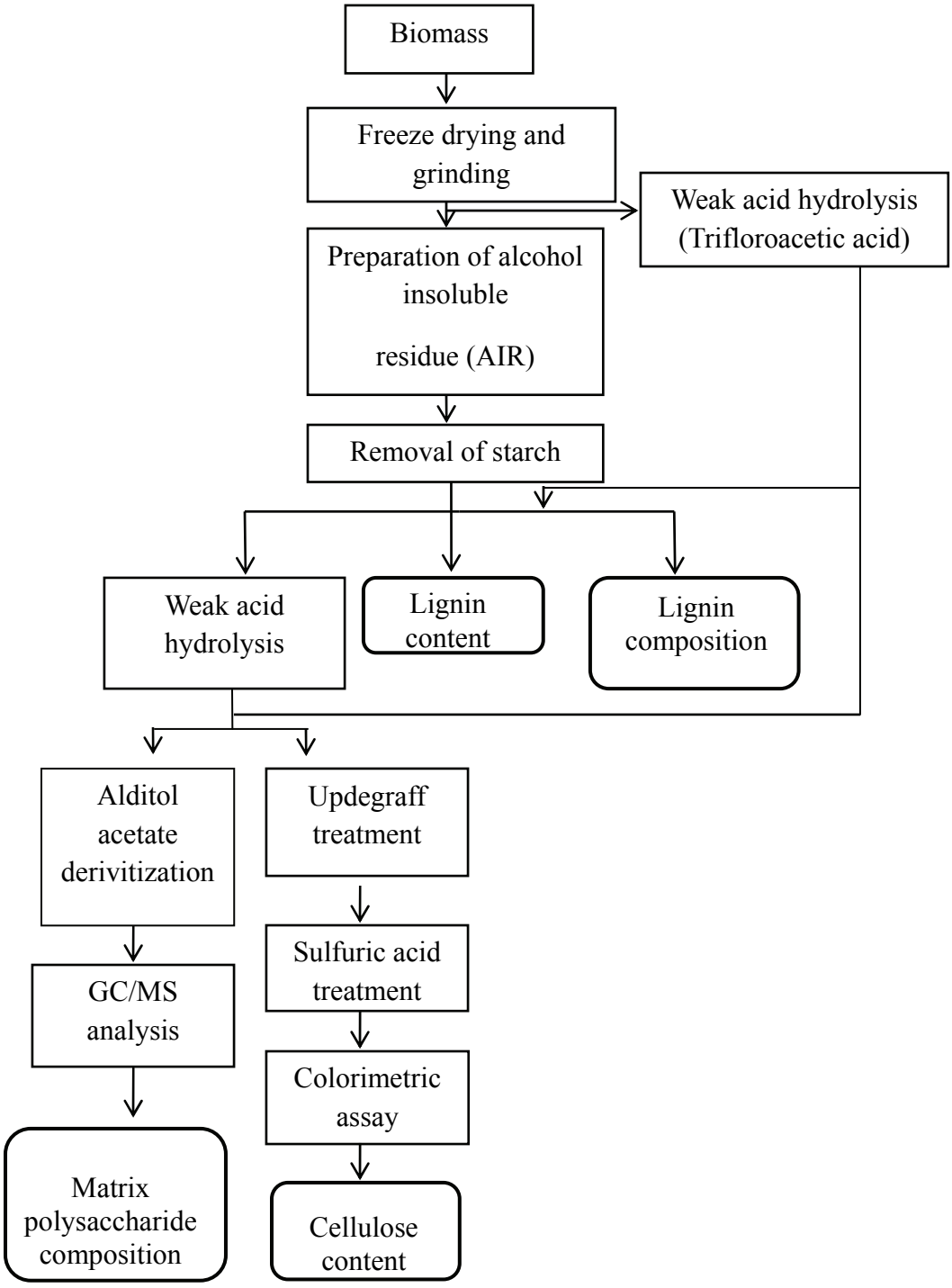


Figure 4.1 Overview of lignocellulosic analysis (Adapted from Foster et al 2010a, b)

4.4 RESULTS

4.4.1 COMPOSITION OF RAW MATERIAL

The proximate composition apple processing by-products are shown in **Table 4.1**. Moisture represented about 79.0% of the total fresh weight of the raw material. The lipid and protein content were 2.3% and 3.8% (DW), respectively. On a freeze dried basis, total reducing sugar content of processing by-products from juice industry was calculated as 18.2%. This represented the sum of total glucose and fructose in the raw material.

The apple processing by products were found to have high total dietary fibre (TDF) content and 35.0% (DW) of it was represented by insoluble dietary fibre (**Table 4.2**). The soluble fibre content of approximately 10% (DW) was represented mainly by pectin. To examine the polysaccharide content of apple processing by-products, acid detergent fibre (ADF) and neutral detergent fibre (NDF) were calculated. The NDF was found to be higher than ADF by approximately 5.2% on a dry basis. This difference between NDF and ADF represented hemicellulose content of apple processing by-products on a dry basis. The pectin content, calculated as the difference between total dietary fibre and NDF, was 9.0% (DW). The total carbohydrates calculated by difference method were found to be 89.4% (DW).

4.4.2 CELL WALL COMPOSITION

The dry matter content of lyophilized raw material was approximately 85%. Since, starch and free glucose content were expected to be present in samples with non-AIR treatment, therefore its glucose content was found to be higher than present in AIR. Unfortunately, the amount of material lost by AIR treatment could not be calculated due to small sample size used.

Table 4.1 Composition of apple pomace from juice industry.

Constituent	Value[§] (wet basis)	Value[§] (dry basis)
Moisture (%)	79.0±0.5	-
Dry matter (%)	20.9±0.5	-
Crude protein (%)	0.8±0.0	3.8±0.1
Crude fat (%)	0.5±0.0	2.3±0.0
Ash (%)	0.7±0.0	3.5±0.0
Calcium (ppm)	≤ 200±0.0	1000±0.0
Phosphorous (ppm)	200±0.0	1000±0.0
Sodium (ppm)	≤ 200±0.0	≤ 200±0.0
Potassium (ppm)	900±0.0	5000±0.0
Magnesium (ppm)	200±0.0	1000±0.0
Manganese (ppm)	4.7±0.1	22.6±0.8
Copper (ppm)	0.4±0.0	1.7±0.2
Zinc (ppm)	1.2±0.3	5.7±1.7
Total carbohydrates (%)*	18.9±0.1	89.4±0.3
Total reducing sugars (%)	3.6±0.8	18.2±4.0

[l]*The results are tabulated in weight percent of apple processing by-products.*
[§] *Values represent means ± standard deviation of three analyses*
**By difference method: total carbohydrates, (crude)=100-%(protein+ash+crude fat+moisture)*

Table 4.2 Fibre content of apple pomace from juice industry.

Constituent (%)	Value[§] (wet basis)	Value[§] (dry basis)
Soluble fibre	2.1± 0.1	10.0±0.5
Insoluble fibre	7.3±0.1	34.8±0.5
Total dietary fibre	9.4±0.0	44.9±0.0
Acid detergent fibre	6.4±0.2	30.5±1.1
Neutral detergent fibre	7.5±0.3	35.7±1.5
Pectin[a]	1.9±0.1	9.1±0.0
Hemicellulose[b]	1.1±0.1	5.2±0.4

[l]*The results are tabulated in weight percent of apple processing by-products.*
[§]*Values represent means ± standard deviation of three analyses*
[a]*Total Dietary Fibre-Neutral Detergent Fibre*
[b]*Neutral Detergent Fibre-Acid Detergent Fibre*

Thus the percentage wall composition as a basis of the original (pre-AIR) sample could not be calculated. The polysaccharide composition of AIR and non-AIR are presented in **Table 4.3**. For non-AIR treatment, results were expressed as mg/g of freeze dried apple processing by-products; whereas for AIR treatement results were expressed in mg/g of the cell wall material.

The non-AIR consisted mainly of glucose, xylose, arabinose and minor contents of mannose, fucose and rhamnose as well. On a freeze dried basis, the non-AIR treated apple pomace from the juice industry was composed of 110 mg/g glucose, 38 mg/g of xylose, 27 mg/g of arabinose and 12 mg/g of galactose. Besides glucose, the neutral saccharides xylose and arabinose, were found to be in high quantities, representing a total of 65 mg/g on freeze dry basis. These three sugars represented more than 85% of all the sugars found. The glucose here refers to hemicellulosic glucose (as released by TFA hydrolysis), whereas crystalline cellulose derived glucose is the material obtained after Updegraff treatment and concentrated sulfuric acid treatment.

Table 4.3 Characterization of cell wall of apple processing by-products

Component	Non-AIR[2] mg/g lyophilizate	AIR[3] mg/g cell wall isolate
Hemicellulose		
Glucose	109.7±9.7	13.4±1.7
Xylose	37.5±3.1	101.7±5.7
Arabinose	27.2±1.2	37.9±5.5
Galactose	11.5±0.2	18.8±2.1
Mannose	5.1±2.4	3.6±0.5
Rhamnose	4.2±0.8	5.7±0.9
Fucose	2.2±0.2	2.7±0.4
Cellulose		
Crystalline cellulose	199.1±25.7	452.9±19.8
Lignin[¥]	193±6.0	111±8.4

[2] *AIR-Alcohol Insoluble* Residue
[§] *Values represent mean±standard deviation of three analyses*
[¥] *General extinction coefficient of 20 $g^{-1}Lcm^{-1}$ used for calculations.*

Since most soluble fractions of apples are removed in the AIR, the remaining insoluble components consist of macromolecular cell wall materials such as cellulose, hemicelluloses and pectin. In contrast to the non-AIR material, the free glucose in AIR was found to be quite low (13.4 mg/g of cell wall material), representing just 1% of cell wall matter. Crystalline glucose content was found to dominate all other polysaccharides present in the cell wall, representing more than 45% of cell wall material. This crystalline glucose represented the glucose that was released from cellulose hydrolysis. Arabinose and rhamnose units (making part of rhamnogalacturonan and arabinoxylan) accounted jointly for 43.2 mg/g of the cell wall material. Xylose, mannose and galactose units, which make part of hemicellulosic polymers (e.g. arabinoxylan and arabinogalactan), accounted for 124.1 mg/g cell wall material. The amount of xylose was found to be (101.7 mg/g cell wall material), which represented approximately 13% of cell wall material. In addition to this, the components of pectin (rhamnose and galactose)

together represented around 2.5% of cell wall material. Overall, the neutral sugar content of cell wall was found to be 177 mg/g of cell wall material. This neutral sugar content was calculated by excluding galacturonic acid composition of cell wall isolates.

4.4.3 LIGNIN CONTENT AND ITS COMPOSITION

Both the AIR and non-AIR samples were subjected to lignin analysis. The lignin content of the non-AIR treated sample was 19.3% of freeze dried apple processing by-products. On the other hand, lignin content of AIR was 11.1% of cell wall material (**Table 4.3**). Apparently, the amount of lignin content of AIR material was lower than non-AIR material because of removal of some mass after washing. The presence of rice hulls in the raw material was thought to have contributed to the total lignin content.

The monomer composition of lignin is presented in **Figure 4.2**. On a freeze-dried basis, the non-alcohol treated apple processing by-products (non-AIR) were found to contain 0.4 mg/g of 4-hydrolxyphenyl, 7 mg/g of guaiacyl and 3 mg/g of syringyl units. On the other hand, the alcohol insoluble fraction (AIR) consisted of 1, 15 and 6 mg/g of 4-hydrolxyphenyl, guaiacyl and syringyl units, respectively. The results showed that 4-hydrolxyphenyl units formed a minor portion of the total lignin content. Dominance of guaiacyl units in both AIR and non-AIR treatments was apparent from the composition of lignin present in apple processing by-products.

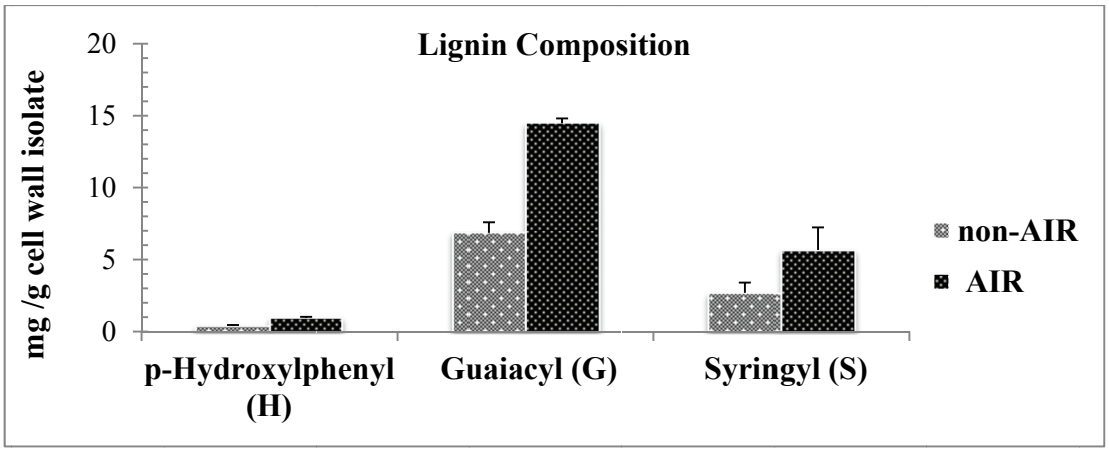


Figure 4.2 Lignin composition of freeze dried apple processing by-products
AIR- Alcohol Insoluble Residue

4.5 DISCUSSION

4.5.1 COMPOSITION OF RAW MATERIAL

The lipid content (2.3%) of apple processing by-products was 23-51% lower than the values ranging between 3.0-4.7 g/100 g as reported by other authors (Cho and Hwang, 2000; Joshi and Attri, 2006; Smock and Neubert, 1950; Sudha et al., 2007). The variation in seed composition could be the most probable source of variation in the lipid content. The protein content in the samples was approximately 3.8%, which a little lower than a range of 4.5-5.7% reported by Smock and Neubert (1950) and considerably lower than the 11.40% reported by Cho and Hwang (2000). The ash content was found to be 3.5% in the present study. Smock and Neubert (1950) reported similar results ranging from 2.1 to 3.5%. However, the ash content of the present study was found to be higher than the range of 0.6-1.7% reported by other studies (Cho and Hwang, 2000; Gullón et al., 2007; Joshi and Attri, 2006). The variation may be due to the difference in cultivars and ripening stage. The data for mineral content (K, Mg, Mn, Cu, Zn and P), except for sodium (≤0.02%) was found to be similar to other studies (Bhushan et al., 2008; Joshi and Attri, 2006). The mineral content of the raw material is important for making fermentation media for further bio-conversion into bio-ethanol and organic acids.

The total dietary fibre (TDF) content of apple processing by-products was similar to the 33.4-51.8% (DW) range for 11 different cultivars of apple pomace as reported by Sato et al. (2010). However, a small part of the total fibre content in the present study may be due to presence of rice husks used as processing aid while pressing apples for juice. In addition to this, it was assumed that rice hull (19-21% silica) contributed to total carbohydrate content and ash content. Acid detergent fibre (ADF) and neutral detergent fibre (NDF) are important in determining the content of cell wall polysacharides. They

measure the possibility of apple processing by-products as a substrate for enzymatic hydrolysis for producing fermentable sugars. NDF from a food sample contains cellulose, hemicellulose and lignin as cell wall constituents, whereas ADF comprises most of the cellulose, lignin, a portion of the pectin and minor amounts of the hemicelluloses (Kurasawa et al., 1982). The pectin content of apple processing by-products as determined by difference method (TDF-NDF) was 9.1% (DW). This value supported the previously reported studies, which described apple pomace to contain 6.7-15.0% of pectin on a dry weight basis (Hours et al., 1988; Toyokawa et al., 1980; Wang and Thomas, 1989). Pectin content is important as it can yield fermentable sugars such as galacturonic acid upon hydrolysis by pectinases. Galacturonic acid can further be fermented into bio-ethanol using genetically engineered strain of *Escherichea coli* KO11 (Grohmann et al 1995). Previous studies by Givens and Barber (1987) reported a cellulose content of 12.0-23.2% of cellulose content in apple pomace.

4.5.2 CELL WALL COMPOSITION

The quantification of monosaccharides following acid hydrolysis is commonly used to obtain an overview of polysaccharide composition (Arnous and Meyer, 2008; De Ruiter et al., 1992; Meseguer et al., 1999). About 11% of glucose content was observed after TFA hydrolysis of lyophilized apple processing by-products (non-AIR). Partial hydrolysis of polysaccharides (mainly cellulose and hemicellulose) could also have contributed to this glucose content. These results agree to the previously reported range 6.1-13.3% DW by Wang and Thomas (1989) and Waugh (1981). Besides glucose, the neutral saccharides arabinose and xylose dominated. Rhamnose was also present as a typical unit of pectin. Other saccharides such as fucose were found in negligible amounts. It is well known that the soluble sugars in apple pomace are fructose (18-31%),

glucose (2.5-12.4%) and sucrose (1.4-5.2%) with fructose being the dominant component (Hours et al., 1988; Lee and Mattick, 1989; Queji et al., 2010; Voget et al., 1985). The crystalline glucose content, represented by cellulose present in the raw material, was found to be approximately 200 mg/g. This contributed about 20% of freeze dried apple processing by-products. This appears to be a considerable amount that can yield glucose monomers after treatment with suitable carbohydrolases such as cellulase. The resultant glucose has a high potential to be converted into bio-ethanol using yeast such as *Saccharomyces cerevisae* under suitable conditions. Previous studies have reported a range of 12.0-23.2% DW for cellulose, depending on the variety of apples used (Given and Barber, 1987). The poor hydrolyzing capability of cellulose by TFA has been described previously (Carnachan and Harris, 2000), therefore crystalline glucose is the amount left after TFA hydrolysis. The TFA hydrolyzate of apple processing by-products includes sugars which would also be extracted during enzymatic hydrolysis. In addition to these, the non-AIR hydrolyzate also contains pectin components such as galacturonides (not measured) and rhamnose. The total pectin content of apple processing by-products was found to be 9.1% (DW). This value supports the pectin range of 5.0-16.0% DW in apple pomace as reported by Marcon et al. (2005).

Alcohol-insoluble residue (AIR) is a suitable way for the characterization cell wall material. The cell wall of apples is composed of different polysaccharides, such as pectin, hemicelluloses, and cellulose (Stevens et al., 1984), as well as structural proteins and lignin. The composition of AIR reveals the potential of bio-conversion of apple processing by-products into bio-ethanol and other value added products such as organic acids. The total amount of alcohol insoluble solids could not be calculated due to smaller

size of sample used. However, it has been previously shown that on a dry weight basis, apple pomace contains 11.0-20.4% of alcohol insoluble solids (Grohmann and Baldwin, 1992). The cell wall matrix analysis performed on the AIR revealed the presence of high amount of cell wall components such as crystalline cellulose and hemicellulose in the raw material, thereby demonstrating their potential to yield fermentable sugars upon enzymatic hydrolysis. The non-crystalline glucose here represented hemicellulosic glucose and/ o glucose derived from amorphous cellulose. The considerably lower amount of glucose in AIR showed that several washing steps during the preparation of AIR led to the removal of majority of free glucose. It has been previously shown that solvent washing could loosen skin material from intercellular liquid, (rich in glucose and fructose) and could remove any free monosaccharides (Larrauri, 1999). Arabinose (38 mg/g) and galactose (19 mg/g) were present in considerable quantities in the cell wall material. A previous study by Fisher and Bipp (2005) indicated the abundance of these two monomers in apple cell walls. The presence of arabinose is an indicator of highly branched R-L-arabinofurans (arabinans) linked to rhamnogalacturonan region of pectin (Schols et al., 1994). The presence of mannose indicated that a minor amount of mannan was also present, and the fucose and xylose content suggested that xyloglucan was a large proportion of the cell wall isolate. Rhamnose was also present as a typical unit of pectin.

Since starch present in immature apples gets converted into simple sugars with the ripenening of fruit, it was assumed that this was the “free” glucose content. In addition to this, the total hemicellulose content of the cell wall (calculated as sum of individual sugars) was found to be higher than the data reported by other studies (Lee

and Mattick, 1989; Neukom et al., 1980). Such difference in the chemical composition of raw material can be expected due to the morphology of the apple cultivar, growing conditions, genetics, the extraction method (Kennedy et al., 1999) and probably drying method used. In addition to this, polyphenols formed as a result of hydrolysis can precipitate on the cell wall matrix (Renard et al., 1995) and these may interfere with the polysaccharide extraction process. Also, the high content of lignin (19%) is noteworthy, suggesting apple processing by-products as lignin-rich biomass with respect to enzymatic degradation. Lignin content in the present study was found to be higher than the value 15.8% DW as reported by Gullón et al. (2007). Rice hulls, used as a processing aid in apple processing industry, are known to contain about 13.5-21.8% lignin (Martín et al., 2006; Vila et al., 2002). The presence of rice hulls in the raw material was expected to have made a major contribution to the overall lignin content. The composition of lignin was comparable to the findings of Campbell and Saderoff (1996), who described that lignins present in softwoods are typically composed predominantly of guaiacyl units with a minor proportion of p-hydroxyphenyl units.

The content and composition of apple processing by-products are markedly different from other lignocellulosic biomass. The cellulose content (around 20% DW) is very low when compared to wood or mature grass tissues in which it is approximately 40-55% and 25-40%, respectively (Sun and Cheng, 2002). The hemicellulose and lignin contents are also low, when compared to other lignified biomass containing around 24-40% and 18-25%, respectively (Jorgenson et al., 2007). The major hemicelluloses contain arabinose and galactose as their monomeric units. The complex composition and interactions between pectin, cellulose, hemicellulose and lignin in lignocellulosic

biomass have been proved by different studies (Oechslin et al., 2003; Selvendran and Ryden, 1990; Villas-Boˆas et al., 2002b). These complexities could hinder the transformation of biomass unless efficient bio-conversion technologies are invented to reduce recalcitrance of biomass.

4.6 CONCLUSIONS

The chemical composition of apple processing by-products was determined in order to evaluate their potential as feedstock for production of bio-ethanol and other value added products (such as acetic acid). Apple processing by products are marked by low content of protein, fat and ash. The mineral content of the raw material favors the conditions required for fermentation media. Alcohol-soluble compounds were mainly made up of monosaccharides such as glucose, xylose and arabinose, thus favoring the fermentation process. In addition to this, the alcohol-insoluble residue was mainly composed of polysaccharides such as cellulose, hemicellulose, pectin and lignin. From the cell wall analysis, it was assured that apple pomace contains considerable amount of polysaccharides which can be broken down to their sugar monomers. The fermentable sugars thus produced can further be fermented into bio-ethanol and organic acids using specific yeast and/or bacteria. The results derived from this study confirmed that apple processing by-products are a promising bio-resource for the manufacture of value-added products.

