

**Bioconversion of Pulp and Paper Mills Sludge and Prehydrolysate Stream into  
Ethanol and Cellulase Enzyme**

by

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## Abstract

In the first part of this dissertation, two different types of paper mill sludges, primary sludge and recycle sludge, were evaluated as a feedstock for bioconversion to ethanol. The sludges were first subjected to enzymatic conversion to sugars by commercial cellulase enzymes. The enzymatic conversion was inefficient because of interference by ash present in the sludges with the enzymatic reaction. The main cause was that the pH level is dictated by the  $\text{CaCO}_3$  in ash, which was two units higher than the pH optimum of cellulase. To alleviate this problem, simultaneous saccharification and co-fermentation (SSCF) using cellulase (Spezyme CP) and recombinant *Escherichia coli* (ATCC-55124), and simultaneous saccharification and fermentation (SSF) using cellulase and *Saccharomyces cerevisiae* (ATCC-200062) were applied to the sludges without any pretreatment. Ethanol yields of 75-81% of the theoretical maximum were obtained from the SSCF on the basis of total carbohydrates. The yield from the SSF was also in the range of 74-80% on the basis of glucan. The SSCF and SSF proceeded under stable condition with the pH staying near 5.5, close to the optimum for cellulase. Decrease of pH occurred due to carbonic acid and other organic acids formed during fermentation. The ash was partially neutralized by the acids produced from the SSCF and SSF and acted as a buffer to stabilize the pH during fermentation. When the SSF and SSCF were operated in fed-batch mode, the ethanol concentration in the broth increased from 25.5 and 32.6 g/L to 42 and 45 g/L, respectively. The ethanol concentration was

limited by the tolerance of the microorganism in case of SSCF. The ethanol yield in fed-batch operation dropped to 68% for SSCF and 70% for SSF.

The second part of this dissertation dealt with the de-ashing process of paper sludges. In the first part, it was demonstrated that the paper sludges with the high ash content were efficiently converted to ethanol in both SSCF and SSF. However, high ash content in the sludge, however, limited the solid loading in the bioreactor causing low product concentration. De-ashing of sludges before SSCF and SSF was attempted to overcome this difficulty. Bioconversion of the low ash content sludges gave an ethanol yield of 80% with cellulase loading of 15 FPU/g-glucan. With lowering the cellulase loading to 10 FPU/g-glucan, the ethanol yield was reduced to 70%. High solids loading in SSF and SSCF decreased the ethanol yield. High agitation and de-ashing of the sludges make up part of the yield loss caused by high solids loading. In addition, substitution of the laboratory fermentation medium (peptone and yeast extract) with corn steep liquor did not bring about any adverse effects in the fermentation. When the SSCF and SSF were operated in fed-batch mode using low-ash content sludges, the ethanol concentration in the broth was increased to 48 and 60 g/L, respectively.

In the third part of the dissertation, production of cellulase from unbleached hardwood pulp and kraft paper sludge was investigated. Cellulase on-site production is a supplementary unit in a kraft paper mill in parallel with or in place of bioethanol. Using cellulase and *Saccharomyces cerevisiae* on kraft paper sludge, which is waste from the kraft pulp making process, it is possible to produce bioethanol. It is feasible and convenient to integrate these two processes without any impact on the output of paper goods and on the production of bioethanol. To explore this potential, the cellulase

enzyme was produced by *Trichoderma reesei* Rut C-30 and was investigated for its characteristics and titers. Experiments were conducted at different conditions to determine the impact of operational factors upon *T. reesei* Rut C-30 growth and cellulase production. Different concentrations of unbleached hardwood pulp were examined to determine the maximum levels of cellulase activity. The highest titer of 7.5 FPU/mL was obtained with use of 4% (w/v) of unbleached kraft processed hardwood pulp. Paper sludge was also considered as a potential feedstock for cellulase production. However, it has a high ash content which is detrimental to cell growth. It is desirable to remove ash as much as possible from the sludge, while retaining carbohydrates. In this study, the de-ashed sludge pre-processed via physical and chemical treatment was used as substrates for cellulase production and ethanol fermentation. The cellulase enzyme produced from de-ashed sludge exhibited cellulase activity as high as 8 FPU/mL. The properties of the crude cellulase were determined and compared with a commercially available cellulase, Spezyme CP, using hardwood pulp and acid-treated corn stover as the substrates. The gross cellulolytic activities and other properties the enzymes produced in this work from the unbleached hardwood pulp and kraft paper sludge were comparable with those from the commercial enzyme, Spezyme CP. The in-house cellulase, however, exhibited higher xylanase activity than Spezyme CP.

In the last part of the dissertation, ethanol production from hemicellulose prehydrolysate was investigated. Most of the hemicellulose fraction of pulp mill feedstock (softwood or hardwood) is released into black liquor during the pulping process. The black liquor is combusted to recover chemicals and to generate steam and electricity. It is feasible to recover this fraction of carbohydrate and enhance its value by

converting it into value-added products. Hemicellulose is selectively converted to soluble sugars (termed as prehydrolysate) by treating it with hot water. The sugars produced from pre-hydrolysis process are mixtures of pentose, hexose, and their oligomers. In this study, pectinase and *Saccharomyces cerevisiae* were used to convert the prehydrolysate into ethanol. The prehydrolysate produced from wood also contains toxins, primarily lignin and sugar degradation products, which strongly inhibit microbial reaction. De-toxification of the prehydrolysates was done by overliming (addition of excess CaO). When hydrolysate is obtained by treating wood, the total sugar concentration is below 4 wt. %. Consequently, when the hydrolysate is used as a fermentation substrate, the ethanol concentration is less than 2 wt. %, which is far below the level acceptable as the distillation feed. Use of the mixture of prehydrolysate and pulp mill sludges as the fermentation feed, however, can increase the product concentration. In bioconversion of sludge, a certain amount of water is added to attain fluidity required for SSF operation. In this study, prehydrolysate, in place of water, was added into the bioreactor along with the sludge. Using this procedure, there was a net increase of total sugar concentration in the bioreactor above that of the base case, which led to an increase in product concentration. The experimental data detailing the proposed bioprocess conversion of the mixed-feed to ethanol are presented in this paper.

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## **I. Introduction**

Increasing energy demand, escalating oil prices, concerns about greenhouse gas emissions from fossil fuels and energy security have led to great interest in the production of fuels from renewable materials. Ethanol has been an economically feasible fuel for transportation for several decades. Currently, ethanol is mainly produced from any feedstock that contains natural sugars or starch that can be readily converted to sugar, such as corn in the United States. However, it is recognized that the corn ethanol cannot meet the requirement without causing a major disruption of the food markets (McElroy, 2006).

Cellulosic ethanol offers promise as cellulose comes from inexpensive and abundant lignocellulosic biomass (Inderwildi and King, 2009). In addition, cellulose does not compete with food related agricultural products. It is estimated that 323 million tons of cellulosic raw materials are thrown away each year in the U.S. alone that could be used to produce ethanol. This includes 36.8 million dry tons of urban wood waste, 90.5 million dry tons of primary mill residues, 45 million dry tons of forest residues, and 150.7 million dry tons of corn stover & wheat straw (Energy Information Administration, 2007).

The increasing need of energy and the dire financial performance of the U.S. pulp and paper industries bring up the possibility of wood based-biorefinery which would look to use all components of wood to make pulp, transportation fuels, power, and chemicals. From the pulp and paper making process, there are three potential opportunities, paper sludge including kraft paper mill sludge and waste news print, hemicellulose, and black

liquor. The pulp and paper industry produces about 4.1 to 5 million dry tons per year of solid waste in U. S., consisting primarily of de-watered sludge, which contains fibers and other materials produced during paper-making or paper recycling (Fan and Lynd, 2006b). Paper sludges are normally disposed by incineration or landfill, which cause substantial financial burden and various environmental problems. A better alternative to landfilling and incineration is to utilize paper sludge in a biorefinery. Sludge contains much more cellulose and much less hemicellulose and lignin than corn stover. Moreover, the short fibers in the sludge are easily digested by cellulase enzymes (Fan and Lynd, 2006a; Jeffries et al., 1999; Fan et al., 2003). These features can potentially make bioconversion of sludges possible without pretreatment. As one of the major processing steps in bioethanol plants, pretreatment is a significant cost factor, second only to feedstock cost which accounts for about 30% of the total production cost of ethanol (Galbe and Zacchi, 2002). Simultaneous saccharification and fermentation (SSF) using cellulase (Spezyme CP) and *Saccharomyces cerevisiae* (ATCC-200062), and simultaneous saccharification and co-fermentation (SSCF) using cellulase and recombinant *Escherichia coli* (ATCC-55124) were separately applied to the sludges without any pretreatment.

Bioconversion of kraft paper sludges with high ash content can be done by both fermentation processes. However, the operation of sludges with high ash content is difficult. Some degree of de-ashing before SSCF and SSF is therefore necessary. Several methods have been applied to recover the fiber and fillers from paper sludge (Leuthold and Leuthold, 1996; Maxham, 1994; Simpson and Lam, 1998), which facilitate the reuse of fiber or fillers instead of the sludge as a whole. The equipment used to separate fiber or

fillers from sludge may be as conventional as screens and cleaners (Dorica and Simandl, 1995).

The high cost from the usage of enzymes in the hydrolysis step is one of the major drawbacks in the production of bioethanol (Steele et al., 2005; Spano et al., 1978). The cost of bioethanol and cellulase production could be reduced when immediate processing is minimized, without compromising the quality of the final product. Cellulase production using substrates directly from the kraft mill could be feasible, convenient, and economically viable. Besides the sulfite wood pulp (Watson et al., 1984), some waste items such as pretreated wood (Juhasz et al., 2005; Shin et al., 2000), sawdust (Lo et al., 2005), waste paper (Royer and Nakas, 1987; Chen and Wayman, 1991; Shin et al., 2000), waste cardboard (Szijártó et al., 2004) and paper sludge (Royer and Nakas, 1987; Maheshwari et al., 1994) could be used as a carbon source for cellulase production.

In kraft pulping, about 80% of the hemicellulose in the wood is dissolved in the black liquor (Biermann, 1996; Sjostrom, 1993). It is technically difficult to separate this hemicellulose part from alkaline black liquor before it is burned to generate heat and power (Sjostrom, 1993). It is technically feasible to insert a new process in front of the digester to extract hemicellulose and convert to ethanol through a fermentation process. Hemicelluloses can be removed from lignocellulosic materials by hot water, alkaline, and solvent extractive processes (Willfor and Holmbom, 2004; Song et al., 2008; Sears et al., 1971; Nguyen et al., 1998; Aziz and Sarkanen, 1989). Through additional processes, ethanol can be produced from hemicellulose.

The overall objective of this study is to investigate the technical feasibility of establishing a wood-based biorefinery to be integrated with existing kraft paper mills.

The following specific objectives were set for this study.

- To investigate the performance of the kraft paper mill sludge using cellulase and *Saccharomyces cerevisiae* (ATCC-200062), or recombinant *Escherichia coli* (ATCC-55124).
- To develop a process for de-ashing of paper mill sludge.
- To develop methods for efficient cellulase production from unbleached hardwood pulp and paper sludge.
- To improve the fermentation yield of prehydrolysate by investigating the factors affecting (inhibiting) the fermentation step.

## II. Literature Review

### 2.1 Background-Bioethanol

Since 2003, the price of the fossil fuels has been increasing and peaked at \$147.30 July 2008 ([http://www.usatoday.com/money/economy/2008-07-11-3815204975\\_x.htm](http://www.usatoday.com/money/economy/2008-07-11-3815204975_x.htm)). As countries develop, industry, rapid urbanization, and higher living standards drive up energy use. Thriving economies such as China and India are quickly becoming large oil consumers. Demand for oil is outstripping the supply of oil and oil depletion is expected to cause crude oil prices to go up over the next 50 years. The days of inexpensive fossil energy are gone, and we have to turn to new sources of energy, such as, solar, tidal, wind, hydro, nuclear and so on. In addition, the dependency on oil has two major drawbacks: burning fossil fuels may contribute to global warming; and for net-consuming countries like the United States, importing oil creates a dependency on oil-producing countries. Therefore, shifting society's dependence away from fossil fuel to alternative renewable biomass fuel has attracted great interest. Ethanol is a renewable resource, can be produced, unlike petroleum which cannot be produced and in time will be gone. Because it is easy to manufacture and process, it is rapidly becoming a common alternative to gasoline in some parts of the world, such as in Brazil.

Ethanol can be mass-produced by fermentation of sugar or by hydration of ethylene ( $\text{CH}_2=\text{CH}_2$ ) from petroleum and other sources. Currently ethanol is being produced mainly from starch or sugar in a wide variety of crops, but there has been considerable debate about "Food vs. fuel". The years 2007–2008 saw a dramatic increase

in world food prices, creating a global crisis and causing political and economical instability and social unrest in both developing and developed nations. Systemic causes for the worldwide increases in food prices continue to be a subject of debate. The increasing use of biofuels in developed countries is thought to be one of the main causes. Recent developments with cellulosic ethanol production and commercialization may allay some of these concerns. Cellulosic ethanol is obtained from cellulose, the main component of wood, straw and much of the structure of plants. Since cellulose cannot be digested by humans, the production of cellulose does not compete with the production of food, other than conversion of land from food production to cellulose production. Cellulose is present in every plant; in the form of straw, grass, and wood. The whole plant can be harvested, unlike corn. It is estimated that 323 million tons of cellulose containing raw materials, which could be used to create ethanol, are thrown away each year in the US alone. This includes 36.8 million dry tons of urban wood wastes, 90.5 million dry tons of primary mill residues, 45 million dry tons of forest residues, and 150.7 million dry tons of corn stover & wheat straw (Energy Information Administration, 2007). Kim and Dale (Kim, S. and Dale, B.E., 2004) estimated that there is enough biomass from wasted crops and crop residues to potentially replace 32% of the global gasoline consumption with bioethanol.

Also, waste paper, paper sludge, and packaging materials comprise a substantial part of the solid waste sent to landfills or incineration in the United States each day alone, which is definitely a solid waste disposal headache. Finding new landfills is difficult and also causes environmental problems. The cost of incineration is not cheap. Reduction of the disposal of solid waste through cellulosic ethanol conversion would reduce solid

waste disposal costs by local and state governments. It is estimated that each person in the US throws away 4.4 lb of trash each day, of which 37% contains waste paper, which is largely cellulose. That computes to 244 thousand tons per day of discarded waste paper. The solid waste raw material that produces cellulosic ethanol is not only free, it has a negative cost i.e., ethanol producers can get paid to take it away. Taking paper sludge as an example, the current cost of disposal ranges from \$0 to \$50 per wet ton according to Boise Paper Mill (Abston, 2008).

Besides energy and economic security, environmental benefits are also one of the main driving forces behind cellulosic ethanol commercialization. According to the U.S. Department of Energy studies conducted by the Argonne Laboratories of the University of Chicago, one of the benefits of cellulosic ethanol is that it reduces greenhouse gas emissions (GHG) by 85% over reformulated gasoline (<http://www1.eere.energy.gov/biomass/news.html>). However, even though the cellulosic biomass is plentiful and low-cost, it is relatively difficult to convert to ethanol (Wyman, 1996). Unlike starch and sugar, cellulosic biomass is made up of complex structure, which has three main biopolymers (cellulose, hemicellulose, and lignin) intertwined chemically and physically, rendering it very difficult to producing alcohol.

## **2.2 Composition and structure of biomass**

### **2.2.1 Cellulose**

Cellulose is the main constituent of wood and other biomass, approximately 38-50% of dry substance and forms the structural framework of the cell walls. Like amylose and the main chains of amylopectin, the cellulose molecule is a linear, unbranched homopolysaccharide with a repeating unit of D-glucose strung together by  $\beta$ -glycosidic

linkages, consisting of 10,000 to 15,000 glucose units (Goldstein, 1981). The  $\beta$ -glycosidic linkages in cellulose form linear chains that are highly stable and resistant to chemical attack because hydrogen bonding between cellulose chains makes the polymers more rigid, inhibiting the flexing of the molecules that must occur in the hydrolytic breaking of the glycosidic linkages. The free hydroxyl groups present in the cellulose macromolecule are likely to be involved in a number of intra and inter molecular hydrogen bonds which may give rise to various ordered crystalline arrangements (Lehninger et al., 2004). Thus, bundles of cellulose molecules are aggregated together to form microfibrils— diameter of an individual microfibril is around 2-5 nm (Zhang et al. 2007), via orderly hydrogen bonds and Van der Waal's forces leading to highly ordered (crystalline) regions alternate with less ordered (amorphous) regions resulting in low accessibility to enzymes. Microfibrils build up fibrils and, finally, cellulose fibers (Sjöström, 1993; Zhang et al. 2007). Crystalline cellulose microfibrils are embedded in a matrix of hemicellulose, pectin and a variety of proteins (Mansfield and Saddler, 2003).

Unlike starch, which when ingested in the diet is easily hydrolyzed by  $\alpha$ -amylases, enzymes in saliva and intestinal secretions that break  $\alpha$ -glycosidic bonds between glucose units, cellulose cannot be used as a fuel source by most animals, because they lack an enzyme to hydrolyze the  $\beta$ -linkages. Termites readily digest cellulose (and therefore wood), but only because their intestinal tract harbors a symbiotic microorganism, *Trichonympha*, that secretes cellulase, which hydrolyzes the  $\beta$ -linkages.

### **2.2.2 Hemicellulose**

Hemicellulose is a highly complex, branched polymer made up of five different monomeric sugars attached through different linkages, approximately 23-32% of dry

substance. It contains five-carbon sugars (usually D-xylose and L-arabinose), six-carbon sugars (D-galactose, D-glucose, and D-mannose), acetic acid, and uronic acid. Hemicellulose has a lower degree of polymerization (DP) than cellulose (about 200 vs. more than 10,000), and its lower limits have not been clearly defined. Hemicellulose can be classified as xylans, mannans, galactans etc. based upon the composition and intra-structural bonding, and these groups can exist separately as single components or collectively (Applegarth and Dutton, 1965; Roelofsen, 1959; Jayme and Tio, 1968). Agricultural residues, such as wheat straw and sugarcane bagasse, contain large amounts of xylan, some arabinan, and only very small amounts of mannan. Hardwood hemicellulose mostly contains xylans while softwood hemicellulose contains glucomannan. Xylan is a complex polysaccharide composed of a backbone of  $\beta$ -1,4-linked xylopyranosyl residues that, depending on the plant source, can be variably substituted by side chains of arabinosyl, glucuronosyl, methylglucuronosyl, acetyl, feruloyl and p-coumaroyl residues. Typically, 4-O-methylglucuronic acid linked at C-2 at every tenth (on average) 1, 4-linked xylose units (Shimizu, 2001), and every seven out of ten xylose units contain an acetyl group attached to them (Bailey and Ollis, 1986).

The mannan can be classified in four subfamilies: linear mannan, glucomannan, galactomannan, and galactoglucomannan (Petkowicz et al., 2001). Each of these polysaccharides presents a  $\beta$ -1, 4-linked backbone containing mannose or a combination of glucose and mannose residues (Liepman et al., 2007). In addition, the mannan backbone can be substituted with side chains of  $\alpha$ -1, 6-linked galactose residues. The major softwood hemicellulose is an O-acetyl-galactoglucomannan (15-25% of wood), which is beta-1,4-linked mannose and glucose residues in the ratio 3:1 often with alpha-

1,6-galactose as side group (Timell, 1967; Ethier et al., 1998; Meier, H. 1961; Katz, 1965; Lindberg et al., 1973). In hardwood a low amount of glucomannan (2-5% of wood) is also present, composed of D-glucose and D-mannose, while softwood also contains arabinoglucuronoxylan, which is composed of L-arabinose, 4-O-methyl-glucuronic acid, and D-xylose in the molar ratio 1.3:2:10 (Sjöström, 1993). Mannan also exists in galactomannan forms in the seeds of leguminous plants (Handford et al., 2003; Buckeridge et al., 2000).

### **2.2.3 Lignin**

Lignin, which is a major non carbohydrate in wood and other native plant material and comprises 15-25% of the dry weight of the biomass that encrusts the cell walls and cements the cells together, is a relatively inexpensive and structurally appealing similar to phenolic polymer (Feldman et al., 2002). It is essentially a random, three-dimensional network polymer comprised of variously linked phenyl propane units (Sjöström, 1993). It is amorphous in nature and plays a critical role in giving structural rigidity to hold plant fibers together. The carbohydrate polymers are tightly bound to the lignin by hydrogen bond and some covalent bonds (Fan et al., 1982). Lignin cannot be separated from biomass without degrading it, and the molecular weight of native lignin still remains unclear. It is estimated to have as much as 50 million Daltons (Rowell, 2005).

Lignin also plays an important role in the transport of water and nutrients and decreases the permeation of water through the cell walls. Lignin is proven as resistant to microbial degradation, playing an important role in a plant's natural defense (Basaglia et al., 1992; Chandler et al., 1980). The lignin in plant cell walls must be separated from carbohydrates during biomass conversion to open the protective lignin structure.

Therefore, a pretreatment is necessary in order to improve the digestibility of the biomass for a subsequent enzymatic treatment. The main target of successful pretreatment is to diminish the protective linkage between lignin and polysaccharides (lignin–carbohydrate complexes, LCC).

Most of the current industrial processes have lignin residue which is combusted for energy since it has a much higher heating value (HHV): 5062 cal/g on oven dry basis (Domalski et al., 1987). A kraft paper mill is one such example; the lignin isolated from pulp-making process is burned for energy generation and chemical recycling. However, its application is limited since the efficiency of lignin as a fuel is very low, producing less than 25% as much energy as fuel oil (Feldman et al., 2002).

The competitiveness of the lignocellulosic ethanol process can be enhanced by creating a market for the lignin. Lignin can be used as a precursor for DMSO, vanilla, phenol, and ethylene (Eckert et al., 2007; Lora and Glasser, 2002; Reddy and Yang, 2005). High-quality lignin has been demonstrated to work as a substitute for polymeric materials, such as resins, surfactants, and dispersants. The National Renewable Energy Laboratory (NREL) has investigated the potential of converting lignin into a hydrocarbon that can be used as a high-octane automobile fuel additive (Montague, 2003). Lignin can also be used as a carbon source for carbon fibers (Feldman et al., 2002; Kadla et al., 2002). The use of lignin-based carbon fiber composite materials could dramatically reduce fuel consumption by lowering the overall weight of the vehicle, and a study in 2002 showed that using 10% lignin can produce enough carbon fiber to replace half of the steel used in domestic passenger vehicles (Leitten et al., 2002).

#### **2.3.4 Extractives and Ash**

Extractive components mainly include terpenes, phenolic compounds, inorganic material, non-structural sugars and nitrogen containing compounds. Terpenes are a large and varied class of hydrocarbons, produced primarily by a wide variety of plants. They serve as antiherbivore defense compounds in plants. Phenolic compounds are found in heartwood which gives dark color to heartwood and make wood resistant to decay, and are also the precursor of lignin. Inorganic material may come from any soluble material that is associated with the biomass, such as soil or fertilizer. Nitrogen containing compounds include alkaloids, phenazines and protein. Ashes are mainly inorganic components such as alkali earth carbonate and oxalates, either in structural or extractable form. Structural ash is an inorganic material that is bound into the physical structure of the biomass, while extractable ash is inorganic material that can be removed by washing or extracting the material.

#### **2.3.5 Acetyl Group**

Acetyl groups are bound through an ester linkage to hemicellulose chains, especially xylans, in wood and other plants. A measure of acetyl content is necessary for biomass containing hemicellulose with a xylan backbone, but not for biomass containing a mannan backbone. Xylan, in native plant cell walls, is extensively acetylated (Holtzapple, 1993). Recent studies indicate that removing acetyl groups from xylan greatly enhanced the biomass digestibility through increased swellability (Weimer et al., 1986), thereby increasing the hydrolysis rate (Grohmann et al., 1989; Kong et al., 1992). The natural moisture present in plants hydrolyzes the acetyl groups to acetic acid, particularly at elevated temperatures. Deacetylation occurs during the pretreatment (Grohmann et al., 1989).

## **2.3 Bioconversion of Lignocellulosic Biomass**

There are four stages to produce ethanol using a biological approach (Fig. II-1.):

1. A pretreatment phase, to make the lignocellulosic biomass amenable to hydrolysis;
2. Enzymatic hydrolysis (cellulose hydrolysis) , to break down the molecules into sugars;
3. Microbial fermentation of the sugar solution;
4. Distillation to produce 99.5% pure alcohol.

### **2.3.1 Pretreatment**

The cellulosic microfibrils are embedded in a matrix of lignin and hemicelluloses and this arrangement presents a major accessibility problem for cellulase enzymes. Therefore, a pretreatment (Fig.II-2.) is necessary in order to improve the digestibility of the biomass for a subsequent enzymatic hydrolysis step (Mosier et al., 2005). Many different pretreatment technologies have been developed. Of these many pretreatment technologies, some are focused on hemicellulose, some are focused on disrupting the highly ordered cellulose, and others are focused on disrupting the lignin-carbohydrate complex (Dale et al., 1984). Most pretreatments are done through physical, chemical, and biological pretreatment. Physical pretreatment technologies do not use the addition of chemicals and usually involve ball-milling, irradiation, steaming, and hydrothermolysis (Hsu, 1996). Chemical pretreatment technologies have been proposed and investigated, e.g. steam treatment with SO<sub>2</sub>, ammonia fiber explosion (AFEX), ammonia-recycled percolation(ARP), lime pretreatment, dilute acid pretreatment , and concentrated acid pretreatment (Gong et al.,1999). Biological pretreatment technologies use lignin

degrading microorganisms to improve substrate accessibility to cellulase enzymes (Taniguchi et al., 2005). In order to achieve higher efficiency, some processes are a combination of two or more pretreatment methods (McMillan, 1994). The economic and environmental concerns, however, limit the applicability of most of known methods.

#### **2.3.1.1 Biological Pretreatment**

Although lignin is proven to be resistant to microbial degradation and therefore plays an important function in a plant's natural defense (Basaglia et al., 1992; Chandler et al., 1980), some microorganisms, particularly white-rot fungi and soft-rot fungi, can release the necessary enzymes, such as lignin peroxidases during secondary metabolism, to break lignin down (Kirk and Farrell, 1987). White-rot fungi are one of the most promising types of organisms for the biological pretreatment (Kirk, 1975). Biological pretreatments require low energy input because of the mild conditions of pretreatment, but its low rate in comparison to the chemical treatments has prevented its usage in commercial scale plants.

#### **2.3.1.2 Physical Pretreatment**

There are two kinds of physical pretreatment, mechanical pretreatment and non-mechanical pretreatment. Mechanical pretreatment includes different modes of milling (ball, two-roll, compression, dry, wet and vibratory milling), chipping, and grinding to reduce the particle size and increase the surface area of the biomass (Caufield et al., 1974; Matsumura et al., 1977; Puri, 1984). It is believed that these methods not only reduces their particle size, but also decrystallizes lignocelluloses. There is some debate about whether the benefits derived from the milling generally result from lower crystallinity or actually from the smaller particle size (Chang and Holtzapple, 2000).

Mechanical pretreatment is energy- intensive and capital-intensive, and hence unattractive on a commercial scale. The non-mechanical pretreatment options include irradiation, pyrolysis, steaming, and microwave treatment (Mandels et al., 1974; Beardmore et al., 1980; Mohan et al., 2006). These methods also require considerable energy and have a high operating cost.

### **2.3.1.3 Chemical Pretreatment**

Most of the chemical pretreatments may need a prior step involving chipping and milling. Different chemical treatments have been used over the past few decades for removing lignin or hemicellulose, destroying the cellulose crystalline structure, and increasing the pore size and surface area, and include steam explosion pretreatment, steam explosion with gas or acid, acid pretreatment, alkaline treatment, AFEX pretreatment, the organosolv processes, liquid hot water pretreatment (LHW), oxidizing reagent pretreatment, cellulose solvent pretreatment, and supercritical fluids etc.

Steam explosion: Steam explosion has been one of the most thoroughly investigated pretreatment technologies involving treating different biomass resources (Boussaid et al., 2000; Pan et al., 2004; Yang et al., 2002). In steam explosion the feedstock is subjected to high steam pressure (usually at 200–450 psig) and high temperatures (usually 150-270°C) for retention times of 20 seconds –10 minutes, followed by suddenly discharging the product to atmospheric pressure. Treated biomass with the high pressure and high temperature of the process causes hydrolysis of acetyl groups of hemicellulose which release acetic acid, and therefore cause the autohydrolysis of hemicellulose. The typical steam explosion pretreatment fractionates feedstock into different components. Most of the cellulose and lignin remain in the solid part after

pretreatment and most of the hemicelluloses, low molecular weight lignin decomposed by-product, and extractives are solubilized in the liquid part, thereby leading to much higher content lignin in the solid part than that of untreated feedstock. Lignin phenolic groups content increases after pretreatment and lignin condensation is observed indicate lignin structure is changed (Shevchenko et al., 2001b).

Depending on different lignocellulosic material, especially for softwood, and modifying pretreatment efficiency, by adding SO<sub>2</sub>, CO<sub>2</sub> and sulfuric acid during steam explosion treatment can facilitate the degradation of biomass and improve the enzymatic digestibility of biomass (Schell et al., 1998; Zheng et al., 1998; Robinson et al., 2002; Shahbazi, et al., 2005; Tucker et al., 2003; Varga et al., 2004). SO<sub>2</sub> forms sulfuric acid and CO<sub>2</sub> forms carbonic acid, which therefore make the pretreatment environment more acidic. Since SO<sub>2</sub> is very toxic, this can lead to safety and environmental problems. Carbon dioxide becomes somewhat attractive because it is a byproduct of ethanol fermentation process. Grous et al found the enzymatic digestibility of steam exploded poplar to be as high as 90% conversion in 24 hr (Grous et al., 1986). However, in the mean time, it also generates different inhibitory compounds for further enzymatic hydrolysis and fermentation steps due to autohydrolysis of lignin and hemicellulose degradation. Adding acid or SO<sub>2</sub> improves pretreatment efficiency, but also leads to expensive corrosion and high pressure proof equipment, safety issues and environmental disposal problems.

Acid pretreatment: Acid pretreatment has been investigated for a long time because of its high pretreatment efficiency with most feedstocks (Grethlein et al., 1984; Schell et al., 1992; Nguyen et al., 1998; Schell et al., 2003). Acids used in this

pretreatment are usually sulfuric acid, hydrochloric acid, and phosphoric acid (Allen et al., 2001; Kalman et al., 2002; Saha et al., 2005a; Saha et al., 2005b; Kim and Mazza, 2008). Usually, sulfuric acid is used because of its low cost. In diluted form (0.1% to 0.7% sulfuric acid as catalyst), it generally performs at relatively high temperatures (100-240°C) for a few seconds to 60 minutes depending on temperature. Generally, diluted acid pretreatment process, similar to steam explosion with an acid catalyst, solubilizes the hemicellulose component of biomass into liquid stream, while leaving most of the cellulose fraction intact in the solid part. Both pretreatments remove the lignin-hemicellulose physical protection and improve the remaining cellulose susceptibility to enzymes (Grethlein et al., 1991). Enzymatic digestibility of remaining cellulose is improved but total sugar yield from diluted acid pretreatment is low due to hemicellulose degradation. To maximize the sugar yields from the hemicellulose and cellulose fractions of biomass, a two-stage acid hydrolysis process has been developed (Nguyen et al., 2000). The first stage is performed under relatively milder conditions to hydrolyze hemicellulose, while the second stage is optimized to hydrolyze the more resistant cellulose fraction under higher temperatures. The disadvantages of the dilute acid process include high investment in equipment associated with acid corrosion, low sugar concentration in liquid stream, and high content inhibitors which makes fermentation very difficult. Since diluted acid pretreatment at high temperature produces large quantities of degradation byproducts and undesirable inhibitory compounds, the concentrated acid pretreatment is employed at temperatures which could be as low as 30 °C at near theoretical yields with little degradation. Arkenol and Masada Resource Group plan to use this process in their bioethanol plants (<http://www.arkenol.com>). The

hydrogen bonding between cellulose chains is disrupted during the concentrated acid pretreatment, and cellulose becomes completely amorphous. The amorphous cellulose is extremely susceptible to hydrolysis. Thus, dilution with water at modest temperatures provides complete and rapid hydrolysis to glucose, with little degradation. Minimizing the use of sulfuric acid and recycling the acid cost-effectively are critical factors in the economic feasibility of the process.

Alkaline treatment: Alkali, such as calcium hydroxide (lime), sodium hydroxide and ammonia, has been employed as a pretreatment method for many years (Tarkow and Feist, 1969; Chang et al., 2001; Kaar and Holtzapple, 2000; Kim and Holtzapple, 2005; Kim and Lee, 2005a and 2005b; Iyer et al., 1996; Kim et al., 2000; Kim and Lee, 1996). Alkaline pretreatment causes a swelling effect on the biomass, which is an intra crystalline swelling action penetrating both the amorphous and crystalline structure of cellulose and resulting in irreversible change in the structure of cellulose. This is accompanied by an inter-crystalline swelling action with water, which is a reversible change of dehydration of the substrate and has an effect only on the amorphous phase (Mishra, 2000). During alkaline pretreatment, the lignin is degraded predominantly by cleavage of lignin-hemicellulose bonds, and delignification brings about changes in the structure of cellulose, whose DP and crystallinity decrease and accessible surface area increases, thus making the biomass more susceptible to enzymatic hydrolysis. A certain amount of alkali is required to combine with the acetyl and carbonyl content of biomass and around 10-50% of the alkali is consumed during the pretreatment, which is a significant economic factor (Wenzl, 1970).

Among the alkaline reagents, calcium hydroxide seems a good choice considering the cost of the chemical, process temperatures of 25-100 °C for 1 day to 2 weeks, but it is difficult to recover (Hsu, 1996). The cost of sodium hydroxide is relatively high, but the recovery has been well documented in the pulp industry for a long time. Aqueous ammonia has also been used as a good choice for pretreatment, due to its relatively low cost, about one-fourth the cost of sulfuric acid on a molar basis, and its high volatility which makes it easy to recover and reuse. Also, ammonia is not severely corrosive, unlike sulfuric acid and hydrochloric acid. The ammonolysis cleaves the C-O-C bonds in lignin and the ether and ester bonds in the lignin-carbohydrate complex. Two processes, based on aqueous ammonia, ammonia recycle percolation (ARP) and soaking in aqueous ammonia (SAA), were used by Gupta et al. to improve the enzymatic digestibility of corn stover and hybrid poplar wood close to the quantitative maximum. Both processes have the high selectivity for lignin reaction and high retention of hemicellulose (Kim, T.H. and Lee, Y.Y., 2005a and 2005b; Iyer et al., 1996; Gupta, 2008). In the ARP process, the ammonia is continuously fed and withdrawn from a flow-through (percolation) reactor. The lignin and other extraneous components are cleanly separated from the biomass structure, which prevents recondensation of lignin in the biomass. The SAA is a batch process used at a relatively low severity conditions. Because of low severity conditions, longer treatment time is required. At a typical condition of 15% NH<sub>3</sub>OH and 60° C, which gives a system pressure of about 1 atm (less than 30 psia), a reaction time of several hours to one day is required to achieve acceptable level of pretreatment effects. Because of low process energy and low equipment cost, the overall processing cost of SAA is substantially lower than ARP.

The Ammonia Fiber Expansion (AFEX) process was developed by Bruce Dale et al. in the early 1980's, where biomass is soaked with liquid anhydrous ammonia at temperatures (60 – 160° C) and at pressures (250-1000 psi) from 5 to 30 min (Dale and Moreira, 1982; Wyman et al., 2005; Teymouri et al., 2005). Then, the pressure is released suddenly (explosion) resulting in a physical action on the substrate thus facilitating its disintegration of the biomass. AFEX decreases the crystallinity of cellulose (Gollapalli et al., 2002), retains most of hemicellulose since there is little water present in the process, disrupts the hemicellulose-lignin links by ammonolysis, and modifies the structure of lignin, therefore reducing non-productive binding between cellulase and lignin (Martinez et al., 1991). After the treatment, most of the ammonia can be recovered for reuse and the retained ammonia in the biomass could be used as a nitrogen source in the following bioconversion (Dale et al., 1985). However, within the reaction conditions of temperature (60°C-180°C), the upper limit of the ammonia pressure is around 450 psia, which is relatively higher than a normal pulp mill digester pressure.

Ammonia works well for feedstocks of low lignin content, especially agricultural residues of annual plants such as corn stover, sugarcane bagasse, and wheat straw, but not for wood species, especially softwood (Iyer et al., 1996).

Organosolv Processes: A lot of organic solvents, mainly alcohol, were originally used to extract lignin from wood with high purity in order to study lignin and carbohydrates (Kleinert, 1974). Organosolv pretreatment breaks down bonds such as  $\alpha$ -aryl ether and aryl glycerol- $\beta$ -aryl ether in the lignin-carbohydrate complex (Kleinert, 1974; Sarkanen et al., 1981), bringing significant changes in the lignin structure and decreasing the average molecular weight (Gilarranz et al., 2000). The breakdown

products, like solubilized lignin, are dissolved in the solvent and can be subsequently used to produce valuable coproducts. The organosolv pretreatment is helpful to produce substrates highly susceptible to enzyme and therefore increase the enzymatic digestibility due to delignification (Pan et al., 2005). The acid or base catalyst is added into an aqueous organic solvent to reduce the reaction time and temperature (Chum et al., 1988; Chum et al., 1990; Thring et al., 1990). Based on organosolv processes, Zhang et al developed a new cellulose and organic-solvent-based lignocelluloses fractionation (COSLIF) technology to separate lignocellulosic biomass into amorphous cellulose, hemicellulose, and lignin under modest reaction conditions (atmospheric pressure and  $\sim 50^{\circ}\text{C}$ ) (Zhang et al. 2007).

However, organosolv processes are still in a developing stage and are not yet ready to be commercialized due to the high cost of solvent recovery. Recycling of the solvent is important for reduction of cost due to high cost of solvents. Removal of the solvent from the pretreated biomass is also important since the solvent could be inhibitory to enzymes and microorganisms.

