Pretreatment and Enzymatic Hydrolysis of Peat and Pine Sawdust for Bio-ethanol Production

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May, 2010

Your file Votre référence
ISBN: 978-0-494-71743-1
Our file Notre référence
ISBN: 978-0-494-71743-1

Abstract

This thesis includes three parts: 1.) effect of hydrogen peroxide on delignification of peat; 2.) pre-treatment of pine sawdust using various methods, including organosolv extraction, ultrasonic treatment, and sodium hydroxide treatment; and 3.) enzymatic hydrolysis of pre-treated pine sawdust for glucose production. The delignification efficiency (DE), pre-treatment efficiency (PE), and glucose yield, total sugar yield, and total weight loss were systematically quantified under different conditions. Effects of pre-treatments on peat and pine sawdust structure were characterized using various analytical tools, such as scanning electron microscope (SEM), X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR) and UV spectrometer. The detailed results are summarized below:

1.) Pretreatment of peat with hydrogen peroxide under alkaline condition was studied for delignification in a batch reactor. Impact of reaction time, reaction temperature, and the concentration of hydrogen peroxide on lignin removal was systematically studied. Peat samples before and after pretreatment were characterized using FTIR. The hydrolyzate solution was characterized using UV-spectrometer. The delignification efficiency increased with an increase in reaction time and hydrogen peroxide concentration. There was an optimal reaction temperature (45°C) for delignification. The experimental results suggested that the optimal reaction condition is 1.5 wt% H_2O_2, pH 11.5, temperature 45 °C, reaction time 12 h and about 40 wt% biomass can be dissolved under optimal conditions. The results indicated that the

hydrogen peroxide pretreatment had significant effects on delignification of peat; however, the pretreatment could not change the lignin's structure.

2.) Pretreatment efficiency (PE) and delignification efficiency (DE) of pine samples with different pretreatment methods were studied, and SEM, FTIR, XRD analysis methods were used to examine the structural changes before and after the pretreatment. All the pretreatment methods, in particular the organosolv extraction, resulted in removal of lignin and hemicellulose. The PE and DE were 51.40%±2% and 76.5%±3% for organosolv extracted pine; 53.3%±1% and 77.20%±2.6% for organosolv extracted +ultrasound treated pine; 57.7%±1.1% and 81.5%±3% for organosolv extracted +NaOH treated pine; 61.6%±1% and 86.4%±3% for organosolv extracted +ultrasound+NaOH treated pine, respectively. Among all the methods tested, organosolv+ultrasound+NaOH achieved the highest pretreatment and delignification efficiencies of 61.6%±1% and 86.4%±3%, respectively, implying that the combination of these three methods did have a significant effect on removal of lignin and dissolution of hemicellulose. Through the observations from SEM figures, FTIR and XRD spectrums, the structural feature changes of the components in cell wall after the pre-treatment were clearly demonstrated: first, the cell wall was disordered, twisted and exposed its inner structure; second, the characteristic function groups in lignin and hemicellulose were removed or reformed; third, the pretreatment did not result in the structure change of cellulose but increased its content, as expected.

3.) Enzymatic hydrolysis of pine samples treated with different pretreatment methods was systematically studied. Different glucose yields, total sugar yields and total weight loss were obtained under various enzyme loading (0~15.56 FPU cellulase) and

reaction time (48 hours). The maximum glucose yield and the maximum total sugar yield were 5.78% and 7.13%, for raw pine, 9.56% and 30.14% for organosolv extracted pine, 10.74% and 24.07% for organosolv extracted + ultrasound treated pine, 13.64% and 26.81% for organosolv extracted and NaOH treated pine, and 19.27% and 22.40% for organosolv extracted + ultrasound + NaOH treated pine, respectively. It was observed that the glucose yields were positively proportional to the values of PE and DE from the pretreatment, while the total sugar yields were inversely proportional to the values of PE and DE. Compared to that of the untreated pine sawdust sample, hydrolysis of the pretreated pine samples led to 2-3 fold increases in the glucose and total sugar yields under the optimal conditions. These results suggest that enzymatic hydrolysis of softwood sawdust could be greatly enhanced by pretreatment of the feedstock by organosolv extraction combined with ultrasound and alkaline treatment.

Acknowledgement

I would like to express my sincere appreciation and deepest gratitude to my supervisors Dr. Baoqiang Liao and Dr. Charles Xu for their enthusiasm in research, effective guidance, and continuous encouragement and support throughout this thesis. Their guidance and patience were most helpful particularly when this project experienced any difficulties. Meanwhile, I would like to show my great appreciation for their help in my study, research, and life during the past two years in Canada.

Financial support provided by Dr. Xu and Dr. Liao from Natural Sciences and Engineering Research Council of Canada (NSERC) through the Discovery Grant Program and funding from CFI through Leaders/New Opportunity Funds is highly appreciated, as well as from the Ontario Ministry of Energy and Ontario Centers of Excellence through the Atikokan Bioenergy Research Center (ABRC) program.

I want to thank many colleagues, Dr. Hongjun Lin, Dr. Zhongshun Yuan, Graduate students Weijue Gao, Linghong Zhang, just to name a few, for their help in some experiments and the preparation of the thesis.

Furthermore, I want to extend my hearted thanks to Dr. Wa Gao, Dr. Lionel Catalan, Mr. Garry Rathje and all the graduate students in the Department of Chemical Engineering and the M.Sc. Program in Environmental Engineering for their invaluable comments and their great patience in my graduate studies at Lakehead University. I am full of gratitude to the reading committee for their time and effort. My special thanks go to the staff of the Lakehead University Instrumentation Lab, particularly to Mr. Allen

Mackenzie, Mr. Ain Raitsakas and Mr. Keith Pringnitz, for their great assistance in various sample analyses.