CHAPTER 5: OPTIMIZATION OF ACID-BASED HYDROTHERMAL PRE-TREATMENT OF APPLE PROCESSING BY-PRODUCTS AND REMOVAL OF POTENTIAL INHIBITORS OF FERMENTATION

5.1 ABSTRACT

Use of underutilized fruit processing waste for production of bio-ethanol and organic acids requires pre-treatment of the material facilitating release of sugars. Conditions employed in pre-treatment of biomass can, however, give rise to by-products that may be inhibitory to enzymes and fermenting micro-organisms and hinder further bio-conversion. Potentially problematic compounds include furans, acetic acid and phenolic compounds. The efficiency of the bio-conversion process is based on removal of inhibitors from hydrolyzates prior to fermentation. A pre-treatment based on dilute sulfuric acid hydrolysis of apple processing by-products was optimized for yield of glucose in relation to three independent variables: acid concentration (0.5-2% w/v), reaction time (5-30 min.) and temperature (80-100 ^{o}C) using response surface method. A central composite design (CCD) was used to code the three variables at five levels. Additionally, laccase, a polyphenol oxidase from *Trametes versicolor*, was used to delignify the acid-treated hydrolyzate by oxidation of polyphenolic compounds. The optimal acid hydrolysis conditions obtained through Canonical analysis of response surface method were: acid concentration, 1.5%; time, 16 min.; and reaction temperature, 91 ^{o}C. The optimized pre-treatment conditions were able to produce 29 mg/g FW of glucose. The results showed a negative effect of acid and laccase treatment on thephenolic compounds, representing a decrease of more than 90%. Acetic acid and hydroxymethyl furfural showed a significant increase ($P<0.05$); whereas no furfural was detected after acid pre-treatment. Accounting for the formation of the inhibitory compounds, the optimized pre-treatment conditions resulted in inhibitors at a lower concentration than what is considered as inhibitory to fermentation. Laccase at an activity of 10 units/25 g FW was found to be sufficient to degrade polyphenolic concentration by more than 90%. Application of commercial laccase did not significantly increase ($P>0.05$) the concentration of acetic acid and furfurals in the hydrolyzates. From this study, it can be concluded that the optimized dilute sulfuric acid pre-treatment can be successfully applied to apple processing by-products to enhance monomeric glucose yields with acceptable concentrations of compounds which could impede enzyme hydrolysis and fermentation.

Keywords: biomass, dilute sulfuric acid pre-treatment, enzymatic hydrolysis, furfural, hydroxymethylfurfural, laccase, phenolic compounds, response surface method

5.2 INTRODUCTION

Bio-conversion of apple processing by-products entails hydrolysis of polysaccharides into sugar monomers, which can be fermented into bio-ethanol using specific bacteria and yeast. Major polysaccharides present in apple processing by-products include cellulose, hemicelluloses, pectin and lignin. During lignocellulosic bio-conversion, the above complex polysaccharide structures should be broken down to make cellulose accessible to enzymes for its conversion into glucose (**Figure 5.1**). However, the inherent recalcitrance of polysaccharides, especially cellulose suggests that they require severe processing. Therefore, pre-treatments causing disruption of the lignin matrix could increase the accessibility of cellulose to enzymes and eventually its hydrolysis rate. Biological, physical, chemical or a combination of these pre-treatments is required to reduce recalcitrance of biomass to enzymatic hydrolysis.

Dilute acid hydrolysis is a chemical pre-treatment that utilizes sulfuric acid, hydrochloric acid, trifloroacetic acid, phosphoric acid or nitric acid at low concentrations up to a maximum of 4% (w/v; Nguyen et al., 2000). Dilute sulfuric acid-based pre-treatment is a simple and fast method with a potential for efficiency improvement (Wright et al., 1987). Poor enzymatic hydrolysis and fermentability are the major concerns associated with dilute acid hydrolysis (Saha et al., 2005; Sun and Cheng, 2005). This is based on the reason that in addition to release of constituent sugars from polysaccharides, dilute acid hydrolysis could also lead to formation of some by-products. The concentration of these by-products depends on the type of conditions involved during the process.

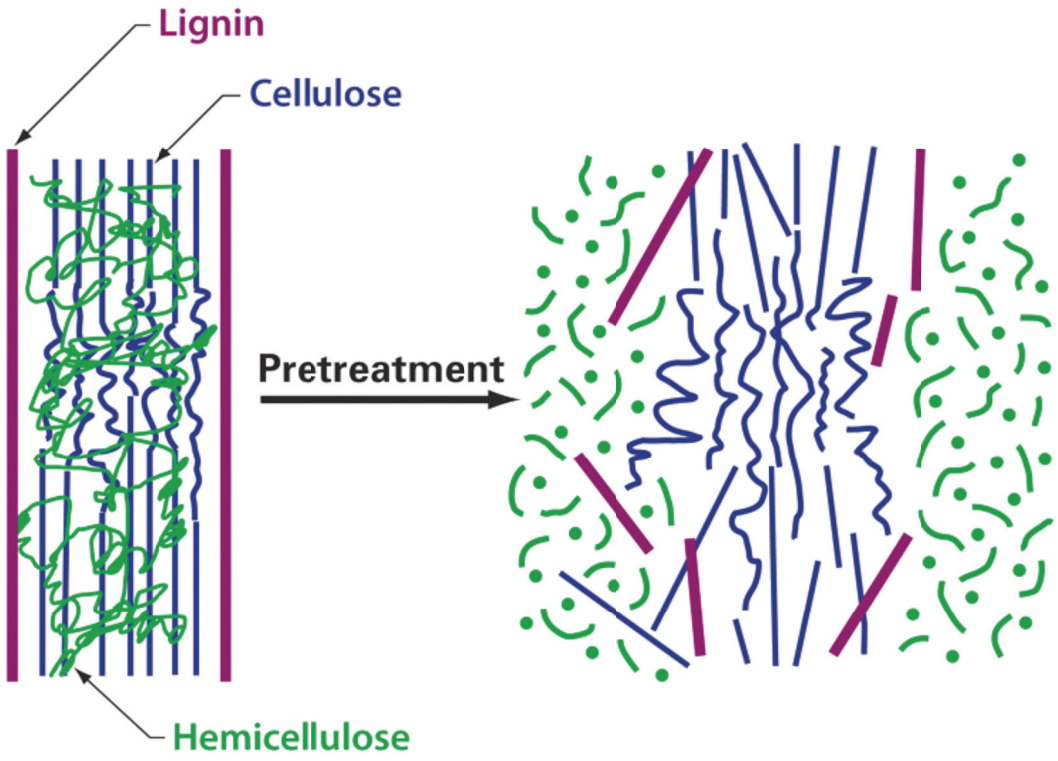


Figure 5.1 Mode of action of acid pre-treatment on lignocellulosic biomass (Adapted from Mosier et al., 2005).

Resulting by-products of biomass pre-treatment can be categorized into three types according to the chemical structure: 1) organic acids (such as acetic acid, formic acid and levulonic acid); 2) furan compounds (such as furfural and hydroxymethyl furfural) and 3) phenolic compounds (released as a result of lignin disruption). Furan compounds are generated as a result of pentose and hexose degradation due to high temperature hydrolysis of cellulosic matter (Larsson et al., 1999). Acetic acid is produced from the acetyl groups of hemicelluloses during hydrolysis (Fan et al., 1982). Phenolic compounds are results of lignin degradation in biomass. These furans, organic acids and phenolic compounds have been demonstrated to significantly inhibit enzymatic hydrolysis and the fermentation process (Axe and Bailey, 1995 Palmqvist et

al., 1999; Ximenes et al., 2010). Thus, the efficiency of the bio-conversion depends on the removal of inhibitors prior to enzymatic hydrolysis and fermentation steps.

Several biological (white rot fungi), physical (rotovaporation) and chemical (sulphite, lime) methods have been developed for the removal of inhibitors from the substrate prior to fermentation. The selection of methods for inhibitor removal depends on the type of substrate and type of pre-treatment method used. Since apple processing by-products have been well demonstrated to contain high concentrations of phenolic compounds such as chlorogenic acid, catechin, epicatechin, phloridzin and quercitin glycosides (Loo and Foo, 1997; Rupasinghe and Kean, 2008), it becomes important to remove these potential inhibitors prior to enzyme hydrolysis and fermentation.

Laccase (*p*-diphenol oxidase; E.C. 1.10.3.2) is an enzyme that can catalyze the oxidation of a variety of phenolic compounds (Chiacchierini et al 2004; Dur´an and Esposito, 2000). Laccase from *Trametes versicolor* has been studied for fruit juice and wine stabilization (Artik et al., 2004; Minussi, 2007). Also, this enzyme finds application in phenolic removal in lignocellulosic bio-conversion (Jönsson et al., 1998).

Optimization of pre-treatment conditions is one of the crucial steps in the development of an efficient and economically feasible bio-conversion method. Response surface methodology (RSM) is an adequate approach for optimization of dilute sulfuric acid hydrolysis of apple processing by-products, thus allowing good use of a commonly discarded waste biomass. RSM provides an insight into the interactions between independent variables and gives optimum conditions in relatively smaller number of runs. There are several types of models or designs in RSM for optimization purpose.

Although, many studies have shown that dilute acid pre-treatment as a well suited process for producing monomeric sugars from cellulosic agricultural waste such as citrus processing waste (Grohmann et al., 1995), banana fruit peels (Arumugum and Manikandan, 2011), palm fruit (Millati et al., 2011), fruits and vegetable residue (Patle and Lal, 2007), hardly any work has been done on pre-treatment of apple processing biomass by dilute sulfuric acid. Therefore, the present study was undertaken to:

(1) understand and optimize the effect of dilute sulfuric acid pre-treatment factors such as acid concentration, temperature and duration for maximizing glucose recovery using response surface methodology (RSM), adopting a central composite design (CCD);

(2) quantify the potential inhibitory compounds such as acetic acid, furfural, hydroxymethyl furfural and phenolic compounds using appropriate techniques;

(3) investigate the effect of commercial laccase from *Trametes versicolor* on phenolic compounds present in hydrolyzate prior to enzymatic hydrolysis.

5.3 MATERIALS AND METHODS

5.3.1 APPLE PROCESSING BY-PRODUCTS

The apple processing by-products were collected from J. W. Mason and Sons Ltd., Windsor, NS, Canada and Apple Valley Foods Inc., Kentville, NS, Canada during the year 2008-2009. One primary batch of apple processing by-products was collected randomly from the two locations. The apple processing by-products were from 'Idared' and 'Northern Spy' cultivars of apples, which were combined for further use. The collected sample was further subsampled for experimental purposes. Apple pomace, residue from juice extraction contained peel, core, seeds and rice husks. Rice husks are added to facilitate pressing of juice and could be present upto 5-10% of the total weight. The apple processing by-products were from 'Idared' and 'Northern Spy' cultivars of apples (combined) and were stored at -20 ^{o}C until use.

Apple processing by-products were homogenized into puree for two minutes using a food processor (Bead Beater; BioSpec Products, Inc. Bartlesville, OK, USA). The pH of the puree was determined using a standardized pH meter (Model Accumet® 10, Denver Instruments Co., Denver, CO, USA).

5.3.2 REAGENTS AND STANDARDS

Laccase from *Trametes versicolor* was obtained from Cedarline, Burlington, ON, Canada. The declared activity of the enzyme was 1000 units per gram. For furfural and hydroxymethyl furfural determination, potassium ferrocynide [$K_4Fe\ (CN)_6 \cdot 3H_2O$] and zinc acetate [$Zn(CH_3COO)_2 \cdot H_2O$] were purchased from Sigma Aldrich (Oakville, ON, Canada). Methanol, ethyl acetate (reagent grade) and acetonitrile (HPLC grade) were bought from Fisher Scientific, Ottawa, ON, Canada. For total phenolic content

assessment, Folin Ciocalteu reagent and standards of gallic acid were acquired from Sigma Aldrich (Oakville, ON, Canada).

The liquid chromatography standards used for the study were purchased as follows: furfural, hydroxymethyl furfural, sugar standards (glucose, fructose, sucrose), acetic acid from Sigma Aldrich (Oakville, ON, Canada); phenolic standards (catechin, epicatechin, epigallocatechin, epigallocatechin gallate, quercitin and quercitin-3-*O*-glucoside from ChromaDex Inc. (Santa Ana, CA, USA), quercitin-3-*O*-rhamnoside and quercitin-3-*O*-galactoside from Indofine Chemical Company (Hillsborough, NJ, USA); phloridzin, chlorogenic acid and phloritin from Sigma Aldrich, Oakville, ON, Canada.

5.3.3 DILUTE SULFURIC ACID PRE-TREATMENT

Dilute acid pre-treatment of apple processing by-products was carried out in 250 mL Wheaton Magna-flex spinner flasks (250 mL capacity; Fisher Scientific, Ottawa, ON, Canada). Apple processing by-products slurry was mixed with concentrated sulfuric acid at different concentrations (0.7%, 1.0%, 1.5%, 2.0%, 2.3% w/v). The slurry (≈ 25 g apple processing by-products on fresh weight basis/100 mL) was loaded into the flasks and treated at the desired temperature and time. The thermal pretreated samples were cooled to 25±2 ^{o}C and used for further analysis. A sample of 1 mL was taken out after each pre-treatment and was analyzed by HPLC to determine its sugar content.

5.3.3.1 SELECTION OF PARAMETERS FOR DILUTE ACID PRE-TREATMENT

The effect of three independent factors, sulfuric acid concentration (0.5-2.0% w/v), reaction temperature (80-100 ^{o}C), reaction time (5-30 min), on glucose yield was studied. **Table 5.1** summarizes the parameters of dilute sulfuric acid pre-treatment of some commonly used biomass such as fruits, vegetable residues and agricultural crop residues.

Table 5.1 Dilute acid pre-treatment parameters commonly used in biomass.

Biomass type	Sulfuric acid concentration	Reaction time (min)	Reaction temperature (^{o}C)	Reference
sugarcane baggase, cassava stalk, peanut shells	2% (w/w)	20-60	122	Martin et al., 2007
Orange peel	0.5% (w/v)	6	150	Ylitervo, 2008
Pectin and citrus waste	0.5% (w/w)	3-9	130-170	Pourbafrani et al., 2010
Fruits and vegetable residues	4% (v/v)	180	37-55	Patle and Lal, 2007
Corn stover	2% (w/v)	30-120	121	Um et al., 2005

Dilute sulfuric acid is the most commonly used acid for dilute acid pre-treatment, which has been commercialized for a variety of biomass (Kim et al., 2000; Martin et al., 2007; Mosier et al., 2005b; Nguyen, 2000). The concentration of sulfuric acid (0.5-2% w/v) for pre-treatment was selected based on literature. The pre-treatment is generally carried out in a steel reactor at high temperatures (>150 ^{o}C). Depending on the combined severity of the pre-treatment, the sugars can be converted to aldehydes such as furfural and HMF. As compared to high temperatures (150-200 ^{o}C) involved during common pre-treatments used for lignocellulosic biomass, application of moderate temperature (80-120 ^{o}C) conditions could results in reduced degradation of sugars and reduced formation of fermentation inhibitors in fruit based feedstock. At the same time, pre-treatment should result in lignin disruption. The disruption of lignin layer reveals the

underlying cellulose fibers, thereby leading to enhanced enzyme accessibility to cellulose. Thus, the temperature was designed to vary between 80 and 100 °C, since the highest level was selected so as to limit heat induced quality degradation. Moreover, the lignin content of apple processing by-products is lesser than the other common lignocellulosic biomass such as corn stover, hardwood and softwood, the present study proposed that moderate temperature (80-100 ^{o}C) could be a better approach for fruit biomass pre-treatment. For reaction time, a range of 5-30 min. was selected based on the literature. The treatment times were selected so as promote the economic feasibility of the process as longer times would need more energy inputs. In addition short treatment times are expected to result in reduced substrate degradation. The most commonly used reaction time ranges between 3-120 min. (**Table 5.1**) depending on the type of substrate used.

5.3.4 OXIDATION OF PHENOLIC COMPOUNDS

After acid pre-treatment, the hydrolyzate was adjusted with NaOH to pH 5.3. Commercial laccase from *T. versicolor* at dose of 10 units/100 mL hydrolyzate was added to oxidize the polyphenolic compounds present. The enzyme activity of the laccase was approximately 1000 units/g (as declared by manufacturer). One unit corresponds to the amount of enzyme which converts 1 μmole of catechol per minute at pH 6.0 and 25 °C. The flasks containing the hydrolyzate and enzyme were kept at 30 ^{o}C for 12 h in an incubator (model HP50, Apollo, San Diego, CA, USA) shaking at 90 rpm as described elsewhere (Jönsson et al., 1998). After enzyme treatment, samples were stored in -20 ^{o}C and later freeze dried (Model FD2085C0000, Kinetics Thermal Systems, Toronto, ON, Canada) for further use.

5.3.5 EXTRACTION OF PHENOLIC COMPOUNDS

After being freeze dried, samples were ground to a fine powder using a coffee grinder (Model DCG-12BCC, Cuisinart, Brampton, ON, Canada). To 1 g of dried sample in a centrifuge tube (50 mL capacity), 10 mL of 100% methanol was added to extract the phenolic compounds. The mixture containing pretreated apple processing by-product powder and the extraction solvent was vortexed and the flasks were placed in an ultrasonic bath (model 750D, VWR, West Chester, PA, USA) for 15 min. durations up to 45 min, with 10 min. interval (Rupasinghe et al., 2008). The sample was centrifuged at 8000 × g (Sorval ST 16 Centrifuge, Thermo Scientific, Ashville, NC, USA) 15 min. The supernatant was evaporated by nitrogen flushing system (Organomation Associates Inc., Berlin, MA, USA) and the residual sugars were dissolved in 10 mL of deionized water. The samples were rendered sugar free through solid phase extraction (SPE) by passing it through a C18 Bond Elut column (Agilent Technologies, Mississauga, ON, Canada) with 6 mL capacity. The column was conditioned and saturated with 3 mL of 100% methanol and 3 mL of deionized water, respectively. A load volume of 10 mL sample, followed by wash volume of 6 mL deionized water was used prior to elution. The phenolic compounds were eluted using 100% methanol as solvent. Sampling consisted of removing 2 mL of the solution, which was filtered (nylon 0.45 micron filters, Waters Co., Milford, MA, USA) before being placed into HPLC auto-sample vials for phenolic analysis.

5.3.6 DETERMINATION OF TOTAL PHENOLIC CONTENT FOLIN-CIOCALTEU ASSAY

The Folin-Ciocalteu assay was used to measure the total phenolic content as described by Singleton and Rossi (1965). The original assay was changed for use in 96-well microtiter plate (Rupasinghe et al., 2009). The results are expressed as gallic acid

equivalents (GAE) per gram of dry matter. Under dark conditions, the Folin-Ciocalteu reagent and methanol extract of freeze dried apple processing by-products were mixed together and placed in the 96-well micro plates. After 5 min, 300 µL of 75 g/L Na_2CO_3 was added and the sample was incubated for two h at 25 ^oC. The absorbance of the sample was read at 760 nm against a blank that consisted of all the reactants except the phenolic extract.

5.3.7 EXTRACTION OF HYDROXYMETHYL FURFURAL (HMF)

After pre-treatment and polyphenol removal, the freeze dried hydrolyzate of apple processing by-products was ground in a coffee grinder (Model DCG-12BCC, Cuisinart, Brampton, ON, Canada). HMF was extracted according to the procedure of Teixido et al. (2006). A sample of 0.5 g was taken into 15 mL centrifuge tubes and acidic water (pH 1.0) was added to each tube and the mixture was vortexed for one minute. To each tube, 1 mL of Carrez solution I containing 15 g of potassium ferrocyanide [$K_4Fe(CN)_6 \cdot 3H_2O$] in 100 mL of deionized water was added. This was followed by the addition of 1 mL of Carrez solution II, that contained 30 g of zinc acetate [$Zn(CH_3COO)_2 \cdot H_2O$] in 100 mL of deionized water. The mixture was then centrifuged at 4000 × g for 10 min. Supernatant from each tube was filtered (nylon 0.45 micron filters, Waters Co., Milford, MA, USA) before being placed into HPLC auto-sample vials for liquid chromatography and tandem mass spectrometry (UPLC-MS/MS) analysis.

5.3.8 EXTRACTION OF FURFURAL

Extraction of furfural from treated samples was carried out using the procedure by Li et al. (2009) with some modifications. Briefly, 0.5 g of freeze dried sample was taken into 15 mL centrifuge tubes and 9 mL of deionized water was added. The sample

was centrifuged for 1 min. and passed through a Bond Elut C18 column (Agilent Technologies, Mississauga, ON, Canada) to remove sugars and furfurals were eluted with 100% acetonitrile as solvent. Sampling consisted of removing 3 mL of the solution, which was filtered (nylon 0.45 micron filters, Waters Co., Milford, MA, USA) before being placed into HPLC auto-sample vials for quantification.

5.3.9 Liquid Chromatography and Mass Spectrometry Analysis

5.3.9.1 Determination of major phenolic profiles

A UPLC H-class system containing a Waters Model code CHA Separations Module and an aquity UPLC BEH C_{18} column (2.1 × 100 mm, 1.7 μm; Waters, Milford, Massachusetts, USA) was used. For the analysis of flavan-3-ols, flavonols, dihydrochalcones, and phenolic acids, gradient elution was carried out with 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B) at a flow rate of 0.2 mL/min. A linear gradient profile was used with the following proportions of Solvent A applied at time t (min); (t, A%): (0, 90%), (6, 50%), (8, 35%), (10, 10%), (15, 90%), (20, 90%). For each sample, an injection volume of 2 μL was used with a run time of 20 min.

MS/MS analysis was performed on a Waters Micromass Quattro *micro* API triple quadruple mass spectrometer controlled by the Masslynx V4.0 data analysis system (Micromass, Cary, USA), as previously described by Rupasinghe et al. (2008). Electrospray ionization in negative ion mode (ESI-) was used for the analysis of the phenolic compounds. In negative mode, the following MS tune settings were used: capillary voltage -3000 V, nebulizer gas temperature (N_2) 375 ^{o}C at a flow rate of 600 L/hr. The MS settings were tuned for each individual compound. Analytes were identified and quantified using multiple reactions monitoring (MRM) mode in

comparison with standards. In the MS/MS experiments, both quadrupoles were operated at unit resolution.

5.3.9.2 DETERMINATION OF HMF

The same UPLC system and column (as described above) were used for the determination of HMF. However, the conditions employed were different for its quantification. Gradient elution was carried out with 0.1% formic acid in water (Solvent A) and 0.1% formic acid in methanol (Solvent B) at a flow rate of 0.150 mL/min. A linear gradient profile was used with the following proportions of Solvent A applied at time t (min); (t, A%): (0, 90%), (6, 50%), (8, 35%), (10, 10%), (15, 90%), (20, 90%). Electrospray ionization in positive ion mode (ESI+) was used with the following settings: capillary voltage at 4.25 V, cone voltage of 20 V, source temperature at 80 ^{o}C, and the nebulizer gas (N_2) at 115 ^{o}C and a flow rate of 500 L/hr. Single ion-monitoring (SIM) mode using the abundance of the protonated molecule $[M+H]^+$ (m/3 =127.1) was used for quantification in comparison with standards. Four-point linear calibration curves of external standards were used for quantification of the compounds.

5.3.9.3 DETERMINATION OF FURFURAL

Furfural content in the samples was determined using a Waters 2695 HPLC system with a Waters 2996 PDA detector (Waters Corp. Milford, MA, USA). The stationary phase was a Synergy C18 column (150 × 4.6 mm, 4 µm particle size, Phenomenex, Torrence, CA, USA), the column temperature was 25 ^{o}C and the detection wavelength was 280 nm. Mobile phase A contained 0.1% formic acid in water and phase B consisted of 0.1% formic acid in acetonitrile. The injection volume was 10 µL.The flow rate was maintained at 0.35 mL/min for 25 min. with a ratio of 94% A and 6% B.