Liquid hot water pretreatment: Water under high pressure penetrates the biomass for up to 15 min at  $200\text{--}230^{\circ}\text{C}$  (Mosier et al., 2005). Liquid hot water pretreatment (LHW) is also called autohydrolysis, because the hydronium ion from water ionization causes the cleavage of the acetyl group under high temperature, which forms the acetic acid catalyzing autohydrolysis of hemicellulose (Casebier et al., 1969; Lora and Wayman, 1978). Acetic acid catalyzes the hydrolysis of glycosidic linkages in hemicellulose and the beta-ether linkages in lignin resulting in the formation of different phenolic acids from lignin and some organic acids, such as formic acid and glucuronic

acid from hemicellulose degradation. Like steam explosion, LHW pretreatment keeps most of cellulose and lignin in the solid part after pretreatment and solubilizes most hemicelluloses, low molecular weight lignin decomposed by-product and extractives into the liquid part. But unlike steam explosion, there is no addition of chemicals in this process, and therefore, formation of inhibitory components is much lower and the requirement for corrosion-resistant materials is also much lower. In order to minimize hydrolysis to monosaccharides, control of pH during pretreatment is employed by adding of alkali to maintain the pH constant above 5 and below 7 (Weil et al., 1998). It also appears to improve enzymatic digestibility of cellulose, decrease in cellulose crystallinity, and lower association of cellulose with lignin (Ladisich et al., 1983; Ladisich et al., 1989; Lin et al., 1985).

Cellulose solvent pretreatment: Cadoxen, an alkali solution of CdO in aqueous ethylenediamine was utilized as a cellulose solvent (Ladisich et al., 1978; Tsao, 1978). Once cellulose is dissolved, not only is the cellulose structure decrystallized by dissolution and subsequent regeneration to a highly active amorphous cellulose form, but lignin the protective shell is also disrupted. However, Cadoxen is very aggressive and time-consuming to prepare. The cost of these solvents and their toxicity may prohibit their commercial application.

Recently, an ionic liquid for pretreatment of cellulosic biomass was developed as a promising green solvent for biomass fractionation. Ionic liquids are nonflammable and recyclable cellulose solvents with very low volatility and high thermal stability. Carbohydrates and lignin can be simultaneously dissolved in ionic liquids with anion activity (e.g. the 1-butyl-3-methylimidazolium cation in the 1-butyl-3-methylimidazolium

chloride (BMIMCl) and 1-allyl-3-methylimidazolium cation in the 1-allyl-3-methylimidazolium chloride (AMIMCl)) (Dadi et al., 2006; Reichert et al., 2001). However, most data showing the effectiveness of ionic liquids has been developed using pure crystalline cellulose, and its applicability to the more complex lignocellulosic biomass, toxicity toward enzymes and microorganisms, and recovery cost is unknown.

Oxidizing reagent: Oxidizing reagents, such as oxygen (McGinnis et al., 1983), ozone (Ben-Ghedalia and Miron, 1981; Ben-Ghedalia and Shefet, 1983; Neely, 1984), hydrogen peroxide (Wei and Cheng, 1985; Azzam et al., 1989; Bjerre et al., 1996; Kim et al., 2000), peracetic acid (Teixeira et al., 2000; Zhao et al., 2007), and chlorine dioxide (Sullivan and Hershberger, 1959) can modify the structure of lignin-carbohydrate complexes, and therefore enhance the enzymatic digestibility of pretreated biomass. Azzam et al. found the enzymatic digestibility of the H<sub>2</sub>O<sub>2</sub> pretreated cane bagasse to be 95%, but a significant amount of hemicellulose was lost (Azzam et al., 1989). However, in the presence of a reducing agent, high concentrations of H<sub>2</sub>O<sub>2</sub> will react violently, and hydrogen peroxide in high concentrations is an aggressive oxidizer and will corrode many materials, including human skin. Chlorine dioxide is very toxic. Ozone was found to have high selectivity for lignin removal with slight effect on hemicellulose, low treatment temperature/pressure and the absence of fermentation inhibitors after pretreatment. However, ozone, like H<sub>2</sub>O<sub>2</sub> and chlorine dioxide, is expensive and cannot be recovered (Vidal and Molinier, 1988). In addition, it is usually more difficult to handle and store a gas, like oxygen and ozone, than a liquid.

Supercritical fluids pretreatment: A supercritical fluid is any substance at a temperature and pressure above its critical point. It behaves as neither a fluid nor a gas,

but has some of the properties of each. It can diffuse through solids like a gas, and dissolve materials like a liquid. Supercritical fluids are suitable as a substitute for organic solvents in a range of industrial and laboratory processes. Carbon dioxide, sulfur dioxide and ammonia have been used to treat lignocellulosic biomass. Carbon dioxide becomes more attractive because it is a byproduct from ethanol fermentation process. Shah et al treated hardwood with supercritical carbon dioxide-sulfur dioxide mixtures to make acetone, butanol, and ethanol (ABE) (Shah et al., 1991). Zheng et al. (Zheng et al., 1998) used supercritical carbon dioxide to treat Avicel, recycled paper mix, sugarcane bagasse and the repulping waste of recycled paper, and then suddenly released the carbon dioxide pressure to increase the accessible surface area of the cellulosic substrate to enzymatic hydrolysis. However, this technology raises serious questions about equipment costs due to high pressure.

### **2.3.2 Enzymatic Hydrolysis of Lignocellulosic Biomass**

The enzymatic hydrolysis of cellulose into glucose is a slow and complex process because of the physical nature of the substrate. Cellulose in its native form has a highly crystalline structure. In addition, the cellulose is embedded in a matrix of lignin and hemicellulose, where the number of active enzyme binding sites available is limited. The factors that affect the enzymatic hydrolysis of lignocellulosic biomass include cellulase property, substrates, and reaction conditions (temperature, pH, etc.).

#### **2.3.2.1 Cellulase**

Cellulases are an important class of industrial enzymes for various industries, such as textile and paper industries, outside the biorefinery area. Cellulase enzymes are found in bacteria, some of which are in the digestive tracts of herbivores through

symbiotic microbes. Fungi and termites also contain the enzymes and are largely responsible for the degradation of decaying trees and plants. Both fungi and bacteria can produce cellulases, including endo- and exoglucanases, for the hydrolysis of lignocellulosics (Tomme et al., 1995).

Of all fungal genera, including *P. chrysosporium* and species of *Trichoderma*, *Aspergillus*, and *Penicillium* (Lynd et al., 2002), *Trichoderma* has been most extensively studied for cellulase production. Cellulase enzymes from *Trichoderma reesei*, the most frequently studied cellulolytic fungus, are composed of three components - exo-1,4- $\beta$ -D-glucanases or cellobiohydrolases or exocellulase, microcrystalline cellulase, avicelase (CBHs, EC 3.2.1.91), endo-1,4- $\beta$ -D-glucanases or endoglucanase, endocellulase, CMCCase (EGs, EC 3.2.1.4) and  $\beta$ -glucosidases or cellobiase ( $\beta$ Gs, EC 3.2.1.21).

In the presence of cellulose, *T. reesei* secretes CBH I (~60%), CBH II (~15%), a number of EGs (~20%), and over 30 other minor proteins (Gritzali et al., 1979). EG I is reported to represent 5 to 10 % of the secreted protein, the major endoglucanase (Penttilä et al., 1987; Nidetzky and Claeysens, 1994), but EG II is reported to account for most of the endoglucanase activity produced by *T. reesei* (Suominen et al., 1993). Vinzant et al. identified five endo-glucanases (EG I/Cel 7B, EG II/Cel 5A, EG III/Cel 12A, EG IV/Cel 61A, and EG V/Cel 45A), two exo-glucanases (CBH I/Cel 7A, CBH II/Cel 6A), two xylanases (XYN I and XYN II), a  $\beta$ -D-glucosidase, an  $\alpha$ -L-arbinofluranosidase, an acetyl xylan esterase, a  $\beta$ -mannanase, and an  $\alpha$ -glucuronidase by 2-D electrophoresis in the cellulase complex of *T. reesei* (Vinzant et al., 2001).

The endoglucanases have two distinct structural domains, the catalytic (tadpole head) and binding (tail) regions, which are separated by a “linker” domain that is usually

glycosylated. Glycosylation is thought to protect the enzyme from proteolytic attack and improves the thermal stability of the protein. It also helps in the adsorption of endoglucanases into the insoluble cellulose (Langsford et al., 1987; Merivuori et al., 1985; Olden et al., 1985). Endoglucanases cleave glycosidic bonds within cellulose microfibrils, acting preferentially at amorphous cellulose regions. EGs fragment cellulose chains to generate reactive ends for CBHs, which act “processively” to degrade cellulose, including crystalline cellulose, from either the reducing (CBH I) or non-reducing (CBH II) ends, to generate mainly small oligosaccharides and cellobiose.  $\beta$ -glucosidase hydrolyzes small oligosaccharides and cellobiose to glucose (Fig.II-3.).

It was found that enzyme preparations containing only endocellulases have little effect on native cellulose. On the other hand, those containing both endo and exocellulases will cause significant degradation of native cellulose. Thus, the endo and exocellulases appear to work in a synergistic or cooperative manner on native high-molecular-size cellulose. According to a recent study, CBHII of *T. reesei* had no detectable effect on the DP of cotton cellulose and caused only a 3% solubilization; however, it rapidly solubilized the lower DP cellulose and acted synergistically with EGI to solubilize cotton (Kleman-Leyer et al., 1996). At high concentrations, cellobiose inhibits CBH activity. Thus  $\beta$ -glucosidase, which converts cellobiose into glucose, is often required for optimal cellulose performance in conditions where cellobiose accumulates, as it relieves end-product inhibition. *T. reesei* cellulase preparations, supplemented with *Aspergillus*  $\beta$ -glucosidase, are used most often for cellulose saccharification on an industrial scale (Sternberg et al., 1977). *T. reesei* produces  $\beta$ -glucosidases at low levels compared to other fungi such as *Aspergillus* species (Reczey et

al., 1998) and are not sufficient for extensive in vitro saccharification of cellulose, though the levels of *T. reesei*  $\beta$ -glucosidase are presumably sufficient for growth on cellulose. In addition, the  $\beta$ -glucosidases of *T. reesei* are subject to product (glucose) inhibition (Chen et al., 1992; Gong et al., 1977), while those of *Aspergillus* species are more glucose tolerant (Gunata and Vallier, 1999; Watanabe et al., 1992; Yan and Lin., 1997).

### **2.3.2.2 Addition of Surfactants**

Enzymatic hydrolysis of cellulose consists of three steps: adsorption of cellulase enzymes onto the surface of the cellulose which takes place at cellulose–liquid interface, the biodegradation of cellulose to water soluble sugars, and the desorption of cellulase. There are many studies on fungal cellulases showing that, once adsorbed on to the surface, desorption of the enzyme is not easy (Otter et al., 1989; Palonen et al., 1999; Nidetzky et al., 1994). Many researchers found that the rate of cellulose hydrolysis decreases dramatically as the hydrolysis proceeds, called rate retardation (Desai and Converse, 1997; Eriksson et al., 2002a). It has been suggested that the rate of hydrolysis is proportional to the amount of adsorbed enzymes (Sattler et al., 1989; Sattler et al., 1988). A major obstacle in the enzymatic conversion of lignocellulose is the adsorption of significant amounts of enzyme on exposed lignin surfaces due to unspecific binding of cellulases by hydrophobic interactions with lignin. Palonen et al. showed that significant amounts of CBH I and EG II could bind to alkali and enzymatic lignin (Palonen et al., 2004). Surfactant adsorption to lignin prevents unproductive binding of enzymes to lignin (Eriksson et al., 2002b). The surfactants used in the enzymatic hydrolysis include nonionic surfactant, such as, Tween 20, 80 (Wu and Ju, 1998), polyoxyethylene glycol (Park et al., 1992), anionic surfactant, such as, sodium dodecylsulphate (Eriksson et al.,

2002b), cationic Q-86W (Ooshima et al., 1986), sophorolipid, rhamnolipid, and bacitracin (Helle et al., 1993). Inhibitory effects have been observed with cationic Q-86W at high concentration and anionic surfactant Neopelex F-25 (Ooshima et al., 1986). Nonionic surfactants are believed to be more effective for enhancing the cellulose hydrolysis. One of the main surfactants that have been investigated for its potential to improve hydrolysis is the Tween series (Polysorbate series). The hydrolysis yield of newspaper was increased from 41% to 55% by the addition of Tween 80 (Castanon and Wilke, 1981). Ooshima et al later showed that Tween 20 enhanced the enzymatic hydrolysis of Avicel from 33% to 45% within 72 hr (Ooshima et al., 1986).

Three explanations have been presented the effectiveness of surfactants such as Tween in improving hydrolysis, including, 1) decreasing the adsorption of cellulases to substrates (Ooshima et al. 1986), 2) increasing the desorption of enzymes from their binding sites (Park et al. 1992), and 3) increasing enzyme stability and prevent denaturation of enzymes during hydrolysis (Kaar and Holtzapple 1998).

Eriksson et al. found that addition of non-ionic and anionic surfactants to a hydrolysis mixture of lignocellulose resulted in a decrease in Cel 7A adsorption to the residual substrate, and, regardless of the addition of Tween 20, cellulase Cel 7A (CBH I) retained 100% of its activity during 96 hr of incubation (Eriksson et al., 2002b). Therefore, it was unlikely that surfactants function as stabilizers during hydrolysis.

Not only surfactants but polymers with various amount of ethylene oxide (EO) content, such as poly (ethylene glycol) (PEG), can also bind to lignin by hydrophobic interaction and hydrogen bonding and can reduce the unproductive binding of enzymes. J. Börjesson et al found that addition of PEG to enzyme hydrolysis of lignocellulose

increased the conversion from 42% to 78% in 16 hr, and decreased desorption of Cel 7A decreased from 81 to 59% (Börjesson et al., 2007).

### **2.3.2.3 Hemicellulase**

Xylanases and mannanases are currently used in the pulp and paper industry where xylanase or mannanase pretreatment facilitates chemical bleaching of pulps (Viikari et al., 1994; Bajpai, 2004; Montiel et al., 1999; Bhat et al., 2000). Xylanases do not remove lignin-based chromophores directly but instead remove the xylan network that interacts with the residual lignin. Similar to this mechanism, xylanases do not work in the cellulose but instead remove the covering of xylan from the cellulose microfibrils by catalyzing the hydrolysis of the xylan backbone, and therefore increase the access of cellulolytic enzymes to cellulose. The effective enzymatic release of D-xylose from xylan requires a large variety of hemicellulases (Biely, 1985): endo-1,4- $\beta$ -xylanase (EC 3.2.1.8) (or xylanases), which cleaves bonds within the xylose backbone and produces xylobiose and xylooligomers, xylan 1,4- $\beta$ -xylosidase (EC 3.2.1.37) (or  $\beta$ -xylosidase) which release xylose units from xylobiose and xylooligomers, and side-chain-splitting enzymes such as  $\alpha$ -L-arabinofuranosidases (EC 3.2.1.55),  $\alpha$ -D-glucuronidases (EC 3.2.1), acetyl xylan esterases (EC 3.1.1.6), hydroxycinnamic acid esterases (ferulic acid esterases EC 3.1.1.73 and p-coumaric acid esterases) (Fig.II-4).

Like the cellulolytic enzyme, synergistic action was also observed among the xylanolytic enzymes, but the synergy is more complex in the cellulolytic enzyme. Different components of xylanases involving the main-chain and side-chain hydrolytic enzymes work synergistically with each other, and this synergy can be defined as homosynergy or heterosynergy (Coughlan et al., 1993). Acetyl groups can hinder the

approach of enzymes that cleave the xylan backbone; hence the removal of these substituents by acetyl xylan esterases facilitates the action of xylanases. McCrae et al found that the feruloyl and p-coumaryl esterases aided the release of xylan (McCrae et al., 1994). Similarly, xylanases greatly enhance the release of arabinose from heteroxylans by the  $\alpha$ -L-arabinofuranosidases (Filho et al., 1993). It was also found that the mixture of xylanase and  $\beta$ -xylosidase was only effective in hydrolyzing the acetyl xylan in the presence of acetylxylan esterase (Clarke, 1997; Biely, 1985). Tenkanen et al. indicated that unlike cellulases, the endoxylanases of fungi do not bind strongly to xylan, mannan, or cellulose (Tenkanen, et al., 1995a). Interestingly, some bacterial endoxylanases possess a cellulose-binding domain (CBD), the function of which is unknown; the binding domain does not bind to xylan, nor does it enhance xylan hydrolysis (Coughlan et al., 1993).

The enzymatic hydrolysis of galacto(gluco)mannans, present particularly in softwoods, is accomplished through the action of endo- $\beta$ -1,4-mannanase ( $\beta$ -mannanase EC 3.2.1.78) which, together with the exo  $\beta$ -glucosidases (EC 3.2.1.21), randomly cleave the beta-mannosidic linkages within the main chain. Additional enzymes, mannan-1, 4- $\beta$ -mannosidases (EC 3.2.1.25) and  $\alpha$ -1, 6-galactosidases (EC 3.2.1.22),  $\beta$ -glucosidases (EC 3.2.1.21), and acetyl mannan esterases (EC 3.1.1.6) are needed to cleave the side chain, which create more sites for subsequent enzymic hydrolysis (Filho, 1998) (Fig.II-5.).

Similar to the cellulolytic enzyme and xylanolytic enzymes, synergistic action also happens in the mannan structure by a variety of main- and side-chain-cleaving enzymes. Gūbitz et al. found an increased production of monomers from the galactomannan by the homeosynergy between  $\beta$ -mannosidase and two  $\beta$ -mannanases

from *Sclerotium rolfsii* (Gübitz et al., 1996). The galactose release from softwood pulp is enhanced by the presence of mannanase in combination with alpha-galactosidase (Clarke et al., 2000).

Since hemicelluloses restrict the access of cellulolytic enzymes by coating cellulose fibers, enzyme mixtures supplementation of so-called “accessory” enzymes - hemicellulase with similar cellulase activity could show differences in performance on lignocellulosic biomass if they differ in hemicellulase composition (Berlin et al., 2005a; Berlin et al., 2005b; Berlin et al., 2006).

#### **2.3.2.4 Substrates**

Many researchers have addressed issues concerning how the structural features of the substrate including lignin content, hemicellulose content, biomass crystallinity and accessible surface area and substrate concentration affect the enzymatic hydrolysis.

Structural features of the substrate: Chang et al observed that lignin can interfere with hydrolysis if it irreversibly binds cellulase or  $\beta$ -glucosidase (Chang et al., 1981). Ooshima et al. observe that cellulase enzymes bind to lignocellulose residue (Ooshima et al., 1990). Tarkow et al. observed that the location and nature of carbohydrate-lignin bond also strongly influence digestibility (Tarkow and Feist, 1969). Delignification causes biomass swelling and modification of the lignin structure, therefore an increase the internal surface area and median pore volume (Fan et al., 1982), which leads to a reduction of irreversible adsorption of enzyme on lignin (Ooshima et al., 1990) and an increase of enzyme accessibility to cellulose (Meunier-Goddik and Penner, 1999; Mooney et al., 1998). Only 20-65% of the lignin need be removed to increase the susceptibility of enzymatic degradation depending on the sources of cellulose (Fan et al.,

1982). However, because most delignification techniques result in significant solubilization of hemicellulose, the delignification may not be the only factor that attributed digestibility increase (Wyman, 1996).

Dilute acid pretreatment studies (Grohmann et al., 1985 and 1986) showed that the removal of hemicellulose improves cellulose digestibility despite the high lignin content. Even though the results of Burns et al. and Converse et al. suggested that hemicellulose removal increases porosity and the specific surface areas accessible to cellulase (Converse et al., 1990; Burns et al., 1989), Torget et al reported that lignin subjected to high-temperature acid treatments was chemically modified and recondensed as an altered lignin polymer, which may play a less detrimental role in digestibility (Torget et al., 1991).

Another factor that may affect enzymatic hydrolysis is biomass crystallinity. Dunlap et al discovered an inverse linear relationship between the crystallinity index (CrI) and digestibility of natural cellulose (Dunlap et al., 1976). Reese et al. found that the cellulose becomes more crystalline with a decrease of the highly accessible amorphous portion. They found an increase in crystallinity during hydrolysis of cellulose and enzymes (Reese et al.1950). Thus, it is important for complete hydrolysis of cellulose to open up the crystalline structure of the cellulose and decrease the resistance to further hydrolysis, i.e., the interaction between cellulose chains (Sasaki et al., 1979).

The CrI is largely influenced by the biomass type and composition. For lignocellulosic biomass, the crystallinity index measures the relative amount of crystalline cellulose in the total biomass. Many investigations have shown that crystallinity correlates inversely with digestibility, but increased CrI after biomass

pretreatment has been observed in many studies (Chang and Holtzapple, 2000; Kasahara et al., 2001; Tanahashi et al., 1983). Caulfield et al found that increased accessible surface and decreased particle size rather than crystallinity affect the rate and extent of the hydrolysis (Caulfield and Moore, 1974.). It appears likely that the percentage of crystallinity is therefore not the main determinant of the enzymatic degradation of cellulose (Puri, 1984; and Sawada et al., 1988).

The accessible surface area of lignocellulose is another key factor that determines susceptibility to enzymatic degradation. It is important because contact between enzyme molecules and the cellulose surface is required for hydrolysis to proceed. Cellulosic particles have both external and internal surfaces, and the external surface is thought to be a small fraction of overall surface area for most substrates. Knappert et al. found that changes in the internal area are primarily due to changes in the pore size region, and hence the increase in accessibility for the enzyme would be significant (Knappert et al., 1980). But Burns et al. (1989) found that the surface area of pores which are too small to be accessible to the enzyme decreases more slowly, presumably because the substrate containing these small pores reacts only at the external surface. Thompson et al. conclude that the surface area of the substrate in relation to the size of the enzymes is the main limiting factor in the enzymatic hydrolysis of lignocellulosic biomass (Thompson et al., 1992).

**Substrate Concentration:** There is a tradeoff between achieving high glucose concentrations and achieving high levels and rates of cellulose conversion. This is likely due to product inhibition, though non-productive enzyme binding could also play a slight

role. Varga et al. (Varga et al., 2002) have reported that product inhibition starts to occur around 15 g/L glucose.

It is believed that substrate inhibition is not present at a low substrate concentration, and Elshafei et al. (Elshafei et al., 1991) mention that substrate loading between 0.5-5% total solids has only a minimal effect on cellulose conversion, though there is a substantial effect on hemicellulose conversion for alkaline treated corn stover samples. Huang et al. (Huang and Penner, 1991) found that the substrate inhibition occurred when the ratio of the microcrystalline substrate Avicel PH 101 to the cellulase from *Trichoderma reesei* (grams of cellulose/FPU of enzyme. FPU is filter paper unit, defined as a micromole of reducing sugar as glucose produced by 1 ml of enzyme per minute.) was greater than 5. Penner et al. found that the optimum substrate to enzyme ratio was 1.25 g of the microcrystalline substrate Avicel per FPU of the cellulase from *T. reesei* (Penner and Liaw, 1994).

### **2.3.3 Fermentation**

Fermentation is the phase of the bioconversion process which converts sugars to ethanol and carbon dioxide or other valuable products such as numerous organic acids by following enzymatic hydrolysis of lignocellulosic biomass. The fermentation reaction is caused by microorganism-yeast or bacteria, which feed on the sugars. Three approaches have been investigated for ethanol fermentation:

Consolidated BioProcessing (CBP): This combines all three processes, cellulase production, cellulose hydrolysis, and fermentation. They are carried out in one reactor with the use of single microorganism or group of microorganisms operating at the same conditions. This is also called Direct Microbial Conversion (DMC). The microorganism

community used in the reactor produces enzymes required for all the steps in the biomass to ethanol process (Lynd et al., 1996). Two strategies can be adopted for engineering the microorganisms for this purpose. It is estimated that CBP can reduce the cost of biological conversion by more than 4 times that of SSCF process, which is an advanced process combination with simultaneous saccharification with co-fermentation of hexose and pentose sugars, because of the reduced number of vessels required, the reduced cost of cellulase production and the reduced time of the process. First, naturally occurring cellulolytic microorganism can be engineered for expressing the enzymes required for ethanol production. Second, ethanol producing organisms can be improved genetically to express the cellulase system (Lynd et al., 2005). However, the ethanol yields are rather low, a lot of metabolic by-products are produced, and the organisms usually do not have high ethanol tolerance (Ravinder et al., 2000; South et al., 1993).

Separate Hydrolysis and Fermentation (SHF): In this process, enzymatic hydrolysis and fermentation is performed sequentially in two different vessels. In this process each step can be conducted at optimal conditions of pH and temperature. Cellulolytic enzymes usually perform better at higher temperatures -  $50 \pm 5^\circ \text{C}$  and a pH of 4.0–5.0 (Saddler and Gregg, 1998), whereas most of the ethanologenic organisms proposed for the fermentation of sugars, e.g. *Saccharomyces cerevisiae* (Olsson and Hahn-Hägerdal, 1993; Tonn et al., 1997) or *Zymomonas mobilis* (Park et al., 1993; Lawford et al., 1997) limit the temperature to below  $40^\circ \text{C}$ , usually at  $30^\circ \text{C}$  (Philippidis et al., 1996). However, glucose and cellobiose accumulation in the hydrolysis step inhibits the activity of the cellulases (Stenberg et al., 2000; Xiao et al., 2004; Tengborg et al.,

2001). The build-up of any of these products negatively affects enzymatic hydrolysis in the SHF process.

Simultaneous Saccharification and Fermentation (SSF): SSF combines cellulose hydrolysis and fermentation in one step. Because the glucose produced by the hydrolysis process is immediately consumed by the microorganism, only very low levels of cellobiose and glucose are observed in the reactor. Therefore, this process can greatly reduce the product inhibition to cellulase, which in turn increases sugar production rates, concentrations, and yields, and decreases enzyme loading requirements and process time. There are resulting cost savings due to the reduced number of vessels required compared to SHF process. In addition, there is another advantage to reducing requirements for sterile conditions because the presence of ethanol in the culture broth helps to avoid undesired microbial contamination in SSF (Szczo drak and Targonski, 1989; Wyman et al., 1992; Grohmann, 1993).

The *Saccharomyces cerevisiae* strains are the most commonly used ethanologens for starch and cellulosic biomass sugars and have high efficient fermentation of glucose to ethanol and rapid fermentation rates at low pH (Wyman, 1996). In addition, they have very high ethanol tolerance. Baker's yeast—*S.cerevisiae*—can produce 96.71g/L ethanol in the fermentation process (Caylak and Vardar, 1996). This microorganism utilizes six-carbon sugars, such as glucose, mannose and galactose, as well as the disaccharides sucrose and maltose by the Embden-Meyerhof Pathway (EM).

However, there are some the disadvantages to SSF. The microorganisms used in SSF are usually the yeast *S. cerevisiae*. The optimal temperature for SSF is around 38 °C, which is a compromise between the optimal temperatures for hydrolysis (50 °C) and

fermentation (35 °C) (Philippidis, 1996). This has a negative effect on the productivity because the enzymatic hydrolysis is the rate-limiting step in SSF (Philippidis and Smith, 1995). Thermotolerant yeasts and bacteria have been developed in the SSF to increase the temperature close to the optimal temperature of enzymatic hydrolysis. Kadam et al found that the thermotolerant yeast, *Candida acidotherophilum*, produced 80% of the theoretical ethanol yield at 40 °C using dilute-acid pretreated poplar as substrate (Kadam and Schmidt, 1997). Ballesteros et al. found *Kluyveromyces marxianus* and *K. fragilis* that have the highest ethanol productivity and an ethanol yield of 0.5 g/g cellulose in 78 hr using Solka Floc 200 as a substrate at 42 °C from 27 yeast strains (Ballesteros et al., 1991). Another disadvantage to SSF is that it is difficult to recover and reuse the yeast in an industrial process because of the difficulty in separating the yeast from the solid residue after SSF (Galbe and Zacchi, 2002). In addition, ethanol was found to be inhibitory to enzymes. Wu et al. found that cellulase lost its original activity dependent on ethanol concentrations (Wu and Lee, 1997).

Simultaneous Saccharification and Co-Fermentation (SSCF): Similar to SSF, SSCF combines cellulose hydrolysis and fermentation in one step by using strains with the ability to convert both hexose and pentose to ethanol. The main advantages of SSCF are that microorganisms can fermentate glucose and xylose into ethanol in one bioreactor, and further improve xylan removal which can improve the access of enzymes to cellulose (Öhgren et al., 2007) and reduce the inhibitory effect of xylose and xylooligomer on the cellulose hydrolysis by cellulase (Xiao et al., 2004; Nigam, and Prabhu, 1991; Kim and Lee, 2005b; Kumar and Wyman, 2008).

As stated above, the most commonly used microorganism is *Saccharomyces cerevisiae*; it has very high ethanol tolerance and is very robust. Some carbonyl compounds – furans and phenolics, which are very inhibitory to most of ethanologen bacteria, are less inhibitory to yeast (Taherzadeh et al., 2000a; and 2000b), since yeast can convert them to the corresponding and less toxic alcohols (Martin and Jönsson, 2003; Sarvari Horvath et al., 2003). However, it cannot ferment pentose sugars; as any hydrolysis process, the pentose fraction is currently discarded. In order to improve process economics, it is desirable to convert both pentoses and hexoses to ethanol (Lynd et al., 1999). Yeasts such as *Pichia stipitis*, *Candida shehatae*, and *Pachysolen tannophilus*, which are naturally capable of fermenting both glucose and xylose to ethanol, are being used in the fermentation process at laboratory scale (Lynd et al., 1999). *Pichia stipitis* CBS 6054 is one of natural xylose-fermenting yeast, which notably ferments xylose to ethanol with reasonable yield and productivity.

However, these yeast strains are inhibited by byproducts generated during pretreatment and enzymatic hydrolysis of the lignocellulose biomass (Hahn-Hägerdal et al., 1994). In addition, Chandakant et al. reported that these yeasts produce about one fifth the ethanol of *S. cerevisiae* (Chandakant and Bisaria, 1998). Therefore, a lot of research has been focused on metabolic engineering strains of bacteria and yeast able to utilize pentose and hexose recently. Anaerobic bacteria *Z. mobilis* (Lawford et al., 1998), the bacteria *Escherichia coli* (Ingram et al., 1997), and *S. cerevisiae* (Tonn et al., 1997) have been developed to meet the requirements of industrial lignocellulosic biomass fermentation. Naturally, *Z. mobilis* can only use the hexose sugars glucose and fructose to produce ethanol with higher yields, due to the production of less biomass, but is less

robust (Rogers et al., 1979; Lawford et al., 1998). *Z. mobilis* degrades hexose sugars to pyruvate using the Entner-Doudoroff pathway (Sprenger, 1996). The pyruvate is then fermented to produce ethanol and carbon dioxide as the only products, similar to yeast. But *Z. mobilis* can not consume pentose. In addition, another big concern in the fermentation process is that *Z. mobilis* is sensitive to various inhibitors. Zhang et al. inserted the genes for xylose assimilation and pentose phosphate pathway enzymes, like xyloase isomerase, xylulokinase, transketolase, and transaldolase from *E. coli* into the bacterium *Zymomonas mobilis* for effective fermentation of xylose to produce ethanol in NREL (Zhang et al., 1995). The researchers at Dupont continued this work. In 2006, DuPont's biofuels technology manager William Provine reported that his group has recently come up with a *Zymomonas* strain capable of tolerating up to 10% ethanol (Service, 2007).

*E. coli* can ferment a number of pentose and hexose sugars into a mixture of acids (lactic, acetic, formic, and succinic) and ethanol (Clark, 1989). *E. coli* KO11 is one of the very first ethanologens engineered by Ingram's group. They transformed pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adh B*) from *Zymomonas mobilis* into the parental strain of *E. coli* KO11, which has the natural ability to utilize several pentoses and hexoses, to produce ethanol with high metabolic yield (Ohta et al., 1991). However, its growth robustness and ethanol tolerance are relatively low compared to yeast (Yomano et al., 1998; Dien et al., 2003; Service, 2007). In order to improve the ethanol tolerance of *E. coli* strain KO11, Ingram's group isolated mutant strains with increased ethanol tolerance by using a novel approach alternating selection for ethanol tolerance in liquid and high antibiotic resistance on solid media. The best of these

mutants, strain *E. coli* LY01, exhibited more resistant than KO11 to inhibitors generated from pretreatment stage (Yomano et al., 1998) and high ethanol tolerance as high as 100 g L<sup>-1</sup> (Zaldivar et al.,1998).

Yeasts are still the most promising metabolic-engineering platforms for cellulosic ethanol processes as can be demonstrated by their usage in biotechnology for hundreds of years, because of their natural tolerance to high ethanol and inhibitors and their low pH growing condition which avoids bacterial contaminations. Ho et al. transformed three xylose-fermenting genes, xylose reductase and xylitol dehydrogenase genes from *Pichia stipitis* and xylulokinase gene from *Saccharomyces cerevisiae*, into *Saccharomyce spp.* for the co-fermentation of glucose and xylose with an ethanol yield of 0.30 g/g of initial total sugar (Ho et al., 1998). In order to increase the ethanol yield and the tolerance to toxic inhibitors, Ho et al further developed stable *Saccharomyces* yeasts 424A (LNH-ST), which can ferment a mixture of pure glucose (7%) and xylose (4%) into ethanol in 30 hours with an ethanol yield of 0.41 g/g of initial total sugar and 80% of the ethanol theoretical yield of initial total sugar (Sedlak, 2004).

#### **2.4 The Integrated Forest Biorefinery**

The pulp and paper industry in United States is facing overseas competitors, primarily from Asia, who have installed new technologies and who also have wood and labor cost advantages. At the same time, due to the digital revolution, the demand for certain grades of papers, particularly newsprint, is decreasing. According to Global Forest the decline in newspaper readership and advertising, among many sectors of the population, particularly young adults, and along with increasing competition from the Internet and other media, has led to consumption falling 10% from 2006 to 2007 (Global

Annual Forest, Paper & Packaging Industry survey - 2008 edition - Survey of 2007 Results). The combination of reduced demand in the US along with the increased supply of cheap paper is causing a great upset to the pulp and paper industry in the United States, which is forcing the pulp and paper industries to find new opportunities in order to improve their competitiveness. The integrated “Biorefinery” process to produce ethanol and cellulase as a supplementary unit in kraft paper mills can be developed. It can transform the pulp and paper industry into a competitive and innovative one, which allow a significant economic growth through new value-added products based on existing byproducts or waste at pulp and paper mills (i.e. hemicelluloses, black liquor, and paper sludge), while still preserving the main product-paper and paper related products.

#### **2.4.1 Background of Pulp and Paper-making**

Pulp and paper-making begins with trees as the main raw material - nearly 90%, though many lignocellulosic non-woody plants can be used, such as cotton, wheat straw, sugar cane waste, flax, bamboo, and linen rags. The main steps in pulp and paper-making are raw material preparation, such as wood debarking and chipping, pulp manufacturing, pulp bleaching, paper manufacturing, and fiber recycling. Not all parts of wood are useful for making pulp. Bark contains relatively high lignin and ash, and has to be removed. Chipping reduces the wood waste to a uniform size. Pulp manufacturing converts wood or lignocellulosic non-woody material to pulp fibers for paper manufacturing by mechanical or chemical means (Biermann, 1996; Sjöström, 1993).

##### **2.4.1.1 Mechanical Pulp or Thermomechanical Pulp (TMP)**

In the TMP making process, the wood chips are steamed with refiner plate. The heat softens the lignin, which allows fiber separation with less damage than in purely

mechanical pulping. The mechanical pulping process usually has a very high yield, in which more than 90% of the wood used in the process is converted into pulp. However, the process doesn't remove much lignin, and therefore most of the lignin remains in the pulp. Mechanical pulp can be used without bleaching to make printing papers for applications in which low brightness is acceptable, primarily newsprint, and other low-strength and low brightness applications, such as cardboard. Mechanical pulp is sometimes blended with kraft pulp to produce a middle-level product with low cost and reasonable strength and color properties, such as magazine. A large amount of energy is required to produce mechanical pulp, but it costs less than chemical pulp (Biermann, 1996; Sjöström, 1993).

#### **2.4.1.2 Chemithermomechanical Pulp (CTMP)**

Chemithermomechanical pulp (CTMP) or chemimechanical making processes involve mechanical action and the use of chemicals. The wood chips are softened with sodium carbonate, sodium hydroxide, sodium sulfite, and other chemical prior to a drastic mechanical action, but not as drastic as a mechanical pulp making. It removes extractives and small amounts of hemicellulose but not much lignin. Therefore, the yield of the chemimechanical pulping process is also high, 85-95%. The CTMP is stronger than TMP, but requires less energy to produce. CTMP can be bleached to produce bleached chemithermomechanical pulps (BCTMP) with high yield. (Biermann, 1996; Sjöström, 1993).

#### **2.4.1.3 Chemical Pulp**

Chemical pulping uses chemicals to dissolve the lignin in the wood under the vigorous conditions, freeing the cellulose fibers.

Sulfite Pulping: The sulfite process uses mixtures of sulfurous acid and/or its alkali salts carried out between pH 1.5 and 5 to solubilize lignin through the formation of lignosulfonates and cleavage of lignin bonds. Sulfite pulping has higher yield than kraft pulping. The byproduct of sulfite pulping, lignosulfonate has a wide variety of applications, such as leather tanning, drilling mud dispersants, ore flotation, and resins. However, sulfite pulp is weaker than kraft pulp. Not all species of wood can be pulped easily. Cooking cycles are long, and chemical recovery is complicated compare to kraft pulping. Therefore, most of sulfite mills have been converted to kraft mills (American Forest & Paper Association, 2002).

Kraft Pulping: In the kraft pulping process, wood chips are cooked with white liquor, a mixture of sodium hydroxide and sodium sulfide. It is heated in a digester which is capable of holding high pressures and high temperatures. The typical condition of delignification is several hours at 130 to 180 °C. Under these conditions 90~95% lignin, all extractive, and some hemicellulose are degraded to give fragments which are soluble in the strongly alkaline liquid. The remaining solid is collected and washed to become pulp. The combined liquid is called “black liquor”. The unbleached kraft pulp at this point is quite brown, called brown stock.

To make white paper, the unbleached kraft pulp must undergo additional bleaching process to remove the remaining lignin and brighten the pulp. Chlorine and hypochlorite were initially used to bleach chemical pulps. Because of environmental considerations about the the release of organochlorine compounds into the environment, chlorine dioxide, oxygen, ozone, hydrogen peroxide, and other chemicals have been used recently

The chemical recovery process is an important part of the kraft pulping process. In this process, the black liquor is concentrated in multiple effect evaporators to 65-70% solids and then sent to the recovery boiler to burn. The degraded organic compounds in the black liquor have significant energy content and are able to produce enough steam for mill operations. The inorganic chemicals ( $\text{Na}_2\text{S}$ ,  $\text{Na}_2\text{CO}_3$ , and any impurities) are recovered at the base of the recovery boiler. Dissolving these inorganic chemicals from the recovery boiler produces green liquor. Converting the  $\text{Na}_2\text{CO}_3$  in the green liquor to  $\text{NaOH}$  using  $\text{Ca}(\text{OH})_2$  regenerates white liquor used in the pulping process. Calcium carbonate precipitates from the white liquor and is heated to become calcium oxide. Calcium oxide is reacted with water to regenerate the calcium hydroxide.