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Chapter One

General Introduction

1.1 Background

The pursuit of bio-ethanol as an alternative energy source has attracted increasing interest due to its renewability, the fast increased demand of energy and the depleted fossil resources. Fossil fuels, mainly coal, petroleum and natural gas, have been the majority of the energy source worldwide. According to Energy Information Administration (EIA) (http://www.eia.doe.gov/aer/ep/ep_frame.html), fossil fuels accounted for approximately 86% share in the primary energy production in the world in 2006 and the world energy consumption has been growing by about 2.3% per year. The burning of fossil fuels emits around 21.3 billion tonne of greenhouse gases annually. As such, it is strategically pivotal to pursue other clean, alternative and renewable energy sources.

Bio-ethanol has emerged to be an alternative, renewable, sustainable and environmentally friendly energy source. Combustion of bio-ethanol produces relatively low emissions of hydrocarbons, carbon monoxide (CO), nitrogen oxides and volatile organic compounds (Bailey, 1996; Wyman, 1999). As a typical bio-renewable fuel, bio-ethanol is considered carbon neutral since the carbon dioxide released from combustion of ethanol produced from renewable lignocellulosic materials is the CO_2 sequestered by the plants during their growth (Chang et al., 1991). Bio-ethanol can be used in various ways, while more commonly as a blended fuel in gasoline. Nowadays, all gasoline engines can use up to 10 wt% ethanol blended fuel without any need of modification. A

high combustion efficiency can be achieved for a transportation fuel when blended with gasoline at a high blending ratio of as high as 10-22 wt% in USA, Brazil, Germany, Spain, France and other EU countries (González-García et. al.2009; Prasad et al. 2006). However, the major challenge for the commercialization of the bio-ethanol technologies may be the combination of the low cost of conventional energy resources and the high biomass processing cost (Vallander and Eriksson, 1990; Duff and Murray, 1995; Kaylen et al. 1999; Prasad et al. 2006).

Compared to the conventional starch-based bio-ethanol manufacture, production of cellulosic ethanol using non-food lignocellulosic feedstock is advantageous as it does not compete with the food industry for feedstock. Typical feedstock for bio-ethanol production includes crop residues, grasses, forest biomass and waste, such as sawdust and wood chips (Sun and Cheng, 2002; Prasad et al. 2006). Lignocellulosic biomass is the most abundant renewable materials with an estimated annual production at 1x 10^{10} million tonnes (Sanchez and Cardona, 2008). Softwoods are the dominant wood species in North America. Softwoods, e.g. pine and spruce, contain about 40-45 wt% cellulose, 20-25 wt% hemicellulose and 25-30 wt% lignin. In addition, there is an abundant peat resource in Canada and USA as well as other countries such as Finland and Malaysia. Peat may be considered as a slowly renewable lignocellulosic material, and it is currently used mainly as an energy source through direct or indirect burning (Cruichshank et al., 1995; Lund, 2007), a soil supplement in horticulture, or a filter for domestic and industrial wastewater treatment (Ho et al., 1995; Brown et al., 2000).

Most of the lignocellulosic materials possess the similar structural compositions mainly consisting of cellulose, hemicellulose and lignin. Cellulose is the most abundant

natural polymer, with the formula of $(C_6H_{12}O_6)_n$, or a polysaccharide with a linear chain of thousands to over ten thousands of β (1-4) linked D-glucose units containing high degree of polymerization and crystallinity. Cellulose can be chemically broken down into its glucose units when hydrolyzed in water catalyzed by a concentrated acid at an elevated temperature or by an enzyme (cellulase). Hemi-cellulose is another major composition in plant's cell wall and always contains several different mono sugars, including xylose, mannose, galactose and arabinose. Compared with cellulose, hemi-cellulose has a random, branched and amorphous structure, consisting of shorter chains with several hundreds to thousands of sugar units. It can be easily hydrolyzed by a dilute acid or base at moderate temperatures as well as by a hemicellulase enzyme. Lignin acts like a glue in the cell wall, and it is covalently linked to hemi-cellulose and cellulose, covering and connecting the hemicellulose and cellulose to protect them from bacteria and maintaining conferring mechanical strength to the cell wall. Lignin has a complex structure with a mixture of branched polymers of polyphenol propane units, which are bonded to each other by a number of different linkages such as β-4-O and α.-4-O ether linkages. Lignin mainly contains three basic mono lignol structures: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. These lignols are incorporated into lignin in the form of the phenylpropanoids p-hydroxyphenyl (H), guaiacyl (G), and syringal (S) structures, respectively. In softwood lignin, the dominant building units are guaiacyl units. While in hardwood, lignin is built mainly of guaiacyl units and syringyl propane units..

Studies on enzymatic hydrolysis processes in bioconversion of lignocellulosic materials to ethanol production have achieved markedly significant progress in the past decade. The enzymatic hydrolysis is carried out under ambient or mild conditions

normally at 45-50°C and pH around 4.5~5, in the presence of cellulase and hemicellulase enzymes. Enzymatic hydrolysis processes are thus more advantageous than the conventional concentrated or dilute acid hydrolysis with respect to energy efficiency. Enzymatic hydrolysis of lignocellulosic materials can achieve a high glucose yield from10% ~ 60%, depending on the type of lignocellulosic materials (Duff and Murry, 1996).