Standard solutions of furfural were made in 100% acetonitrile and kept in dark at 4-5 ^{o}C until use. Data was collected and analyzed using Mass Lynx 4.1 software.

5.3.10 DETERMINATION OF SUGARS AND ACETIC ACID

Samples were centrifuged for 20 min. at 8000g (Sorvall Legend Micro-17 Centrifuge, Thermo Scientific, Ottawa, ON, Canada), filtered through a 0.45 micron nylon filters (Chromaspec, Chicago, IL, USA). Concentrations of sugars and acetic acid present in supernatant were determined by High Performance Liquid Chromatography using Waters Alliance 2695 separation module with a ROA-organic acid column (300 x 7.8 mm; Phenomenex, Torrence, CA, USA) and refractive index (RI) detector. Samples were run at following conditions: column temperature of 65 °C, detector temperature of 30 ^{o}C, run time of 25 min. and flow rate of 0.6 mL/min. with 0.005N sulfuric acid as solvent. Calibration curves were obtained using standard solutions of glucose, fructose and acetic acid using concentration range of 50-200 mg/L. The peaks of glucose, fructose and acetic acid eluted at retention times of 10.1, 10.8 and 15.6 min., respectively.

5.3.11 EXPERIMENTAL DESIGN AND STATISTICAL ANALYSES

To determine the optimal levels for the given factors of pre-treatment, a central composite design (CCD) was used and data was analyzed using response surface methodology (RSM; Montgomery, 2005). This design with the actual and coded levels of variables is shown in **Table 5.2**. This design was preferred because few experimental combinations of the variables are adequate to estimate the complex response functions including interaction between different factors. The response function was glucose concentration (mg/g FW). Under this methodology, the obtained data were subjected to Canonical analysis using RSREG procedure of SAS Institute, Inc. (1999) and contour

plots were drawn. The steps followed in conducting Canonical analysis using RSM are given in **Figure 5.2**. The strategy of RSM contains four steps in optimising the variable conditions. Firstly, RSM procedure was designed to move into the optimum region. Then, RSM determined the behavior of the response in the optimum region; this was followed by the estimation of the optimum conditions of the process, and lastly followed by the verification step (Vitali, 2000).

In order to estimate standard error, five replicates at the center of design (acid concentration of 1.5% (w/v), temperature of 90 ^{o}C and time of 15 min.) were used. The nature of the response can be determined from the stationery point and the signs and magnitudes of the Eigen values. If the Eigen values are all positive, stationary point is point of minimum response; if Eigen values are all negative, stationery point is a point of maximum response; and if Eigen values have different signs, the stationery point is a saddle point. When the results showed a saddle point in response surfaces, the ridge analysis of SAS RSREG procedure was used to compute the estimated ridge of the optimum response. The examination of contour plots further enables the study of relative effect of two of the independent factors on the response variable, while keeping the third factor constant. Based on that, additional experimentation was carried out to obtain a point of maximum yield of glucose within the given levels of all factors. Completely randomized designs (CRD) were used for all other experiments and the assumptions of independence, constant variance, and normality were tested for the obtained data using Minitab15 (Montgomery, 2005). The data were analyzed using ANOVA methods to compare the factor levels in terms of the mean response, using the general linear model

(GLM) procedure of the SAS Institute, Inc. (1999). Differences among means were tested by the Tukey's Studentized Range test at $\alpha = 0.05$.

Table 5.2 The acid pre-treatment variables and their experimental design levels

	Levels				
Coded value	**-1.68**	**-1**	**0**	**+1**	**+1.68**
Uncoded variables					
Sulfuric acid concentration (%w/v)	0.7	1	1.5	2	2.3
Reaction time (min)	7	10	15	20	23
Reaction temperature (^{o}C)	81.3	85	90	95	98.7

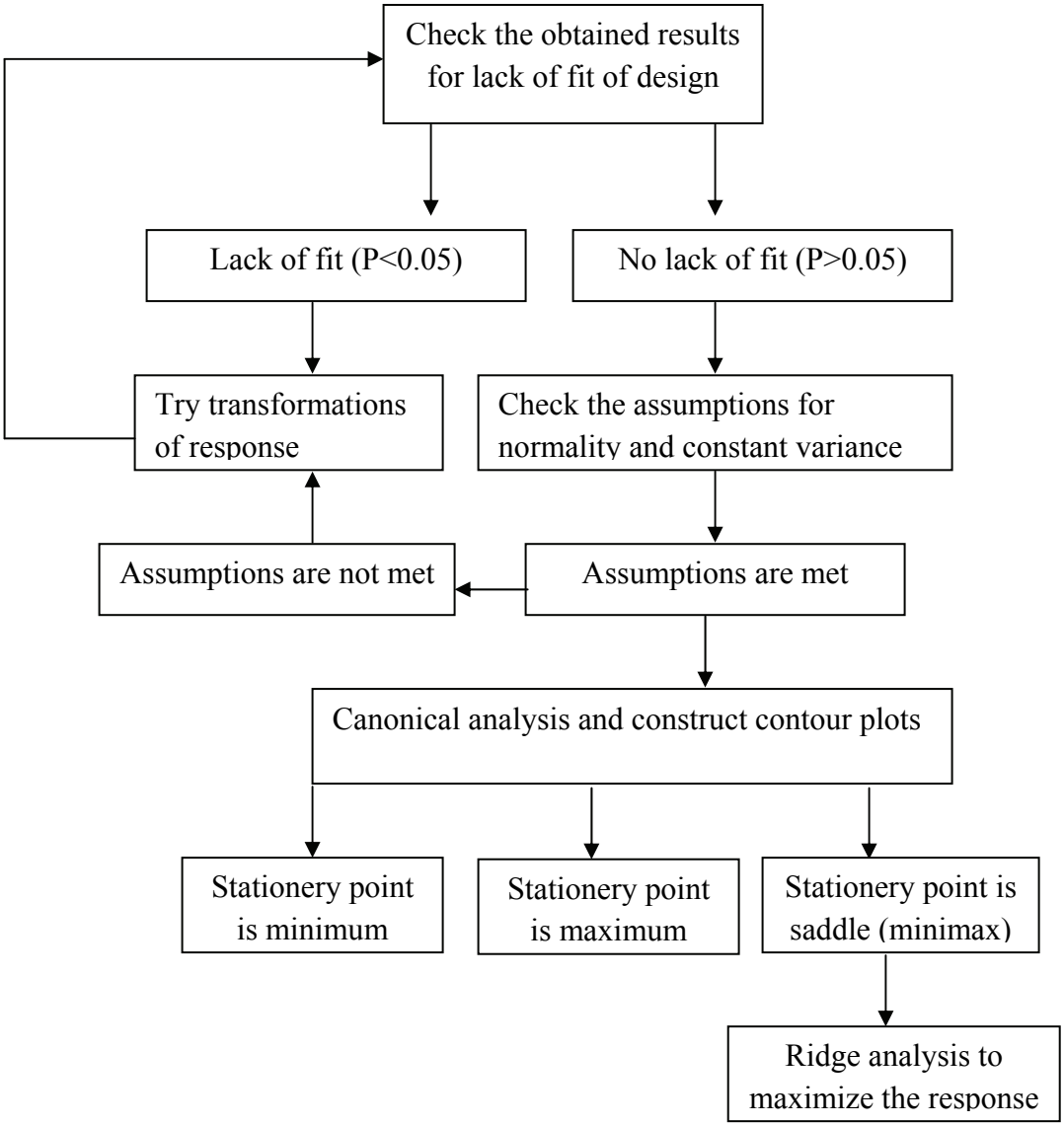


Figure 5.2 The steps followed for performing Canonical analysis using RSM.

5.4 RESULTS

5.4.1 DILUTE ACID PRE-TREATMENT

Glucose concentration in apple processing by-products pretreated with dilute acid was used to assess the extent of pre-treatment process. The pre-treatment was optimized to maximize the glucose production at the desired operating conditions. The response values of glucose concentration obtained by running the central composite design using RSM are given in **Table 5.3**. Canonical analysis was further conducted (**Table 5.4**). The models showed lack of fit to be insignificant (P>0.05) for all the parameters studied, thereby indicating that the number of experimental combinations formed in the design of experiment was enough to determine the effect of independent variables on the response. From the analysis of variance, it was clear that the interaction of acid concentration, treatment time and reaction temperature had a strong influence on pre-treatment process. The Eigen values given by RSEG canonical procedure were a mixture of positive and negative values, which indicated that the stationary point for the optimization was a saddle point. This could be a point of maximum or minimum values (Montgomery, 2005). The critical values at coded levels of variables studied were: acid concentration of 1.5%, reaction temperature of 90.5 ^{o}C and reaction duration of 15.3 min. The response contour of glucose production using central composite design is shown in **Figure 5.3.**

Following the steepest ascent in the ridge analysis (**Figure 5.4; APPENDIX A**), additional experimental runs were carried out after fixing the acid concentration to 1.5%. This was done in order to find out the exact stationary point with the pre-treatment conditions. This time a two factor central composite design (CCD) was used with reaction temperature and duration as independent factors, keeping the center point values same as in CCD with three independent variables. The RSEG analysis gave a maximum

point for glucose concentration (**Figure 5.5**). The examination of surface plot illustrates the relationship between experimental factors and response in three-dimensional representation generated by the models for response variable. The uncoded values for analyzed parameters as determined by canonical procedure of RSEG were: acid concentration of 1.5%; reaction temperatureof 91.1^{o}C and reaction time of 16.8 min. The response surface plot showed that the optimal values of glucose concentration in the hydrolyzate can be found when the acid concentration, treatment time and reaction temperature were close to their respective center points

Table 5.3 Response values resulting from central composite design with three independent variables.

Acid conc. (% w/v)	Time (min)	Temperature (^{o}C)	Glucose (mg/g FW)	Fructose (mg/g FW)
0.7	15.0	90.0	28.2	44.0
1.0	10.0	85.0	26.2	77.7
1.0	10.0	95.0	22.0	46.8
1.0	20.0	85.0	27.5	74.2
1.0	20.0	95.0	25.7	67.2
1.5	15.0	90.0	29.2	52.2
1.5	15.0	90.0	29.2	52.2
1.5	15.0	90.0	29.1	57.2
1.5	15.0	90.0	29.7	51.1
1.5	15.0	90.0	26.1	65.5
1.5	23.4	90.0	22.2	56.3
1.5	6.6	90.0	19.5	54.1
1.5	15.0	98.4	36.6	60.3
1.5	15.0	81.6	26.7	56.9
2.0	10.0	85.0	19.5	54.3
2.0	10.0	95.0	37.7	59.0
2.0	20.0	85.0	21.1	63.8
2.0	20.0	95.0	30.6	55.6
2.3	15.0	90.0	29.4	66.6

Table 5.4 Canonical analysis of the response surface for glucose concentration after pre-treatment

Variable	Three factor CCD*	Two factor CCD[§]
P – value for model adequacy (Lack of fit)	0.06	0.09
Eigen values	120.1 -52.7 -120.6	- -89.9 -100.7
Critical values at coded level of variables		
Acid concentration	1.5	-
Reaction duration	15.3	15.8
Reaction temperature	90.5	91.1
Critical values at actual level of variables		
Acid concentration	1.5	-
Reaction time	15.3	16.1
Reaction temperature	90.5	90.8
Predicted response value	26.6	29.3
Stationery point	Saddle point	Maxima

**CCD-central composite design*

[§]*Central Composite Design with two independent factors (Reaction Time and Reaction Duration) after fixing acid concentration to 1.5%*

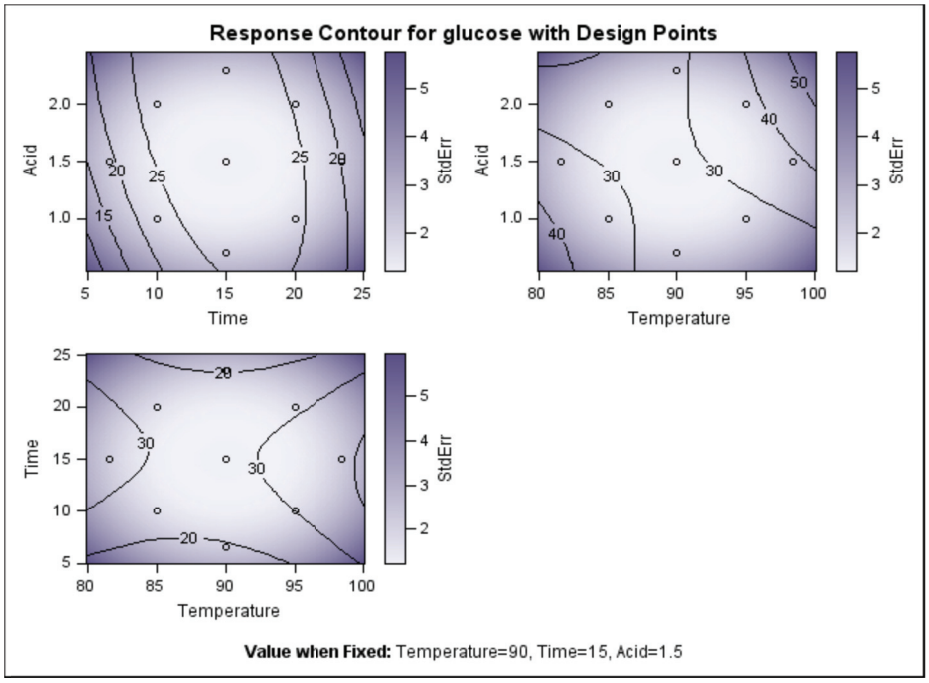


Figure 5.3 Response contour for glucose using central composite design.

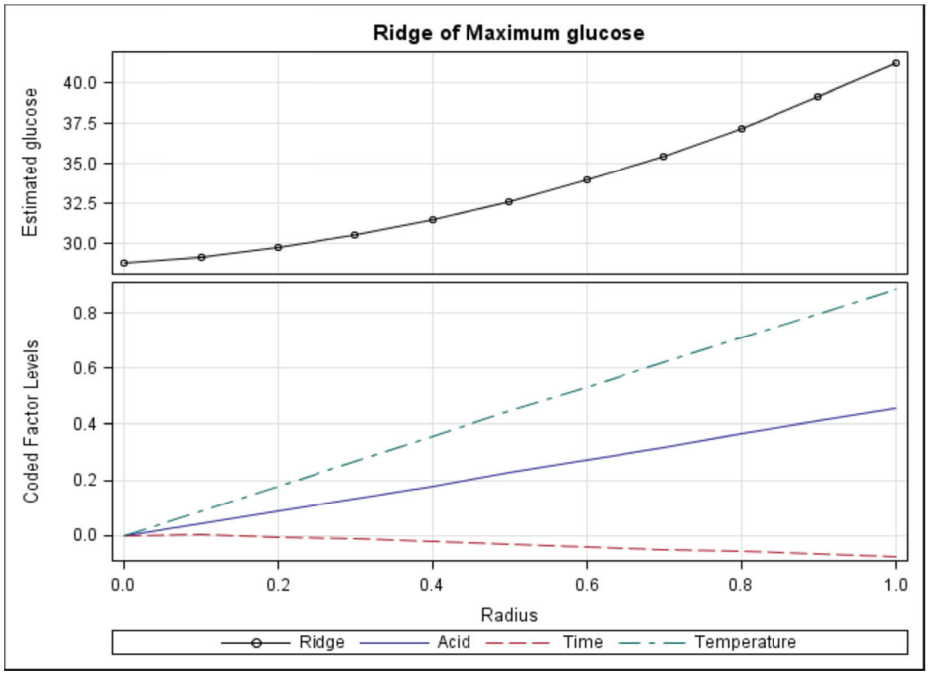


Figure 5.4 Ridge analyses for glucose using central composite design with three independent variables.

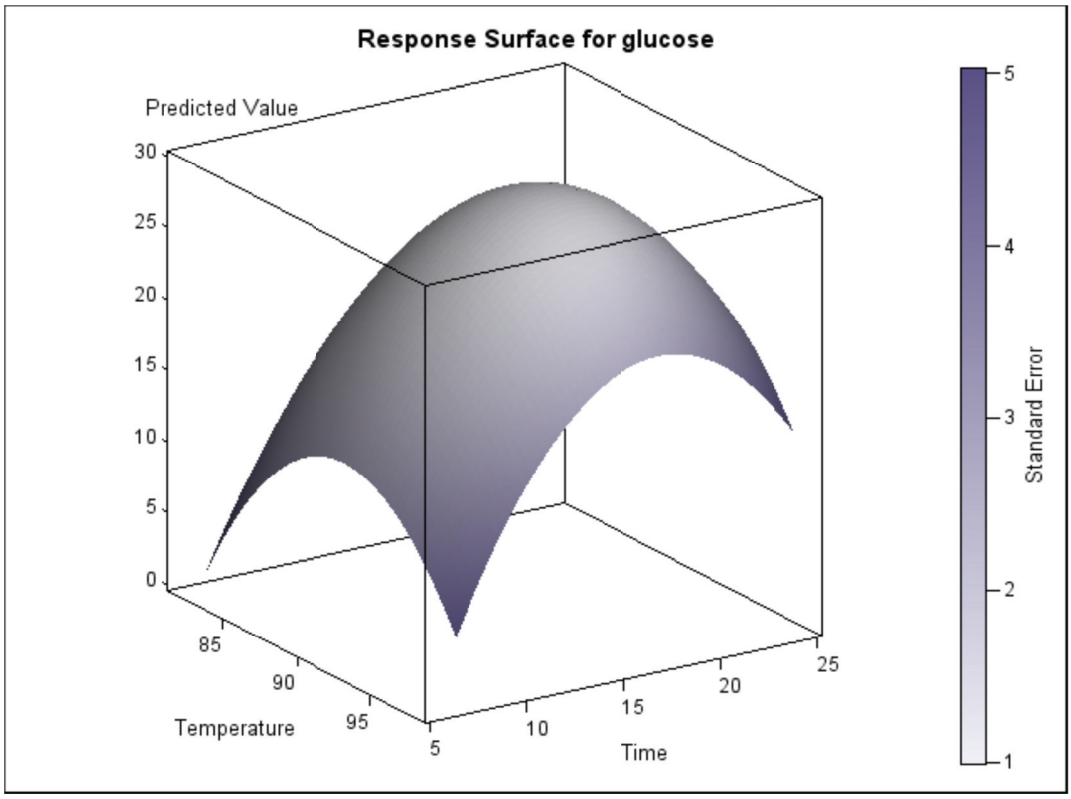


Figure 5.5 Response surface for glucose showing maxima in Canonical analysis conducted for dilute acid pre-treatment of apple processing by-products.

5.4.2 POTENTIAL INHIBITORS TO ENZYMATIC HYDROLYSIS AND FERMENTATION

5.4.2.1 CONCENTRATION OF BIO-CONVERSION INHIBITORS AND EFFECT OF COMMERCIAL LACCASE

In order to assess potential risk for inhibition of hydrolytic enzymes and fermenting micro-organisms (**Table 5.5**), hydrolyzates were analyzed for the presence of inhibitory compounds such as acetic acid, furfural, HMF and total phenolics. Acetic acid (3.6 mg/g lyophilizate) was produced as a result of acid pre-treatment of apple processing by-products. The concentration of acetic acid as a function of acid pre-treatment coincided with the maximal HMF content. However, furfural, a by-product of pentose sugars was not detected in any of the pretreated samples. As expected, the concentration of acetic acid did not change significantly ($P>0.05$) when acid pretreated hydrolyzate was subjected to laccase treatment. Conversly, the concentration of HMF was found to be significantly lower ($P<0.05$) in acid-laccase treated biomass than in acid pretreated biomass. During acid pre-treatment, the total phenolic content was found to be degraded due to high temperatures and low pH. Total phenolic content of the untreated samples was found to be 1.1 mg GAE/ g of lyophilizate, which decreased to almost half (0.6 mg GAE/ g) after acid hydrolysis. Treatment with laccase at an enzyme activity of 10 units/25 g FW for 12 h led to significant loss (more than 80 %) of total phenolic content of apple processing by-products.

5.4.2.2 EFFECT OF ACID AND LACCASE TREATMENT ON CONCENTRATION OF SPECIFIC PHENOLIC COMPOUNDS

The concentrations of some phenolic compounds, found mostly in all types of apple pomace, as affected by acid and laccase treatment are summarized in **Table 5.6.** Dilute acid and laccase treatment had a significant effect ($P<0.05$) on the concentrations of selected phenolic compounds. Among phenolic acids, phloridzin content was the

highest (17.6 mg/ 100 g lyophilizate) in untreated biomass sample. However, after pre-treatment, the amount decreased to 0.07 mg/g lyophilizate, representing a drop of 59%. The concentration of phloridzin was not significantly different ($P>0.05$) when acid hydrolyzates were further treated by polyphenol oxidase. The same was true for chlorogenic acid, which decreased by more than 75% after dilute acid pre-treatment. However, the concentrations of ferulic acid and caffeic acid in control were found to have increased by 33 and 50% respectively.

The flavanol concentration of apple processing by-products showed a large variation in response to the different treatments applied. Interestingly, quercetin, which was not detected initially in the control, was observed after acid pre-treatment (0.01 mg/g lyophilizate). On the other hand, quercitin with attached sugar moieties such as quercitin-3-*O*-glucoside, quercitin-3-*O*-galactoside and quercitin-3-*O*-rhamnoside were found to have degraded significantly ($P<0.05$) after acid and laccase treatments. This indicated the splitting of sugar moieties from quercetin during acid pre-treatment, thereby forming their corresponding aglycones. Most of the phenolic compounds (>90%) were observed to have oxidized and further polymerized into high molecular weight complexes after the application of laccase to acid pre-treated hydrolyzates (**Figure 5.6**).

Table 5.5 Mean concentration of potential inhibitors of enzymes and fermentation present in apple processing by-products

Sample	Acetic acid (mg/g lyophilizate)	Furfural (mg/g lyophilizate)	Hydroxymethyl furfural (mg/g lyophilizate)	Total phenolics[1] (mg GAE/g lyophilizate)
Untreated	0.0±0.0 [b]	ND	0.1±0.02 [c]	1.1±0.06 [a]
Acid pretreated*	3.4±0.4 [a]	ND	2.1±0.01 [a]	0.6±0.03 [b]
Acid-laccase pretreated**	3.6±0.4 [a]	ND	1.4±0.05 [b]	0.1±0.00 [c]

**Acid pre-treatment: acid, 1.5% (w/v); temperature, 91 °C and time, 15 min.*
***Laccase from Trametes versicolor at 10 units/100 mL*
GAE, gallic acid equivalents; ND, not detected

Table 5.6 Mean concentration of phenolic compounds present in apple processing by-products.