Kraft chemical recovery processes consume an estimated 8.04 million BTU/ ton of pulp. However, the recovery boiler in the chemical recovery processes can produce 1 - 2 times more energy in the form of high pressure steam, which is used to reduce the steam pressure for the mill use and generate electricity (Martin et al., 2000). Bark and wood residues are often burned in a separate power boiler to generate steam. In addition, the water from multiple effect evaporators is usually clean enough to be used in other parts of the mill.

Kraft pulping produces a stronger pulp from a wide variety of tree species, compared to mechanical, semichemical, or sulfite pulp and is used in high-strength papers, such packaging bag and shipping containers (Biermann, 1996; Sjöström, 1993).

#### **2.4.1.4 Recycled Paper Pulp**

Since paper contains the same fibers as the original plants, these fibers can be recycled to make new paper. In the US, approximately 27% of paper is recycled and used

in applications which require less brightening, such as containerboard and paperboard. (American Forest & Paper Association, Paper, Paperboard & Wood Pulp: 2002 Statistics, Data through 2001, (2002)).

Paper recycling processes can use either chemical or mechanical pulp produced by mechanical energy with chemicals and water. Paper recycling typically includes repulping, screening, and cleaning process. These processes remove contaminants, such as plastics staples, and glues. Sometimes, Processes that produce newsprint, tissue or other “bright” grades must undergo a deinking process, which remove printing ink and other unwanted elements by adding chemicals (surfactant) during pulping. If white recycled paper is needed, the pulp may need to be bleached with hydrogen peroxide, chlorine dioxide, or oxygen to increase the brightness of deinked pulp. The recycled pulp can be used alone or blended with chemical pulp to give it extra strength or smoothness.

#### **2.4.1.5 Paper Making**

The pulp is diluted with water to make it 99% water. This slurry enters the headbox, and then is sprayed onto a fast-moving, continuous wire screen. Water starts to drain from the pulp on the screen, and the fibers quickly begin to bond together to form the paper sheet.

At this point, the paper is uncoated. For printed paper, it may be coated with fillers, such as calcium carbonate or clay and titanium dioxide, to increase its strength, water resistance, opacity and brightness (Biermann, 1996).

#### **2.4.2 The Development of an integrated wood- based Biorefinery**

In the emerging cellulosic ethanol market, the opportunity to repurpose existing U.S. pulp and paper mills as biorefineries has been brought up. The pulp and paper

industry in the U.S. is facing global competition and reduced profit. In addition, there has been a lack of capital investment in recent years. Therefore, the performance of the U.S. pulp and paper sector has been obsolete for the last 10 years. In the last 5 years the closures of pulp mills, and the devastation of the local communities where they are based, has accelerated. However, the pulp and paper industries have some advantages for cellulosic ethanol production. They are large biomass handlers with the know-how and highly skilled workforce to operate a complex process industry, and therefore they are a natural host for cellulosic ethanol production. A wood-based biorefinery is a plant that produces multiple value added chemical products from wood, chemicals and materials. From the pulp and paper making process, there are several potential opportunities, hemicellulose, black liquor, paper sludge, and waste paper.

#### **2.4.2.1 Hemicellulose Pre-extraction**

In the United States, 80% of total pulping capacity is chemical pulp. Approximately 98% of chemical pulp capacity is kraft pulping, with the remaining 2% being the sulfite pulping (American Forest & Paper Association, Paper, Paper board & Wood Pulp: 2002 Statistics, Data through 2001, (2002)).

In kraft pulping, around 80% of the hemicellulose in the wood is dissolved in the black liquor (Biermann, 1996; Sjöström, 1993). It is difficult to separate this hemicellulose part from alkaline black liquor before it is burned to generate heat and power and to recover pulping chemicals, since part of the hemicellulose is degraded at alkaline conditions (Sjostrom, 1993).

It is technically feasible to insert a new process before the digester stage to extract hemicellulose and convert to ethanol through a fermentation process. Hemicelluloses can

be removed from lignocellulosic materials by hot water, alkaline, and solvent extractive processes (Willfor and Holmbom, 2004; Song et al., 2008; Sears et al., 1971; Nguyen et al., 1998; Aziz and Sarkanen, 1989). It has been reported that during alkaline extraction, hemicellulose is partially degraded to aliphatic acids (Glasser et al., 2000). Hemicellulose exists in an amorphous form and is effectively hydrolyzed into soluble, monomeric sugars by dilute-acid pretreatment (Wyman, 1996). Hemicellulose hydrolyzates from water or dilute-acid pretreatment can be utilized to produce value-added products, such as ethanol, xylitol, (Buhner and Agblevor, 2004; Mussatto et al., 2005; Walther et al., 2001; Werpy and Petersen, 2004) and so on. Hemicelluloses have been utilized as plant gum for thickeners, adhesives, protective colloids, emulsifiers, and stabilizers as well as a biodegradable oxygen barrier films (Hartman et al., 2006; Kamm and Kamm, 2004; Kilpeläinen et al., 2007). Oligosaccharides may provide a source of higher value-added products, such as animal feed additives (Davis et al., 2002; Fernandez et al., 2002). Furfural, a xylose degradation product, can be utilized for production of lubricants, coatings, adhesives, and furan resins.

The optimization of pre-extraction technology for optimal removal of hemicellulose should be investigated. Hemicellulose preextraction has to be done without reducing pulp yield and strength, since the existing ways of pulp and paper making will still remain as heart of wood based biorefinery (Closset et al., 2005; Mensink et al., 2007). Cellulose fiber is the main component of paper; the minimizing the hydrolysis of cellulose should be considered. The fiber strength of the final paper is a key physical parameter in the production of paper. Some researchers have noted hemicellulose content is related to fiber strength (Schönberg et al., 2001). The inhibitors to microorganism, such

as furans, HMF, furfural, organic acid and phenolic compounds form during the preextraction process (Taherzadeh et al.,2000a; Taherzadeh et al., 2000b;Palmqvist et al.,2000a; Palmqvist et al.,2000b; Klinke et al.,2004). If the main target of prehydrolysate is ethanol production, the minimizing and removal of inhibitors should be done before the fermentation step.

#### **2.4.2.1 Black Liquor**

During the kraft process, 90~95% lignin, all extractives and 80% of the hemicellulose in the wood are degraded to give fragments which are soluble in the black liquor. The black liquor is concentrated, and then is combusted to produce steam and power and to recover the pulping chemicals, NaOH and Na<sub>2</sub>S.(Biermann, 1996). Lignin removed from the black liquor by acid precipitation may be used for chemicals. Gasification of black liquor is another approach. Gasification of the black liquor produces synthesis gas, which may be used as feedstock to produce transportation fuels. Two demonstration plants of black liquor gasification are operating in the United States (Closset et al., 2004).

#### **2.4.2.3 Pulp and Paper Waste**

The Pulp and Paper industry generates huge amounts of waste, mainly from the papermaking process and waste paper and paperboard. According to the U.S. Environmental Protection Agency, waste paper and paperboard made up the largest component of all municipal solid waste generated, about 32.7 percent and around 50% of them is recovered to make new paper (Municipal Solid Waste Generation, Recycling and Disposal in the United States: Facts and Figures, 2007). However, wood fibers can only be recovered 5 to 7 times before they become too short and weak to be made into new

paper. The pulp and paper industry also produces about 4.1 to 5 million dry tons per year of solid waste in United States, consisting primarily of de-watered sludges, which contain fibers and other materials produced during paper-making or paper recycling (Fan and Lynd, 2006b). The paper sludges are normally disposed by incineration or landfill, which causes substantial financial burden and a source of various environmental problems. Fiber in the sludge decomposes and produces methane, a greenhouse gas with 23 times the heat-trapping power of carbon dioxide. More than one-third of municipal landfill solid waste is waste paper and paper sludge, and municipal landfills account for 34 percent of human related methane emissions to the atmosphere, making landfills the single largest source of such emissions, according to EPA's study (Environmental Paper Network, 2007).

The cost of incineration is high because the burn efficiency of sludge is low, as a result of high moisture content (Fan and Lynd, 2006; Scott et al., 1995; Sjöde et al., 2007). A better alternative to landfilling and incineration is to incorporate paper sludge in a biorefinery. Paper sludge has high carbohydrate content. Table II-1 lists the typical compositions of primary and recycled sludge. Compared with corn stover, sludge contains much higher cellulose, and much less hemicellulose as well as lignin. The short fibers in the sludge are easily digested by cellulase enzymes (Fan and Lynd, 2006a; Jeffries et al., 1999; Fan et al., 2003). These features can potentially make bioconversion of sludges possible without pretreatment. As one of the major processing units in bioethanol plant, pretreatment is a significant cost factor, second only to feedstock cost which accounts for about 30% of the total production cost of ethanol (Galbe and Zacchi, 2002). As a feedstock for bioethanol, paper mill sludges offer two significant economic

benefits. First, the cost of sludge is essentially zero and may even be a negative number considering that the cost of disposal is about \$20 per wet ton (Randy Abston, 2008). Second, elimination of pretreatment can reduce the production cost of ethanol.

### **2.4.3 Cellulase Production in Kraft Pulp Mills**

One of the drawbacks in the production of bioethanol is the high cost from the usage of the enzymes utilized in the hydrolysis step (Steele et al., 2005; Spano et al., 1978).

Cellulase production in the microorganism is induced by crystalline cellulose and other known inducers (lactose and sepharose). Microbial uptake of the substrate is more complex than enzymatic hydrolysis of it. Although there is a similarity between the two cases, the microbial reaction is much more complex because the cellulase enzyme is excreted from it and works synergistically with the microorganism. The microbial process is also influenced by oxygen uptake in the bioreactor since this organism is highly aerobic, thus affected by oxygen mass transfer in the process of cell growth and enzyme production. Cellulase production is also accompanied by xylanase production. How the xylan content in the feedstock affects xylanase production is also an important issue in cellulase production.

The best-known host culture in cellulose production is the softrot filamentous fungus *-Trichoderma reesei*. *T. reesei* holds the greatest potential for reducing the cost in the production of enzymes for lignocellulosic biomass conversion (Egyhazi et al. 2004; Gadgil et al. 1995; Juhasz et al., 2004; Kadam and Keutzer, 1995). *T. reesei* is relatively easy and inexpensive to cultivate and it is not easily susceptible to contamination (Penttilä, 1998). It is a safe organism, because it is non-pathogenic and non-toxic to

healthy laboratory animals (Hjortkjaer et al., 1986) and to humans. It does not produce mycotoxins or antibiotics under the conditions used for enzyme production (Nevalainen et al., 1998; Nevalainen et al., 1994) and it is a low-risk to microorganisms (Penttilä, 1998). *T. reesei* is used industrially for enzyme production (Esterbauer et al., 1991; Kubicek, 1992). Most of the previous work is based on a variation of *T. reesei*-*T. reesei* Rut-C30 (ATCC-56765), which was isolated using a combination of ultraviolet irradiation and nitrosomethyl guanidine (NTG) by Montenecourt and Eveleigh (Montenecourt and Eveleigh, 1979). The industrial strain, although not publically revealed, is also originated from this culture and modified genetically.

In many investigations of cellulase production, soluble forms of modified cellulose and pure cellulose have been used as the carbon source. Mixtures of glucose and lactose have also been tested as a feasible substrate for cellulase production (Domingues *et al.*, 2001). Pourquoi (Pourquie *et al.*, 1989) and Chaudhuri (Chaudhuri *et al.*, 1993) reported cellulase production using lactose as the co-substrate. The soluble carbon sources are convenient to use and acceptable purely on a technical stand point. However the cost is prohibitively high. For many researchers, the preferred substrates have been pure commercial celluloses such as Solka Floc and Avicel (McClean *et al.*, 1986; Shiang *et al.*, 1991; Hayward *et al.*, 2000; Yu *et al.*, 1999). Hayward et al reported a cellulase yield above 150 FPU/g - cellulose and productivity above 55 FPU/L-hr obtained using Sloka Floc as the substrate. This appears to be the benchmark values of laboratory scale cellulase production. By fed-batch, relatively higher yield and productivity were achieved (Yu *et al.*, 1998; Yu *et al.*, 1999).

To convert a kraft process paper mill to an integrated biorefinery, the cellulase production has to be feasible, convenient, and economically viable. The substrate directly from paper and pulp industries could be considered. Sulfite wood pulp (Watson et al., 1984), pretreated wood (Juhasz et al., 2005; Shin et al., 2000), sawdust (Lo et al., 2005), waste paper (Royer and Nakas, 1987; Chen and Wayman, 1991; Shin et al., 2000), waste cardboard (Szijártó et al., 2004), and paper sludge (Royer and Nakas, 1987; Maheshwari et al., 1994) could be used as a carbon source for cellulase production. In general, the performance of cellulase production with these substrates was far inferior to the cellulose production obtained with glucose and pure cellulose. The individual results would vary depending upon what was used as the feedstock and what type of pretreatment had been applied. Generally, the presence of acetic acid, furfural, and lignin in the pretreated biomass caused delays in enzyme production and this in turn inhibited the enzyme production by *T. reesei* (Vinzant et al., 1998; Hayward et al., 2000; Szengyel and Zacchi, 2000; Bigelow and Wyman, 2002). Kraft paper sludge showed quite promising results. However, kraft paper sludge usually had high ash content, which is detrimental to cell growth. The solution to this problem is to remove as much of the ash as much as possible while retaining the carbohydrates (Wang et al., 2010).

Using kraft pulp while on site has the benefit of providing a high quality carbon source for cellulase production. In alkaline delignification of biomass such as kraft pulping, lignin is predominantly degraded by various cleavage reactions of aryl ether linkages (Sjöström, 1993). The Kkraft pulping process also removes about 50% hemicellulose and brings about changes in the structure of fiber (Sjöström, 1993). The kraft pulp could encourage the growth of the *T. reesei* and allows greater production of

cellulases and faster secretion into the culture medium. Cellulase cost contributes 12.5–43.4% to the total cost of cellulosic ethanol production when procured from external sources (Galbe and Zacchi, 2002; Ryu and Mandels, 1980; Wooley et al., 1999). However, the cost is drastically reduced once the in-house produced enzymes are used for saccharification purpose (Kadam, 1996). In addition, the extra-cellular enzymes are obtained from the fermenter broth, not inside the *T. reesei* cell body. The crude enzyme solution (fermenter broth) can be used without further purification.

The cost of bioethanol and cellulase production could be reduced if the immediate processing steps were minimized. That is, a process can be economized to its maximum extent when the process steps are minimized, without compromising the quality of the final product. Thus, process integration is a powerful tool in realizing the possible commercial production of bioethanol. A bioethanol plant could be integrated within an operational kraft process paper mill. This would save considerably on the transportation costs of moving the bulk paper sludge to a bioethanol plant. Kraft paper sludges work well in ethanol production process without pretreatment. The substrate of cellulase production can be obtained directly from a kraft paper mill itself. In addition, in the kraft process paper mill, one of the by-products is a high-calorific-value lignin, which could be used to generate energy for supporting the bioethanol production and the cellulase process.

Table II-1. Composition of sludges and corn stover

	Primary sludge	Recycle sludge	Corn stover
Glucan (%)	44.5	49.6	36.1
Xylan (%)	9.9	13.7	21.4
Lignin (%)	8.1	4.6	17.2
Total Ash (%)	36.0	30.0	7.1
Acid-soluble Ash (%)	26%	22%	-
Acid-insoluble Ash (%)	10%	8%	-
Others (%)	1.5	2.1	18.2
Alkalinity (milliliters of 1 N H <sub>2</sub> SO <sub>4</sub> /10 g dry sludge)	60	50	-

- a. All data in table are based on oven-dry original feedstocks
- b. Data of sludges are the mean value of triplicate (n=3; standard deviation<0.5 for glucan, xylan, and lignin)
- c. The composition of the corn stover was determined by the National Bioenergy Center, NREL, Golden, CO).

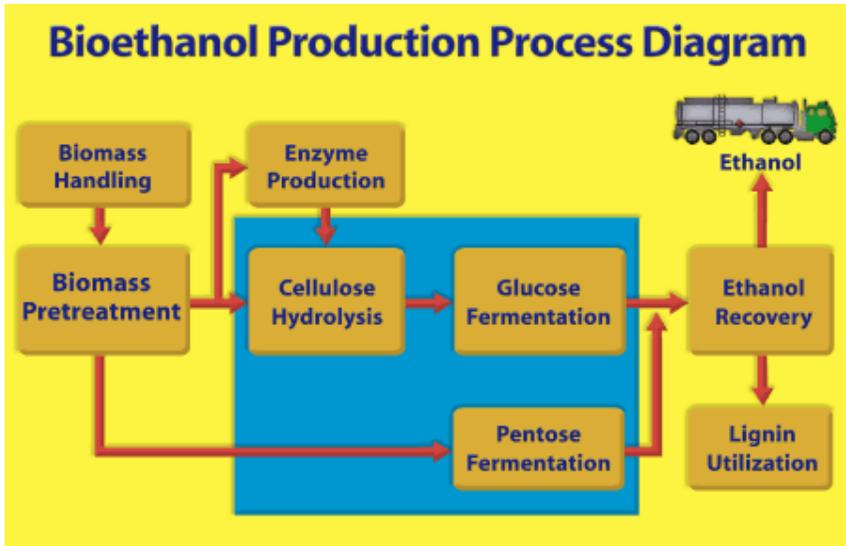


Fig. II-1. Bioethanol production process diagram (Biomass program, NREL, [http://www1.eere.energy.gov/biomass/abcs\\_biofuels.html](http://www1.eere.energy.gov/biomass/abcs_biofuels.html))

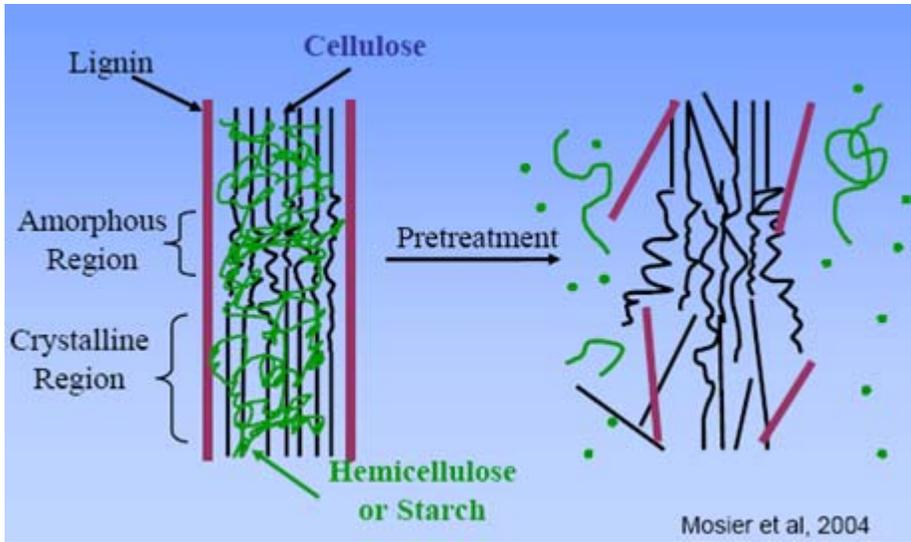


Fig.II-2. Pretreatment of biomass (Mosier et al, 2004)

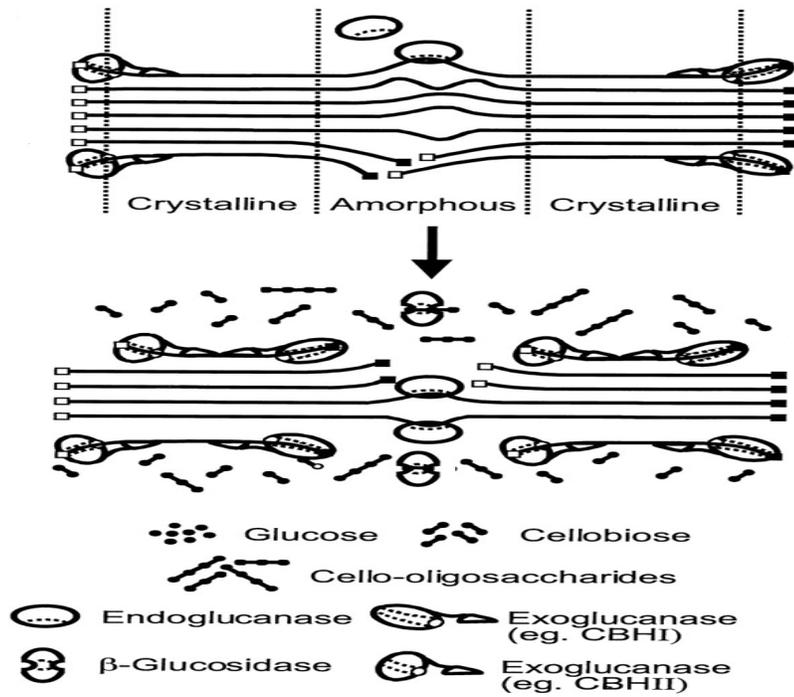


Fig. II-3. Cellulose hydrolysis by cellulases from *Trichoderma reesei*. (Lynd et al., 2002)

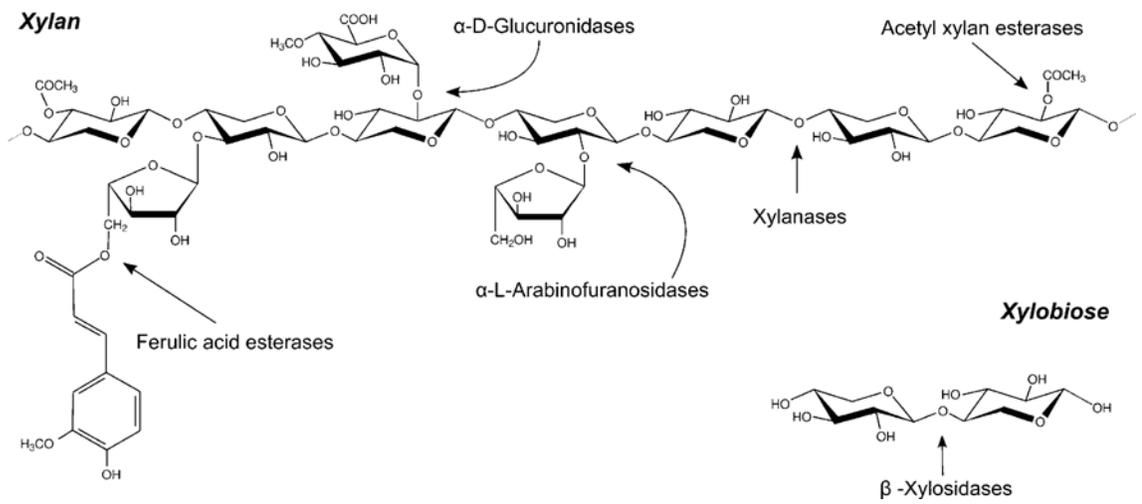


Fig. II-4. Structure of xylan and the sites of attack by xylanolytic enzymes

(Javier et al., 2007)

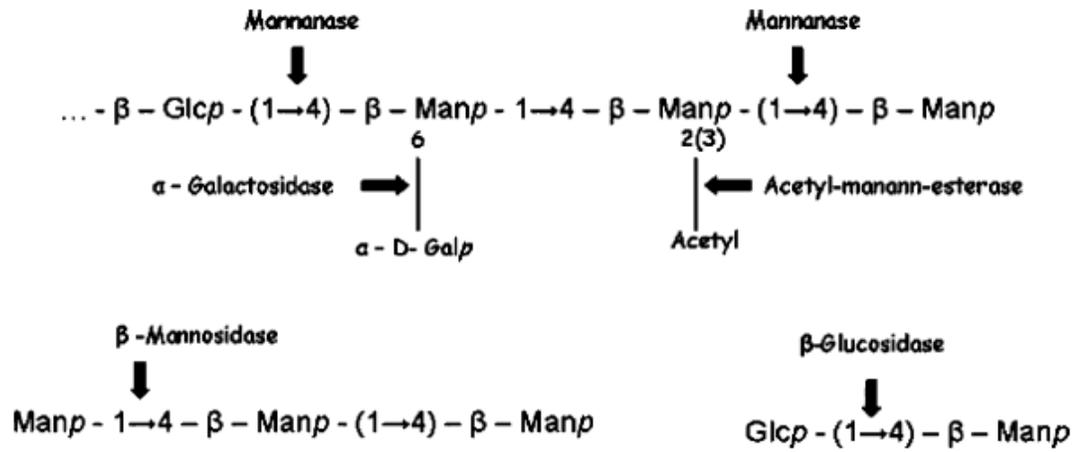


Fig. II-5. Mannanses attack on galactoglucomanna (Puls and Schuseil, 1993)

### **III. Bioconversion of Kraft Paper Mill Sludges to Ethanol by SSF and SSCF**

#### **3.1 Abstract**

Paper mill sludge is a solid waste material composed of pulp residues and ash generated from pulping and paper making processes. The carbohydrate portion of the sludge has chemical and physical characteristics similar to pulp. Because of its high carbohydrate content and well dispersed structure, the sludges can be biologically converted to value-added products without pretreatment. In this study, two different types of paper mill sludges, primary sludge and recycle sludge, were evaluated as a feedstock for bioconversion to ethanol. The sludges were first subjected to enzymatic conversion to sugars by commercial cellulase enzymes. The enzymatic conversion was inefficient because of interference by ash in the sludges with the enzymatic reaction. The main cause was that the pH level is dictated by  $\text{CaCO}_3$  in ash, which is two units higher than the pH optimum of cellulase. To alleviate this problem, simultaneous saccharification and co-fermentation (SSCF) using cellulase (Spezyme CP) and recombinant *Escherichia coli* (ATCC-55124), and simultaneous saccharification and fermentation (SSF) using cellulase and *Saccharomyces cerevisiae* (ATCC-200062) were applied to the sludges without any pretreatment. Ethanol yields of 75-81% of the theoretical maximum were obtained from the SSCF on the basis of total carbohydrates. The yield from the SSF was also in the range of 74-80% on the basis of glucan. The SSCF and SSF proceeded under

stable condition with the pH staying near 5.0, close to the optimum for cellulase. Decrease of pH occurred due to carbonic acid and other organic acids formed during fermentation. The ash was partially neutralized by the acids produced from the SSCF and SSF and acted as a buffer to stabilize the pH during fermentation. When the SSF and SSCF were operated in fed-batch mode, the ethanol concentration in the broth increased from 25.5 and 32.6 g/L to 42 and 45 g/L, respectively. The ethanol concentration was limited by the tolerance of the microorganism in case of SSCF. The ethanol yield in fed-batch operation dropped to 68% for SSCF and 70% for SSF. The high solids condition in the bioreactor appears to create adverse effects on the cellulase reaction.

### **3.2 Introduction**

The pulp and paper industry generates 4-5 million tons of sludges per year in the US (Fan and Lynd, 2006a). The disposal of sludges by incineration or landfill causes substantial financial burden and is a source of various environmental problems. Incineration is a significant cost factor because of low energy efficiency caused by high moisture content (Fan and Lynd, 2006a; Scott et al., 1995; Sjöde et al., 2007). The fibers from the paper mill sludge have characteristics suitable for conversion to value-added products. Sludges from kraft paper mill have high carbohydrate and low lignin contents in comparison to natural lignocellulosic substrates. The composition of the organic portion of the sludge is very similar to that of commercial grade pulp: mostly cellulose with low amounts of hemicelluloses and lignin. It also has well-dispersed structure and high surface area. It has been proven to be highly efficient for bioconversion to ethanol (Lark et al., 1997). Although it is projected that a majority of ethanol is to be produced

from natural lignocellulosic feedstock, paper sludge is a resource that may have opportunities for commercialization.

As a feedstock for bioethanol, paper mill sludges offer two significant economic benefits. First, the cost of sludge is essentially zero and may even be a negative number considering that the cost of disposal is about \$20 per wet ton (Randy Abston, Boise Paper, Jackson, AL, USA, personal communication, 2008). Second, elimination of pretreatment can significantly reduce the production cost of ethanol. Pretreatment opens up the biomass structure and makes it more amenable for enzymatic hydrolysis. It is also a major processing units in bioethanol plant accounting for about 30% of the total production cost of ethanol, second only to the raw material (Galbe and Zacchi, 2002). In alkaline delignification of biomass such as Kraft pulping, lignin is predominantly degraded by various cleavage reactions of aryl ether linkages (Sjöström, 1993). The Kraft pulping process also removes about 50% hemicellulose and brings about changes in the structure of fiber (Sjöström, 1993). The fibers in the sludge are short because they are screen rejects from paper machine and the digester, and are easily digested by cellulase enzymes (Fan and Lynd, 2006a; Fan and Lynd, 2006b; Scott et al., 1995; Jeffries and Schartman, 1999; Fan, et al., 2003).

In this study, sludge generated from a paper mill was investigated as a feedstock for ethanol production. The main objectives of the investigation were to characterize the sludges in terms of composition and physical properties and to test the technical feasibility of converting the feedstock to ethanol without pretreatment. Bioconversion of pulp mill sludge has been previously investigated, wherein the pH of the sludge was pre-adjusted by hydrochloric acid (Marques et al., 2008) or by sulfuric acid (Lynd et al.,

2001). This strategy may encounter a problem when the sludge has a large amount of ash, mainly  $\text{CaCO}_3$ , such as the one used in this work since the amount of acid to neutralize the ash would be excessive. SSF of paper mill sludge was also investigated by Fan, et al. (Fan and Lynd, 2006b), in which pH in the medium was adjusted by sulfuric acid. However, the effect of ash on the pH during the course of SSF was not addressed. The high ash content in paper mill sludges, specially  $\text{CaCO}_3$ , causes several problems in the bioconversion process as it interferes with pH control, limits solid loading capacity in the bioreactor and reduces the efficiency of enzyme in a bioprocess. Additional technical issues associated with the contaminated substrate were also addressed in this investigation.

### **3.3 Materials and Methods**

#### **3.3.1 Materials**

Feedstock: The paper mill sludges were collected from a Kraft paper mill, Boise Paper Company (Jackson, AL, USA). There were two different types of sludges. One was primary sludge (PS), discharged from the paper machine and the digester, which was collected from the primary wastewater clarifier unit. The other one was recycle sludge (RS), which came from a reprocessing unit of recycle paper. Both were stored at 4o C. A sample quantity of wet hardwood pulp (HP) was also obtained from Boise Paper Company. It was used as a reference substrate in bioconversion studies. The paper sludge and hardwood pulp were analyzed for carbohydrates, moisture content, and ash content according to the National Renewable Energy Laboratory (NREL) standard procedure (NREL, 2008). HP was analyzed to contain 76.0% glucan, 21.1% xylan, and 2.9 wt. % unaccounted for (lignin, acetyl group, ash, and protein).

Enzymes: Cellulase enzyme (Spezyme CP, Lot No. 301-00348-257) was a kind gift from Danisco Genencor (Palo Alto, CA, USA) and its activity was measured to be 59 FPU/mL.  $\beta$ -glucosidase (Novozyme188, Cat. No. C-6150) was purchased from Sigma (St. Louis, MO, USA) and its activity was measured to be 750 CBU/mL.

Microorganism: The microorganism used in the SSF was *Saccharomyces cerevisiae* ATCC-200062 (NREL-D5A). The growth media was YP medium, which contained 1% yeast extract (Sigma, Y-0500) and 2% peptone (Sigma, P-6588). Recombinant *Escherichia coli* ATCC-55124 (KO11) was for the SSCF experiments. This organism was grown in LB medium (Sigma, L-3152), which contained 1% tryptone, 0.5% yeast extract, 1% NaCl, and 40 mg/L chloroamphenicol.

### **3.3.2 Contaminant Microorganism Test**

A YPD agar (Sigma, Y-1500) or LB agar (Sigma, L-3027) plate was placed on a turntable, and 10  $\mu$ L of sludge solution was dispersed evenly on the plate. Tetracycline and cycloheximide were added to another plate for comparison purpose. The YPD or LB agar plate was then incubated at 30 °C for 48 h. Samples from each flask were streaked on an YPD or LB agar plate to check microbial contamination. The gram stain was used to differentiate gram-positive and gram-negative bacteria. Colony forming unit (CFU) tests were performed to determine the concentration of microorganism cells. YPD or LB agar plates were prepared for this purpose by diluting the culture with sterile saline (0.89% NaCl solution) to obtain a spread plate cell count of 30-300 cells per plate.

### **3.3.3 Digestibility Test**

The enzymatic digestibility of the sludge was determined according to NREL Chemical Analysis and Testing Standard Procedures (NREL, 2008). Screw-capped 250

mL Erlenmeyer flasks were used as the hydrolysis reactor. The sludge samples were suspended in DI water and put into the flasks to reach a total working volume of 100 mL with sludges loading such that the glucan content became 1% (w/v) in the reactor. Two different sterilization methods were applied. Sludges were steam sterilized by autoclaving at 121 °C for 15 min, or tetracycline and cycloheximide were added into the reactor to maintain sterile condition. The enzymatic digestibility tests were carried out at 50 °C in an incubator shaker (New Brunswick Scientific, Innova-4080) agitated at 150 RPM. For hardwood pulp, enzymatic hydrolysis was carried out under three different conditions: pH 4.8 (0.05 M sodium citrate buffer), pH 7.0 (0.02 M sodium phosphate buffer), and with addition of CaCO<sub>3</sub> to the level of 0.5 g/g-glucan matching that of sludge. Cellulase and β-glucosidase were also added to maintain enzyme loading at 15 FPU and 30 CBU/g-glucan, respectively. The cellulase enzyme was supplemented with β-glucosidase at a ratio of 2 CBU/FPU. The enzymes were filter-sterilized. Hydrolyzate samples were taken at 6, 12, 24, 48, and 72 h and analyzed for glucose, xylose, and cellobiose. Total released glucose and cellobiose after 72 h of hydrolysis were used to calculate the enzymatic digestibility:

$$Digestibility(\%) = \frac{Glucose\_released(g) + 1.053 \times Cellobiose\_released(g)}{1.111 \times Glucan\_added(g)} \times 100$$

Hardwood pulp was put through the same procedure as a control. The digestibilities for xylan content were determined in a similar manner. For xylan digestibility, hydration factor of 1.136 was used in the equation. Reported digestibility value are the average of duplicate test with standard deviation (SD) smaller than 1.0.

### 3.3.4 Simultaneous Saccharification and Fermentation /Co-fermentation

A 250-mL Erlenmeyer flask was used as the bioreactor. It was operated in an incubator shaker (New Brunswick Scientific, Innova-4080) at 37°C with 150 RPM with 100 mL working volume. Sludges and growth medium were added such that the glucan content becomes 3% w/v or 6% w/v. Hardwood pulp was also put through the same procedure as the control. The sludge samples were steam sterilized at 121 °C for 15 min. The growth media for SSF was YP medium. The growth media for SSCF was LB medium (Sigma, L-3152). For SSCF, 40 mg/L aseptic chloroamphenicol was added after autoclaving.

SSF and SSCF of the sludges were carried out at 37 °C without pH control. SSF and SSCF of the hardwood pulp were carried out at 37 °C with pH 4.8 (0.05 M sodium citrate buffer) and pH 6 (0.05 M sodium phosphate buffer), respectively. Cellulase and  $\beta$ -glucosidase were also added to achieve enzyme loading at 15 FPU and 30 CBU/g-glucan, respectively. Optical density (OD) was measured by UV Spectrophotometer (BioTek Synergy HT Multidetector Microplate Readers) at 600 nm for yeast (NREL-D5A) and 550 nm for recombinant *E. coli* (KO11). The initial OD after inoculation was 0.05, equivalent to 16 mg dry cell weight of KO11/L and 50 mg of NREL-D5A/L. In all of the microbial experiments, a sample from each flask was taken at the end of the run and streaked on an YPD plate to check for contamination and checked under an optical microscope. CFU tests were performed using YPD plates or LB agar to check microorganism viability. The SSF and SSCF experiments requiring pH monitoring were carried out using a 1-L bioreactor (Multifors IHORS HT 2×1L) with 400 mL working volume.

The ethanol yield was calculated as follows:

$$\text{Ethanol yield}[\% \text{ of theoretical maximum}] = \frac{\text{Ethanol produced (g) in reactor}}{\text{Initial Sugar (g) in reactor} \times 0.511} \times 100$$

Sugar is interpreted as glucose in the SSF and glucose plus xylose in the SSCF.

The fed-batch experiments were started with 100 mL initial working volume and 6% w/v glucan loading. Squeezed sludge cakes equivalent to 6 g-glucan were added to the bioreactors at desired times. The cellulase and  $\beta$ -glucosidase were also added at beginning of fermentation process to achieve overall enzyme loading at 15 FPU and 30 CBU/g-glucan based on total glucan. Addition of sludge was done at 24 h point for SSCF and at 24 and 48 h points for SSF. Samples were analyzed for glucose, xylose, organic acid, and ethanol by high pressure liquid chromatography (HPLC). Aseptic conditions were maintained in all of the microbial experiments. The fed-batch fermentation runs were made in triplicate.

### **3.3.5 Analytical Methods**

The solid samples were analyzed for carbohydrates and Klason lignin following the NREL standard procedures (NREL, 2008). The moisture content was measured by an infrared moisture balance (Denver Instrument, IR-30). Sugars were determined by HPLC using a HPX-87P column. For the SSF or SSCF tests, BioRad-HPX-87H column was used for measurement of sugar, organic acid and ethanol. A refractive index detector was used with the HPLC. The acid-insoluble ash was determined following the Tappi test method (Test Method T 244 cm-99). Liquid sample analysis and ash determination were done in triplicate.

The alkalinity of the sludge was determined by titration with 1N H<sub>2</sub>SO<sub>4</sub> to pH 5.0 and expressed in terms of milliliters of 1N H<sub>2</sub>SO<sub>4</sub>/10 g dry sludge.

The buffering capacity of the paper sludges was determined by titration. The experiments requiring pH monitoring were carried out using 1-L bioreactor (Multifors IHORS HT 2×1L) with 400 mL working volume at 37 °C and 150 RPM. The sludges were added such that the glucan content becomes 3% (w/v). The sludge slurries were titrated with 20 g/L acetic acid. Deionized water was used as a reference. Where applicable, statistical computation including mean value and standard deviation was performed using Microsoft Office Excel 2003.

### **3.4 Results and Discussion**

#### **3.4.1 Composition**

The compositions of primary sludge and recycle sludge are shown in Table III-1. The composition of corn stover is also provided as a reference. Paper mill sludges are high in glucan and low in lignin in comparison to corn stover. They contain a very high amount of ash, about one third of the total mass. The majority of ash content in sludge is from filler material added during the paper making process: clay, TiO<sub>2</sub> and CaCO<sub>3</sub>. It is added as fine particles of 0.1 - 10 micrometer (Biermann, 1996). The ash was further analyzed to determine acid-soluble fraction and acid-insoluble fraction. Based on the Boise Paper manufacturing process and the analysis data, we conclude that the acid-soluble ash is mainly CaCO<sub>3</sub>, and acid-insoluble ash is mainly TiO<sub>2</sub> and clay.