Cellulase enzymes can be produced form both bacteria and fungi. These microorganisms can be aerobic or anaerobic, mesophilic or thermophilic. Bacteria belonging to *Clostridium*, *Bacteriodes*, *Thermomonospora*, *Ruminococcus*, *Bacteriodes*, *Erwinia*, *Acetovibio*, *Microbispora*, and *Streptomyces* can produce cellulases (Sun and Cheng, 2002; Bisaria, 1991). Because the anaerobes have a very low growth rate and require anaerobic growth conditions, most efforts and research on commercial cellulase production has been focused on using fungi (Sternberg, 1976; Sun and Cheng, 2002; Duff and Murry, 1996). Lignocellulosic enzymes-producing fungi are widespread, and include species from the ascomycetes (eg. *T.reesei*), basisiomecetes containing white-rot fungi (e.g. *P.chrysosporium*), brown-rot fungi (e.g. *Fomitopsis palustris*). Generally cellulases are a mixture of several enzymes such as Endo-1, 4-β-glucanases (EC 3.2.1.4, endocellulase), Cellobiohydrolases (EC 3.3.1.91, exocellulase) and β-glucosidase (EC3.2.1.21). These enzymes have their own characteristics and functions. Endo-1, 4-β-glucanases (EG) can attack the amorphous part of the cellulose, and then it initiates the breakdown of cellulose, providing new free chain ends, which can lead more accessible for cellobiohydrolases (Percival et al. 2006). Cellubiohydrolases preferentially hydrolyze β-1, 4-glycosidic bonds from the chain end, producing cellubiose as the main product

(Coughlan and Ljungdahl et al., 1985; Coughlan and Ljungdahl et al., 1988). The optimal conditions in most of the studies are the pH ~4.8 and 45 ~ 50 °C.

The factors that influence the sugar yield during enzymatic hydrolysis can be divided into two groups: enzyme-related factors (ER) and substrate-related factors (SR):

- Cellulose crystallinity
- Cellulose polymerization degree
- Substrate available surface area
- Lignin
- Hemicellulose
- Feedstock particle size
- Porosity
- Cell wall thickness
- Temperature
- pH
- Reaction time
- Substrate concentration
- Enzyme dosage

Some of the factors listed above, such as the degree of polymerization and crystallinity for cellulose, are related to the properties of lignocellulosic feedstock. Cellulose's high degree of polymerization and crystallinity would prevent enzymes from attacking to hydrolyze the cellulosic materials. Surface area and porosity will also be important factors determining the efficiency of enzyme in hydrolyzing cellulose. In

addition, lignin and hemicellulose are also considered to be the most recalcitrant structure by connecting to each other to maintain a tight cell wall structure. In most research or practical cases, lignocellulosic feedstock needs to be per-treated by various methods (physical, physical-chemical, chemical and biological) before the enzymatic hydrolysis in order to enhance the accessibility of cellulose to the enzymes and increase the hydrolysis efficiency. Specifically, the pretreatment can reduce the particle size, decrease the degree of polymerization of cellulose, lower the crystallinity of cellulose and remove lignin and hemicellulose, all of which could promote the accessibility of cellulose to enzyme. Consequently, pretreatment plays a critical role in the process of enzymatic hydrolysis. The lignocellulosic feedstock pre-treatment methods will be reviewed in details in the following section "1.2 Literature Review".

In the present thesis work, pine sawdust as a typical softwood biomass was pre-treated using various methods, including organosolv extraction, ultrasonic treatment, and sodium hydroxide treatment, and the pre-treated pine sawdust was enzymatically hydrolyzed for glucose production. The delignification efficiency, pre-treatment efficiency and effects of pre-treatments on hydrolysis of pine sawdust were discussed with respect to glucose yield, total sugar yield, and total weight loss.

1.2 Literature review

Because of the structural characteristics (large polymeric molecules and high crystallinity) of the cell wall and the presence of hemicellulose and lignin, enzymatic hydrolysis of cellulose into glucose for bio-ethanol production is challenging due to its

low accessibility to enzymes. As a result, pretreatment of lignocellulosic biomass to loosen the cellulose crystalline structures and increase its accessibility is necessary, important, and is the key in the bioconversion process. There are many different pretreatment methods and can be generally classified into the following types: physical pretreatment, physical-chemical pretreatment, chemical pretreatment and biological pretreatment, whose details will be overviewed as follows.

1.2.1 Physical pretreatment

Physical pretreatment such as comminution of lignocellulosic materials through a combination of chipping, grinding, and milling can be applied to deduce cellulose crytallinity (Sun and Cheng, 2002). After chipping and milling/grinding the size of the biomass materials could be reduced significantly to 10-30 mm and 0.2-2 mm, respectively. Zhu et al. (2009) investigated the effectiveness of different millings as the pretreatment methods in enzymatic hydrolysis, and they found that thermo-mechanical disk milling at 134°C, 2.4 bar to obtain 0.25 mm particles was more effective than the high pressure thermomechanical disk milling at 166°C and 7.2 bar, while the former consumed more energy (460 Wh/kg) than the latter method of milling (151Wh/kg) (Zhu et al., 2009). Yeh et al. (2010) applied a media mill (0.94 kW) for milling the cotton particles to 0.3 mm, and the yield of glucose was significantly increased by 5-folds or more. Ball milling was found to be effective in reducing cellulose crystallinity of the lignocellulosic materials such as woody biomass (Hideno et al., 2009; Millet et al., 1976). For the physical pretreatment method, energy consumption is always an undesirable issue. Obviously, the power requirement for mechanical comminution of lignocellulosic

materials is determined by the final particle size and the biomass characteristics. As reported by Cadoche and Lopez (1989), the energy consumption for size reduction of hardwoods and agricultural wastes was a function of final particle size and the comminution ratio. It was estimated that the energy input for comminution can be lower than 30 kWh per ton of biomass if the final particle size is in the range of 3-6 mm, while in most cases the energy consumption could be even higher than the theoretical energy content of the biomass treated.

