HIGH CONSISTENCY ENZYMATIC HYDROLYSIS OF LIGNOCELLULOSE

by

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ABSTRACT

The work described in this thesis focused on the development of a practical, high consistency hydrolysis and fermentation processes utilizing existing pulp mill equipment. Carrying out enzymatic hydrolysis at high substrate loading provided a practical means of reducing the overall cost of a lignocellulose to ethanol bioconversion process. A laboratory peg mixer was used to carry out high consistency hydrolysis of several lignocellulosic substrate including an unbleached hardwood pulp (UBHW), an unbleached softwood pulp (UBSW), and an organosolv pretreated poplar (OPP) pulp. Enzymatic hydrolysis of OPP for 48 hours resulted in a hydrolysate with a glucose content of 158 g/L. This is among the highest glucose concentration reported for the enzymatic hydrolysis of lignocellulosic substrates. The fermentation of UBHW and OPP hydrolysates with high glucose content led to high ethanol concentrations in the final fermentation broth (50.4 and 63.1 g/L, respectively). These values were again as high as any values reported previously in the literature.

To overcome end-product inhibition caused by the high glucose concentration resulting from hydrolysis at high substrate concentration, a new hydrolysis and fermentation configuration, (liquefaction followed by simultaneous saccharification and fermentation (LSSF)), was developed and evaluated using the OPP substrate. Applying LSSF led to a production of 63 g/L ethanol from OPP. The influence of enzyme loading and β-glucosidase addition on ethanol yield from the LSSF process was also investigated. It was found that, at higher enzyme loading (10FPU or higher), the ethanol production from LSSF was superior to that of the SHF process. It was apparent that the LSSF process could significantly reduce end-product inhibition when compared to a Separate Hydrolysis and Fermentation (SHF) process.

It was also apparent that β-glucosidase addition was necessary to achieve efficient ethanol production when using the LSSF process. A 10CBU β-glucosidase supplement was enough for the effective conversion of the 20% consistency OPP by LSSF.

The rheological property change of the different substrates at the liquefaction stage was also examined using the rheometer technique.

The use of a fed-batch hydrolysis process to further improve the high consistency hydrolysis efficiency was also assessed.

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LIST OF ABBREVIATIONS

α	alpha
β	beta
^{o}C	degrees Celsius
Å	amstrong
BSA	bovine serum albumin
CBH	cellobiohydrolase
CBM	carbohydrate-binding module
CBU	cellobiase units
cm	centimeter
CO_2	carbon dioxide
DNS	dinitrosalicylic acid
DP	degree of polymerization
ED	electrochemical detector
EG	endoglucanase
FID	flame ionization detector
FPU	filter paper units
g	gram
GC	gas chromatography
h	hour(s)
HCH	high consistency hydrolysis
H_2SO_4	sulphuric acid
HPLC	high performance liquid chromatography

L	liter
LSSF	liquefaction and simultaneous saccharification and fermentation
M	molar
min	minute(s)
mL	milliliter
mM	millimolar
NaOH	sodium hydroxide
nm	nanometer
OPP	organosolv poplar pulp
rpm	revolutions per minute
SHF	separate hydrolysis and fermentation
SSF	simultaneous saccharification and fermentation
t	time
T	temperature
μm	micrometer
μl	microliter
UBHW	unbleached hardwood
UBSW	unbleached softwood
UV	ultraviolet light
w/v	weight per volume

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CHAPTER 1 INTRODUCTION

Energy supply plays an important role in the modern world. It not only restricts the nation's energy security, but also affects sustainable development. The inevitable depletion of the world's fossil fuel （oil）supply and the increasing problem of greenhouse gas effects have resulted in an increasing worldwide interest in alternative, non-petroleum based sources of energy. The transportation sector is, in reality, entirely dependent on oil. According to International Energy Agency statistics, the transportation sector accounts for about 60% of the world's total oil consumption (IEA, 2008) and is responsible for half of the total Global CO_2 emissions (Mielenz, 2001). Currently, the USA is the largest single emitter of greenhouse gases, with oil as its largest energy source. The USA uses about 28% of the world's oil supply, 2/3 of which is consumed by car transportation. In Canada, transportation is also the largest single source of greenhouse gas emission (OEE, 2007). Thus, increasing the market share of renewable biofuels, including fuel ethanol, is a topical issue worldwide and particularly in North America. The use of fuel ethanol will significantly reduce net carbon dioxide emission once it replaces fossil fuels. Fermentation-derived ethanol is already a part of the global carbon cycle (Wyman, 1994). The European Commission has decided to raise the market share of renewable energy to 12% by 2010 according to the Kyoto target, and a strategy has been developed to increase the market share of biofuel to 20% (Vermeersch, 2002). The USA has decided to intensify the market position of E10 fuels, which are oxygenated fuel additives-improved gasoline containing the bioethanol. However, these currently only represent 12% of the present market (Knapp *et al.*, 1998). Brazil is a world leader in ethanol

production and 20-25% content of ethanol in gasoline is imposed by the Brazilian federal government (Forge, 2007).

Thus, it is important to find an alternative for petroleum, and reduce our dependence on it. Bioconversion of renewable biomass to ethanol has attracted worldwide interest as a renewable liquid fuel, especially for transportation.

1.1 Background- biofuels

1.1.1 Global status and potential

Applying biology to build a new bioenergy industry can benefit our energy security, economy, and environment in many different ways. During the world oil crisis in the 70's the interest in the use of cellulases to produce fermentable sugars from cellulosic wastes began both in the United States and in Europe (Urbanchuk, 2001; Lynd *et al*., 2003; Samson *et al*., 1998). The aim was to become less dependent on oil and reduce oil imports. At present, the need is even greater, not only because of the increasing cost of oil, but also to reduce greenhouse gas emissions, in order to maintain and improve the quality of life for present and future generations. Biofuels, especially ethanol from plant materials (biomass), have the potential to reduce our dependency on foreign oil in the transportation sector and diversify the energy-technology portfolio. As renewable alternatives that can be harvested on a recurring basis, bioenergy crops (e.g., poplar trees and switchgrass) and agricultural residues (e.g., corn stover and wheat straw) can provide farmers with important new sources of revenue. Consumption of biofuels produces no net CO_2 emissions, releases no sulfur, and has much lower particulate and toxic emissions than do fossil fuels (Greene *et al*., 2004).

Today, there are special programs in a number of countries targetting biofuel productions from renewable resources, for example biogas, bioethanol, biodiesel and fuel

cells (Smeets *et al*., 2005; Yuan *et al*, 2008). Global production of bioethanol increased from 17.25 billion liters in 2000 (Balat, 2007) to over 46 billion liters in 2007, which represented about 4% of the 1300 billion liters of gasoline consumed globally (REN21, 2008). With all of the new government programs in America, Asia, and Europe in place, total global fuel bioethanol demand could grow to exceed 125 billion liters by 2020 (Demirbas, 2007).

Bioenergy ranks second (to hydropower) in renewable U.S. primary energy production and accounts for 3% of the U.S. primary energy production (James *et al*., 2007). The United States is the world's largest producer of bioethanol fuel, accounting for nearly 47% of global bioethanol production in 2005 and 2006 (Balat *et al*., 2009). The "Biofuels Initiative" in the U.S. (US Department of Energy), strives to make cellulosic ethanol cost-competitive by 2012 and supposedly to correspond and account for one third of the U.S. fuel consumption by 2030. In 2007, the U.S. president signed the Energy Independence and Security Act of 2007 (EISA, 2007), which requires 34 billion liters of bio-fuels (mainly bioethanol) in 2008, increasing steadily to 57.5 billion liters in 2012 and to 136 billion liters in 2022.

The EU has also adopted a Biomass Action Plan that sets out measures to increase the development of biomass energy from wood, wastes and agricultural crops by creating market-based incentives and removing barriers to the development of markets. Implementation of the plan will help the EU to cut its dependence on fossil fuels, reduce greenhouse gas emissions, and stimulate economic activity in rural areas. In 2003, the European Union adopted two biofuel directives. These directives set targets for the share of renewable fuels in the transport fuel market (2% by the end of 2005 and 5.75% by the end of 2010) (EC Directive, 2003). The 2005 target was not achieved but the industry is growing rapidly and it is expected that the 2010 target will be achieved. On 23 January 2008, the European Commission proposed a binding minimum target of 10% for the share of biofuels in transport that envisages a 20%

share of all renewable energy sources in total energy consumption by 2020 (EC, 2008). The bioethanol sectors in many EU member states have responded to policy initiatives and have started growing rapidly. Bioethanol production increased by 71% and consumption reached 2.44 billion liters in 2007 (Tokgoz, 2008). The potential demand for bioethanol as a transportation fuel in the EU is estimated at about 12.6 billion liters in 2010 (Zarzyycki *et al*., 2007).

Brazil is the world's largest exporter of bioethanol and second largest producer after the United States. Production is expected to rise from 15.4 billion litres in 2004 to 26.0 billion litres by 2010. Ethanol from sugarcane provides 40% of automobile fuel in Brazil and approximately 20% is exported to the U.S., EU, and other markets (Greenergy, 2007).

There are more than 10 ethanol biofuel facilities either in operation or under construction in Canada and 130 plants in the United States as of 2006 (Allan *et al*, 2006; Parcell *et al*., 2006). In eastern Canada and the U.S., corn is used as the feedstock while in western Canada wheat is used. Brazil produces a large amount of ethanol from sugarcane, and many vehicles in that country have been built to run directly on ethanol fuel. In Europe, ethanol is produced in Sweden, Denmark, Germany, the United Kingdom, France, Italy and Spain. Many Asian countries such as China, India, Japan, and Indonesia are also developing ethanol production capacity (Yang *et al*., 2007; Worldwatch Institute, 2006; Allan *et al*., 2006)

1.1.2 Canadian status and potential

From an energy policy point of view, public interest in renewable resources emerged and grew during the oil supply crises of the 1970s and early 1980s. Canadians, like citizens of other International Energy Agency (IEA) member countries, have been keenly interested in

renewable energy for a long time. Even though many Canadian provinces had been deriving most of their electricity from hydroelectric power, the first oil crises of the 1970s created a strong interest in all forms of renewable energy. In the late 1970s the Government of Canada and most provincial governments responded to public demand for the substitution of oil and other fossil fuels with renewable energy sources (Allan *et al*., 2006).

In Canada, the federal government and provinces have developed policies and programs to stimulate the production and use of biofuels. These include investment tax credits, capital grants, guaranteed prices, consumer rebates, excise tax exemptions and a wide variety of subsidies for production, consumption and research (Allan *et al*., 2006).

The Government of Canada recently announced that a 5% national renewable fuel standard will be in place by 2010. To meet this target and targets of the Kyoto Protocol (Martineau, 2002), it is projected that Canada would need to produce 3.1 billion litres of renewable fuel — a volume that far exceeds the capacity of current and proposed domestic production facilities and represents a twelve-fold increase in biofuel production. In 2007, Canada announced to invest up to 1.5 billion over 9 years to boost Canada's production of biofuels (www.ecoaction.gc.ca/ECOENERGY-ECOENERGIE/biofuelsincentive).

At the same time, each year, the biomass harvest from Canada's forestry and agricultural sectors is about 143 million tons of carbon, which would be abundant renewable resources used for producion ethanol and biodiesel (Wood *et al*., 2003). According to the Canadian Renewable Fuels Association (CRFA), this huge energy source is equal to an annual supply of 30 million barrels of renewable fuels. Biomass feedstocks in Canada include: fuel-wood, wood processing residues (often called "hog fuel"); landfill methane; municipal solid wastes (MSW); industrial wastes; and sewage biogas. There is also interest in developing additional energy supplies and liquid fuels from crop residues, short rotation

energy plantations and agricultural crops such as willow, poplar, and switchgrass, and agricultural crops.

Moreover, Canada's papermaking industry is now facing increasing challenge from developing countries, and needs to increase its competitiveness through innovation. A biorefinery may be one pathway that the pulp and paper industry might follow since both uses lignocellulosic materials. The largest market for biorefinery applications are in the area of transportation fuels, such as bioethanol.

1.2 Bioconversion of lignocellulosic materials to ethanol

Production of ethanol from renewable lignocellulosic sources, such as wood and agricultural residues, is a promising means to decrease the accumulation of greenhouse gas and alleviate pressure on fossil fuel shortage (Wyman & Hinman, 1990; Galbe & Zacchi, 2002). However, the ethanol produced is currently not cost competitive with gasoline. Currently, the raw material and enzyme production are the two main contributors to the overall costs.

Ethanol is commonly produced from corn grain (starch) or sugar cane (sucrose) (MacDonald *et al*., 2001). Sucrose can be fermented directly to ethanol, but starch must be hydrolyzed to glucose before it can be fermented by yeast, generally by Saccharomyces cerevisiae (Lin and Tanaka, 2006). However, starch biomass materials result in severe competition between energy and food supplies, as well as sugar cane is planted mainly from the warm temperate to tropical areas. For renewable biofuel to be able to compete with fossil fuel, a cost-efficient process for an even more abundant renewable resource is needed. In an effort to reduce the cost of producing ethanol, research is underway to develop technologies for the production of ethanol from plentiful, low-cost lignocellulosic biomass such as wood or

agricultural crop waste. The global production of plant biomass, of which over 90% is lignocellulose, amounts to about $200x10^9$ tons per year (Polman, 1994), which are available in large enough quantities to be considered for large-scale production of alcohol-based fuels. Urban wastes are an additional source of biomass. It is estimated that cellulose accounts for 40% of municipal solid waste (Burell *et al*., 2004). Substantial savings would arise from the reduced cost of such feedstock. Using lignocellulosic materials can significantly reduce the cost of raw materials (compared to corn), which comprise more than 20% of the ethanol production cost (Kaylen *et al*., 2000). Biorefinery technology uses raw material in an optimal manner to derive a wide range of fuels and chemicals. Current targets are to produce 200 to 400 million liters/year of ethanol from this source within 10 to 15 years at a cost equivalent to gasoline produced from oil at $32/barrel (approx. US$20/barrel). Unfortunately, because of the complex and crystalline structure of lignocellulose, this material is much more difficult to hydrolyze than starch (Somerville *et al*., 2004). Efficient processes for conversion of lignocellulosic material to fermentable sugars are needed.

1.2.1 Feedstock lignocellulosic biomass

Lignocellulosic biomass such as corn stover, sugarcane bagasse, wheat or rice straw, forestry and paper mill residues and municipal waste, is abundant, domestic and renewable, and has long been recognized as a potential low-cost source that can be converted to bio-ethanol. In contrast to sugar-containing crops, the utilization of lignocellulose as a substrate for ethanol production is difficult because of its complex structure, which resists degradation. Lignocellulose is composed of three main fractions: cellulose (~45% of dry weight), hemicellulose (~30% of dry weight), and lignin (~25% of dry weight) (Wiselogel *et al*., 1996).

Cellulose is found almost exclusively in plant cell walls. It is a linear polymer of glucose, composed of thousands of molecules of anhydroglucose linked by β (1,4)-glycosidic bonds. The basic repeating unit is the disaccharide cellobiose. The secondary and tertiary conformation of cellulose, as well as its close association with lignin, hemicellulose, starch, protein and mineral elements, makes cellulose resistant to hydrolysis. Cellulose can be hydrolyzed chemically by diluted or concentrated acid, or enzymatically. During hydrolysis the polysaccharide is broken down to free sugars by the addition of water (also called saccharification).

Hemicelluloses (20-40% of lignocellulose) are highly branched heteropolymers containing sugar residues such as hexoses (D-galactose, L-galactose, D-mannose, L-rhamnose, L-fucose), pentoses (D-xylose, L-arabinose), and uronic acids (D-glucuronic acid). They also contain smaller amounts of nonsugars such as acetyl groups (Lynd *et al*., 1999). The composition of hemicellulose depends on the source of the raw material (Wiselogel *et al*., 1996). Hemicelluloses in hardwood contain mainly xylans (15-30%) while in softwood galactoglucomannans (15-20%) and xylans (7-10%) predominant. There are various enzymes responsible for hydrolysis of hemicellulose. Because of their branched, amorphous nature, hemicelluloses are easier to hydrolyze than cellulose (Brigham *et al*., 1996).

Lignin (10-30%) is a complex, hydrophobic, cross-linked aromatic polymer in nature. Lignins are polymers of phenylpropane units: guaiacyl (G) units from the precursor trans-coniferyl-alcohol, syringyl (S) from trans-sinapyl-alcohol, and p-hydroxyphenyl (H) units from the precursor trans-p-coumaryl alcohol (Kirk *et al*., 1977). The exact composition of lignin varies widely with species. Softwood contains mainly guaiacyl units while hardwood contains both guaiacyl and syringyl units. It has been suggested that guaiacyl lignin restricts fibre swelling and thus the enzymatic accessibility more than syringyl lignin. The residual

substrate remaining after extensive hydrolysis of steam pretreated aspen and eucalyptus is mainly composed of guaiacyl (Ramos *et al*., 1992).

The combination of hemicellulose and lignin provide a protective sheath around cellulose, which must be modified or removed before efficient hydrolysis of cellulose can occur. Furthermore, the crystalline structure of cellulose makes it highly insoluble and resistant to attack. Therefore, to economically hydrolyze cellulose, more advanced pretreatment technologies are required than in processing sugar crops.

1.2.2 Biomass-to-ethanol process

Typical lignocellulose-to-ethanol processes consist of at least four steps: pretreatment to enhance biomass digestibility, hydrolysis of cellulose to sugar monomers, fermentation of sugars to ethanol, and recovery of ethanol by distillation/evaporation from process stream.

Since enzymatic hydrolysis of native lignocellulose usually results in solubilization less than 20% of the originally present glucan, some form of pretreatment to increase amenability to enzymatic hydrolysis is included in most processes for biological conversion of lignocellulose. The main objective of pretreatment is to produce a solid substrate with high yield, significantly more susceptible to enzyme action than the original feedstock. It retains nearly all of the cellulose present in the original material. Current pretreatment processes employ physical, chemical and biological methods to break down the lignocellulosic structure. Typical processes including hot water, dilute acid, steam explosion, ammonia fiber explosion (AFEX), strong alkali process, as well as mechanical treatment such as hammer and ball milling (Pan *et al*., 2005; Wyman *et al*., 2005) have been tried.

After the pretreatment process, there are two types of processes to hydrolyze the feedstocks for fermentation into ethanol, most commonly used are acid (dilute and concentrated) and enzymatic hydrolysis.

1.2.2.1 Acid hydrolysis

Acid hydrolysis is only applied in so-called two-stage acid processes, following acid pretreatment. The dilute acid process is the oldest technology for converting cellulose biomass to ethanol (first commercial plant in 1898). The first stage is essentially hemicellulose hydrolysis. The sugars produced can be further converted into other chemicals - typically furfural. The sugar degradation not only reduces the sugar yield, but the furfural and other by-products can inhibit the fermentation process. Therefore, the first stage is conducted under mild conditions (e.g. 0.7% sulphuric acid, 190°C) to recover the 5-carbon sugars, while in the second stage the more resistant cellulose is hydrolyzed under harsher conditions (215°C, but a milder 0.4% acid) to produce 6-carbon sugars. Both stages have a short residence time. Yields are 89% for mannose, 82% for galactose, but only 50% for glucose. The hydrolysed solutions are recovered from both stages and fermented to alcohol (Vane, 2005).

The concentrated acid process uses a 70% sulfuric acid at low temperature for 2 to 6 hours, can handle diverse feedstock, and is relatively rapid. The low temperatures and pressures minimize the degradation of sugar. The primary advantage of the concentrated process is the high sugar yield (90% quantitative of both hemicellulose and cellulose sugars). It is critical for the economic viability of this process to minimize the amount of acid, by cost effectively separating the acid for recycling. As early as 1948, membrane separation already achieved 80% acid recovery, continuous ion exchange now recovers over 97% of the acid, 2% of the sugar is lost. However the required equipment is more expensive than for dilute acid.

1.2.2.2 Enzymatic hydrolysis

The hydrolysis of cellulose by cellulolytic enzymes has been investigated intensively since the early 1970s, with the objective of developing a process for the production of ethanol. Figure 1- 1 shows a simplified overview of a "generic" bioconversion process. Over the past decades, a great amount of research interest and effort has been generated in this area (Bjerre *et al.*, 1996; Coughlan, 1992; Duff & Murray, 1996; Himmel *et al.*, 1999; Schwald *et al.*, 1989; Tan *et al.*, 1987; Wright, 1998). Enzymatic hydrolysis methods have shown distinct advantages over acid based hydrolysis methods; the very mild process conditions give potentially higher yields, the utility cost is low (no corrosion problems), Therefore this is the method of choice for future wood-to-ethanol processes (Duff & Murray, 1996; Hsu, 1996).

Enzymatic hydrolysis involves soluble enzymes working on insoluble substrates, so a better understanding of the action of cellulase enzyme systems and their substrates is required as this complex reaction involves multiple cellulose-hydrolyzing activities and substrate features.

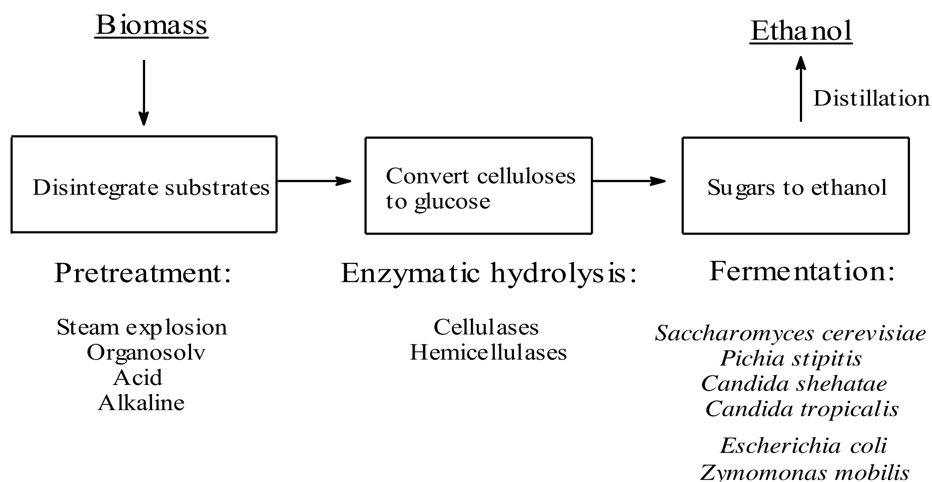


Figure 1- 1. Schematic diagram of a bioconversion biomass-to-ethanol process.

1.2.2.2.1 Cellulase system reaction mechanism

Cellulases play a significant role in the enzymatic process by catalyzing the hydrolysis of cellulose to soluble, fermentable sugars. Cellulases are synthesized by fungi, bacteria and plants. The science of cellulase has come a long way since World War II when the U.S. Army mounted a basic research program to understand the causes of deterioration of military clothing and equipment in the jungles (Sheehan and Himmel, 1999). It has grown in conjunction with the monumental changes that have occurred in molecular biology, protein chemistry, and enzymology over the past 60 years. The cellulase cost has been reduced dramatically from US$5.40 per gallon of ethanol to approximately 20 cents per gallon of ethanol (Moreira, 2005); further efforts are focused on lower costs for bioconversion to below 5 cents per US gallon ethanol (US Department of Energy, 2004).

Reese *et al* (1950) proposed a C_1-Cx concept regarding the enzymatic mechanism of cellulose degradation. It was postulated that crystalline substrates were first rendered susceptible to hydrolysis by a C_1-component. This component was suggested to be a nonhydrolytic chain-separating enzyme. The separation of the cellulose chains was suggested to take place by splitting of hydrogen bonds. Cellulose modified in this way is then hydrolyzed by the Cx-enzyme fraction and by β-glucosidases. Since then this field had attracted the most interest and this early concept of cellulase reaction mechanism has been modified, added to, and argued about for the past 50 years.

At least, three major type of cellulase enzymatic activities are believed to be involved in cellulose hydrolysis based on their structural properties: endoglucanases or 1,4-β-D-glucan-4-glucanohydrolases (EC 3.2.1.4), exoglucanases, including 1,4-β-D-glucan glucanohydrolases (also known as cellodextrinases) (EC 3.2.1.74) and 1,4-β-D-glucan

cellobiohydrolases (cellobiohydrolases) (EC 3.2.1.91), and β-glucosidases or β-glucoside glucohydrolasess (EC 3.2.1.21) (Lynd *et al*., 2002).

Endoglucanases cut at random internal sites in the amorphous cellulose polysaccharide chain, generating oligosaccharides of various lengths and consequently new chain ends. Exoglucanases act in a processive manner on the reducing or nonreducing ends of cellulose polysaccharide chains, liberating either glucose or cellobiose as major products. Exoglucanases can also act on microcrystalline cellulose, presumably peeling cellulose chains from the microcrystalline structure. β-Glucosidases hydrolyze soluble cellodextrins and cellobiose to glucose (Lynd *et al*., 2002).

A general feature of most cellulases is a modular structure often including both catalytic and carbohydrate-binding modules (CBMs). The CBM effects binding to the cellulose surface, presumably to facilitate cellulose hydrolysis by bringing the catalytic domain in close proximity to the substrate, insoluble cellulose. The presence of CBMs is particularly important for the initiation and processivity of exoglucanases (Teeri, 1997).

Cellulase enzyme systems exhibit higher collective activity than the sum of the activities of individual enzymes, a phenomenon known as synergism. 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.

1.2.2.2.2 The cellulase enzyme system of *Trichoderma reesei*

The most frequently reported source of cellulases is the fungus *Trichoderma reesei* (Persson *et al*., 1991; Saddler *et al*., 1998), the most studied cellulolytic microorganism during the last 60 years. Among the various microorganisms capable of synthesizing cellulase enzymes, T. reesei produces an extracellular, stable, and efficient cellulase enzyme system (Jana *et al*., 1994). However, the low-glucosidase activity of the enzyme system from T. reesei leads to incomplete hydrolysis of cellobiose in the reaction mixture and, as a result, to serious inhibition of the enzymes (Holtzapple *et al*., 1990). This can be overcome by genetic modification of T. reesei leading to high glucosidase activity or through the addition of extra β-glucosidase, e.g., from the fungus Aspergillusniger (Wright *et al*., 1986). For most fungally derived cellulases, maximum cellulase activity is observed at 50-55°C and a pH of 4.0-5.0 (Saddler *et al*., 1998).

The cellulase system of T. reesei which contains enzyme with catalytic domain and carbohydrate-binding modules connected by a flexible linker peptide (Beguin, 1994; van Tilbeurgh, 1986) is the characteristic molecular arrangement of this cellulase. This fungus produces at least two exoglucanases (CBHI and CBHII), five endoglucanases (EGI, EGII, EGIII, EGIV, and EGV) and two β-glucosidases (BGLI and BGLII). CBHI and CBHII are the principal components of the T. reesei cellulase system, representing 60 and 20% (Pakula *et al.,* 2000), respectively, of the total cellulase protein produced by the fungus on a mass basis. CBHI works from the reducing end of the cellulose, whereas CBHII from the non-reducing end (Divne *et al*., 1994; 1998). In this way the enzymes support each other in the overall catalysis. CBHI is thought to be processive (Rouvinen *et al*., 1990; Vrsanska and Biely, 1992; Barr *et al*., 1996), moving along a crystalline cellulose chain, `pulling up` that chain and feeding it into the catalytic domain where cellobiose is formed by hydrolyzing alternate β-1,4-

glycosidic linkages. Cellobiohydrolase activity is essential for the hydrolysis of microcrystalline cellulose, although it is not clear why T. reesei produces more CBHI than CBHII. The endoglucanases generally do not act synergistically with each other (Baker *et al*., 1995).

The collective activity of enzyme systems is much more efficient than the sum of individual activities of each enzyme. How do different enzymes work together as a synergistic system to decrystallize? The cellulose structure and the function of cellulase cocktails, the principles and strategies governing the combination of cellulase components for effective hydrolysis and the function of each fraction still require further new scientific insights (Zhang *et al*., 2004).

1.2.2.3 Factors affecting the enzymatic hydrolysis of lignocellulosic materials

It is apparent that various characteristics within the lignocellulosic substrates can limit both the rate and degree of hydrolysis by the cellulose system. However, the action of cellulases also alters the inherent characteristics of lignocellolosic substrates as hydrolysis proceeds. Several workers have shown that the efficiency of such enzyme-substrate interactions is influenced by various physiochemical properties of the substrate at different levels, i.e., microfibril (e.g., crystallinity and degree of polymerization), fibril (e.g., lignin content and distribution), and fiber (pore size and distribution, available surface area, and degree of swelling). It has also been suggested that enzyme-related factors, such as segregation of different enzyme components due to diffusion into substrate pores, the tightness of enzyme binding, and the gradual loss of enzyme activity during the course of the reaction, all influence the rate and extent of the cellulose hydrolysis reaction.