Sample	Flavonols				Flavan-3-ols	Phenolic acids			Chalcones
	Q3Gal	Q3Glu	Q3Rha	Quercetin	Epicatechin	Chlorogenic Acid	Caffeic Acid	Ferulic Acid	Phloridzin
Untreated	0.1±0.7 [a]	4.4±0.4 [a]	5.7±0.3 [a]	ND	2.1±0.5 [a]	1.7±0.0 [a]	0.1±0 [a]	0.2±0.0 [a]	17.6±0.6 [a]
Acid pretreated[y]	0.2±0.0 [b]	0.3±0.0 [b]	0.6±0.2 [b]	1.0±0.0 [a]	ND	0.4±0.0 [b]	0.2±0 [a]	0.3±0.0 [a]	7.4±0.0 [b]
Acid-laccase pretreated[¢]	ND	ND	0.1±0.0	ND	ND	0.1±0.0 [b]	0.0±0 [b]	ND	6.2±0.5 [b]

[y]*Acid pre-treatment: acid, 1.5% (w/v); temperature, 91 °C and time, 15 min.*
[¢]*Laccase from Trametes versicolor at 10 units/100 mL*
Q3Gal, quercitin-3-galactoside; Q3Glu, quercitin-3-glucoside; Q3Rha, quercitin-3-rhamnoside
ND- Not detected
[ǂ]*Amounts are expressed as mg/100 g lyophilizate*
[¥]*Means with different letters are significantly different (P<0.05) and letter grouping is between treatments.*

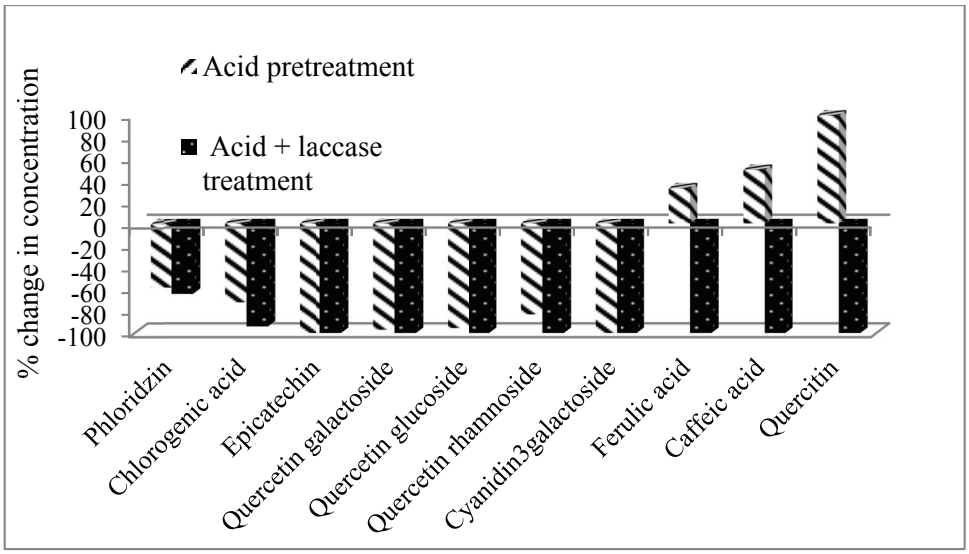


Figure 5.6 Percentage changes in concentration of phenolic compounds resulting from acid pre-treatment and laccase treatment.

5.5 DISCUSSION

5.5.1 PRE-TREATMENT WITH DILUTE SULFURIC ACID

Production of bio-products from renewable biomass faces several technical challenges. Success of using biomass for biofuel production depends largely upon physic-chemical properties of the biomass, pre-treatment methods, efficient micro-organisms, and optimization of processing conditions. The purpose of the acid pre-treatment is to disrupt the lignin-carbohydrate matrix. This can decrease cellulose crystallinity and increase substrate porosity, thereby enhancing cellulose hydrolysis by cellulase (Zheng et al., 2009). With the equipment and procedure used in the present study, the optimum conditions obtained for dilute acid pre-treatment can be applied to obtain enhanced glucose yields. However, these conditions did not lead to high cellulose conversions into glucose due to low temperature (80-100 ^{o}C) and low concentration of acid (0.5-2%) used. Apple processing by-products contain high amounts of fructose also. However, the concentration of fructose was quite variable when biomass was subjected to dilute acid pre-treatment (**Table 5.3**). Under acidic condtions combined with high temperatures, hexose sugars can dehydrate into hydroxymethyl furfural. Since fructose is not present in cell wall, therefore it was not expected to be released as a result of acid hydrolysis of apple processing by-products. Hence, it was not a response of interest. Furthermore, previous studies have shown the ability of dilute acid pre-treatment to solubilize hemicellulose into corresponding sugars in substrates like orange peel and corn stover (Qing and Wyman, 2011; Vaccarino et al., 1989). However, the present study did not detect any peaks in chromatograms other than glucose and fructose in the hydrolyzates.

The type of substrate also plays a crucial role in determining the product yield. The quality of hydrolyzates is also affected with the severity of the pre-treatment conditions (Hernandez, et al 2002). Formation of sugar degradation products, such as furfural compounds, produces dark color in the hydrolyzates. The present study however, did not show generation of high amount of furfural compound as by-products of acid pre-treatment. The desired dilute-acid pre-treatment conditions are those that produce the highest sugar yield and by-products with lowest toxicity. A study by Um et al. (2003) on corn stover as the substrate demonstrated that increasing sulfuric acid concentration from 0.5 to 2% (w/v) increased the degree of substrate digestibility to enzymes from 56 to 80%, after pre-treatment at 121 °C for 120 min. According to this study, acid pre-treatment played a vital role in solubilizing hemicellulose that surrounds the cellulose layer, thereby promoting enhanced access for hydrolytic enzymes.

5.5.2 PRESENCE OF ENZYME AND FERMENTATION INHIBITORY COMPOUNDS IN PRETREATED HYDROLYZATE

During dilute acid pre-treatment, by-products such as acetic acid, phenols and hydroxymethyl furfural (HMF) were generated. The presence of these toxic compounds in pretreated biomass is a significant impediment to its further bio-conversion using enzymes and micro-organisms (Saha et al., 2005; Sun and Cheng, 2005).

Acetic acid is a well know by-product of acid hydrolysis (Grohman et al., 1984; Kong et al., 1992). However, the concentration of acetic acid in the present study was found to be 3.4 mg/g DW. A study by Larsson et al. (1999) reported that acetic acid concentration below 6 mg/g did not inhibit ethanol fermentation using softwood as feedstock. An earlier study by Janshekhar (1983) demonstrated that acetic acid up to 0.1 mg/g increased the yield in similar ethanol fermentation. Therefore, it is expected that

optimized conditions of dilute acid pre-treatment at moderate conditions can be applied to bio-conversion of apple processing by-products without serious concerns of inhibitory compounds.

The inhibitory effect of furan aldehydes such as furfural and HMF has been shown to be synergistic (Taherzadeh et al., 2000; Zaldivar et al., 1999). However, no furfural content was observed in the hydrolyzates. This could be due to the fact that pentoses (mainly xylose) are present in low amounts and bound form in cell walls of apple-processing by-products. The range of the total concentration of HMF was lower than the concentration considered generally to inhibit fermentation (Nichols et al., 2008). The low concentration of inhibitors could presumably be due to moderate conditions used for pre-treatment, which did not lead to any major degradation of released sugars. In general, lignocellulosic biomass employs harsh reaction conditions, especially temperatures greater than 150 ^{o}C, which leads to the formation of significant concentration of furan compounds. The degree to which toxic compounds change during processing depends on the sensitivity of the compound to modification or degradation, and severity of processing conditions (Breene, 1994).

Furfural is reduced by yeast cells to furfuryl alcohol (Villa et al., 1992). Weak acids such as acetic acid affect the fermentation by dissipating the proton-motive force across the membrane due to an influx. This causes them to uncouple energy conservation from biomass formation of the undissociated acid (Pampulha and Dias, 1989). This causes drop in intracellular pH that can be neutralized by pumping the protons out of the cell. ATP is required for the removal of the proton and it is achieved by increased ethanol formation (Verdyn et al., 1990). However, very high concentrations of acetic

acid could deplete the energy reserves of the cell, resulting in acidification of the cytoplasm and cell death (Russel et al., 1992).

The above results showed that polyphenolic compounds present in apple processing by-products are heat labile and their content decreased substantialy during acid based hydrothermal pre-treatment process. This degradation of phenolic compounds could be due to oxidation into their corresponding quinones. Also, polymerization of these compounds could lead to formation of insoluble quinones. However, it was also observed that some phenolic acids such as ferulic acid and caffeic acid were released after acid pre-treatment. The dilute acid can cleave acetal or hemiacetal bond between carbohydrate moieties and the hydroxyl groups off the aromatic ring, thereby releasing phenolic acids.

5.5.3 POLYPHENOL OXIDATION WITH COMMERCIAL LACCASE

More than a 90% decrease in concentration was observed for most of the phenolic compounds after pre-treatments. However, phloridzin was an exception with just 16% decrease after laccase application. This observation suggested that phloridzin might not be a preferred substrate for this particular enzyme. Overall, the results suggest that commercial laccase at an activity of 10 units/100 mL FW was sufficient to degrade polyphenolics from apple processing by-products to a level at which they can be non-toxic to enzymes and fermenting micro-organisms. Also, a significant reduction in the total phenolic content of acid hydrolyzate following laccase treatment indicates the efficiency of laccase in oxidizing lignin. According to the manufacturers, laccase from *T. versicolor* has low specificity and prefers substrates such as *o*-and *p*-diphenols, aminophenols, methoxy-substituted phenols, benzenethiols, polyphenols, polyamines and hydroxyindols.

During catalysis, laccase has been shown to work by reducing molecular oxygen to water and simultaneously carrying out one-electron oxidation of polyphenolic substrates (Thurston, 1994). The initial intermediate reaction products are oxygen-centred radicals (or cation radicals), which are unstable and hence undergo second enzyme-catalysed oxidation converting phenol to quinone with many substrates. These products may also undergo further changes through non-enzymatic routes such as hydrogen ion, disproportionation and/or polymerization. Laccase-assisted reactions strongly depend on the redox potential of enzyme, temperature and pH of the reaction medium (Xu et al., 1999). Minussi et al. (2007) demonstrated the great potential of laccase from *T. versicolor* for degradation of phenolic compound in wines. Reductions higher than 90% of ferulic acid in a model solution and 34% of phenolic compounds in wines were obtained. An earlier study by Cantarreli (1986) showed that a mutant laccase from *Polyporus versicolor* eliminated around 70% catechin and 90% of anthocyanidines from model solutions after 3 h of treatment.

5.6 CONCLUSIONS

Dilute sulfuric acid-based hydrothermal pre-treatment of apple processing by-products was optimized using RSM with the goal of disintegrating the lignin-cellulose complex, thereby enhancing the substrate digestibility. The optimal conditions were selected as acid concentration of 1.5%, reaction temperature of 91 ^{o}C and reaction duration of 16 min. Approximately 30 mg/g of glucose concentration was obtained using these conditions. The conditions optimized in the present study were moderate as compared to commonly used pre-treatment conditions. The application of commercial laccase at 10 units/100 mL significantly reduced the concentration of phenolic compounds in the hydrolyzates. The concentrations of acetic acid, furfurals and HMF in

the hydrolyzates were found to be lower than what can lead to inhibition of cellulase enzymes and fermentation micro-organisms. The present study showed that acid pre-treatment at mild conditions will generate lower amount of problematic compounds, at the same time will enhance the sugar yield in the hydrolyzate. Besides, apple processing by-products, the potential applications of the pre-treatment conditions optimized in the present study can be extended for other fruit biomass with similar polysaccharide profiles.

CHAPTER 6: ASSESSMENT OF ENZYMATIC HYDROLYSIS OF POLYSACCHARIDES PRESENT IN APPLE PROCESSING BY-PRODUCTS BY COMMERCIAL CARBOHYDROLASES

6.1 ABSTRACT

Recent understanding of the importance of second generation bio-fuels has led to increased incentive to identify alternative and ideally renewable feedstock such as lignocellulosic biomass. Processing of apples for numerous products generates several thousand tons of by-products in Nova Scotia which remain underutilized. A process has been investigated for enzymatic hydrolysis of polysaccharides present in waste biomass from the apple processing industry to convert them into simple sugars. The present study assessed the effects of process variables such as enzyme types, enzyme ratio, incubation temperature, pH, hydrolysis time, pre-treatment and solid-liquid separation on the bio-conversion of polysaccharides present in apple processing by-products. Optimization of enzymatic hydrolysis of polysaccharides using commercial pectinase, cellulase and β-glucosidase was carried out to assess the potential of manufacturing simple sugars and bio-ethanol. The result showed that disruption of pectin-cellulose network was critical to bio-conversion of apple processing waste, yielding substantial amount of glucose ($P<0.05$). The interaction of enzyme dosage, incubation temperature, pH and hydrolysis time had a significant effect ($P<0.05$) on the production of glucose. Dilute acid based hydrothermal pre-treatment and solid-liquid separation of hydrolyzate also favored high concentration of glucose ($P<0.05$). The optimum conditions of enzymatic saccharification were: enzyme activity of commercial cellulase (9 units), pectinase (38 units) and β-glucosidase (8 units) per gram of apple processing by-products (FW), operating temperatures of 40-45 ^{o}C, pH of 4.0 and reaction time of 24 h. The fermentable sugars such as glucose and galacturonic acid, obtained from enzymatic hydrolysis can be further converted to bio-ethanol using specific strains of yeast such as *Saccharomyces cerevisiae* and bacteria such as *Escherichia coli*.

Keywords: enzyme hydrolysis, carbohydrolases, lignocellulosic biomass, cellulase, pectinase, β-glucosidase

6.2 INTRODUCTION

Production of bio-ethanol from renewable lignocellulosic sources has been regarded as a promising means of decreasing pressure on fossil fuel (Galbe and Zacchi, 2002; Wyman and Hinman, 1990). However, the bio-ethanol produced is currently not cost-competitive with petroleum products. To date, the raw material, pre-treatment and enzyme production are the two main contributors to the overall costs. In an approach to reduce these costs, research is underway to develop technologies for the efficient bio-conversion of lignocellulosic biomass into ethanol.

Apple processing is carried out for the manufacture of numerous food products such as apple pies, sauces and juice, thereby resulting in significant volume of waste by-products (Rupasinghe and Kean, 2008). In Nova Scotia, the apple processing industry generates about 5 to 6 thousand tonnes of apple pomace, skin and cores in every year (Rupasinghe, 2003). Currently, apple processing by-products are under-utilized and apple processing industry faces disposal problems. In this context, bio-conversion of apple pomace into fermentable sugars is an interesting possibility as apple pomace has been described as an excellent source of bio-conversion to produce value-added compounds (Vendruscolo et al., 2008).

Pectin, cellulose and hemicelluloses and lignin are the major polysaccharides present in apple processing by-products. A covalent cross-link between pectin and cellulose has been suggested by a study by Oechslin et al. (2003). This cross-link is suggested to bind the cellulosic residues in a strong manner. Therefore, break down of pectin network would facilitate access of enzymes to the cellulose and consequently release more glucose. Commercial hydrolytic enzymes such as pectinases, cellulases and hemicellulases are commonly used in the fruit processing industry to hydrolyze

polysaccharides into oligosaccharides and monosaccharides. Maximum cellulase and β-glucosidase activities occur at 40-60 °C and pH of 4.0 to 5.0. However, optimal conditions may change with hydrolysis time (Teherzadeh and Niklasson, 2004) as well as type of substrate used. The synergistic action of pectinase, cellulase, and β-glucosidase is the crucial part of liquefaction and saccharification process of apple pomace (Capek et al., 1995; Will et al., 2000).

High amount of polysaccharides (pectin, cellulose and hemicellulose) present in apple processing by-products makes them a useful substrate for enzymatic hydrolysis to yield fermentable sugars such as glucose, fructose and galacturonic acid. While glucose and galacturonic acid result from hydrolysis of cellulose and pectin, respectively; fructose entrapped in the fruit tissues can be released during hydrolysis. Glucose and fructose can be further converted into bio-ethanol using specific strains of *Sacchromyces cerevisiae* and galacturonic acid can be fermented using recomninant *Escherichia coli* KO11. Owing to the advantages of using enzymes in terms of relatively higher conversion rates as compared to chemical conversion, this study has been attempted to understand the feasibility of enzymatic hydrolysis of apple processing by-products to enhance glucose yield.

The overall goal of this study was to apply commercial pectinolytic and cellulolytic enzymes for hydrolysis of polysaccharides present in apple processing by-products and to optimize the parameters of enzyme dosage, reaction temperature, working pH and duration of reaction required for maximum conversion of polysaccharides to simple sugars.

The specific objectives were to:

1. investigate the effect of commercial pectinase, cellulase and β-glucosidase on the release of simple sugars when used individually and in combinations;
2. optimize enzymatic hydrolysis process for glucose yield by factors such as enzyme dosage, duration, operating temperature and pH;
3. investigate the effect of pre-treatment, polyphenol oxidation and solid-liquid separation of hydrolyzate on sugar yield as obtained by enzymatic hydrolysis.

6.3 MATERIALS AND METHODS

6.3.1 APPLE PROCESSING BY-PRODUCTS

The apple processing by-products were collected from J. W. Mason and Sons Ltd., Windsor, NS, Canada and Apple Valley Foods Inc., Kentville, NS, Canada during the year 2008-2009. One primary batch of apple processing by-products was collected randomly from the two locations. The apple processing by-products were from 'Idared' and 'Northern Spy' cultivars of apples, which were combined for further use. The collected sample was further subsampled for experimental purposes. Apple pomace, residue from juice extraction contained peel, core, seeds and rice husks. Rice husks are added to facilitate pressing of juice and could be present upto 5-10% of the total weight. The apple processing by-products were from 'Idared' and 'Northern Spy' cultivars of apples (combined) and were stored at -20 ^{o}C until use.

Apple processing by-products were homogenized into a puree for two minutes using a food processor (Bead Beater; BioSpec Products, Inc. Bartlesville, OK, USA). The pH of the puree was determined using a standardized pH meter (Model Accumet® 10, Denver Instruments Co., Denver, CO, USA).

6.3.2 REAGENTS AND STANDARDS

Glacial acetic acid (reagent grade) and sodium acetate were purchased from Fisher Scientific (Ottawa, ON, Canada). HPLC grade acetonitrile was purchased from Fisher Scientific, Canada. Analytical grade sugar standards of glucose, fructose and sucrose were obtained from Sigma Aldrich (Oakville, ON, Canada).

6.3.3 ENZYME PREPARATIONS

Commercial enzyme concentrates of pectinase (Pectinex 3XL®) and cellulase (Celluclast 1.5L®), were obtained from Novozymes North America Inc. (Franklinton, NC, USA), while β-glucosidase (Novozyme 188) was purchased from Sigma Aldrich Canada. All the enzyme preparations were stored at 4 ^{o}C until use. The details of enzymes used in the study are summarized in **Table 6.1**.

6.3.4 ENZYMATIC HYDROLYSIS OF APPLE PROCESSING BY-PRODUCTS

Assessment of enzymatic hydrolysis of apple processing by-products was carried out according to the experimental plan given in **Table 6.2**. The enzyme cocktail was suspended in 25 mL of buffer for 10 min. prior to its addition to the puree of apple processing by-products. Enzymatic hydrolysis was carried out in 125 mL Erlenmeyer flasks with a working volume of 50 mL puree (equal to 12.5 g apple processing by-products) for all hydrolysis experiments. To prevent possible microbial spoilage of the produced sugars, 100 µL of 2% sodium azide was added before enzymes. The experiments were carried out under continuous shaking (150 rpm; Model Apollo HP50, San Diego, California, USA). The flasks were covered with aluminum foil to avoid evaporation. The substrate was allowed to reach the desired operating conditions and time zero h samples were collected before addition of the enzymes. Addition of enzyme cocktail marked the initiation of trial. Enzymes were added as per the requirement of each individual experiment. A micropipette was used for sampling, using a pipette tip with the end cut off, since the hydrolyzate was viscous. From each flask, 1 mL of sample was collected at the desired time point and placed in a micro centrifuge tube. The samples were immediately placed in the freezer (-20 ^{o}C) to quench the reaction. Enzymatic hydrolysis procedure was standardized for apple processing by-products.

Based on literature and content of major polysaccharides present, commercial cellulase, pectinase and β-glucosidase were selected for standardizing the process. Four factors including enzyme dose, temperature, pH and time were selected at different levels as described in **Table 6.2**.

6.3.4.1 EFFECT OF CHANGE IN ENZYME ACTIVITY OF CELLULASE AND B-GLUCOSIDASE

The standardized enzyme cocktail consisting of cellulase, pectinase and β-glucosidase was varied in activity (EU/50 mL puree). Cellulase and pectinase were added at 2x and 4x concentrations than standard dose of x (112 EU and 480 EU for cellulase and pectinase, respectively). Hydrolysis of substrate was carried out at pH 4.0, temperature of 40 ^{o}C for 24 h and samples were collected at different time intervals (1, 6, 12 and 24 h) for HPLC analysis.

6.3.4.2 EFFECT OF DILUTE ACID PRE-TREATMENT AND REMOVAL OF PHENOLIC COMPOUNDS ON ENZYMATIC HYDROLYSIS OF APPLE PROCESSING BY-PRODUCTS

Investigation of dilute sulfuric acid pre-treatment on the enzymatic hydrolysis of apple processing by-products was carried out by pretreating raw material under previously determined conditions of 1.5% sulfuric acid (w/v), reaction temperature of 91 ^{o}C for 16 min. Dilute acid pre-treatment was conducted out in Magna flex spinner glass flasks with continuous spinning of hydrolyzate (Wheaton Science Products, Millville, NJ, USA). This was followed by oxidation of polyphenolic compounds using commercial laccase (10 units/100 mL) from *T. versivolor* at a pH of 5.3. The pH of the hydrolyzate was adjusted with acetate buffer (pH 4.0) after pre-treatment and polyphenol oxidation. Subsequent enzymatic hydrolysis followed according to the standardized hydrolysis conditions.

Table 6.1 Summary of enzyme preparation characteristics.

Commercial enzyme name	Main activity[a]	Source[a]	Declared activity (EU/mL)	Optimal pH of main activity[a]	Optimal temperature of main activity (^{o}C)[a]
Celluclast 1.5 L	Cellulase	*Trichoderma reesei*	700	4.5 to 6.0	50-60
Pectinex 3XL	Pectinase	*Aspergillus niger*	3000	4.0-5.0	40-50
Novozyme 188	β-Glucosidase	*Aspergillus niger*	665	4.3[e]	50[e]

[a]*Information supplied by product sheets from Novozymes North America Inc.(Franklinton, NC, USA)*
[b]*EU- Enzyme Unit*
[e]*Adapted from Bravo et al. (2001)*

Table 6.2 Experimental plan for the enzymatic hydrolysis of apple processing by-products.

Experiment	Factor studied	Enzymes				Temperature (°C)	pH	Time (h)
		Combination[a]	Activity (EU/50 mL)[b]					
			P	C	N			
I	enzyme type	P, C, N	60	14	13.3	45	4.6	32
II	enzyme combination	CP, CN, CPN	60	14	13.3	45	4.6	32
III	enzyme dosage, temperature, pH, time	CPN	60, 240, 480	14, 56, 112	13.3, 53.2, 106.4	40, 45, 50	4.0, 4.5, 5.0	24

[a]*P, Pectinase (Pectinex 3XL), C, Cellulase (Celluclast 1.5L), N, β-glucosidase (Novozyme 188)*
[b]A volume of 50 mL puree contained 12.5 g of apple processing by-products (FW).