1.2.2 Physical-chemical pretreatment

1.2.2.1 Steam explosion

Steam explosion is the most commonly used physical-chemical method for the pretreatment of lignocellulosic materials in particular for hardwood and agricultural biomass (Millan, 1994). In this method, biomass is treated with high pressure saturated steam and then the pressure is suddenly reduced to make the materials undergo an explosive decompression. Steam explosion is typically carried out at 160-260°C and 0.69-4.83 MPa for several seconds to a few minutes before the materials are exposed to the atmospheric pressure (Kumar et al., 2009). Like a hydrothermal pretreatment, steam explosion could remove hemicellulose mainly by autohydrolysis and partially by the chemical effects and mechanical forces (Alvira et al., 2009). The high temperature and pressure promote the acetyl groups present in hemicellulose to be hydrolyzed automatically into acetic acid; on the other hand, the water may act as an acid under such high temperature condition. All of these acids formed in the steam explosion process

could thus hydrolyze hemicellulose. Removal of hemicellulose exposes the cellulose surface and increases enzyme accessibility to the cellulose micro fibrils (Kabel et al, 2007). Mechanical effects are caused when the temperature and pressure are suddenly decreased to ambient pressure which causes the lignocellulosic materials to explode from the inside. Compared with the physical method (milling), the steam explosion method to defibrate materials is more advantageous with lower energy consumption. In the stream explosion process, lignin can also be removed to a certain extent, but is redistributed on the fiber surfaces as a result of melting and depolymeriation/repolymerization reactions (Li et al., 2007). The removal and redistribution of hemicellulose and lignin could swell the pretreated sample and increase its accessible surface area (Duff et al., 1996). The main drawback of this method is that many enzyme-inhibitors are produced in the pretreatment. For example, the pentoses and hexoses formed from the hydrolysed hemicellulose and cellulose can be further degraded to furfural, 5-hydroxymethylfurfural (HMF), levullinic acid and formic acid, which would deactivate the enzymes used in the consecutive enzymatic hydrolysis process.

Cara et al. (2006, 2008) investigated the enzymatic hydrolysis of olive tree wood pretreated by steam explosion under 190, 210, 230 and 240°C for 5 minutes followed by alkaline treatment. It was shown that up to 80% of the lignin in the origin wood was removed, leaving a cellulose-rich residue. The pretreated sample could be efficiently hydrolyzed after 72 hours enzymatic hydrolysis at around 50°C, leading to approximately 7 folds increase in the glucose yield, d wood. Also, the efficiency of enzymatic hydrolysis increased as the temperature in the steam explosion pretreatment process increased and 230 °C was found to be the optimal temperature to achieve the highest

glucose yield. Similar results were obtained in the pretreatment of sunflower (Ruiz et. al., 2008). In the study, the glucose yield in the enzymatic hydrolysis increased up to 72% (corresponding to a glucose concentration of 43.7 g/l) in 96 h for the sample pretreated by steam explosion at 220 °C, compared with a glucose yield of 16.7% for the untreated materials at the same conditions. It is possible that the hemicellulose-derived compounds (such as furfural) were lost through volatilization of the degradation products and through the recondensation reactions (Allen et. al., 1996). However, the furfural formation was found to be significant in the work of Kaar et al. (1995) and Alfani et al. (2000). The toxic compounds such as furfural, HMF (hydroxymethylfurfural), weak acids and phenolic compounds could be detrimental to both hydrolysis and fermentation (Oliva et al., 2003; Palmqvist and Hahn-Hagerdal, 2000). The inhibitors formed in the steam explosion process can be removed by water washing, but it could decrease the overall saccharification yields by typically 20-25% (Millan, 1994; Mes-Hartree et al., 1984; Tengborg et. al., 2001).

Addition of H_2SO_4 (SO_2) or CO_2 (typically 0.3-3 w/w%) in steam explosion can decrease the reaction time and temperature, improve hydrolysis effectively, decrease the production of inhibitors and lead to complete removal of hemicellulose (Ballesteros et al., 2004; Stenberg et al., 1998). The most important pretreatment parameters that affect the sugar yield may be the sulphur dioxide (sulfuric acid) level, the pretreatment time and the temperature (Clark and Mackie, 1987; Schwald et al., 1988; Brownell et al., 1986; Stenberg et al., 1997; Ohgren et al., 2005; Soderstrom et al., 2002; Linde et al., 2006; Linde et al., 2007). The efficiency of enzymatic hydrolysis of softwoods (spruce, pine) pretreated by steam explosion with catalysts of SO_2 (1-6 wt% of biomass dry matter)

H_2SO_4 (0.5-4.4 wt% of dry matter) was studied (Stenberg et al., 1997; Tengborg et al., 1998). The yield of reducing sugar achieved as high as 42.1 g per 100 g dry matter for the sample pretreated by SO_2-steam explosion at 210 °C for 5.5 minutes, and 40g per 100 g dry matter for the sample pretreated by steam explosion 210 °C for 1 minute with 2.4 wt% H_2SO_4 (Stenberg et al., 1997; Tengborg et al., 1998). To increase the efficiency of enzymatic hydrolysis and fermentation for ethanol, a two-step steam pretreatment process with dilute H_2SO_4 impregnation was investigated by many researchers (Soderstrom et al., 2002; Tucker et al., 2003; Nguyen et al., 1999; Kim et al., 2001). The rational of this two-step process is that hemicellulose can be removed in the first pretreatment step and cellulose partially hydrolyzed in the second step, making the cellulose more accessible to the enzymatic hydrolysis. The subsequent enzymatic hydrolysis process could obtain very high (up to 70-80%) yield of total fermentable sugars.

1.2.2.2 Ammonia fiber explosion (AFEX)

In Ammonia fiber explosion process (AFEX) pretreatment, lignocellulosic materials were treated with liquid ammonia at the temperature between 60 and 100 °C under high pressure for a certain period of time, normally tens of minutes, before the pressure is released swiftly, which would cause explosion of the materials from inside mechanically. . Recycling of ammonia in the system after the pretreatment is economically feasible due to the ammonia's high volatility at the atmospheric pressure (Teymouri et al., 2005). The AFEX process was employed for the pretreatment of a variety of lignocellulosic matetials such as alfalfa, wheat straw, wheat chaff, barley straw, corn stover, rice straw, municipal solid waste, softwood newspaper, kenaf newspaper, coastal Bermuda grass, switchgrass,

aspen chips and bagasse (Mes-Hartree et al., 1988; Holtzapple et al., 1992; Vlasenko et al., 1997; Holtzapple et al., 1991; Reshamwala et al., 1995).