Thus, the factors influencing enzymatic hydrolysis can be divided into substrate related factors and enzyme related factors. The relationship between structural features of cellulose and rates of enzymatic hydrolysis has been the subject of extensive study and several reviews have been published (Converse, 1993; Mansfield *et al*., 1999; Lynd *et al*., 2002; Zhang and Lynd, 2004).

1.2.2.3.1 Substrate-related factors

The chemical properties of potential lignocellulosic substrates for biomass conversion will vary considerably, depending on the nature of the original feedstock and the conditions used for pretreatment. For example, Avicel is nearly pure cellulose, and the dilute-acid treatment used in its preparation removes both the hemicelluloses and the more extensive amorphous regions of the cellulose fiber. Several substrate characteristics have been suggested to play key roles in determining both the rates and the efficiency of hydrolysis, including crystallinity, degree of polymerization, lignin content and distribution, and pore size and surface area (Mansfield, *et al*. 1999; Zhang and Lynd, 2004).

Cellulose crystallinity used to be thought to play a major role in limiting hydrolysis, because the rate of hydrolysis of amorphous cellulose is 3-30 times faster than that of high crystalline cellulose (Fan *et al*., 1980, 1981; Lynd *et al*., 2002). It would be expected that crystallinity should increase over the course of cellulose hydrolysis as a result of preferential reaction of amorphous cellulose. However, several studies have shown that crystallinity does not increase during enzymatic hydrolysis (Lenze *et al*., 1990; Ohmine *et al*., 1983; Sinitsyn *et al*., 1989), and when all other substrate factors are the same, the degree of crystallinity has no effect on hydrolysis (Puri, 1984). Considering both the uncertainty of methodologies for measuring crystallinity as well as conflicting results on the change of crystallinity during

hydrolysis, it is difficult to draw a conclusion at this time that crystallinity play a dominant role in enzymatic hydrolysis (Mansfield *et al*., 1999; Lynd *et al*., 2002; Zhang and Lynd, 2004).

The lignin content and distribution may influence the enzymatic hydrolysis in two major ways. 1) Lignin prevents enzymes from effective binding to the cellulose (Ucar, 1988). 2) Lignin irreversibly adsorbs the cellulase enzymes, thus preventing their reaction with substrates. The removal of lignin leaves the cellulose more accessible and more open to swelling on contact with cellulase (Grethlein *et al*., 1984; Stone *et al*., 1969; Ahlgren *et al*., 1971; Mooney *et al*., 1998). For example, high enzymatic conversions of cellulose have been obtained from extensively delignified softwood kraft pulp, containing 4% lignin or delignified refiner mechanical pulp, containing 8% lignin; while partial lignin removal (with a final lignin content of 32-36%) has resulted in decreased hydrolysis yields (Schell *et al*., 1998). The extent to which the lignin adsorbs enzymes depends very much on the nature of the lignin itself (Sutcliffe and Saddler, 1986; Tu *et al*, 2008), the degree that lignin adsorption of enzymes is decreased depends on the severity of pretreatment and the resulting decrease in lignin content (Ooshima *et al*., 1983, 1990, 1991).

The degree of polymerization (DP, number of glucosyl residues per cellulose chain) of cellulosic substrates varies greatly, depending on substrate origin and preparation. The DP of wood after pulping is reduced to 500-1500 (Bertran and Dale, 1985; Lee *et al*., 1982) compared to DP in the original wood. It is still unclear if the DP of cellulose is a contributing limiting factor that influences the efficiency of enzymatic hydrolysis, because different conclusions have been drawn. Some of the results show that the DP of wood-derived cellulose fragments decreased with increasing enzyme hydrolysis time (Puls and Wood, 1991; Puri, 1984; Ramos *et al*., 1993a), while others showed that the molecular weight of residual

material remains unchanged after hydrolysis (Walseth, 1952) which indicates that the DP is relatively unimportant (Sinitsyn *et al*., 1991).

Particle size associated with accessible surface area has a significant impact on the saccharification of plant cell walls by cellulolytic enzymes and is thought to be a controlling factor for conversion rates and yield (Zeng *et al*., 2007; Jeoh *et al*., 2007). Since enzyme adsorption is a prerequisite step in the hydrolytic process, it seems that specific surface area would have an effect on hydrolysis rates since a higher surface area-to-weight ratio should mean more available adsorption sites per mass of substrate (Mansfield *et al*., 1999). It was hypothesized that initial rate of hydrolysis is a function of cellulose's accessible surface area (Stone *et al*., 1969). Grethlein *et al*. (1984) found a linear relationship between the initial hydrolyzability of a lignocellulosic substrate and its accessibility to a molecule of nominal diameter 51 Å. All of these experiments provide evidence for a relationship between the size of the enzymes and the relevant biomass accessibility. Pore volume distribution changes for different pretreatments have been measured and initial rates of enzymatic hydrolysis or the effectiveness of cellulose utilization by cellulolytic microbes was correlated to pore volume accessible to enzymes, which can have molecular weights ranging from 40 to 60 kDa (Ladisch *et al*., 1983; Grethlein, 1985; Lin *et al*., 1987).

Small particle sizes of untreated cellulosic substrate are more readily hydrolyzed than large ones because of higher specific surface area (Jackson *et al*., 1993; Mansfield *et al*., 1996; Laivins *et al*., 1996). Pretreatment increases accessible and susceptible surface area leading to enhanced enzymatic cellulose hydrolysis. With steam-pretreated substrates, it has been shown that when the severity of the pretreatment is increased, the average particle size is decreased and the hydrolysis yields are increased (Sawada *et al*., 1995; Tanahashi 1990).

As for pure cellulose, Zhang and Lynd (Zhang and Lynd, 2006) used their functionally based mathematical model and suggested that increasing cellulose accessibility to cellulase is the most influential for increasing the rate of enzymatic cellulose hydrolysis.

1.2.2.3.2 Enzyme-related factors

End-product inhibition of the cellulase complex, thermal inactivation and irreversible adsorption of the enzymes as well as the enzyme synergism are suggested to be the factors associated with the nature of the cellulase enzyme system that affect the enzymatic hydrolysis process (Mansfield *et al*., 1999).

End-product inhibition is a major enzymatic factor that limits cellulase hydrolysis (Xiao *et al*., 2004). Adding extra β-glucosidase, which hydrolyzes cellobiose to glucose, thereby preventing inhibition of cellobiohydrolases by cellobiose (Breuil *et al*., 1992), increasing cellulase loading, removing sugars during hydrolysis by filtration (Gan *et al*., 2005) or using simultaneous saccharification and fermentation (SSF) (Vinzant *et al*.,1994) are strategies designed to resolve this problem.

The adsorption reaction between lignocellulosic substrates and cellulase is important for an efficient hydrolysis process. It has been shown that cellulases interact with the cellulose surface with the cellulose-binding domain (CBD) and the catalytic domain (CD). The overall adsorption binding efficiency of cellulase is markedly enhanced by the presence of CBDs, while the role of CBDs in hydrolysis has not been precisely ascribed due to our current limited understanding of the binding reaction (Mansfield *et al*., 1999). Structural differences, for example, in the hydrophobicity of the surface of these enzymes may have an effect on the general adsorption affinity (Gusakov *et al*., 2000). Several authors have suggested that

cellulases adsorb to the lignin (Sutcliffe and Saddler, 1986; Converse *et al*., 1990; Ooshima *et al*., 1990).

Enzyme synergism, the combined action of two or more enzymes leads to a higher rate of action than the sum of their individual actions. Synergism seems to be particularly important for crystalline cellulose hydrolysis. Amorphous cellulose can be hydrolyzed by both endoglucanases and cellobiohydrolases, while crystalline cellulose is largely hydrolyzed by cellobiohydrolases. Thus, crystallinity probably influences hydrolysis when synergism is lacking due to an incomplete cellulase system or an insufficient enzyme loading (Mansfield *et al*., 1999).

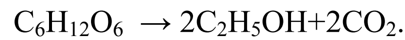
1.2.3 Fermentation and process configurations

Approximately 80% of the ethanol produced in the world is still obtained from the fermentation, the remainder comes largely by synthesis from the petroleum product, ethylene (Lin and Tanaka, 2006).

After enzymatic hydrolysis, the lignocellulosic substrates are converted to monosaccharides, which are further fermented to ethanol by microorganisms. There are a variety of microorganisms, generally either bacteria or yeast, which have been reported for the use of production of ethanol under oxygen-free conditions. They do so to obtain energy and to grow (Lynd 1990; Lin and Tanaka, 2006). Historically, yeasts are the most commonly used microbe, among the yeasts, *Saccharomyces cerevisiae*, which can produce ethanol at concentrations as high as 18% of the fermentation broth, is the preferred microorganism for most ethanol fermentations. This yeast can ferment monosaccharides, such as glucose, to ethanol. In addition, *Saccharomyces cerevisiae* is generally recognized as safe as a food additive for human consumption and is therefore ideal for producing ethanol. According to

the reactions, the theoretical maximum yield is 0.51 kg ethanol and 0.49kg of ethanol per kg of C6 or C5 sugar:

$$3C_5H_{10}O_5 \rightarrow 5C_2H_5OH+5CO_2,$$



$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH+2CO_2.$$

Saccharomyces cerevisiae is not able to ferment xylose. Therefore, metabolic engineering of xylose fermentation in *Saccharomyces cerevisiae* is an attractive approach (Sonderegger and Sauer 2003). Obtaining ethanol from pentoses (of which xylose is the major component) is particularly important, especially when they are present in relatively high amounts, such as in hardwood hemicellulose.

The fermentation step involves the conversion of sugars from hemicellulose and cellulose and some groups have used metabolically engineered microorganisms for the conversion of hexoses and pentoses from the cellulose (glucose) and hemicellulose (released by pretreatment) to ethanol. For the hydrolysis of the cellulose component, an enzymatic treatment is preferred. There are a few options when conducting the hydrolysis and fermentation steps: (a) separate hydrolysis and fermentation (SHF) involves four discrete process steps, (b) simultaneous saccharification and fermentation (SSF), which consolidates hydrolysis and fermentation of cellulose hydrolysis products into one process step, (c) simultaneous saccharification and cofermentation (SSCF) involves two process steps: cellulase production and a second step in which cellulose hydrolysis and fermentation of both cellulose and hemicellulose hydrolysis products occurs, (d) consolidated bioprocessing (CBP), also known as direct microbial conversion (DMC), cellulase production, hydrolysis, and fermentation of products of both cellulose and hemicellulose hydrolysis are accomplished in a single process step (Figure 1-2).

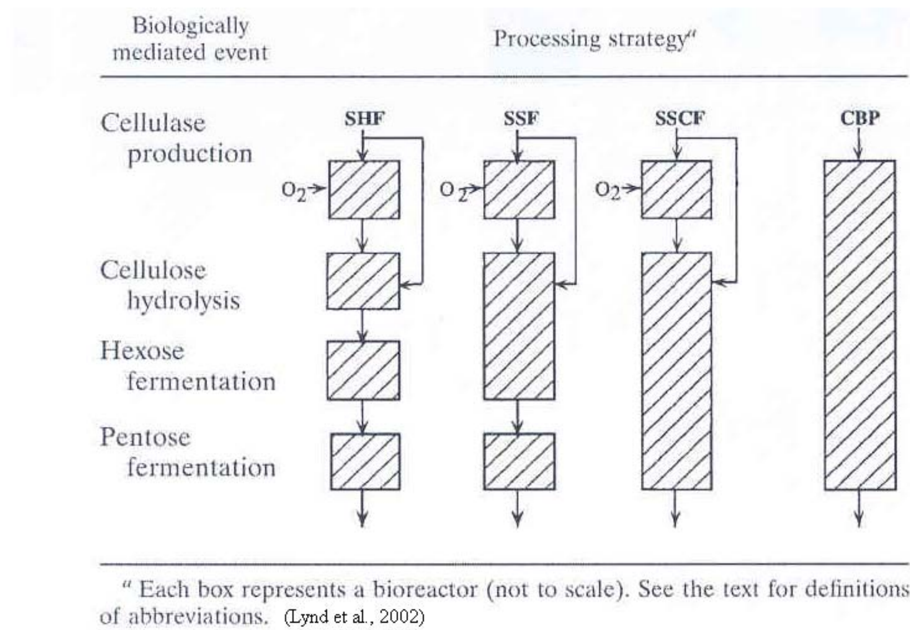


Figure 1- 2. Evolution of biomass-processing schemes featuring enzymatic hydrolysis

The first application of enzymes for hydrolysis of wood in an ethanol process was obvious: simply replace the acid hydrolysis step with an enzyme hydrolysis step. This configuration is now often referred to as "separate hydrolysis and fermentation" (SHF) (Hamelinck *et al*., 2005). During SHF, each operation can be conducted at optimal conditions of pH and temperature, but the accumulation of the end product of hydrolysis, glucose, inhibits the activity of the cellulases.

Simultaneous saccharification and fermentation (SSF), which integrates cellulose hydrolysis to glucose with glucose fermentation to ethanol in a single step, enhances the kinetics and economics of cellulosic biomass conversion to ethanol (Wright *et al*., 1988). During the SSF process, cellulose is hydrolyzed by the cellulase enzyme complex to cellobiose and eventually to glucose through the action of β-glucosidase. Glucose, in turn, provides a carbon/energy source for yeast cell growth and maintenance with concomitant production of ethanol and carbon dioxide. SSF requires less capital equipment than separate

hydrolysis and fermentation, reduces the risk of contamination because of the presence of ethanol, and circumvents enzyme inhibition by hydrolysis products (cellobiose, glucose). It has an enhanced rate of hydrolysis, needs lower enzyme loading, results in higher ethanol yields and improved ethanol productivities, and associated economics. Previous work in the areas of SSF and cellulase enzymes allows us to draw some conclusions regarding the choice of enzyme and yeast strain (Gonde *et al*., 1984; Lastick *et al*., 1984; Shoemaker, 1984; Spindler *et al*., 1988; 1989a；1989b; Wyman *et al*., 1986). The proper choice of cellulase is critical to the performance of the SSF process, and a cellulase with well-balanced activities can result in improved SSF performance. In particular, the relative ratio of β-glucosidase activity in the cellulase mixture seems to affect ethanol yields and rates significantly. Supplementation of β-glucosidase reportedly increased the yields and rates of ethanol production significantly for *Saccharomyces cerevisiae*.

In spite of the obvious advantages presented by the SSF, it has some drawbacks. These lie mainly in different temperature optima for hydrolysis (45-50°C) and fermentation (28-35°C) (Ballesteros *et al*., 2004; Jeffries and Jin, 2000). The yeast *Saccharomyces cerevisiae*, often proposed as the best organism for the fermentation of lignocellulosic hydrolysates (Hahn-Hagerdal *et al*.1991; Olsson and Hahn-Hagerdal, 1993), limits the temperature to 37°C. At this temperature, the cellulases have a low activity, which in turn results in lower hydrolysis rate (Novo Nordisk A/S product information; Huang and Chen, 1988). Besides, ethanol itself, some toxic substances arising from pretreatment of the lignocellulose inhibit the action of fermenting microorganisms, as well as the cellulase activity (Yu and Zhang, 2004). Achieving microorganism-enzyme compatibility becomes a major issue in the SSF, since some compounds (e.g., proteolytic enzymes) that are released on cell lysis or are

secreted by a particular strain can degrade the cellulases, alternatively, compounds in the enzyme preparation, can reduce microbial viability leading to cell lysis (Lin *et al*., 2006).

When the SHF and SSF processes are compared, it is evident that the advantage of SHF is that each step can be performed under optimal conditions, whereas in SSF a compromise must be made regarding operational temperature (Philippidis 1996). The major drawback of SHF is that the sugars released inhibit the enzymes during hydrolysis: end-product inhibition of β-glucosidase occurs. In SSF, the sugars are immediately consumed by the yeast and converted to ethanol. Previous studies on one-step steam pretreatment have shown that SSF gives higher yields than SHF when performed under the same conditions. In previous screening studies of a two-step steam pretreatment process, SHF proved to give higher yields. Wingren *et al* (2003) evaluated and compared the SHF and SSF processes from a technical and economic point of view. They found that the ethanol production costs for SSF was lower than that for the SHF, especially at higher solid material concentrations.

It has been shown that direct microbial conversion/consolidated bioprocessing (DMC/CBP), using anaerobic Clostridia (Wiegel *et al*. 1979; Zeikus 1980; Ahring *et al*. 1996; Lynd 1996) when grown at high temperatures, produce cellulolytic enzymes that hydrolyze the substrate and the generated sugars are immediately converted to ethanol. The disadvantages are, however, low ethanol yields, caused by byproduct formation (acetate, lactate), low tolerance of the microorganism to ethanol (3.5% w/v), and limited growth in hydrolysate syrups.

Overall, the performance of the fermentation step depends strongly on further development of cheaper and more efficient microorganisms and enzymes. Newer microorganisms may also allow for combining more process steps in one vessel, such as fermentation of different sugars, and enzyme production. Thus, despite the drawbacks, CBP

still demonstrates the trend toward the biomass processing technology development (Lynd *et al*., 2002).

1.3 Potential of high consistency enzymatic hydrolysis and fermentation

Despite intensive research over the few past decades, (Bjerre *et al*., 1996; Coughlan, 1992; Duff & Murray, 1996; Himmel *et al*., 1999; Schwald *et al*., 1989; Tan *et al*., 1987; Wright, 1998), the enzyme hydrolysis step remains as a major techno-economic bottleneck in lignocellose biomass-to-ethanol bioconversion process. This is partially due to the high cost of enzyme, thus the current fuel grade ethanol produced from lignocellulosic material is still not able to compete with gasoline (Sun & Cheng, 2002; Van Wyk, 2001). Although enzyme costs have decreased in the last few years, this is still true in 2009 (Simpson T., 2009).

Conventional enzymatic hydrolysis of lignocellulosic materials is typically carried out at a substrate consistency below 5% solids content. This results in a sugar concentration below 5% in the hydrolysate and, subsequently, a final ethanol concentration less than 2% (w/w) after fermentation. In contrast, enzymatic hydrolysis and fermentation of starch based substrates (e.g. corn) is commonly performed at a substrate loading above 20% of dry matter and over 10% (w/w) final ethanol concentration can be obtained after fermentation. Increasing substrate loading during hydrolysis of lignocellulose will lead to increased sugar concentration and higher final ethanol content after fermentation. This approach will bring about significant economic savings to the bioconversion process, such as reducing capital and operating cost for hydrolysis and fermentation, and minimizing energy consumption during distillation/evaporation and other downstream processes (Mohagheghi *et al*., 1992). Previous techno-economic assessments have suggested that an increase in substrate loading from 5% to

8% (w/w) can reduce the total production cost by nearly 20% (Stenberg *et al*., 2000; Wingren *et al*., 2003). A further increase in substrate loading will provide even more significant cost savings. However, using high substrate concentration in the form of fibrous, solid materials poses another problem: high viscosity prevents efficient mixing. A previous study has shown that high solid concentration (> 10%) resulted in poorer ethanol yield due to inefficient mass transfer (Spindler *et al*., 1988; Mohagheghi *et al*., 1992). It was also observed that once the dry matter content increased to 10%, no fermentation products was detected using steam-pretreated softwoods (Stenberg *et al*., 2000); Fermenting pretreated herbaceous crops and wheat straw at high dry material content encountered the same problem (Spindler *et al*., 1989b; 1990). To maximize the solids concentration, a prehydrolysis step was carried out in a fed batch way to obtain better mixing conditions by some liquefaction of the cellulase containing substrate. A maximum of 15% solid concentration of pretreated corn stover can be efficiently fermented to ethanol via the SSF process. A further increase of substrate concentration reduced the ethanol yield significantly as a result of insufficient mass transfer (Varga *et al*., 2004). Although all the studies carried out to date did not achieve an effective hydrolysis at a substrate consistency above 10% using either separate hydrolysis or fermentation (SHF) or simultaneous saccharification and fermentation (SSF) approaches, hydrolysing at higher substrate consistency is the trend for bioethanol production.

1.4 Problems addressed and thesis objectives

1.4.1 Problems to be addressed

It is apparent that the implementation of high consistency hydrolysis (HCH) process can bring an enormous economic benefit to the bioconversion process. At the same time, it is

anticipated high consistency hydrolysis will also cause a major impact on hydrolysis yield, process configuration, and productivity etc. Therefore, my thesis was designed to address a number of process-related technical barriers that may be ecountered during the implementation of HCH. The specfic issues to be addressed by my research are: 1) high concentration of fibrous materials reduces mass transfer rate and cause rheological problem; 2) high substrate consistency leads to high concentration of inhibitory substances, which in turn leads to severe end-products inhibition effects**.**

The more specfic objectives for my thesie are: 1) understand the rheological problem associated with fiber matrix consistency and identify industrial process/equipment to overcome rheological problems and faciliate enzymatic hydrolysis at high substrate consistency, 2) determine the hydrolysability of the lignocellulosic substrates at high solids loading, 3) investigate the end-products inhibition effects during high consistency hydrolysis and develop strategy to alleviate end-products inhibition.

1) Understand the rheological problem associated with fiber matrix consistency and identifying industrial process/equipment to overcome rheological problems and facilitate enzymatic hydrolysis at high substrate consistency.

Water exists in fibre matrix either as absorbed (free water) or adsorbed (bound water). Absorbed water is also called free water. At moisture content below 25-30% (fibre saturation point), the majority of the water is present as bound water within the cell wall. Above the fibre saturation point, waters starts to occupy the cell wall lumens (and/or inter fibre capillary) under the capillary force until full saturation of fibre matrix, which is typically reached at moisture content between 60%-70%. Water becomes mobile above this moisture content.

Increasing the substrate loading to obtain a concentrated solution after hydrolysis appears to be a straightforward approach. However, as most of the laboratory hydrolysis

testing has been carried out in shake flasks, rheology problems are typically encountered once the substrate consistency is increased above 12%. Rheological problems are caused by the increased viscosity of the matrix. It was observed that increasing pulp consistency resulted in a decrease in the amount of free water in the substrate matrix, the viscosity of the matrix increased and the mass transfer rate was reduced. As a result, the mixing provided by shake flasks is not effective in breaking down and liquefying the matrix. In consequence, the hydrolysis rate is significantly hindered. This rheology problem is the first obstacle that needs to be overcome to implement high consistency hydrolysis.

2) Determine the hydrolysability of the lignocellulosic substrates at high solid content

The goal for high consistency hydrolysis is to provide concentrated glucose solutions for ethanol fermentation. This approach will significantly reduce the cost of fermentation as well as the subsequent ethanol distillation and recovery processes (Olsson and Hahn-Hagerdal, 1996). However, many microorganisms have a limited tolerance to either the substrate or ethanol product (Loyd *et al*., 1993). At increasing levels of solids, sugar inhibition of enzymes becomes more important (Xiao *et al*., 2004). At the same time, it is expected that high substrate loading will lead to an increased level of inhibitory compounds (e.g. lignin and extractives) derived from the degradation of the substrates. It is also likely that high glucose concentration generated from high consistency hydrolysis will in turn cause an elevated end product inhibition effect. Therefore, it is important to determine the hydrolysis efficiency at high substrate consistency and investigate the impact of these factors on cellulase enzyme performance.

3) Investigate the end-products inhibition effects during high consistency hydrolysis and develop strategy to allivate end-products inhibition.

Using a separate hydrolysis and fermentation (SHF) process even at high substrate loading, the conversion efficiency is still relatively low. There is still a lot of cellulose not hydrolyzed due to the strong inhibition by hydrolysis products: glucose and short cellulose chains, thus the recovery of the unused cellulose is a problem. One possible way to overcome cellulase end-product inhibition is to ferment the glucose to ethanol as soon as it appears in solution. Simultaneous sacharification and fermentation (SSF) combines enzymatic hydrolysis with ethanol fermentation to keep the concentration of glucose low by fermenting the glucose to ethanol as soon as it appears in solution, overcome cellulase end-product inhibition. The accumulation of ethanol in the fermentor does not inhibit cellulase as much as high concentrations of glucose. SSF also provides a means of reducing enzyme dosage.

Therefore an SSF approach should provide a good strategy for increasing the overall rate of cellulose to ethanol conversion. In SSF the ethanol production rate is controlled by the cellulase hydrolysis rate not the glucose fermentation, so steps to increase the cellulase hydrolysis will lower the cost of ethanol production via SSF.

1.4.2 Thesis objectives

Since the bottleneck of bioconversion of lignocellulosic materials to bioethanol remains the low efficiency of enzymatic hydrolysis, and high consistency loading may significantly increase the productivity of ethanol, the purpose of the thesis research can be divided into two principal objectives. The first is to investigate high consistency liquefaction and hydrolysis (20% or higher) to produce high concentrations of ethanol. To achieve this objective, the application of pulping equipment to the biomass conversion process will be assessed. The second objective is to restrict end product inhibition by using an SSF approach.

Achieving these objectives should bring high consistency hydrolysis a step closer to industrial implementation.

CHAPTER 2 MATERIALS AND METHODS

Substrates

Unbleached hardwood kraft pulp (UBHW) and unbleached softwood kraft pulp (UBSW) were obtained from a Canadian kraft pulp mill. Organosolv pretreated poplar (OPP) was prepared in the Paprican pilot plant by cooking poplar wood chips in 50% (w/w) aqueous ethanol solution with 1.25% H_2SO_4 (w/w) as catalyst at 170°C for 60 minutes (Pan, *et al*. 2006). These substrates were chosen because they are representative of delignified material available from Canadian wood processing operative.

The extractives content of UBHW, UBSW, and OPP were determined by a PAPTAC (Pulp and Paper Technical Association of Canada) standard procedure (STANDARD G.13 and G.20) using acteone as a solvent. The total lignin content (acid soluble lignin and acid insoluble lignin) of UBHW, UBSW, and OPP was measured following a PAPTAC standard procedure G.8 and G.9. The filtrate obtained from lignin analysis was collected and used for sugar analysis. The sugar monomers in the filtrate, including arabinose, galactose, glucose, xylose and mannose, were separated by an anion exchange column (Dionex CarboPac™ PA1) on a Dionex DX-600 Ion Chromatograph system (Dionex, Sunnyvale, CA) equipped with an AS50 autosampler and a GP50 gradient pump. De-ionized water was used as an eluent at a flow rate of 1 ml/min; 1M NaOH was used to equilibrate the column after elution of sugars. To optimize baseline stability and detector sensitivity, 0.2M NaOH was added post column. After being filtered through 0.45 µm nylon syringe filters (Chromatographic Specialties Inc.), a 20 µl sample was injected on the column. The sugars were monitored by a ED50 electrochemical detector with parameters set for pulsed amperometric detection. Sugar

standards were prepared and analyzed using the same procedure in order to calibrate the instrument before sample analysis. The Chromeleon 6.5 software was used to control the chromatograph system and quantify sugar concentrations.

Enzymes

Celluclast 1.5L (cellulase) and Novozyme 188 (β-glucosidase) used in this study were obtained from Novozymes North America (Franklinton, NC). The Celluclast contained the following hydrolytic activities: 80 filter paper units per milliliter (FPU/mL). The activity of Novozyme 188 was 450 cellobiase units per milliliter (CBU/mL). The enzyme dosage was 20 FPU cellulase supplemented with 80 CBU of β-glucosidase per gram of cellulose in the substrate.

Enzymatic hydrolysis in shake flasks

The batch hydrolysis experiments were carried out in 500-mL flasks. The reaction solution contained 200 mM acetate buffer (pH 4.8) with differing concentrations of the substrates and enzyme dosages described above. All the flasks were fixed in a controlled environment incubator shaker (New Brunswick Scientific Co., Edison, NJ. USA). The enzymatic hydrolyses were carried out at a temperature of 50˚C and a rotating speed of 200 rpm for up to 96 h at various substrate consistencies.

Enzymatic hydrolysis in peg mixer

Enzymatic hydrolysis in peg mixer was also carried out under the same treatment conditions (temperature, pH and enzyme dosage) except that the mixing speed was set at 20 rpm. Prior to the hydrolysis, the substrate, enzyme and buffer were mixed thoroughly in a Hobart mixer before they were transferred to the peg mixer.