6.3.4.3 EFFECT OF END PRODUCT REMOVAL ON RESIDUAL POLYSACCHARIDE HYDROLYSIS

A separate experiment was conducted to assess the effect of removal of end products of enzyme hydrolysis on further hydrolysis of the residual polysaccharides. A puree of apple processing by-products which was enzymatically hydrolyzed according to standardized conditions (112, 480 and 106.3 EU of cellulase, pectinase and β-glucosidase at 40 ^{o}C, pH 4.0 for 24 h) was centrifuged at 8000 × g for 10 min. This was followed by solid-liquid separation by decanting the supernatant off into another tube. The residual solid content was supplemented with acetate buffer (pH 4.0) to make upto 50 mL volume. Enzymes were then added and the mixture was again subjected to hydrolysis for another 24 h. The liquid content from each fraction was filtered with 0.45 micron filter and transferred into HPLC vials for sugar analysis.

6.3.5 QUANTIFICATION OF SUGARS

Samples (1 mL) were removed from each flask at different time intervals. Samples were centrifuged for 20 min. at 8000 × g (Sorvall Legend Micro-17 centrifuge, Thermo Scientific, Ottawa, ON, Canada), filtered through a 0.45 micron nylon filter (Chromaspec, Brockville, ON, Canada) and the supernatant was analyzed for glucose, fructose and sucrose by HPLC using a Waters Corporation HPLC system connected to a Waters 2414 refractive index (RI) detector. A monosaccharide amino column (250 x 4.6 mm; Phenomenex, Torrence, CA, USA) was used and the analysis conditions were: temperature of 40 °C and 30 °C for column and detector, respectively; mobile phase of acetonitrile and deionized water (75:25); run time 15 minutes and flow rate 1.0 mL/min. An injector volume of 20 μL was used for each run. Calibration curves were obtained by

using standard solutions of glucose, fructose and sucrose (25, 50, 100 and 200 ppm). The MassLynx 4.1 software was used to control the chromatograph system and quantify sugar concentrations.

6.3.6 EXPERIMENTAL DESIGN AND STATISTICAL ANALYSES

Experiment I and II (**Table 6.2**) were carried out with two replicates per treatment. Experiment III was carried out with three replicates per treatment using a four factor factorial design for assessing the interaction effects. All other experiments were carried out using completely randomized design with three replicates per treatment. Treatments were separated using repeated measures PROC MIXED model of SAS with statistical significant difference at $P \leq 0.05$ using least square means (SAS Institute, 1999). The assumption of normal probability distribution was verified by using normal probability plots of residuals. The assumption of constant variance was verified by a scatter diagram by plotting residuals versus fits (Montgomery, 2005).

6.4 RESULTS

Enzymatic hydrolysis was carried out to hydrolyze cellulose, hemicelluloses, pectin, cellobiose and oligosacharides. Concentrations of glucose, fructose and sucrose concentrations from the various trials were tracked as a function of hydrolysis.

6.4.1 EFFECT OF INDIVIDUAL ENZYMES ON SUBSTRATE

The preliminary study was carried out using one enzyme at a time. The trials were performed with a working volume of 50 mL in duplicates at a temperature of 45 ^{o}C and pH 4.6 under continuous agitation of 150 rpm for 32 h. Commercial cellulase, pectinase and β-glucosidase were used at 14, 60 and 13.3 EU, respectively per 12.5 g FW apple processing by-products. The enzyme loading was selected based on their commercial application in apple mash treatment (Novozymes North America Inc., Franklinton, NC, USA). Enzymatic hydrolysis using single enzyme at a time showed significant effect ($P<0.05$) of the enzyme type and hydrolysis time on the production of glucose in hydrolyzates (**Table 6.3**).

Table 6.3 ANOVA for single enzyme at a time.

Effect	P value	
	Glucose	**Fructose**
Treatment	<0.0001	0.3790
Time	<0.0001	<0.0001
Treatment*time	<0.0089	<0.5140
SEM	0.74	2.42

SEM-Standard Error of Mean

Commercial cellulase yielded significantly higher ($P<0.05$) amount of glucose (10 mg/g FW) than produced by pectinase (6 mg/g FW) and β-glucosidase (3 mg/g FW) after 32 h of hydrolysis (**Figure 6.1**). It was interesting to note that the amount of glucose generated by cellulase increased significantly after 16^{th} h, when compared to hydrolysis by pectinase. Enhancement in glucose content of pectinase hydrolyzate indicated that glucose being trapped in the pectin network was released by pectinase. Besides, the results suggested that commercial preparation of cellulase and pectinase could contain some additional activities of other enzymes such as cellobiohydrolases, exo-glucanase and β-glucosidase. Owing to the absence of free cellobiose in the hydrolyzate, commercial β-glucosidase was found to yield least amount of glucose after 32 h of hydrolysis.

Analysis of variance (ANOVA) showed that interaction between two factors (enzyme type and duration of hydrolysis) was insignificant for fructose content in the hydrolyzate. However, significant changes were observed for fructose with respect to time of the reaction. Reaction time of 32 h showed maximum fructose concentration followed by values at 16 h. Concentration of fructose was found to be 54.5-68.7 mg/g FW after 32 h of hydrolysis (**APPENDIX B**).

Before enzymatic treatment, amount of sucrose present in 50 mL puree of apple processing by-products was measured as 5.2-6.3 mg/g FW. Sucrose content decreased with the increase in hydrolysis time and hence was not detected by the end of 32 h, except for control. Sucrose, a disaccharide, under enhanced temperatures is inverted into its component monomers glucose and fructose during hydrolysis.

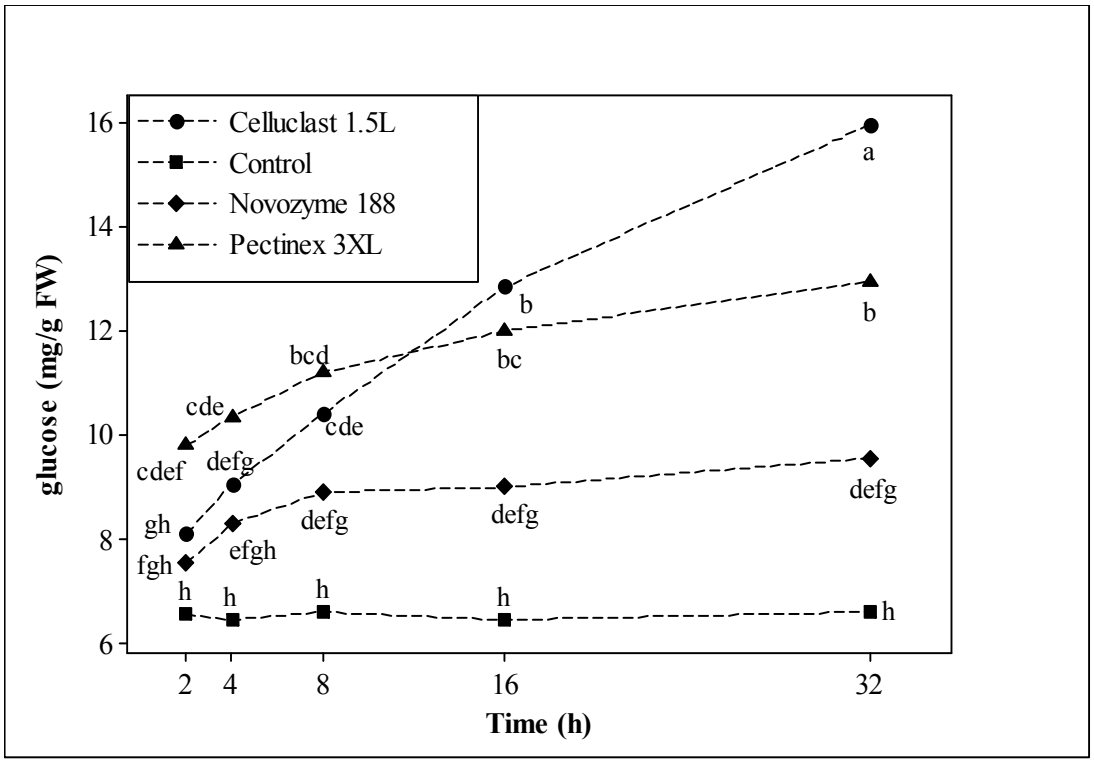


Figure 6.1 Production of glucose in response to hydrolysis of apple processing by-products by cellulase, pectinase, and β-glucosidase at pH4.6 and temperature of 45 ^{o}C.

[¥]*Data points represent the mean of two analyses.*
[ǂ]*Means with different letters are significantly different (P<0.05) and letter grouping is between treatments.*
[*]*Celluclast 1.5L, cellulase; Pectinex 3XL, pectinase and Novozyme 188, β-glucosidase*
[4]*Substrate concentration was 12.5 g apple processing by-products per trial (50 mL puree)*
[†]*Enzyme activity: 14 EU for Celluclast 1.5L, 60 EU for Pectinex 3XL and 13.3 EU for Novozyme 188.*

6.4.2 EFFECT OF ENZYME COMBINATIONS ON SUGAR YIELD

Commercial cellulase, pectinase and β-glucosidase were used in different combinations, while keeping their enzyme units same as above. The interaction between enzyme combinations and hydrolysis time was found to be significant ($P<0.001$) for glucose (**Table 6.4**). As seen from **Figure 6.2**, hydrolysis of apple processing by-products with a combination of three enzymes (CPN) yielded more glucose ($P<0.05$) than produced by the other treatment combinations (CP and CN). Additive and synergistic effect of all three enzymes together (CPN) produced 24.4 mg/g FW glucose after 32 h, followed by 21.1 mg/g FW produced after 16 h of hydrolysis. After 32 h, enzyme hydrolysis using cellulase and β-glucosidase (CN) was able to produce only 15.7 mg/g FW glucose, which was not different from the 15.1 mg/g FW glucose as produced after 16 h of hydrolysis. Similarly, cellulase and pectinase (CP) together did not produce high amount of glucose after 32 h of hydrolysis. Only 13 mg/g FW of glucose was observed after hydrolysis with combined cellulase and pectinase (CP).

Table 6.4 ANOVA for enzyme combinations.

Effect	**P value**		
	Glucose	**Fructose**	**Sucrose**
Treatment (enzyme combinations)	<0.0001	0.0397	<0.0001
Time	<0.0001	0.0003	<0.0001
Treatment*time	<0.0001	0.1247	<0.0001
SEM	0.346	2.696	0.270

SEM-Standard Error of Mean

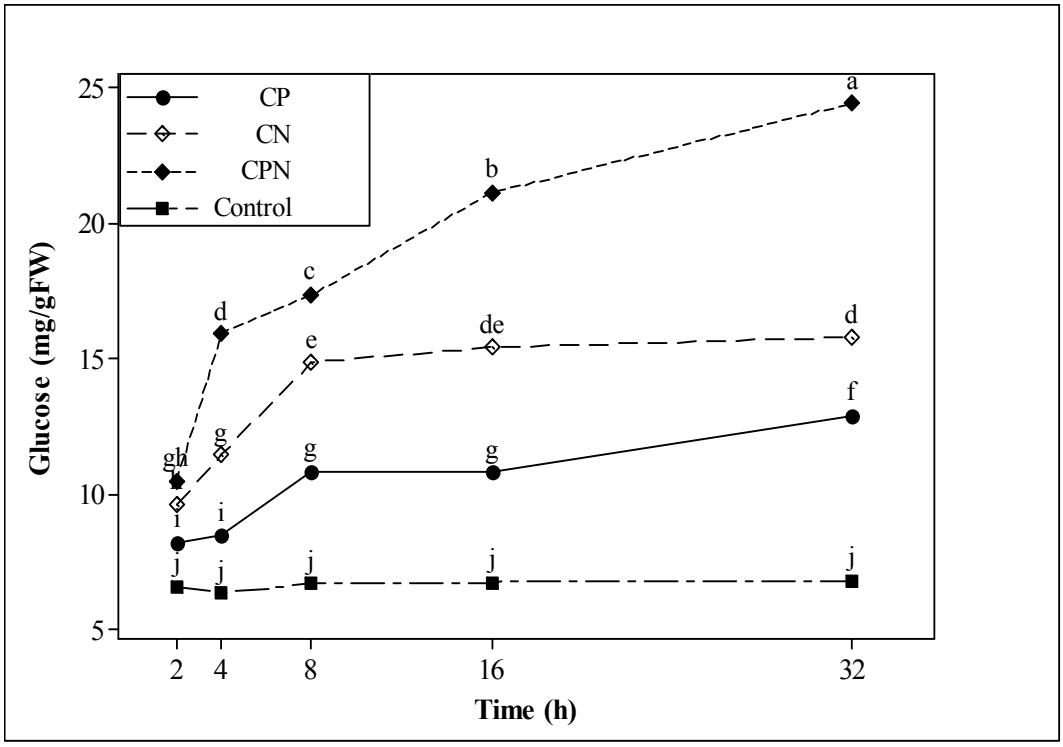


Figure 6.2 Production of glucose in response to hydrolysis of apple processing by-products by enzyme combinations of cellulase, pectinase, and β-glucosidase at pH of 4.6 and temperature at 45 ^{o}C.

[*]C, Celluclast 1.5L (cellulase); P, Pectinex 3XL (pectinase) and N, Novozyme 188(β-Glucosidase)

[¥]Data points represent the mean of two analyses.

[ǂ]Means with different letters are significantly different (P<0.05) and letter grouping is between treatments.

[€]Means separated by Tukey's studentized range test (P<0.05)

[†]Enzyme activity: 14 EU for Celluclast 1.5L, 60 EU for Pectinex 3XL and 13.3 EU for Novozyme 188.

[ɬ]Substrate concentration was 12.5 g apple processing by-products per trial (50 mL puree)

However, higher yields of glucose were observed when pectinase was combined with cellulase than when only pectinase was added to the substrate, thereby indicating cellulose-pectin interactions.

Fructose content was not affected by enzyme combinations. However, a high amount of free fructose, ranging between 63-75 mg/g FW, was measured after 32 hours of hydrolysis (**APPENDIX C**). By contrast, the decrease in sucrose content seems to be directly correlated to increase in hydrolysis time using different enzyme combinations (**APPENDIX D**). However, sucrose concentration in the control did not decrease as compared to other hydrolyzates. After two h of hydrolysis, the sucrose content in the hydrolyzates was found to lie in the range 4.9-5.7 mg/g FW, which decreased to almost zero after 32 h. Concentration followed a significant ($P<0.001$) decreasing trend after four hours of hydrolysis.

6.4.3 EFFECT OF TEMPERATURE, PH, TIME AND ENZYME DOSAGE ON HYDROLYSIS

In general, the efficiency of the enzyme-aided hydrolysis of biomass is influenced by several variables, such as enzyme type, enzyme activity, reaction temperature, time, and pH. The hydrolysis of apple processing by-products was standardized for enzyme activity, pH, incubation temperature and time. Preliminary experiment (**Appendix E**) showed that at enzyme activity above 112, 480 and 106.4 EU for Celluclast 1.5L, Pectinex 3XL and Novozyme 188, respectively, resulted in no significant improvement in glucose yields at constant conditions of temperature, pH and time. Therefore, enzyme activities of 112 EU (cellulase), 480 EU (pectinase) and 106.4 EU (β-glucosidase) were selected as the highest level. Based on optimum activity of enzymes (as recommended by the manufacturer), the temperature (40, 45 and 50 ^{o}C) and pH levels (4.0, 4.5 and 5.0) were selected. These process conditions were used to refine

the range of each independent factor to be used as the basis for all further trials with pretreated substrates.

The results from the ANOVA showed that the main effects of enzyme dosage and time had a significant effect ($P<0.001$) on the release of glucose as a result of hydrolysis of apple processing by-products (**Table 6.5)**. In addition, the interactions between all the four factors (temperature, pH, time and enzyme activity) exerted significant impact on the hydrolysis of the substrate in terms of glucose production. **Figure 6.3** shows the effect of hydrolysis conditions (temperature, pH, enzyme dosage and time) on glucose release. Glucose was generated at a high rate by action of polysaccharide hydrolases. Production of glucose increased progressively when the enzyme activity was increased from lower level to higher level at 40 ^{o}C and pH 4.0 (**Figure 6.4**). Buffer pH affected yield of glucose hydrolysis of apple processing by-products at incubation temperature of 45 ^{o}C and enzyme activity of 56, 240 and 53.2 EU for Celluclast 1.5L, Pectinex 3XL and Novozyme 188, respectively, after 24 h. These conditions produced the highest amount of glucose (44.1 mg/g FW). However, this was not significantly different ($P>0.05$) from glucose concentration (42 mg/g FW) found at pH 4.0-4.5, temperature 45 ^{o}C and enzyme activity of 112, 480 and 106.4 EU for Celluclast 1.5L, Pectinex 3XL and Novozyme 188, respectively, after the same duration of reaction.

Table 6.5 ANOVA for glucose, fructose and sucrose as a result of four factor factorial design used for hydrolysis of apple processing by-products.

Effect	P value		
	Glucose	**Fructose**	**Sucrose**
Temperature	0.9064	<.0001	<0.0001
pH	0.1889	0.2623	<0.0001
Temperature*pH	<0.0001	<.0001	<0.0001
Enzyme dosage	<0.0001	<.0001	<0.0001
temperature*enzyme dosage	0.0001	0.0249	0.0014
pH* enzyme dosage	0.0005	0.0064	0.0043
temperature*pH* enzyme dosage	0.0049	0.0806	<0.0001
time	<0.0001	<.0001	<0.0001
temperature*time	<0.0001	<.0001	0.0007
pH*time	<0.0001	<.0001	0.0080
temperature*pH*time	0.0001	<.0001	<0.0001
enzyme dosage*time	0.2118	0.1853	<.0001
temperature* enzyme dosage*time	0.0216	0.1649	<0.0001
pH* enzyme dosage*time	0.0004	0.0008	<0.0001
temperature*pH* enzyme dosage*time	<0.0001	0.2306	<0.0001

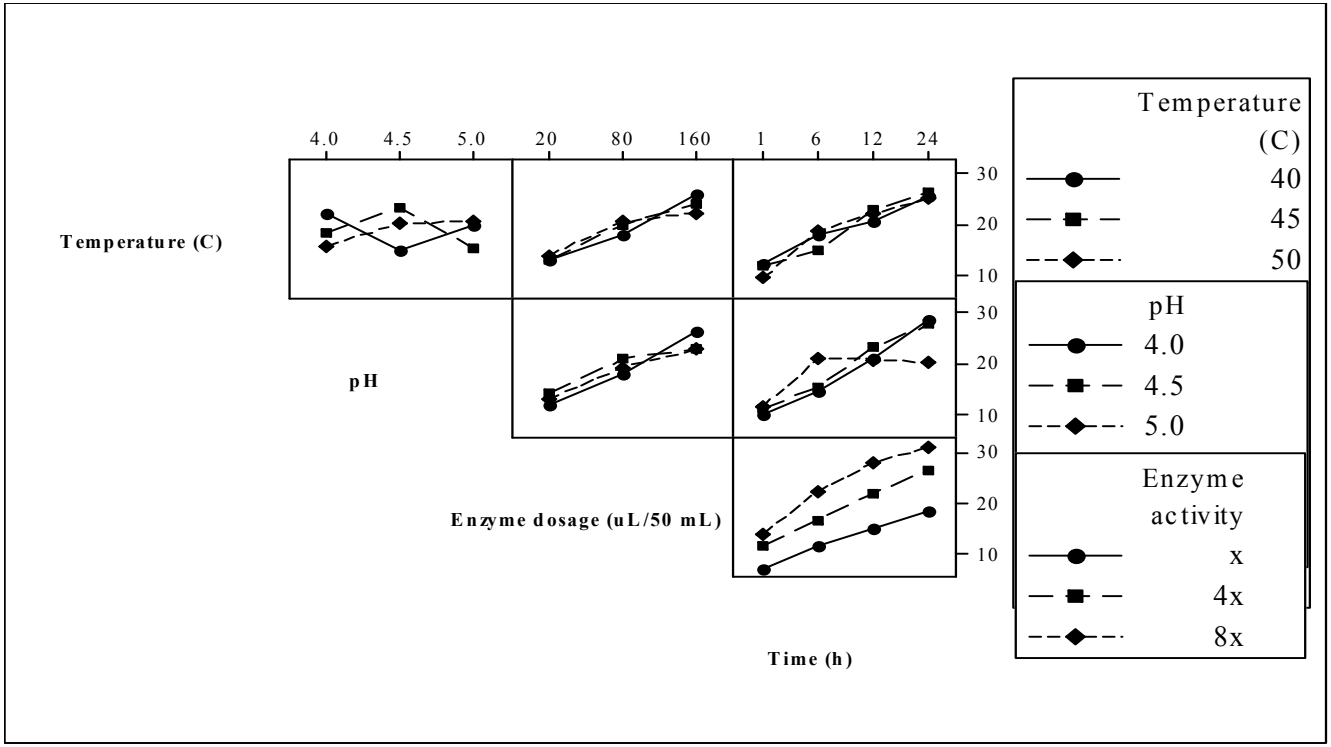


Figure 6.3 Interaction profile for glucose (mg/g FW) as affected by enzyme activities of of cellulase (14, 56 and 112 EU), pectinase (60, 240 and 600 EU) and β-glucosidase (13.3, 53.2 and 106.4 EU), temperature (40- 50^{o}C), reaction duration (1-24 h) and pH (4.0-5.0).

[$] *Enzyme activity of x corresponds to 14 EU (Celluclast 1.5L), 60 EU (Pectinxe 3XL) and 13.3 EU (Novozyme 188); 4x corresponds to 56 EU (Celluclast 1.5L), 240 EU (Pectinxe x3XL) and 53.2 EU (Novozyme 188); 8x corresponds to 112 EU (Celluclast 1.5L), 480 EU (Pectinxe 3XL) and 106.4 EU (Novozyme 188).*

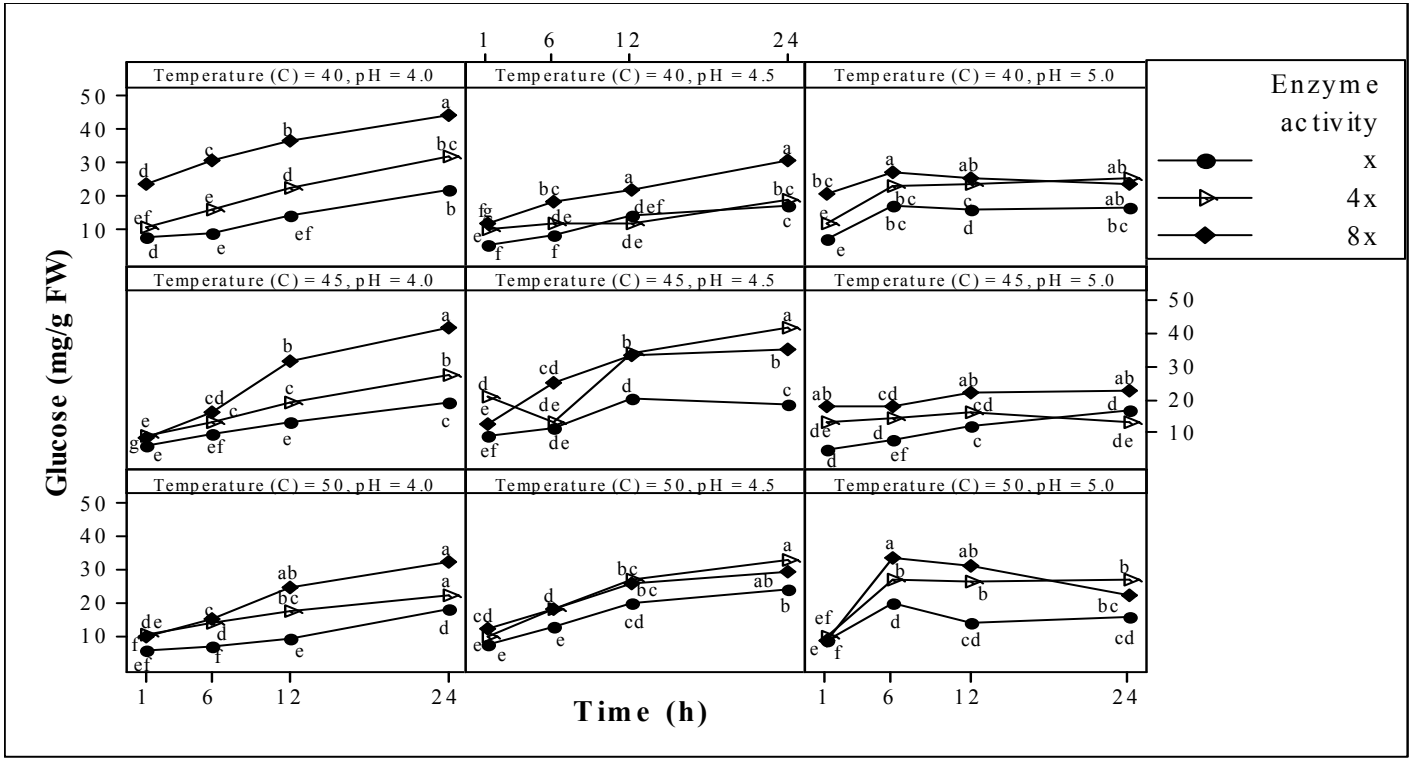


Figure 6.4 Scatterplot of glucose production in response to hydrolysis of apple processing by-products by enzyme combinations of cellulase (14, 56 and 112 EU), pectinase (60, 240 and 600 EU) and β-glucosidase (13.3, 53.2 and 106.4 EU) at pH 4.0-5.0 and temperature of 40-50 ^{o}C.