AFEX pretreatment does not remove much hemicellulose and lignin, but it can decrease th cellulose crystallinity, disrupt the lignin-carbohydrate linkages and remove the acetyl groups from hemicellulose (Wyman et al., 2005; Kumar et al., 2009). The ammonia pretreatment is thus not as effective as other chemicals pretreatments, such as acid and alkaline treatment, and it mainly limits the ability of lignin to adsorb enzyme (Chandra et al., 2007). After pretreatment, the enzymatic digestibility of lignocellulosic materials can increase. Moniruzzaman et al. (1996) achieved more than 80% of the theoretical sugar yield from corn fiber pretreated using AFEX at 90°C, ammonia-to-corn fiber mass ratio of 1:1 and 200 psi for 30 minutes. Except for the reduction in protein content, there is no chemical interaction involved during the AFEX pretreatment process between ammonia and lignocellulosic materials. The effectiveness of AFEX treatment may be accounted for by the fact that ammonia could effectively penetrate the lignocellulosic materials' matrix and react with interior cellular components of the corn fiber to increase its surface area by splitting fiber bundles axially and physically (Moniruzzaman et al., 1996; Laureano-Perez et al., 2005; Wyman et al., 2005; Kumar et al., 2009). AFEX removes the least of hemicellulose and quite little lignin compared with other pretreatment methods such as SO_2-catalyzed steam explosion, dilute acid pretreatment (Kumar et al., 2009). Superior to the steam explosion pretreatment where many inhibitors are formed from the degradation of hemicellulose and cellulose, the AFEX pretreatment is likely more advantageous as no toxic byproducts are formed

except for some phenolic fragments from lignin (Dale et al., 1984; Mes-Hartree et al., 1988; Sun and Cheng, 2002).

In a study by Alizadeh et al. (2005), switchgrass was AFEX pretreated in a bench-top 300 ml reactor (pressure vessel). The study showed that the AFEX accelerated the rate of enzymatic hydrolysis, led to glucose conversion of 93% in and xylan conversion f 70% at a very low enzyme loading (15FPU/g of glucan), compared with 16% and 3% for the untreated switchgrass, respectively. As a matter of fact, much lower enzyme loading, as low as 5 FPU, was employed and demonstrated to be effective or enzymatic hydrolysis of lignocellulosic materials after AFEX pretreatment (Holtzapple et al., 1991; Teymouri et al, 2004; Wyman et al., 2005). However, the AFEX process was not very effective for biomass with higher lignin content such as newspaper and woody biomass (Millian, 1994; Kumar et al., 2009).

1.2.2.3 Microwave pretreatment

Microwave pretreatment may be considered a physico-chemical process since it involves both non-thermal and thermal effects. The microwave method has proved to be effective for improving enzymatic hydrolysis of many agricultural residues/biomass such as rice straw and wheat straw (Zhu et al., 2005; Zhu et al., 2006; Zhu et al., 2006; Ma et al., 2009), bagasse (Ooshima et al., 1984) and switchgrass (Keshwani et al., 2009). It was believed that microwave and microwave based pretreatment could hydrolyze hemicellulose and solubilize lignin. Moreover, the thermal and non thermal effects arising from the heating could enlarge the pore size of the lignocellulosic materials, and enhance the accessibility of the cellulose to enzymes (Banik et al., 2003). During the

microwave treatment, the thermal effects could lead to the formation of acid (e.g., acetic acid) as a catalyst for hemicellulose hydrolysis. It was also found that microwave-based pretreatment can be carried out more effectively when combined with some chemical reagents such as acid and alkaline compounds (Caddick, 1995). .

Zhu et al. (2005, 2006) studied effects of various microwave-based pretreatment methods (such as microwave/alkaline, microwave/acid and microwave/alkaline/acid/ H_2O_2) on hydrolysis of rice/wheat straw materials. It is generally accepted that microwave/alkaline pretreatment improves the removal of hemicellulose and lignin, which would hence promote the enzymatic hydrolysis rate, compared with alkaline treatment alone. It was also found that the treatment of microwave/acid/alkali/H_2O_2 led to the highest total weight loss, the greatest cellulose content in the residue, and the lowest contents of moisture, ash, lignin and hemicellulose. In the subsequent enzymatic hydrolysis process, biomass pretreated by microwave/acid/alkaline/ H_2O_2 achieved the highest yield of reducing sugars. On the other hand, Ma et al. (2009) systemically optimized the pretreatment conditions for rice straw hydrolysis, and studied the effects of microwave intensity, irradiation time and substrate concentration on the hydrolytic conversion of cellulose and hemicellulose.

1.2.2.4 Ultrasonic pretreatment

Ultrasonic pretreatment could be another promising method for the removal of hemicellulose and lignin, and it has been widely used in extraction of hemicelluloses and enzyme proteins from biomass and organic waste materials such as bio-sludge (Sun and Tomkinson, 2002; Ebringerova and Hromadkova, 1997; Hromadkova et al., 1998; Yin et

al., 2004), although there is very limited published literature with respect to the effects of ultrasonic pretreatment on the glucose yield and carbohydrate conversion in enzymatic hydrolysis of lignocellulosic materials. Yachmenev et al. (2009) reported that saccharification of cellulose was enhanced considerably by ultrasonic pretreatment. The increased enzymatic hydrolysis yields after ultrasonic pretreatment could be explained by the effects of ultrasonic pretreatment: cracking of the cell wall, dislocation of the secondary wall of the middle layer of cell wall, and exposure of the middle layer to enzymes (Tang and Liang, 2000). These effects increase the accessibility of the cellulose microfibrils to enzymes. Additionally, the high frequency used in the ultrasonic pretreatment could introduce more violent cavitation, which would enhance the transportation of enzymes macromolecules toward the substrate surface. Moreover, mechanical impacts produced by the collapse of cavitation bubbles, could yield an important benefit to the hydrolysis process by exposing the surface of solid substrates to the enzymes. Yu et al.(2009) investigated the effects of ultrasonic pretreatment on enzymatic hydrolysis of rice hull using 250 W, 40 kHz at 25 °C for different period of time ranging from 10 ~ 60 minutes. Their results showed that the yields of total sugar and glucose increased from 11.7% and 10.9% to 16.3% and 15.8%, respectively, by the ultrasonic pretreatment for 30 min. In a study by Satish et al. (2009), ultrasonic pretreatment of grain sorghum resulted in an increase in saccharification efficiency by 8%.