#### **3.4.2 Enzymatic Digestibility**

The enzymatic digestibility data of sludges and a reference substrate are summarized in Table III-2. Hardwood pulp was taken as the reference substrate because the composition is similar to the organic portion of the sludges. Digestibility tests were made at two different temperatures of 37° C and 50° C. Although the optimum

temperature for enzymatic hydrolysis was 50° C, tests at 37° C were also made in consideration of SSF/SSCF investigated in this work. Digestibilities were determined for glucan and xylan separately. For sludges, tests were carried out without pH control because of the high ash content which could neutralize any acid. The pH of sludge digestion remained near neutral. For hardwood pulp, enzymatic hydrolysis was carried out under three different conditions: pH 4.8 (optimum for cellulase), pH 7.0, and with addition of CaCO<sub>3</sub> to the level of 0.5 g/g-glucan matching that of sludge. The enzymatic digestion of sludges was dismal giving sugar yields of only 8 - 32% of theoretical maximum. The highest digestibility observed was that of hardwood conducted at pH 4.8 and 50° C which stood at 94.6% and 66.3% for glucan and xylan, respectively. The data clearly indicate that the digestibilities of sludges are far below to those of hardwood pulp tested at pH 4.8. Hardwood pulp tested at pH 7.0, although much lower than that tested at pH 4.8, was significantly higher than that tested with addition of CaCO<sub>3</sub>. These data collectively indicate that the inefficient digestion of sludges is mainly due to the presence of ash in the sludges. The high level of ash in sludge made it difficult to control pH because of the buffering action of CaCO<sub>3</sub>. Also to be noted here is that the sugar yield obtained at 37° C was higher than that obtained at 50° C. This result is unexpected since 50° C is close to the optimum temperature for the cellulase enzymes. We have no clear explanation to offer at this time. It may be that the cellulose-enzyme reaction may be hampered by certain ionic components released from the sludges, and more of such components are released at high temperature.

The cellulase enzyme used in the test was supplemented with β-glucosidase at the level of 2 CBU/FPU, to suppress inhibition by cellobiose. Cellobiose was nonexistent in

the enzymatic hydrolysis of hardwood pulp at pH 4.8 with this enzyme formulation, but its accumulation was significant in all of the neutral pH runs. Cellobiose was higher also at 37 °C, versus 50 °C where two conditions were not favorable for fungal cellulases and  $\beta$ -glucosidase. Their activities peak at  $50 \pm 5$  °C and pH of 4.0 - 5.0 (Saddler and Gregg, 1998). This perhaps is an indication that  $\beta$ -glucosidase is more sensitive to pH and temperature than the other cellulose components. Marques et al. found the optimum condition of enzymatic hydrolysis of recycle sludge is at 35 °C and pH 5.5. The recycle sludge used in their study was neutralized with hydrochloric acid before enzymatic hydrolysis (Marques et al., 2008).

### **3.4.3 Microbial Contamination of Sludges**

Enzymatic hydrolysis of sludges conducted with addition of antibiotics without sterilization has shown very poor results giving digestibility of less than 10% (Table III-2). To test bacterial contamination, sludge solution was sampled on YPD agar plate. After the incubation, the gram-negative bacteria and molds were identified from the gram stain test and the morphology of the microorganism. It appears that tetracycline and cycloheximide provide only a partial protection as we observed that some microorganisms still grew during the enzymatic hydrolysis. Steam sterilization of sludges was therefore necessary to eliminate the contamination problem.

### **3.4.4 Simultaneous Saccharification and Fermentation**

Recognizing the difficulty of enzymatic hydrolysis of sludges, other means of improving the process were sought. The first was to remove the ash content of the sludges before subjecting them to bioconversion. This work is currently in progress in our laboratory. The second approach was to apply a different and more advanced

bioconversion scheme for the sludges, namely, the simultaneous saccharification and fermentation. The SSF is now considered as the norm for the bioconversion of lignocellulosic feedstocks for a number of reasons. One reason is that the enzymatic conversion becomes more efficient because of the inhibition of sugars to the enzyme can be removed. Simultaneous use of cellulase and microorganism makes the SSF proceed under sugar-limited conditions so that the sugar concentration in the bioreactor is kept below the inhibition threshold. According to Varga et al (Varga et al., 2002) product inhibition starts to occur at 15 g/L of glucose. In addition, the sugar yield obtained from the enzymatic digestion of sludges was very low since the pH of sludge digestion remained near neutral because of high ash content in the sludge. Some ethanologen microorganisms, especially yeast, have a fairly broad pH range, 3.0 - 6.0. The pH range for *E.coli* is near the neutral point. Therefore, the fermentation of sludges by ethanologen microorganisms could perform differently.

With this understanding, a number SSF runs were made using the paper mill sludges without removing the ash. The enzyme mix and loading were identical to those of the digestibility test. *S. cerevisiae*, ATCC-200062 (NREL-D5A) was used for the conversion of glucose to ethanol. The SSF of sludges was run at 37° C without pH control. Two different substrate loadings were applied: 3% and 6% glucan (*w/v*). The time-course results of the SSF run are shown in Fig.III-1, and the yield data are shown in Table III-3. The data summarized in Table III-3 shows that approximately 80% of the maximum theoretical yield is achieved with 3% glucan loading and 75% yield with 6% glucan loading, corresponding to ethanol concentration of 13.6 and 25.5 g/L, respectively. The yield of SSF is calculated on the basis of glucan in the sludge because

*S. cerevisiae* can only convert glucan, xylan being left unconverted. The 1:1 mixture of the two sludges gave about the same ethanol yield.

The results show that conversion of sludges through SSF is far superior to enzymatic digestion. The 75-80% yield of ethanol from the SSF of the sludges is a drastic improvement over the 8-32% sugar yield obtained from the enzymatic digestion. It is surprising because the front part of the SSF is the enzymatic digestion. The profiles of glucose, xylose, and ethanol follow the pattern of well-behaving SSF (Fig. 1). The glucose concentrations remained below 0.1 g/L after 24 h signifying that the yeast was rapidly consuming any available glucose. The xylose concentrations remained unchanged after 24 h since the yeast NREL-D5A cannot convert xylose to ethanol. The two different sludges showed identical conversion pattern. Although the terminal ethanol yields were the same, hardwood pulp was converted somewhat faster than the sludges.

The main reason for the improved performance of SSF appears to be the low pH level observed in the SSF. The SSF run started at a pH of 7.05 for primary sludge and 7.12 for recycle sludge and decreased to pH of 5.73 and 5.69, respectively, a level close to the optimum for cellulase and for *S. cerevisiae*. After the initial drop, the pH stayed relatively constant throughout the SSF. Since pH is a critical factor in bioconversion of sludges, we investigated further on it to verify the reason for the pH drop. We have measured the level of acetic acid and lactic acid in our SSF experiments. The maximum acetic acid level was 1.8 g/L, and lactic acid was 2.8 g/L. We speculated that these acids may be the reason for the pH drop. These organic acids produced in quantities are not enough to dissolve all acid-soluble ash. But they may interact with  $\text{CaCO}_3$  in the sludge forming buffers, Ca-acetate or Ca-lactate, eventually lowering the pH.

To test this possibility, we have carried out titrations adding acetic acid into deionized water and water-sludge mixtures, simulating the SSF condition. As shown in Fig. 2, pH drop in water was rather drastic changing from 7.0 to 3.5 with addition of 45 mL of 20 g/L of acetic acid to 500 mL deionized water (equivalent to 4.5 g acetic acid per liter matching the total acid produced in the SSF). In the same test with water-sludge mixtures, the pH drop was much slower, decreasing from 7.0 to 6.6 with addition of same amount of acetic acid. The results indicate that acid production by yeast, although contributing to pH drop, cannot explain the entire pH drop in the SSF.

Another reason one can deduce is carbon dioxide produced by yeast. It is produced in large quantities; about the same amount as ethanol, although most of it escapes from the broth during the SSF due to its low solubility in water. Carbon dioxide in the broth may exist in a supersaturated condition before it is released from the broth. One sample of supersaturated carbon dioxide is carbonated soft drinks, which show pH in the range of 2.5 - 4.2. The extent of supersaturation during fermentation can be quite high even under atmosphere pressure because it has to overcome the surface tension before it is released. If so, contribution of carbon dioxide to pH change is further increased to the level that it can explain the observed pH drop. Kraemer et al. found that CO<sub>2</sub> was supersaturated during fermentative hydrogen production regardless of N<sub>2</sub> sparging (Kraemer and Bagley, 2006). To what extent carbonic acid contributes to the pH drop of SSF is unknown at this time. The organic acid and carbonic acid may interact to give additive effects to account for the observed pH drop. Whatever the reason may be, we emphasize that a normal operation of SSF of untreated paper mill sludge proceeds well

without pH control to give satisfactory performance as a process converting the feedstock to ethanol.

### **3.4.5 Simultaneous Saccharification and Co-fermentation**

In order to utilize the xylan content in the sludge, another process scheme was investigated, namely, simultaneous saccharification and co-fermentation. This process uses recombinant *E. coli* ATCC<sup>®</sup> 55124 (KO11) in place of the yeast in SSF. This organism is known to convert xylose as well as glucose to ethanol with high efficiency (Ohta et al., 1991). The overall performance data and the time-course data of SSCF are presented in Table III-3 and Fig. III-3. The SSCF of sludges was also run at 37° C without pH control. Two different substrate loadings were applied: 3% and 6% glucan (*w/v*). The ethanol yields obtained from SSCF are similar to those of SSF ranging from 75% to 80%. These yields are calculated on the basis of total carbohydrates (glucan and xylan) for obvious reason. The yields of SSCF were also calculated on the basis of glucan only for direct comparison with the SSF. The 3% glucan-based yield of ethanol was 98.3% for primary sludge and 98.9% for recycle sludge. 6% glucan-based yield of ethanol was 95.8% for primary sludge and 96.3% for recycle sludge, corresponding to ethanol concentration of 32.5 and 32.7 g/L, respectively. In theory, this yield can surpass 100%. On the average, SSCF can produce 25% more ethanol from the same feedstock primarily because of the ability of KO11 to convert xylose to ethanol. The yields were slightly higher with 3% glucan loading than with 6% glucan loading. Elshafei et al. reported that substrate loading between 0.5-5% total solids in SSF was not affected by substrate or ethanol inhibition (Elshafei, et al., 1991). In this work, total solid loading was much

higher, ranging from 6% to 14% (w/v). Here again the presence of high ash content in the sludges was not a problem in operation of the SSCF.

The time-course profiles of various observable parameters are shown in Fig. 3. There were discernable differences in sugar and pH profiles from those of SSF. The pH again dropped sharply in the early phase of the SSCF, and remained relatively constant afterwards. The SSCF was started at pH 7.0, decreased to pH of 5.93 for primary sludge and 5.89 for recycle sludge, which is close to the optimum for KO11 (pH of 6) but somewhat higher than those of SSF. The same argument made for the pH drop of SSF also applies here. During the SSCF, acetic acid and lactic acid were detected at the level of 3.0 and 1.0 g/L, respectively. The acid-soluble ash can dissolve under weak acidic condition and acts as a buffer. The ethanol conversion rate of hardwood pulp was faster than the sludges, but the terminal ethanol yields were essentially the same. The xylose concentration of both sludges decreased after 12 h. This is a positive proof that the xylan fraction is utilized in the SSCF by KO11.

The profiles of glucose in Figures 1 and 3 showed that both SSF and SSCF proceed under a glucose-limited condition, which means the enzymatic reaction is the rate-limiting step. In the SSCF, xylose consumption occurred shortly after depletion of glucose. This is a classic example of diauxic consumption of substrates wherein glucose is preferred to xylose. The SSCF applies only to a feedstock that contains both glucan and xylan. It also requires cellulase enzyme that has built-in xylanase activity. Most of the commercial “cellulases” including Spezyme CP have xylanase activity. The reacted residues after SSF and SSCF were analyzed for glucan and xylan contents (Table III-4). For both sludges, the SSF residues have higher unreacted glucan and xylan than SSCF

residues. The fact that two different microorganism are used has no bearing in this observation because the microbial actions are not the rate-controlling step. Therefore, the main difference between the two processes is that SSF has a buildup of xylose. It is well known that xylose has an inhibitory effect on xylanase (Paul and Varma, 1990; Copapatiño et al., 1993; Rapp and Wagner, 1986). Low xylanase activity results in less xylan removal. Unreacted xylan in solid influences the glucan hydrolysis because xylan physically surrounds cellulose fibrils in the structure of lignocelluloses and becomes a barrier impeding glucanase action. It has indeed been proven that xylan removal improves the access of cellulase enzyme to cellulose and consequently the activity of cellulase (Öhgren et al., 2007). It has also been reported that xylose has a direct inhibitory effect on cellulase (Xiao et al., 2004; Nigam and Prabhu, 1991 Kim and Lee, 2005).

#### **3.4.6 Fed-Batch Operation**

The concentration of ethanol in the bioreactor significantly affects the cost of the downstream separation process. The threshold level of ethanol concentration for economically feasible distillation is reported to be about 4 wt. % (Hahn-Hägerdal et al., 2006; Wingren et al., 2003). We have attempted the fed-batch operation (intermittent input of feed) of SSF and SSCF in an effort to increase the terminal ethanol concentration in the bioreactor. The bioreaction was started with a sludge loading of 60 g-glucan/L. Additional feedstock of the same level was put into the reactor at 24 h point (one additional input) for SSCF and at 24 and 48 h points for SSF (two additional inputs). At these input points, the reaction has proceeded far enough to liquefy the dense solid slurry and retain fluidity high enough to accept additional solid feed. The ethanol production from the fed-batch runs are shown in Fig. 4. In the case of SSCF with one additional

feeding, ethanol concentration of 42 g/L was obtained at the end of the run. The yields were identical for both sludges. This is equivalent to ethanol yield of 68% on the basis of total sugar, which is substantially lower than the yield of single batch with 6% glucan loading. *E. coli* KO11 is known to utilize several pentoses and hexoses for producing ethanol with high metabolic yield (Ohta et al., 1991). However, its growth robustness and ethanol tolerance are relatively low compared to yeast (Dien et al., 2003). We believe it is the main reason for the low ethanol yield observed from the fed-batch SSCF. Additional buildup of ash from the sludge may also have played a role in lowering the ethanol yield. Xylose utilization in the fed-batch SSCF was somewhat inefficient compare to single-feed runs, as evidenced by relatively high xylose concentration in the reactor which remained at 2 g/L even after 72 h. On the other hand, glucose had a short initial buildup and then remained below 0.1 g/L after 24 h. In the fed-batch SSF runs with two additional feedings, the final ethanol concentration of 45 g/L was achieved. The overall ethanol yield in the fed-batch SSF was approximately 70% based on glucan, which was also lower than the yield of single batch with only 6% glucan loading. In fed-batch SSF using yeast (ATCC-200062), ethanol concentration higher than SSCF was obtained, yet the overall yield was lower. Viability of the cells at the end of fed-batch SSF was tested by measurement of cell count on agar plate. The measured cell concentration of  $5 \times 10^7$  CFU /mL indicated that the cells remained highly viable at the end of the operation. It appears that the cellulase enzyme somehow becomes less effective under high solid loading. The interference of high ash with the enzyme, unproductive binding with increased lignin content after successive feeding, product inhibition by ethanol and/or acids, and substrate inhibition by unreacted xylose are the potential reasons for the low

yield of the fed-batch SSF. Mass transfer resistance at the solid - liquid interface and within the solids may further increase the local concentration of some of the inhibitory components. The exact reasons and the mechanisms associated with these phenomena are yet to be determined.

### **3.5 Conclusion**

Kraft paper mill sludges have features desirable for bioconversion to value-added products. The organic component of the sludges is essentially the same as commercial grade pulp, high in carbohydrates and low in lignin. Its small size and high surface area make it amenable for biological conversion without pretreatment. However, enzymatic hydrolysis of the sludges is inefficient because of the high ash content. The buffering action of the calcium carbonate in the ash keeps the pH near to the neutral point and two units higher than the optimum pH of cellulase.

SSF based on Spezyme CP and *S. cerevisiae* (D<sub>5</sub>A), and the SSCF based on the same enzyme and the recombinant *E. coli*-KO11 performed well despite the high ash content. The main reason for the improved performance is the pH drop to 5.5 and 5.8 that occurred in the SSF and SSCF. The decrease of pH was caused by carbonic acid and organic acids produced during fermentation. The glucan-based ethanol yield for the primary sludge and recycle sludge in the SSF were 79.5% and 80.0%, respectively. In the SSCF using recombinant *E. coli* KO11, xylose was effectively utilized. The ethanol yield based on glucan and xylan was 80.1% and 77.1 %, respectively, for the primary sludge and recycle sludge. Fed-batch SSF and SSCF were attempted to increase the ethanol concentration. The ethanol concentration in the broth was increased to 42 and 45 g/L, for two-feed SSCF and three-feed SSF, respectively. The yields of the fed-batch runs were

lower at 68% for SSF and 70% SSCF. Further increase of ethanol concentration in the SSCF was difficult because of the low ethanol tolerance of *E.coli* KO11. Yields are lower for multiple-feed fed-batch runs than the single-feed runs for both SSF and SSCF. The low conversion yield is related to the cellulase enzyme reaction rather than microbial reaction. High-solid conditions in the bioreactor and the product and substrate inhibition are believed to be the major factors suppressing the cellulase enzyme reaction.

Table III-1 Composition of sludges and corn stover

	Primary sludge	Recycle sludge	Corn stover
Glucan (%)	44.5	49.6	36.1
Xylan (%)	9.9	13.7	21.4
Lignin (%)	8.1	4.6	17.2
Total Ash (%)	36.0	30.0	7.1
Acid-soluble Ash (%)	26	22	-
Acid-insoluble Ash (%)	10	8	-
Others (%)	1.5	2.1	18.2
Alkalinity (milliliters of 1 N H <sub>2</sub> SO <sub>4</sub> /10 g dry sludge)	60	50	-

- a. All data in table are based on oven-dry original feedstocks.
- b. Data of sludges are the mean value of triplicate (n=3; standard deviation<0.5 for glucan, xylan, and lignin).
- c. The composition of the corn stover was determined by the National Bioenergy Center, NREL, Golden, CO).

Table III-2 Enzymatic digestibility of paper sludges

	Temperature (°C)	Sugar released at 72-h enzymatic hydrolysis (g/L)			72-h digestibility (%)	
		Glucose	Cellobiose	Xylose	Glucan	Xylan
Primary sludge <sup>a</sup>	50	0.6	0.3	0.2	8.3	7.9
	37	2.5	1	0.8	32.0	31.7
Recycle sludge <sup>a</sup>	50	0.7	0.3	0.3	9.2	9.6
	37	2.4	0.9	0.9	30.2	28.7
Hardwood pulp (HP) with 0.5 g CaCO <sub>3</sub> / g- glucan <sup>a</sup>	50	1.4	0.7	0.7	19.3	22.1
	37	2.6	1	0.8	32.9	25.2
HP with 0.05 M citrate buffer (pH=4.8) <sup>a</sup>	50	10.5	0	2.1	94.6	66.3
	37	9.1	0	1.8	82.0	56.8
HP with 0.05 M phosphate buffer (pH = 7) <sup>a</sup>	50	3.6	0.6	0.8	38.1	25.2
	37	4.8	0.5	0.9	48.0	28.4
Primary sludge <sup>b</sup>	37	0.7	0.3	0.1	9.2	4.0
Recycle sludge <sup>b</sup>	37	0.6	0.3	0.1	8.3	3.2

Data of sludges and hardwood pulp is the mean value of duplicate (n = 2; SD < 1.0).

- a. Sterilization methods applied by autoclaving.
- b. Sterilization methods applied by antibiotics.

Table III-3 Ethanol yield from SSF and SSCF of paper sludges

	Primary sludge (PS)		Recycle sludge (RS)		Mixture of PS and RS (1:1)	
	6 % glucan loading	3 % glucan loading	6 % glucan loading	3 % glucan loading	6 % glucan loading	3 % glucan loading
SSF ethanol yield(%) <sup>a</sup>	74.5	79.5	75.0	80.0	74.6	80.0
SSCF ethanol yield (%) <sup>b</sup>	78.0	80.1	75.1	77.1	77.0	80.5
SSCF ethanol yield (%) <sup>c</sup>	95.8	98.3	96.3	98.9	94.5	98.8

Data of sludges is the mean value of triplicate (n = 3; SD < 3.0).

- a. The ethanol yield of SSF based on glucan only.
- b. The ethanol yield of SSCF based on glucan and xylan.
- c. The ethanol yield of SSCF based on glucan only.

Table III-4 Composition analysis of fermentation residues

Component (%)	Primary sludge	Primary sludge residue <sup>a</sup>		Recycle sludge	Recycle sludge residue <sup>a</sup>	
		SSF <sup>b</sup>	SSCF <sup>b</sup>		SSF <sup>b</sup>	SSCF <sup>b</sup>
Glucan	44.5	1.6	0.5	49.6	1.9	0.5
Xylan	9.9	2.4	0.8	13.7	4.1	0.9

Data of sludges is the mean value of triplicate (n = 3; SD < 0.5 for glucan and xylan).

- a. All data in table are based on oven-dry original sludges.
- b. SSF and SSCF were based on 3% (w/v) glucan loading.

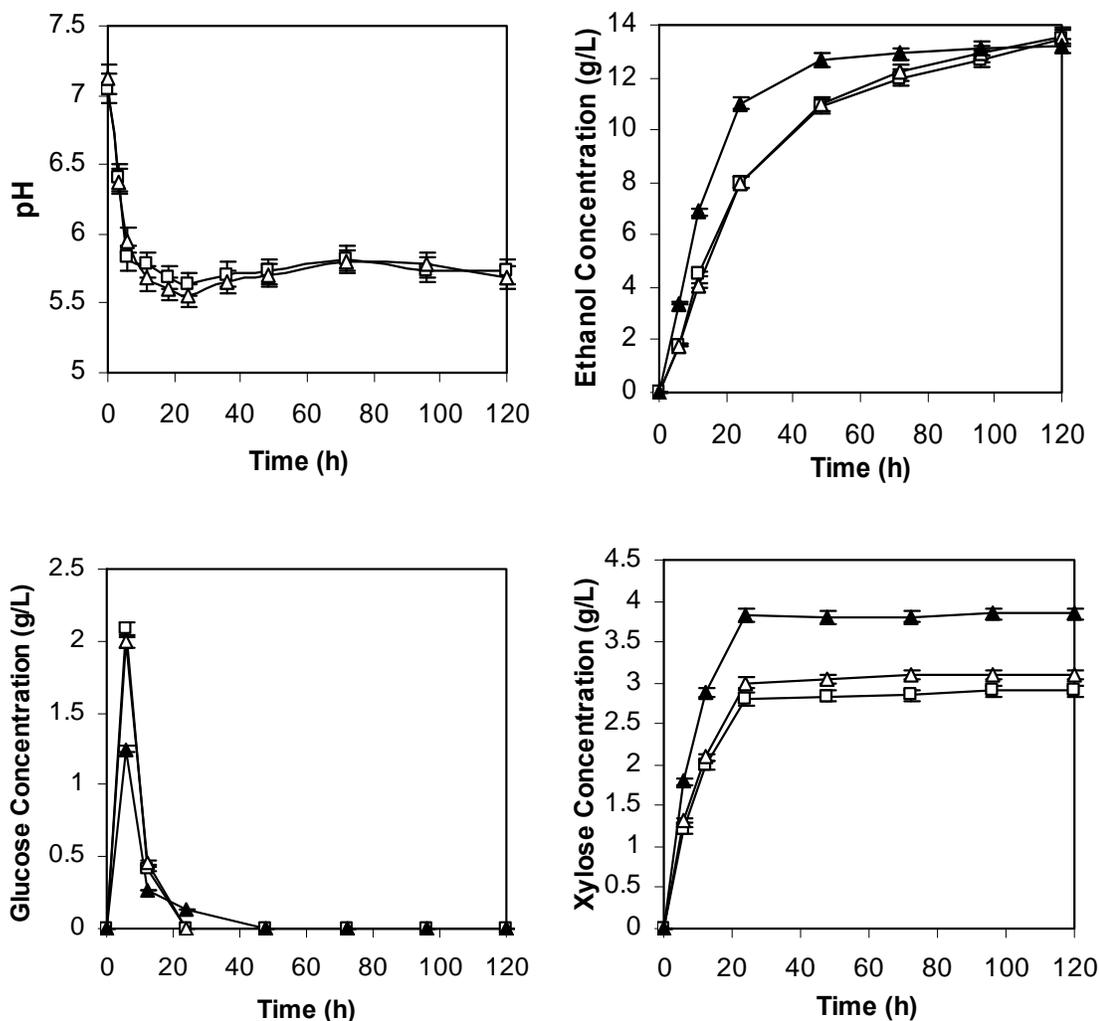


Fig.III-1 Simultaneous saccharification and fermentation (SSF) of sludges by *Saccharomyces cerevisiae* (ATCC-200062)

Squares with connecting lines represent primary sludge. Triangles with connecting lines represent recycle sludge. Filled triangles with connecting lines represent hardwood pulp.

The data points represent average of triplicate runs. The pH was controlled at 4.8 for runs with hardwood pulp. Other conditions of the SSF were: 3% (w/v) glucan loading, 15 FPU Spezyme CP + 30 CBU of Novozyme-188/g-glucan.

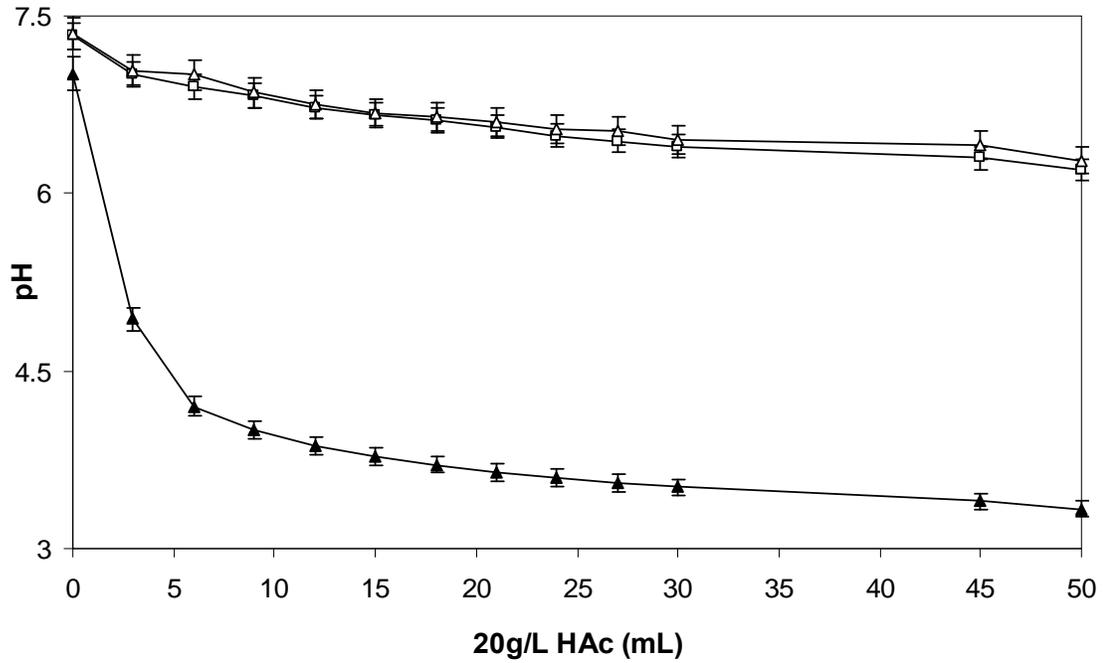


Fig.III-2 Buffer capacity titration curves with acetic acid for paper sludge slurries and deionized water

Squares with connecting lines represent primary sludge. Triangles with connecting lines represent recycle sludge. Filled triangles with connecting lines represent water.

The data points represent average of duplicate runs.

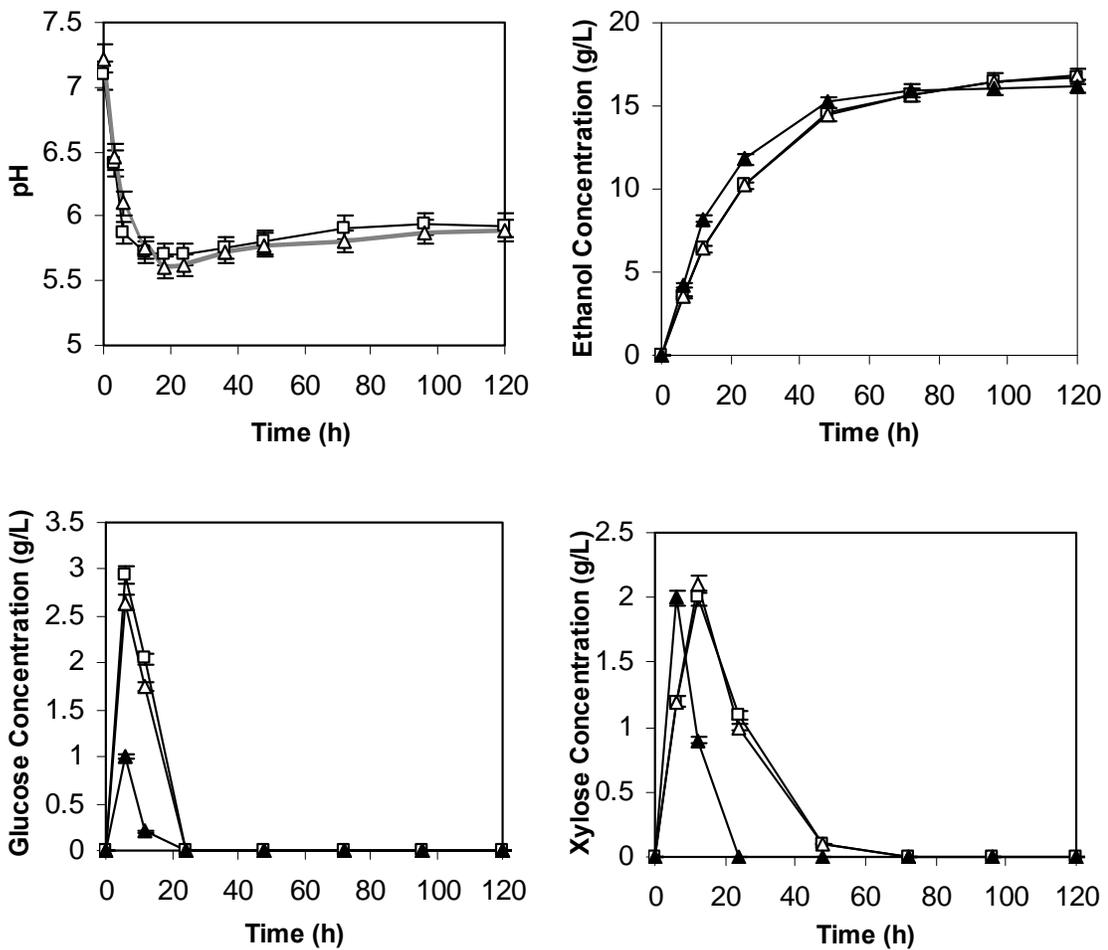


Fig.III-3. Simultaneous saccharification and cofermentation (SSCF) of sludge by recombinant *E.coli* (KO11)

Squares with connecting lines represent primary sludge. Triangles with connecting lines represent recycle sludge. Filled triangles with connecting lines represent hardwood pulp. The data points represent average of triplicate runs. The pH was controlled at 6.0 for runs with hardwood pulp. Other conditions of the SSCF were: 3% (w/v) glucan loading, 15 FPU Spezyme CP + 30 CBU of Novozyme-188/g-glucan.

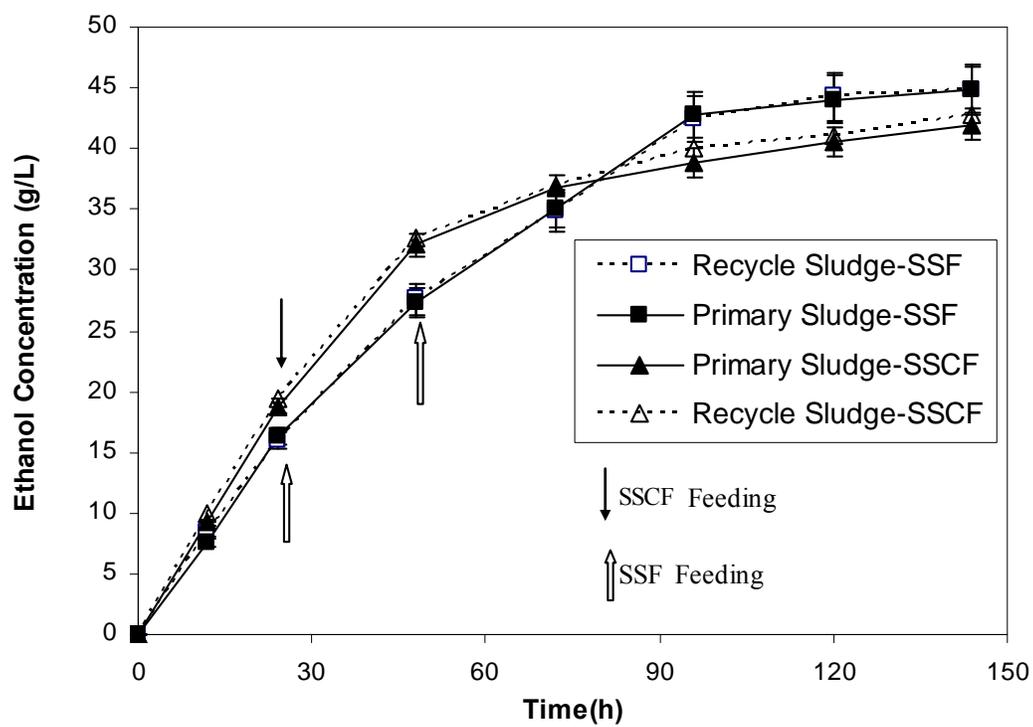


Fig.III-4. Fed-batch SSF and SSCF of sludges

The data points represent average of triplicate runs. Other conditions of the SSF were:

6% (w/v) glucan loading, 15 FPU Spezyme CP + 30 CBU of Novozyme-188/g-glucan.

## **IV. De-ashing Treatment of Paper Sludges and Enhancing Ethanol**

### **Production by SSF and SSCF of De-ashing Paper Sludge**

#### **4.1 Abstract**

The previous study in our lab, the “Bioconversion of Kraft Paper Mill Sludges to Ethanol by SSF and SSCF”, has demonstrated that paper sludges with high ash content work well in both Simultaneous Saccharification and Co-Fermentation (SSCF) and Simultaneous Saccharification and Fermentation (SSF). However, operations of using sludges with high ash content are difficult. In this work, de-ashing processes before SSCF and SSF were developed to overcome the difficulty. Low ash content sludges provide relatively higher ethanol yields, a result that was achieved with enzyme loading of 15 FPU/g-glucan. Reduction in enzyme loading to 10 FPU/g-glucan decreased the ethanol yield, but a 70% yield was still obtained. The effect of different levels of solid loading in the fermentation test was also investigated. In addition, different low-cost fermentation nutrients were evaluated for their effectiveness in the fermentation of paper sludges to produce ethanol. When the SSCF and SSF were operated in fed-batch mode by using low-ash content sludges, the ethanol concentration in the broth was increased to 48 g/L and 60 g/L, respectively.

#### **4.2 Introduction**

Feedstock and the enzymes were identified as two primary cost items in the biomass conversion processes (Nguyen and Saddler, 1991; Aden and Foust, 2009). In our

previous study, we have demonstrated that the SSF based on Spezyme CP and *Saccharomyces cerevisiae* (NREL-D5A), and the SSCF based on the same enzyme and the recombinant *Escherichia coli* -KO11 performed reasonably well in bioconversion of untreated kraft paper sludges to ethanol in spite of the high ash content (Kang et al.,2010). SSCF performed with 13.5% solid loading of primary paper sludge resulted ethanol concentration of 3.25 wt. %; and SSF with the same feed resulted ethanol concentration 2.65 wt. %. Higher ethanol concentration is achieved with high solid loading. However, operation of high solid with high ash content creates extremely high viscosity causing difficulty in mixing and pumping. High viscosity and non-Newtonian behavior of the broth require strong agitation in order to provide adequate mixing during fermentation. The ash content comes primarily from paper filler materials such as clay (kaolin) and  $\text{CaCO}_3$ , etc. In our previous study, we found that the sludge hampers the enzymatic reaction primarily by the buffering action of the  $\text{CaCO}_3$  in the ash, which drifts the pH away from the optimum value for cellulase (Kang et al., 2010). Nikolov et al., (2000) reported that fillers and other additives in the paper form an adhesive “envelope” around the cellulose fiber to obstruct the access of the enzymes to the cellulose substrate. Clay minerals are known to form aggregates with organic molecules, and the formation of enzyme-clay complexes could alter the level of enzyme activity (Tietjen and Wetzel, 2003; Cabezas et al., 1991; Haska, 1981). Additional evidences were also found that clay had an inhibitory effect on cellulase activity (Hamzehi and Pflug, 1981; Pflug, 1982; Tothill et al., 1993). The high ash content in paper mill sludges also limits the cellulose loading capacity in the bioreactor since it lowers the cellulosic portion of the feedstock. Much of these problems can be alleviated by de-ashing of the sludges.

A number of pulp and paper mills have implemented reclamation of fiber, fillers, or both from the sludges to reuse the separated materials and also as a means to reduce sludge volume in the pulp and papermaking processes (Hardesty and Beer, 1993; Rosenqvist, 1978; and Soderhjelm, 1976). Several different methods were used to recover the fiber and/or fillers from paper sludge (Leuthold and Leuthold, 1996; Maxham, 1994; Simpson and Lam, 1998). The equipment used to separate fiber or fillers from sludge varies but it can be as conventional as screens and cleaners (Dorica and Simandl, 1995). Reclamation and reuse of sludge in pulp and papermaking processes, however, has not been practiced widely because of the concern that it may lower the quality of the final product. In normal process of de-ashing, the purity of the fiber has inverse relationship with the recovery yield of the fiber. For bioconversion purpose, the de-ashing operation needs to be optimized in order to recover as much fiber as possible while keeping the ash content low enough to be accepted as a fermentation feedstock. Various chemical treatments were also used for de-ashing (Lynd et al., 2001; Marques et al., 2008). However, they are not deemed appropriate for application in bioconversion feedstock because of high processing cost and safety issues.