Fermentation of the hydrolysate

T1 yeast cells (*Saccharomyces cerevisiae*, provided by Tembec Inc, Témiscaming, Québec) were inoculated into 250 mL of YEPD medium (Yeast extract 1%, Peptone 2% and glucose 2%), incubated at 30˚C in a rotary shaker (200 rpm) for 24 h. The yeast cells were collected by centrifugation at 5000 g for 10 min at 4˚C. The pellet was washed three times with sterile deionized water. Yeast cells from this preparation were then inoculated into 60 mL of pre-hydrolysate or pure glucose solution. The final cell concentration was 5.5 g/mL.

The pH of the glucose controls and the hydrolysates was adjusted to 6.0 using 50% NaOH prior to the fermentation after the addition of 0.3% yeast extract, 0.5% peptone and $(NH_4)_2HPO_4$ to a final concentration of 20 mM. The fermentation experiment was carried out in 125 mL serum bottles containing 60 mL of hydrolysate. The serum bottles were vented using a syringe needle and placed in a rotary shaker (New Brunswick Scientific Co., Edison, NJ USA) at 30˚C for up to 96 h. All fermentations were carried out in duplicate and the mean value is reported.

Sugar, ethanol and inhibitors analysis

During the hydrolysis, aliquots of 0.5 mL were taken at different reaction times, and immediately filtered through a 0.45 μm membrane filter. The glucose concentration in the resulting filtrate was then determined by the above mentioned ion chromatograph method. All values are averages obtained from experiments performed in duplicate.

Aliquots of 0.5 mL were taken periodically from the fermentation broth to determine the ethanol and glucose concentration. Samples were first centrifuged to remove the yeast cells and then filtered through a 0.45 μm membrane filter. The ethanol concentration was determined by gas chromatography method equipped with a flame ionization detector

(GC/FID) and glucose concentration was measured by the same HPLC method described above.

Liquefaction and Simultaneous Saccharification and Fermentation (LSSF)

LSSF experiments were performed under semisterile conditions in two steps.

In the first step of LSSF, liquefaction step, 800 g dry substrate was prehydrolyzed in a Peg mixer at 50°C for the desired time with enzyme loading of 3–20 FPU/g cellulose at 20% consistency under semisterile conditions. Before the prehydrolysis, all the flasks, bottles, solutions and substrates were autoclaved at 120°C for 20min. The enzyme solutions were not sterilized. The liquid fractions were not sterilized to avoid further high-temperature decomposition of the material (Felby *et al*., 2003).

Prehydrolysates liquefied for different design times were collected for the subsequent SSF step. No cellulase was added in this step. β-Glucosidase was added as a supplement at a ratio of 1:4 of FPU cellulase to CBU β-glucosidase both in the liquefaction and in the SSF step. Baker's yeast was simultaneously added at a final yeast concentration of 5.5 g/L.

The SSF step was carried out in duplicate at 37°C and agitated at 200 rpm for 120 h.

Fed-batch enzymatic hydrolysis

Fed-batch enzymatic hydrolysis was carried out at 20% consistency, pH 4.8 and 50°C in a Peg mixer with a working volume of 12L. Experiments were started with 700g (dry weight) OPP substrate and enzyme loading of 3FPU/12CBU/g cellulose. Same amount of substrates and enzymes were added twice at 2h and 4h of hydrolysis, respectively.

Calculation of the ethanol theoretical yield

The samples of fermentation were collected and centrifuged at 5,000 rpm for 10 min. Ethanol concentration and the remaining monosaccharides were determined by GC and HPLC under the previously described conditions. The ethanol yield (Y_{EtOH}) was calculated assuming that 1 g of glucose present in the liquid theoretically gives 0.511 g of ethanol and 1 g of cellulose gives 1.11 g of glucose (due to the addition of water when the glycosidic bonds are hydrolysed). This yield is always less than 100%, as part of the sugars is needed for cell growth and synthesis of other byproducts, such as glycerol and acetic acid.

Enzyme activity measurement

The activity of the cellulolytic enzymes was measured in filter paper units (FPU). A 1× 6 cm strip of a Whatman No. 1 filter paper was added to 1.5 mL enzyme solution containing 0.05M Na-citrate buffer, pH 4.8. The samples were incubated 1 h at 50°C. Reducing sugars were determined after stopping the hydrolysis by addition of 3 mL DNS solution followed by 5 min boiling. After cooling, 20.0 mL distilled water was added and the UV-absorbance was read at 540 nm (Ghose, 1987).

Viscosity test

A stress-controlled rheometer (Viscometer Haake RS100-5Nm) equipped with an open cup coaxial cylinder (Couette) geometry, with 22mm inner diameter and a gap of 1 mm, was used for shear viscosity measurements. All the shear viscosity measurements were performed at 50°C which was the liquefaction temperature. Before measurements, the rheometer was calibrated using two standard oils (Cannon N26, N100 standard) under various temperatures. The measuring system was thermostatted and silicone oil circulated from a

temperature controlled bath through the thermostat around the cup to maintain a constant temperature for measurement. Temperature was maintained at a constant value of 50°C within ±0.1°C or less.

Each sample was warmed in 50°C-water bath for 10 minutes while the viscometer sample cup was warmed at 50°C at the same time. After transferring the sample to the cup, the rotor was re-installed. (The rotor had to be removed to load the viscous sample.) After another 30 minutes, measurement was made at constant shear rate for 30 minutes.

Cellulose viscosity determination

The degree of polymerization of cellulose was determined by the standard Cupriethylenediamine (CED) viscosity method, as described in CPPA the standard method G.24p.

CHAPTER 3 PULP RHEOLOGICAL PROBLEM ENCOUNTERED AT HIGH SUBSTRATES LOADING HYDROLYSIS

3.1 The influence of different substrate consistencies on hydrolysis in shake flasks

The hydrolysability of UBHW at different substrate consistencies, from 2 % to 20 % at 3% intervals in shake flasks was first examined (Table 3-1).

Table 3- 1. The influence of substrate consistencies on liquefaction time during hydrolysis of UBHW in shake flasks and peg mixer.

	shake flasks							**Peg mixer**	
Substrate consistency (%)	2	5	8	11	14	17	20	2	20
Liquefaction time (hours)	0	0	2.5	6	12	28	40	0	1
Glucose content after 48 h hydrolysis, g/L	17	41	64.7	86.9	103	108	113	17.2	125
48 h cellulose-to-glucose conversion rate, %	100	97	95.9	93.7	87.3	75.4	67	100	74

It was observed that increasing pulp consistency resulted in a decrease in the amount of free water in the substrate matrix. At 2% and 5% consistency, the substrates can be sufficiently suspended in the water solution, while upon increasing the consistency to above 8%, there is little free water present in the substrate matrix, and mixing provided by the

shaking bath is not effective to break down and liquefy the matrix. In consequence, the hydrolysis rate was significantly hindered and very little glucose was detected at the beginning of the hydrolysis of UBHW. The higher the initial consistency, the longer it took to liquefy the substrate matrix; the 48 h cellulose-to-glucose conversion rate decreased with increasing consistency of the substrate (Table 3-1). Increasing the substrate from 2% to 20% consistency, the 48 h cellulose-to-glucose conversion rate decreased from 100% to 67%. In the shake flasks, at 8%, 11% , 14%, and 17% consistency, it takes 2.5 h, 6 h, 12 h and 28 h, respectively, to liquefy the substrate, and at 20% substrate consistency the UBHW did not liquefy even after 40 h incubation in the presence of cellulase enzymes. UBSW took even longer times to liquefy, 48 h, which may be contributed to the different fiber characteristic and higher lignin content from UBHW. These experiments demonstrated that the shake-flask method is not suitable for evaluating high consistency hydrolysis of lignocellulosic feedstock. Increase the consistency of the substrate, resulted in an increase of the matrix viscosity. This creates the so called "rheological problem" during mixing and significantly reduces the amount of free water available for hydrolysis, and made it impossible for hydrolysis reactions to occur. It has been reported repeatedly that solid concentrations above 10% resulted in poor ethanol yield due to inefficient mass transfer (Spindler *et al*., 1988; Mohagheghi *et al*., 1992).

3.2 Pulp rheological problem encountered during hydrolysis at high substrate loadings

3.2.1 Pulp network characteristics and rheology

Pulp fiber suspensions have an inherent tendency to flocculate and form three-dimensional fibre networks. Pulp suspensions are continuous fiber networks which possess

structure and strength resulting from interaction between neighboring fibers. In suspension having consistencies greater than 0.5%, cohesive strength occurs from mechanical forces caused by bending and hooking of fibers (Kerekes *et al*., 1985). As the consistency of the fiber suspension increases, the number of fiber/fiber interactions increases which in turn increases the network strength. A consequence of the development of three-dimensional fiber networks is that networks possess properties similar to those normally encountered in solid materials. When lignocellulosic substrates are present at low consistencies (0 – 4 %) in water solution, the fibrous materials are suspended in abundant free water which makes the suspensions easy to be mixed and transferred. There is a minimum amount of fibre floc or fibre network formation at low consistency, and pulp dispersed as single fiber or small fiber aggregates helps to assure a more even distribution of enzyme within the fibers (Nutt *et al*., 1993; Osawa *et al*., 1963). However, once the substrate consistency increases up to 8 %, a greater degree of fibre interactions occur, and this leads to a substantial increase in the strength of the fibre network. As a result, the character of the suspension changes from one mass of fibres in water to wet fibre aggregates surrounded by gas (Duffy *et al*., 1975). At high consistency (20-40%), the suspension becomes a network of damp fiber aggregates surrounded by gas. The void ratio in this range is sufficiently great that the network is a permeable medium having a much lower resistance to gas flow in the inter-floc spaces than in the intra-floc passages. Thus, fiber flocs in this consistency range present an "aerodynamic specific surface" to a flowing gas substantially less than the specific surface of an individual fibers, approximately 15-60 m^2/kg compared with approximately 350-1000 m^2/kg (Garner, 1978). Thus, although a liquid readily flows through the suspension, there may be little contact between the liquid and most fibers unless the flocs are broken up in some manner. The increase in pulp consistency consequently increases the fiber interaction, the matrix

viscosity increases which creates the so called "rheological problem" during mixing and significantly reduces the amount of free water available for hydrolysis. In this case, the enzyme can only relatively reach the inter-floc spaces but not the intra-floc passages which will subsequently affect the enzymatic hydrolysis efficiency.

3.2.2 Mass transfer processes at high substrate consistency

The transfer of enzyme to the active site in the fiber takes place by convection in the liquid phase in which moist fibers are dispersed, dissolution in the water layer surrounding the individual fibers and, finally diffusion to the reaction site.

According to Osawa and Schuerch's (1963) model (Figure 3-1), at low consistency, under the exterior force of shaking or agitation, enzymes are easily transported to the reaction site of the fiber by convection across the mobile water layer (d_1) and by diffusion across the immobile water layer (d_2) immediately surrounding the fiber (Osawa *et al*., 1963; Bouchard *et al*., 1995). At low consistency, the immobile water layer is of maximum thickness. Diffusion across d_2 is the rate-determining step because convective transport across d_1 is faster.

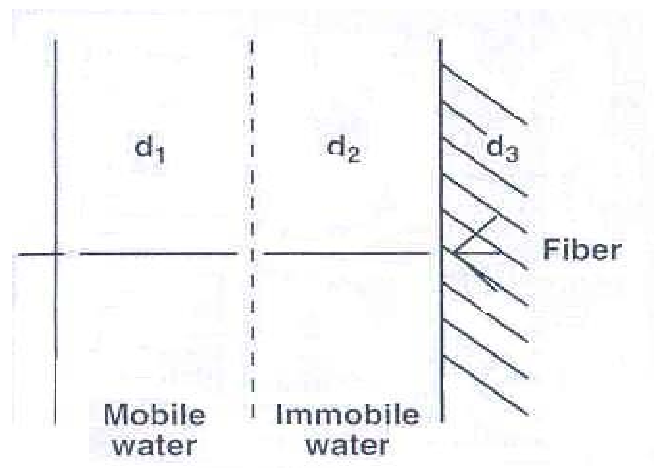


Figure 3-1. Mass transfer process model.

As the consistency is increased from low to medium (about 10%), the mobile layer is progressively eliminated leaving only the immobile layer. Water layer thickness now becomes the rate-determining step. It is suggested that in a high-intensity mixing system, fluidization of a fiber suspension makes it possible to effectively set the d_2 layer in motion (Laxen *et al*., 1990; Reeve *et al*., 1986; Kappel *et al*., 1994) and changes the environment so that the mass can be transported by convection instead of the more sluggish diffusion process. In the high consistency range (> 20%), most of the water is stored within the fiber and only a thin mobile water layer envelopes the fiber, thus decreasing considerably the diffusion path length of enzyme to the fiber. However, due to the disappearance of the mobile layer, enzyme cannot freely disperse to all fiber sites and as a result the enzyme may be concentrated in a smaller area of the fiber aggregates.

Maximum exposure of the fiber surface to the enzyme is achieved by finely shredding or fluffing the pulp to separate fiber aggregates to the greatest extent possible before contacting the fiber with enzyme. Enzyme can diffuse quickly through the diminished immobile water layer. Consequently, relatively mild agitation such as in a Hobart or Peg mixer (see below) may be sufficient at high consistency to facilitate the transport of enzyme to the fiber surface.

3.2.3 Peg mixer

The fiber network and fiber flocs cause the rheology problem of high consistency enzymatic hydrolysis and the accessibility of the fiber to enzyme has been considered more important than the reaction between the enzyme and the fiber itself. Disrupting the fiber network and exposing more fiber to the enzyme and water is crucial for the high consistency hydrolysis. Proper shear mixing was demonstrated to help to achieve this purpose (Laxen *et*

al., 1990; Sixta *et al*., 1991; Bennington *et al*., 1989). Mixing can be achieved by creating surface area within the suspension to facilitate contact between cellulose fibers and enzymes or by subjecting the suspension to cycles of compression, relaxation and shear to distribute enzymes through the suspension.

A peg mixer (Diagram 3-1), has a shaft with attached pegs. When the rotating bars shear the substrate suspension against the stationary elements, the shearing action creates transport through the suspension and thus exposes new fiber surfaces.

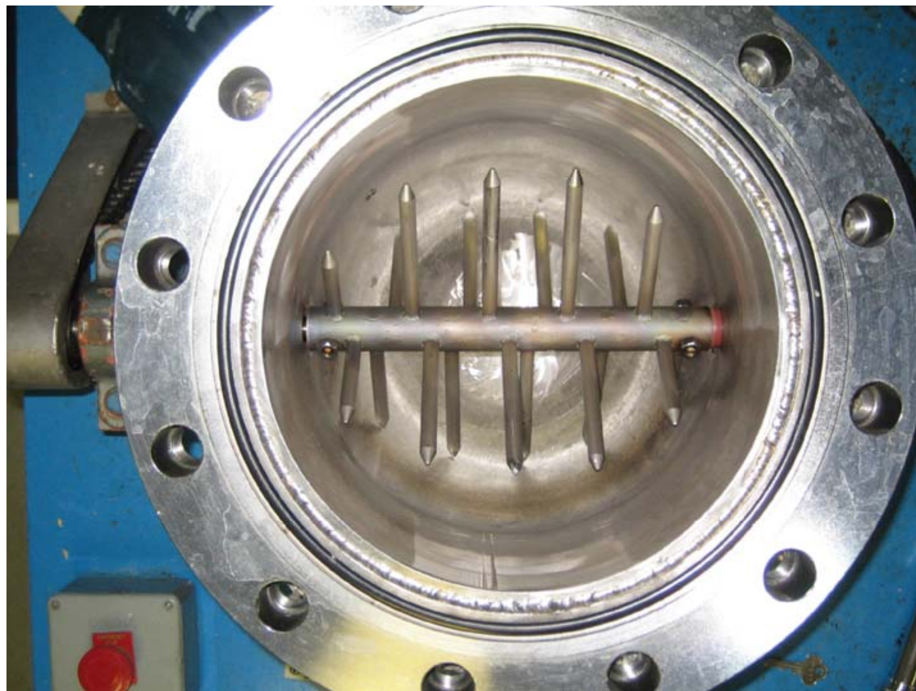


Diagram 3-1. The inner chamber of a laboratory peg mixer.

A peg mixer is standard equipment to achieve effective mixing of medium-consistency pulp which is commonly used for oxygen delignification, and was found to be capable of providing effective mixing of UBHW at high consistency. It has been shown previously that high shear mixing (>200 rpm) can deactivate cellulases and results in reduced

efficiency in cellulose hydrolysis (Mukataka *et al*., 1983; Cao *et al*., 2004). In an agitated batch reactor, the intensity of agitation has little effect on cellulose hydrolysis as long as cellulose fibers are completely suspended (Huang, 1975). Therefore, high shear is not necessary, and a lower shear rate was selected, as provided by the Peg mixer maintained at a low speed of 20 rpm during the high consistency hydrolysis process.

The first issue to be resolved in high consistency hydrolysis is to achieve an effective mixing of the substrate matrix. Effective mixing will facilitate mass transfer and reduce the viscosity of the matrix (liquefaction). As shown in Table 3-1, a significant increasing in the liquefaction time as determined by visual observation was found during the enzymatic hydrolysis in the shake flasks at higher substrate consistency. A complete liquefaction of UBHW at 20% consistency was observed after mixing at 20 rpm for 1 h at 50ºC, compared to 40 h in flasks shaken at 200rpm. At the same enzyme loading, the liquefaction time is reduced dramatically. The glucose conversion rate is also higher than that obtained in shake flasks after 48 h incubation time. This shows that mixing can significantly improve the substrate liquefaction process and subsequently increase the hydrolysis rate. It is evident that this equipment can greatly improve the liquefaction rate of UBHW substrate in the presence of cellulase.

The results indicate that a peg mixer is suitable for the high consistency enzymatic hydrolysis. Good mixing breaks the fiber network and the fiber flocs, increasing the fiber and enzyme contact area, and facilitating the fiber liquefaction process. Therefore the rheological and mixing problems of high consistency hydrolysis could be overcome by existing commercial pulp mixers. This result suggests that there is potential to enhance the hydrolysis efficiency through improving the substrate liquefaction with proper mixing.

When we look at actual industrial processes, it is apparent that low substrate consistencies are not economically feasible. In the pulp and paper industry, except for the pulp transportation processes, all the reactions are carried out at consistency higher than 10% (w/v). High consistency pulp bleaching has operated commercially for two decades (Dence and Reeve, 1996). For example, in ozone bleaching, the consistency of the pulp can be as high as 30-40%. In the starch industry, the hydrolysis can also be carried out at as high as a 40% consistency. So, high consistency enzymatic hydrolysis combined with the existing industry equipment might be an attractive way to achieve commercial enzymatic hydrolysis.

3.3 The factors influencing the liquefaction of substrate during high consistency hydrolysis (HCH)

During the high consistency hydrolysis of lignocellulosic substrates, through effective mixing the fibre network is first broken down and substrate starts to liquefy. Liquefaction reduces the mass viscosity, favouring the mixing and facilitating the mass transfer, so liquefaction is an important step during high consistency hydrolysis. When the substrate is in a totally liquefied state, the efficiency of enzymatic hydrolysis is improved because access to the substrate is easier. The factors influencing the liquefaction of substrate during high consistency enzymatic hydrolysis (HCH) were evaluated.

3.3.1 The effect of mixing on liquefaction and hydrolysis

Table 3-2. The influence of enzyme and mixing on substrates liquefaction time.

Conditions	Liquefaction time, h		
Substrates	OPP	UBHW	UBSW
Mixing without enzyme	96, not liquefied	96, not liquefied	96, not liquefied
Mixing with enzyme	1	1	2
With enzyme without mixing	36	40	48

It has been shown previously that high speed shear mixing (>200 rpm) can deactivate cellulases and result in reduced efficiency in cellulose hydrolysis (Mukataka *et al*, 1983; Cao *et al*, 2004). Therefore, the Peg mixer shear speed was maintained at a low speed of 20 rpm during the hydrolysis process.

All of the substrates, from Table 3-2, OPP, UBHW, and UBSW, when hydrolyzed at 20% consistency with the same enzyme loading at 50ºC, were completely liquefied after mixing at 20 rpm in Peg mixer for 1 h or 2 h, respectively. When hydrolyzed under the same conditions without mixing (shaking in flasks at 200rpm and 20% consistency), all three substrates could be liquefied, but required a longer time, 36 h, 40 h and 48 h, respectively. When these three substrates were hydrolyzed under the same conditions in the Peg mixer with mixing without cellulase enzyme loading, all the volumes of the substrates in the mixer decreased significantly, but still did not liquefy even after 96 h incubation time. These results show that it is the enzyme that causes the substrate liquefaction, but proper mixing significantly facilitates the liquefaction process and dramatically reduces the liquefaction

time. Thus both mixing and enzymes are crucial factors for the efficient hydrolysis of high solids substrates.

3.3.2 Influence of enzyme components on substrate liquefaction

The Celluclast 1.5CL preparation contains three types of enzyme activities: endoglucanases (1, 4-β-D-glucan-4-glucanohydrolase), exoglucanase (cellobiohydrolase) and β-glucosidase (β-glucoside glucohydrolase). Although cellulase preparations contain β-glucosidase activity, the activities of this enzyme are generally insufficient to prevent the accumulation of cellobiose. Consequently, cellulase preparations are typically supplemented with extra β-glucosidase. The influence of enzymatic components on OPP substrate liquefaction at different enzyme loadings in the Peg mixer at 20% consistency were assessed (Table 3-3).

Table 3-3. The influence of enzyme components on OPP substrate liquefaction.

Enzyme dosage Celluclast (FPU)/ β-G (CBU)	Liquefaction time, h
5 FPU/ 20 CBU	2
20 FPU/ 0 CBU	1
20 FPU/ 20 CBU	1
20 FPU/ 80 CBU	1
Endo-glucanase	48 , no liquefaction

From Table 3-3, it is apparent that the higher the amount of Celluclast applied, the shorter the time needed for liquefaction. It took two hours to liquefy the 20% consistency OPP substrate at 5 FPU/ 20 CBU enzyme loading while only one hour was needed for the

same substrate at 20 FPU/ 20 CBU loading. The results indicate that the dosage of Celluclast enzyme has an impact on the rate of liquefaction.

We then evaluated the effect of supplemental β-glucosidase activity on the liquefaction of the OPP substrate at 20% consistency. Table 3-3 shows that at the given dosage of Celluclast, supplementing with β-glucosidase seemed to have no effect on the liquefaction of OPP. The substrate supplement of β-glucosidase at 20CBU and 80CBU loading liquefied at the same rate as the substrate without supplement of β-glucosidase after 1 h incubation. Based on these results, the β-glucosidase did not significantly contribute to the substrate liquefaction stage.

The effects of endoglucanase on liquefaction were also examined using a commercial endoglucanase preparation Novozyme 613. When Novozyme 613 was used alone for hydrolyzing OPP at 20% consistency substrate in a PEG mixer, the substrate did not liquefy after 48 h. The liquefaction process probably requires the synergistic effects of exoglucanases and other cellulase enzymes.

3.4 Establishing a protocol to measure substrate viscosity and determine liquefcation rate

The first stage in high consistency hydrolysis is liquefaction. During the course of liquefaction, the substrate volume reduces dramatically, and the phase of the substrate also changes from solid suspension to liquid slurry. Although the liquefaction phenomenon can be observed by visual examination, a sound scientific method is required to quantify the rate of liquefaction. Essentially, liquefaction is a process of substrate viscosity reduction. Therefore, viscosity is an important factor to evaluate the changes in substrate characteristics during the high consistency enzymatic hydrolysis process. Viscosity is a measure of the resistance of a

fluid which is being deformed by either shear stress or extensional stress. In general terms it is the resistance of a liquid to flow, or its "thickness". Viscosity describes a fluid's internal resistance to flow and may be thought of as a measure of fluid friction. The study of viscosity is known as rheology. It is critical to get better understanding of the substrate's rheological properties.

Traditionally, cupriethylenediamine (CED) solubilization is used to measure the average degree of polymerization or average molecular weight of the cellulose molecules in any particular pulp. But there is a limitation of the sample that the lignin content cannot be over 0.5%, otherwise it will affect the resulting viscosity value. In addition, a relatively complex sample pretreatment process is needed. Unfortunately, the lignin content of most lignocellulosic materials we use for enzymatic hydrolysis is higher than 2%, where the CED method is not suitable. At Paprican, a rheometer is used more practically to measure the viscosity of black liquor. This is a fast, feasible method and no sample pretreatment is required when measuring the viscosity. Accordingly, we tried to establish a protocol to measure the substrate viscosity by using a rheometer to determine the trend of viscosity change during the time course of hydrolysis.

3.4.1 Stability test

Prior to establish a protocol to determine the viscosity, a thermal stability test was first carried out to identify suitable testing conditions.

The thermal stability of the hydrolysate samples was first tested at 20°C with a shear rate of 10 s^{-1}. A hydrolysate sample obtained after six hours of enzyme hydrolysis of OPP was used. As shown in Figure 3-2, the viscosity of hydrolysate increased with time and then levelled off after 40 minutes. The initial increase in viscosity within the first 40 minutes is

probably due to the decrease of the sample temperature (The room temperature is 23°C). Then, the viscosity stays almost constant (around 4500cp) for about an hour. It appears that the hydrolysate has very good thermal stability at 20°C. However, it requires some time (up to 60 minutes) to reach to this steady state.

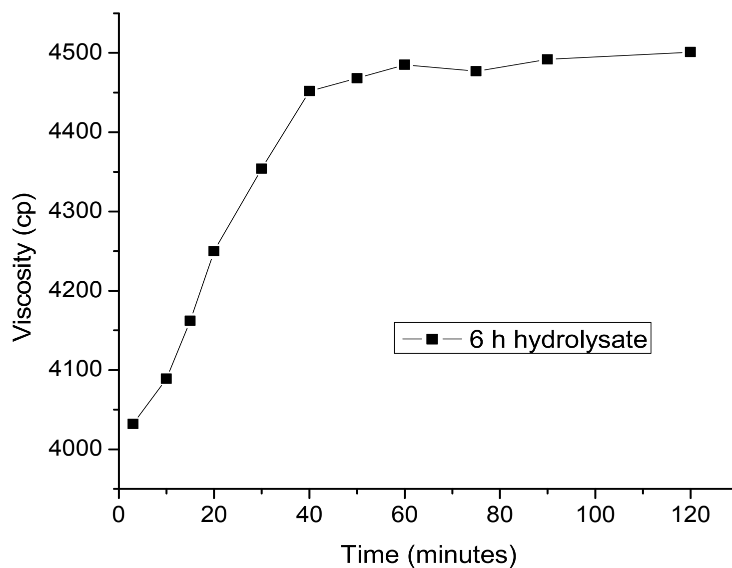


Figure 3-2. Thermal stability test of OPP hydrolysate at 20°C.

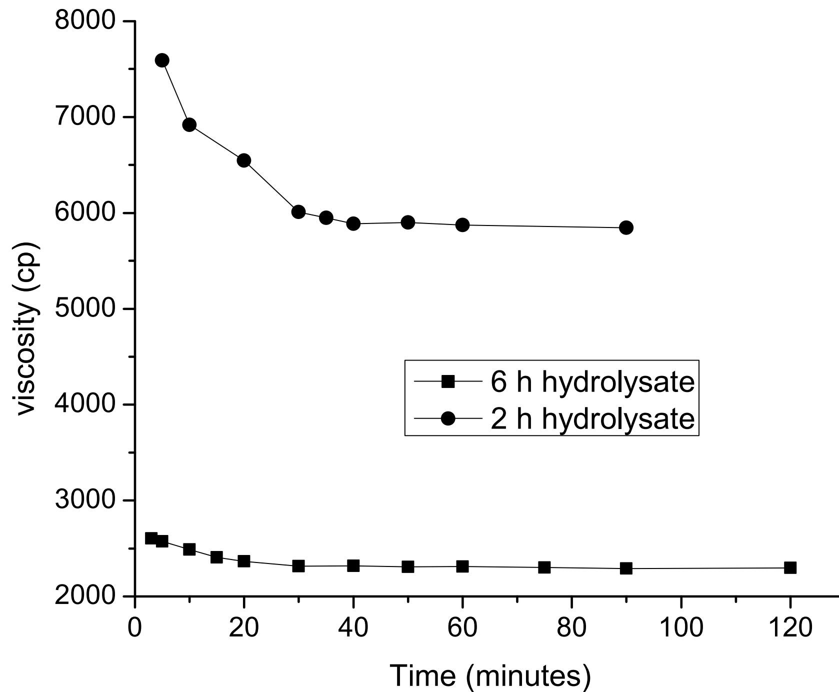


Figure 3-3. Thermal stability test of OPP hydrolysates at 50°C.