In summary, ultrasonic treatment can cause cavitation, crash the cell wall structure, and provide more accessible surfaces in the substrate. Ultrasonic pretreatment can thus be a potential method for pretreatment of lignocellulosic materials due to its lower energy

consumption. Due to the limited research in this respect, it is of interest to examine the effects of ultrasonic pretreatment on enzymatic hydrolysis of lignocellulosic materials, in particular when combining it with other chemical pre-treatment approaches such as organosolv and alkaline methods.

1.2.3 Chemical pretreatment

1.2.3.1 Acid pretreatment

The main object of acid pretreatment is to hydrolyze hemicellulose in lignocellulosic materials into fermentable sugars and to make the cellulose more accessible to enzymes, while acid pretreatment has less effect on lignin degradation or removal. Acid pretreatment can be performed with a concentrated acid such as hydrochloride acid, sulfuric acid, phosphoric acid and nitric acid, or a dilute acid (such as dilute sulfuric acid). Although concentrated acid proved to be very effective for hydrolysis of hemicellulose, it is less attractive for practical application to ethanol production, due to the significant formation of high concentration of inhibiting compounds (such as furfural, HMF, etc.). In addition, pretreatment using concentrated acid under a high temperature (>180°C) may cause decomposition of cellulose. Moreover, a strong acid of a high concentration would cause corrosive issue for the reactor and the acid recovery issue, which would lead to high operational and maintenance costs (Alvira et al., 2009). Unlike the concentrated acid pretreatment, dilute acid pretreatment has been successfully developed for the pretreatment of lignocellulosic materials under relatively moderate experimental conditions. It can be used either for pretreatment of

lignocellulosic materials before enzymatic hydrolysis or for directly hydrolyzing of biomass into fermentable sugars. Typically, dilute acid pretreatment can achieve high reaction rate and obtain nearly 100% hemicellulose removal under the optimal conditions: 180°C for a short reaction time (e.g. 5 min) or at 120 °C for a comparatively long reaction time (e.g. 30-90 min). Due to the possible decomposition of cellulose and formation of inhibitors at severe condition, many studies have focused on the conditions of a lower temperature.

The very positive effects of dilute acid pretreatment on enzymatic hydrolysis were demonstrated in many studies. For example, the dilute acid pretreatment of rye straw and Bermuda grass at 121 °C with a 1.2% dilute sulfuric acid for 60 min, led to conversion of more than 50% of the hemicellulose of rye straw into monomeric sugars (Sun and Cheng, 2002) found that. Dilute acid pretreatment caused minimal (at ~ 10 wt%) conversion of cellulose/glucan and lignin. Zhu et al. (2005) conducted a research on effects of dilute acid pretreatment of corn stover using various acid concentrations (0.2-1.0% w/w) and various temperatures, leading to a high xylose yield up to 73%. Dilute acid pretreatment of wheat straw using dilute H_2SO_4 (0.75%, v/v) was found to promote enzymatic hydrolysis and fermentation efficiencies while producing less measurable quantities of furfural and hydroxymethyl furfural as the inhibitors (Saha et al., 2005). Guo et al. (2008) attained a high yield of xylose (70% and 75%) from silvergrass when treated with 1%, 2% and 3% dilute sulfuric acid at 121 °C for 30 min. The dilute acid pretreatment process also produced glucose at a yield of 10%, and converted more than 90% of the acetyl groups into acetic acid and 4-5% of furfural, which are the inhibitors for the fermentation process. Lu et al. (2007) investigated pretreatment of corn stover with dilute sulfuric acid,

and the optimal conditions for corn stover pretreatment were found to be at 120°C for 43 minutes with 2.0 wt% H_2SO_4, producing 77% xylose yield with a low glucose yield of 8.4%. The subsequent enzymatic hydrolysis led to up to 42.1 g of glucose/100 g of substrate (Lu et al., 2007). Yat et al. (2007) investigated pretreatment of 28-10/20 mesh woody species (aspen, balsam fir, basswood and red maple) and switchgrass using sulfuric acid concentrations (0.25-1.0 % w/w) at various temperatures (160-190 °C). As expected, the formation reaction rates of the xylose and glucose were found to depend strongly on both temperature and acid concentration. The maximum yields ranged from 70% (balsam) to 94% (switchgrass) for xylose, from 10.6% (balsam) to 13.6% (switchgrass) for glucose. While furfural formation was confirmed, xylose degradation varied linearly as a function of acid concentration (Yat et al., 2007). However, the highest hemicellulosic sugars recovery in the pretreatment does not necessarily lead to the highest enzymatic hydrolysis efficiency (Taherzadeh and Karimi, 2008; Cara et al., 2007). For example, Cara et al. (2007) reported that the maximum hemicellulose recovery (83%) from olive tree biomass was obtained at 170 °C and 1% sulfuric acid concentration, but the maximum enzymatic hydrolysis sugar yield (76.5%) was obtained when pretreated at 210 °C with 1.4% acid concentration.

Organic acids such as fumaric or maleic acids are appealing as alternatives to the aforementioned inorganic acids to enhance cellulose hydrolysis for ethanol production. In a recent study by Kootstra et al. (2009), fumaric and maleic acids were tested in comparison to dilute sulfuric acid for pretreatment of wheat straw, and the study demonstrated that the organic acids effectively raised the enzymatic digestibility even

with a higher solid loading ratio up to 30%, while the formation of the inhibitors such as furfural in the pretreatment was less significant.