Fermentation nutrient is a significant factor in bioconversion of lignocellulosic biomass to ethanol. Expensive complex nutrients, such as yeast extract and peptone, are utilized in most laboratory studies, the cost of which is prohibitively high for commercial production. Corn steep liquor is a byproduct of corn wet-milling and has been used as a fermentation nutrient in several different commercial fermentation processes (Kadam and Newman, 1997; Lawford and Rousseau, 1997; Amartey and Jeffries, 1994; Tang et al., 2006; Underwood et al., 2002).

This investigation was undertaken to improve the process of converting the kraft paper mill sludges to ethanol from the level we have previously developed in our laboratory. The focus of the work was on three main issues. The first was to develop a method to de-ash kraft paper mill primary sludge. The second was to ascertain the effects of de-ashing on the efficiency of bioconversion to ethanol. The third was to improve the process economics deploying low-cost fermentation nutrients. As tools of bioconversion tests, the Simultaneous Saccharification and Co-Fermentation (SSCF) and Simultaneous Saccharification and Fermentation (SSF) were operated under batch and fed-batch modes. Special attention was paid on the product yield, concentration, and enzyme dosage in order to assess the overall process performance.

#### **4.3 Materials and Methods**

##### **4.3.1 Materials**

Feedstocks: The paper primary sludge (PS) was collected from the primary wastewater clarifier unit of a Kraft paper mill, Boise Paper Company (Jackson, AL, USA). The PS was washed by tap water three times and further thickened to 30% consistency using a vacuum filter, and stored at 4° C. A sample quantity of wet hardwood pulp (HP) was also obtained from Boise Paper Company. It was used as a reference substrate in bioconversion studies. The paper sludge and hardwood pulp were analyzed for carbohydrates, moisture content, and ash content according to the National Renewable Energy Laboratory (NREL) standard procedure (NREL, 2008). Hardwood pulp was analyzed to contain 76.0% glucan, 21.1% xylan, and 2.9 wt. % unaccounted for (lignin, acetyl group, ash, and protein). Primary sludge was analyzed to contain 44.5 % glucan, 9.9 % xylan, 8.1% lignin and 36.0 wt. % ash, which includes 26.0% acid-soluble

ash and 10.0% acid-insoluble ash. De-ashing was done by treating by screening. It was then thickened to about 30% consistency using a vacuum filter. The screening procedure was that of Dorica et al., (Dorica and Simandl, 1995) with a slight modification that CO<sub>2</sub> was used instead of air. One liter of resuspended paper sludge with 3% consistency was placed in a 2 L beaker and mixed at 300 RPM using a laboratory stirrer (LR400A, Fisher Scientific) for 30 minutes with carbon dioxide bubbles through two glass tubings, and put through 100 mesh screen. This procedure was repeated three times and thickened to about 45% consistency using a vacuum filter. The resulting solid was referred as S-PS hereafter and was subjected to bioconversion tests. The PS after the two consecutive screenings was referred to as S-PS-1 and the PS after three consecutive screenings referred to as S-PS-2 (Fig.IV-1.). S-PS-1 was analyzed to contain 64.8% glucan, 13.5% xylan, 5.6% lignin and 14.0 wt. % ash. S-PS-2 was analyzed to contain 71.2% glucan, 14.8% xylan, 6.2% lignin and 6.1 wt. % ash.

Enzymes: Cellulase enzyme (Spezyme CP, Lot No. 301-00348-257) was a kind gift from Genencor-Danisco (Palo Alto, CA, USA). The specific activity of Spezyme CP was 59 FPU/mL, as determined by NREL standard procedure (NREL, 2008).  $\beta$ -glucosidase (Novozyme188, Cat. No. C-6150) was purchased from Sigma (St. Louis, MO, USA). Its specific activity was measured to be 750 CBU/mL.

Microorganism: The microorganism used in the SSF was *Saccharomyces cerevisiae* ATCC-200062 (NREL-D<sub>5</sub>A). This organism was grown on YPD agar plates containing solid YPD medium, which contained 2.0% peptone (Sigma, P-6588), 1.0% yeast extract (Sigma, Y-0500), 1.5% agar (Sigma, A-1296) and 2.0% (w/v) glucose (Sigma, G-8270). The recombinant *Escherichia coli* ATCC-55124 (KO11) was used for

the SSCF. This organism was grown on LB agar plates containing solid LB medium (Sigma, L-3152) , which contained 1% tryptone, 0.5% yeast extract, and 1% NaCl, supplemented with 2% xylose (Sigma, X1500), 1.5% agar (Sigma, A-1296) and 40 mg/L or 600 mg/L chloroamphenicol (Fluka, 23275) (Yomano et al.,1998).

#### **4.3.2 Colony Forming Unit Test**

The colony forming unit (CFU) tests were performed to determine the viable cell population. The YPD or LB agar plate was prepared for this purpose diluting the culture with sterile saline (0.89% NaCl solution) to obtain a spread plate cell count of 30-300 cells/plate.

#### **4.3.3 Simultaneous Saccharification and Fermentation /Co-fermentation**

A 250 mL Erlenmeyer flask was used as a bioreactor with 100 mL total working volume. It was operated in an incubator shaker (New Brunswick Scientific, Innova-4080) at 37 °C. Sludges and growth medium were added such that the glucan content becomes 3% or 6% (w/v). Hardwood pulp (HP-I) and hardwood pulp with 20% CaCO<sub>3</sub> based on dry basis of hardwood pulp (HP-II) were used as control and put through the same bioconversion procedures. The sludge samples were steam sterilized at 121 °C for 15 min. The growth media for SSF was YP medium. The growth media for SSCF was LB medium (Sigma, L-3152). For SSCF, 40 mg/L of chloroamphenicol was added after autoclaving to prevent bacterial contamination (Yomano et al., 1998).

The SSF and SSCF of hardwood pulp were carried out at initial pH of 4.8 in 0.05 M sodium citrate buffer and pH 6.0 in 0.05 M sodium phosphate buffer, respectively, without further control of pH during fermentation. Three levels of cellulase loadings were applied: 5, 10 and 15 FPU /g-glucan in paper sludge. The cellulase enzyme was

supplemented with  $\beta$ -glucosidase at a ratio of 2 CBU/FPU. The optical density (OD) was measured by a UV Spectrophotometer (BioTek Synergy HT Multidetector Microplate Readers) at 600 nm for yeast (NREL-D<sub>5</sub>A) and 550 nm for Recombinant *E. coli* (KO11). The initial OD after inoculation was 0.05, equivalent to 16 mg dry cell weight of KO11/L and 50 mg of NREL-D<sub>5</sub>A/L. In all of the microbial experiments, a sample from each flask was taken at the end of the run and streaked on an YPD plate to check for contamination. The presence of contaminants was also checked under an optical microscope. CFU tests were performed to check microorganism viability. The SSF and SSCF experiments requiring pH monitoring were carried out using a 1-L bioreactor (Multifors IHORS HT 2×1L) with 400 mL working volume.

The ethanol yield was calculated as follows:

$$\text{Ethanol yield}[\% \text{ of theoretical maximum}] = \frac{\text{Ethanol produced (g) in reactor}}{\text{Initial Sugar (g) in reactor} \times 0.511} \times 100$$

Sugar is interpreted as glucose in the SSF and glucose plus xylose in the SSCF.

The fed-batch experiments were started with 100 mL initial working volume, and squeezed sludge cakes were added to the bioreactor at the desired time to achieve the total glucan content of 6% (w/v). Addition of sludge was done at the 24 h for SSCF and at 12, 24 and 48 h for SSF. The cellulase and  $\beta$ -glucosidase were also added to maintain overall enzyme loading at 10 FPU and 20 CBU/g-glucan. Samples were analyzed for glucose, xylose, organic acid, and ethanol by HPLC. Aseptic conditions were maintained in all of the microbial experiments. The fed-batch fermentation runs were made in triplicates.

The fermentation runs for the low-cost medium study were operated in an incubator shaker (New Brunswick Scientific, Innova-4080) at 37 °C with 150 RPM. The

cellulase and  $\beta$ -glucosidase were also added to maintain enzyme loading at 10 FPU and 20 CBU/g-glucan. Two growth media were used for the SSF test of screen de-ashed paper sludges: (I) “YP medium” containing 10 g/L yeast extract (Difco, Detroit, MI) and 20 g/L peptone (Difco), and (II) “low-cost medium” containing 0.45% by volume corn steep liquor (CSL) (Sigma C4648, 50% solid) and 5 mM  $\text{MgSO}_4$  as described by Newman et al. (Kadam and Newman, 1997).

For the SSCF test of screening de-ashed paper sludges, seven growth media were used: (A) a “LB medium” containing 5 g/L yeast extract (Difco, Detroit, MI) and 10 g/L peptone (Difco), and (B) 1.0% CSL by volume (Sigma C4648, 50% solid) without mineral supplement; (C) 1.0% CSL by volume with mineral supplement as described by Martinez et al. (Martinez, A. et al. 1999); (D) 3.0% CSL by volume without mineral supplement; (E) 3.0% CSL by volume with mineral supplement; (F) 5.0 % CSL by volume without mineral supplement ; (G) 5.0 % CSL by volume with mineral supplement. Mineral supplements per liter for CSL + M medium were: 1 g of  $\text{KH}_2\text{PO}_4$ , 0.5 g of  $\text{K}_2\text{HPO}_4$ , 3.1 g of  $(\text{NH}_4)_2\text{SO}_4$ , 0.4 g of  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  and 20 mg of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ .

#### **4.3.4 Analytical Methods**

The solid samples were analyzed for carbohydrates and Klason lignin following the NREL CAT standard procedures (NREL, 2008). The moisture content was measured by an infrared moisture balance (Denver Instrument, IR-30). Sugars were determined by HPLC using an HPX-87P column. For the SSF or SSCF tests, a BioRad-HPX-87H column was used for measurement of sugar, organic acid and ethanol. A refractive index detector was used with the HPLC. The acid-insoluble ash was determined following the Tappi test method (T 244 cm-99). Liquid sample analysis and ash determination were

done in triplicates. Where applicable, statistical data including mean value and standard deviation were computed using Microsoft Office Excel 2003.

## **4.4 Results and Discussion**

### **4.4.1 De-ashing of Sludges by Screen-Washing**

The PS was further analyzed for the ash contents to give 26% acid soluble fraction and 10% acid-insoluble fraction based on oven-dry untreated sludge. In reference to the Boise Paper making process and the analysis of data, we conclude that acid soluble ash is primarily from  $\text{CaCO}_3$ , and acid-insoluble ash is from  $\text{TiO}_2$  and clay. Part of the insoluble ash content was physically associated with the fibers concurring to the findings of Middleton and Scallan (1991). The suspended fine particles not associated with fibers are presumed to pass through the screen with the filtrate. Separation of fiber or fillers from sludge has been practiced in pulp and paper industry using commercially available screens and cleaners (Dorica and Simandl, 1995). Table IV-1 shows the glucan/xylan content and their loss in the screening process. With S-PS-1, the glucan/xylan loss was 11.2%/17.2% when the ash removal was 76.4%. As indicated by the data on screening of S-PS-2, increase of ash removal caused increase of the glucan/xylan loss; glucan/xylan loss corresponding to 90.6% ash removal was 25.6%/34.3%. The  $\text{CO}_2$  bubbles enhance the extent of separation of the ash from the fibers. The gas bubbles rise to the top surface carrying the particle of low density, such as fine fiber, to the surface of the liquid phase. Carbon dioxide was used in place of air since a large amount of it is produced during the fermentation process, and it is also a by-product in the manufacture of lime from calcium carbonate during the kraft chemical recovery process (Biermann, 1996). Use of  $\text{CO}_2$  has additional benefit that it increases the dissolution of  $\text{CaCO}_3$ . The solubility of  $\text{CO}_2$  in the

water is much higher than air (88 mL CO<sub>2</sub>/100 mL of water at 20 °C) and its aqueous solution is carbonic acid, a weak unstable acid. The water insoluble calcium carbonate in the presence of water and carbon dioxide dissolves as water soluble calcium bicarbonate which is a compound existing only in solution. Fig.IV-2.A through C shows increasing degree of separation after successive treatments of mechanical means (screening).

#### **4.4.2 SSF and SSCF of Screened De-ashing of Sludges**

Data on S-PS-1 and PS in Table IV-2 clearly indicate that ash removal increases the ethanol fermentation yield. The ethanol fermentation yields of the S-PS-2 with the lowest ash content at very low enzyme loading of 5 FPU cellulase /mL were higher than those of the original sludge at medium enzyme loading of 10 FPU cellulase /mL. The adverse effect of ash in the sludge is primarily on the enzymatic reaction part of the SSF/SSCF rather than the microbial reaction. A number of researchers found that various components in the ash interfere with cellulase reaction (Nikolov et al., 2000; Tietjen and Wetzel, 2003; Cabezas et al., 1991; Haska, 1981; Hamzehi and Pflug, 1981; Pflug, 1982; Tothill et al., 1993). Improvement of yield by de-ashing is therefore due to improvement in the enzymatic hydrolysis. It is quite obvious that de-ashing removed the chemicals that inhibit enzymatic hydrolysis and/or fermentation process. The fact that SSF/SSCF proceeds under glucose limited condition (Fig. IV-3 and IV-4) further proves that the hydrolysis is the rate-limiting step of the SSF/SSCF. On the other hand, ash removal causes loss of fine fibers, therefore loss the glucan/xylan. As shown in the case of S-PS-2, 90% removal of ash accompanied loss of glucan/xylan as high as 25.6%/34.3% during the screen de-ashing. Considering large glucan/xylan loss during the screening of S-PS-2, and that only 6% difference in ethanol yield between the two enzyme loading of 15

and 10 FPU cellulase /g-glucan), S-PS-1 was used in the subsequent bioconversion tests in which medium enzyme loading of 10 FPU cellulase/g-glucan was applied.

Ash removal also decreases the total bulk solid loading for a given glucan content in the feedstock. As an example, for 6% glucan loading, the total solid loading of the original primary sludge, S-PS-1 and S-PS-2 were 13.5 wt.%, 9.3 wt.% and 8.4 wt.%, respectively. Fermentation of high viscosity broth requires strong agitation to provide adequate mixing and mass transfer. Agitation intensity as it relates to solid loading thus became of our interest. When sludge and enzyme loadings increase, the viscosity of the reaction mixture increases accordingly. Fermentation results indicate that the dense sludges (high solid loading) require longer time for liquefaction (breakdown of viscosity). The ethanol yields from fermentation were also lower at higher solid loading (Table IV-3). With high solid loading (21.6% w/v for PS), the enzymatic hydrolysis was insufficient to liquefy the sludge, resulting in a very low ethanol yield (data not shown here). In a separate research in our laboratory, we found that the binding capacity of cellulase decreased when the substrate concentration increased (Wang et al., 2011). This has a negative effect on the productivity because the enzymatic hydrolysis is the rate-limiting step in the SSF. There are a number of reports that suggests agitation enhances the hydrolysis yield of cellulosic substrates. The literature information along these lines collectively indicate that agitation enhances the adsorption of cellulase to the substrate, therefore increasing the activities of the endoglucanases and the cellobiohydrolases (Cavaco-Paulo and Almeida, 1994; Cavaco-Paulo et al.,1996; Cavaco-Paulo et al.,1998; and Sakata et al.,1985). Most of data in Table IV-4, except the ethanol yield from SSCF of PS at relatively high agitation (250 RPM), agree clearly show that agitation has a

positive effect on ethanol production in the SSF and SSCF, which agree with the previous findings. Enhanced interaction between enzyme and the solid substrates of sludge due to high mixing is a plausible reason for improvement of ethanol production. In addition, agitation may also improved mass transfer of sugar into microorganisms.

However, the ethanol yield from SSCF of PS at relatively high agitation (250 RPM) was lower than the medium agitation (150 RPM) (Table IV-4). It was probably due to the fact that the *E.coli* is less viable under high agitation as measured by CFU tests. It is well known that growth robustness of *E.coli* is low compared to yeast (Dien et al., 2003). The yeast used in this work is very robust and can withstand high agitation under anaerobic condition. It was reaffirmed by our CFU tests that showed no observable damage after the SSF. Mechanical damage of the *E.coli* by shear stress could result in a slower growth under high agitation. Glass bead milling is frequently used for cell lysis for production of intracellular products from microorganisms, in which the harvested cells are agitated with glass beads in a closed chamber (Kura and Schütte, 1987). In our sludge processing, 4.9% (w/v) ash content was present in the fermentation broth of 6% (w/v) glucan loading of untreated PS. This ash may have exerted the same effect as glass beads.

#### **4.4.3 Evaluation of Low-cost Fermentation Medium for Fermentation**

The yeast extract and peptone are high quality nitrogen sources containing vitamin B complex and amino acids, yet the cost is prohibitively high to be used as nutrient for industrial fermentation. Replacement of the laboratory medium with practical nutrients is one of the major technical hurdles to be cleared for the development of industrial fermentation from laboratory work. The fermentation route to produce ethanol

from biomass is no exception. Substitution of the laboratory medium with alternative inexpensive nitrogen source medium, however, has often resulted slow rates and low yield in ethanol production. Corn steep liquor (CSL), which is low in carbohydrates and rich in proteins, is considered as a suitable replacement for yeast extract in fermentation media (Thomsen, 2006). It is currently used widely in industrial fermentation processes.

A number of SSF runs were made using screen de-ashed paper mill sludges without pH control. The enzyme loadings were: 10 FPU/g-glucan and 20 CBU/g-glucan. *Saccharomyces cerevisiae*, ATCC-200062 (NREL-D<sub>5</sub>A) was used for the conversion of glucose to ethanol. Two different substrate loadings were applied: 3% and 6% glucan (w/v). The ethanol yield of S-PS-1 from the SSF with lean medium (II) was 71.1% of theoretical maximum with 6% glucan loading. It is very close to the ethanol yield achieved with the rich medium (I) -72.8% under the same conditions. This is in agreement with the previous findings that CSL is a good substitute for *yeast* extract and peptone (Kadam and Newman, 1997; Amartey and Jeffries, 1994; and Tang et al., 2006).

In our previous study (Kang et al., 2010), the ash in the sludge, especially calcium carbonate, was partially neutralized by the acids produced from the SSCF and SSF and acted as a buffer to stabilize the pH during fermentation. After screening, S-PS-1 still contained 8.0% acid-soluble ash, which is primarily CaCO<sub>3</sub>. The time-course results of the SSF run are shown in Fig. IV-3.

During the fermentation of S-PS-1, HP-I and HP-II, the pH quickly dropped and reached the final values (at 120 h) of 5.7, 4.3 and 5.6, respectively. The decrease of pH occurred due to carbonic acid (CO<sub>2</sub> dissolved in water) and other organic acids formed during fermentation. This pH drop by CO<sub>2</sub> and organic acid is counteracted by the

buffering action of ash in the S-PS-1, calcium carbonate in the HP, and CSL in the lean medium to reach the respective final pH values in the bioreactor. Since CSL contains proteins, peptides and amino acids, the addition of CSL may strengthen the buffering capacity of the medium (Stanburg and Whitaker, 1984).

In general, the activity of *Saccharomyces cerevisiae* is stable under neutral or slightly acidic and anaerobic conditions. Under the anaerobic conditions, the intracellular pH of *Saccharomyces cerevisiae* is usually maintained between 5.5 and 5.75 when the external pH is 3.0 (Imai and Ohno, 1995a) or between 5.9 and 6.75 when the external pH is between 6.0 and 10.0 (Imai and Ohno, 1995b). The various enzymes involved in yeast metabolism of sugars are located within the yeast cell, the enzyme activity is unaffected if the internal pH of the yeast cell is stable. The ethanol yields of S-PS-1, HP-I, and HP-II with 3% glucan loading are essentially the same: 73.7%, 73.7%, and 72.4%, respectively. This yield level is also very close to that from fermentation of hardwood pulp under pH 4.8 (73.0%). Our previous study (Kang et al., 2010) has shown that although the terminal ethanol yields were the same, hardwood pulp was converted faster than the untreated sludges. After screen de-ashing, S-PS-1 attained almost identical ethanol producing rate as that of HP.

In order to utilize the xylan content in the sludge, the SSCF was also performed. In this process, recombinant *E.coli* ATCC 55124 (KO11) was used in place of the yeast. This organism is known to convert xylose as well as glucose to ethanol with high efficiency (Ohta et al., 1991). This strain has performed well with high metabolic yields in rich media. However, the yields of both strains declined in a minimal media (Martinez et al., 1999; York and Ingram., 1996). The poor performance in a minimal media may be

attributed to NADH-mediated inhibition of citrate synthase, limiting the availability of glutamate, a protective osmolyte (Underwood et al. 2002a and 2002b). In this work, different media were tested for their performance in ethanol fermentation by *E.coli* KO11 from the screen de-ashed paper sludges. The ethanol yield data are shown in Table IV-5. The yields were calculated on the basis of total carbohydrates (glucan and xylan). The yields of SSCF were also calculated on the basis of glucan only for direct comparison with the SSF. On the average, the SSCF produced 15% more ethanol than the SSF from the same feedstock because KO11 can convert both glucose and xylose to ethanol.

Our initial approach was to use CSL as a source of protein and vitamin,  $\text{NH}_4\text{Cl}$  as an inorganic nitrogen source with other minerals. For 1% CSL supplementation, addition of minerals increased the ethanol yield from 53.1% to 57.4%. At CSL levels above 1%, supplementation minerals showed no significant improvement in ethanol production. With 1% CSL and very low amount of yeast extract (0.05%) (medium H), the ethanol yield reached 73.5%, which was almost identical to that of rich medium. Although the terminal ethanol yields were almost identical, S-PS-1 in the rich medium was converted faster than that in medium H. The 3% CSL alone (medium F) appears to provide sufficient nutrients to achieve ethanol yield of 71.2 %, which is comparable to that from rich medium A (73.6%). There was no apparent improvement in ethanol yields with CSL level above 3%. These results support the findings of previous studies that the recombinant strain of *E. coli* KO11 does not produce ethanol efficiently without the addition of large amounts of complex nutrients (Asghari et al., 1996; York and Ingram, 1996). It is also noteworthy that the initial cell density is low, 16 mg dry cell weight of KO11/L. In this study, CSL was tested as a nutritional supplement for *E.Coli*-KO11. The

3% CSL was shown to meet the nutrient requirement, enough to attain cell growth to the level obtainable from rich medium A. The economic feasibility of using medium G and H in this process is yet to be investigated. There are some technical issues concerning the use of CSL as fermentation media; 1) need for sterilization to remove bacterial contaminants, 2) difficulty of product separation caused by impurities in the broth that are originated from CSL; 3) increase of waste treatment burden due to high BOD in the spent fermentation liquor, and 4) inconsistent composition of CSL that varies with the source (Lee, 2005; Lawford and Rousseau, 1997). Handling of CSL is not easy because it is highly viscous and tends to gelate turning into sticky liquid. In view of the aforementioned problems associated with CSL, the subsequent tests were performed using medium H containing low CSL (1%) and low amount of yeast extract (0.05%). The time-course profiles of various observable parameters of SSCF based on medium H are shown in Fig. IV-4.

Of our keen interest here was to observe how the pH profiles vary with different nutrient media and substrates. The pH dropped sharply in the early phase of the SSCF due to production of carbonic acid ( $\text{CO}_2$  dissolved in water) and other organic acids, and remained relatively constant afterwards. The SSCF was started with pH 7, but decreased to 5.9 for S-PS-1. In the HP-I run, however, the pH quickly dropped from 6.8 to 4.2, similar to the pH profile of the SSF process. Much like CSL, the yeast extract and peptone also have a certain level of buffering capacity (Terzaghi and Sandine, 1975; Hugo and Lund, 1968). However, at low level, their buffer capacity was not high enough to significantly affect the pH behavior of the SSCF of the HP-I. Presence of  $\text{CaCO}_3$  enhances the buffering capacity of the medium. The pH of SSCF of HP-II with  $\text{CaCO}_3$

dropped from 6.8 (0 h) to 5.8 (120 h), which is similar to the pH profile of the SSCF of S-PS-1. Ethanogenic derivatives of *E. coli* B function efficiently between pH 5.8 and pH 7.5 (Beall et al., 1991). Often pH 6.0 is used as a practical optimum because it lowers solubilization of CO<sub>2</sub> (Moniruzzaman et al., 1998). The pH in the fermentation HP-II remained below 5 after 6 h. Insufficient use of xylose, even glucose, and low ethanol yield indicates that *E. coli* KO11 was inhibited under low pH. Moniruzzaman et al also found that only 2 h exposure at pH 3 could cause long delays in cell growth and low ethanol yield (Moniruzzaman et al., 1998).

#### **4.4.4 Fed-batch Operation**

The concentration of ethanol in the bioreactor is a factor significantly affecting the cost of the downstream separation process. Alcohol concentration of 40 g/L has been mentioned as a target value in biomass fermentations (Hohmann and Rendleman, 1993). We attempted the fed-batch operation (intermittent input of feed) of SSF and SSCF in an effort to increase the terminal ethanol concentration in the bioreactor. The bioreaction was started with the initial sludge loading of 60 g-glucan/L. Additional feedstock of the same level was put into the reactor at 24 h for SSCF and at 12, 24 and 48 h for SSF. At these input points the reaction has proceeded far enough to liquefy the dense solid slurry and retain fluidity high enough to accept additional solid feed. The ethanol production from the fed-batch runs are shown in Fig.IV-5.

In the case of SSCF with one additional feeding, ethanol concentration of 47.8 g/L was obtained at the end of the run. This is equivalent to an ethanol yield of 70% on the basis of total sugar with 10% w/v total glucan loading, which was slightly lower than the yield of single-batch with 6% glucan loading. However, it was still higher than the

ethanol yield of 68% from untreated PS even with a lower enzyme loading and a low-cost medium. This is a significant benefit in the SSF gained specifically by de-ashing of the sludge. The reason for the low ethanol yield observed from the fed-batch SSCF is that the ethanol tolerance of *E. coli* KO11 is relatively low compared to yeast (Dien et al., 2003, Kang et al., 2010). In the fed-batch SSF runs with three additional feedings, the final ethanol concentration of 60 g/L was achieved, which was much higher than that of the fed-batch SSF of untreated primary sludge, 45 g/L, with two additional feedings. The overall ethanol yield in the fed-batch SSF was approximately 70% based on glucan - 15%w/v (23.1%w/v total solid loading), which was also lower than the yield of single batch with 6% glucan loading, yet comparable to that of the fed-batch SSF of untreated PS with a lower enzyme loading and a low-cost medium. The yield and product concentration data collectively indicate that the removal of ash and other extraneous components has positive effects on the bioconversion process.

#### **4.5 Conclusion**

Kraft paper mill sludges have features desirable for bioconversion to value-added products. Without any cleaning, the sludges can be converted to ethanol by SSF or SSCF with reasonable efficiency (Kang et al., 2010). The bioconversion processes, however, can be significantly improved by de-ashing and deinking of the sludges. The major benefits of de-ashing are: (1) the ethanol yields in the SSF or SSCF are significantly improved, (2) the solid loadings into the bioreactor are increased, the latter leading to higher ethanol concentration. The ethanol yields for the de-ashed primary sludge (S-PS-1) reached 72.8% and 73.6% of the theoretical maximum for SSF and SSCF, respectively. It is particularly notable that these yields were achieved with relatively low

enzyme loading of 10 FPU/g-glucan and 20 CBU/g-glucan. The often used laboratory fermentation media, yeast extract and peptone can be replaced by CSL or at least supplemented at a reduced level, without adversely affecting the ethanol yield. Fed-batch SSF and SSCF were attempted to increase the ethanol concentration. The ethanol concentration in the broth was increased to 47.8 g/L and 60 g/L, for 2-feed SSCF and 4-feed SSF, respectively.

Table IV-1 Composition of de-ashed sludges

(%)	PS	S-PS-1 <sup>d</sup>	S-PS-1 <sup>e</sup>	S-PS-2 <sup>d</sup>	S-PS-2 <sup>e</sup>
Glucan	44.5	38.4	39.5	32.4	33.1
Xylan	9.9	7.8	8.2	5.9	6.5
Ash	36.0	10.7	8.5	4.5	3.4
Glucan loss	-	13.7	11.2	27.2	25.6
Xylan loss	-	21.2	17.2	40.4	34.3
Ash removal	-	70.3	76.4	87.5	90.6

- a. The data of glucan, xylan and ash in table are based on oven-dry untreated primary sludge.
- b. The data of glucan loss, xylan loss and ash removal in table are based on the initial glucan, xylan and ash of oven-dry untreated primary sludge.
- c. All Data are the mean value of duplicate (n=2; standard deviation<1.0).
- d. Without carbon dioxide.
- e. With carbon dioxide.

Table IV-2 Ethanol yield from SSF and SSCF of treated paper sludges

Enzyme loading	15 FPU of Cellulase + 30 CBU of $\beta$ -glucosidase /g-glucan			10 FPU of Cellulase + 20 CBU of $\beta$ -glucosidase/g-glucan			5 FPU of Cellulase + 10 CBU of $\beta$ -glucosidase/g-glucan		
	P.S.	S-PS-1	S-PS-2	P.S.	S-PS-1	S-PS-2	P.S.	S-PS-1	S-PS-2
SSF									
Ethanol Yield(%) <sup>a</sup>	74.5	77.6	81.2	67.1	72.8	75.7	60.5	64.5	70.2
SSCF									
Ethanol Yield(%) <sup>b</sup>	78	80.4	82.8	68.2	73.6	76	59.6	65.1	71.8
SSCF									
Ethanol Yield(%) <sup>c</sup>	95.8	97.5	100.4	83.7	89.3	92.2	73.2	78.9	87.1

Data of sludges are the mean value of duplicate (n=2; standard deviation<0.1).

- a. The ethanol yield of SSF based on glucan only.
- b. The ethanol yield of SSCF based on glucan and xylan.
- c. The ethanol yield of SSCF based on glucan only.
- d. SSF and SSCF based on 6% (w/v) glucan loading/ 100 mL working volume.

Table IV-3 Ethanol yield from SSF and SSCF of paper sludges at different solid loading

Sludge	PS			S-PS-1		
Solid loading (%w/v)	6.7	13.5	20.2	4.6	9.3	13.9
Glucan loading (%w/v)	3.0	6.0	9.0	3.0	6.0	9.0
SSF Ethanol Yield(%) a	68.8	66.1	-	74.3	72.8	71
SSCF Ethanol Yield(%) b	72.4	69.8	-	75.1	73.6	70.8
SSCF Ethanol Yield(%) c	87.8	85.7	-	91.1	89.3	85.9

- a. Simultaneous Saccharification and Fermentation (SSF) based on glucan only.
- b. Simultaneous Saccharification and co-Fermentation (SSCF) based on glucan and xylan.
- c. Simultaneous Saccharification and co-Fermentation (SSCF) based on glucan.
- d. Data of sludges are the mean value of duplicate (n=2; standard deviation<0.1).
- e. Enzyme loading, 10 FPU of Cellulase + 20 CBU of  $\beta$ -glucosidase /g-glucan.

Table IV-4 Ethanol yield from SSF and SSCF of sludges at different agitation intensity

Sludge	PS			S-PS-1		
Agitation intensity (rpm)	250	150	100	250	150	100
SSF Ethanol Yield(%) a	67.7	66.1	58.4	74.4	72.8	69.0
SSCF Ethanol Yield(%) b	64.4	69.8	61.1	74.9	73.6	70.1
SSCF Ethanol Yield(%) c	79.1	85.7	75.0	90.9	89.3	85.0

- a. Simultaneous Saccharification and Fermentation (SSF) based on glucan only.
- b. Simultaneous Saccharification and co-Fermentation (SSCF) based on glucan and xylan.
- c. Simultaneous Saccharification and co-Fermentation (SSCF) based on glucan.
- d. Data of sludges are the mean value of duplicate (n=2; standard deviation<0.1).
- e. Enzyme loading, 10 FPU of Cellulase + 20 CBU of  $\beta$ -glucosidase /g-glucan.
- f. 6% (w/v) glucan loading / 100 mL working volume.

Table IV-5 Ethanol yield from SSCF of de-ashed paper sludges on different medium

Medium	Yeast Extract (%w/v)	Peptone (%w/v)	CSL (%v/v)	Mineral Supplement	SSCF Ethanol Yield(%) <sup>a</sup>	SSCF Ethanol Yield(%) <sup>b</sup>
A	0.5	1	0	0	73.6	89.3
B	0	0	1	0	53.1	64.4
C	0	0	1	Yes	57.4	69.6
D	0	0	2	0	64.4	78.1
E	0	0	2	Yes	65.6	79.6
F	0	0	3	0	72.1	87.5
G	0	0	3	Yes	71.2	86.4
H	0.05	0	1	0	73.5	89.2

All Data are the mean value of duplicate (n=2; standard deviation<1.0). The Enzyme loading was: 10 FPU of Cellulase + 20 CBU of  $\beta$ -glucosidase /g-glucan.

- a. The ethanol yield of SSCF based on glucan and xylan.
- b. The ethanol yield of SSCF based on glucan only.
- c. SSCF based on 6% (w/v) glucan loading.

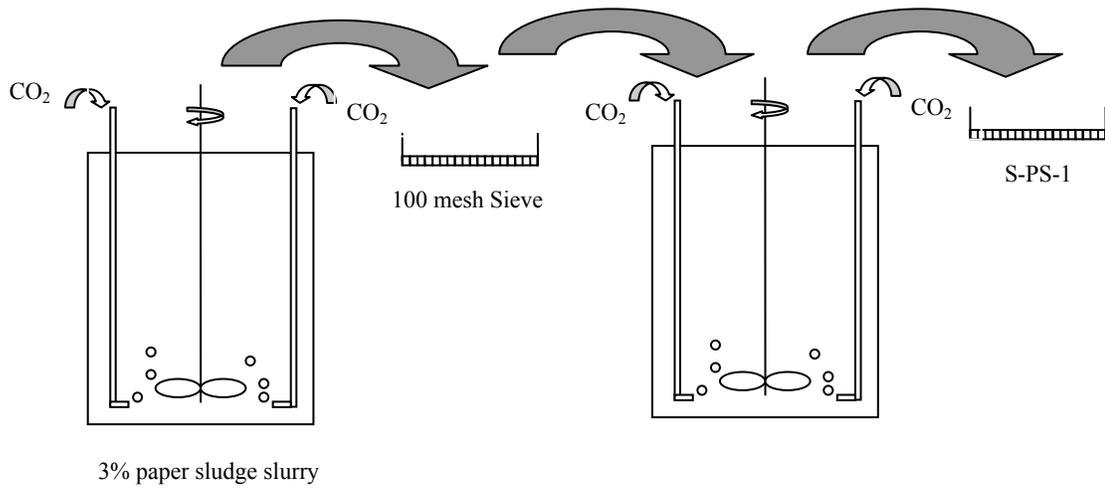


Fig.IV-1. De-ashing process of the primary sludge.

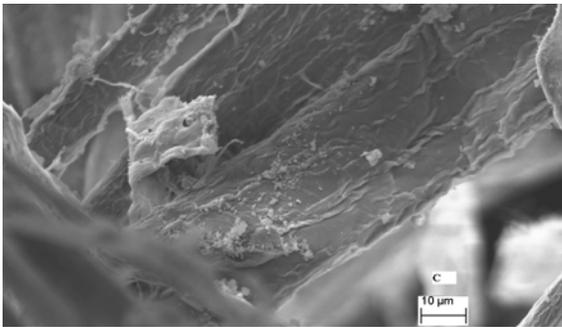
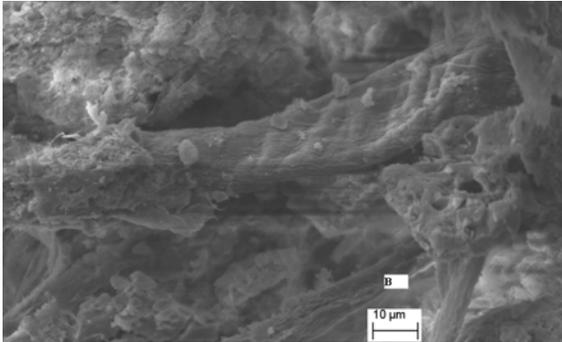
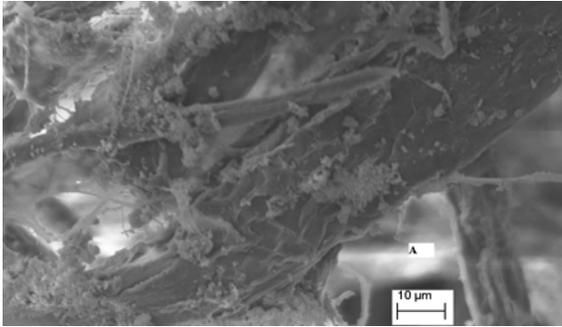


Fig.IV-2. Scanning electron micrograph (SEM) of de-ashed and untreated sludge  
A. untreated primary sludge ( $\times 2000$ ).  
B. primary sludge after two consecutive screenings ( $\times 2000$ ).  
C. primary sludge after two consecutive screenings ( $\times 2000$ ).