Enzymatic hydrolysis is typically carried out at 50°C. Therefore it is more relevant to test the thermal stability at 50°C. As shown in Figure 3-3 the viscosities of both 2 h and 6 h hydrolysates decreased dramatically with time and then levelled off at a longer time. The decrease in viscosity is due to increase of sample temperature during the first 30 minutes (starting temperature is about 23°C) to reach 50°C and stable the system. Once the sample reaches the target temperature, the viscosity becomes almost constant, but still slightly decreases. This may due to the possible enzymatic degradation at 50°C.

It is common knowledge that viscosity varies with temperature. In general, the viscosity of a simple liquid decreases with the increase in temperature (and vice versa). As the temperature rises, the average speed of the molecules in a liquid increases and the amount of time they spend "in contact" with their nearest neighbors decreases. Thus, as temperature

increases, the average intermolecular forces decrease. The exact manner in which the two quantities vary is nonlinear and changes abruptly when the liquid phase changes.

The results in Figure 3-2 and Figure 3-3 show that the hydrolysates have a good thermal stability both at 20°C and 50°C.

3.4.2 Rheological test

Another factor can significantly affect substrate viscosity is the shear rate applied during viscosity testing. Since the shear rates inside the reactor are different, depending on the speed of the impeller, the distance between the impeller and the internal wall of the reactor, and how far it is from the impeller, it would be of great interest to measure the viscosity of the substrate under various shear rates. However, due to the limitation of the apparatus, only viscosity under higher shear rate could be obtained. Three different shear rates were choosen, the lowest shear rate tested was 1 s^{-1}, and the other two rates were 10 s^{-1} and 100 s^{-1}.

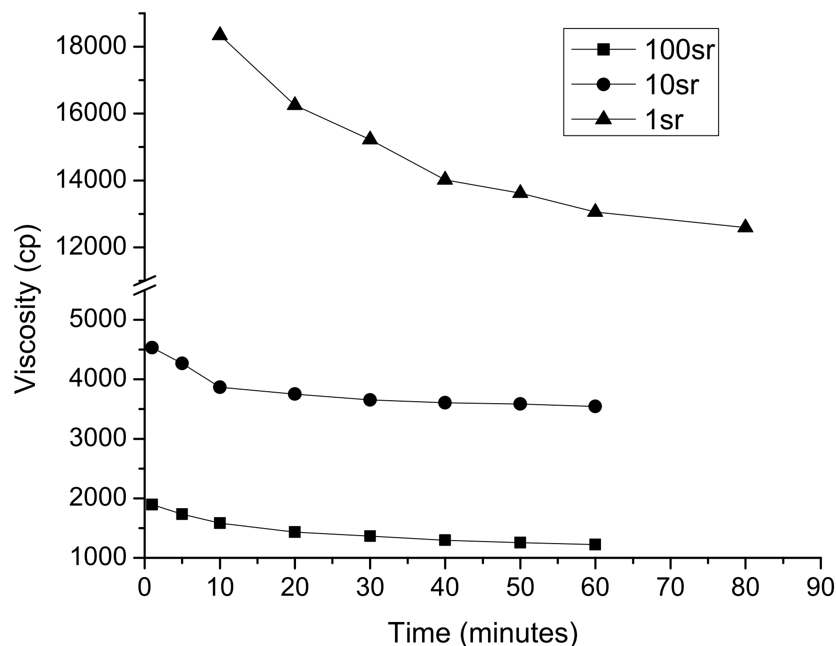


Figure 3-4. The viscosity of 4h OPP hydrolysate obtained at different shear rate.

As shown in Figure 3-4, the shear rate had a significant impact on viscosity as expected. At the low shear rate, 1 s^{-1}, a high viscosity obtained, and the viscosity value kept decreasing during the time course tested. While at high shear rate, 100 s^{-1}, the viscocity quickly dropped to a very low level (<2000); At the medium shear rate, 10 s^{-1}, the viscosity of the substrate can maintain at a relative constant value, around 3700cp, after 20 minutes of the testing.

From these experiments, the optimum conditions for testing the viscosity of substrates during high consistency enzymatic hydrolysis (HCH) can be established and used for measuring the viscosity of the hydrolysate: In all the subsequent liquefaction testings, substrate samples were pre-heated and sheared at 50 ^{o}C and 10 s^{-1} for 30 minutes, then started to record for about 40 minutes.

Another important factor is temperature. Arrhenius and Williams-Landel-Ferry (WLF) equations are normally used to describe temperature dependence of polymer solution and polymer melt. Arrhenius equation can be expressed as follows:

$$\mathbf{a_T = \exp\left[\frac{E_a}{R}\left(\frac{1}{T} - \frac{1}{T_r}\right)\right]} \qquad (1)$$

Where: a_T is the temperature shift factor, E_a, the activation energy, R, gas constant and T_r is the reference temperature.

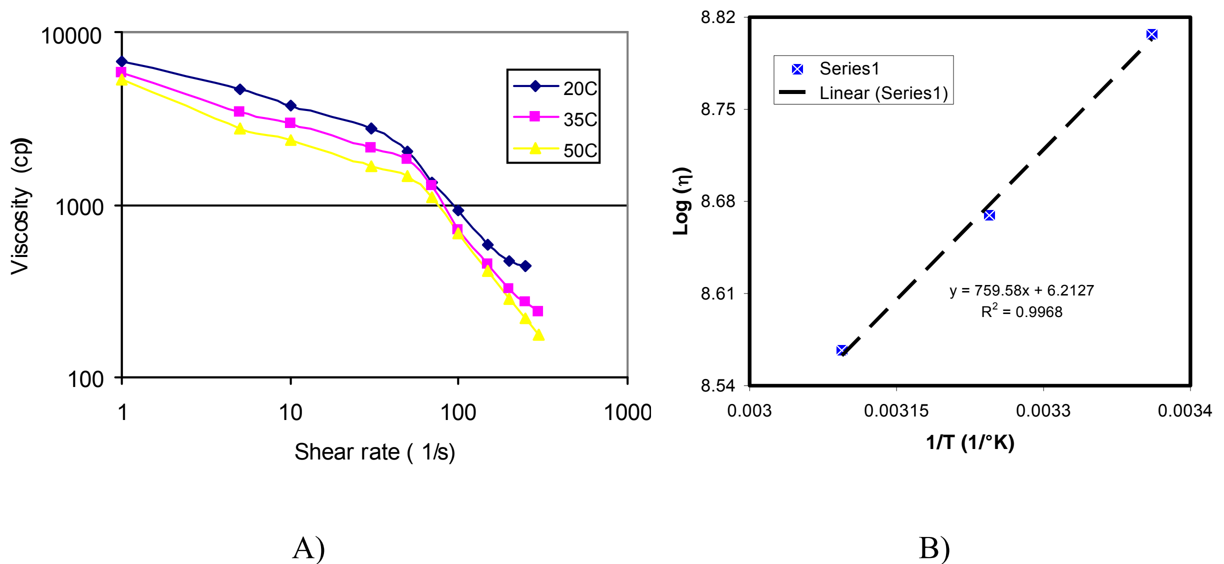
The WLF equation can be describes as follows:

$$\mathbf{\log_e a_T = \frac{-C_1(T - T_0)}{C_2 + (T - T_0)}} \qquad (2)$$

Where C_1 and C_2 are constants and T_0 is the reference temperature.

The Arrhenius equation is normally used when the application temperature is 100°C higher than the polymer glass transition temperature (T_g), whereas WLF equation is used when the application temperature is close to T_g.

The glass transition temperature of hydrolysate was not determined. It is apparent that the hydrolysate is similar to polymer solution than to a water solution, especially at earlier stages of liquefaction. Therefore, the Arrhenius equation was chosen in this study. Figure 3-5(a) shows the viscosity versus shear at various temperatures for the hydrolysate. As seen in Figure 3-5(a), the hydrolysate exhibits pseudoplastic (shear thinning) behavior. The lowest shear rate tested was $1s^{-1}$, which was used for the calculation of the activation energy. Figure 3-5(b) shows the logη versus 1/T and the linear regression is based on Arrhenius equation. The activation energy can be obtained from the slope (E_R/R), and can be used to evaluate the sensitivity of viscosity to the temperature change.



A) B)

Figure 3-5. A) Viscosity of hydrolysate versus shear rate under various temperatures. B) logη versus 1/T fitting by Arrhenius equation.

3.4.3 The viscosity of the hydrolysate at different liquefaction times

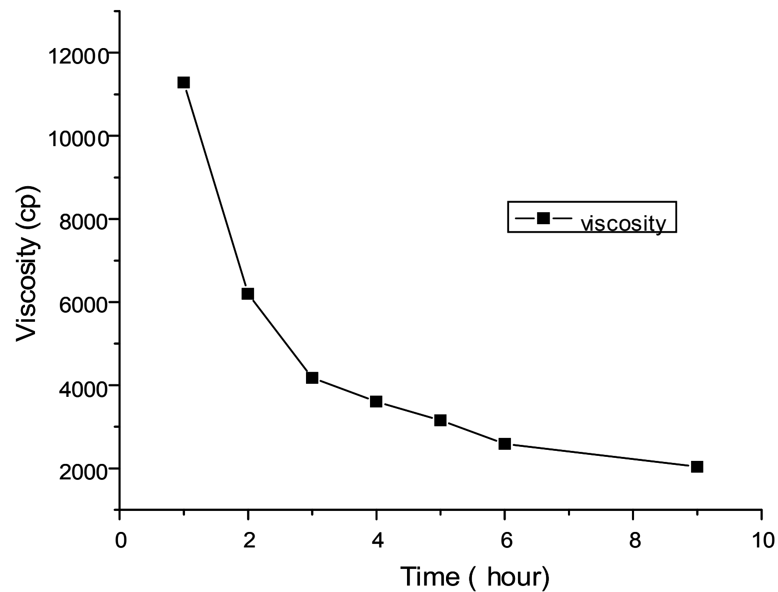


Figure 3-6. Viscosity of the samples collected at different liquefaction times.

Based on the conditions identified from above experiments, vicosity values of substrate samples collected at 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, and 9 h during enzyme hydrolysis of OPP at 20% consistency were determined. With the hydrolysis process proceeding, the viscosity of the hydrolysates decreased. Especially during the first 2 h of hydrolysis, the viscosity decreased significantly, from about 11000cp after one hour decreased to 6000cp after two hours hydrolysis. We define liquefcation point as the substrate viscosity drops to 6000cp when it becomes feasible for pumping the substrate slurry in an industrial process.

3.5 Conclusions

The results indicate that a peg mixer is suitable for high consistency enzymatic hydrolysis. The rheological and mixing problems of high consistency hydrolysis can be overcome by using the already existing commercial pulp and papermaking mixers.

Both mixing and enzymes are crucial factors for the efficient hydrolysis of high solid substrates. Celluclast and its dosage are important for substrate liquefaction, while β-glucosidase seems to have no significant contribution to the liquefaction stage. Endoglucanase also requires the synergistic effects of exoglucanases and other cellulase enzymes.

An experimental protocol to quantify liquefaction rate is thus established. The viscosity of the substrate decrease with the liquefaction proceeding.

CHAPTER 4 HIGH CONSISTENCY ENZYMATIC HYDROLYSIS OF LIGNOCELLULOSIC SUBSTRATES

"High consistency" enzymatic hydrolysis can be roughly defined as beginning at the insoluble solids level where significant levels of free liquid are no longer present in the slurry such that the separation of a liquid and solid phase from the suspension is not spontaneous (Hodge *et al*., 2009).

Unlike starch-based feedstock, a lignocellulosic substrate is mainly fibrous material with a high degree of polymerization (DP). In water suspension, fibrous substrates can interact with each other and form fibre flocs or, on a larger scale, fibre networks. This leads to a considerable increase in the viscosity of the substrate matrix and creates a so called "rheological problem" where the mass transfer rate in the substrate matrix is significantly hindered. Due to the limited amount of free water present in the matrix, it takes a much longer time to liquefy the matrix and carry out effective hydrolysis. Rheological problems associated with mixing pulp fibre suspensions have long been recognized in pulp and paper manufacturing. Dealing with high substrate consistency is a common practice in wood pulp bleaching. Industrial bleaching equipment is designed to handle pulps at various consistencies, typically up to a 35% consistency. Medium or high consistency mixing devices can effectively break down fibre flocs and networks formed in pulp suspensions above 20% consistency.

In this chapter, we examine the feasibility of using a peg mixer to carry out enzyme hydrolysis of lignocelluloses at high substrate consistency. Three substrates were used,

unbleached hardwood kraft pulp (UBHW), unbleached softwood kraft pulp (UBSW), and organosolv pretreated poplar pulp (OPP).

4.1 Chemical composition of pulps

As shown in Table 4-1, we found that unbleached hardwood (UBHW) has a cellulose content of approximately 80% with 19.6% of xylan. This pulp contains a small amount of lignin and low extractives content. The cellulose and lignin content of unbleached softwood (UBSW) pulp are 82% and 4.62% respectively, higher than those of the UBHW pulp, while xylan content is 10%, mannose contains about 5%.

Table 4-1. The chemical compositions of UBHW, OPP, and UBSW pulps.

Component, % w/w	UBHW	OPP	UBSW
Acetone extractives	0.15 ± 0.02	8.17 ± 0.06	0.07± 0.01
Cellulose (as glucan)	79.1 ± 0.4	86.5 ± 0.4	82.0± 0.3
Cellulose (as glucose)	84.3 ± 0.4	92.3 ± 0.4	87.5± 0.3
Xylan	19.6 ± 0.4	1.46 ± 0.03	10.0± 0.2
Mannose			4.83± 0.04
Lignin			
Acid soluble	0.63 ± 0.04	0.34 ± 0.01	4.04± 0.05
Acid in-soluble	1.06 ± 0.05	2.08 ± 0.01	0.58± 0.02

The organosolv pretreated poplar (OPP) pulp contains approximately 87% cellulose, the highest cellulose content of the three substrates and with little xylan (~1.5%). The lignin content of OPP is 2.4% which is slightly higher than that of UBHW but lower than UBSW.

The most distinctive difference among the OPP, UBHW and UBSW pulps is the significant amount of acetone extractives detected in OPP, as high as 8.2%, while UBHW and UBSW only contain trace amount of extractives. The acetone extractives were further analyzed by GC/FID. It was found the predominant extractive compounds are low molecular weight phenolic compounds such as lignans (data not shown).

4.2 Hydrolysis of UBHW and UBSW at three consistencies in shake flasks

4.2.1 Hydrolysis of UBHW and UBSW at 2%, 5% and 20% consistency in shake flasks at higher enzyme loading

The hydrolysability of UBHW and UBSW were first determined in conventional shake flasks at 2%, 5%, and 20% (w/w) consistencies with high enzyme loading, 20 FPU/g and 80 CBU/g of cellulose.

The monosugars obtained from UBHW are mainly glucose (Figure 4-1A), together with a small amount of xylose. Hydrolysis at 2% substrate consistency for 24 h resulted in a glucose concentration of about 17 g/L, while hydrolysis at 5% substrate consistency produced approximately 41 g/L glucose in the final hydrolysate. When the percent cellulose-to-glucose conversion was determined, it was found that most of the cellulose present in 2% and 5% UBHW substrate were converted to glucose within 24 h of incubation. Increasing substrate consistency to 5 % led to a slightly lower cellulose-to-glucose conversion rate, approximately 97% after 48 h. The is probably due to end-product inhibition by the glucose and cellobiose (Xiao *et al*., 2004).

When hydrolysing the UBHW at 20% consistency in shake flasks, due to the pulp rheology problem, the 20% consistency substrate is like a solid. In this case, shaking is not enough to achieve good mixing between enzyme and substrate, so substrate at 20% consistency was more difficult to hydrolyze than at low consistency. It took about 40 h incubation for the complete liquefaction of the substrate. The cellulose-to-glucose conversion rate at 48 h was about 64%, which is significantly lower than that obtained at low consistency hydrolysis (2% and 5% substrate loading).

The hydrolysability of UBSW is shown in Figure 4-2. Hydrolysis of UBSW at 2% substrate consistency for 48 h resulted in a glucose and xylose concentration of about 18 g/L and 1.4 g/L respectively, whereas hydrolysis at 5% substrate consistency produced approximately 42 g/L glucose and 3.4 g/L xylose in the final hydrolysate. Most of the cellulose and hemicellulose present in 2% UBSW substrate were converted to glucose and xylose within 24 h of incubation. Five percent substrate consistency also led to a slightly lower cellulose-to-glucose conversion rate, approximately 96% after 48 h, but the hemicellulose-to-xylose conversion rate only reached 68%, lower than that of 2% hydrolysis. It seems that, at low consistency, the cellulose hydrolysability of UBSW and UBHW are similar.

During hydrolysis of the UBSW at 20% consistency in shake flasks, the same situation was encountered as with the UBHW. Due to the rheological problem, nearly 48 h incubation time was required for the complete liquefaction of the substrate.

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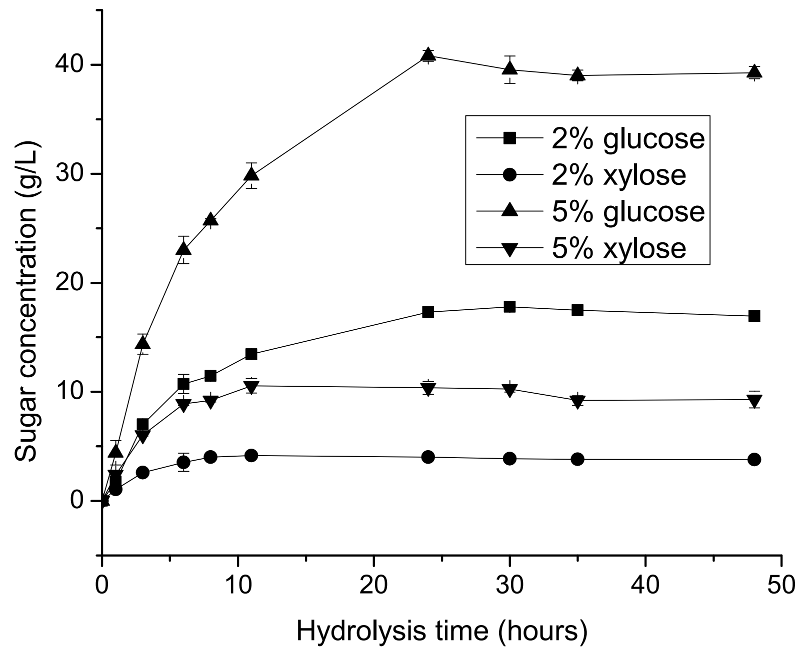

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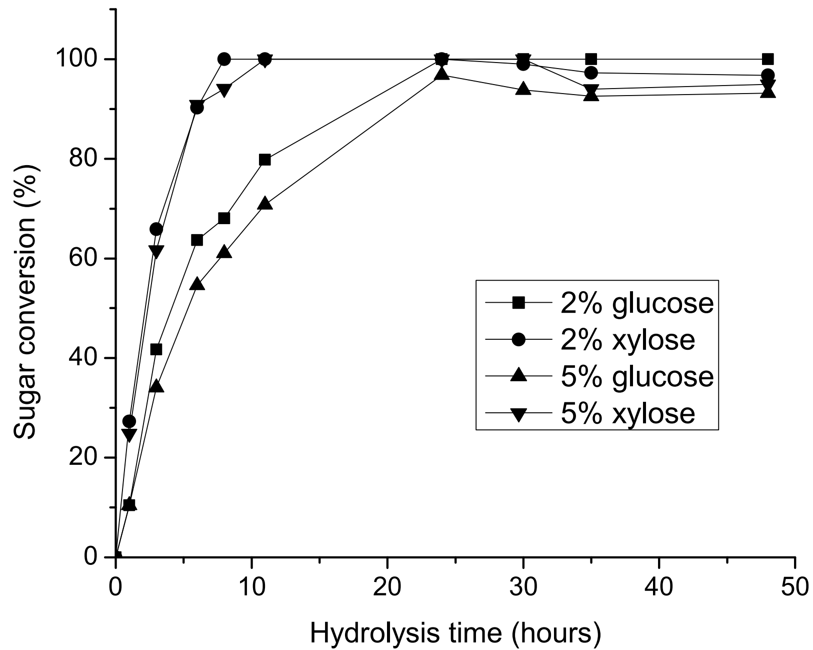
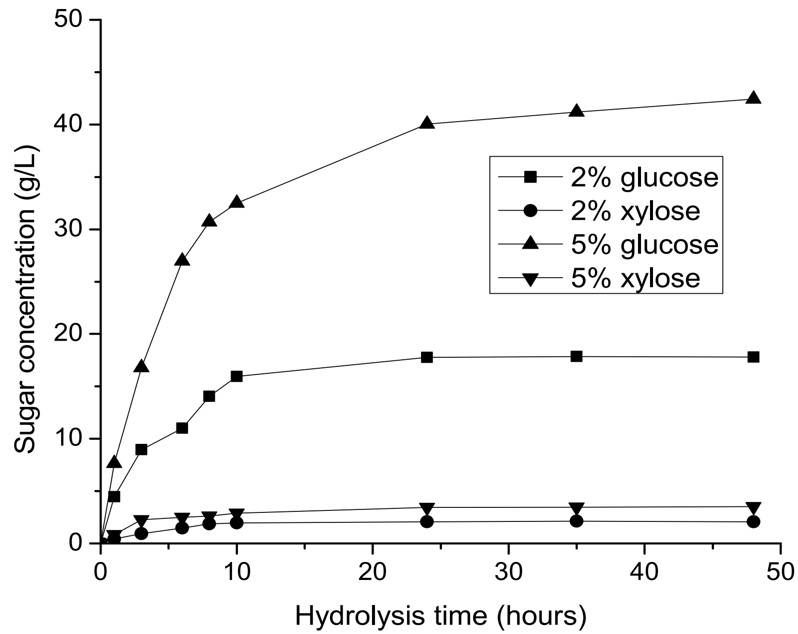


Figure 4-1. Enzymatic hydrolysis of UBHW at 2% and 5% substrate consistencies in shake flasks at 20FPU/80CBU/g of cellulose enzyme loading, based on A) glucose and xylose concentration formed and B) percent sugar conversion.

A:



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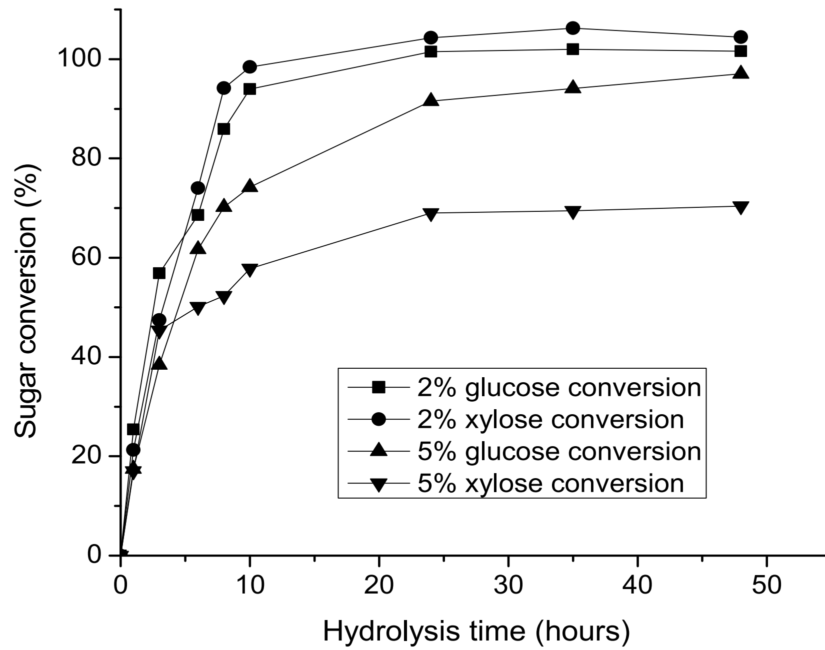


Figure 4-2. Enzymatic hydrolysis of UBSW at 2% and 5% substrate consistencies in shake flasks at 20FPU/80CBU/g of cellulose enzyme loading, based on A) glucose and xylose concentration formed and B) percent sugar conversion.

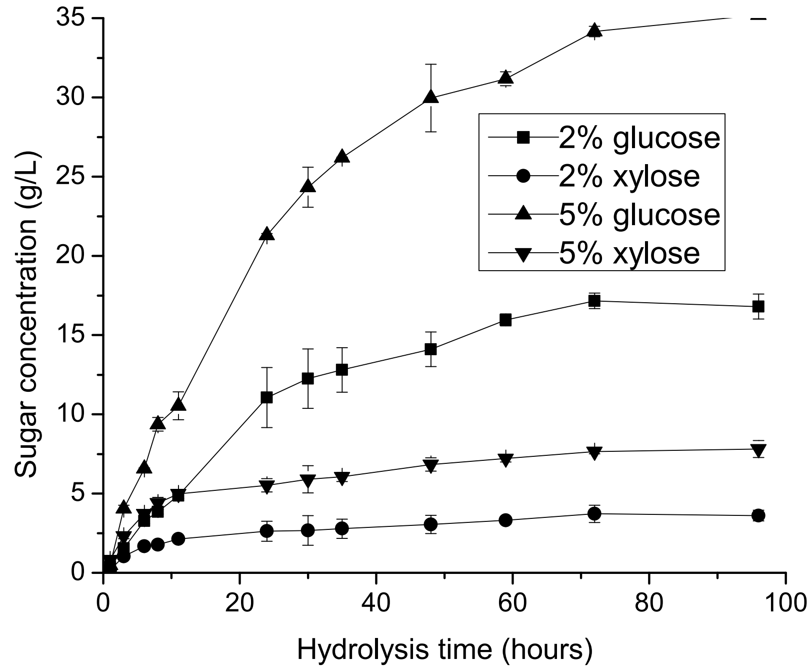
4.2.2 Hydrolysis of UBHW and UBSW at 2% and 5% consistency in shake flasks at lower enzyme loading

The hydrolysability of UBHW and UBSW was then determined in conventional shake flasks at 2% and 5% consistencies at a lower enzyme loading, 5FPU/20CBU/g cellulose.

Hydrolysis of UBHW at 2% consistency with lower enzyme loading resulted in almost all the cellulose in the substrate being converted to glucose (see Figure 4-3). For hydrolysis at 5% consistency, both the final glucose content and cellulose-to-glucose conversion rate were relatively low compared with 20FPU cellulase enzyme loading (Figure 4-1). The results indicate that the amount of enzyme applied may not be enough for effective hydrolysis of the UBHW at 5% consistency.

When UBSW was used as the substrate and hydrolyzed at 2% and 5% consistency with lower enzyme loading (Figure 4-4), similar trends as with UBHW were observed. Both the final glucose content and cellulose-to-glucose conversion rate were decreased, even when hydrolyzed at 2% consistency. It seems that UBSW is more resistant to hydrolysis than UBHW, and 5FPU cellulase enzyme loading is not sufficient to hydrolyze UBSW at these consistencies.