**Substrate concentration was kept at 12.5 g apple processing by-products per 50 mL*

[§]Means with different letters within each panel are significantly different (P<0.05).

[€]Means separated by Tukey's Studentized range test (P<0.05)

[$]Enzyme activity of x corresponds to 14 EU (Celluclast 1.5L), 60 EU (Pectinxe 3XL) and 13.3 EU (Novozyme 188); 4x corresponds to 56 EU (Celluclast 1.5L), 240 EU (Pectinxex 3XL) and 53.2 EU (Novozyme 188); 8x corresponds to 112 EU (Celluclast 1.5L), 480 EU (Pectinxe 3XL) and 106.4 EU (Novozyme 188).

It was noted that in most cases, hydrolysis with enzyme activity of 112 EGU (cellulase), 480 FDU (pectinase) and 106.4 CBU (β-glucosidase) demonstrated the production of high glucose amount. Increase in enzyme activity in the ratio x: 4x: 8x did not lead to increase in glucose concentration in the same ratio. The three selected levels of enzyme activity of cellulose (14, 56 and 112 EU), pectinase (60, 240 and 480 EU) and β-glucosidase (13.3, 53.2 and 106.4 EU), hydrolysis temperature of 40 ^{o}C with pH 4.0 after 24 h produced glucose concentration of 21, 31 and 44 mg/g FW, respectively. Increase of glucose concentration between 12 and 24 h using the highest level of enzyme activity (112 EGU, 480 FDU and 106.4 CBU for Celluclast 1.5L, Pectinex 3XL and Novozyme 188, respectively) was found to be quite variable, ranging from 3-12 mg/g FW, depending on the conditions applied. During the last 12 h of hydrolysis, the highest levels of enzyme activity for cellulase, pectinase and β-glucosidase, produced glucose in the range between 2-10 mg/g FW in the hydrolyzate. At the same time, the intermediate level of enzyme activity (56, 240 and 53.2 EU for Celluclast 1.5L, Pectinex 3XL and Novozyme 188, respectively) generated 3-9 mg/g FW glucose, while the lowest level produced 4-8 mg/g FW glucose. In most cases, the reaction duration of 24 h resulted in high amount of glucose relative to other reaction durations. However, in some cases, glucose concentration was found to decrease with the increase in reaction duration from 12 to 24 h. This trend was observed in hydrolyzates with pH 5.0, which were incubated at 50 ^{o}C for 24 h. With the lowest, intermediate and highest enzyme activity levels, hydrolysis period of six h produced 20, 27 and 34 mg/g FW of glucose, respectively. It was also observed that the highest temperature (50 ^{o}C) combined with highest pH level (5.0) did not favor glucose production. It was also interesting to note that enzyme

intermediate enzyme activity levels for all the three enzymes produced significantly higher amounts of glucose than the highest enzyme activity level at pH 4.5, with temperatures 45 and 50 ^{o}C.

With respect to fructose concentration, two of the three-way interactions, (pH*temperature*time and pH*enzyme dosage*temperature) were observed to be significant (**Table 6.5)**. Fructose concentration after 24 h of hydrolysis at incubation temperature 50 ^{o}C and pH 5.0 was about 89 mg/g FW, which was significantly greater than 56 mg/g FW produced at pH 4.5 and the same temperature (**Figure 6.5a, b**). Interestingly, the trend observed for increasing fructose concentration in the hydrolyzates was opposite to the one observed for glucose. This was because of the fact that highest level of temperature (50 ^{o}C) favored increase in fructose concentration.

Similar to glucose, a four-way interaction between all the selected independent factors was highly significant for sucrose (**Table 6.5**). However, amount of sucrose was found to decrease with the hydrolysis process. On a fresh weight basis, the apple processing by-products contained an average of 7.6 mg/g of sucrose before enzymatic hydrolysis, which dropped to non-detectable limit after 24 h of hydrolysis at the applied conditions (**Figure 6.6**). The decrease in sucrose concentration was observed to occur at a high rate between first and sixth h at all selected pH and temperatures using enzyme acitivities of intermediate and highest level. However, this drop was observed to take place at a slower rate in the case of hydrolysis using the lowest level of enzyme activity.

From the above results, it is clear that glucose was the major sugar released after enzymatic hydrolysis of polysaccharides present in apple processing by-products.

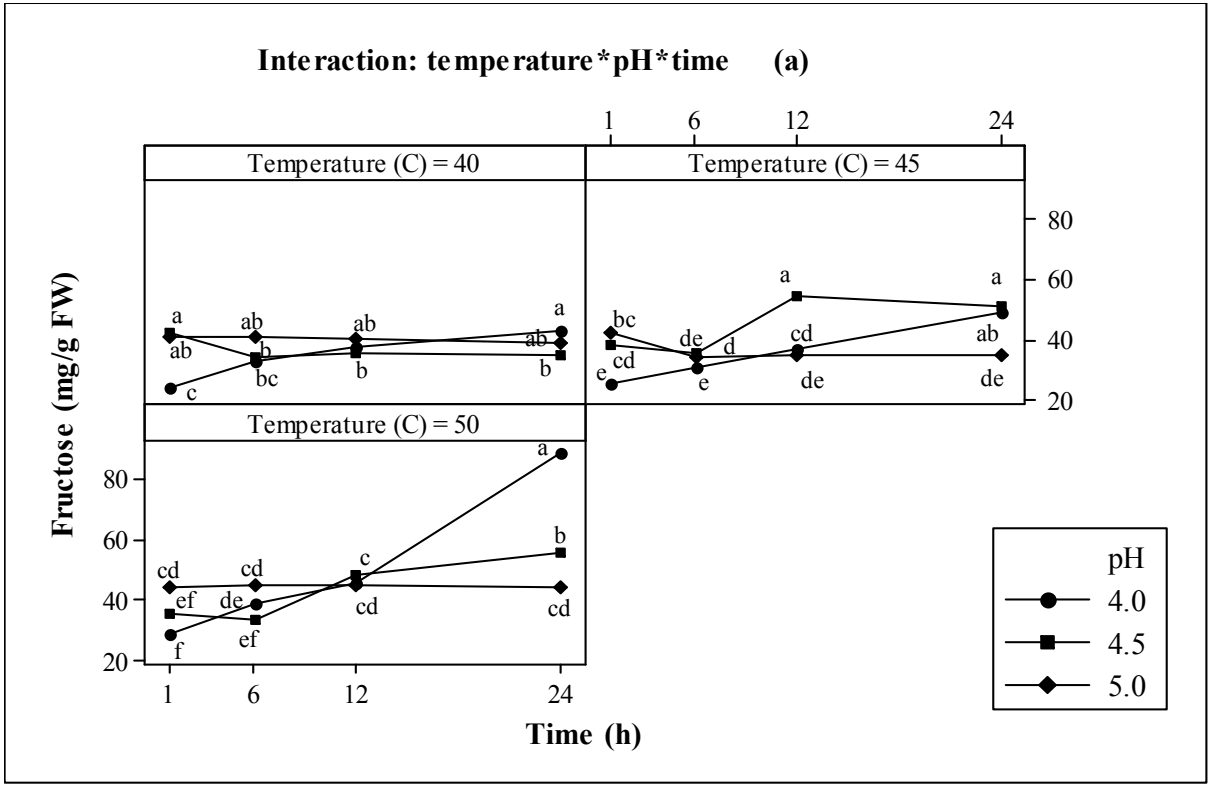


Figure 6.5a Interaction profile for fructose (mg/g FW) as affected by temperature (^{o}C), time (h) and pH, using a four factor factorial design.

**Substrate concentration was kept at 12.5 g apple processing by-products per 50 mL*
[§]Means with different letters within each panel are significantly different (P<0.05).
[€]Means separated by Tukey's Studentized range test (P<0.05)
[$]Enzyme activity of x corresponds to 14 EU (Celluclast 1.5L), 60 EU (Pectinxe 3XL) and 13.3 EU (Novozyme 188); 4x corresponds to 56 EU (Celluclast 1.5L), 240 EU (Pectinxex 3XL) and 53.2 EU (Novozyme 188); 8x corresponds to 112 EU (Celluclast 1.5L), 480 EU (Pectinxe 3XL) and 106.4 EU (Novozyme 188).

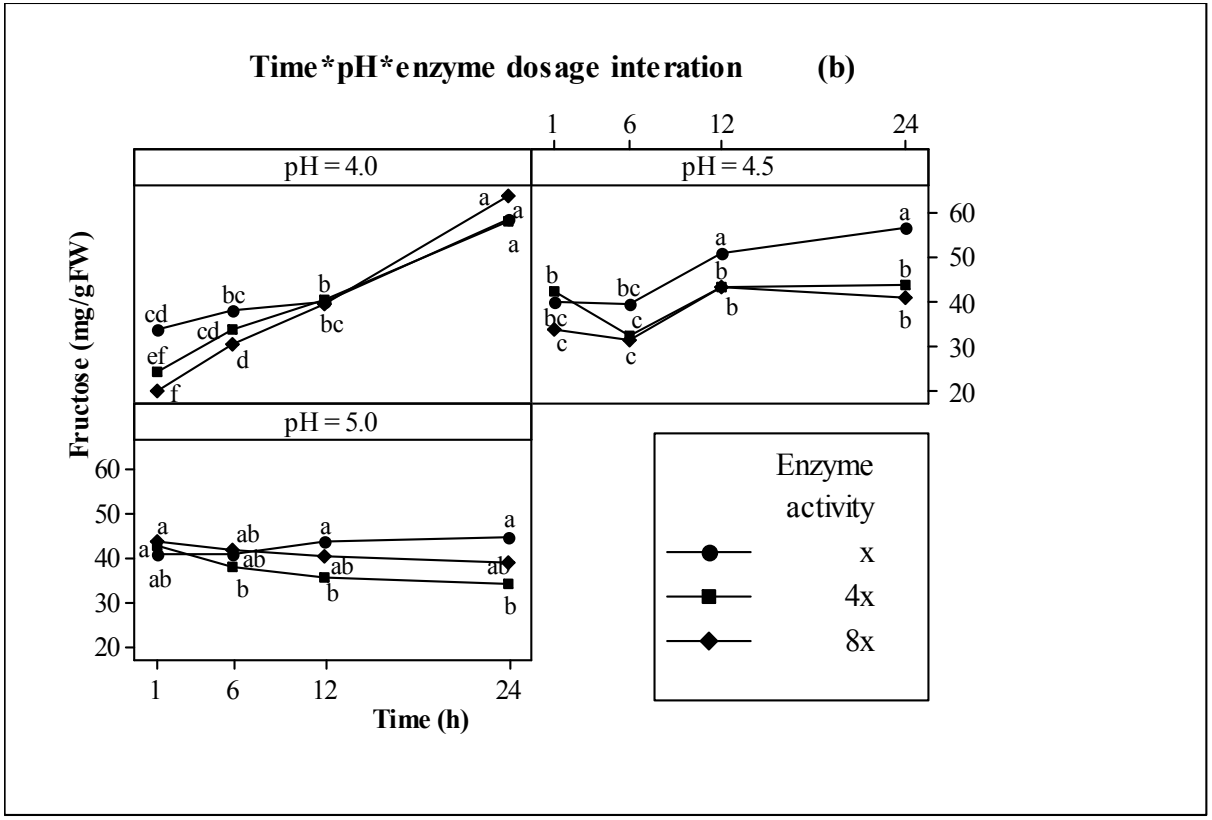


Figure 6.5b Interaction profile for fructose (mg/g FW) as affected by enzyme activity (cellulase, pectinase and β-glucosidase), reaction duration (h) and pH, using a four factor factorial design.

**Substrate concentration was kept at 12.5 g apple processing by-products per 50 mL*
[§]Means with different letters within each panel are significantly different (P<0.05).
[€]Means separated by Tukey's Studentized range test (P<0.05)
[$]Enzyme activity of x corresponds to 14 EU (Celluclast 1.5L), 60 EU (Pectinxe 3XL) and 13.3 EU (Novozyme 188); 4x corresponds to 56 EU (Celluclast 1.5L), 240 EU (Pectinxe 3XL) and 53.2 EU (Novozyme 188); 8x corresponds to 112 EU (Celluclast 1.5L), 480 EU (Pectinxe 3XL) and 106.4 EU (Novozyme 188).

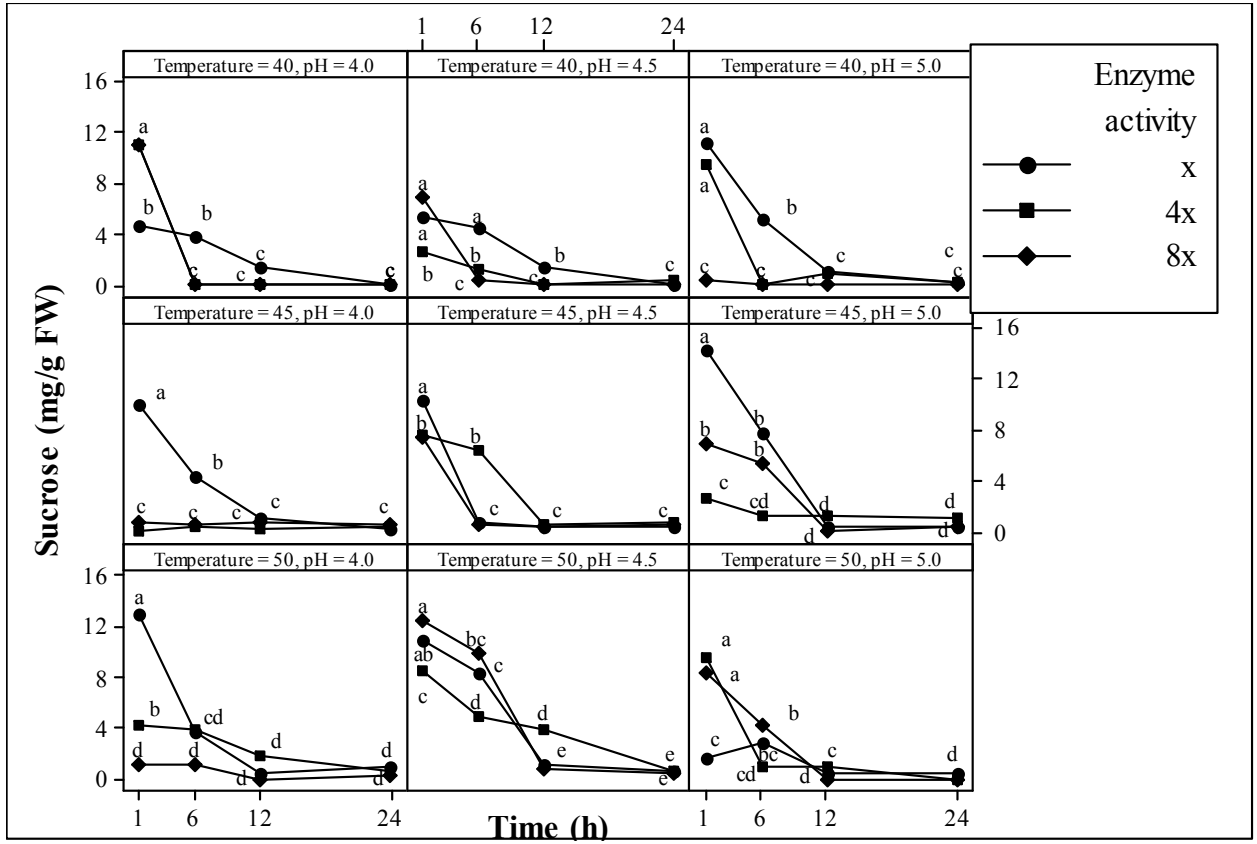


Figure 6.6 Scatterplot of sucrose in response to hydrolysis of apple processing by-products by enzyme combinations of cellulase (14, 56 and 112 EU), pectinase (60, 240 and 600 EU) and β-glucosidase (13.3, 53.2 and 106.4 EU) at pH 4.0-5.0 and temperature of 40-50 ^{o}C.

**Substrate concentration was kept at 12.5 g apple processing by-products per 50 mL*

[§]Means with different letters within each panel are significantly different (P<0.05).

[€]Means separated by Tukey's Studentized range test (P<0.05

[$]Enzyme activity of x corresponds to 14 EU (Celluclast 1.5L), 60 EU (Pectinxe 3XL) and 13.3 EU (Novozyme 188); 4x corresponds to 56 EU (Celluclast 1.5L), 240 EU (Pectinxe 3XL) and 53.2 EU (Novozyme 188); 8x corresponds to 112 EU (Celluclast 1.5L), 480 EU (Pectinxe 3XL) and 106.4 EU (Novozyme 188).

Considering the highest amount of glucose obtained and economical aspects of operating conditions, enzymatic hydrolysis of apple processing by-products was standardized as follows: enzyme activity of 112 EU (Celluclast 1.5L), 480 EU (Pectinex 3XL) and 106.4 EU (Novozyme 188); incubation temperature of 40 ^{o}C, pH of 4.0 and duration of 24 h.

6.4.4 Effect of change in activity of cellulase and b-glucosidase on sugar yield

In order to evaluate the effect of change in activity of the two key enzymes (cellulase and β-glucosidase) on glucose production, puree of apple processing by-products was subjected to different activities of these two enzymes, while keeping the pectinase at the same activity level that was optimized previously (480 EU/50 mL). The pH and temperature were kept as 4.0 and 40 ^{o}C, respectively (standardized as above). Change of dosage and hence activity of cellulase and β-glucosidase had a significant effect ($P<0.05$) on the response glucose concentration in the hydrolyzate. Increasing the activity of β-glucosidase by twice (223 EU) and four times (426 EU) than the standard activity of 112 EU produced 31 and 36 mg/g FW of glucose, respectively after 24 h of hydrolysis (**Figure 6.7**). This suggests the accumulation of cellobiose in the hydrolyzate which got converted into glucose after the addition of sufficient amount of β-glucosidase. However, the presence of cellobiose in the hydrolyzate was not observed in the chromatograms generated by HPLC at standardized conditions. It was suspected that more cellulose would be hydrolyzed by increased activity of cellulase in the hydrolyzate, thus producing more glucose. However, increasing the cellulase activy by two or four times did not enhance glucose production as compared to standard cellulase activity of 112 EU/50 mL.

Changes in enzyme activity were observed to have variable effects on fructose concentration. Hydrolysis of apple processing by-products with increased activity of cellulase led to a drop in fructose content by 6-7 mg/g FW (**Figure 6.8**), when compared to the content prior to hydrolysis (Table 5.6). However, no significant change in fructose was found after hydrolysis with increased β-glucosidase activity. On the other hand, the hydrolysis conditions led to total depletion of sucrose content from the hydrolyzate, making it non-detectable after six h, with all enzyme treatments.

6.4.6 EFFECT OF END PRODUCT REMOVAL ON RESIDUAL POLYSACCHARIDE HYDROLYSIS

In a separate experiment, the influence of end product removal from hydrolyzate was assessed on the hydrolysis of residue in terms of glucose yield. After 24 h of hydrolysis using standard conditions, soluble sugars were removed from the hydrolyzate by centrifugation. The remaining residue was again suspended in the buffer (pH 4.0), supplemented with the enzyme cocktail and hydrolyzed for another 24 h. Approximately 7-10 mg/g FW glucose was produced after end product removal (**Figure 6.9**). Concentration of glucose after end product removal (48 mg/g FW) was significantly higher ($P<0.001$) than observed before (40 mg/g FW). These results indicated the inactivation of cellulase/β-glucosidase during the course of hydrolysis and the need to reload them to continue hydrolysis.

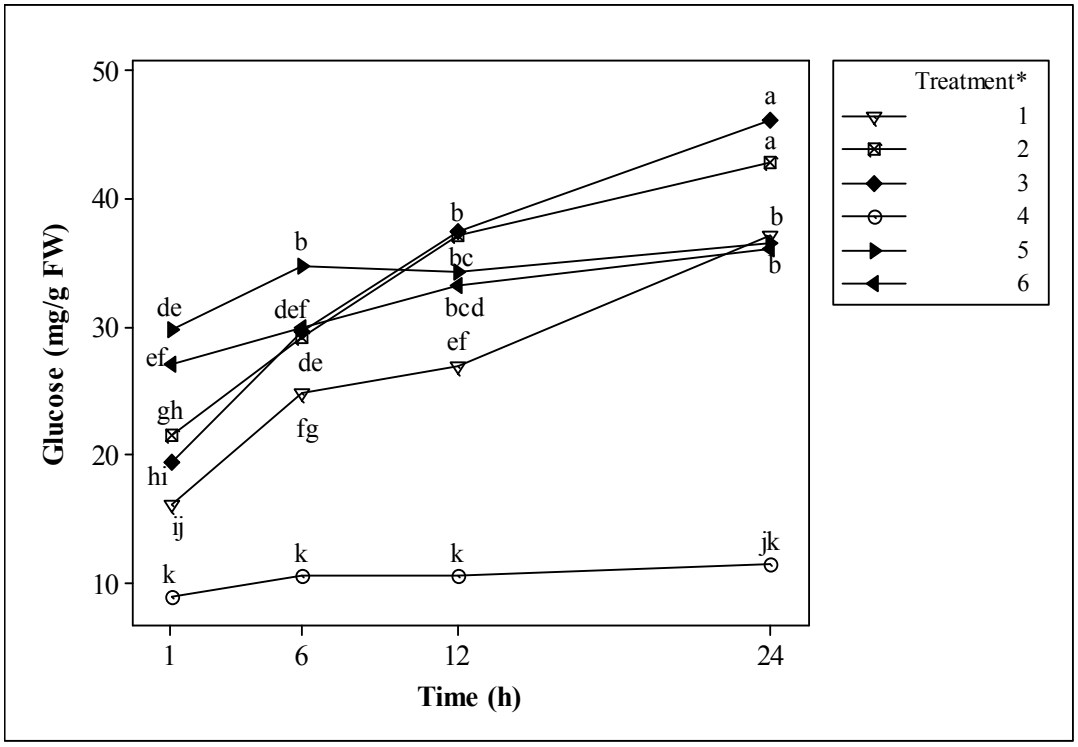


Figure 6.7 Glucose produced in response to various pectinase, β-glucosidase and cellulase activity at pH 4.0 and temperature 40 ^{o}C.