1.2.3.2 Alkaline pretreatment

Alkaline and alkaline solutions such as NaOH, $Ca(OH)_2$ have been applied for effectively removing lignin and hemicellulose from lignocellulosic materials in the pulping processes. They were also proved to be effective reagents in pre-treating lignocellulosic materials to increase the accessibility of enzymes to cellulose for bio-ethanol production. Compared with the steam explosion approach as introduced previously, alkaline pretreatment can be carried out in ambient or mild conditions. In addition, unlike the acid pretreatment with the issues of corrosion and inhibitor formation, alkaline pretreatment causes less sugar degradation to form toxic compounds, and many of caustic salts may be recovered or regenerated to achieve a better economics of the process. However, a drawback of alkaline pretreatment may be that it normally needs a longer pretreatment time ranging from hours to days.

Alkaline pretreatments can increase cellulose digestibility and they are more effective for solubilization and removal of lignin, while having minimum effects on the holocellulose, for example, cellulose and hemicellulose (Carvalheiro et al., 2008). The mechanism of alkaline hydrolysis is believed to involve saponification of intermolecular ester linkages in xylan of hemicellulose and lignin. The porosity of the lignocellulosic materials increased with the removal of these ester crosslinks (Tarkow and Feist, 1969), and this could eliminate the nonproductive adsorption sites for enzymes and hence improve the digestibility of cellulose.

Alkaline pretreatment using sodium hydroxide has been widely reported in a number of publications. NaOH treatment of lignocellulosic materials was found to cause swelling, an increase in internal surface area, decreases in the crystallinity and the degree of polymerization of cellulose, separation of the structural linkages between lignin and carbohydrates, and disruption of the lignin structure (Kumar et al., 2000; Taherzaheh and Karimi, 2008). Zhao et al. (2007) compared the effects of pretreatments using mild sulfuric acid and NaOH on enzymatic hydrolysis of crofton weed stem, and they concluded that the optimal pretreatment conditions using NaOH was at 110°C for 120 min with NaOH loading of 10 wt% and a liquid to solid ratio of 6:1 (w/w). Under these optimal conditions, 30% of the raw material and 25% of the lignin were removed in the pretreatment. Compared to mild sulfuric acid pretreatment, the NaOH pretreatment obtained a higher enzymatic conversion ratio of cellulose. Silverstein et al. (2006) studied the effects of sulfuric acid, sodium hydroxide, H_2O_2, and ozone pretreatments for enzymatic conversion of cotton stalks and found that sodium hydroxide pretreatment was the most effective method. The treatment using 2% NaOH solution at 121 °C for 90 min resulted in the highest level of delignification of 65%, and the cellulose conversion under this condition was as high as 60.8%. Chosdu et al. (1993) combined the irradiation and 2% NaOH pretreatment methods to treat corn stalk, cassava bark and peanut husk, producing 43% glucose yield of corn stalk after the pretreatment, almost doubling the glucose yield of the untreated corn stalk. Xu et al. (2010) employed NaOH to pretreat switchgrass and achieved 45 g of total reducing sugars per 100g of raw biomass with the glucan and xylan conversion ratios as high as 74.4% and 62.8%, respectively, at the optimal conditions (e.g. 50°C, 12 h and 1.0% NaOH), which were nearly 4 times that of

the untreated biomass. More recently, Wang et al. (2010) also reported a higher lignin removal efficiency of 86% and very high total glucose and xylose yield of 90.43% and 65.11%, respectively, in the enzymatic hydrolysis of coastal Bermuda grass after NaOH pretreatment under the pretreatment conditions of 0.75%, NaOH, 121°C for 15 min. NaOH and NaOH/urea pretreatment at low temperatures also proved to be effective for enzymatic hydrolysis of woody biomass, e.g., spruce (Zhao et al., 2007). In their study, the best pretreatment conditions were 3% NaOH/12% urea, −15 °C for 7 days, the glucose conversion was between 60~70%. Such a low-temperature process is appealing due to the improved energy efficiency. At such a frozen temperature, the structure of cell wall is more fragile and easy to break down, but a too low temperature can reduce the reaction rate and increase the time of the pretreatment process (several days) compared with minutes or hours for the high–temperature processes.

Calcium hydroxide, $Ca(OH)_2$, also converted into lime, can be an alternative pretreatment reagent to NaOH, to remove amorphous substances such as lignin. Compared with NaOH, the lignin removing efficiency of $Ca(OH)_2$ is normally lower probably due to the formation of a calcium–lignin complex. Specifically, calcium cations, each carrying two positive charges, tend to crosslink lignin molecules which are negatively charged under the alkaline conditions due to the ionization of functional groups (e.g. carboxyl, methoxy, and hydroxyl) to form stoichiometric bonds, thus preventing from solubilization of lignin during the $Ca(OH)_2$ pretreatment (Torre et al., 1992; Xu et al., 2008). The $Ca(OH)_2$ pretreatment could also remove acetyl groups from hemicellulose, thus reducing steric hindrance of enzymes and enhancing cellulose digestibility (Mosier et al., 2005). Lime pretreatment of corn stover with 7.5 wt% lime

loading for 4 h at 120°C led to conversion of glucan, xylan and arabinan at 88%, 87.7% and 92.1%, respectively, in the enzymatic hydrolysis (Kaar and Holtzapple, 2000). Also, Saha and Cotta (2007) employed lime pretreatment of rice hull at 121 °C for 1 hour with various lime loading, and obtained 32% sugar yield in the enzymatic hydrolysis. High glucose and xylose yields of 91.3% and 51.8%, respectively, were reported by Kim and Holtzapple (2005) in the enzymatic hydrolysis of biomass after lime pretreatment with a lime loading of 0.5 g/g raw biomass at 55 °C for 4 weeks, removing 87.5% of the lignin in the original biomass.