A:



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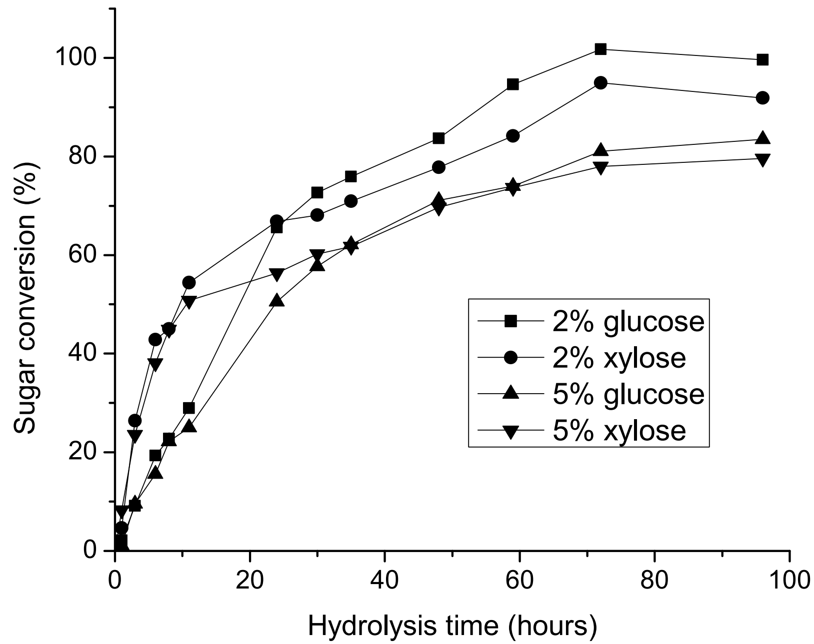
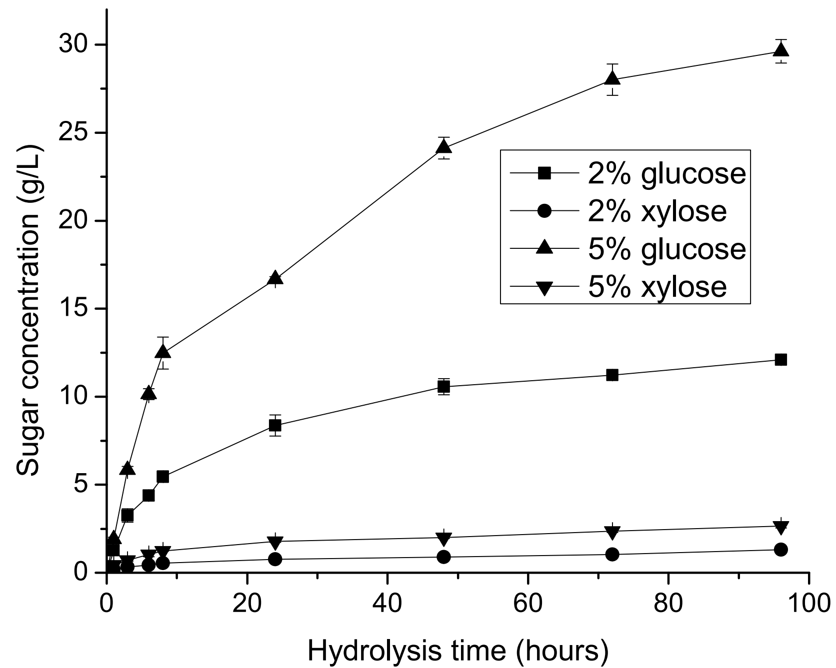


Figure 4-3. Enzymatic hydrolysis of UBHW at 2% and 5% substrate consistencies in shake flasks at 5FPU/20CBU/g of cellulose enzyme loading, based on A) glucose and xylose concentration formed and B) percent sugar conversion.

A:



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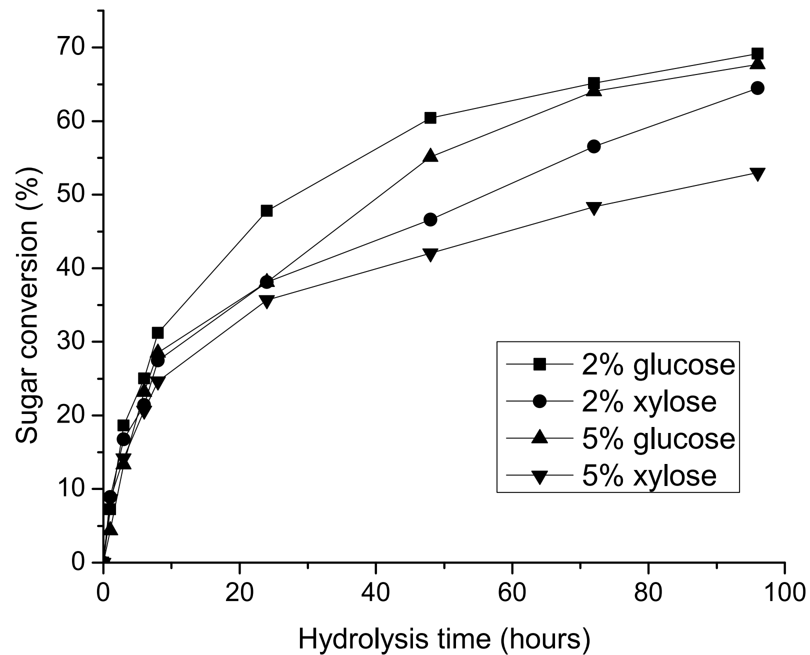


Figure 4-4. Enzymatic hydrolysis of UBSW at 2% and 5% substrate consistencies in shake flasks at 5FPU/20CBU/g of cellulose enzyme loading, based on A) glucose and xylose concentration formed and B) percent sugar conversion.

4.3 High consistency enzymatic hydrolysis of UBHW, UBSW and OPP

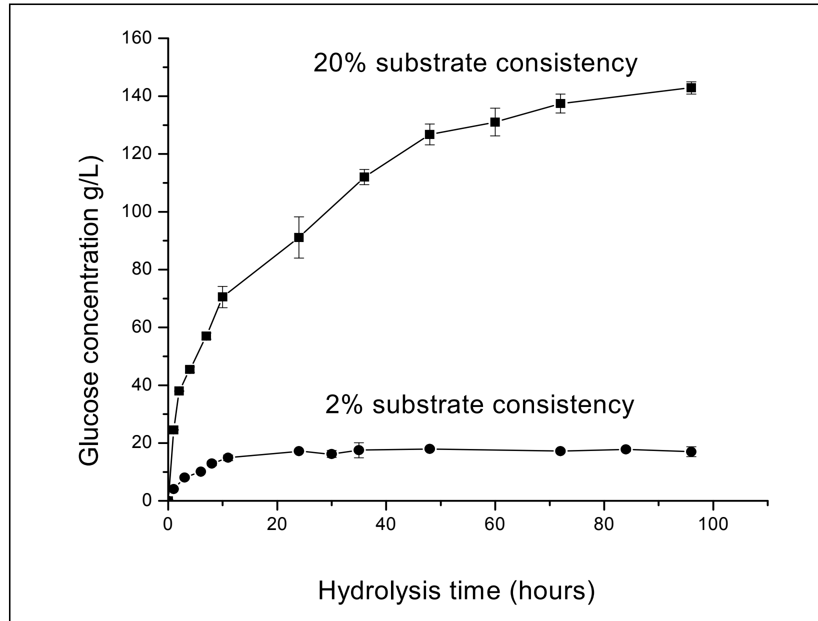
High consistency hydrolysis of lignocellulose is an attractive approach to obtaining a high sugar concentration for fermentation, thus reducing both capital and operating costs in the hydrolysis, fermentation and evaporation/distillation process steps. From a practical perspective, it is a common practice to carried out hydrolysis and fermentation at high solids content (20%w/v and above) in current starch-based fuel ethanol production. The pulp consistency in most modern pulp and papermaking unit operations is typically 10% and above. High consistency bleaching, such as ozone bleaching, has already commercially operated for two decades. The consistency of the pulp can be as high as 40%. There are many mixers for the medium or high consistency bleaching. The hydrolysability of UBHW, UBSW and OPP substrate at 20 % consistency and 20FPU/80CBU/g cellulose enzyme loading were then evaluated in the peg mixer.

4.3.1 High consistency hydrolysis of unbleached hardwood pulp (UBHW)

It was anticipated that a high substrate loading will raise the cellobiose concentration in the hydrolysate which will in turn elevate the end-product inhibition effects on cellulase enzymes (cellobiohydrolases and endoglucanases). Therefore, we chose a higher cellulase and β-glucosidase dosage of 20 FPU cellulase with 80 CBU of β-glucosidase per gram of cellulose. Hydrolysis of UBHW at 2% consistency was also carried out in peg mixer to compare with the results obtained from shake flask experiments. As shown in Figure 4-5A, a significant increase in glucose concentration was obtained during hydrolysis of UBHW at 20% consistency. The glucose content reached 144 g/L after 96 hours of incubation. This is the highest glucose concentration reported from batch hydrolysis of a lignocellulose substrate. Hydrolyzing UBHW at 2% consistency in a peg mixer showed a similar hydrolysis profile

(Figure 4-5) to that obtained from shake-flask experiments (Figure 4-1) with 100% cellulose-to-glucose conversion obtained after 24 h of incubation. However, at 20% substrate consistency, the cellulose-to-glucose conversion rate is reduced to about 85% after 96 h of hydrolysis. Extending the hydrolysis to longer times resulted in little increase in the glucose concentration (data not shown). Typically, cellulase hydrolysis of cellulose follows a two-phase curve, with an initial logarithmic phase and a subsequent asymptotic phase (Ramos *et al*., 1993b). A number of factors contribute to the slower conversion rate in the later hydrolysis phase. Among these factors, end-products such as cellobiose and glucose were shown to play a major role in hindering hydrolysis (Tengborg *et al*., 2001a). It is anticipated that the end-product inhibition effect will become severe at high substrate loading. A previous study has demonstrated that the presence of 100 g/L glucose in the hydrolysate can reduce the efficiency of cellulase hydrolysis by 80% (Xiao *et al*., 2004). The lower conversion rate at 20% consistency compared to 2% is mainly due to the inhibition effects from the high glucose content in the hydrolysate (Xiao *et al.*, 2004).

A:



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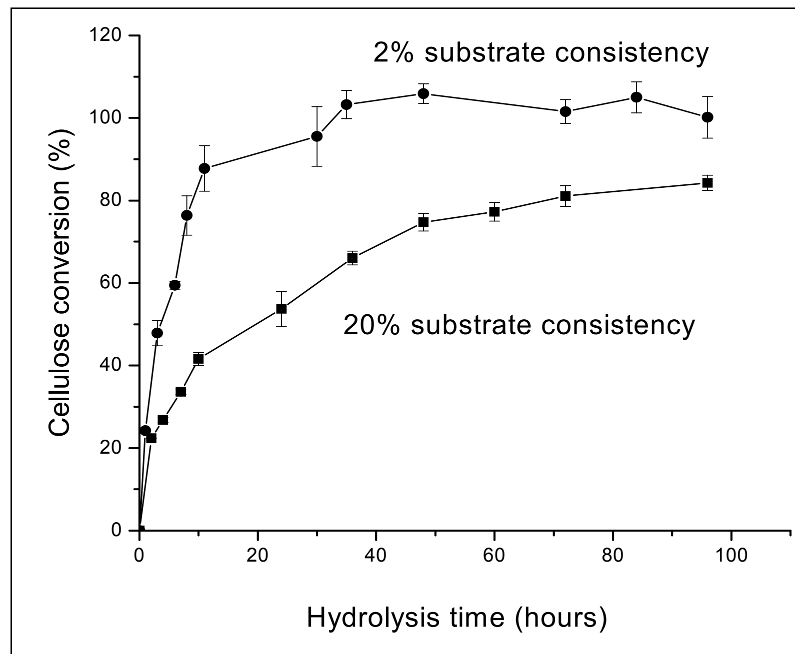

Figure 4-5. Enzymatic hydrolysis of UBHW at 2% and 20% substrate consistencies in a peg mixer, based on A) glucose concentration formed and B) percent cellulose conversion.

As mentioned earlier, the UBHW was used as an "ideal" pretreated wood substrate that has a minimum content of lignin and other contaminants. To test whether this approach can be applied to other substrates, unbleached softwood kraft pulp (UBSW) and an organosolv pretreated poplar (OPP) samples using the pretreatment condition described previously (Pan *et al*. 2006) were also hydrolyzed at the same condition as UBHW. The chemical composition of the organosolv pretreated poplar (OPP) and UBSW are shown in Table 4-1.

4.3.2 High consistency hydrolysis of unbleached softwood kraft pulp (UBSW)

UBSW was then hydrolyzed in a peg mixer at both 2% and 20% substrate consistencies under the same conditions as applied to UBHW. The monosugars contained in the UBSW hydrolysate obtained from enzymatic hydrolysis at 20% consistency mainly included glucose, xylose and mannose, trace amounts of arabinose and galactose. As shown in Figure 4-6, the UBSW is easily hydrolyzed at 2% substrate consistency. The substrate released ~18 g/L of glucose after 24 h enzymatic hydrolysis (Figure 4-6A) which is a complete conversion of all the cellulose to glucose (Figure 4-6B). Hydrolysis of UBSW at 20% substrate consistency also yielded a high glucose concentration. The glucose content reached 140 g/L in the hydrolysate after 96 h of enzymatic hydrolysis, corresponded to cellulose to glucose conversion of about 80%. Compared to hydrolysis UBHW at 20% consistency, UBSW had a lower cellulose-to-glucose conversion rate which may due to the higher lignin content. Maekawa (1996) previously reported that softwood enzymatic hydrolysis is less efficient due to the recalcitrant lignin.

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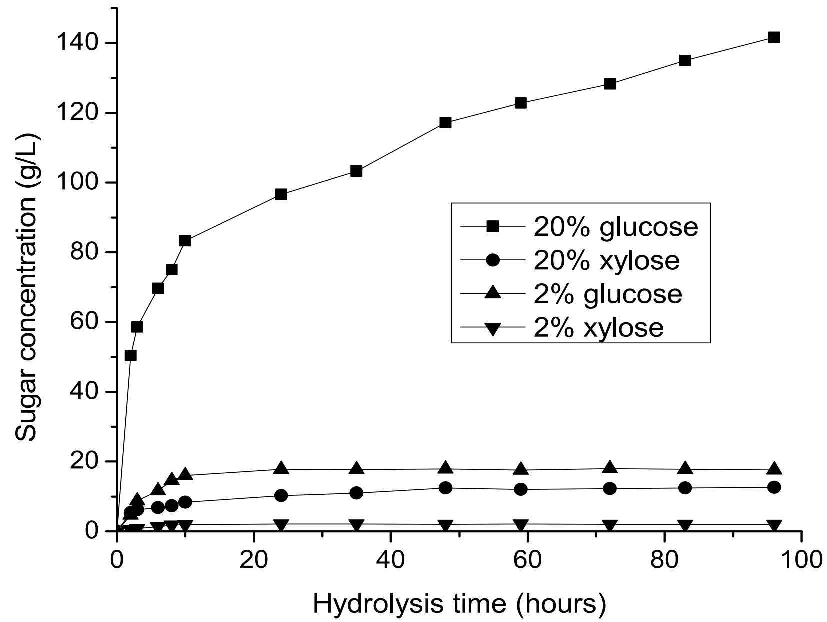

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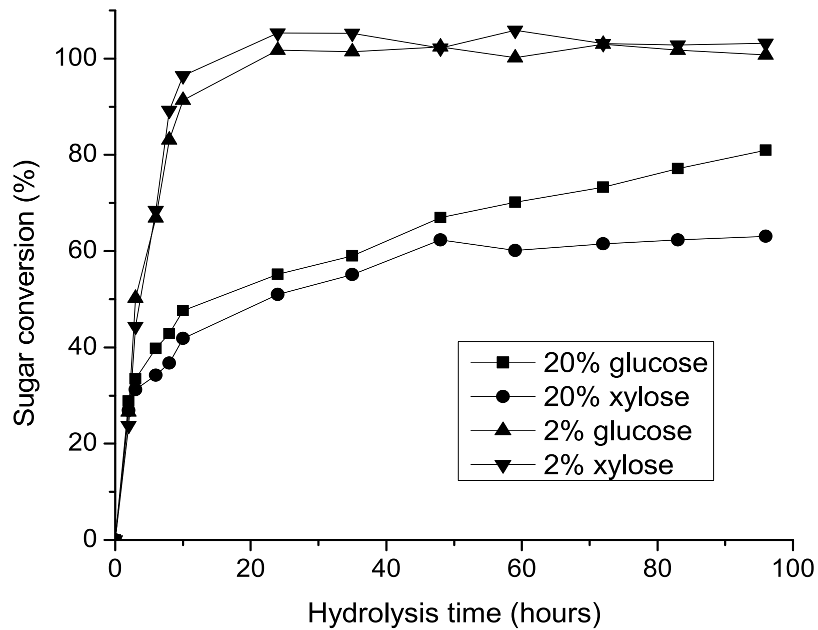
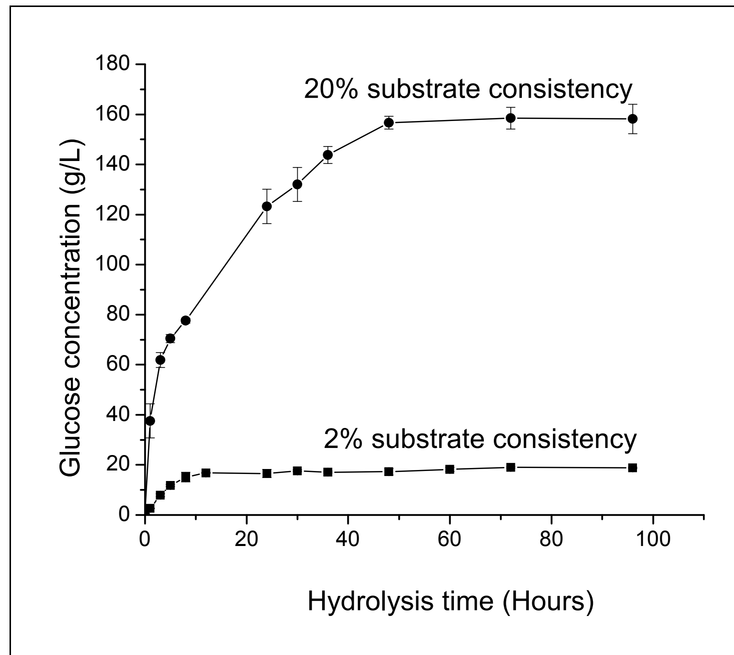


Figure 4-6. Hydrolysis of unbleached softwood kraft pulp (UBSW) at 2% and 20% substrate consistency in a peg mixer, based on A) monosaccharide concentration formed and B) percent sugar conversion.

4.3.3 High consistency hydrolysis of organosolv pretreated hardwood (OPP)

The OPP was also hydrolyzed in a peg mixer at both 2% and 20% substrate consistencies under the same conditions as applied to UBHW and UBSW. As shown in Figure 4-7, the OPP demonstrates a high hydrolysability at 2 % substrate consistency. The substrate released 16.8 g/L of glucose after 12 h enzymatic hydrolysis (Figure 4-7A) which represents 91% of the available cellulose (as glucose) in the OPP (Figure 4-7B). A complete conversion of all the cellulose to glucose was obtained after 60 h of enzymatic hydrolysis. Hydrolysis of OPP at 20% substrate consistency yielded a significantly higher glucose concentration. The glucose content reached 158 g/L in the hydrolysate after 48 h of enzymatic hydrolysis which is even higher than that obtained from UBHW. The amount of glucose released after 48 h of hydrolysis corresponded to a cellulose-to-glucose conversion of about 85%. There seemed to be little increase in sugar concentration after 48 h hydrolysis of OPP at this substrate consistency.

A:



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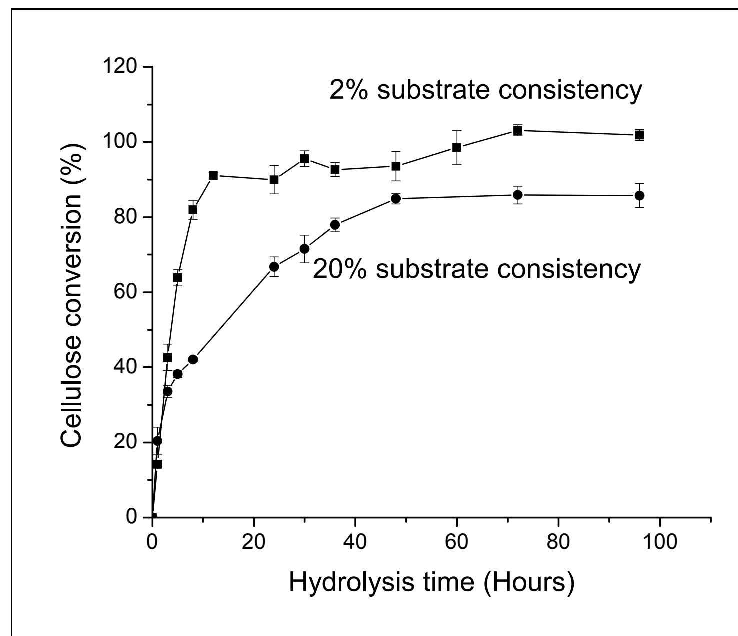


Figure 4-7. Hydrolysis of organosolv pretreated poplar (OPP) at 2% and 20% substrate consistency in a peg mixer, based on A) glucose concentration formed and B) percent cellulose conversion.

4.3.4 The effect of substrate DP on enzymatic hydrolysis

It was surprising to find that OPP demonstrated a better hydrolysability at high consistency than UBHW. The initial high cellulose content likely contributed to the high glucose concentration observed during OPP hydrolysis. Although a similar cellulose-to-glucose yield (84% *vs.* 85%) was obtained after 96 h hydrolysis of UBHW and OPP at high consistency, OPP demonstrated a higher initial reaction rate during the hydrolysis. The initial velocity (Vi) calculated based on the reaction rate obtained during the first hour of hydrolysis of OPP is 0.204 g $g^{-1}h^{-1}$ (gram of glucose produced per gram of cellulose per hour), whereas the Vi obtained from UBHW is 0.146 g $g^{-1}h^{-1}$. In order to understand this difference, we analyzed the CED viscosity and determined the DP (degree of polymerization) of cellulose present in both substrates. It was found that OPP cellulose has an extremely low viscosity (2.67 mPa.s) and DP (207), while UBHW cellulose has a viscosity of 40.3 mPa.s and a DP of 1643. The degree of polymerization (DP) of cellulosic substrates determines the relative abundance of terminal and interior β-glucosidic bonds, substrates for exo-acting and endo-acting enzymes, respectively (Zhang and Lynd, 2004). Exoglucanases act on chain ends, and thus decrease DP only marginally (Kleman-Leyer *et al*., 1992, 1996), while endoglucanases act on interior portions of the chain, leading to a rapid decrease in DP (Kleman-Leyer *et al*., 1992, 1994; Srisodsuk *et al*., 1998) and an increase in chain ends without resulting in appreciable solubilization (Irwin *et al*., 1993). DP represents the fraction of chain end and lower DP would be expected given the greater availability of chain ends, and exoglucanase has been found to have a marked preference for substrates with lower DP (Wood, 1975). OPP was obtained from pulping at high temperature under acidic conditions. Therefore, the pretreatment has significantly degraded the cellulose macromolecules making them

susceptible to cellulase hydrolysis. Our results using UBHW and OPP show that the DP of the substrate affects the hydrolysis rate in agreement with the earlier results of Puri (1984).

4.4 Enzymatic hydrolysis of OPP at 30% consistency

With increasing hydrolysis consistency, the viscosity of a cellulose slurry increases sharply. While in-situ native cellulase systems in wood-degrading microorganisms have been reported to hydrolyze cellulose at insoluble solids concentrations as high as 68-76% (Mandels and Reese, 1965), industrial enzymatic hydrolysis is ultimately limited by processing constraints.

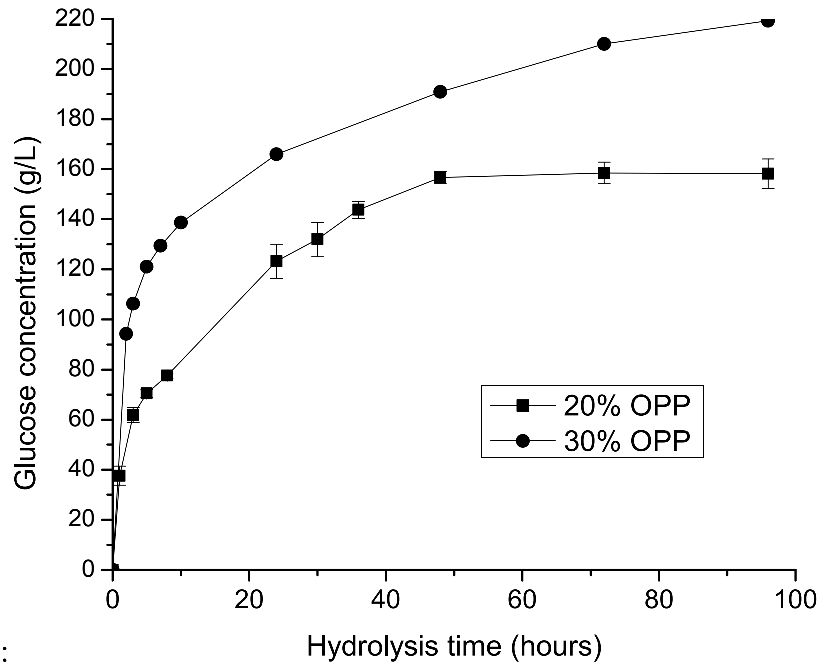
Recently, two studies dealing with the topic of high consistency hydrolysis were published in the literature. One study carried out by Jorgensen and colleagues (Jorgensen *et al*., 2007) employed an in-house chamber to carry out liquefaction of wheat straw at 40% w/v consistency. After the liquefaction the straw slurry was subjected to subsequent saccharification and fermentation in either SHF or SSF configuration. Forty percent substrate consistency is apparently the highest solid loading that has been attempted so far. It should be noted that earlier workers, looking at in-situ native cellulase systems, reported that enzymes could function at solids levels as high as 76% w/v (Mandels and Reese, 1965). However, in a practical fibre processing industry (e.g. pulp and paper industry), a pulp consistency between 20% and 25% w/v is typically encountered. Therefore, we chose to use a 20% w/v substrate consistency to examine hydrolysability in a peg mixer. As shown in Diagram 3-1, the mixing mechanism used in the peg mixer and the chamber designed by Jorgensen *et al* is similar, with both applying a rotating shaft to break down fibre floc and disintegrate the fibre networks. In our study, although a lower substrate loading was used, significantly higher glucose concentrations were obtained from the hydrolysis of the UBHW and OPP substrates,

respectively 144 g/L (or 144 g/kg based on w/w), and 158 g/L (or 158 g/kg) after 96 h. In Jorgensen's study, 86 g/kg and 76 g/kg glucose were produced by hydrolysing wheat straw at 40% and 20% substrate consistency, respectively.

In another recent study (Cara *et al*., 2007), the authors carried out enzymatic hydrolysis of pretreated olive tree biomass at a substrate consistency of up to 30%. The study reported production of 73 g/L of glucose after 72 h hydrolysis of delignified LHW (liquid hot water)-pretreated olive tree biomass. It should be noted that the substrates used in these two studies contain different amounts of cellulose from UBHW and OPP.

Higher consistency enzymatic hydrolysis of OPP was next carried out at 30% consistency under the same conditions as the 20% consistency hydrolysis. The glucose content and conversion rate are shown in Figure 4-8.

A:



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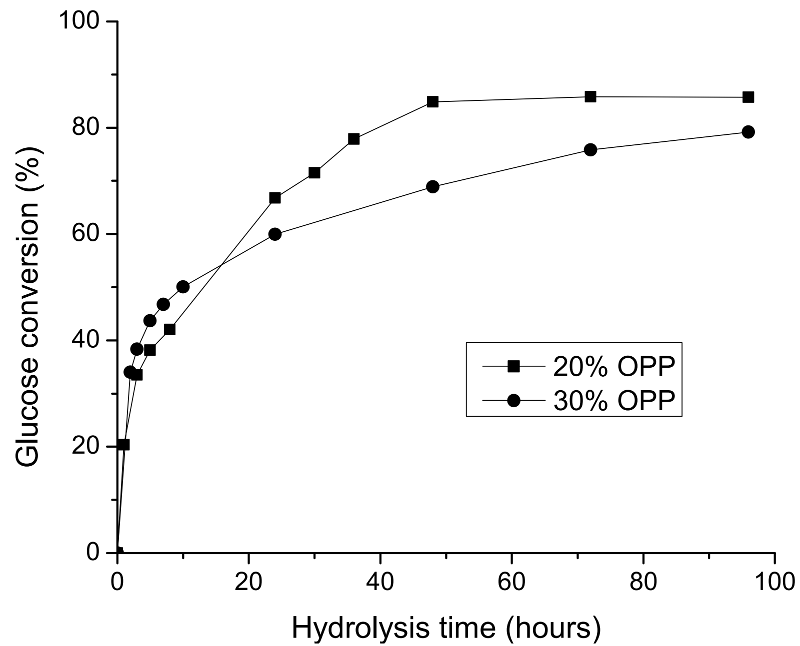

Figure 4-8. Enzymatic hydrolysis of OPP at 30% substrate consistencies in a peg mixer at 20FPU/80CBU/g of cellulose enzyme loading, based on A) glucose concentration formed and B) percent cellulose conversion.