[*]1= Cellulase-Pectinase-β-glucosidase: 1:1:1(112 EU: 480 EU: 106.4 EU); 2= Cellulase-Pectinase-β-glucosidase: 1:1:2 (112 EU: 480 EU: 212.8 EU); 3= Cellulase-Pectinase-β-glucosidase: 1:1:4 (112 EU: 480 EU: 425.6 EU); 4= control; 5= Cellulase-Pectinase-β-glucosidase: 2:1:1(224 EU: 480 EU: 106.4 EU); 6= Cellulase-Pectinase-β-glucosidase: 4:1:1(448 EU: 480 EU: 106.4 EU);

**Substrate concentration was kept at 12.5 g apple processing by-products per 50 mL*

[‡]Means with different letters are significantly different (P<0.05) and letter grouping is between treatments.

[€]Means separated by Tukey's Studentized range test (P<0.05)

[¥]Data was transformed to achieve normality. Untransformed data is shown.

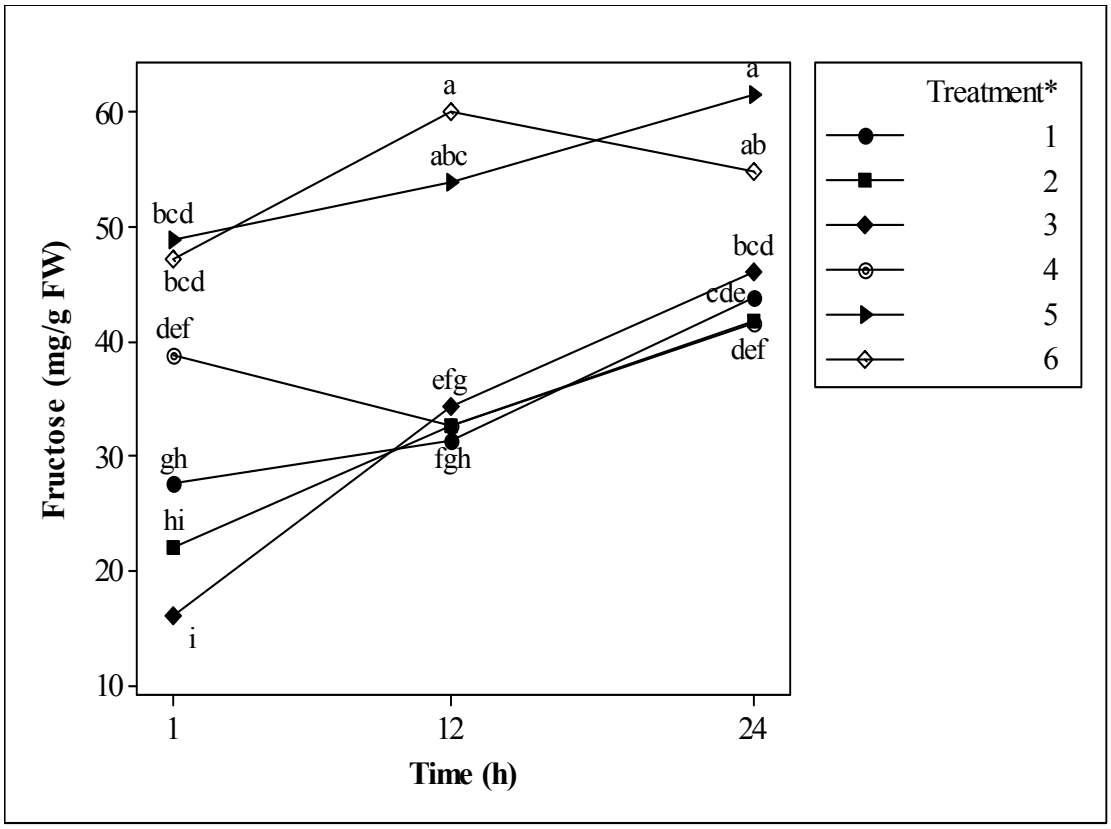


Figure 6.8 Fructose produced in response to increasing β-glucosidase and cellulase activity at pH 4.0 and temperature 40 ^{o}C.

[*]1= Cellulase-Pectinase-β-glucosidase: 1:1:1(112 EU: 480 EU: 106.4 EU); 2= Cellulase-Pectinase-β-glucosidase: 1:1:2 (112 EU: 480 EU: 212.8 EU); 3= Cellulase-Pectinase-β-glucosidase: 1:1:4 (112 EU: 480 EU: 425.6 EU); 4= control; 5= Cellulase-Pectinase-β-glucosidase: 2:1:1(224 EU: 480 EU: 106.4 EU); 6= Cellulase-Pectinase-β-glucosidase: 4:1:1(448 EU: 480 EU: 106.4 CBU);

**Substrate concentration was kept at 12.5 g apple processing by-products per 50 mL*

[ǂ]Means with different letters are significantly different (P<0.05) and letter grouping is between treatments.

[ϵ]Means separated by Tukey's Studentized range test (P<0.05)

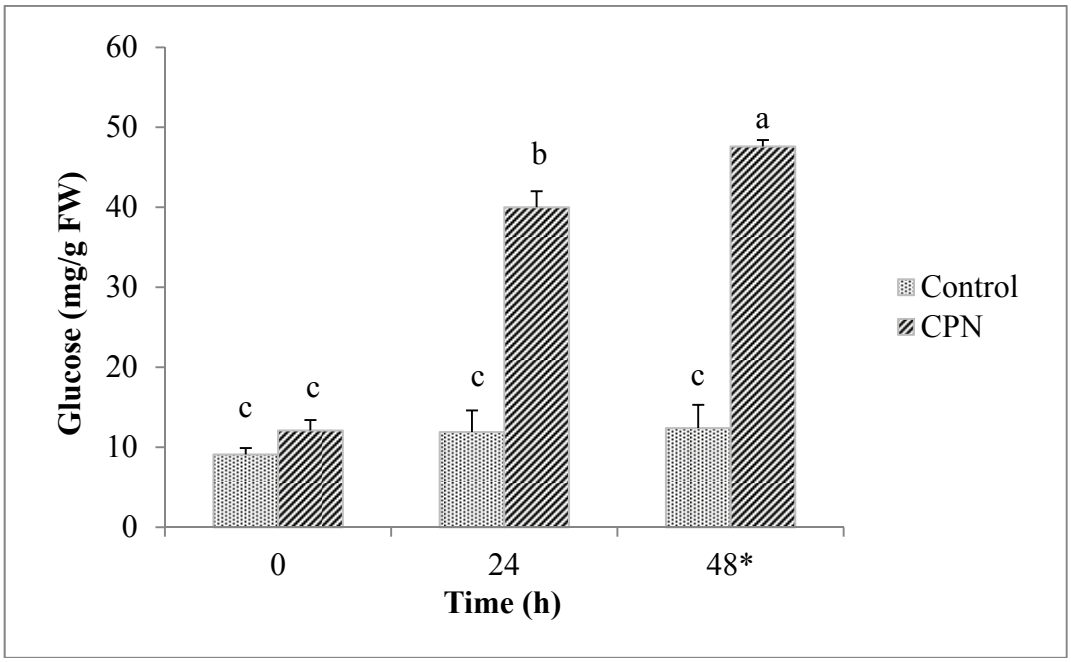


Figure 6.9 Concentration of glucose when puree of apple processing by-products was incubated with a mixture of commercial cellulose, pectinase and β-glucosidase at pH 4.0 and temperature 40 ^{o}C.

CPN, combination of Celluclast 1.5L, Pectinex 3XL and Novozyme 188
[¥] *Activity of Celluclast 1.5L=112 EU, Pectinex 3XL=480 EU and Novozyme 188=106.4 EU per 50 mL puree*
[*] *End product removal from hydrolyzate was done after 24 h. Data at 48 h represents values calculated by adding concentration after 24 and 48th h of hydrolysis.*
[¢] *Hydrolysis was conducted using 12.5 g FW in 50 mL volume.*
[¥] *Data presented as means of triplicates with standard error bars on top*
[§] *Means with different letters in the same pattern are significantly different (P<0.05).*
[€] *Means separated by Tukey's studentized range test (P<0.05)*

6.4.5 Effect of dilute acid pre-treatment and polyphenol oxidation on enzyme hydrolysis

Acid pre-treatment and polyphenol removal had a remarkably significant effect ($P<0.001$) on the glucose production in hydrolyzates (**Figure 6.10**). In comparison to glucose content of control (13.4 mg/g FW), treatments such as dilute acid pre-treatment, polyphenol oxidation and enzymatic hydrolysis enhanced glucose concentration significantly from 24 mg/g to 56 mg/g FW. Enzymatic hydrolysis followed by dilute acid pre-treatment of apple processing by-products enhanced the glucose content by 33 mg/g FW. Furthermore, inclusion of a polyphenol oxidation step by subjecting dilute acid pretreated hydrolyzate to laccase treatment prior to enzymatic hydrolysis (under previously standardized conditions) led to an increment of another 10 mg/g FW of glucose. It was apparent from these results that the increase of accessible surface area of substrate to the enzymes, lignin degradation played an important role in increasing the hydrolysis efficiency. However, concentration of fructose dropped significantly ($P<0.05$) from 36 mg/g to 23 mg/g as the treatments proceeded.

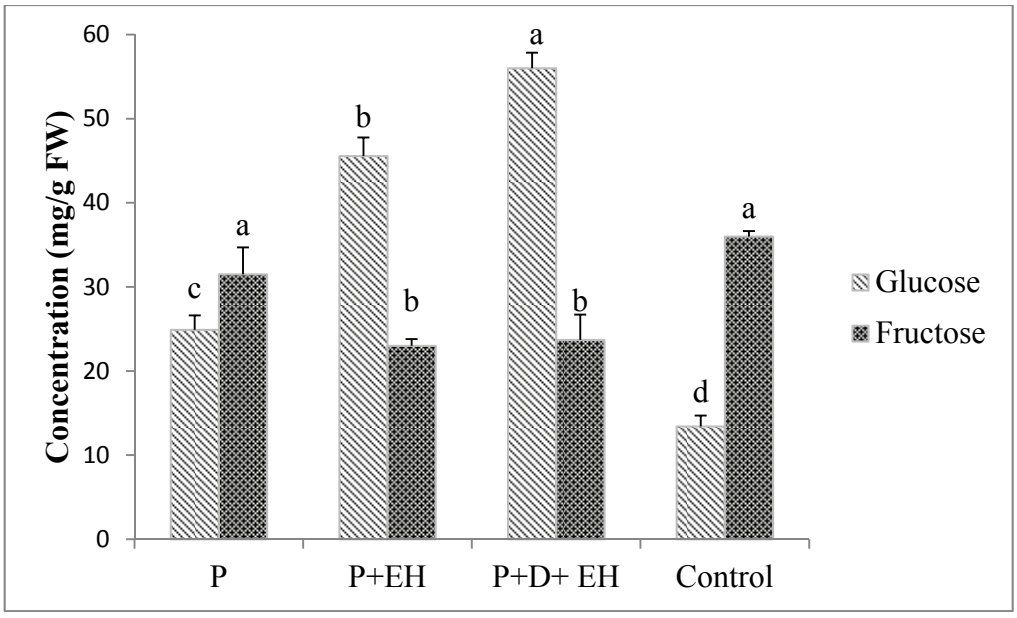


Figure 6.10 Glucose and fructose produced in response to acid pre-treatment, polyphenol oxidation and enzymatic hydrolysis.

P, Acid pre-treatment; D, Detoxification by commercial laccase and EH, Enzyme Hydrolysis
[¥]Data presented as means of triplicates with standard error bars on top
[§]Means with different letters in the same pattern are significantly different (P<0.05).
[€]Means separated by Tukey's studentized range test (P<0.05)
[þ]Data for glucose was transformed (reciprocal) to achieve normality. Untransformed data shown.

6.5 DISCUSSION

Apple processing by-products were hydrolyzed using commercial preparations of cellulase, pectinase and β-glucosidase. The above results showed an additive and synergistic action of three carbohydrolases in hydrolyzing polysaccharides such as cellulose and pectin present in cell wall of apple processing by-products. These results supported the study by Voragen et al. (1980) who reported high release of monosaccharides from apple cell walls by using a combination of pectolytic enzymes and cellulase. The results suggested that glucose release from polysaccharide network was dependent more frequently on pectin breakdown. This was evident from the fact that the highest amount of glucose (44 mg/g FW) was produced at temperature range 40-45 ^{o}C and pH of 4.0 which, according to manufacturers, were the optimal conditions for Pectinex 3XL. The results from present study clearly demonstrated that pectinase can promote the activity of cellulase by breaking the pectin network surrounding cellulose, thereby facilitating release of glucose monomers. Carpita and Gibeaut (1993) proposed a cell wall model for flowering plants and stated that the pectin matrix envelopes cellulose fibres coated with hemicelluloses. This suggests that pectin hinders the hydrolysis of cellulose and hemicelluloses in the apple cell wall, thus making pectinase necessary in addition to cellulase and β-glucosidase to facilitate release of sugars from polysaccharides. Cellulase has partial cellobiose cleaving activity and thus cellobiose accumulation occurs in the initial stages of hydrolysis inhibiting the activity of cellulase (Grohmann et al., 1994). Beta-glucosidase has been reported to regulate the cellulolytic process and is the rate limiting factor during enzymatic hydrolysis of cellulose, since both endoglucanase and cellobiohydrolase activities can be inhibited by cellobiose (Calsavara et al., 1999).

Since enzyme cost is one of the major limitations to large-scale production of bio-ethanol, this was an important consideration when determining enzyme dose. In most cases an enzyme activity of 112, 480 and 106.4 EU for cellulase, pectinase and β-glucosidase, respectively, was observed to yield high concentrations of glucose in the hydrolyzates. The hydrolysis was carried out with constant shaking of the reaction vessel and at a temperature higher than room temperature. Therefore, it was an energy consuming process and it would not be economical to prolong the reaction beyond a certain time period. Overall, it seems that lower temperature (40-45 ^{o}C) and lower pH (4.0) of the system favored higher cellulose conversion into glucose. Optimum pH range for glucose yields from apple processing by-products hydrolysis was pH 4.0-4.5. Since the normal pH range of apple processing by-products is 3.2-3.4, pH 4.0 is favored for enzyme hydrolysis as this will require less addition of buffer. However, the pH optimal ranges for fermenting micro-organisms, *S. cerevisiae* and *E. coli K011* are pH 5.0-5.2 (Russell, 2003) and pH 5.8-6.2 (Grohmann et al., 1995a), respectively. Therefore, pH 4.5 is recommended since it is closest to the pH optimal ranges of fermentation.

Furthermore, it was observed that although the glucose concentration in the hydrolyzate at 50 ^{o}C and pH 5.0 increased initially with time, there was a drop in the rate of glucose production after few hours of hydrolysis. This could be due to deactivation of the enzyme at high temperature combined with high pH. A previous study has shown that presence of lignin in the substrate prevents cellulases from effective binding to cellulose (Ucar, 1998). This was in agreement with some earlier studies that described irreversible adsorption of cellulase to lignin, thereby deactivating it (Grethlein et al.,

1984; Mooney et al., 1998; Stone et al., 1969). The extent to which lignin adsorbs itself to enzyme depends on the nature of lignin and reaction conditions (Tu et al., 2008).

By contrast, concentration of fructose in the hydrolyzates was quite variable during the course of hydrolysis. This could be due to sucrose inversion into fructose and glucose at hydrolysis conditions. Also, fructose content in the hydrolyzate could be dependent on the efficiency of pectinase in disintegrating the pectin network and consequently releasing sugars entrained in the tissues. According to previously reported studies, treatment with pectinase leads to a substantial degradation of the middle-lamella and cell wall pectin by polygalacturonase, pectinmethylesterase and pectinlyase activities (Grassin et al., 1996; Will et al., 2000).

The results of hydrolysis without pre-treatment were used as a baseline to establish if dilute acid pre-treatment method was effective in increasing glucose yields. As expected, dilute acid pre-treatment (1.5% w/v, 91 ^{o}C for 16 min.) was able to substantially enhance enzymatic conversion through the combined chemical and physical effects of lignin relocation and pectin disintegration of biomass material. The results also suggested that pre-treatment increased the accessible surface area of the substrate, thereby leading to enhanced enzyme hydrolysis. The need for pre-treatment has been supported by the fact that enzyme hydrolysis without any pre-treatment leads to hydrolysis of less than 20% of total glucan present in biomass (Grohmann and Bothast, 1994; Mosier et al., 2005). Cellulose fibres in orange peel have been found to resist dilute mineral acid hydrolysis (Grohmann and Bothast, 1994). Dilute acid pre-treatment combined with oxidation of polyphenols present in the substrate led to a significant improvement in hydrolysis yields of glucose, yielding approximately 56 mg/g of glucose

on a fresh weight. Biological oxidation of phenolic compounds by commercial laccase led to selective and virtually complete oxidation of phenolic compounds from the hydrolyzates, thereby enhancing the glucose concentration by 10 mg/g. The concentrations of phenolic compounds in the hydrolyzate are a function of biomass type, pre-treatment conditions, and ratio of biomass to water (Ximenes et al., 2010). Therefore, efficient polyphenol oxidation procedure is required for enhanced bio-conversion of substrate.

The end product removal from hydrolyzate followed by hydrolysis of residue could be another way to overcome enzyme inhibition and enhance the bio-conversion process. Overall, end product removal after 24 h of hydrolysis increased the yields of glucose by 8 mg/g FW. It is also possible that initial action of pectinolytic and cellulolytic enzymes released the glucose content from amorphous region of cellulose and the amount observed after solid-liquid separation was from crystalline region of cellulose. It has been demonstrated previously that the rate of hydrolysis of amorphous cellulose is 3-30 times faster than that of crystalline cellulose (Fan et al., 1980, 1981; Lynd et al., 2002). End product removal before 24 h would decrease the time required to reach the highest glucose production by optimal conversion of cellulose. At a commercial scale for production of bio-ethanol, this corresponds to faster product formation, decreased energy input and most importantly, economic savings.

In this study, maximum amount of glucose observed in the hydrolyzate using commercial carbohydrolases from Novozymes was 56 mg/g FW. Celluclast 1.5L used in this study mainly contained activity of endo-glucanase and a small activity of exo-glucanase (Novozyme, North America Inc. Franklinton, NC, USA). Therefore, there

might be a lack of synergy between both enzyme activities (endo and exo glucanase) for complete conversion of cellulose to glucose. It has been previously described that endo-glucanase is mostly responsible for hydrolysis of amorphous cellulose; whereas exo-glucanase is the main activity required for the hydrolysis of micro-crystalline cellulose (Lynd et al., 2002). Thus, high activity of exo-glucanase is desirable for efficient conversion of cellulose into product. A study by Mansfield et al. (1999) emphasized a synergism between endo and exo-glucanase. According to this study, hydrolysis is influenced by crystallinity of cellulose, when insufficient cellulase system is used or insufficient enzyme is loaded. Purity of enzymes also plays important role in determining the final conversion of cellulose into glucose. Another study by Demers et al. (2009) showed that carbohydrolases from Novozymes such as cellulase (Celluclast 1.5L) and β-glucosidase (Novozyme 188) produced lower amounts of glucose from apple pomace than produced by corresponding enzymes from another manufacturer (Genencor, Paulo Alto, CA, USA).

6.6 CONCLUSIONS

Apple processing by-products have the capability to produce a usable amount of glucose for further fermentation into bio-ethanol and organic acids. Furthermore, high content of fructose favors their bio-conversion into value-added products. The determination of optimum conditions for enzymatic hydrolysis of apple processing by-products was based on economics as well as yields of sugars. Based on the above results, incubation at pH 4.0 and temperature of 40-45 ^{o}C with continuous shaking (150 rpm) is recommended for enzymatic hydrolysis of apple processing by-products. Since enzyme cost is deterrent to the large-scale production of ethanol (Barta et al., 2010), this was an important consideration while determining enzyme dose. Therefore, enzyme activities of 112, 480 and 106.4 EU for Celluclast 1.5L, Pectinex 3XL and Novozyme 188, respectively, are recommended for enhancing the glucose yields in apple processing by-products. Although solid-liquid separation of hydrolyzate yielded significantly high amounts of glucose, hydrolysis time of 24 h was sufficient to yield high amount of fermentable sugars in the hydrolyzate, followed by dilute acid pre-treatment and polyphenol oxidation. Enzyme hydrolysis after dilute acid pre-treatment and polyphenol oxidation yielded approximately 56 mg/g glucose on fresh weight basis. It was interesting to note that high yields of glucose (45 mg/g FW) were observed by increasing the dose ratio of β-glucosidase by four as compared to other two enzymes. Considerably high conversion yields of polysaccharides with dilute acid pre-treatment and polyphenol oxidation demonstrated that pre-treatment of biomass is necessary to disintegrate lignin barrier and increase enzyme accessibility to cellulose. Without pre-treatment, an excess of enzyme would need to be added in order to achieve the same conversion rates.

CHAPTER 7: CONCLUSIONS

7.1 CONCLUSIONS

The goal of the current research was to obtain fermentable sugars by hydrolysis of polysaccharides present in processing by-products obtained from apple juice industry. The study preceded with characterization of cell wall components of apple processing by-products, followed by dilute acid based hydrothermal pre-treatment and polyphenol oxidation prior to hydrolysis of polysaccharides by commercial cellulase, pectinase and β-glucosidase. Methods were explored to improve polysaccharide hydrolysis to yield greater amount of sugar monomers.

Information on the composition of apple processing by-products was obtained by determining its fibre content, monosaccharide composition and polysaccharide content. The high (45% w/w DW) fibre content of the material was an indication of the considerable amount of polysaccharides present. Weak acid hydrolysis by trifluroacetic acid (TFA) demonstrated the presence of monosaccharides such as glucose, xylose and arabinose, whereas the alcohol-insoluble residue was mainly made up of polysaccharides such as cellulose, hemicellulose, pectin and lignin. From the cell wall analysis, it was evident that apple processing by-products contain considerable amount of polysaccharides that have the potential for bio-conversion into their sugar monomers.

Optimization of dilute sulfuric acid based hydrothermal pre-treatment of apple processing by-products was carried out for glucose yield at mild conditions (acid concentration 0.5-2% w/v, time 5-30 min., and reaction temperature 80-100 ^{o}C). The maximal yield of monomeric glucose (29 mg/g FW) was produced by an acid concentration of 1.5 % at a reaction temperature of 91 ^{o}C in 16 min. The results gave an

insight into effect of lignin disruption by pre-treatment, thereby enhancing the substrate digestibility. Furthermore, polyphenol oxidation by commercial laccase led to a significant improvement in glucose yield. In addition to sugars, dilute acid pre-treatment also generated by-products such as hydroxymethyl furfural and acetic acid; however, at lower concentrations which have been described inhibitory to cellulases and fermenting micro-organisms.