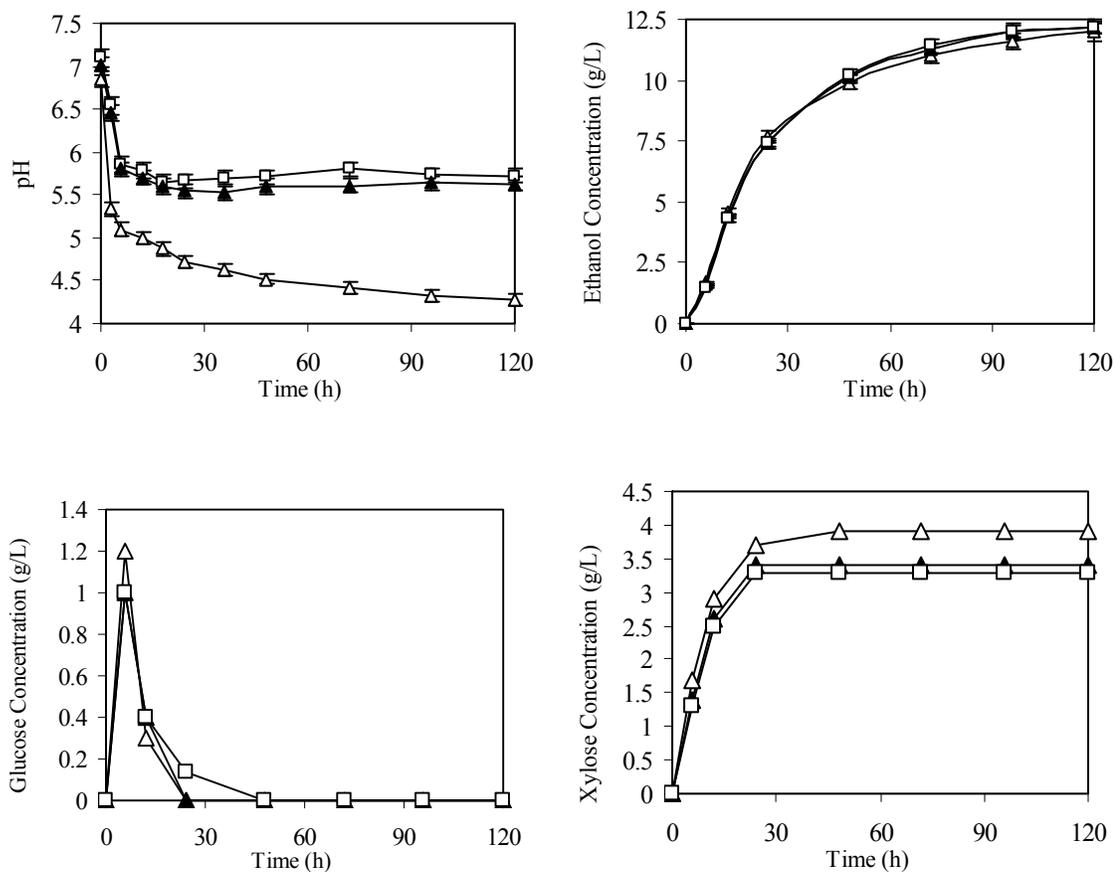


Fig.IV-3. Simultaneous Saccharification and Fermentation (SSF) of de-ashed Sludges by *Saccharomyces cerevisiae* (ATCC-200062)

Squares represent de-ashed primary sludge (S-PS-1). Triangles represent hardwood pulp (HP-I). Filled triangles represent hardwood pulp with 20% CaCO<sub>3</sub> on dry basis of hardwood pulp (HP-II).

The data points represent the average of triplicate runs. The pH was not controlled. Other conditions of the SSF were: 3% (w/v) glucan loading, 37°C, 10 FPU Spezyme CP + 20 CBU of Novozyme-188/g-glucan.

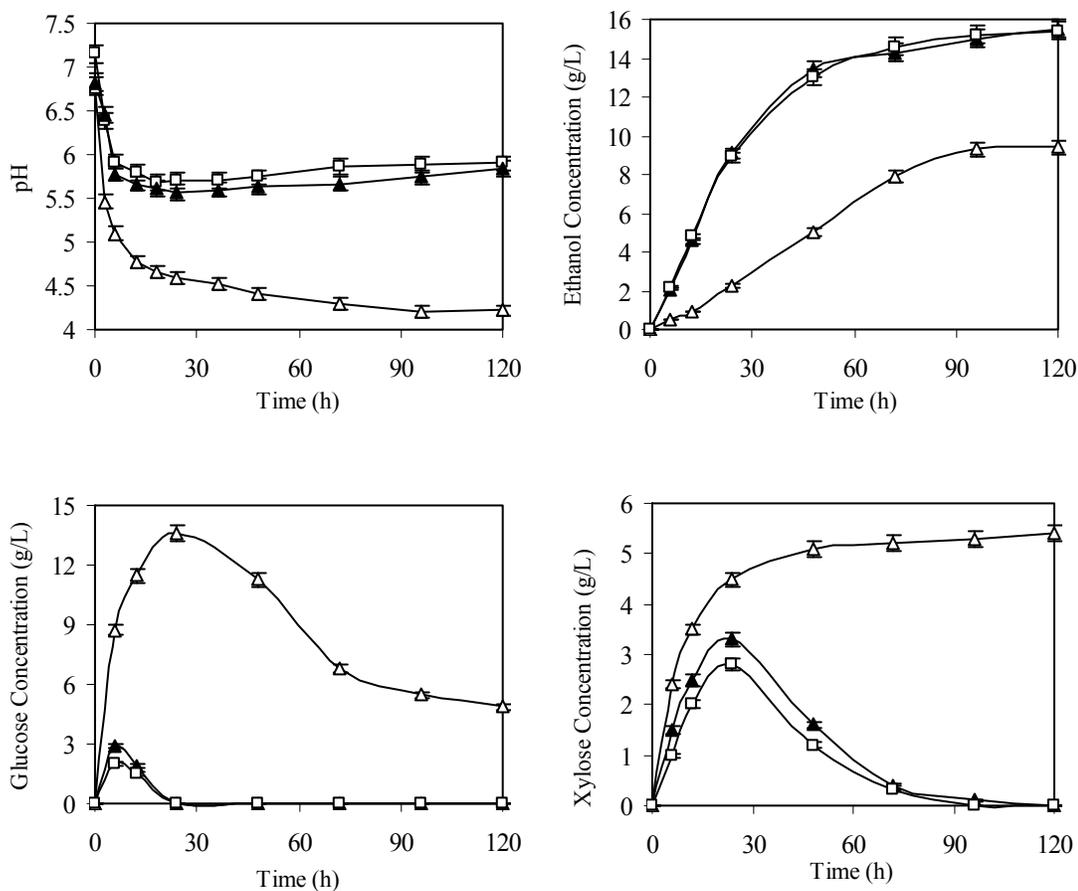


Fig.IV-4. Simultaneous saccharification and co-fermentation (SSCF) of de-ashed sludges by *Escherichia coli* KO11 (ATCC-55124)

Squares lines represent de-ashed primary sludge (S-PS-1). Triangles represent hardwood pulp (HP-I). Filled triangles represent hardwood pulp with 20% CaCO<sub>3</sub> on the basis of dry hardwood pulp (HP-II).

The data points represent the average of triplicate runs. The pH was not controlled. Other conditions of the SSF were: 3% (w/v) glucan loading, 37°C, 10 FPU Spezyme CP + 20 CBU of Novozyme-188/g-glucan.

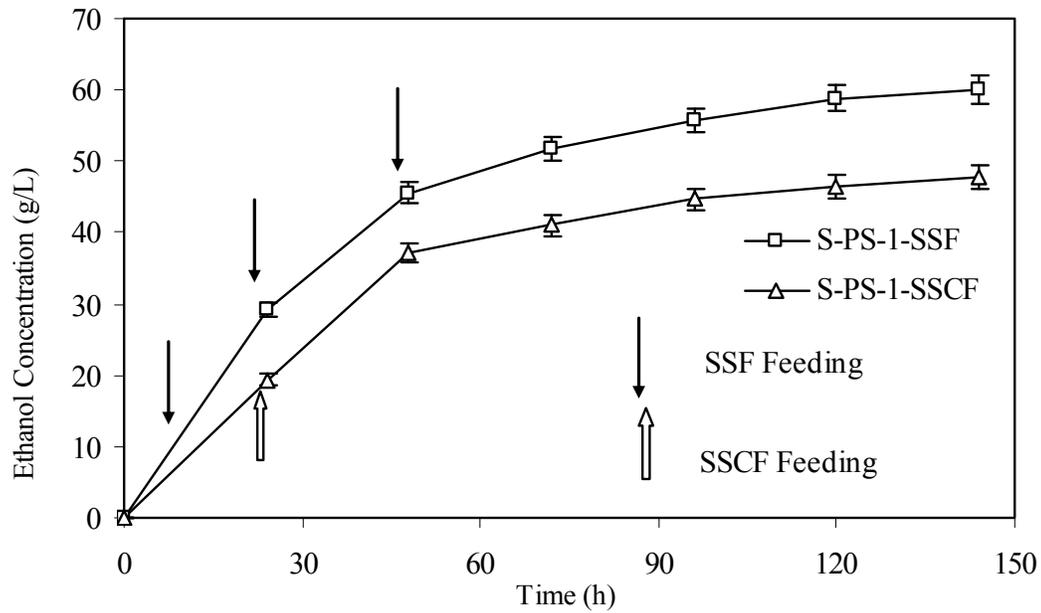


Fig.IV-5. Fed-Batch SSF and SSCF of S-PS-1

The data points represent the average of triplicate runs. Other conditions of the SSF were: 6g initial and additional feeding of glucan loading (9.3 g dry basis S-PS-1), 37°C, and 10 FPU Spezyme CP + 20 CBU of Novozyme-188/g-glucan. Total glucan loading for SSCF was 10% w/v and for SSF was 15% w/v.

## **V. Cellulase Production from Unbleached Karft Hardwood Pulp and Paper Sludge**

### **5.1 Abstract**

Cellulase on-site production can be a supplementary unit in a kraft paper mill production system. By using cellulase and *Saccharomyces cerevisiae* on kraft paper sludge, which is waste from the kraft pulp making process, it is possible to produce bioethanol. It is feasible and convenient to integrate these two processes without any impact on the production of paper goods and on the production of bioethanol. The cellulase enzyme was produced by *Trichoderma reesei* Rut C-30 and was investigated in a 3 L fermenter for its productivities and titers. Experiments were conducted over a range of possible pH values to determine the impact upon *T. reesei* Rut C-30 growth and cellulase production. Different concentrations of unbleached kraft processed hardwood pulp were examined to determine the maximum levels of cellulase activity obtainable. The highest titer of 7.5 FPU/mL was obtained when using 4% (w/v) hardwood pulp. Paper sludge was also considered as a potential feedstock for cellulase production. However, it also has a high ash content which is detrimental to cell growth. It is desirable to remove ash as much as possible from the sludge, while retaining carbohydrates. In this study, the de-ashed sludge pre-processed via physical and chemical treatment was used as substrates for cellulase production and ethanol fermentation. The cellulase enzyme

produced from de-ashed sludge exhibited cellulase activity as high as 8.0 FPU/mL. The sugar and ethanol yields using the crude enzyme solutions in the enzymatic hydrolysis and fermentation test were compared with a commercially available enzyme, Spezyme CP. In addition, cellulase produced from both substrates showed slightly higher xylanase activity.

## **5.2 Introduction**

The bioconversion of paper sludge to bioethanol is an intriguing possibility in the wood-based biorefinery industry. In our previous research (Kang et al., 2010), we found that ethanol production could be a supplementary part of an integrated biorefinery in a kraft process paper mill, while still preserving the main products, paper and paper related products. One of the bottlenecks in the production of bioethanol is the high cost from the usage of the enzymes utilized in the hydrolysis step (Steele et al., 2005; Spano et al., 1978). The cost of bioethanol and cellulase production could be reduced by simplifying the processing steps. The operating cost and capital investment of this supplementary unit can be reduced by sharing the existing facilities of the pulp mill thus utilizing the utilities and the power that may be available at low cost from the pulp mill operation.

The soft-rot filamentous fungus, *Trichoderma reesei*, holds the greatest potential for reducing the cost in the production of enzymes for lignocellulosic biomass conversion (Egyhazi et al. 2004; Gadgil et al. 1995; Juhasz et al., 2004; Kadam and Keutzer, 1995). *T. reesei* Rut-C30, a mutant of *T. reesei*, was isolated using a combination of ultraviolet irradiation and nitrosomethyl guanidine (NTG) by Montenecourt and Eveleigh (Montenecourt and Eveleigh, 1979). Most previous works are based on this mutant.

The current laboratory investigations primarily have been devoted to reduce the cost of enzyme production. Carbon source is one of the main factors in microbial cellulase production (Ryu and Mandels, 1980; Campbell and Laherrehe, 1998). In order to convert a kraft paper mill to an integrated biorefinery, cellulase production using carbon sources directly from the kraft mill could be feasible, convenient, and economically viable. Sulfite wood pulp (Watson et al., 1984), pretreated wood (Juhasz et al., 2005; Shin et al., 2000), sawdust (Lo et al., 2005), waste paper (Royer and Nakas, 1987; Chen and Wayman, 1991; Shin et al., 2000), waste cardboard (Szijártó et al., 2004) and paper sludge (Royer and Nakas, 1987; Maheshwari et al., 1994) could be used as carbon sources for cellulase production. Shin et al. reported production of cellulase by *T. reesei* Rut C-30 on various lignocellulosic substrates (the newspaper sludge, office paper sludge, and steam-exploded woods treated in various ways). The steam-exploded wood showed the best properties for substrate in cellulase production. Chen and Wayman used waste newspaper and sorbose in the *T. reesei* Rut C-30 cellulase production and partially enzymatic saccharified newspaper was found to be most effective. The enzymatic hydrolyzate of acid pretreated wastepaper was also used as soluble-inducing substrate for cellulase production in continuous culture of *T. reesei* Rut C-30 (Ju and Afolabi, 1999). Chahal used NaOH treated chemithermo mechanical pulp as a carbon source in the solid-state fermentation for the *T. reesei* Rut C-30 cellulase production (Chahal et al, 1985).

Using kraft pulp on site has the benefit of providing a high quality carbon source for cellulase production. The alkaline delignification of wood causes the lignin degradation predominantly by the cleavage of lignin-hemicellulose bonds (Wang et al., 1967). In addition, delignification causes changes in the structure of the cellulose fiber

(Lewin and Roland, 1971). One advantage of using kraft pulp is that the fungus can easily assimilate the substrate and quickly initiates the cellulase production. Kraft paper maybe a good substrate for cellulase production because the fibers in the sludge are fine kraft pulp because they are screen rejects from paper machine and the digester, and are easily digested by cellulase enzymes (Fan and Lynd, 2006a; Fan and Lynd, 2006b; Scott et al., 1995; Jeffries and Schartman ,1999; Fan, et al.,2003). However, using paper mill sludge as a source for cellulase production is rarely studied. Maheshwari et al used paper mill sludge as a potential source for cellulase production, in which a mixed cultivation of *T. reesei* QM 9123 and *Aspergillus niger* was used (Maheshwari et al., 1994) , however, scaling up cellulase fermentation in bioreactors and properties of the cellulase produced from sludge have not been well studied.

There are several factors which govern the cellulase production. pH is one of the main factors that has a significant role in cellulase production (Ryu and Mandels, 1980; Xiong et al., 2004; Mukhopadhyay and Nandi, 1999; Wayman and Chen, 1992; Kadam and Keutzer, 1995). The impact of carbon source on cellulase production has been well documented. In addition to pH, this study also investigated on how different concentrations of unbleached kraft processed hardwood pulp could have an impact upon the efficiency of cellulase enzyme production by *T. reesei*. The production of cellulase using industrial wastes, de-ashed and chemical treated paper mill sludge, as the main carbon source, was also investigated. The objective of this study was to evaluate the paper mill sludge for its applicability in economical cellulase production. The enzymatic hydrolysis experiments were intended to be performed by using cellulase produced by

both substrate and by Spezyme CP at the same enzyme loading based on the substrate (filter paper units (FPU)/g of cellulose).

## **5.3 Materials and Methods**

### **5.3.1 Materials**

Feedstock: The unbleached hardwood pulp and primary paper sludge were collected from a kraft paper mill (Boise Paper Company Jackson, AL, USA). Primary sludge (PS), discharged from the paper machine and the digester, was collected from the primary wastewater clarifier unit. Both were stored at 4° C. The paper sludge and hardwood pulp were analyzed for carbohydrates, moisture content, and ash content according to the standard biomass analytical procedure of the National Renewable Energy Laboratory (NREL) (NREL, 2008). Hardwood pulp was analyzed to contain 76.0% glucan, 21.1% xylan, and 2.9 wt. % unaccounted for (lignin, acetyl group, ash, and protein). Unbleached hardwood pulp was ground in a Oster 14-Speed Blender, and then milled in an IKA MF 10 basic mill (IKA Werke GmbH & Co., Statufen, Germany) to give a particle size lower than 20 mesh. Primary paper sludge was analyzed to contain 44.5 % glucan, 9.9 % xylan, 8.1% lignin and 36.0 wt. % ash, which includes 26.0% acid-soluble ash and 10.0% acid-insoluble ash. De-ashing was done by treating by screening according to the previous chapter. The deashed paper sludge was analyzed to contain 64.8% glucan, 13.5% xylan, 5.6% lignin and 14.0 wt. % ash. De-ashed sludge was leached with 1 N sulfuric acid and further water washing until pH around 6.0. Acid leaching sludge was analyzed to contain 72.6% glucan, 15.8% xylan, 4.4% lignin and 4.7 wt. % ash. The acid leaching sludge was leached with 1 N sodium hydroxide and further

water washing until pH around 7.5. Alkaline leaching sludge analyzed to contain 74.5% glucan, 15.0% xylan, 3.7% lignin and 3.8 wt. % ash.

Enzymes: The cellulase enzyme (Spezyme CP, Lot No. 301-00348-257) was a kind gift from Danisco Genencor (Palo Alto, CA, USA). The activity of Spezyme CP was 59 FPU/mL, as determined by NREL standard procedure (NREL, 2008).

Microorganism: The microorganism used in the Simultaneous Saccharification and Fermentation (SSF) was *Saccharomyces cerevisiae* ATCC-200062 (NREL-D5A). The growth media was YP medium, which contained 1% yeast extract (Sigma, Y-0500) and 2% peptone (Sigma, P-6588).

*T. reesei* Rut C-30 (ATCC 56765) was used in the study of cellulase production. *T. reesei* was grown on potato dextrose agar (Difco Lab., Detroit, USA) slants. 5 days after inoculation at 28 °C, the greenish conidia were harvested and suspended in sterile water. The spore suspension was ready for inoculation to the media of cellulase production.

Seed culture of *T. reesei* was grown in a 500 mL Erlenmeyer flask on a medium containing: 2 g/L KH<sub>2</sub>PO<sub>4</sub>, 1.4 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 0.3 g/L MgSO<sub>4</sub>; 0.005 g/L FeSO<sub>4</sub>; 0.075 g/L MnSO<sub>4</sub>; 0.0015 g/L ZnSO<sub>4</sub>; 0.002 g/L CoCl<sub>2</sub>; 0.3 g/L CaCl<sub>2</sub>; 0.3 g/L urea; 0.015 g/L Tween 80; 0.1 g/L peptone; 5g/L glucose; 10 g/L unbleached kraft hardwood pulp. Silicone sponge closures (part 2004-00005; Bellco Glass, Vineland, NJ) were used to cap the flasks.

### **5.3.2 Cellulase Production**

A seed culture containing 150 mL of medium in a 500 mL Erlenmeyer flask was inoculated with 150 µL of spore suspension (107 spores/mL) and incubated at 28 °C on a rotary shaker at 180 rpm. After two days of pre-cultivation, a 150 mL inoculum from the

seed culture was inoculated into the production medium in order to initiate the cellulase production. The scale up enzyme production experiments were performed in a 3 L fermenter (New Brunswick, USA) with an operating volume of 1.5 L. The temperature, pH, agitation, aeration and dissolved O<sub>2</sub> were automatically monitored and controlled in this fermenter. The composition of production medium for cellulase was the same as that of the corresponding growth medium, except that it was supplemented with different concentration of hardwood pulp (from 10 to 50 g/L) or different concentration of de-ashed sludge instead of 10 g/L grounded hardwood pulp and 5 g/L glucose as the carbon source. Sterile diluted (1:10) antifoam (BYK-019 water defoamer) was added manually to control foaming. Temperature was maintained at 28 °C. The pH was allowed to drop from initial pH 6.0 to three different values, 3.5, 4.0 and 5.0, after which it was adjusted not to fall below those values. The pH was controlled by the automatic addition of NH<sub>4</sub>OH, which also supplied the nitrogen necessary for the synthesis of excreted proteins. Dissolved O<sub>2</sub> was maintained above 25% of the saturation by varying the agitation and aeration rates. Approximately 15 mL of fermentation broth were taken aseptically from the vessel, and it was immediately subjected to phase separation. Supernatants collected by centrifugation (5600 × g, 10 min) were assayed for reducing sugar content, dry cell weight, extracellular protein, and cellulase activity.

### **5.3.3 Enzyme Analysis**

Cellulase activity of the samples was determined as filter paper activity (FPA) expressed in filter paper units (FPU) according to Mandels' procedure (Mandels et al., 1976). 3,5- dinitrosalicylic acid (DNS) method (Miller ,1959) was used to estimate the reducing sugar released in 60 minutes from a mixture of 0.5 mL appropriately diluted

enzyme solution (supernatant of culture broth), 1 mL 0.1 M acetate buffer (pH 4.8), and 50 mg Whatman No.1 filter paper (Whatman Lab., Hillsboro, USA), incubated at 50 °C. One FPU was defined as the amount of enzyme that releases 1 µmol glucose/min. based on 2 mg glucose released in 60 minutes, according to National Renewable Energy Lab procedure (NREL, 2008). Activities were reported as FPU/mL. Carboxymethylcellulase (CMCase; endoglucanase) activity was determined by measuring the release of reducing sugars from 1% carboxymethylcellulose (Sigma) at 50 °C and pH 4.8 (0.1 M acetate buffer) for 10 min. (Wood and Bhat, 1988).

The xylanase activity was determined by following the release of reducing sugars from a 1% birchwood xylan (Sigma) solution at 50 °C for 5 min (Bailey et al., 1992). Xylanase activity was defined as the amount of enzyme that released either 1 µmol of glucose or xylose as reducing sugar equivalents per min.

The β-Glucosidase activity was determined by measuring the release of nitrophenol from 4-nitrophenyl-β-D-glucopyranoside (Sigma) for 15 minutes at 50 °C and pH 4.8 (0.05 M acetate buffer) (Hägerdal et al., 1979). The reaction was stopped by the addition of 1 M Na<sub>2</sub>CO<sub>3</sub>, and the absorbance was measured at 410 nm from a nitrophenol standard curve. One unit of activity was defined as the amount of enzyme that released 1 µmol of 4-nitrophenol per min.

Extracellular protein was assayed according to Bradford's method (Bradford, 1976), using bovine serum albumin as the standard. Dry cell weight (DCW) was assayed according to Morikawa's method (Moedcawa et al., 1985), measuring optical density after treatment with 1 N perchloric acid. One gram of mycelial dry weight per liter corresponded to 0.65x absorbance at 260 nm.

### 5.3.4 Enzymatic Hydrolysis Test

The enzymatic digestibility of the sludge was determined according to NREL Chemical Analysis and Testing Standard Procedures (NREL, 2008). Two different substrates, unbleached hardwood pulp and dilute-acid-pretreated corn stover (NREL) were each tested for enzymatic hydrolysis. The dilute-acid pretreated corn stover had the composition of: 60.0% glucan, 2.5% xylan, 33.6% Klason lignin, 1.2% acid soluble lignin, and 5.7 wt. % unaccounted for acetyl group, ash, and protein. The enzyme loaded was 15 FPU/g-glucan of Spezyme CP or the cellulase produced in this work from both substrates. Screw-capped 250 mL Erlenmeyer flasks were used as the hydrolysis reactor. The samples were suspended in DI water, and put into the flasks to reach a total volume of 100 mL such that the glucan content of the substrates become 1% (w/v) in the reactor. The enzymatic digestibility tests were carried out at 50°C or 36°C, and pH 4.8 using 0.05 M sodium citrate buffer in an incubator shaker (New Brunswick Scientific, Innova-4080) agitated at 150 rpm. The hydrolyzate samples were taken at 6, 12, 24, 48, and 72 h, and analyzed for glucose, xylose, and cellobiose. Total released glucose and cellobiose after 72 h of hydrolysis were used to calculate the enzymatic digestibility:

$$Digestibility(\%) = \frac{Glucose\_released(g) + 1.053 \times Cellobiose\_released(g)}{1.111 \times Glucan\_added(g)} \times 100$$

The digestibilities for xylan contents were also determined in a similar manner. For xylan digestibilities, hydration factor of 1.136 was used in the equation.

### 5.3.5 Simultaneous Saccharification and Fermentation (SSF)

A 250 mL Erlenmeyer flask was used as the bioreactor. It was operated in an incubator shaker (New Brunswick Scientific, Innova-4080) at 37°C with 150 rpm with 100 mL working volume. Sludges and growth medium were added such that the glucan

content becomes 3% or 6% (w/v). The hardwood pulp also underwent the same procedure as a control. The sludge samples were steam sterilized at 121 °C for 15 min. The growth media for SSF was YP medium, which contained 1% yeast extract (Sigma, Y-0500) and 2% peptone (Sigma, P-6588).

The SSF of sludges was carried out 37 °C without pH control. The cellulase enzyme loading was 15 FPU of Enzyme from both substrates or Spezyme CP and 30 CBU of Novozyme-188/g-glucan. Optical density (OD) was measured by UV Spectrophotometer (BioTek Synergy HT Multidetector Microplate Readers) at 600 nm for yeast (NREL-D5A). The initial OD after inoculation was 0.05, equivalent to 50 mg of NREL-D5A/L. In all of the microbial experiments, a sample from each flask was taken at the end of the run and streaked on an YPD plate to check for contamination. Presence of contaminants was also checked under an optical microscope. The ethanol yield was calculated as follows:

$$\text{Ethanol yield}[\% \text{ of theoretical maximum}] = \frac{\text{Ethanol produced (g) in reactor}}{\text{Initial Sugar (g) in reactor} \times 0.511} \times 100$$

Sugar is interpreted as glucose in the SSF.

Fed-batch fermentation was carried out at 37° C in a 250 mL Erlenmeyer flask. Experiments were started with 100 mL initial working volume, and squeezed sludge cakes were added to the bioreactor at desired time to achieve the total glucan content of 6% (w/v). Addition of sludge was done at the 24 h point for the SSF. Cellulase enzymes were also added to achieve overall enzyme loading at 15 FPU and 30 CBU/g glucan. Samples were analyzed for glucose, xylose, organic acid, and ethanol by HPLC. Aseptic

conditions were maintained in all of the microbial experiments. The fed-batch fermentations runs were made in triplicates.

### **5.3.6 Analytical Methods**

The solid samples were analyzed for carbohydrates and Klason lignin in triplicates following the NREL standard procedures (NREL, 2008). The moisture content was measured by an infrared moisture balance (Denver Instrument, IR-30). Sugars were determined by HPLC using an HPX-87P column. For the SSF tests, a BioRad-HPX-87H column was used for measurement of sugar, organic acid and ethanol. A refractive index detector was used in the HPLC. Liquid sample analysis was done in triplicates. The element analysis was done by Soil Testing Laboratory in Auburn University. The metals were analyzed with a Varian Vista Axial ICP. Where applicable, statistical computation including mean value and standard deviation was performed using Microsoft Office Excel 2003.

## **5.4 Results and Discussion**

### **5.4.1 Effect of Different pH on Cellulase Production from Hardwood Pulp**

pH has a major impact on the amount of enzymes produced by *T. reesei*. A combination of pH values and type of carbon sources will result in different yields of cellulose. For example, fermentation under pH 3.0 to 4.0 gave the best result on pure cellulose carbon source (Ryu and Mandels, 1980), while the optimum pH was 4.0 to 5.0 on lactose carbon source. Higher initial pH turned to be better when working with lignocelluloses (Xiong et al., 2004). R. Doppelbauer et al. (R. Doppelbauer et al., 1987) found a constant pH 6.0 during the fermentation gave optimal yields in the case of wheat straw, waste paper and sulfite pulp as substrates.

In this study, the pH was allowed to drop from an initial pH of approximately 6.0 to three different values of 3.5 (Condition I), 4.0 (Condition II), and 5.0 (Condition III). Once the desired “floor” pH was reached, the environment was adjusted to prevent any further decline.

Fig.V-1 shows the pH of cultivation strongly influenced the production of cellulase. The highest cellulase activity was obtained at Condition I. With the increase in pH the cellulase activity decreased gradually. The cellulase activity reached its maximum of 4.6 FPU/mL for Condition I at 96 h, 4.2 FPU/mL for Condition II at 96 h and 3.5 FPU/mL for Condition III at 84 h, respectively. These are shown as in Fig.V-1. Table V-1 shows these values in a tabular form.

During the first day of the fermentation process, a rapid drop in the pH was observed. Sternberg reported the phenomenon and ascribed this to liberation of the hydrogen ions, which resulted from the depletion of the inorganic nitrogen source (Sternberg, 1976). A rapid drop in the dissolved oxygen (DO) level and an increase in the agitation and the aeration rates (data not shown here) were also observed. To address this, the DO level was kept above 25% of the saturation value by varying the agitation and the aeration rates in response to changes in the amount of DO. These issues merely demonstrated that the fungal growth started immediately after inoculation. To confirm this educated guess, measurements of the reducing sugars in the hardwood pulp were taken. The results showed that the concentration of reducing sugars was less than 1.5 g/L during whole fermentation process, which indicated that fungus used most of the reducing sugars.

Enzyme secretion displayed a lag-phase of about 24 h at a pH of 5.0. After that, the rate of enzyme secretion rapidly increased and most of the enzyme secretion was obtained during the stationary phase. At the stationary phase, the DO level was still controlled at 25%, however, the air flow rate decreased slightly during fermentation as compared to the air flow rate during the cell growth phase. About 10% of cellulase was continuing to be excreted when the pH was increased from 3.5 to 5.4 under Condition I from 72 h to 96 h. About 5 % of FPU was continuing to be excreted when the pH increased from 4.0 to 5.4 under Condition II from 72 h to hour 84 h. It is probable that the lower cellulase amount came from being physically adsorbed on the remaining fermentation solids, which mainly consisted of fungal mycelium. The pH values increased from 5.0 to 6.7 during 72 h to hour 96 h. The DCW decreased during this time, which may be due to autolysis of the fungus at the higher pH. The cellulase activity decreased after 84 h under Condition II and after 72 h under Condition III, which may be due to the deactivation of the enzyme as a result of the higher pH. Another possibility for the reduced cellulase activity could be proteolysis of cellulase, which tends to occur in the late station stage.

Observations were made of the cellulase production growth profile. It was found that the time required for the fungus to reach maximum cell concentration was approximately 36 h under Condition I and Condition II, and 48 h under Condition III. It is interesting to note that under Condition III, the DCW was much higher than the other conditions. Reducing sugars were measured at the same time and it was noticed that under Condition I, the amount was low, i.e. less than 1 g/L concentration of reducing sugars. At the same time, maximum DCW was 3.8 g/l. Although the pH was relatively

low at this condition, it was still high enough for the fungus to grow and to take part in the fermentation. Under Condition II, clearly the fungus was able to grow better, the maximum DCW increased to 4.4 g/L (see Fig.V-1). Under Condition III, the maximum DCW was 5.8 g/L.

The optimal pH for enzymatic hydrolysis of cellulose is 4.8 (Ghose, 1987). Some researchers used the pH of 4.8 for their cellulase studies (Olsson et al., 2003), but 4.8 is not the optimal pH when the carbon source is hardwood pulp.

Although *T. reesei* grew faster at the higher pH (Condition III), the obtained final cellulase activities were 20% lower. The reason may be that lower pH produces slow growth and prolongs the period of enzyme production. Inside the cell, where the pH is neutral, immediate dissociation of the acids occurs, causing a decrease in the intracellular pH, which can inhibit cell growth. Because these compounds are weak acids, the concentration of the associated acids increased and thus inhibited cell growth. If the pH beyond the cell's walls is low, then the growth is inhibited even more (Lambert and Stratford, 1999). It is known that microorganisms often have multiple systems for a single function. Therefore, different metabolic pathways could be used depending on the pH conditions. The level of cellulase increase in acidic medium suggests that *T.reesei* cells may use different metabolic pathways depending on the pH of the extracellular environments. Sternberg et al. (1976) suggested that the fungi should be grown in an environment where the pH values are sub-optimal for enzymatic hydrolysis in order to gain benefits such as reduction in the efficiency of cellulose with the reduced accumulation of extracellular sugars and reduction in the impact of catabolite repression of enzyme synthesis.

In addition to cellulase, a certain amount of xylanase and glucosidase are also produced from *T. reesei*. With hardwood pulp containing 20% xylan, higher  $\beta$ -glucosidase and xylanase activities were expected to be achieved than from pure cellulose. Chaudhary and Tauro (Chaudhary and Tauro et al., 1982) found that xylan (a good carbon source) positively affects the production of  $\beta$ -glucosidase, and Bailey (Bailey et al., 1993) found that xylan was an effective inducer of xylanases. The measurements supported this expectation. The highest  $\beta$ -glucosidase and xylanase activities were obtained under Condition III. When the pH was reduced, the  $\beta$ -glucosidase and xylanase activities were gradually decreased. Other researchers had similar results (Tangnu et al., 1981; Bailey et al., 1993). Xiong found that at a low pH cellulase was prevalent and at a high pH xylanase was prevalent. However, they found that cellulase activities did not change much when the pH values were in the range of 4.0 to 5.5 and they noticed that the cellulase activities actually decreased significantly when the pH was 3.5 (Xiong et al., 2004). The different results might be due to the usage of lactose as a carbon source (Xiong et al., 2004). The results are presented in Table V-1.

#### **5.4.2 Effect of Different Hardwood Pulp Concentration**

Previous work indicates that higher concentration of the carbon source resulted in higher enzyme activities (Esterbauer et al., 1991; Tangnu et al., 1981; Yu et al., 1998). Fig.V-2. shows the impact of different hardwood pulp concentrations on cellulase production in the 3 L fermentor.

Enzyme formation commenced about one day after inoculation. The maximum enzyme activity was reached after 4 to 5 days of incubation. The pH was allowed to drop from the initial pH value of 6.0 to 3.5 and was adjusted not to fall below this value. The

pH values increased after 3 to 5 days of incubation (data not shown here). As shown in Fig.V-2., the enzyme activities and DCW increased in proportion to substrate concentration, up to 40 g/L. The highest productivity was also obtained at substrate concentration of 40 g/L, however, yield was highest by using 20 g/L hardwood pulp as a substrate (see Table V-2). This result agreed with previous studies (Esterbauer et al., 1991; Tangnu et al., 1981; Yu et al., 1998). A maximum of 7.5 FPU/mL was found when the concentration is 40 g/L of hardwood pulp, which is comparable to the results with pure cellulose such as Solka Floc (Velkovska et al., 1997; Domingues et al., 2000) and the results with glucose or lactose (Ahamed and Vermette, 2008; Domingues et al., 2001).

Several studies speculated that lignin on the fiber surface would impair inter-fiber bonding due to the hydrophobic nature of lignin (Shao and Li, 2006; Li and Reeve, 2002). Since chemical pulping dissolves the lignin and other materials of the inter-fiber matrix material and dissolves most of the fiber wall lignin, the pulp fibers become more hydrophilic and acquire surface groups that are capable of hydrogen bonding resulting in improved inter-fiber bonding. Therefore, the adjacent fibers in the pulp tend to held together by the hydrogen bond formation between the cellulosic surfaces (Sixta, 2006; Nissan and Batten, 1990). In this context, it seems that further increase in substrate concentration would result in decreased production levels due to the creation of very thick slurry. As expected, at 50 g/L concentration of hardwood pulp, the cellulase activity markedly decreased since the mixture was too thick to achieve effective mixing or aeration. The fungus growth rate was also found to be low. As Fig.V-2 shows, at 50 g/L hardwood pulp concentration, the fungus grew more slowly and maximum DCW was

lower than with other concentrations. Schugerl (Schugerl et al., 1981) reported that high viscosity unfavorably influences mixing and oxygen transfer. When higher viscosity mixtures are used, higher power inputs are required to achieve similar levels of mixing in the gas/liquid interfacial area. The stirring efficiency is reduced since the impeller is more easily flooded. The mass transfer coefficient decreases. Researchers found that with better designs in the aerator and mixer stages in a larger scale fermentor, higher cellulase activity could be obtained as a result of the more efficient oxygen mass transfer. Any such design would avoid oxygen supplementation since any operation that involved oxygen supplementation would make the final products more expensive (Hayward et al, 2000).

#### **5.4.3 Enzyme Production from De-ashed and Chemical Treated Primary Sludge**

The composition analysis of the primary sludge indicates that the hemicellulose and lignin content is low compared to other raw materials. This property of the sludge should be favorable for cellulase production. Unfortunately, high ash content is detrimental to the growth of microorganisms. Moreover, all residues retained from each grade of operation in the paper making process made a great negative effect on the cellulase production. In our study, using original untreated sludge as substrate, the fungus was not able to grow in the fermentation medium. Apparently ash and some other chemicals in the sludge greatly inhibit the cellulase production, even the cell growth. So, it is desirable to remove ash from the feedstock, while retaining carbohydrates.

However, cellulase activity measured for the production culture in a 3-L fermenter was still low (less than 2 FPU/mL) by using de-ashed sludge with ash content of 14.0% (data not shown here). It is worth noting that an increase in the substrate concentration from 25 g/L to 35 g/L did not improve, but instead inhibit the cellulase

production. All these results indicated that the primary sludge, with high cellulose content due to hemicellulose and lignin removed in pulping process, was not effective in the cellulase production. The cellulase production was inhibited by the by-products generated during the paper and pulping process. Ash and some other insoluble chemicals such as clay,  $\text{CaCO}_3$  are really detrimental to the microorganism and inhibitory against the cellulase production. Further treatment of sludge is required to enhance the cellulase production.

De-ashed sludge was leached with 1 N sulfuric acid and further water washing. In this case,  $\text{CaCO}_3$ , clay could be partly removed. Cellulase production with acid treated sludge as the substrate performed better than the previous runs (Fig.V-3). The cellulase production commenced within 24 hr, which indicated the lag phase was shortened due to the better substrate and cell growth. The Filter paper activity and productivity reached its maximum of 3.2 FPU/mL and 37.5 FPU/L·h at 96h, respectively.

Seeking further improvement of cellulase production, the acid treated sludge was treated with 1% (w/v) NaOH solution and further water washing. The ash in the alkaline treated sludge was further decreased to 3.8 %, the glucan content was increased as shown in Table V-3. Most of the inhibitory chemicals against the cellulase production such as aluminum and copper were further removed by this NaOH based treatment. This further treatment provided the sludge good characteristics as carbon source for cellulase production. The strain grew well, and produced 8 FPU/mL of cellulase at 132h with 35g-glucan/L (47g/L sludge) concentration (Fig.V-4). The yield of cellulase enzyme and productivity reached 307 FPU/g of glucan and 60.6 FPU/L·h, respectively. And the specific cellulase activity was 8 FPU/ mg protein. Table V-3 shows the activities of

cellulolytic and hemicellulolytic enzymes of the fermentation broth. The cellulase titers, productivities of all runs above were summarized in Table V-4.

The specific cellulase activity was obtained from 40 g/L hardwood pulp is 7.7 FPU/mg protein. Although the protein content of enzyme was measured by the Bradford method, without TCA precipitation, the value was much higher than the reported specific activity of commercial cellulases. These numbers, however, do not necessarily reflect the molecular activities of the respective enzymes. It may have more to do with the fact that some of the commercial enzymes contain non-enzyme proteins mixed in as a nutrient during fermentation. In the cellulase production from both substrates, nitrogen sources were mostly inorganic salts, therefore, background protein levels were very low in the broth. Additionally, it suggests a possibility of developing a simpler purification process.