It is apparent that the sugar content of the hydrolysate from OPP at 30% consistency is higher than that obtained from 20% consistency. The amount of glucose reached 220 g/L after 96 h hydrolysis at 30% consistency. A cellulose-to-glucose conversion rate of ~78% conversion rate was achieved after hydrolysing OPP at 30% for 96 h, which was lower than hydrolysis at 20% consistency. The glucose conversion rate decreases as increasing hydrolysis substrate consistency. Some reports have suggested that the mechanism behind the decreasing conversion is product inhibition (Mohagheghi *et al*., 1992; Cara *et al*., 2007; Hodge *et al*., 2008). Others have suggested it may be explained by mass transfer limitations or other effects related to the increased content of insoluble solids, such as non-productive adsorption of enzymes (Rosgaard *et al*., 2007; Sorensen *et al*., 2006). The specific mechanisms responsible for the decreasing hydrolytic efficiency are still unclear. Hydrolysis of OPP substrate at higher hydrolysis consistency required longer hydrolysis reaction time to reach the highest glucose content. The glucose yield started to level off at around 48 h for the hydrolysis at 20% consistency, whereas for the 30% consistency, the glucose yield seemed still to increase after 96 h hydrolysis.

4.5 Fermentation of the hydrolysate obtained from high consistency hydrolysis of UBHW and OPP

The hydrolysates obtained from hydrolysis of UBHW and OPP at 20% consistency represent the highest glucose concentrations obtained to date from batch enzymatic hydrolysis of lignocelluloses. There has been little information on the fermentability of "realistic" hydrolysates with such high sugar concentrations. It can be expected that high substrate loading may lead to an increased amount of potential inhibitors in the hydrolysate. Therefore, it is critical to determine how well yeast will ferment sugars in these hydrolysates.

4.5.1 Fermentation of the hydrolysates obtained from low consistency hydrolysis of UBHW

Firstly, fermentation of hydrolysates obtained from hydrolysis of the 2% and 5% consistency UBHW at 48 h was evaluated. Two glucose solutions were prepared as controls at the sugar concentrations present in 2% and 5% UBHW hydrolysates. The initial glucose concentration of 2% and 5% UBHW hydrolysates prior to fermentation were about 14 g/L and 35 g/L, and the control pure glucose solutions were 14.6 g/L and 37.2 g/L respectively. The glucose consumption and ethanol production were determined during the fermentations. The yeast fermented both hydrolysates and pure glucose solutions well (Figure 4-10). Nearly all sugars of the 2% and 5% hydrolysates and glucose controls were metabolized after 6 h fermentation.

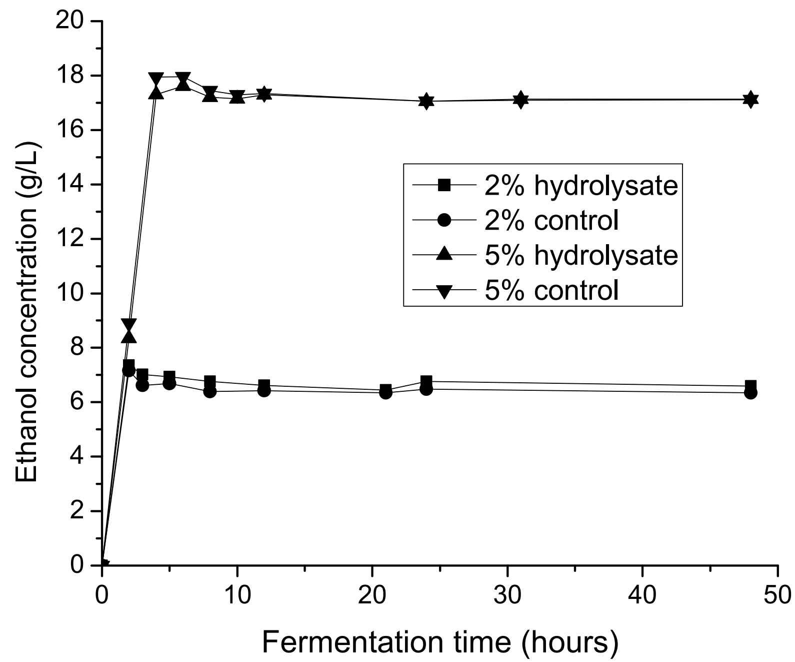

Figure 4-9. The production of ethanol during *Saccharomyces cerevisiae* fermentation of 2% and 5% UBHW hydrolysates.

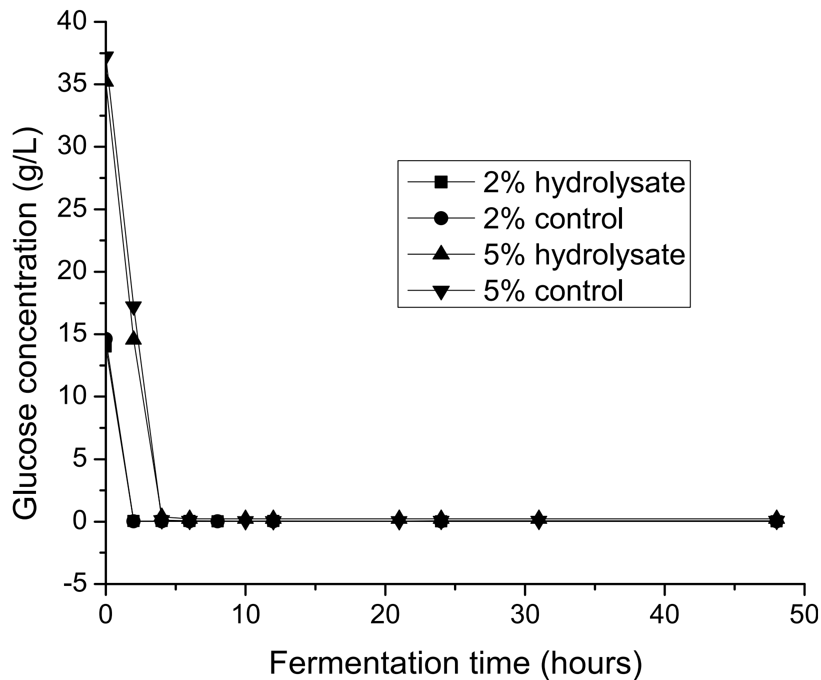

Figure 4-10. The decrease in glucose concentration during fermentation of 2% and 5% UBHW hydrolysates to ethanol by *Saccharomyces cerevisiae*.

The ethanol production was highest at this time and started to level off afterwords (Figure 4-9). For 2% hydrolysate and control, approximately 7 g/L and 7.12 g/L ethanol were produced after 4 h which are near 100% theoretical glucose-to-ethanol conversion yield, (based on a theoretical yield of 0.51 g ethanol / g glucose). For 5% hydrolysate and control, approximately 17.4 g/L and 17.8 g/L ethanol were produced after 6 h, also near 100% of the theoretical glucose-to-ethanol conversion yield. The yeast was able to effectively utilize glucose in UBHW hydrolysate and a high glucose to ethanol yield was obtained.

Comparing the fermentation curves, both pure glucose controls had similar fermentation profiles as the hydrolysates obtained at both 2% and 5% consistency. This indicates that the UBHW hydrolysates obtained at low consistency hydrolysis have no negative inhibition effects on the subsequent fermentation process. Both the final ethanol production and the reaction velocity were not affected during the fermentation.

4.5.2 Fermentation of the hydrolysate obtained from high consistency hydrolysis of UBHW

The hydrolysates obtained from 48 h hydrolysis of the UBHW and OPP substrates at 20% w/v consistency were collected and used for the subsequent fermentation experiments. Two glucose solutions were prepared as controls at the sugar concentrations present in respective UBHW and OPP hydrolysates. The initial glucose concentration of the UBHW hydrolysate prior to fermentation was about 112 g/L and the control pure glucose solution was 110 g/L. The fermentation experiment was carried out for 96 h and the glucose consumption and ethanol production were determined during the fermentation. The yeast showed a high fermentability with pure glucose solution. Nearly all the sugars were metabolized after 12 h fermentation (Figure 4-11). The ethanol production reached approximately 44 g/L at this time and then started to level off (Figure 4-12). The final ethanol concentration (after 96 h) was 48.4 g/L which is about 86% of the theoretical glucose-to-ethanol conversion yield (based on a theoretical yield of 0.51 g ethanol / g glucose). The yeast was also able to effectively utilize glucose in UBHW hydrolysate to produce a significant amount of ethanol. Compared to the glucose control, there was an initial lag phase observed in the glucose decrease and ethanol production during UBHW hydrolysate fermentation. The depletion of glucose occurred after 36 h of fermentation with an ethanol production of 46 g/L at that time. The final ethanol concentration (after 96 h) was 50.4 g/L which is 88% of the theoretical yield.

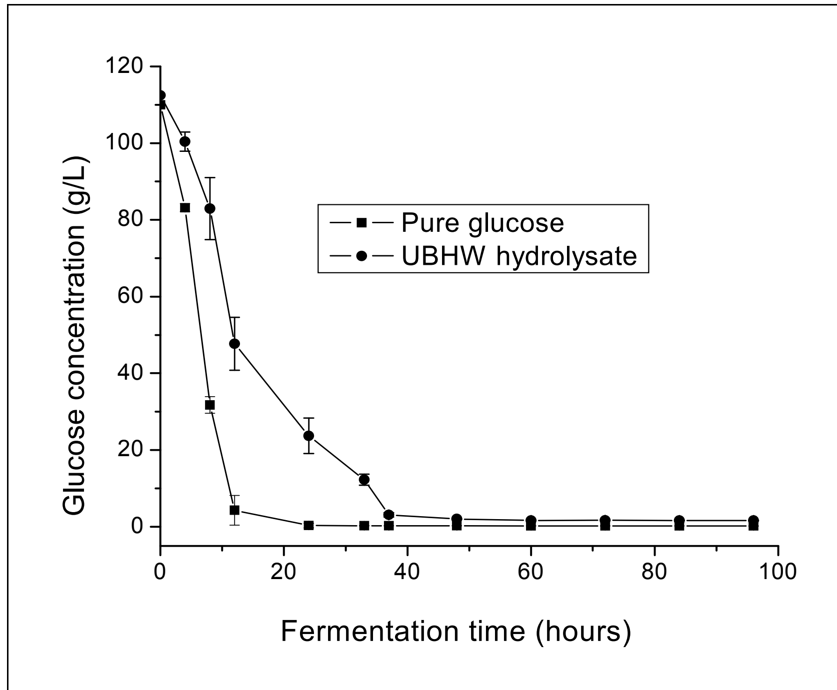

Figure 4-11. The decrease in glucose concentration during fermentation of 20% UBHW hydrolysate to ethanol by *Saccharomyces cerevisiae.*

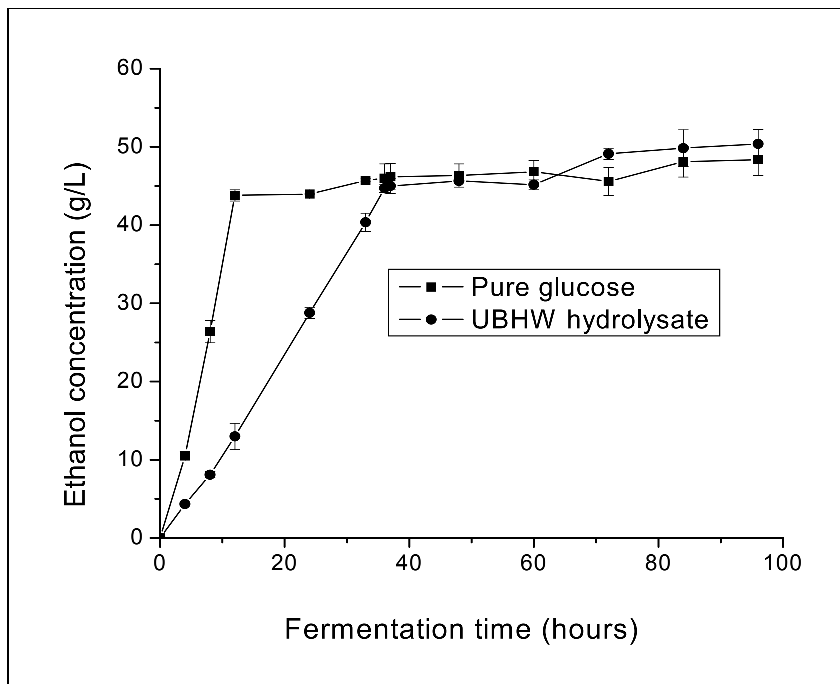

Figure 4-12. The production of ethanol during *Saccharomyces cerevisiae* fermentation of 20% UBHW hydrolysate.

4.5.3 Fermentation of the hydrolysate obtained from high consistency hydrolysis of OPP

The fermentation of the OPP hydrolysate was tested under the same conditions and compared to a control containing 150 g/L of pure glucose. The initial glucose concentration in OPP hydrolysate was about 149 g/L. The yeast again demonstrated a good capability to ferment concentrated glucose solution. Almost all the glucose was used up within the initial 12 h of fermentation, with an ethanol production of nearly 60 g/L (Figure 4-13 and 4-14). A higher final ethanol concentration (after 96 h), 62.3 g/L, was obtained compared to the previous glucose control (Figure 4-14). However the conversion yield was lower, 81% *vs.* 86% of the theoretical yield. Again, an initial lag phase was observed during OPP hydrolysate fermentation compared to the control media with similar glucose content. The maximum ethanol production was achieved after 24 h of fermentation. The final ethanol concentration (after 96 h) from fermenting OPP hydrolysate was 63.1 g/L which is equivalent to 83% of the theoretical yield.

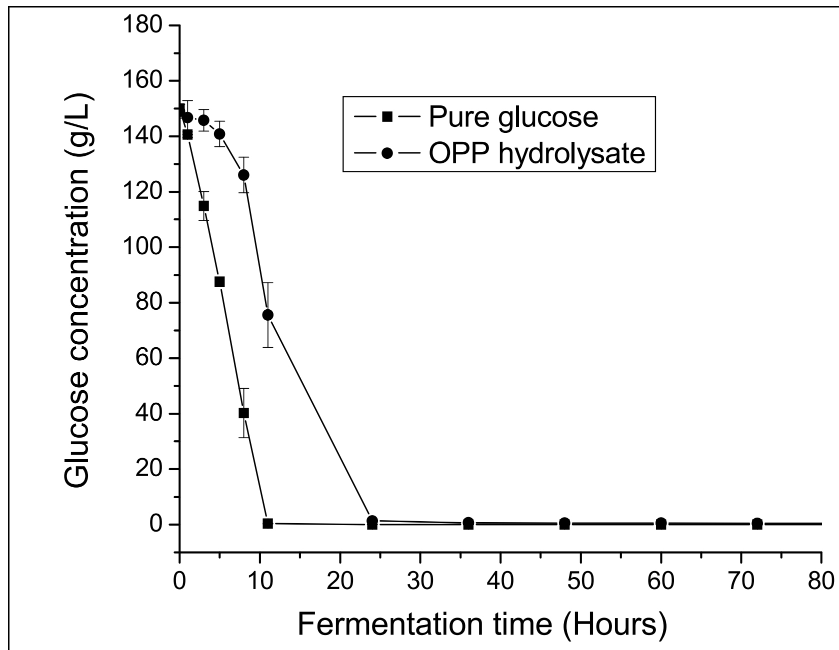

Figure 4-13. The decrease in glucose concentration during fermentation of 20% OPP hydrolysate.

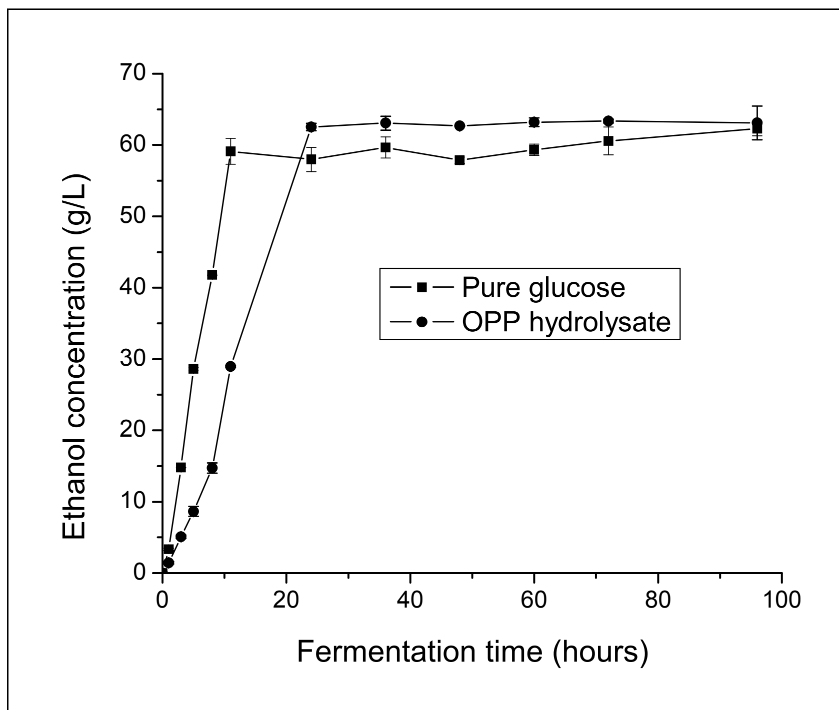

Figure 4-14. The production of ethanol during *Saccharomyces cerevisiae* fermentation of 20% OPP hydrolysate

The final glucose to ethanol conversion yield obtained from UBHW and OPP hydrolysates was slightly higher than their respective glucose controls. Besides glucose, both hydrolysates were found to contain some cellobiose and other cellulose oligomers (data not shown), and these sugars are presumably also degraded during fermentation. Although it is generally accepted that *S. cerevisiae* cannot ferment cellobiose to ethanol, there has been speculation that this industrial yeast may have adapted to convert some oligomers to ethanol. In fact the ethanol concentration obtained from this study is the highest that has ever been reported in the literature from lignocellulose based feedstock. The high consistency hydrolysis and fermentation presents a new approach to lignocellulose hydrolysis and fermentation. This also opens up new opportunities to examine substrate-enzyme interactions which are the subject of our ongoing studies.

4.5.4 The effect of inhibitors in high consistency hydrolysate on yeast fermentation

High consistency hydrolysis not only significantly reduces the capital cost for installation of a hydrolysis vessel, but more importantly it also produces a concentrated glucose stream for the subsequent fermentation. The yeast strain used in this study was a *Saccharomyces cerevisiae* strain adapted to spent sulphite liquor, obtained from an industrial ethanol plant in Eastern Canada. *Saccharomyces cerevisiae* has been shown to tolerate high ethanol concentrations up to 180 g/L (Lin and Tanaka, 2006). Therefore, it was not surprising that this industrial adapted yeast can effectively ferment the two pure glucose controls. The yeast achieved the maximum glucose conversion in the two controls within 12 h. The increase in initial glucose concentration from 110 g/L to 150 g/L lowered the final ethanol yield from 0.44 g/g (gram of ethanol per gram of glucose) to 0.415 g/g due to the high substrate

inhibition (Thatipamala *et al*., 1992). Fermentation of both of the lignocellulosic hydrolysates followed a slower initial rate when compared to the pure glucose controls. This can be attributed to the presence of inhibitory compounds in the hydrolysates.

The amount of potential inhibitors, including acetic acid, phenolic compounds, furfural and hydroxymethylfurfural, were determined. As shown in Table 4-2, there is an appreciable amount of acetic acid and phenolic compounds present in both hydrolysates. No furfural or hydroxymethylfurfural was detected in the hydrolysate prior to the fermentation. Although the OPP hydrolysate appeared to have a higher acetic acid and total phenolic content than that of the UBHW substrate, its fermentability was not affected by these compounds. The maximum ethanol yield was obtained after 24 h fermentation of OPP hydrolysate, while it took 36 h to reach to ethanol production peak in UBHW hydrolysate. Although it is generally accepted (Palmqvist *et al*., 2000; Klinke *et al*., 2004) that weak acids and phenolic compounds can inhibit yeast growth and ethanol production, the concentration effects of these compounds on yeast fermentation is still under debate. For example, the presence of 100 mM acetic acid in the media was shown to increase rather than decrease the ethanol yield from approximately 0.41 g/g to 0.45 g/g by *Saccharomyces cerevisiae* (Larsson *et al*., 1999). Also different types of phenolic compounds exhibit different effects on *S. cerevisiae* fermentation (Palmqvist and Hahn-Hagerdal, 2000). As mentioned earlier, this particular *S. cerevisiae* strain has been adapted to spent sulphite liquor which has a high phenolic and acetic acid content. Therefore, it is not surprising that it can generate a high ethanol yield from sugars in the two hydrolysates which have a relatively low phenolic and acetic acid content compared to a typical spent sulphite liquor.

Table 4-2. The amount of potential inhibitory compounds present in UBHW and OPP hydrolysates.

Concentration (g/L)	UBHW hydrolysate	OPP hydrolysate
Acetic acid content	3.22	6.57
Total phenolic content	2.1	5.2

4.6 The influence of enzyme dosage on high consistency hydrolysis and fermentation

4.6.1 High consistency hydrolysis with different enzyme dosages

Comparing our high consistency hydrolysis with other studies, the specific cellulase loadings on cellulose in Jorgensen's（2007）study, was 7FPU per gram of dry matter added to wheat straw which has a cellulose content of 52%. A high β-glucosidase dosage was supplied (ratio of 5:1 between CBU and FPU) in Jorgensen's study. In another recent study, Cara et al. (2007) carried out enzymatic hydrolysis of pretreated olive tree biomass at a substrate consistency of up to 30%. Cara employed 15FPU cellulase with 15CBU of β-glucosidase per gram of substrate on delignified LHW-pretreated olive tree biomass, which has cellulose content of 56.7 %, therefore the FPU and CBU loadings based on cellulose are approximately 26.5. In our study, a cellulase loading of 20FPU with 80CBU β-glucosidase per gram of cellulose in the substrate was used. This enzyme laoding is in a comparable to those used in the previsous study. The results show that 20FPU/80CBU enzyme loading for the 20% consistency is reasonable as about 84% glucose conversion rate was obtained after 96 h hydrolysis. As the cost of cellulases contribute significantly to the total cost of the

bioconversion process, the cellulase dosage should be minimized as much as possible. To achieve this goal we further investigated the effect of cellulase dosages on the high consistency hydrolysis. Four different enzyme loadings were applied to hydrolyse OPP substrate, while the ratio of cellulase and β-glucosidase was kept at 1:4 (20FPU/80CBU, 10FPU/40CBU, 5FPU/20CBU, and 3FPU/12CBU).

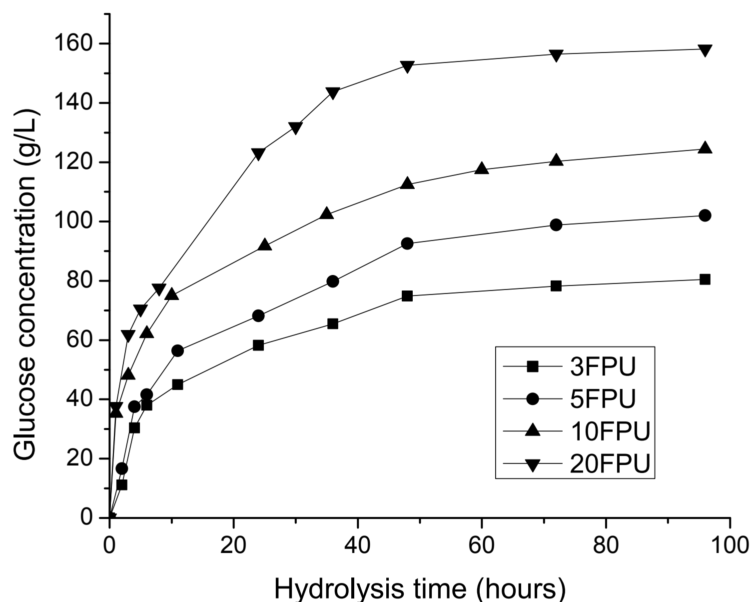

Figure 4-15. Glucose production during hydrolysis of OPP at 20% consistency with different enzyme loadings.

As seen in Figure 4-15 the dosage of cellulase enzyme has a significant effect on the glucose production at 20% consistency hydrolysis of OPP. It is apparent that higher enzyme loading resulted in higher glucose concentration. The glucose content of the 3FPU/12CBU enzyme loading hydrolysate reached about 65 g/L at 48 h, the glucose content of the 10FPU/40CBU and 20FPU/80CBU hydrolysate reached 115 g/L and 150 g/L respectively.

The enzyme loading of 20FPU/80CBU gave the highest glucose content and glucose conversion rate.

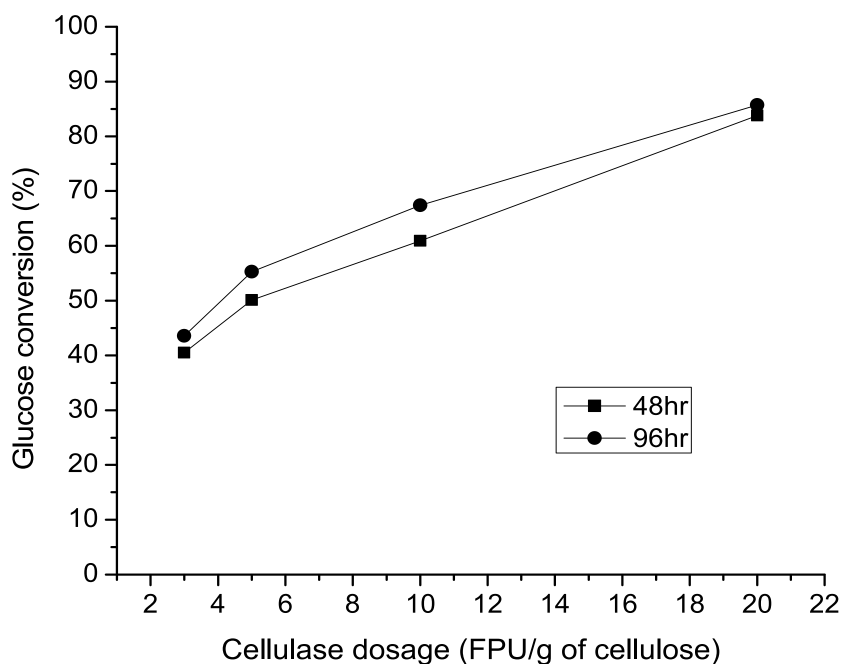

Figure 4-16. Hydrolysis of OPP at 20% consistency with 4 different cellulase loadings after 48 h and 96 h.

Increased enzyme loading also led to an increase in cellulose-to-glucose conversion yield. The glucose yield increased from 40% at 3FPU/12CBU load to 83% at 20FPU/80CBU load. The glucose contents resulting from the different enzyme loadings all started to level off after about 48 h incubation. Further prolonging the hydrolysis time to 96 h increased the glucose content and glucose yield respectively, but by no more than 5%. It is apparent that the glucose yield curves continue to increase, which implies that 20 FPU may not be the sufficient cellulase dosage for the 20% consistency hydrolysis.

4.6.2 Fermentation of the hydrolysates from different cellulase dosages

As the cost of cellulase contributes significantly to the total cost of bioconversion processes, the cellulase dosage should be minimized.

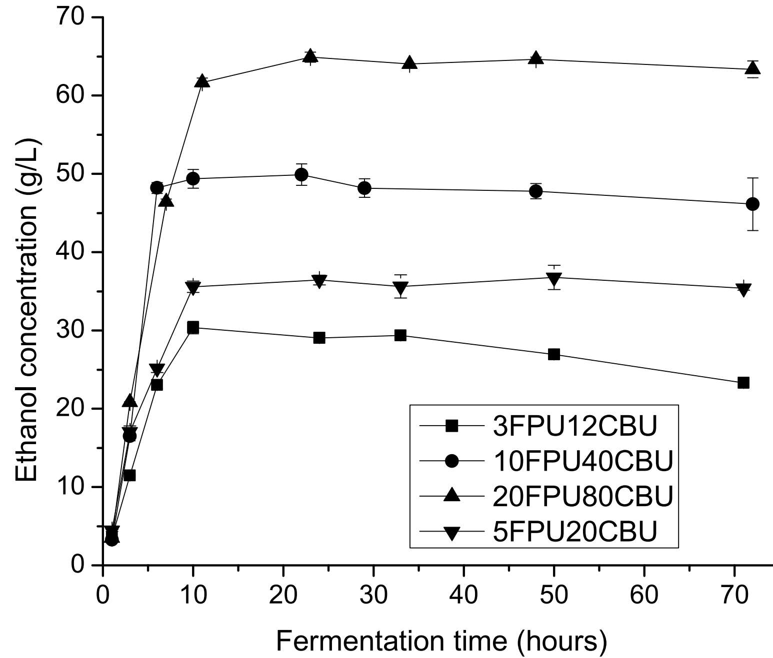

Figure 4-17. Ethanol contents of SHF processes at different enzyme loadings.