It is well known that enzymatic conversion of lignocellulosic biomass into value-added products provides alternative ways to utilize biomass for practical purposes. Commercial enzymes such as Celluclast 1.5L, Pectinex 3XL and Novozyme 188 were applied to obtain fermentable sugars from apple processing by-products. It was investigated how the factors such as temperature, pH, enzyme dosage and time affect the resulting glucose concentration during hydrolysis. It became apparent from this study that effective hydrolysis of apple processing by-products can be achieved by synergistic action of pectinases, endo-glucanase, exo-glucanase and β-glucosidase combined with pre-treatment and polyphenol oxidation. Overcoming polyphenol inhibition of cellulases by commercial laccase treatment gave insight into increasing bioprocess efficiency. After 24 h, hydrolysis of apple processing by-products without any pre-treatment at pH 4.0-4.5, temperature of 40-45 ^{o}C using commercial cellulase, pectinase and β-glucosidase generated approximately 36-44 mg/g FW glucose. These results were observed at enzyme activities of 112, 480 and 106.4 enzyme units of Celluclast 1.5L, Pectinex 3XL and Novozyme 188. The addition of dilute acid pre-treatment and polyphenol oxidation step significantly enhanced the glucose yield by 10 mg/g FW, resulting in 56 mg/g glucose on a fresh weight basis. However, the cause of significant

variability in the fructose concentration after subjecting apple processing by-products these treatments was unclear.

The present study showed higher values of glucose (154 mg/g DW) after hydrolysis than demonstrated by Demers et al. (2009), who showed that approximately 105 mg/g DW of glucose can be produced at optimum levels. The observed difference in the two studies could be due to several reasons including difference in cultivars, growing conditions, fruit maturity, enzyme sources, enzyme activities, pre-treatment and hydrolysis conditions used.

Apparently, liquefaction of apple processing by-products can depolymerize polysaccharides such as cellulose and pectin and thus enhance the monomer yield. It was conceivable that pectin disruption was remarkably important for releasing sugar monomers, thereby enhancing the process efficiency. It was found from this study that both endo-glucanase and exo-glucanase with high activites are essential for enhanced bio-conversion of the cellulose into glucose. In addition to this, β-glucosidase also had a significant effect on the yield of glucose in the hydrolyzate. It was evident from this study that a high β-glucosidase activity is crucial for achieving high cellulose to glucose conversion. For 24 h of hydrolysis at different enzyme loadings, it was observed that the higher the enzyme dosage, the higher the glucose yields.

The multi-step process for enhancing glucose yield by optimizing dilute acid-based pre-treatment, polyphenol oxidation and enzymatic treatment appears to be encouraging enhanced hydrolysis of polysaccharides into glucose in apple processing by-products. This process was able to produce a maximum of 56 mg glucose/ g FW and 46 mg fructose/g FW of apple processing by-produts, totaling to about 102 mg/g of

reducing sugars on fresh weight basis. Bio-ethanol can be produced with a theoretical yield of 52.1 g/kg FW of apple processing by-products. In addition to this, considerable amount of galacturonic acid (18.5 mg/g FW) was released as a result of enzymatic hydrolysis (**APPENDIX F**). The total amount obtained from the multi-step process corresponds to 12.7% sugars on fresh weight basis (120.5 mg/g FW), which appears substantial amount for obtaining enhanced bio-ethanol yields. Results obtained in present study were in agreement with a previous study carried out by Patle and Lal (2007), who demonstrated that about 122 mg/g FW of reducing sugars can be produced with a two-step process employing acid and enzymatic hydrolysis.

With specific microbial sources for glucose, fructose and galacturonic acid, these sugars can be fermented to produce bio-ethanol. Overall, the present study demonstrated that by-products resulting from apple processing industry are promising sources of bio-conversion into bio-ethanol and other value added products. However, the economic feasibility of the process needs to be evaluated.

7.2 ECONOMIC FEASIBILITY

Despite their large potential to produce bio-ethanol and organic acids, the feasibility of using apple processing by-products is limited by the costs involved in each step. The costs are relatively high based on the current technologies. Although new biotechnologies have increased the efficiency of the process, there are numerous challenges that must be addressed before the large-scale use of these by-products as a feedstock for bio-ethanol and organic acid manufacture. In order to make this economically and technologically viable, further research is required to investigate its application beyond the laboratory-scale and to develop necessary biotechnologies. For instance, novel enzyme sources with enhanced acitivities should be identified and

engineered to improve cellulosic bio-conversion to maximize enzymatic hydrolysis and fermentation processes. Methods to recover enzymes, the use of simultaneous saccharification and fermentation techniques should be developed to achieve higher reaction rates at lower costs. These integral approaches would also aid in development and execution of this waste management strategy.

7.3 FUTURE RECOMMENDATIONS

The current study addresses issues regarding utilization of waste by-products from apple industry, by obtaining fermentable sugars such as glucose, that have the potential to be used as a fermentation source for bio-fuel and organic acid production. However, numerous challenges remain. The following list of experiments describes approaches that would build on current work that could lead to further advances to maximize sugar yield as well as final bio-ethanol production for an economically viable bio-conversion process.

7.3.1 STEAM PRE-TREATMENT OF BIOMASS

It is well known that pre-treatment is critical to the economics of bio-conversion processes. Although the present study has shown the efficiency of dilute acid-pre-treatment at mild temperatures in enhancing the glucose yield, it would be interesting to investigate the effect of steam pre-treatment. Since steam pre-treatment does not employ any corrosive acid, use of this pre-treatment method does not require any neutralization prior to enzyme addition. In addition to this, it could lead to release of hemicellulosic sugars along with cellulosic conversion. Therefore, it is important to assess the effectiveness of steam pre-treatment on apple processing by-products which could be cost effective.

7.3.2 HYDROLYSIS USING DIFFERENT ENZYME SOURCES

The present study used Celluclast 1.5L, Pectinex 3XL and Novozyme 188 from Novozymes North America Inc. (Franlinton, NC, USA). Since Celluclast 1.5L contains low activity of exo-glucanase, it is recommended to investigate the effect of cellulytic enzymes from other sources such as Accelerase 1000 (Genencor, Paulo Alto, CA, USA) on resulting glucose yield. This could facilitate high cellulose conversion by enhancing the synergy between exo and endo glucanases.

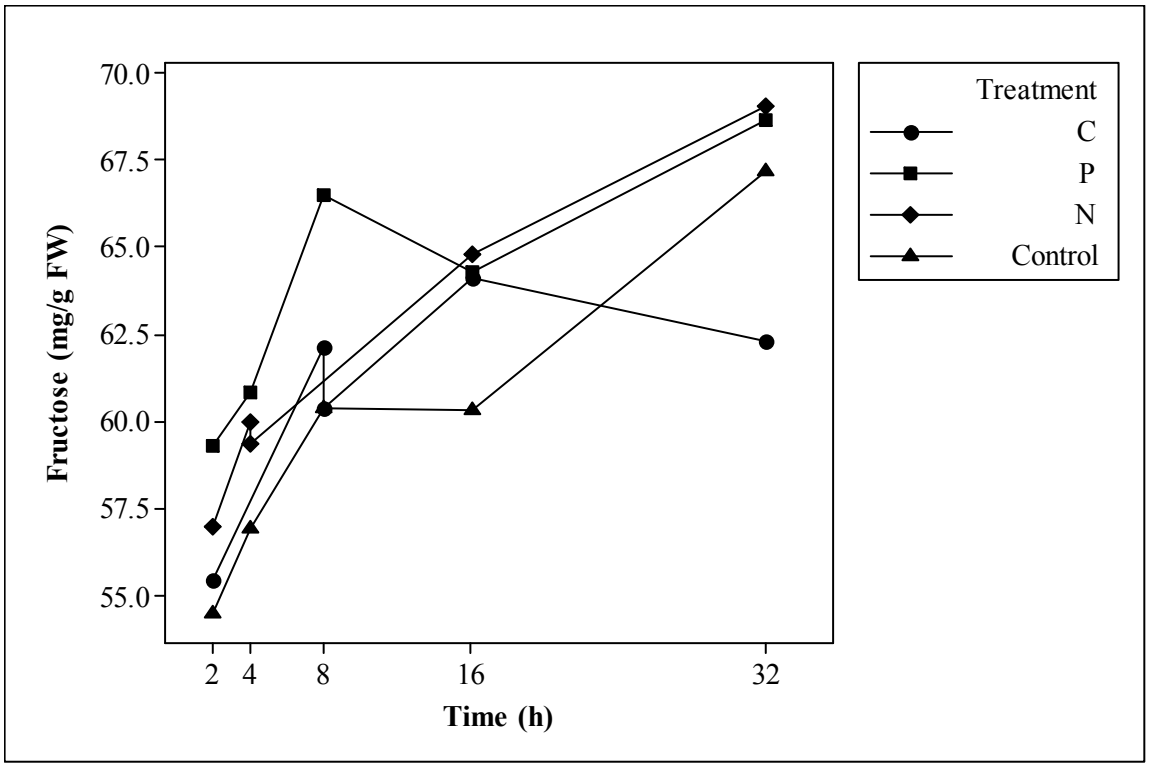
7.3.3 EFFICIENT FERMENTATION OF SUGARS FROM APPLE PROCESSING BY-PRODUCTS

Apart from glucose and fructose, galacturonic acid has been known to be a significant component of the monosaccharides yield after enzyme hydrolysis of pectin-rich residues. In order to obtain high yield of bio-ethanol, all three sugars should be utilized by fermenting micro-organisms. In some previous studies based on citrus waste, genetically engineered bacteria called *Escherichia coli K011* has been used to directly convert galacturonic acid into ethanol. Therefore, it could be beneficial to use *E. coli K011* and *S. cerevisae* together to achieve high rates of bio-conversion.

APPENDIX A: Ridge analysis for maximizing glucose

Coded radius	Temperature (°C)	Time (min)	Acid concentration (% w/v)	Glucose concentration (mg/g FW)
0.0	90.0	15.0	1.5	28.8
0.1	90.8	15.0	1.5	29.2
0.2	91.5	15.0	1.6	29.8
0.3	92.3	14.9	1.6	30.5
0.4	93.0	14.8	1.6	31.6
0.5	93.7	14.7	1.7	32.6
0.6	94.5	14.7	1.7	34.0
0.7	95.2	14.6	1.8	35.5
0.8	95.9	14.5	1.8	37.2
0.9	96.7	14.4	1.8	39.1
1.0	97.4	14.4	1.8	41.2

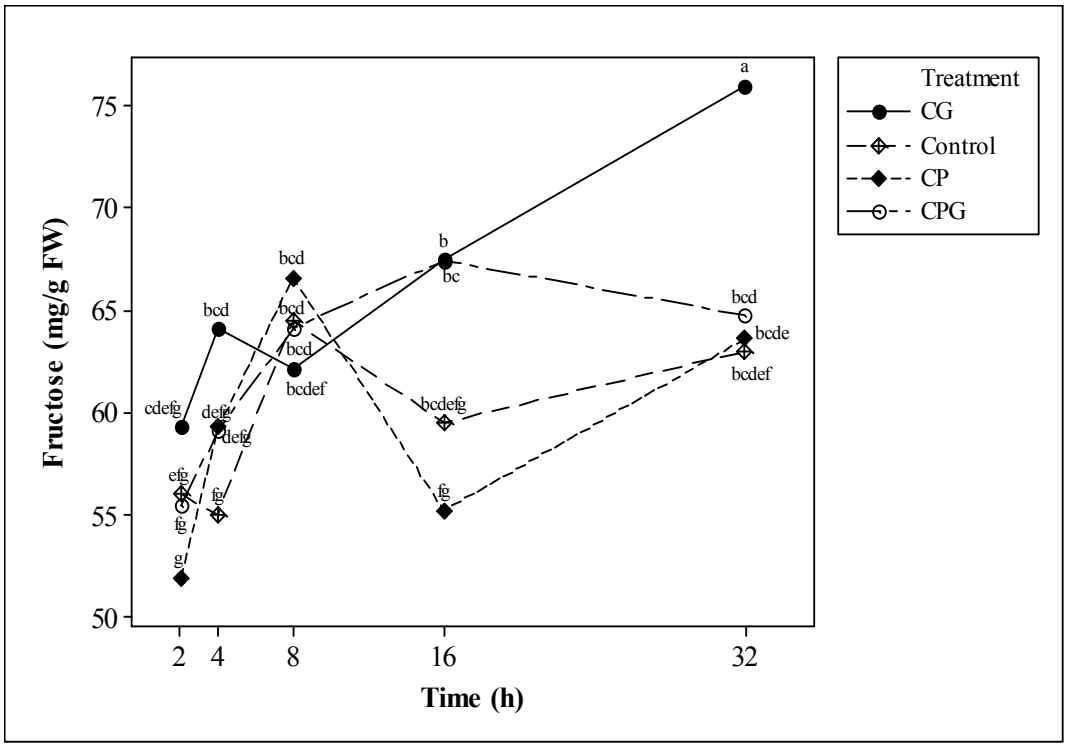
APPENDIX B: Supplementary data for fructose in response to enzyme hydrolysis



Release of fructose in response to hydrolysis of apple processing by-products by commercial cellulase, pectinase, and β-glucosidase at pH 4.6 and temperature of 45 ^{o}C.

[1]Data points represent the mean of two analyses..
[2]Means with different letters in the same column are significantly different ($p<0.05$).
[3]C-Celluclast 1.5L (cellulase); P-Pectinex 3XL(pectinase) and N-Novozyme 188(β-glucosidase)
[4]Enzyme activities used: 14, 60 and 13.3 EU for cellulose, pectinase and β-glucosidase
[5]Substrate concentration was 12.5 g apple processing by-products per trial (50 mL puree)

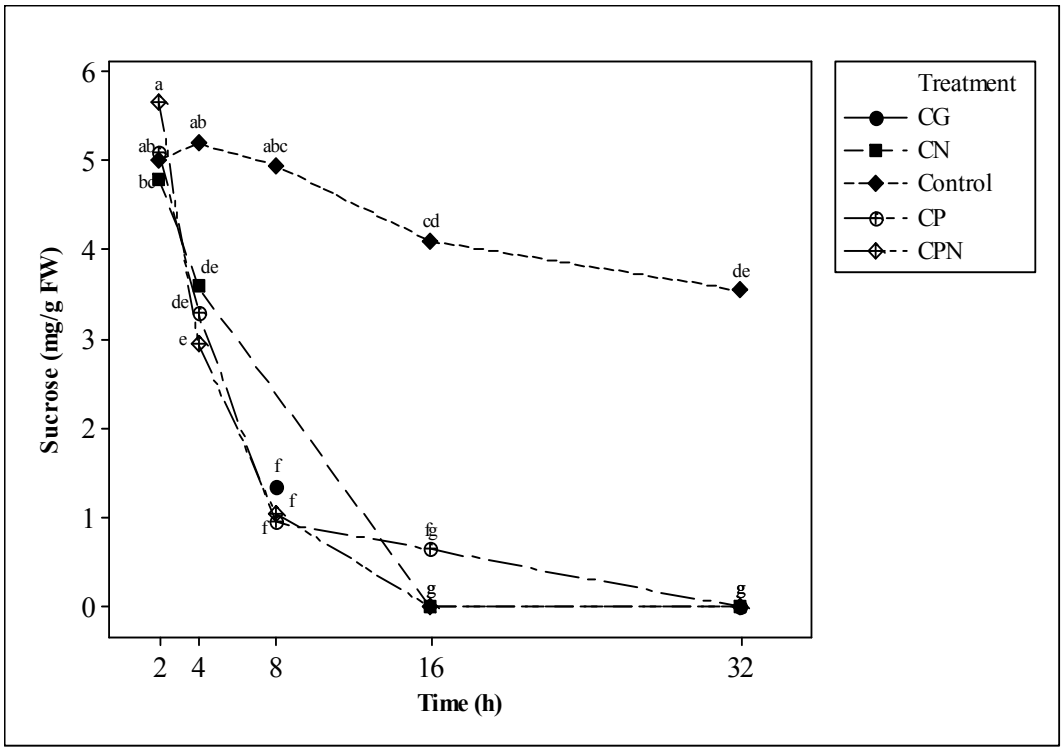
APPENDIX C: Supplementary data for using enzyme combinations



Scatterplot of fructose in response to hydrolysis of apple processing by-products by enzyme combinations of cellulase, pectinase, and β-glucosidase at pH 4.6 and temperature of 45 ^{o}C.

[1]Data points represent the mean of two analyses.
[2]Means with different letters in the same column are significantly different ($p<0.05$).
[3] C-Celluclast 1.5L (cellulase); P-Pectinex 3XL (pectinase) and N-Novozyme 188 (β-glucosidase)
[4]Enzyme activities used: 14, 60 and 13.3 EU for cellulose, pectinase and β-glucosidase
[5]Substrate concentration was 12.5 g apple processing by-products per trial (50 mL puree)

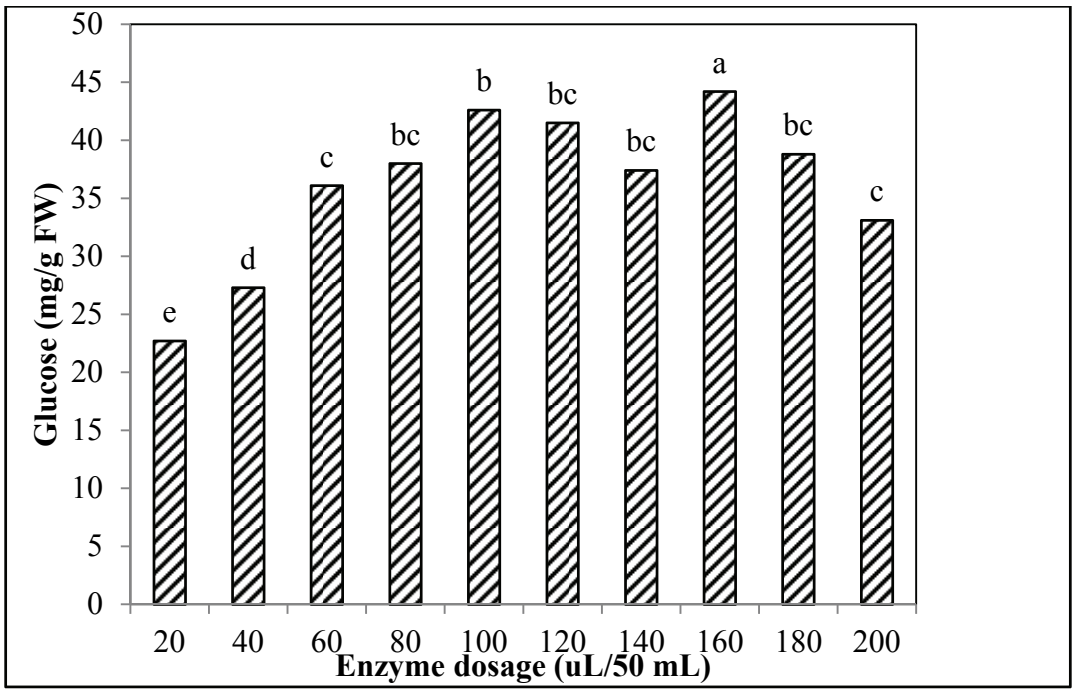
APPENDIX D: Supplementary data for sucrose using enzyme combinations



Scatterplot of sucrose in response to hydrolysis of apple processing by-products by enzyme combinations of cellulase, pectinase, and β-glucosidase at pH 4.6 and temperature of 45°C.

[1]Data points represent the mean of two analyses.
[2]Means with different letters in the same column are significantly different (p<0.05).
[3] C-Celluclast 1.5L (cellulase); P-Pectinex 3XL (pectinase) and N-Novozyme 188-(β-glucosidase)
[4]Enzyme activities used: 14, 60 and 13.3 EU for cellulose, pectinase and β-glucosidase
[5]Substrate concentration was 12.5 g apple processing by-products per trial (50 mL puree)

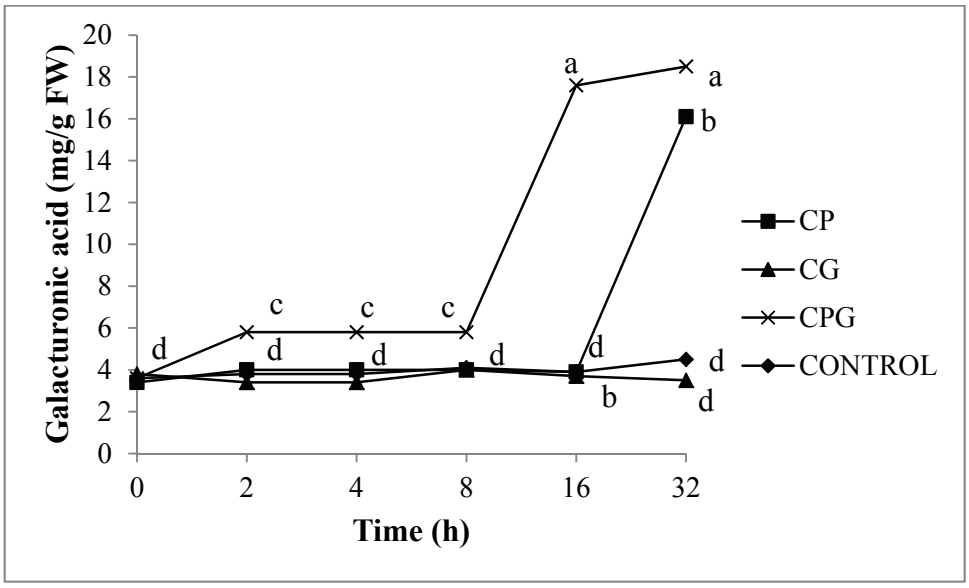
APPENDIX E: Preliminary data for glucose in response to enzyme hydrolysis



Production of glucose from apple processing by-products in response to increase in enzyme dosage from 20-200 µL/50 mL at temperature of 45 ^{o}C and pH 4.5

[1]Data points represent the mean of three analyses.
[2]Means with different letters are significantly different ($p<0.05$).
[3] Enzyme added were Celluclast 1.5L (cellulase); Pectinex 3XL (pectinase) and Novozyme 188 (β-glucosidase)
[4]All enzymes used at dosage of 20 µL/50 mL
[5]Substrate concentration was 12.5 g apple processing by-products per trial (50 mL puree)

APPENDIX F: Release of galacturonic acid in response to enzyme hydrolysis



Scatterplot of galacturonic acid from apple processing by-products in response to enzyme combinations of cellulase (20, 56 and 112 EU), pectinase (60, 240 and 600 EU) and β-glucosidase (13.3, 53.2 and 106.4 EU) at temperature of 40 ^{o}C and pH 4.0

[1]Data points represent the mean of three analyses.
[2]Means with different letters are significantly different ($p<0.05$).
[3] Enzyme added were Celluclast 1.5L (cellulase); Pectinex 3XL (pectinase) and Novozyme 188 (β-glucosidase)
[4]All enzymes used at dosage of 20 µL/50 mL
[5]Substrate concentration was 12.5 g apple processing by-products per trial (50 mL puree)

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