This inhibitory effect may be due to metal ions in the sludge. Stutzenberger and Sterpu found that the metals in municipal refuse compost, such as aluminium and calcium, appeared to specifically inhibit cellulase production at high concentration (Stutzenberger and Sterpu, 1978). The data in this study agreed with it. The calcium content was reduced from 8.9 % (untreated sludge) to 4.7% (deashed sludge), 1.5% (acid treated sludge) and 1.1 wt. % (alkaline treated sludge). The aluminum content was reduced from 0.55 % (untreated sludge) to 0.44 % (deashed sludge), 0.37% (acid treated sludge) and 0.003 wt. % (alkaline treated sludge).

#### **5.4.4 Enzymatic Hydrolysis Test**

Performance of hydrolysis tests were conducted for hardwood pulp enzyme (HP-enzyme) and sludge enzyme using two different substrates: Corn stover pretreated by dilute-acid and kraft processed hardwood pulp. The same hydrolysis test was done for

Spezyme CP (Genencor, Lot No. 301-00348-257) for comparison purposes. A glucan loading of 1% (w/v) and 15 FPU/g-glucan of enzyme loading were used. The hydrolysis profiles are shown in Fig.V-5. Only data that were from the hydrolysis and fermentation tests by using HP-enzyme are shown here, since both enzymes produced from hardwood pulp enzyme (HP-enzyme) and sludge enzyme had the same performance.

In side by side comparison between HP-enzyme and Spezyme CP for enzyme hydrolysis of pretreated corn stover, the yield and the glucose profile were almost equal, 91.0% for Spezyme CP and 90.2% for HP-enzyme. Since both enzymes were supplemented without  $\beta$ -glucosidase, the difference in gross activity indicates that there is a very slight difference in endo and exo-glucanase activities between the two.

The same tests were repeated using hardwood pulp as a substrate. The results were slightly different from the previous test as shown in Fig.V-5. The HP-enzyme showed higher overall glucose yield than Spezyme CP. The 72 h yields were 88.2% (1% glucan loading) for HP-enzyme and 85.2% (1% glucan loading) for Spezyme CP. The major difference between the two substrates is the xylan content: 2.5% in acid-treated corn stover versus 21.1% xylan in hardwood pulp. We speculate that the difference in yield has to do with the xylanase activity. This was confirmed by the xylan digestibility data from the same experiments and the xylanase activity of the two enzymes. The xylan digestibility profiles for the two enzymes are shown in Fig.V-5. The results confirmed that HP-enzyme has higher xylanase activity than Spezyme CP. The high xylanase activity of HP-enzyme may result from the fact that hardwood pulp has relatively high xylan (21.1%) which may have acted as an effective inducer for xylanase production by *T. reesei*. Other researchers have proposed that xylan removal improves the access of

cellulase enzyme to cellulose and thus improves cellulose activity (Öhgren et al., 2007). Furthermore, it was also found that xylose has a direct inhibitory effect on cellulase production (Xiao et al., 2004; Nigam and Prabhu, 1991; Todorovic and Grujic, 1987; Kim and Lee, 2005).

#### **5.4.5 SSF Experiments**

Batch and fed-batch SSF experiments that used paper sludge as the substrate were performed over a 5-day period and ethanol concentration was monitored as a function of time. Fig.V-6. shows the ethanol data obtained from the SSF tests. In the batch SSF experiments, the 96 h measurement for a cellulase from the hardwood pulp loading of 15 FPU/g-glucan revealed that the ethanol concentrations had reached 25.2 g/L, which is similar to what was obtained when using a commercial cellulase (25.1 g/L). The commercial cellulase had almost the same amount of ethanol yield, which was 74% (based on 6% (w/v) glucan).

In the fed-batch SSF experiments, the ethanol concentration could achieve 45 g/L, however only 68% yield was obtained (data not shown). The low conversion is attributed to the cellulase enzyme reaction rather than to the microbial reaction. *Saccharomyces cerevisiae* (D<sub>5</sub>A) is very robust and has a high ethanol tolerance. We believe that the high-solid conditions in the bioreactor and in the product plus substrate inhibition are major factors for suppressing the cellulase enzyme reaction.

The projected cellulase plant is a low-investment supplementary unit that can be built within a kraft paper mill. On-site cellulase production can be promoted as a stand-alone unit or as a unit within an ethanol production system supplying the enzyme in-house, thus reducing the overall production cost.

## 5.5 Conclusion

To reduce the cost of pretreatment and to simplify the bioconversion process, unbleached kraft processed hardwood pulp and treated paper sludge were tested as feedstocks for production of cellulase enzymes using *T. reesei* Rut C-30. The *T. reesei* strains can produce cellulase enzymes in an efficient manner. The cellulase production by this scheme yielded 246.7 FPU/g-glucan of hardwood pulp, and a productivity of 56.8 FPU/L.h. The titer of cellulase produced from 4% hardwood pulp loading was 7.5 FPU/mL, which is comparable to those produced from pure cellulosic substrates. The cellulase production yield of 307 FPU/g glucan of sludge, and productivity of 60.6 FPU/L h were achievable from alkaline treated sludge. The titer of enzyme was 8.0 FPU/mL. The specific activities of cellulase from both substrates were higher than that of the commercial cellulases. The main reason is that cellulase was produced here using inorganic salts as a main nitrogen source, thereby resulting in very little non-enzyme proteins in the broth. Cellulase produced from has both substrates has higher xylanase activity than Spezyme CP. No difference was found between cellulase produced from unbleached hardwood pulp and Spezyme CP in their performance in the ethanol production from primary paper sludge.

Table V-1. Enzyme activities and productivity obtained in the cultivation of *T. reesei* Rut C-30 on 20 g/L hardwood pulp under different pH control conditions

pH control conditions	Maximum FPA Time (hr)	FPA (FPU/mL)	Productivity (FPU/L/h)	Yield (FPU/g)	$\beta$ -Glucosidase (pNPG U/mg)	Xylanase (IU/mg)
Condition I	96	4.6	47.9	230	0.35	49.3
Condition II	96	4.2	43.8	140	0.39	56.1
Condition III	84	3.5	41.7	87.5	0.51	78.9

Table V-2. Enzyme activity, yield and productivity obtained in the cultivation of *T. reesei* Rut C-30 on different concentration of hardwood pulp as substrates

Hardwood pulp concentration (g/L)	Time (h)	FPA (FPU/mL)	Productivity (FPU/L/h)	Yield (FPU/g glucan)
20	96	4.6	47.9	302.6
30	120	6	50.0	263.2
40	132	7.5	56.8	246.7
50	120	4.5	37.5	118.4

Table V-3. Enzyme activities obtained in the cultivation of *T. reesei* Rut C-30 on 40 g/L hardwood pulp

Filter Paper Activity (FPU/mL)	$\beta$ – Glucosidase (pNPG U/mg)	Xylanase Activity (IU/mg)	CMCase Activity (IU/mg)
7.5	0.4	156.2	4.2

Table V-4. Specific activities of cellulolytic and hemicellulolytic enzymes of fermentation broth of alkaline treated sludge

Filter Paper Activity ( FPU/mL)	$\beta$ – Glucosidase (pNPG U/mg)	Xylanase Activity (IU/mg)	CMCase Activity (IU/mg)
8.0	0.5	170.2	4.4

Table V-5. Cellulase activity and productivity of *T. reesei* Rut C-30 in 3-L fermenter using acid and alkaline treated paper mill sludge as substrates

	FPA (FPU/mL)	Productivity (FPU/L/h)	Yield (FPU/g glucan)
Acid Treated Sludge	3.2	37.5	176.3
Alkaline Treated Sludge	8.0	60.6	306.8

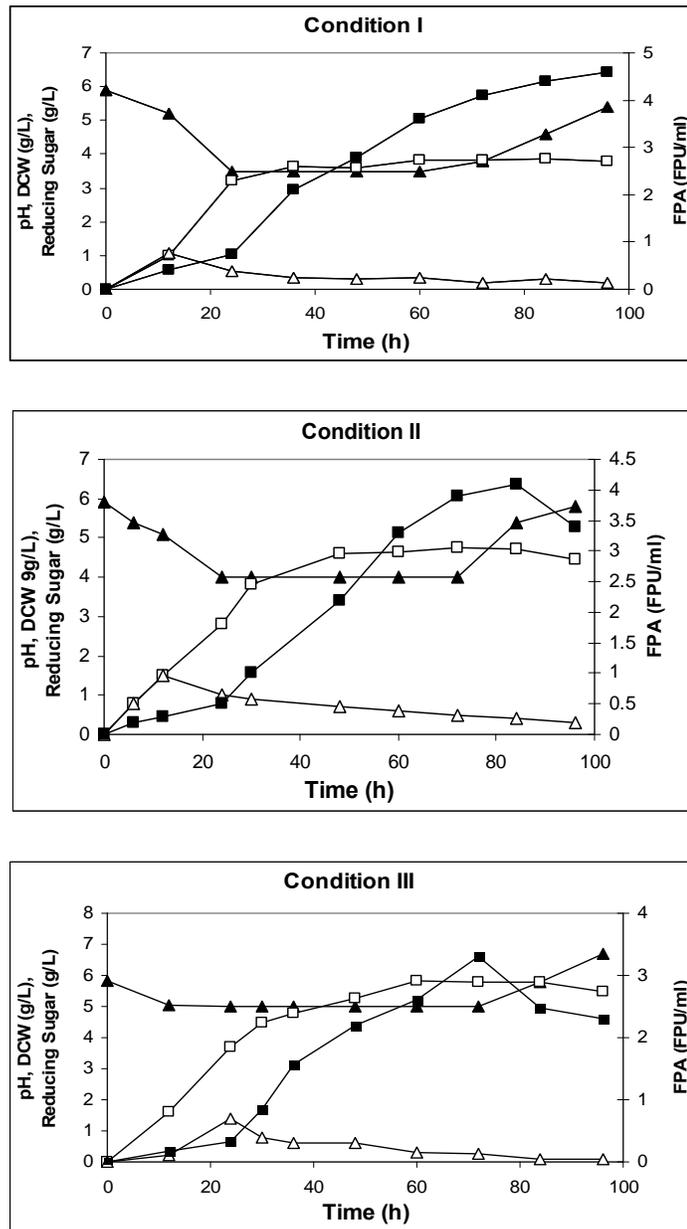
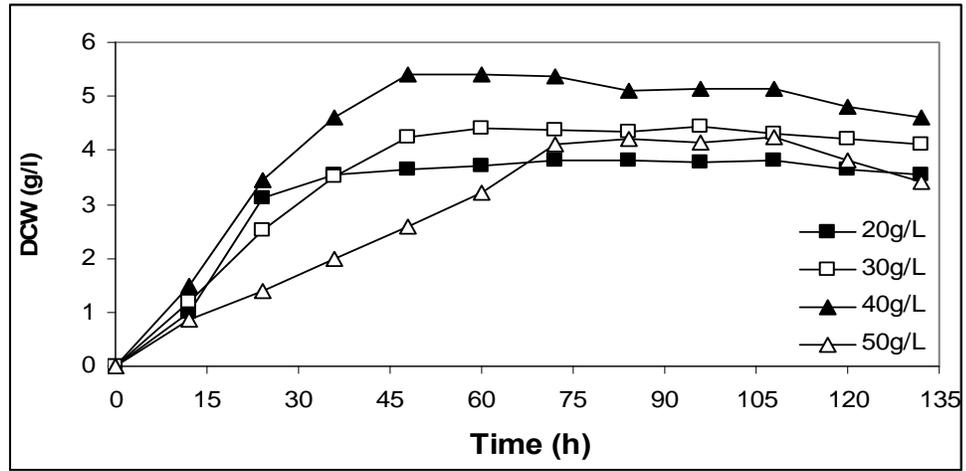


Fig.V-1. Cellulase production by *T. reesei* Rut C-30 under different pH control conditions. Squares with connecting lines represent Dry Cell Weight (DCW, g/L). Triangles with connecting lines represent Reducing Sugar (g/L). Filled triangles with connecting lines represent pH. Filled squares with connecting lines represent Filter Paper Activity (FPA, FPU/ml).

A



B

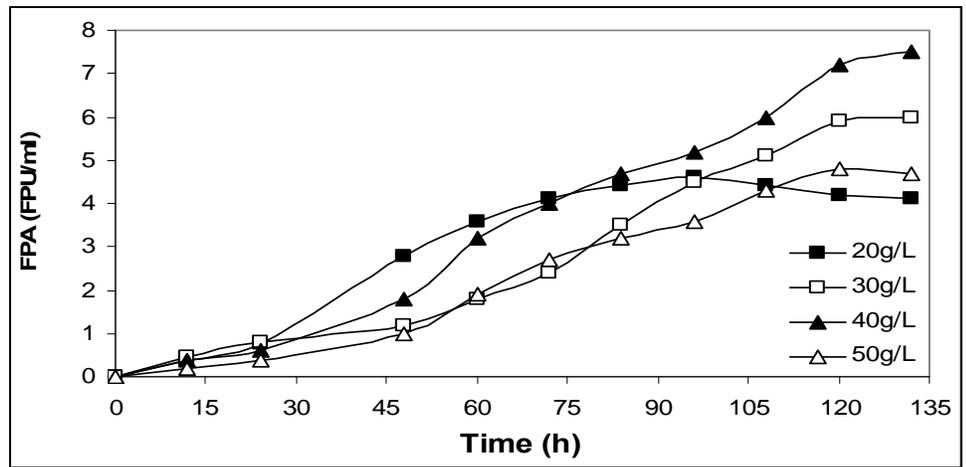


Fig.V-2. Cellulase production by *T. reesei* Rut C-30 at different concentration of hardwood pulp

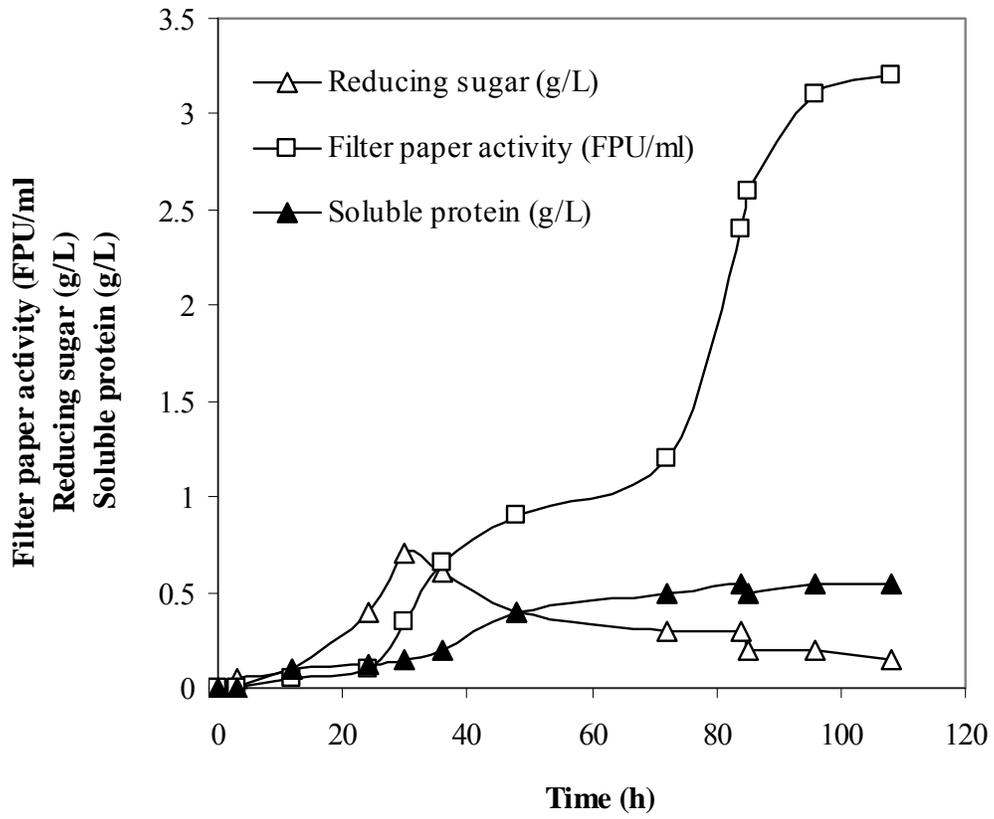


Fig.V-3. Cellulase production by *T. reesei* using acid leached sludge

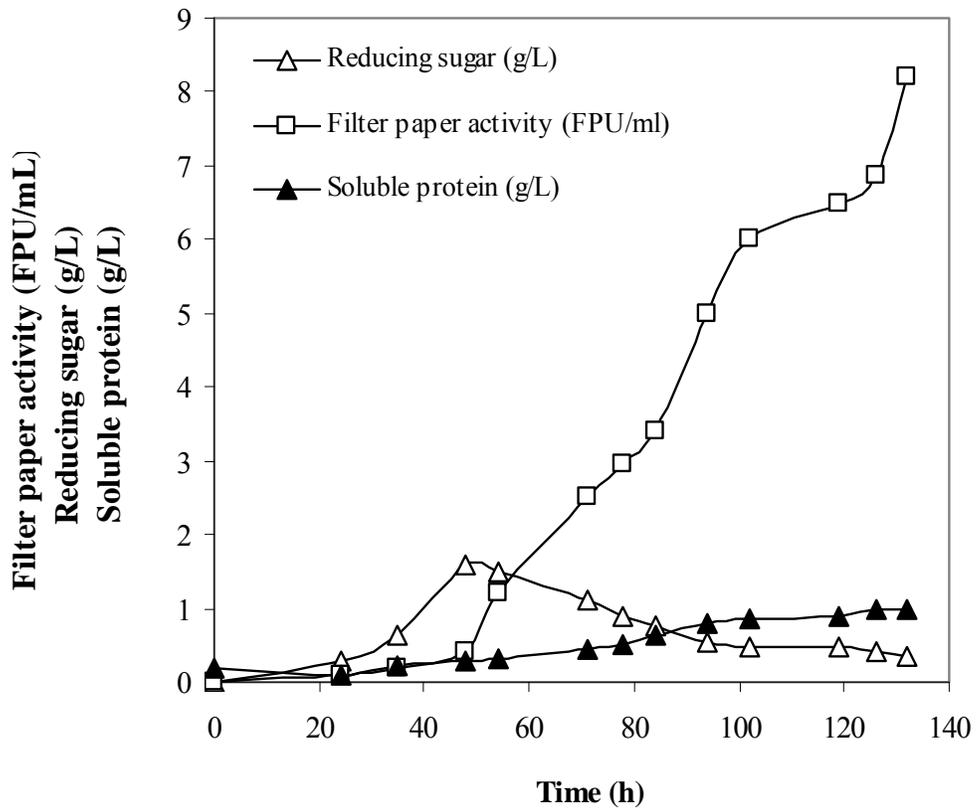
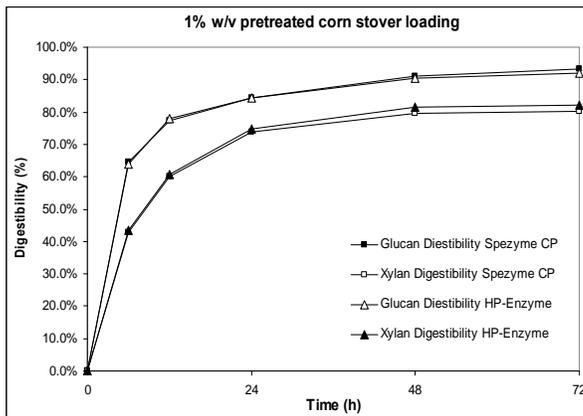
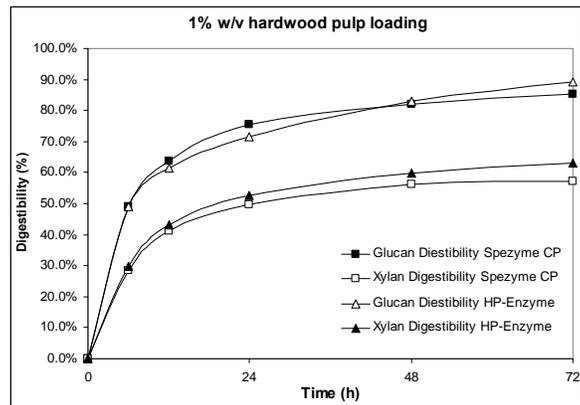


Fig.V-4. Cellulase production by *T. reesei* using alkaline treated sludge



A



B

Fig.V-5. Enzymatic hydrolysis of different substrates by HP-enzyme and Spezyme CP

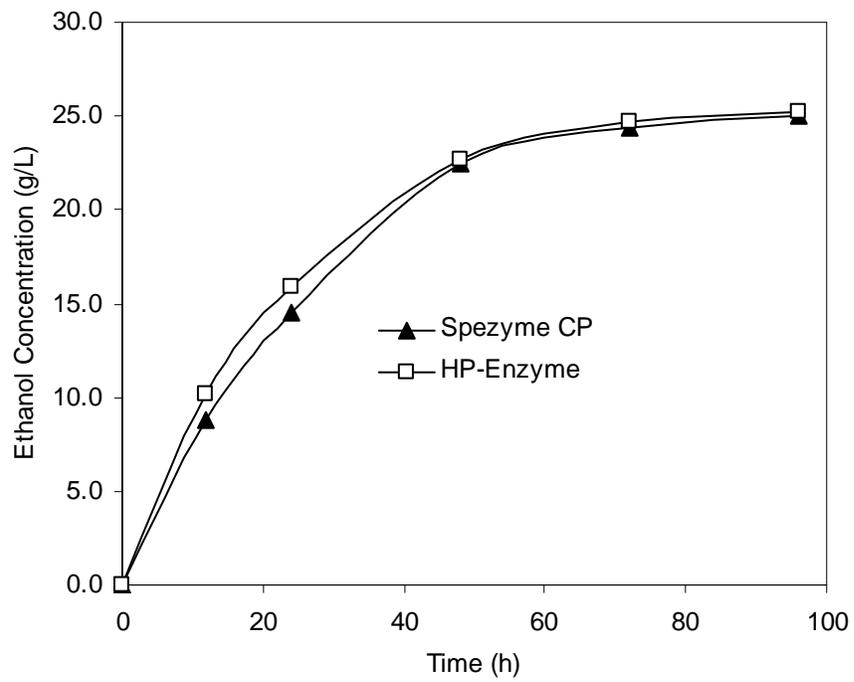


Fig.V-6. Ethanol production from untreated primary sludge by HP-enzyme and Spezyme CP

## **VI. Ethanol Production from the Mixture of Hemicellulose Prehydrolysate and Paper Sludge, Integrated with Hemicellulose Pre- extraction of Southern Pine**

### **6.1 Abstract**

Most of the hemicellulose fraction of pulp mill feedstock (softwood or hardwood) is released into black liquor during the pulping process. The black liquor is combusted to recover chemicals and to generate steam and electricity. It is technically feasible to recover this fraction of carbohydrate and enhance its value by converting it into value-added products. Hemicellulose is selectively converted to soluble sugars (termed as prehydrolysate) by treating it with hot water. The sugars produced from pre-hydrolysis process are mixtures of pentose, hexose, and their oligomers. In this study, pectinase and *Saccharomyces cerevisiae* were used to convert the prehydrolysate into ethanol. The prehydrolysate produced from wood also contains toxins, primarily lignin and sugar degradation products, which strongly inhibit microbial reaction. De-toxification of the prehydrolysates was done by overliming (addition of excess CaO). When hydrolysate is obtained by treating wood, the total sugar concentration is below 4 wt. %. Consequently, when the hydrolysate is used as a fermentation substrate, the ethanol concentration is less than 2%, which is far below the level acceptable as distillation feed. Use of the mixture of

prehydrolysate and pulp mill sludges as the fermentation feed, however, can increase the product concentration. In bioconversion of sludge, a certain amount of water is added to attain fluidity required for Simultaneous Saccharification and Fermentation (SSF) operation. In this study, prehydrolysate, in place of water, was added into the bioreactor along with the sludge. Using this procedure, there was a net increase of total sugar concentration in the bioreactor above that of the base case, which led to an increase in product concentration. The experimental data detailing the proposed bioprocess converting the mixed-feed to ethanol are presented in this paper.

## **6.2 Introduction**

While the commercialization of bio-ethanol production is still emerging from the cradle, another industry is struggling in the United States, namely the pulp and paper industry. The combination of reduced demand for paper in the domestic market along with the increased supply of cheap foreign paper, primarily from Asia, is causing great difficulty for the pulp and paper industry in the United States, forcing the pulp and paper industries to find new opportunities in order to improve their competitiveness. An integrated bio-refinery which can extract hemicellulose while preserving cellulose for pulp production can be developed. Such a process can transform the pulp and paper industry into a competitive and innovative one, which would allow significant economic growth through new value-added products (i.e. ethanol), while still preserving the main product-paper.

In the United States, 80% of total pulping capacity is chemical pulp. Approximately 98% of chemical pulp capacity is kraft pulping while the remaining 2% is sulfite pulping (American Forest & Paper Association, 2002). Eighty percent (80%) of

the hemicellulose fraction of pulp mill feedstock (softwood or hardwood) is released into black liquor during the kraft pulping process. The black liquor is burned to recover chemicals and generate steam and electricity. Since the heating value of hemicellulose (13.6 MJ/kg) is only half of that of lignin (27 MJ/kg), and it is technically difficult to separate this hemicellulose part from alkaline black liquor before it is burned to generate heat and power (Sjostrom, 1993), pre-extracting the hemicellulose prior to the pulping process for producing high value products has the potential to improve the overall economics of the process.

It is technically feasible to remove part of the hemicellulose before pulping. Hemicellulose is selectively converted to soluble sugars (termed as prehydrolysate) in treatment with hot water (Yoon et al., 2008; Yoon et al., 2010 ), diluted acid (Springer and Harris, 1982) or mild alkaline hydrolysis (Al-dajani and Tschirner, 2008). Hot water extraction is attractive for wood hemicellulose removal due to savings in the cost of mineral acid or alkaline. The sugars produced from prehydrolysis contain mixtures of pentose, hexose, and their oligosaccharides.

Hemicellulose prehydrolysate can be converted to ethanol from lignocellulosic biomass through hydrolysis and downstream fermentation processes (Walton et al., 2010a). Besides ethanol, other value added chemicals are also produced from hemicellulose prehydrolysate (Walton et al, 2010b; Soderqvist et al., 2001). Oligosaccharides may further prove to be a source of higher value-added products, such as animal feed additives (Davis et al., 2002; Fernandez et al., 2002).

The major softwood hemicellulose is an O-acetyl-galactoglucomannan (15-25% of wood) which is beta-1,4-linked mannose and glucose residues in the ratio 3:1 often

with alpha-1,6-galactose as side group (Timell, 1967; Ethier et al.,1998;Meier, H. 1961; Katz, 1965; Lindberg et al., 1973). Because softwood hydrolysates contain much more mannose than xylose, there is less need for a xylose-fermenting organism when this raw material is used. Regarding softwood hydrolysates, *S. cerevisiae* has a great advantage over Ethanologenic Bacteria, such as, *E-coli*, since *S. cerevisiae* has higher tolerance to toxin and ethanol. *Saccharomyces cerevisiae* was used to convert the hexose in the prehydrolysate into ethanol in this study. The prehydrolysate produced from lignocellulosic biomass contains some toxic compounds such as furan, organic acids, and various phenolic compounds that inhibit microbial fermentation to obtain the desired products (Klinke et al. 2004; Palmqvist and Hahn-Hägerdal 2000). The prehydrolysate, therefore, has to be detoxified for efficient conversion to ethanol. Several detoxification processes can be used for removing the inhibitory compounds from wood hydrolysates, such as over liming (Martinez A et al, 2001), ammonium hydroxide detoxification (Alriksson et al, 2005), enzymatic detoxification (Jönsson et al., 1998), ion exchange resins (Fein et al., 1984), active charcoal (Roberto et al., 1991), extraction (Fein et al., 1984), and adsorption (Roberto et al., 1991). The detoxification of pine wood prehydrolysate was developed in this study.

To avoid excessive damage to the fiber quality, hemicellulose can not be totally removed. Therefore, the pre-extraction process gave a low concentration of sugars. If the prehydrolysate is used as a fermentation substrate, the ethanol concentration is less than 2.0%, which is far below the level acceptable as distillation feed. In bioconversion of the sludge, the sludge needs to be diluted with water to attain the fluidity required for the fermentation operation. In this study, prehydrolysate, in place of water, was added into

the bioreactor along with the paper sludge. Under the proposed scheme, there is a net increase of total sugar concentration in the bioreactor compared to prehydrolysate alone, thereby reducing the cost of ethanol distillation.

## **6.3 Materials and Methods**

### **6.3.1 Materials**

Feedstock: Fresh southern pine chips were obtained from Rock-Tenn Company in Demopolis, Alabama. Chips with major defects including bark, knots, and decayed parts were removed prior to screening on a chip class laboratory screen equipped with a stack from top to bottom of 45-mm round screens, 8-mm bar screens, 6-mm bar screens, and 4-mm round screens. The wood fraction passing 45-mm round screens and 8-mm bar screens and retained on 6-mm bar screens was collected, well mixed, and air-dried before use.

The paper primary sludge (PS) was collected from the primary wastewater clarifier unit of a kraft paper mill, Boise Paper Company (Jackson, AL, USA). The sludge was washed with tap water three times and was further thickened to 38% consistency using a vacuum filter, and stored at 4° C. The primary sludge was analyzed for carbohydrates, moisture content, and ash content according to the National Renewable Energy Laboratory (NREL) standard procedure (NREL, 2008). The primary sludge was analyzed and found to contain 44.5 % glucan, 9.9 % xylan, 8.1% lignin and 36.0 wt. % ash, which includes 26.0% acid-soluble ash and 10.0% acid-insoluble ash.

Enzymes: Cellulase enzyme (Spezyme CP, Lot No. 301-00348-257, 59 FPU/mL, 123mg protein/mL), Xylanase (Multifect Xylanase, Lot No. 301-04021-015; 42 mg protein/mL), Pectinase (Multifect Pectinase FE, Lot No. A21-03356-001, 82 mg

protein/mL), and measurements of their protein content/ specific activities were generously provided by Genencor Division of Danisco US, Inc. (Palo Alto, CA, USA).  $\beta$ -glucosidase (Novozyme 188, Cat. No. C-6150, 665 CBU/mL, 140mg protein/mL) was purchased from Sigma (St. Louis, MO, USA). The activities present in commercial enzyme preparations are shown in Table VI-1 which is based on the publication by Dien et al (Dien et al., 2008; Berlin et al., 2007).

Microorganism: The microorganism used in the SSF was *Saccharomyces cerevisiae* ATCC-200062 (NREL-D5A). The growth medium was YP medium, which contained 1% yeast extract (Sigma, Y-0500) and 2% peptone (Sigma, P-6588).

### **6.3.2 Pre extraction**

Hot water has proved to be an effective solvent for the extraction of hemicellulose from wood (Werpy and Petersen,2004). Three stage extractions were done to increase the sugar content in the extracted liquor and to simulate the continuous extraction process. The extractions were conducted using a 500 mL cylindrical bomb digester that was placed inside a computer-profiled M/K laboratory digester filled with water as a heat transfer fluid. In every water-extraction stage, seventy grams of oven-dried untreated softwood chips were used in the bomb digester at a liquor-to-wood ratio is 5.8 to 1. The digester temperature was ramped from room temperature to a preset maximum temperature of 170 °C at a rate of 3.2 °C per minute. At the end of the digester operation, each bomb was then quenched in a cold water bath. Extraction times at the preset extraction temperature (170 °C) varied from 0 to 90 min to attain various wood weight loss levels ranging from 0 to 15% based on oven-dry wood. After completion of water extraction, extract was drained from the wood chips (70% of the total liquor) and

collected to be used for chemical analysis. The liquor used in the first stage was deionized (DI) water. The liquor used in the second stage was mixed with the extract liquor from the first stage extraction (70% volume of the total first stage liquor) and additional DI water to make up lost volume (30% volume of the total first stage liquor) since the extracted wood chip absorbed around 30% volume of total liquor. The liquor used in the third stage was mixed with the extract liquor from the second stage extraction (70% volume of the total second stage liquor) and DI water (30% volume of the total second stage liquor). As wood chips are extracted, their weight can be expected to decrease due to components dissolved and diffused from the wood into the extraction liquor (termed as prehydrolysate). The wood weight loss data were calculated from the difference between the weight of fresh wood chips and that of thoroughly washed wood residue based on its dry weight. The sugar contents of the extract were determined according to methods published by the National Renewable Energy Laboratory (NREL, 2008). The total amount of cellulose, and hemicelluloses, which led to these sugar concentrations, were then calculated using the following equations described by van Heiningen et al. (Yoon and van Heiningen, 2010b).

$$\text{Cellulose} = \text{Glu} * \left(\frac{162}{180}\right) - \frac{\text{Man}}{b} * \left(\frac{162}{180}\right)$$

Where  $b = 4.15$  (This is the average value for number of mannose units per glucose unit in hemicellulose of pine/spruce wood, based on Janson (Janson, 1974) :

$$\text{Hemicellulose} = (\text{Ara} + \text{Xyl}) * \left(\frac{132}{150}\right) + (\text{Gal} + \text{Glu} + \text{Man}) * \left(\frac{162}{180}\right) - \text{Cellulose}$$

In order to determine the intensity of the pre extraction, the combined effects of time and temperature were measured in terms of the H-factor (H), defined as:

$$H = \int_0^t \left( \exp\left(43.20 - \frac{16.113}{T}\right) \right) dT$$

Where t is the time (hours) needed for achieving the desired temperature (T).

Table VI-2 shows the first stage of water-extraction condition, weight loss of wood chips, and the concentrations of glucose, xylose, galactose, arabinose, and mannose. The water-extracted wood chips were used in the subsequent treatment and kraft pulping experiments (Yoon et al., 2010).

### 6.3.3 Composition Analysis of Prehydrolysate

The liquid samples were analyzed for carbohydrates following the NREL standard procedures (NREL, 2008). The extracted liquor was centrifuged to separate the solid part from the liquid. Then, the solid-free liquid was incubated for 1 h with 4% sulfuric acid at 121°C in an autoclave along with sugar recovery standards determined according to methods published by the National Renewable Energy Laboratory (NREL, 2008). The liquid was neutralized with CaCO<sub>3</sub>, and the total amount of sugar monomers was analyzed by high pressure liquid chromatography (HPLC). The concentration of oligosaccharides with a degree of polymerization larger than 2 was calculated as follows:

$$\text{Hexose}_{-}\text{Oligomer} (g / L) = \frac{\text{Hexose}_{-}\text{After}_{-}\text{Acid}_{-}\text{Hydrolysis} (g / L) - \text{Hexose}_{-}\text{Before}_{-}\text{Acid}_{-}\text{Hydrolysis} (g / L)}{1.11}$$

$$\text{Pentose}_{-}\text{Oligomer} (g / L) = \frac{\text{Pentose}_{-}\text{After}_{-}\text{Acid}_{-}\text{Hydrolysis} (g / L) - \text{Pentose}_{-}\text{Before}_{-}\text{Acid}_{-}\text{Hydrolysis} (g / L)}{1.136}$$

Hexose oligomer here is interpreted as mannose oligomer (Man 2), galactose oligomer (Gal 2), and glucose oligomer (Glu 2). Pentose oligomer here is interpreted as arabinose oligomer (Ara 2) and xylose oligomer (Xyl 2).

Hexose here is interpreted as mannose (Man), galactose (Gal), and glucose (Glu).

Pentose here is interpreted as arabinose (Ara), and xylose (Xyl).

The concentration of acetyl group linked with carbohydrate was calculated as follows:

$$\text{Acetyl\_Group (g/L)} = \frac{\text{Acetic\_Acid\_After\_Acid\_Hydrolysis (g/L)} - \text{Acetic\_Acid\_Before\_Acid\_Hydrolysis (g/L)}}{1.394}$$

#### **6.3.4 Contaminant Microorganism Test**

A YPD agar (Sigma, Y-1500) plate was placed on a turntable, and 10  $\mu\text{L}$  of sludge solution was dispersed evenly on the plate. The YPD agar plate was then incubated at 30 °C for 48 h. Samples from each flask were streaked on an YPD agar plate to check for microbial contamination. The gram stain was used to differentiate gram-positive and gram-negative bacteria. Colony forming unit (CFU) tests were performed to determine the concentration of microorganism cells. YPD agar plates were prepared for this purpose by diluting the culture with sterile saline (0.89% NaCl solution) to obtain a spread plate cell count of 30-300 cells per plate.

#### **6.3.5 Treatment of Prehydrolysate with CaO**

Prehydrolysate (100 mL) was held at either 25 °C or 60 °C in an incubator shaker (New Brunswick Scientific, Innova-4080) with 150 RPM during the rapid addition of anhydrous CaO (Fisher Scientific) until pH around 10.0. After 30 min of incubation, the hydrolysate was cooled to ambient temperature in a second water bath, followed by pH readjustment to 5.5 with 85% phosphoric acid and used immediately for analyses or in fermentations without further storage. Approximately 80% of the treated hydrolysate was decanted for use in fermentation experiments. Residual suspended particulates were removed by centrifuge at 5000 RPM for 5 min.

#### **6.3.6 Enzymatic Hydrolysis Test**

The enzymatic digestibility of the extracted liquor was determined according to NREL Chemical Analysis and Testing Standard Procedures (NREL, 2008). To each 20

mL glass scintillation vial, 0.5 mL 1 M sodium citrate buffer (pH 4.5), 0.1 mL of a 2% sodium azide solution was added to prevent the growth of organisms during the digestion. The extracted liquor was neutralized by calcium carbonate until pH reached 5.0, and then 9.4 mL prehydrolysate was added to the 20 mL glass scintillation vial. A 100 uL enzyme solution was diluted by DI water so that the enzyme was added to maintain enzyme loading at 25 mg protein/g-mannose oligomer. It was operated in an incubator shaker (New Brunswick Scientific, Innova-4080) at 50 °C with 150 RPM. The samples were taken at 6, 12, 24, 48 h and analyzed for glucose, xylose, galactose and mannose. The sugar digestibilities of released sugars, such as glucose, xylose, galactose and mannose, were used to calculate the enzymatic digestibility of sugar respectively:

$$\text{Sugar\_Digestibility}(\%) = \frac{\text{Sugar\_released}(g)}{\text{Total\_Sugar\_added}(g)} \times 100$$

Reported digestibility values are the average of duplicate tests with standard deviation (SD) smaller than 1.0.