Fermentation of the 48 h hydrolysates was performed at four different enzyme loadings. The results shown in Figure 4-17 indicate that the ethanol production shows the same trend as that of the glucose production. That is, the higher the enzyme loading, the higher the ethanol yields. The ethanol content of 20FPU/80CBU loading SHF process reached ~65 g/L after 24 h, equivalent to 82% of the theoretical ethanol yield. Whereas for the 3FPU/12CBU loading process, the ethanol content was only 30 g/L, which is 76% of the theoretical ethanol yield.

Almost all the glucose from the different enzyme loading hydrolysates was consumed during the first 24 h of fermentation. It is interesting that from Table 4-3, the ethanol conversion reached to 89% of the theoretical yield at a 10 FPU enzyme loading.

Table 4-3. Theoretical ethanol yield of SHF process at different enzyme loadings.

Cellulase dosage, FPU	3	5	10	20
Initial glucose content, g/L	74.8	92.5	109.5	156
Ethanol content at 24 h, g/L	29	36.5	49.8	64.9
Ethanol theoretical yield, %	76	77.4	89	82

4.7 Conclusions

In conclusion, we have demonstrated that a peg mixer, commonly employed in pulping processes, can be used for successful high consistency hydrolysis of lignocellulosic substrates. Hydrolysis of unbleached hardwood pulp (UBHW) and organosolv pretreated poplar (OPP) at 20% substrate consistency led to a high glucose concentration in the hydrolysate. Enzymatic hydrolysis of OPP for 48 h resulted in a hydrolysate with 158 g/L of glucose content. This is the highest glucose concentration that has been obtained from enzymatic hydrolysis of lignocellulosic substrate.

Further increasing the hydrolysis consistency to 30%, gave higher glucose content. However, the cellulose-to-glucose conversion rate decreased from 100% at 2% consistency to 78% at 30% consistency which is likely due to increasing end-product inhibition. Moreover, higher hydrolysis consistency required a longer hydrolysis reaction time to reach the highest glucose content.

The dosage of cellulase enzyme has a significant impact on glucose production from high consistency hydrolysis of OPP. The higher the amount of enzyme, the more glucose content in the final hydrolysate. For fermentation of the 48 h hydrolysates obtained from different cellulase enzyme loadings, the higher the enzyme usage, the higher the ethanol yield. Almost all the glucose from the different enzyme loading hydrolysates was consumed during the first 24 h of fermentation.

The UBHW hydrolysates obtained at low consistency had similar fermentation profiles as the pure glucose controls, no negative inhibition effects was found on the subsequent fermentation process. The yeast demonstrated a good fermentability for both the UBHW and OPP hydrolysates. Fermentation of UBHW and OPP hydrolysates with high glucose content led to high ethanol concentrations in the final fermentation broth, much higher than those reported in previous literature. Due to the presence of inhibitory compounds in the high consistency hydrolysates, there was an initial lag phase during UBHW and OPP hydrolysate fermentation compared to the control media with similar glucose content, but the final ethanol production (after 96 h) from fermenting both hydrolysates were not affected. Potential inhibitors included acetic acid and phenolic compounds.

Applying existing pulping equipment designed for high and medium consistency pulp mixing to carry out high consistency hydrolysis provides a practical means to overcome the rheological problems encountered in laboratory shake flask experiments, and brings biomass conversion a step closer to industrial implementation.

CHAPTER 5 HIGH CONSISTENCY SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF LIQUEFIED OPP SUBSTRATES (LSSF)

At high cellulosic substrate loading, due to rheological problems, both HCH and SHF are difficult to practically operate and thus the glucose concentration available for fermentation is limited (Linde *et al*., 2007). The results from the previous chapters show that the peg mixer could be used to resolve many of the technical issues related to mixing during high consistency hydrolysis. We showed that a 20% w/v consistency OPP substrate could be liquefied by cellulases within 1 h, resulting in a high concentration of glucose. However, using a separate hydrolysis process even at high substrate loading, the final cellulose-to-glucose conversion efficiency was still relatively low (only about 75% glucose conversion rate after 48 h) due to enzyme inhibition by the high concentration of hydrolysis products, namely glucose and short cellulose chains.

One way to overcome cellulase end-product inhibition is to ferment the glucose to ethanol in situ. Simultaneous saccharification and fermentation (SSF) combines enzymatic hydrolysis with ethanol fermentation to keep the concentration of glucose low. The accumulation of ethanol in the fermenter inhibits cellulase less than high concentrations of glucose. It was also recognized that SSF is superior to separate hydrolysis and fermentation (SHF) for the product efficiency and cost saving at elevated substrate consistency (Stenberg *et al*., 2000; Soderstrom *et al*., 2005).

As mentioned, liquefaction is the first stage in the high consistency bioconversion process. The objective of this part of the thesis is to develop and examine LSSF (Liquefaction

followed by Simultaneous Saccharification and Fermentation) to convert pretreated biomass to ethanol. The aim is to increase the final ethanol yield and reduce the overall reaction time, thereby decreasing the overall product cost.

In this chapter, the feasibility of treating organosolv pretreated pulp (OPP) using LSSF at high substrate consistency was examined. The influence of β-glucosidase on the LSSF process was also investigated.

5.1 The effect of liquefaction time on the SSF process

Due to the inefficient mass transfer caused by stirring hindrance (shaking in flask), enzyme could not liquefy pretreated 20% consistency corn stover and the poor saccharification rate resulted in low ethanol yields by SSF, averaging only around of 5% (Varga *et al*., 2004). If a high consistency substrate can be liquefied, the viscosity of the slurry will be reduced and which will facilitate the stirring during SSF. However, previous results showed that a 24 h prehydrolysis of barley straw prior to SSF resulted in a lower final ethanol yield compared to the SSF without prehydrolysis at 7.5% consistency (Linde *et al*., 2007). To determine whether the liquefaction time would affect the final SSF ethanol yield, prehydrolysates collected at 2 h, 4 h, 6 h and 9 h after liquefaction with 20FPU/80 CBU/g of cellulose enzyme loading were evaluated. The results are shown in Figure 5-1.

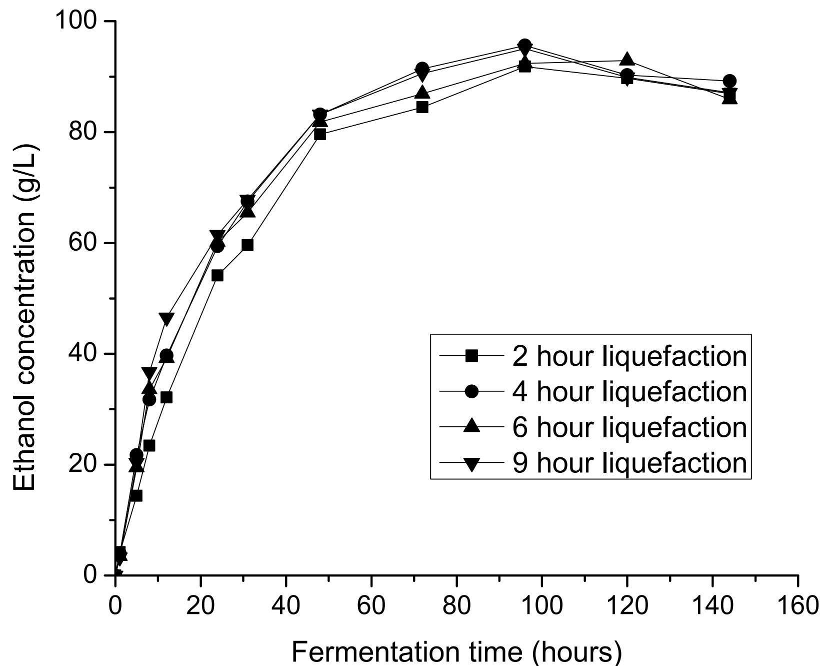

Figure 5-1. The effect of liquefaction time on ethanol yield of SSF process at 20FPU/80CBU enzyme loading.

It was apparent (Figure 5-1) that there was only a small difference between the four liquefied substrates in terms of final ethanol yields. The 2 h liquefied substrate had a slightly lower ethanol concentration than the others, while the 4 h, 6 h, and 9 h liquefied prehydrolysates were almost the same. It seems that as long as the high consistency substrate is liquefied adequately, the liquefaction time has no obvious effect on the final ethanol yield of the LSSF at 20FPU/80CBU enzyme loading. All of the liquefied substrates reached the highest ethanol yield at 96 h incubation time. Thus increasing the liquefaction time further will have no benefit for the subsequent SSF process for ethanol production.

Next the effect of β-glucosidase dosage on the production of ethanol from LSSF was investigated. With 20FPU cellulase loading, the dosage of β-glucosidase was reduced from 80CBU to 20CBU, as shown in Figure 5-2. At lower β-glucosidase supplement, the

prehydrolysate liquefaction time gave a similar fermentation profile as obtained from higher β-glucosidase supplement. Again, the 2 h liquefied prehydrolysate gave the lowest ethanol content, while 4 h, 6 h, and 9 h liquefied prehydrolysates had similar ethanol contents during the time course.

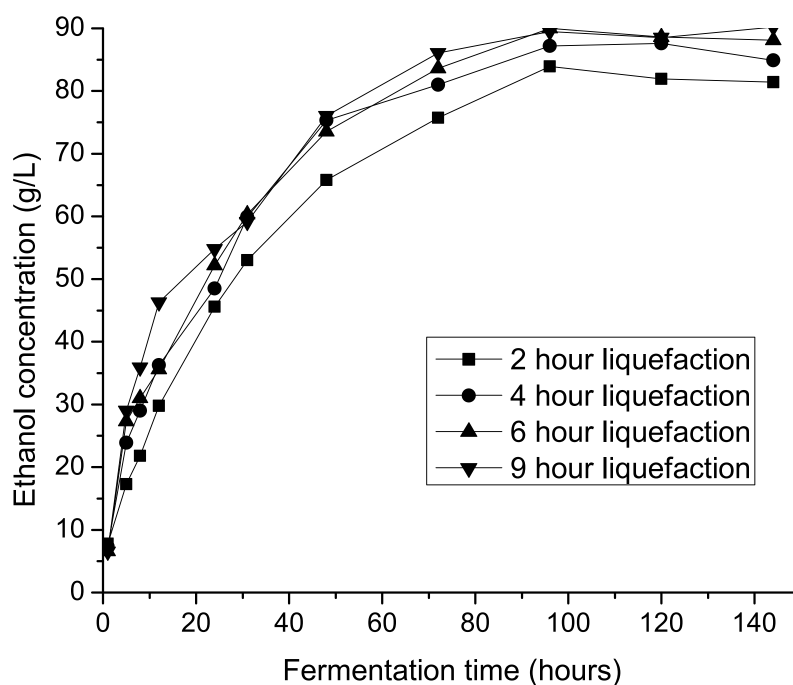

Figure 5-2. The effect of liquefaction time on ethanol yield of SSF process at 20FPU/20CBU enzyme loading.

During SSF, the ethanol production rate is controlled by the cellulase hydrolysis rate not by the glucose fermentation rate. Increasing the cellulase hydrolysis rate will benefit the cost of ethanol production via SSF. Having enough glucose available for fermentation is important. From Figure 3-5 we know that the viscosity of 6 h liquefied substrate is more feasible for industrial operation. Therefore, considering the total conversion time, and for practicality, 6 h was chosen as the time for the liquefaction stage of the LSSF process.

5.2 Influence of β-glucosidase addition on hydrolysis and fermentation

5.2.1 Influence of β-glucosidase addition sequence on HCH

The previous results in Table 3-3 show that adding only 20FPU cellulase without supplemental β-glucosidase is enough to liquefy the solid substrate. Since β-glucosidase is more sensitive to shear forces than cellulase (Gusakov *et al*., 1996; Tengborg *et al*., 2001b), supplementing β-glucosidase after liquefaction of the substrate may favor this enzyme activity during ethanol production. In order to maximum the activity of β-glucosidase, we evaluated the effect of supplemental β-glucosidase prior to and after substrate liquefaction on the ethanol production from LSSF (Figure 5-3).

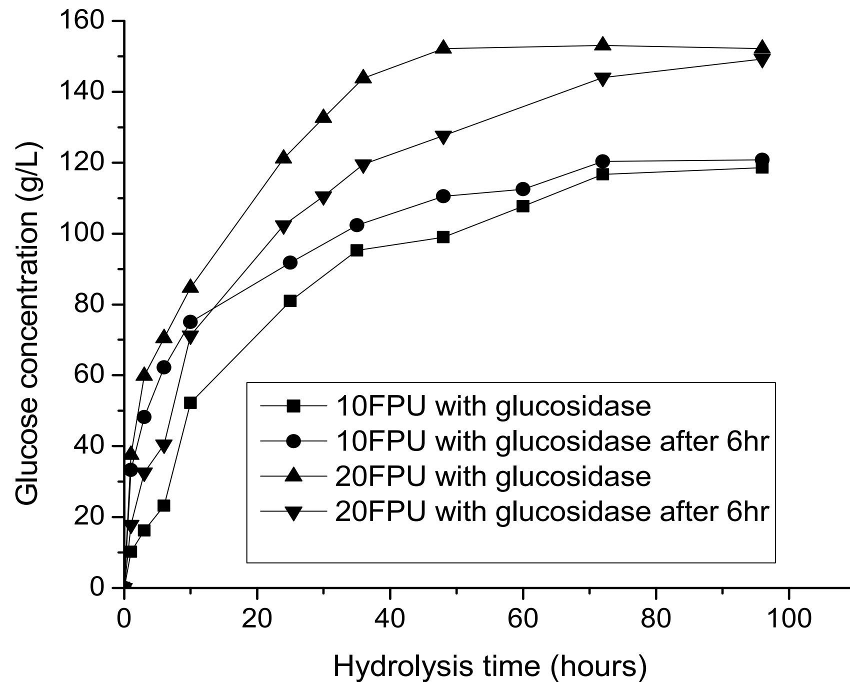

Figure 5-3. Influence of β-glucosidase addition sequence on glucose content.

Figure 5-3 shows that when the β-glucosidase is added with the cellulase at the beginning of the hydrolysis, the glucose content is higher compared to β-glucosidase addition

after 6 h liquefaction. The hydrolysis reaction rate was also higher for both the 10FPU and 20FPU enzyme loading processes. After 48 h, both of the curves where β-glucosidase was added initially with the cellulase started to level off, whereas it took a longer time, 96 h, for the curves where β-glucosidase was added after 6 h liquefaction to reach the same glucose content. After 96 h incubation time, the final glucose content was almost the same for both processes showing that, given enough incubation time, β-glucosidase addition sequence does not affect the final hydrolysis glucose production.

5.2.2 Influence of β-glucosidase addition sequence on fermentation

Two 6 h prehydrolysates, one obtained by hydrolysis of OPP with 10FPU cellulase and 40CBU β-glucosidase, the other by hydrolysis only with 10FPU cellulase without supplementing with β-glucosidase, were used for the subsequent SSF.

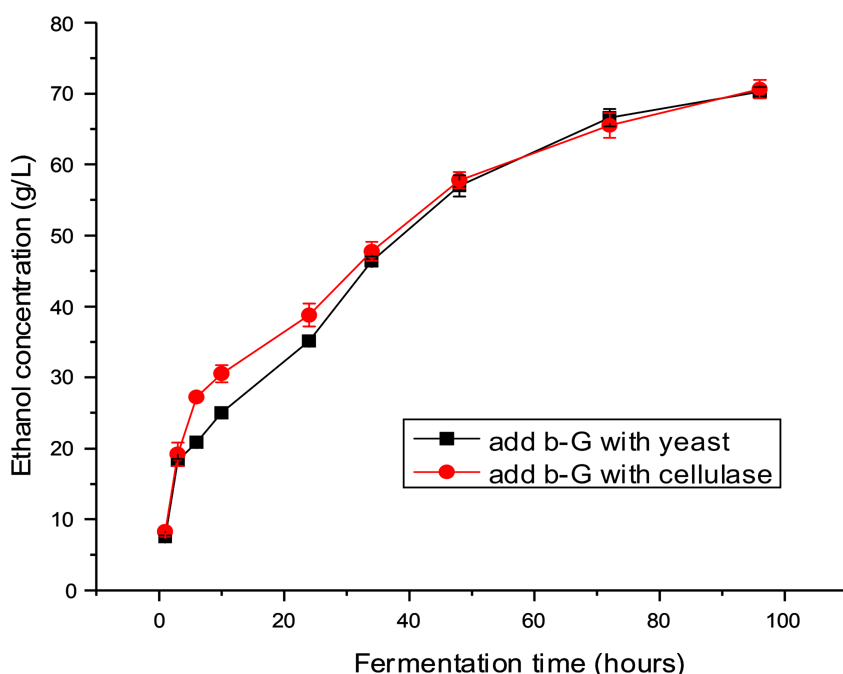

Figure 5-4. Influence of β-glucosidase addition sequence on ethanol production of LSSF.

The results in Figure 5-4 show that the same amount of β-glucosidase added before substrate liquefaction (adding β-glucosidase with cellulase) has a higher ethanol production than when the β-glucosidase was added after liquefaction (adding β-glucosidase with yeast) during the initial 40 h incubation. After 40 h of SSF, the ethanol content in the two processes streams was similar, and the final ethanol content after 96 h incubation was the same. The β-glucosidase addition sequence does not affect the final ethanol production of the LSSF process.

5.2.3 Influence of β-glucosidase dosage on LSSF

The influence of β-glucosidase dosage on LSSF was studied at 10FPU Celluclast loading. After liquefaction at 50°C for 6 h, the hydrolysate was collected and six different β-glucosidase dosages were added with the yeast for the subsequent SSF process. The ethanol content during the fermentation is shown in Figure 5-5.

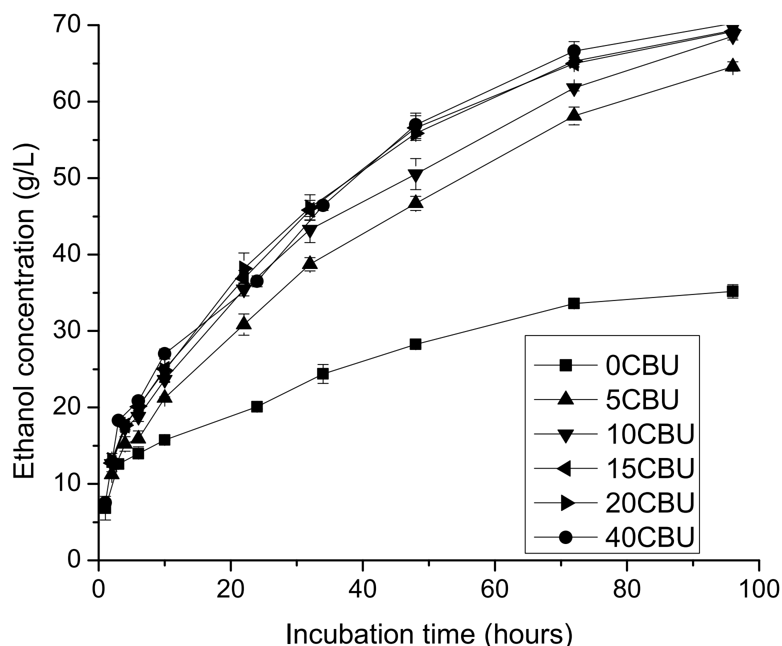

Figure 5-5. The influence of β-glucosidase dosages on ethanol yield by LSSF.

In Figure 5-5 we can see that the effect of β-glucosidase on ethanol yield by the LSSF process is important. Without supplemental β-glucosidase (0CBU) a lower ethanol yield was obtained when compared to β-glucosidase supplementation. Without β-glucosidase, the ethanol content after 96 h is about 35 g/L, while with 5CBU of β-glucosidase, the ethanol production was dramatically increased to about 62 g/L.

Increasing the amount of β-glucosidase increased the content of ethanol (Figure 5-6). For example, increasing the β-glucosidase dosage to 10 CBU, increased the ethanol content to about 66 g/L; further increasing the β-glucosidase dosages gave little increase in ethanol yield. After 96 h incubation times the final ethanol yield of all processes reached ~70 g/L. Increasing the β-glucosidase dosage form 5CBU to 40CBU, only resulted in ~8 g/L ethanol yield increase, compared to the dosage of β-glucosidase increased, the ethanol yield gained was relatively low. Taking final ethanol yield and ethanol yield gain into account, 10CBU of β-glucosidase is probably the optimum supplement dosage (Figure 5-6).

The results from Figure 5-5 and Figure 5-6 demonstrate that the β-glucosidase activity has significant influence on the ethanol yield of LSSF. Previous studies (Spindler *et al.*, 1989b) showed that β-glucosidase supplementation is necessary to achieve efficient cellulose conversion. However, the final ethanol yield is not proportional to the β-glucosidase dosage. When the amount of β-glucosidase is sufficient, further increase has no benefit for the final ethanol production.

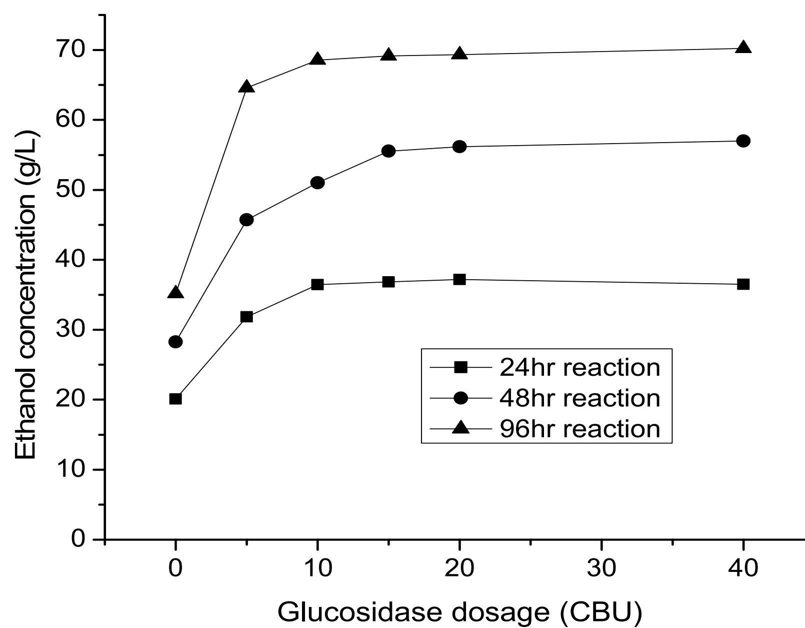

Figure 5-6. Ethanol yield of LSSF processes at different β-glucosidase dosages and different incubation times.

5.3 The influence of enzyme dosages on LSSF

We next investigated the fermentability of the LSSF process at different cellulase enzyme loadings. The hydrolysates obtained from 6 h liquefaction of OPP substrate at 20% consistency and four different enzyme loadings were collected for the subsequent LSSF experiments. The glucose reduction and ethanol production were determined during each LSSF processes (Figure 5-7 and 5-8).

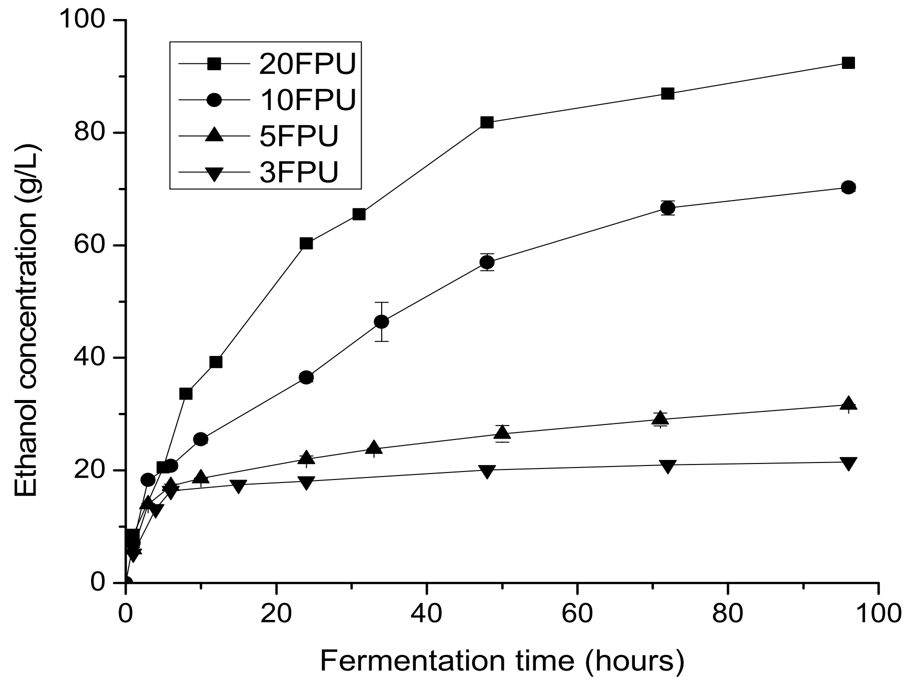

Figure 5-7. Ethanol content at different enzymatic loadings from LSSF processes.

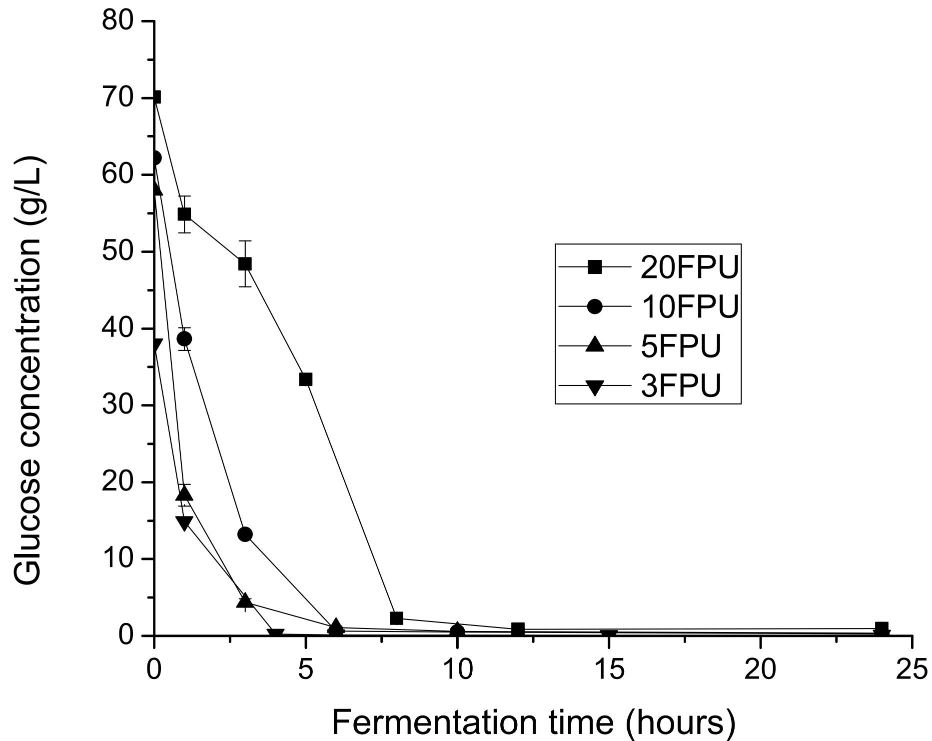

Figure 5-8. Glucose consumption during LSSF at different cellulase loadings.

The amount of cellulase clearly influences the ethanol production during the LSSF processes. The higher the amount of cellulase enzyme used, the more ethanol is produced. For 20 FPU/g cellulose loading, the final ethanol yield reached 90 g/L after 96 h, corresponding to the theoretical ethanol yield of 95%. However for the LSSF process carried out under the same conditions but with lower enzyme loading, 5 FPU/g cellulose, ethanol yield only reached 30 g/L after 96 h reaction, corresponding to theoretical ethanol yield of 37 %. For LSSF processes carried out at 3 FPU/g and 10 FPU/g cellulose, the ethanol theoretical yields were 21% and 69% respectively after 96 h incubation.

Decreasing the enzyme loading will reduce the ethanol production costs, though decreasing enzyme loading obviously decreases the ethanol yield (Linde *et al*., 2006; Chen *et al*., 2007). On the other hand, due to the current high prices of commercial cellulases a reduction of the amount of cellulases added may improve the process economy more than an increase in ethanol productivity. It is important to find a compromise to achieve an economic-technical practically bioconversion process.