### **6.3.7 Separation Hydrolysis and Fermentation (SHF) and SSF of Prehydrolysate**

In the SHF process, the treated prehydrolysate was subjected to enzymatic hydrolysis first for 48 h at 50 °C and then to the fermentation step at 32 °C. The SSF process was carried out at 37 °C. A 20 mL serum bottle was used as the bioreactor. To each serum bottle, 9.3 mL treated or untreated extracted liquor was added. The samples were steam sterilized at 121 °C for 15 min. Then 0.1 mL enzyme solution was diluted by DI water in such a way that the enzyme was added to maintain enzyme loading at 25 mg protein/g-mannose oligomer. Our previous study shows that acid soluble ash in the paper sludge, such as calcium carbonate, could act as a buffer to stabilize the pH during fermentation (Kang et al., 2010). Therefore, calcium carbonate was used in the SSF

process of softwood prehydrolysate. Fermentation was carried out under two different conditions: 0.5mL 1M sodium citrate buffer (pH 4.5) for SHF and 0.5 mL deionised water with addition of the CaCO<sub>3</sub> to the level of 0.05 g/10mL for SSF. To each serum bottle, 0.1mL concentrated yeast was added along with the hydrolysate discussed above to make the initial yeast concentration of each sample 50 mg/L. The serum bottles were then sealed with rubber septa, vented by a needle. It was operated in an incubator shaker (New Brunswick Scientific, Innova-4080) at 36 °C with 150 RPM. Hydrolyzate samples were taken at 6, 12, 24, 48 h and analyzed for glucose, xylose, galactose, arabinose, mannose and ethanol. In all of the microbial experiments, a sample from each flask was taken at the end of the run and streaked on an YPD plate to check for contamination and checked under an optical microscope. CFU tests were performed using YPD agar plates to check microorganism viability. A mixture of 0.2 mL of the aliquots and the yeast was transferred into a 1.5 mL centrifuge tube and centrifuged at 5000 RPM for 5 min. After the centrifuge, 0.1 mL of the supernatant was withdrawn and mixed with 0.9 mL DI water for chemical analysis). Aseptic conditions were maintained in all of the microbial experiments

The ethanol yield was calculated as follows:

$$\text{Ethanol yield [\% of theoretic al maximum]} = \frac{\text{Ethanol produced (g) in reactor}}{\text{Initial Total Sugar (g) in reactor} \times 0.511} \times 100$$

Sugar is interpreted as hexose in the reactor.

Reported digestibility values are the average of duplicate test with standard deviation (SD) smaller than 1.0.

### 6.3.8 SSF of Paper Primary Sludge with or without Prehydrolysate

The SSF test of the sludge was determined according to NREL Chemical Analysis and Testing Standard Procedures (NREL, 2008). Screw-capped 250 mL Erlenmeyer flasks were used as the hydrolysis reactor. The sludge samples were suspended in DI water or prehydrolysate and put into the flasks to reach a total working volume of 100 mL with sludges loading such that the glucan content became 6% (w/v) in the reactor. Samples were steam sterilized by autoclaving at 121 °C for 15 min. The test was conducted in an incubator shaker (New Brunswick Scientific, Innova-4080) at 37°C with 150 RPM. The growth medium for SSF was YP medium. SSF of the sludges was carried out at 37 °C without pH control. Cellulase and β-glucosidase were also added to achieve enzyme loading at 15 FPU and 30 CBU/g-glucan, respectively. Multifect Pectinase FE was added as 25 mg protein/g-mannose oligomer for the SSF of paper primary sludge with prehydrolysate. For comparison, the same amount of Multifect Pectinase FE was also added for the SSF of only paper primary sludge. Optical density (OD) was measured by UV Spectrophotometer (BioTek Synergy HT Multidetector Microplate Readers) at 600 nm for yeast (NREL-D<sub>5</sub>A). The initial OD after inoculation was 0.05, equivalent to 50 mg of NREL-D<sub>5</sub>A/L. In all of the microbial experiments, a sample from each flask was taken at the end of the run and streaked on a YPD plate to check for contamination and checked under an optical microscope. CFU tests were performed using YPD agar plates to check microorganism viability.

The ethanol yield was calculated as follows:

$$\text{Ethanol yield}[\% \text{ of theoretical maximum}] = \frac{\text{Ethanol produced (g) in reactor}}{\text{Initial Total Sugar (g) in reactor} \times 0.511} \times 100$$

Sugar is interpreted as hexose in the SSF.

Samples were analyzed for glucose, xylose, organic acid, and ethanol by high pressure liquid chromatography (HPLC). Aseptic conditions were maintained in all of the microbial experiments.

### **6.3.9 Analytical Methods**

The solid samples were analyzed for carbohydrates, acid insoluble lignin (AIL) and acid soluble lignin (ASL) following the NREL standard procedures (NREL, 2008). The moisture content was measured by an infrared moisture balance (Denver Instrument, IR-30). Sugars were determined by HPLC using a BioRad-HPX-87P column. Ethanol, HMF, levulinic acid, acetic acid and Furfural were determined by HPLC using a BioRad-HPX-87H column. A refractive index detector was used with the HPLC. The acid-insoluble ash was determined following the Tappi test method (Test Method T 244 cm-99). Liquid sample analysis and ash determination were done in triplicate. The total phenolics in the prehydrolysate before and after treatment were determined using Folin-Ciocalteu method as described by Kujala et al. (2000).

Where applicable, statistical computation, including mean value and standard deviation, was performed using Microsoft Office Excel 2003.

## **6.4 Results and Discussion**

### **6.4.1 Pre-extraction of Softwood**

Water under high pressure and high temperature (160-180°C) was used selectively to remove the hemicelluloses from the biomass (Springer and Harris, 1982). As wood chips were extracted, their weight decreased as components dissolved and diffused from the wood into the extraction liquor (termed as prehydrolysate). The weight

loss increased with extraction time from almost 4% at the mildest extraction condition (0 min, 115 H-factor hours) to about 15% at the most severe condition (80 min, 1356 H-factor hours) (Table VI-2). With the rising of the H-factor, the extracted amount of hemicellulose increased up to about 11%. In the case of the extracted amount of cellulose, however, only a small trace (0.03%) was detected over a higher H-factor of 1300 h. This indicates that there is almost no change in the cellulose content of wood chips because the sugar contribution is dominated by hemicellulose within this weight loss range. Total sugar content represents the sum of cellulose and hemicelluloses dissolved at a given weight loss. The purpose of pre-extraction is to extract hemicellulose while still preserving cellulose for quality pulp production. To avoid damaging the fiber quality, cellulose was not to be removed at all and hemicellulose was not to be totally removed. Therefore, the extraction time of 66 minutes was chosen to extract as much hemicellulose as possible without damaging fiber in this experiment. To increase the sugar content in the extracted liquor and simulate the continuous extraction, a specified time was used in the second and third stages of water extraction. The goal was to extract 10% hemicellulose from fresh wood.

#### **6.4.2 Composition of Softwood Prehydrolysate**

During the pre-extraction process, the hydronium ion from water ionization causes the cleavage of the acetyl group, which forms acetic acid acting as a catalyst for the hydrolysis of glycosidic bonds in hemicellulose. The hemicellulose chain is decomposed first into oligosaccharides and then into monomers. Hemicellulose sugars can degrade into furfural, hydroxymethylfurfural (HMF), and some organic acids, such as levulinic acid.

Since 70% of liquor used in the second and third stages was derived directly from the last stage, the sugar and organic compounds content of the liquor did increase substantially with each stage (Table VI-3 and VI-4). However, there were not much difference between the three stages in the resulting pH of the pre-hydrolyzate: 3.50, 3.43 and 3.39, respectively, in spite of the concentration of acetic acid increasing. The pH did not decrease much possibly because ash in the wood is mainly an inorganic salt, which dissolved in the water during extraction to form a buffer. In addition, the sugar oligomers released during pre-extraction of pine wood were acetylated. Only part of acetyl groups in the original wood was released as free acetic acid in the prehydrolysate (Table VI-4), possibly due to the moderate stability of ester linkage towards acid.

The pre-extraction converted most of the hemicellulose (10% based on dry wood) to oligomers and monomers. It can be seen from Table VI-3 that most of the dissolved sugars were already present in oligomer form rather than as monomers. However, the proportion of oligomers was significant and dependent on the type of sugar (Table VI-3). As anticipated, the oligomer/monomer ratio was higher for the hexose which had previously been shown to be more resistant to acid-catalyzed degradation (BeMiller, 1967). The arabinose substituents in the arabinoglucuronoxylan – another major hemicellulose in softwood which is extremely labile toward acid hydrolysis, were almost completely depolymerized during pre- extraction.

Furfural and HMF can be produced in the hemicellulose extraction process and acid hydrolysis, due to the acidic condition and the intense pressure (Nguyen, et al., 1998). The increase of HMF and furfural (Table VI-4) shows that hexose and pentose

underwent intense degradation with each additional stage of extraction as solution acidity slightly increases.

Some studies have suggested that covalent linkages between lignin and hemicellulose exist in native wood (Björkman, 1957; Eriksson and Lindgren, 1977). These structures are typically referred to as lignin-carbohydrate complexes (LCC). The lignin is covalently bound to the hemicellulose which, in turn, is bound to cellulose through extensive hydrogen bonding. Lignin-carbohydrate bonds and some inter-unit lignin bonds, mainly derived from the benzyl alkyl ether type, may be cleaved during water pre-hydrolysis. Acetic acid catalyzes the hydrolysis of the  $\beta$ -ether linkages in lignin resulting in the formation of different phenolic products from lignin. Part of the dissolved lignin precipitated during the cooling of the prehydrolysate and appeared to be very fine. This lignin was separated from the prehydrolysate by centrifugation and denoted as a solid fraction. The compositions of the solid fraction (Table VI-5) contained about 6% (w/w) carbohydrate and 85% lignin, which show that this part of lignin compounds was released in LCC form. Pre-extraction could facilitate subsequent alkaline delignification because of the partial hydrolytic degradation of lignin compounds, the cleavage of alkali-stable carbohydrate-lignin bonds, and the improvement in the accessibility of the cooking liquor (Sixta, Herbert (2006) Handbook of pulp, Wiley-VCH, Weinheim).

#### **6.4.3 Enzymatic Hydrolysis of Softwood Prehydrolysate**

Secondary hydrolysis of softwood prehydrolysate to monosaccharides can be accomplished by enzymatic or diluted acid hydrolysis. However, dilute acid hydrolysis produces toxins that negatively affect biocatalyst growth and metabolism (Klinke, et al., 2004). These toxins can be minimized by the enzymatic hydrolysis process which is also

more environmentally friendly. Moreover, the enzymatic hydrolysis process reduces the consumption of acids and alkalis (Hashimoto and Nakata, 2003). In this study, four different commercial enzymes were tested, Cellulase enzyme (Spezyme CP), Multifect Xylanase, Multifect Pectinase FE, and  $\beta$ -glucosidase (Novozyme 188), based on the same loading protein number (25 mg protein/g mannose oligomer) to evaluate their abilities to undergo hydrolysis of softwood prehydrolysate. The results are shown in Fig.VI-1.

There was relatively low sugar yield by using Spezyme CP and Novozyme-188. Over 70% xylose yield was achieved using Multifect Xylanase since it has the highest xylanase specific activity among the four enzymes. Multifect Pectinase FE, however, appeared to be the most effective. Adding Multifect Pectinase FE significantly improved the yields of glucose, galactose and mannose.

It was speculated that adding pectinase might aid saccharification of hemicellulose oligomer because these types of preparations also demonstrated hemicellulase related activities, even though only traces of pectin substances are found in the cell walls of pine wood. In addition, Berlin et al. found the Multifect Pectinase FE shows relatively high specific activity of mannase (3.0 U/mg protein), compared to Multifect Xylanase, Spezyme CP and Novozyme- 188 (Berlin et al.,2007). From Table VI-1, it can be seen that Multifect Pectinase FE also shows relatively high specific activity of  $\beta$ -Glucosidase, Xylanase,  $\beta$ -Xylosidase, and  $\alpha$ -Galactosidase.

Similar to the cellulolytic enzymes, synergistic action also occurs in the mannan structure as a result of a variety of main- and side-chain-cleaving enzymes. Due to the complexity of the galactoglucomannan structure, both main-and side-chain cleaving enzyme activities are required to hydrolyze the oligomer into monomer sugars (Filhom,

1998). For instance,  $\beta$ -Glucosidase, an exo-type enzyme, hydrolyzes 1,4- $\beta$ -D-glucopyranose at the non-reducing end of the oligosaccharides released from glucomannan and galactoglucomannan by  $\beta$ -mannanase. A side-chain-cleaving enzyme,  $\alpha$ -Galactosidase, catalyzes the hydrolysis of  $\alpha$ -1, 6-linked D-galactopyranosyl side chains of galactomannan and galactoglucomannan (Moreira and Filho, 2008).

The highest galactose yield (over 70%) achieved by using Multifect Pectinase FE also showed the synergistic action of different enzymes. Even though Novozyme-188 has the highest  $\alpha$ -Galactosidase specific activity among four enzymes, only 50% galactose yield was reached by using  $\beta$ -glucosidases (Novozyme-188). Clarke et al. also found that the galactose release from softwood pulp is enhanced by the presence of mannanase in combination with  $\alpha$ -galactosidase (Clarke et al., 2000). Over 60% xylose yield was also achieved, even though Multifect Pectinase FE has slightly lower specific activity of xylanase than that of Spezyme CP, and 30 times lower than that of Multifect Xylanase. However, Multifect Pectinase FE contains the highest  $\beta$ -Xylosidase activity. It was suggested that supplementation of  $\beta$ -xylosidase improved glucose release during hydrolysis by decreasing the accumulation of xylose oligomers, which are found to inhibit cellulase activity (Kumar and Wyman, 2009).

#### **6.4.4 De-toxification and SHF of Softwood Prehydrolysate**

Detoxification of lignocellulosic hydrolysates by adding CaO or Ca(OH)<sub>2</sub> to increase pH to 10 (over liming) followed by pH readjustment to 5.5 with acid was reported to remove certain inhibitory compounds generated in the hydrolysis process (Larsson et al., 1999).

Two different detoxification conditions were performed in this study: temperature at 25 °C or 60 °C at 30 min with 10 g CaO/L followed by pH readjustment to 5.5 with 72% sulphuric acid.

None of the organic acids concentrations was affected by either condition. Phenolic compounds, HMF and furfural were partially removed. A small decrease in concentration of total sugars (8% to 12%) and fermentable sugars (glucose and mannose, 6% to 9%) was observed because of dilution with acid when pH was adjusted to 5.5 and because of sugar degradation under alkaline condition. Overall, at a relatively high temperature (60 °C), the concentrations of all compounds decrease slightly more than at 25 °C, as shown in Fig.VI-2.

The treated prehydrolysate was subjected first to enzymatic hydrolysis by Multifect Pectinase FE for 48 h at 50 °C and then to the fermentation step at 32 °C. The overall performance data and the time-course data of SHF are presented in Fig. VI-3. The concentration of xylose, galactose and arabinose remained unchanged during the fermentation process (data not shown). Although galactose is also a hexose, the concentration of galactose remained unchanged during the fermentation process. In the yeast *Saccharomyces cerevisiae*, the flux through the galactose utilization pathway is threefold lower than the rate of glucose utilization (Ostergaard et al., 2000). Therefore only the uptake of glucose and mannose was calculated in ethanol yield.

Excellent ethanol production was observed after detoxification. The ethanol yields based on initial fermentable sugars (glucose and mannose) were 83.9% and 85.0% at 48 h, respectively for the detoxification condition of the pre-hydrolysate at 25 °C and 60 °C, compared to only 56.9% ethanol yield for the untreated prehydrolysate. The ethanol

concentrations at 96 h increased from 6.0 to 10.4 after 25 °C and to 10.6 g/L after 60 °C detoxification condition. Although the loss of fermentable sugars is more at 60 °C than at 25 °C, the former temperature was more appropriate for detoxification since it removed more HMF, furfural and phenolic compounds. In addition, it is more convenient and economical to do detoxification at the higher temperature because of the time and energy consumed in cooling down the prehydrolysate from the pre-extraction temperature (170 °C) to room temperature, such as 25 °C. Therefore 60 °C was used during the detoxification process for the rest of the study.

It was very interesting to see that the sugars concentrations from enzymatic hydrolysis also increased after detoxification. The glucose concentrations at 48 h increased from 4.1 to 4.3 after 25 °C and 4.4 g/L after the 60 °C detoxification condition. The mannose concentrations at 48 h increased from 10.8 to 11.3 after 25 °C and 11.5 g/L after the 60 °C detoxification condition. The study also found the enzymatic yields of all five sugars increased after detoxification under different enzymes, such as Multifect Xylanase, Spezyme CP and Novozyme -188 (data not shown here). Apparently, the toxins in the prehydrolysate are inhibitory to enzymes.

The glucose was consumed quickly within 12 h even for untreated prehydrolysate. However, the mannose consumption rate was much slower for untreated hydrolysate. Most glucose could be consumed within 12 h, while mannose needed a longer time, about 24 h. Within the first 12 h of fermentation of glucose and mannose, glucose tended to be used preferentially compared to mannose by *Saccharomyces cerevisiae* in the prehydrolysate. It has also been reported that *Saccharomyces cerevisiae* would consume glucose faster than mannose (Smith et al., 1997).

The inhibition of ethanol fermentation was most likely not from furfural and HMF, because the concentration of furfural and HMF after detoxification were less than 1 g/L and 0.6 g/L, respectively. Low concentrations of furfural and HMF do not affect the fermentation of lignocellulosic hydrolysate (Tu et al., 2009; Taherzadeh et al., 1997). *Saccharomyces cerevisiae* can convert furfural and HMF to their corresponding alcohols via NADH-dependent alcohol dehydrogenase (Palmqvist et al., 1999). Although different conditions were used in this study, similar results were determined, that after 24 h of fermentation the furfural and HMF were quickly consumed by yeast in the fermentation.

The organic acids, acetic, levulinic and formic acid, inhibit yeast fermentation by reducing biomass formation and ethanol yields (Larsson et al., 1999). The concentration of organic acids did not change after detoxification. However, Van Zyl et al. (1991) found that the degree of inhibition caused by acetic acid depended not only on its concentration, but also on the pH of the medium. When the pH of the medium is low, acetic acid (pKa=4.75 at 25 °C), formic acid (pKa =3.75 at 20 °C) and levulinic acid (pKa=4.66 at 25 °C) appear in the undissociated form, diffuse across the plasma membrane, and dissociate due to higher intracellular pH, thus decreasing the cytosolic pH (Pampulha et al., 1989). As a consequence, the internal pH drops, thus inhibiting cell activity and even causing the death of cell. According to the Henderson–Hasselbalch equation, at relatively high pH, the concentration of the undissociated form is much lower than that at low pH. Therefore the inhibition of organic acids could be lower at relatively higher pH when the pH was adjusted to 5.5 while fermenting above pH 7 is unfavourable for ethanol production.

Phenolic compounds could be the major inhibitors in the hydrolysate. Phenolic compounds are known to increase biological membrane fluidity and can cause loss of cellular integrity, thereby affecting its role in acting as selective barriers and enzyme matrices (Heipieper et al., 1991; Heipieper et al., 1994).

#### **6.4.5 SSF of Softwood Prehydrolysate**

SSF of prehydrolysate after detoxification was also investigated in this study. SSF combines enzymatic hydrolysis and fermentation in one step. This process can greatly reduce the product inhibition to enzymes, which in turn increases sugar production rates, concentrations, and yields, and also decreases process time. There is a resulting cost saving due to the reduced number of vessels required compared to SHF process.

The profile of ethanol production with SSF is shown in Fig.VI-4. The observed trends for sugar consumption and ethanol production in the SSF process of softwood prehydrolysate were similar to the SHF process of prehydrolysate. In the SHF, the fermentation of this prehydrolysate produced ethanol at a concentration of 10.51 g/L after a total reaction time of 72 h (hydrolysis 48 h and fermentation 24 h) and 10.56 g/L after 96 h (hydrolysis 48 h and fermentation 48 h), respectively, for 84.6% and 85.0% of the ethanol yield. The slightly lower ethanol yield of 82.6% and ethanol concentration of 10.3 g/L was reached with SSF at 48 h, however there was a gain of 24 h using SSF compared with SHF.

#### **6.4.6 SSF of Prehydrolysate with Paper Sludge**

The ethanol concentration is slightly over 1 % for either SHF or SSF processes of prehydrolysate, which is far below the acceptable level as distillation feed. The concentration of ethanol in the bioreactor significantly affects the cost of the downstream

separation process. Use of the prehydrolysate and pulp mill sludges mixture as the fermentation feed can increase the product concentration. Our previous study demonstrated that the ethanol production based on Spezyme CP and *S. cerevisiae* (D<sub>5</sub>A) performed well by using untreated kraft paper sludges (Kang et al., 2010). The profile of ethanol production of SSF of prehydrolysate with paper sludge is also shown in Fig.VI-4. The bioreaction was started with a total sludge loading of 150 g/L, which was 66.75 g-glucan/L. The ethanol concentration increased from 10.3 g/L (prehydrolysate only) and 27.7 g/L (paper sludge only) to 31.0 g/L (mixture of prehydrolysate and paper sludge). It was noticed that the ethanol yield of the mixture - 70.1% was lower than that of paper sludge (73.2%) or prehydrolysate (82.6%). The cellulase enzyme becomes less effective by inhibition of ethanol (Wu and Lee, 1997), phenolic compounds (Ximenes et al., 2010), furan compounds (Hodge et al., 2008), sugar monomers and sugar oligomers (Xiao et al., 2004; Nigam and Prabhu, 1991; Kim and Lee, 2005; Kumar and Wyman, 2009).

## **6.5 Conclusion**

It is technically feasible to remove part of the hemicellulose before pulping. Hemicellulose prehydrolysate can be converted to ethanol from lignocellulosic biomass through detoxification, enzymatic hydrolysis and downstream fermentation processes. Detoxification removed the part of toxin which strongly inhibits microbial action, and therefore increased the efficiency of ethanol conversion. Different commercial enzyme preparation has a different effect on the hydrolysis of prehydrolysate. The Multifect Pectinase FE was shown to be most efficient. The overliming detoxification increased microbial ethanol conversion. The ethanol yield based on initial fermentable sugars was 83.9% and 85.0%, respectively for 25 and 60 °C detoxification treatment. The

detoxification treatment also increased the enzymatic yield of prehydrolysate. In the SHF of this prehydrolysate, the ethanol concentration of 10.51 g/L and the ethanol yield of 84.6% were reached after a total reaction time of 72h (hydrolysis 48 h and fermentation 24h). The slightly lower ethanol yield of 82.6% and the ethanol concentration of 10.3g/L were reached with SSF for 48 h, however there is a gain of 24 h using SSF compared with SHF Use of the mixture of prehydrolysate and kraft pulp mill sludges as the fermentation feed was attempted to increase the ethanol concentration. The ethanol concentration in the broth was increased to 31.5 g/L. The yield of the mixture run was lower at 71.5%. The low conversion yield is related to the cellulase enzyme reaction rather than microbial reaction.

Table VI-1. Enzyme activities detected in commercial enzyme preparations

Activity (units/mg)	Spezyme CP	Novozyme 188	Multifect Xylanase	Multifect Pectinase FE
Cellulase (FPU) <sup>a</sup>	0.47	0.06	0.02	0.05
$\beta$ -Glucosidase	1.04	4.75	0.85	4.22
Xylanase (OSX) <sup>b</sup>	21.32	0.88	600.07	20.29
$\alpha$ -Arbinofuransoidase	0.18	0.21	0.22	22.71
$\beta$ -Xylosidase	0.06	0.12	0.54	2.27
$\alpha$ -Galactosidase	0.00	0.83	0.06	0.39
Feruloyl esterase	0.00	0.00	0.00	0.12
<i>p</i> -Coumaroyl esterase	nm <sup>c</sup>	nm <sup>c</sup>	0.03	0.26

- a. Filter paper units.
- b. Oat spelt xylan.
- c. Not measured.

Table VI-2. Sugar content of first stage prehydrolysate at different time periods

Water Extraction at 170 °C			Total Sugars in Prehydrolysate (g/L)					Polysaccharides (% dry basis)	
Time (min)	H	Wt. Loss (%)	Glu	Xyl	Gal	Ara	Man	C	H
0	115	4.24	0.33	0.74	0.91	0.53	1.81	0.00	2.24
11	277	5.83	0.56	1.20	1.18	0.69	2.39	0.00	3.12
25	483	8.57	1.07	2.23	1.79	1.05	4.47	0.00	5.50
45	839	11.64	1.60	3.13	2.53	1.48	6.69	0.00	8.00
66	1124	14.00	1.79	3.55	2.81	1.57	9.60	0.00	10.03
80	1356	14.94	2.40	4.05	3.11	1.83	9.71	0.03	10.91

All data in the table represent the mean values of duplicate runs (n=2; standard deviation<0.5).”Total Sugars” in the prehydrolysate were present as monomers.

C: Cellulose.

H: Hemicellulose.

Table VI-3. Sugar content of three stage prehydrolysates

	Monomer Sugars in Prehydrolysate (g/L)					Oligomer Sugars in Prehydrolysate (g/L)					Total Sugars (g/L)
	Glu	Xyl	Gal	Ara	Man	Glu2	Xyl2	Gal2	Ara2	Man2	
1st Stage	0.40	1.14	0.73	1.46	0.55	1.60	2.46	2.07	0.27	8.15	19.32
2 nd Stage	1.07	2.65	1.59	2.27	1.60	2.88	3.02	3.60	0.33	12.97	31.99
3rd Stage	2.41	3.76	2.61	3.54	3.20	2.97	2.65	3.93	0.10	15.53	40.70

All data in the table represent the mean value of duplicate runs (n=2; standard deviation<0.5).

Table VI-4. Organic compounds content of three stage prehydrolysates

(g/L)	Acetic acid	Acetyl group	Levulinic acid	HMF	Furfural
1st Stage	0.75	0.25	0.07	0.20	0.37
2nd Stage	1.58	0.37	0.12	0.57	0.98
3rd Stage	2.61	0.48	0.20	1.02	1.41

All data in the table represent the mean value of duplicate runs (n=2; standard deviation<0.5).

Table VI-5. Composition of solid fraction in three stage prehydrolysates

	Solid Fraction Conc.(g/L)	Glu (Wt%)	Xyl (Wt%)	Gal (Wt%)	Ara (Wt%)	Man (Wt%)	AIL (Wt%)	ASL (Wt%)
1st Stage	5.5	2.0	2.1	0.3	0.2	1.8	84.7	2.0
2nd Stage	4.6	1.8	2.0	0.4	0.2	2.0	85.1	2.4
3rd Stage	4.2	2.1	2.4	0.4	0.2	2.1	84.5	2.7

All data in table are the mean value of duplicate runs (n=2; standard deviation<0.5).

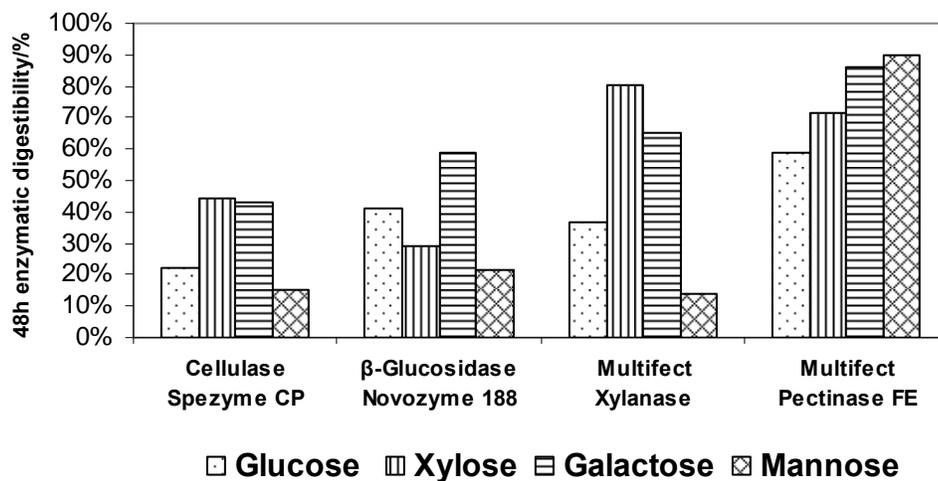


Fig. VI-1. Different commercial enzyme preparation undergo hydrolysis of softwood prehydrolysate

The data represent the average of duplicate runs. The enzyme loading was 25 mg protein/g-mannose oligomer.

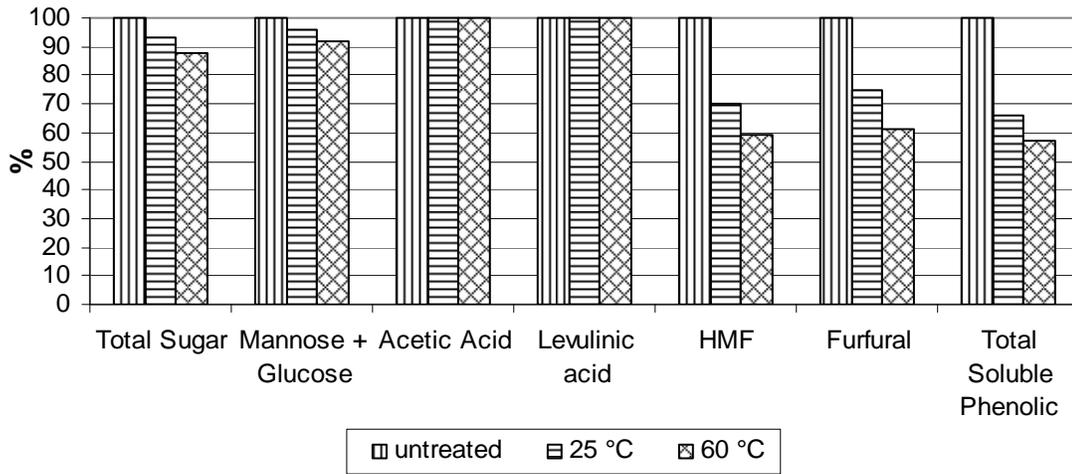


Fig.VI-2. Composition change of prehydrolysate after de-toxification

The data represent average of duplicate runs and are expressed as percentage of the concentration of compounds after detoxification.

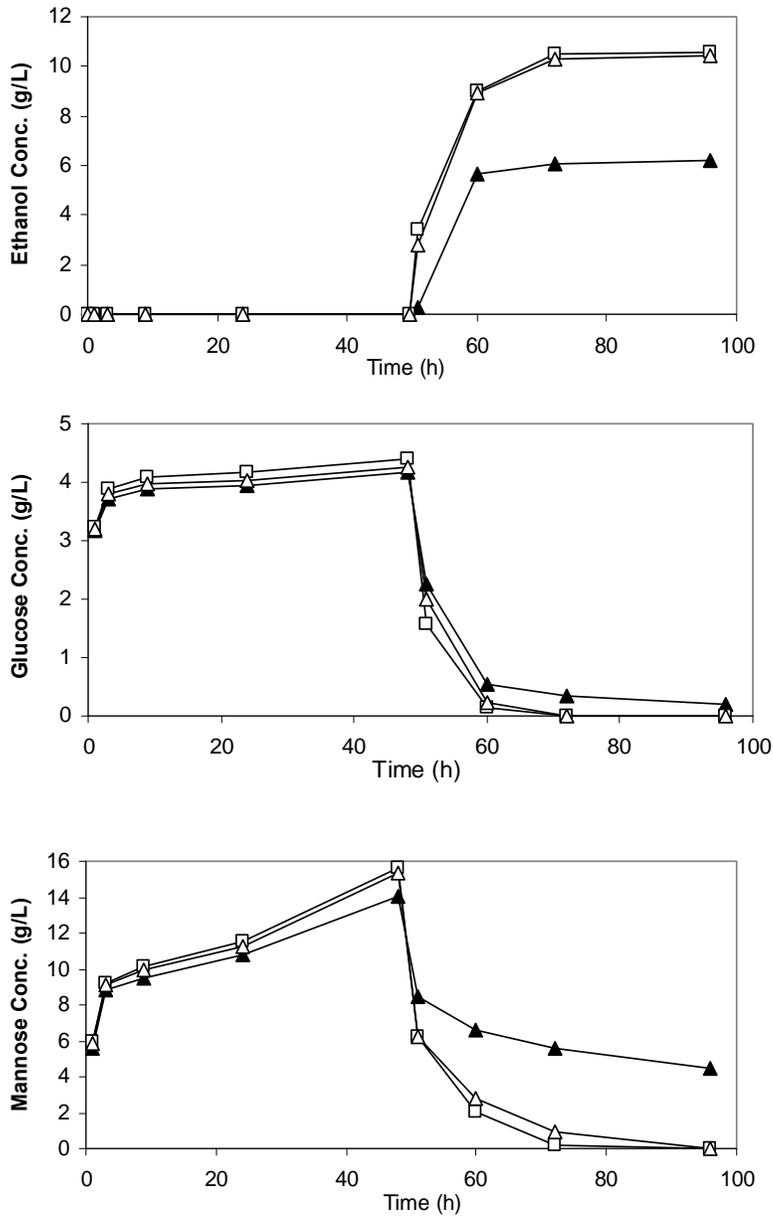


Fig.VI-3. Separation hydrolysis and fermentation (SHF) of prehydrolysate by Multifect Pectinase FE and *Saccharomyces cerevisiae* (ATCC-200062)

Squares with connecting lines represent prehydrolysate after 60 °C detoxification treatment. Triangles with connecting lines represent prehydrolysate after 25 °C detoxification treatment. Filled triangles with connecting lines represent untreated prehydrolysate. The data points represent the average of duplicate runs.

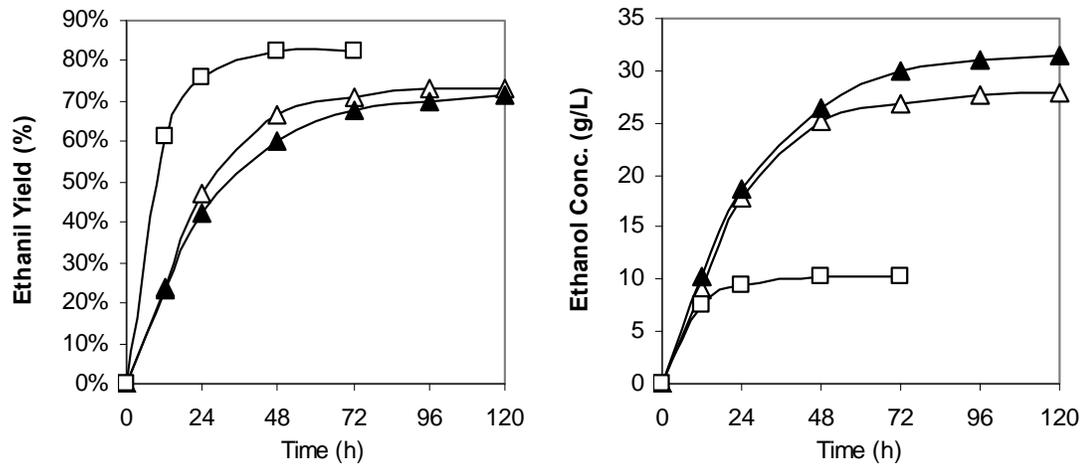


Fig.VI-4. Simultaneous saccharification and fermentation (SSF) of prehydrolysate by *Saccharomyces cerevisiae* (ATCC-200062) and Multifect Pectinase FE

Filled triangles with connecting lines represent paper sludge with prehydrolysate. Triangles with connecting lines represent paper sludge only. Squares with connecting lines represent prehydrolysate only. The data points represent the average of duplicate runs.

## **VI. Future Work**

The focus of this dissertation was to study the technical feasibility of establishing a biorefinery based on existing paper mills especially those of kraft paper mills. The concept of a wood based-biorefinery is to use all components of wood to make pulp and paper, while also creating transportation fuels, power, and chemicals. A wood based biorefinery could transform the pulp and paper industry, making it sustainable, competitive, and innovative. It would promote significant economic growth through new value-added products based on existing byproducts or waste at pulp and paper mills (i.e. hemicelluloses, black liquor, paper sludge and wood chip rejects), while still preserving the main paper and paper-related products. Ethanol production from paper sludge and hemicellulose prehydrolysate, and cellulase production from paper sludge and hardwood pulp have been investigated.

The following points are suggested for the future study aimed at establishing a biorefinery based on existing paper mills:

- Butanol production from paper sludge: Butanol can be derived from lignocellulosic materials, such as paper sludge. Butanol is considered to be a better biofuel than bioethanol because it's less corrosive and has a higher caloric value, giving it a higher energy value. Like bioethanol, butanol is being considered as an additive to gasoline. Certain clostridial species are well known for their ability to produce butanol via ABE fermentation, such as, wild-type

strain *Clostridium acetobutylicum* (ATCC 824), *Clostridium acetobutylicum* M5, *Clostridium beijerinckii* NCIMB 8052 and *Clostridium beijerinckii* BA101 (Parekh and Blaschek, 1999; Ezeji et al., 2007; Lee et al., 2009; Gu et al., 2009). In the future research, *Clostridium acetobutylicum* (ATCC 824) will be used to convert paper sludge and the prehydrolysate into butanol.

- Lactic acid production from hemicellulose prehydrolysate: Lactic acid (LA) is another value added product which could be produced in the paper mills with applications in food industry, cosmetics, polymers, and pharmaceuticals. Recently, the manufacture of biodegradable polylactate polymers, an environment-friendly alternative to conventional non-biodegradable plastics derived from petrochemicals has, received much attention. Presently, 90% of the total LA produced annually worldwide is based on microbial fermentation of starch-derived glucose or sucrose (Litchfield, 1996) and the remainder is produced by the hydrolysis of lactonitrile (Nolasco-Hipolito et al., 2002). With the concern on feedstock cost, the use of lignocellulosic materials, hemicellulose hydrolyzate and paper sludge as an inexpensive carbon source for LA production have been pursued (Garde et al., 2002; Lee et al., 2004; Neureiter et al., 2004). Bioconversion of the sugars in the hemicellulose prehydrolysate to LA would be helpful in improving the economics of biorefinery.
- Study of lignin-carbohydrate complex (LCC) from hemicellulose prehydrolysate: Lignin can be partly removed from wood during the hot water pre-hydrolysis process. However, lignins are rarely isolated as pure materials, and are always associated with carbohydrate linkages to varying extents as lower molecular

weight segments or lignin-carbohydrate complex (LCC). LCC components present in hemicellulose prehydrolysate liquor could be characterized by using Nuclear magnetic resonance (NMR), Fourier transform infrared spectroscopy (FTIR), HPLC, thermal analysis, elemental analysis, atomic absorption spectroscopy, and treatment with different commercial enzymes. These diverse experimental techniques could give us a deeper insight into the structures and properties of LCC and will be valuable in evaluating LCC for new applications. In addition, identification and tracking the release of LCC would provide the information of basic mechanism during hot water pre-hydrolysis.

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