Due to the different cellulase and β-glucosidase loadings, the 20% consistency hydrolysates resulted in different glucose contents after 6 h prehydrolysis (Figure 4-15), with the highest enzyme dosage used resulting in the highest glucose yields, 71 g/L.

Yeast was able to ferment efficiently in the LSSF process solution. Nearly all the sugars obtained from the 4 different dosage of enzyme loadings were metabolized after 12 h fermentation (Figure 5-8), including the initial glucose and the glucose produced during the period of fermentation. Corresponding to the glucose consumption, the ethanol content increased quickly, reaching approximately 40 g/L for the 20FPU loading LSSF process.

Between 12 h and 48 h incubation time, the glucose concentration remained low, while the ethanol content increased gradually. For example, 20FPU loading LSSF process,

ethanol content increased from 40 g/L to 80 g/L, indicating that glucose was still produced by the cellulase, and the glucose produced was simultaneously converted to ethanol by the yeast. When the hydrolysis rate slowed, the rate of fermentation also slowed down.

It has been reported (Linde *et al*., 2007; Stenberg *et al*., 2000) that at the beginning of SSF, the glucose concentration increases and a long lag phase in ethanol production is observed at 7.5% and 10% substrate concentration. The duration of the lag phase increased with increasing solid concentration. As we can see from Figure 5-1 to Figure 5-8, there is no lag phase in ethanol production during the LSSF process at as high as 20% solids concentration, at any enzyme loading. It is likely that substances presented in the hydrolysate such as HMF and furfural, are metabolized by the yeast, reducing the ethanol productivity until all the inhibitors had been consumed thus creating a lag in fermentation (Wright *et al*., 1987; Taherzadeh *et al*., 1999). Due to the different substrate employed, no furfural or HMF were detected in the hydrolysate that was used in our study (see table 4-2).

5.4 Comparison of SHF with LSSF at different enzyme dosages

The goal of the SSF study was to overcome end-product inhibition caused by the high glucose content at high substrate loading, thus further improving the final ethanol concentration. The ethanol contents during LSSF and SHF of OPP at different enzyme dosages were determined (Figures 5-9, 5-10)

For the OPP substrate, from Figure 4-15 it was found that the glucose content in the 48 h hydrolysate gave almost the highest glucose content for the 20% consistency hydrolysis at all four different cellulase enzyme dosages. Further prolonging the reaction time has no distinct benefit for increasing glucose. Taking the glucose yield and total reaction time into account, the 48 h hydrolysates were chosen as the substrates for the SHF processes.

For all four SHF processes, almost all the glucose is converted to ethanol after about 60 h reaction times and at the same time ethanol content is the highest and starts to level off. At low enzyme dosages, 3FPU and 5FPU, from Figure 5-9, the ethanol contents obtained from LSSF were lower than that of the SHF processes after 60 h, even after longer incubation time, 100 h. The results indicate that at low enzyme loading, the SHF process has a higher ethanol concentration and shorter incubation time than the LSSF process, so SHF is superior to the LSSF process in terms of ethanol production.

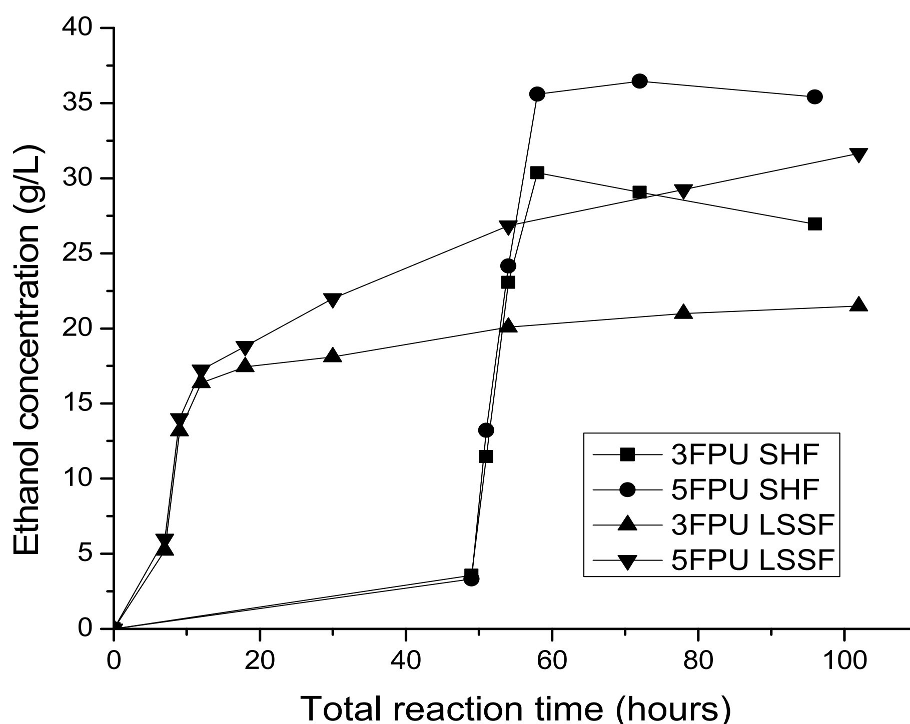

Figure 5-9. Comparison of production of ethanol from LSSF and SHF at 3FPU and 5FPU enzyme dosages. (The time accounted in SHF process included the initial 48 h hydrolysis time)

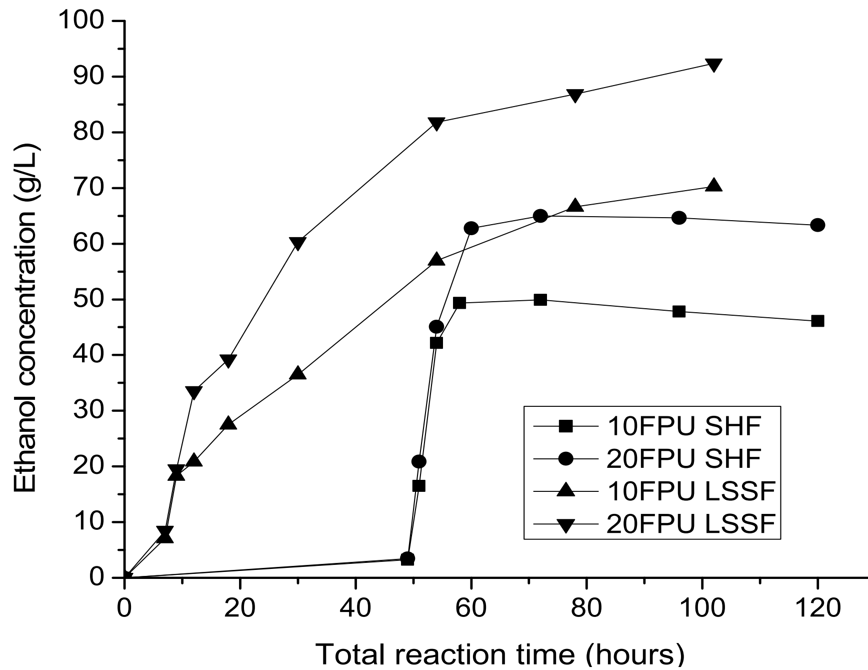

Figure 5-10. Comparison of production of ethanol from LSSF and SHF at 10FPU and 20FPU enzyme dosages. (The time accounted in SHF process included the initial 48 h hydrolysis time)

At higher enzyme loading (Figure 5-10), 10FPU and 20FPU, when SHF processes reached the highest ethanol concentration after 72 h, at this time the ethanol concentration obtained from LSSF processes were higher than for the SHF processes after 54 h. Further prolonging the incubation time, resulted in the ethanol content of LSSF processes increased further. After 102 h incubation, the ethanol content reached 90 g/L and 70 g/L for 20FPU and 10FPU enzyme loading respectively, whereas for the SHF processes at the same enzyme loading, the ethanol contents only reached 65 g/L and 48 g/L respectively after 96 h. The results show that at higher enzyme loading, the ethanol production from LSSF is superior to that of the SHF process.

The highest glucose content of 20% OPP hydrolysis is 158 g/L. Thus for the SHF process, the corresponding highest theoretically yield of ethanol should be no more than 80

g/L, which is less than the ethanol content of LSSF process at 60 h reaction. This shows that LSSF can reduce the end-product inhibition effect and produce higher ethanol content compared with the SHF process.

It is reported that during SSF there is often a lag phase in fermentation due to the change from cultivation to fermenting conditions. The lag phase increases the total time required for SSF and thus increases the production cost (Linde *et al*., 2007). The depletion of glucose occurred after 36 h of fermentation with an ethanol production of 46 g/L at this time. The final ethanol concentration (after 96 h) was 50.4 g/L which is 88% of the theoretical yield. The ethanol production in LSSF is like that of the pure glucose fermentation impling that the inhibition effect was significantly reduced in LSSF process compared to that of the SHF process. This is similar to the results obtained by acid hydrolysis of spruce where a complete fermentation was achieved without any detoxification treatment but was strongly inhibiting in batch fermentation. Adding the substrate at low rate in fed-batch fermentation keeps the concentrations of bioconvertible inhibitors in the fermentor low, and the inhibiting effect therefore decreases (Taherzadeh *et al*., 1999; Palmqvist *et al*., 2000).

5.5 Low enzyme high substrate loading for batch and fed-batch simultaneous saccharification and fermentation of OPP

Traditionally, "fed-batch" saccharification is used to increase the cumulative insoluble substrate level during hydrolysis to overcome reactor mixing (rheological problem), and achieve higher consistency hydrolysis (Hodge *et al*., 2009; Varga *et al*., 2004; Fan *et al*., 2003). By using a Peg mixer, all the problems encountered previously could be effectively avoided. The specific aim of the fed-batch loading used in this study was to increase the final ethanol productivity and the whole LSSF process efficiency.

During high consistency hydrolysis, the volume of substrate decreased dramatically, with only one-third of the original volume remaining after the substrate was liquefied. To optimize the capacity of the reactor vessel, in this part, the applicability of a "fed-batch" strategy, that is, sequential loading of substrate plus enzymes during enzymatic hydrolysis was evaluated.

5.5.1 Single-batch and fed-batch low enzyme high substrate loading hydrolysis

Single-batch and fed-batch hydrolysis were performed at 20% consistency and 3FPU/12CBU/g cellulose loading with OPP substrate. For the fed-batch experiment, substrate was added in three batches over six hours. The results are shown in Figure 5-11.

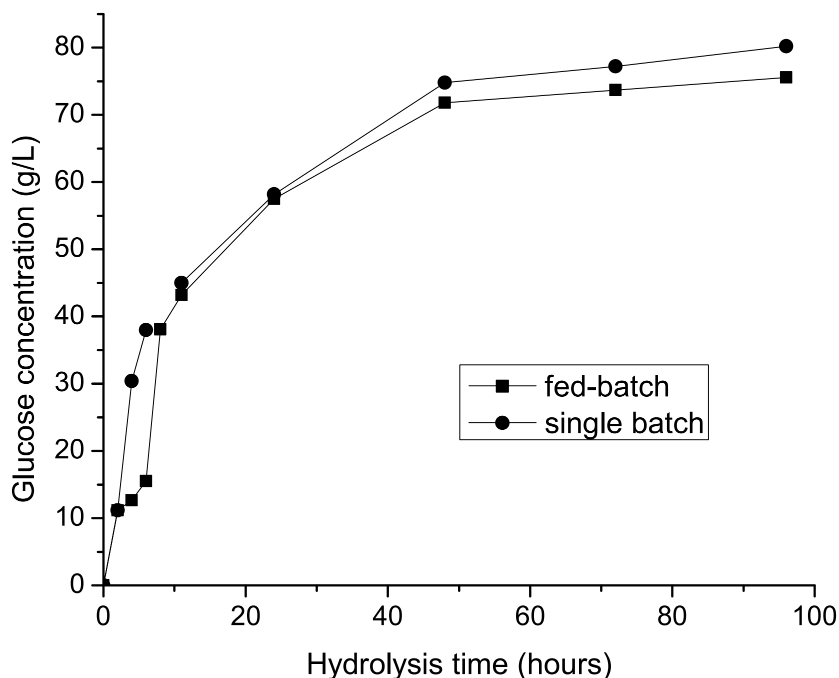

Figure 5-11. Single-batch and fed-batch hydrolysis at low enzyme high substrate loading.

For the fed-batch process, during the period from 2 to 6 h when extra substrate was added, the hydrolysis efficiency decreased temporarily. The final glucose content reached about 72 g/L after 48 h reaction and started to level off. The single-batch process showed a similar trend and reached 74 g/L glucose content after the same reaction time. The final glucose content after 48 h reaction is similar for the two processes, so the efficiency of the fed-batch hydrolysis is the same as the single-batch hydrolysis process. Comparing to single batch, fed-batch involved in the amount of substrates equal to three times of single batch, but these two kinds of process reached both the same highest glucose concentration at the same time. It means that when a given amount of glucose need to be produced, the fed-batch process may shorten the required reaction time three times than the single batch process, therefore enhanced the productivity greatly compared with batch hydrolysis.

5.5.2 Fermentibility of the single-batch and fed-batch hydrolysate

Single-batch 6 h hydrolysate and fed-batch 8 h hydrolysate gave similar glucose contents, and were selected for the subsequent SSF process.

The results in Figure 5-12 and Figure 5-13 indicated that the hydrolysates from single-batch and fed-batch loading have the same fermentability. The ethanol production and glucose consumption during the SSF process have similar profile; the final ethanol yield after 72 h reaction is around 20 g/L for both sequences.

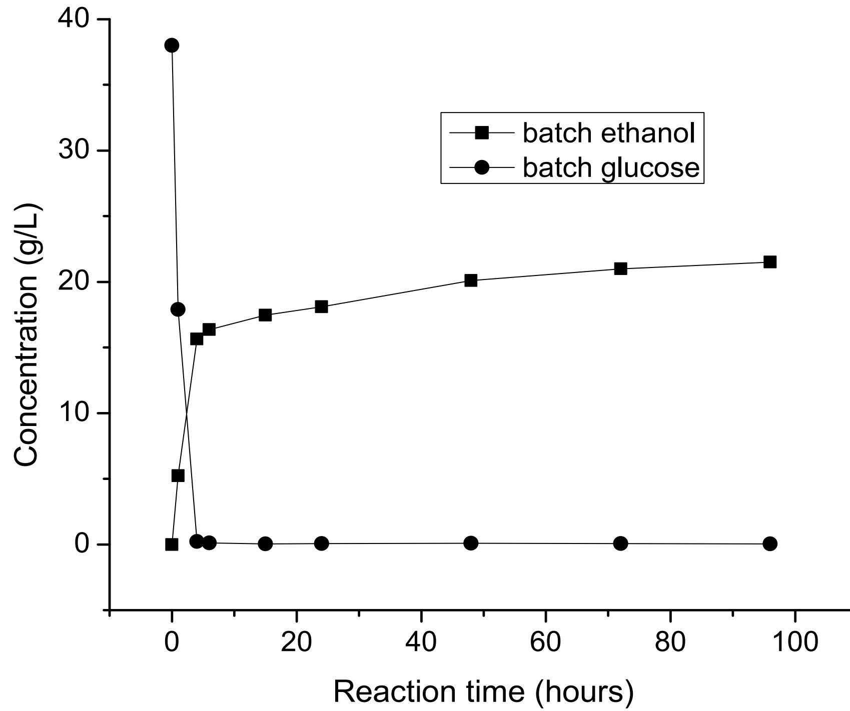

Figure 5-12. Fermentation of hydrolysate from the single-batch hydrolysis.

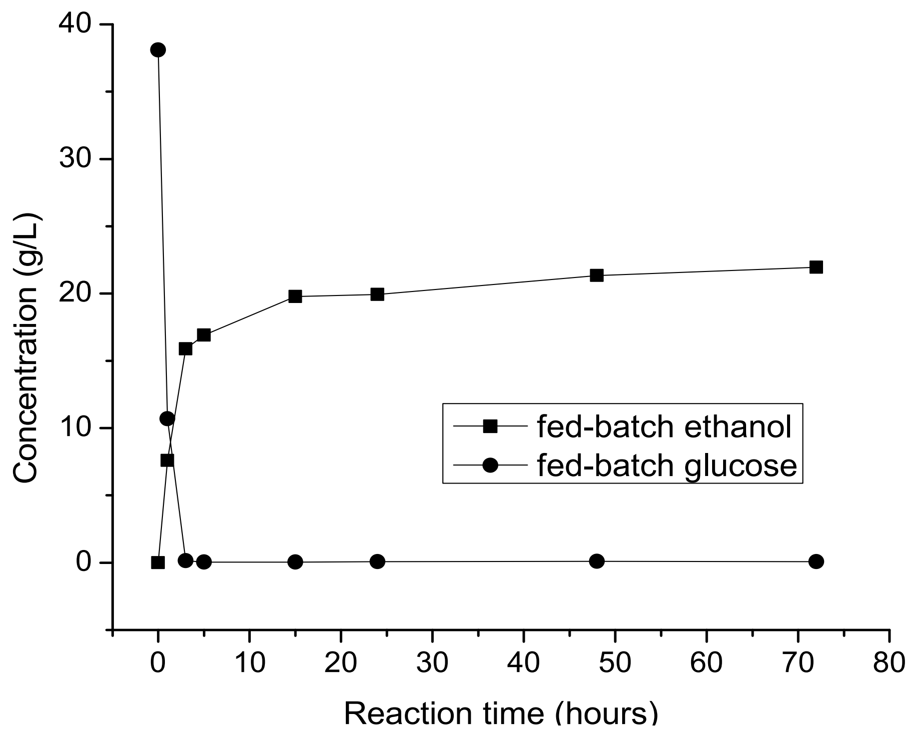

Figure 5-13. Fermentation of hydrolysate from fed-batch hydrolysis.

5.5.3 Fed-batch SHF and LSSF processes

In order to increase the ethanol productivity further, clearly the rate of enzymatic hydrolysis has to be increased. Due to the current high price of commercial cellulase preparations, addition of more enzymes is not an attractive option. Alternatively, the rate of hydrolysis can be accelerated by raising the temperature. An increase from 37°C to 50°C can result in a 29% increase in enzymatic activity (Rudolf A., *et al*. 2005). An option which may combine the advantages of SSF and SHF could be to run a short hydrolysis at elevated temperature and when the hydrolysis becomes end product inhibited, switch to SSF by adding yeast and lowering the temperature. Three different prehydrolysates with hydrolysis at higher temperature for 8 h, 24 h and 48 h were selected and compared for fermentability by the SSF processes (Figure 5-14).

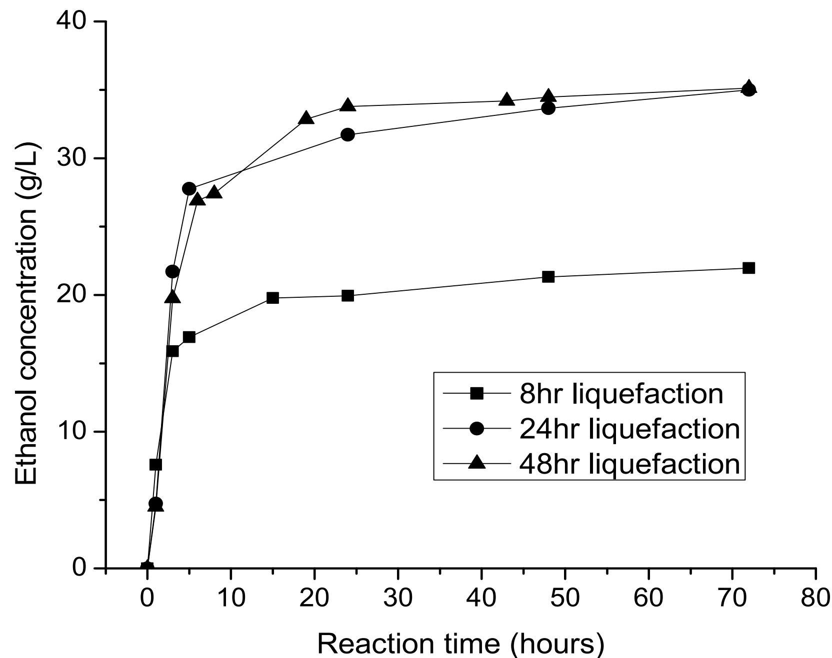

Figure 5-14. Fed-batch LSSF at different liquefaction time.

When the liquefaction time increased from 8 h to 24 h, which means of hydrolyzing was carried out at 50°C for a longer time, the ethanol yield increased from 20 g/L to about 33 g/L and started to level off after 24 h. The ethanol yield obtained from the SHF process with a 48 h prehydrolysate was around 34 g/L after 24 h incubation. It seems that at low enzyme loading, sufficient prehydrolysis time is important. Optimizing the prehydrolysis or the liquefaction time not only increases the final ethanol yield but also shortens the overall process time.

5.6 Conclusions

Supplementing β-glucosidase prior to or after substrate liquefaction does not affect the final hydrolysis glucose production and ethanol production obtained from the LSSF process, if given enough incubation time.

The β-glucosidase activity is necessary to achieve efficient ethanol production from LSSF. However, the final ethanol yield is not proportional to the β-glucosidase dosage. When the amount of β-glucosidase reaches a certain dosage, further increasing has no benefit for the final ethanol production. Taking final ethanol yield and ethanol yield gain into account, 20CBU β-glucosidase supplement is enough for the 20% consistency OPP LSSF.

Cellulase enzyme dosage has a different influence on SHF and LSSF processes. At low enzyme dosage (5FPU or below), the SHF process has a higher ethanol concentration and shorter incubation time than the LSSF process, so SHF is superior to the LSSF process in terms of ethanol production. At higher enzyme loading (10FPU or higher), the ethanol production from LSSF is superior to that of the SHF process, and LSSF can reduce the end-product inhibition compared with SHF.

No major differences in performance between batch and fed-batch hydrolysis and subsequent SSF process were observed. For degradation of equivalent substrates, fed-batch loading during hydrolysis (combining three batches hydrolysis) shortened the reaction time, and therefore enhanced the productivity greatly compared with batch hydrolysis without decreasing the final glucose yield. It seemed that at low enzyme and high substrate loading, optimizing the prehydrolysis or the liquefaction time not only can increase the final ethanol yield but also shortens the overall process time.

CHAPTER 6 CONCLUSIONS AND PROPOSED FUTURE WORK

6.1 Conclusions

It becomes apparent from my thesis study that effective hydrolysis of lignocellulosic substrtates can be acheieved by using existing equipment employed in pulping processes, such as a peg mixer. Overcoming rheological and mixing problems associated with high consistency fibrous matrix is the key to obtain an effective high consistency hydrolysis of unbleached hardwood pulp (UBHW), unbleached softwood pulp (UBSW), and organosolv pretreated poplar (OPP). Hydrolysis at 20% substrate consistency in a peg mixer led to very high glucose concentrations in the hydrolysates. For example, enzymatic hydrolysis of the OPP substrate for 48 h resulted in a hydrolysate with a glucose concentration of 158 g/L. A review of the literature indicated that this is among the highest glucose concentrations that have been reported from the enzymatic hydrolysis of lignocellulosic substrate. Further increase in substrate consistency to 30% w/v led to an even higher glucose content. The cellulose-to-glucose conversion rate decreases along with the increase in substrate consistencies, e.g. from 100% at 2% consistency to 78% at 30% consistency. This is likely due to the elevated end-product inhibition effect caused by increasing in the sugar concentration.

The hydrolysates obtained from high consistency hydrolysis have a similar fermentation profile to the pure glucose controls, no major negative inhibition effect was found during fermentation process. Fermentation of UBHW and OPP hydrolysates with high

glucose content led to high ethanol concentrations in the final fermentation broth. Although, there was an initial lag phase during UBHW and OPP hydrolysate fermentation compared to the control media with similar glucose content, the final ethanol production (after 96 h) from fermenting both hydrolysates were not affected.

It is conceivable that liquefaction is a process to depolymerize cellulose and thus reduce substrate viscosity. It was found from my thesis study that both endo-glucanase and exo-glucanse are essential for reducing the viscosity of the ligncellulosic matrix at high solid loadings, whereas β-glucosidase has little effect on changing the rheological properties of the substrate matrix during the initial liquefaction stage. It is therefore recommended to add the β-glucosidase after the liquefaction stage which will likely help preserve its activity.

It is evident that a high β-glucosidase acitivity is crucial for achieving high cellulose-to-glucsoe conversion yield. High β-glucosidase will inevitably lead to significant increase in hydrolysis cost. Although enzyme recycling is one way of reducing enzyme dosage, my thesis study examined the approach of using SSF to minimize enzyme loading for ethanol production.

The final glucose or ethanol yield is not affected by the initial β-glucosidase addition either. When the amount of β-glucosidase reaches a certain dosage, further increasing has no benefit for the final ethanol production. Taking final sugar and ethanol yield into account, 10 CBU/g β-glucosidase additions is sufficient for the 20% consistency OPP LSSF. Supplemental β-glucosidase prior to and after substrate liquefaction does not affect the final hydrolysis glucose production and ethanol production from LSSF process if given enough incubation time.

Cellulase enzyme dosage had a significant impact on both glucose production and subsequent SHF and LSSF processes. The higher enzyme dosage lead to higher glucose

content in the final hydrolysate. For fermentation of the 48 h hydrolysates obtained at different cellulase enzyme loadings, the higher the enzyme usage, the higher the ethanol yield. Almost all the glucose from the different enzyme loading hydrolysates was consumed during the first 24h of fermentation. At a low enzyme dosage (5FPU or below), the SHF process has a higher ethanol concentration and shorter incubation time than the LSSF process, so the SHF was superior to the LSSF process in terms of ethanol production. At higher enzyme loadings (10FPU or higher), the ethanol production from the LSSF substrate was superior to that of the SHF process. The LSSF process could reduce end-product inhibition when compared to the SHF process.

No major difference in performance between batch and fed-batch hydrolysis and subsequent SSF process was observed. For degradation of equivalent substrates, fed-batch loading hydrolysis (combined three same batch hydrolysis) shortened the reaction time, and therefore enhanced the productivity greatly compared with batch hydrolysis without decreasing the final glucose yield. It seems that at low enzyme and high substrate loading, optimizing the prehydrolysis or the liquefaction time not only can increase the final ethanol yield but also shortens the overall process time.

Applying existing pulping equipment designed for high and medium consistency pulp mixing to carry out high consistency hydrolysis provided a practical means to overcome the rheological problems encountered in laboratory shake flask experiments. The results provided realistic data for further practical operations that could bring biomass conversion a step closer to industrial implementation.

6.2 Future work

Inhibitory effect on the high consistency hydrolysis and ethanol production

It was observed that the hydrolysis efficiency decreased with increasing substrate consistency, which partly decreased the advantage of running at high consistency. In order to facilitate high glucose conversion at high consistency hydrolysis, a better understanding of the mechanism involved in high product inhibition (glucose and cellobiose) should be further investigated.

The substrates used in this study were well washed, with only trace amounts of acetic acid and phenolic compounds, produced during the hydrolysis, detected in the prehydrolysate. Thus little inhibition on ethanol production was observed. Typical prehydrolysates normally contain inhibitors such as HMF, furfural, acetic acid and phenolic compounds, etc. With high substrate loadings, the inhibitor concentration derived from the pretreatment could also be higher. Therefore further investigation of the inhibition behaviour during the SHF or SSF process is required.

Fermentation process integration

The most important factor for the economic outcome of a wood-to-ethanol process is the overall ethanol yield. As a consequence it is important to maximize the overall sugar conversion to ethanol. The UBHW and UBSW substrates contain a significant amount of hemicellulose, especially pentose sugars. The yeast used in this study, *Saccharomyces cerevisiae*, is unable to ferment pentose. To achieve the commercialization of biomass bioconversion, it is also important to improve the final ethanol yield by utilizing pentoses to reduce the overall cost. Consequently, obtaining ethanol from pentoses (of which xylose is the

major component) is particularly important especially when they are present in relatively high amounts.

Choosing a more consolidated fermentation process, such as the simultaneous saccharification and cofermentation (SSCF) or consolidated bioprocessing (CBP) approaches in which the cellulose hydrolysis and fermentation of both cellulose and hemicellulose hydrolysis products is performed could also enhance the overall process.

Improve the accessibility of the substrate

The hydrolysability of the different substrates, UBHW, UBSW and OPP, is different due to the different pulping methods used which results in substrates with different chemical composition and structures. Substrate accessibility and degree of adsorption of cellulase are factors limiting the final glucose yield. Pretreatment to improve fiber swelling is one way to achieve this goal.

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