The addition of an oxidant agent, such as H_2O_2, to alkaline pretreatment can improve the performance by favoring lignin removal. Alkaline peroxide is an effective method, typically the lignocellulosic materials are soaked in alkaline solution at optimally pH 11~12 (pretreatment efficiency may change at different pH values), then adding low concentration of H_2O_2 into the solution at low temperatures normally 30~70°C for a period of time typically several hours. The ability of H_2O_2 for delignification is attributed to its ability to react with several carbonyl-containing structures in lignin, which can be explained through the reactions of the hydro-peroxide anion (HOO-), formed in an alkaline medium according to the equilibrium (Sun, R. C. and X. F. Sun, 2002):

$$H_2O_2 + HO^- \leftrightarrow HOO^- + H_2O, \text{ pKa} = 11.6 \text{ at } 25\ ^\circ C \quad (1)$$

This anion is a strong nucleophile that preferentially attacks ethylenic and carbonyl groups present in lignin. As a consequence, such chromophores as quinines, cinnamaldehyde and ring conjugated ketones could be converted to nonchromophoric species in the alkaline solution (Sun and Fan, et al., 2000). On the other hand, the H_2O_2 itself is unstable in alkaline condition and can readily decompose as the temperature

increases. At the same time, catalyzed by some metal ions as manganese, iron and copper presence in the biomass, the H_2O_2 would release more active radicals, e.g,, HO• and O^{2-}, to facilitate the delignification of biomass (Sun, R. C. and X. F. Sun, 2002; Pan et al., 1998). The use of H_2O_2 has resulted in a high pretreatment efficiency in many previous studies. Gould (1985) demonstrated the effectiveness of delignification of agricultural residues such as wheat straw using 1% H_2O_2 at pH 11.5 and 25°C for 18–24 h. Under this condition, more than half of the lignin and most of hemicellulose were solubilized and removed. These results were higher than those of NaOH treatment (without H_2O_2 addition) by several times. Enzymatic hydrolysis of H_2O_2 (at pH 11.5)-treated wheat straw also showed an obvious enhancement where nearly 100% conversion was attained. The treatment with NaOH alone under identical condition exhibited a significant increase in cellulose digestibility only at pH > 12 and the maximum cellulose conversion efficiency did not exceed 65%. Sun et al. (2000) reported the delignification efficiency of rye straw using H_2O_2 under different temperatures from 20 °C to 70 °C with 2% H_2O_2 at pH 11.5 for 12 h. the maximum delignification efficiency was calculated to be 87.8% at 70 °C and correspondingly 71.9% of hemicellulose was removed too. Mishima et al. (2006) examined twenty chemical pretreatment compounds most of which were acids and bases, in order to improve the efficiency of enzymatic hydrolysis of water hyacinth and water lettuce. It was shown that the alkaline/oxidative pretreatment, in which NaOH and H_2O_2 were used, was the most effective method for improving the enzymatic hydrolysis. Yamashita et al. (2009) conducted a comparison of different pretreatment methods for enzymatic hydrolysis of bamboo: a relatively large amount of glucose and reducing sugar of 399 and 568 mg/g raw biomass was achieved with the pretreatment using 1% (v/v)

H_2O_2 and 1 wt. % NaOH at 90 °C for 60 min as that with steam explosion or sodium hydroxide pretreatment. Saha and Cotta (2007) pre-treated rice hull using 7.5% (v/v) H_2O_2 at pH 11.5, 35 °C for 24 h and reached the yield of sugars in the enzymatic hydrolysis was 428 mg/g and a theoretical sugar yield of 90%, where no measurable furfural and hydroxymethylfurfural (HMF) were detected in the process. Yanez et al. (2005) reported that 67.9% ~80% of cellulose conversion was achieved in enzymatic hydrolysis of rice husks after the H_2O_2 pretreatment at 80 °C for 4 h.

1.2.3.3 Organosolv pretreatment

Organosolv pretreatment is another promising pretreatment method suitable for enzymatic hydrolysis of lignocellulosic materials. In general, it uses an organic or aqueous organic solvent to remove or cleave the linkages between lignin, hemicellulose and cellulose. In this process, lignocellulosic materials are mixed with an organic solvent with or without water and heated up to a temperature (typically 150~250°C) and high pressure, although some literature studies revealed that it can be operated at a mild condition too. However, for a mild-temperature operation, mineral acids are normally needed to act as catalysts. Mineral acids (hydrochloric acid, sulfuric acid and phosphoric acid) proved to be good catalysts to accelerate delignification and xylan degradation, while some organic acids such as formic acid, oxalic, acetylsalicylic, and salicylic acid could also be used as the catalysts (Sun and Cheng, 2002). The mechanism has been proposed that under high temperature and pressure, organic reagents can cleave the bonding of different function groups (such as the α-O-4, β-O-4 ethers) in lignin and fragment very huge lignin molecule into smaller ones; it also accompanies the formation

of acetic acid or other acids or byproducts from the acetyl groups in hemicellulose under severe conditions. These acids can act as catalysts accelerating the rapture of lignin-carbohydrate complex (Duff and Murray, 1996). At the same time, water acts like a medium to dissolve these products. At the end of the reaction, it produces three phases (oily phase and water soluble phase, and solid residue). Cellulose can be recovered in the solid phase and hemicellulose and lignin fractions can be obtained after extraction or distillation of the liquid phase (Pan et al., 2006; Rolz et al., 1986; Pan et al., 2005). The final products contain an enriched cellulose fraction, lignin and an aqueous stream mainly from degraded hemicellulose (Duff and Murray, 1996). Many organic solvents such as alcohols, esters, ketones, glycols, organic acids, phenols, and ethers have been tested as the organosolv pretreatment solvents for the pulping processes and enzymatic bio-ethanol production (Zhao et al., 2009; Aittamaa and Sundquist, 2000). From the economic perspective, the use of low-molecular-weight alcohols such as ethanol and methanol, are more favorable as these solvents are easy for recovery due to their lower boiling points.

Pasquini et al. (2005) extracted lignin using ethanol-water mixtures and CO_2 from sugar cane bagasse and pinus tarda wood chips, and achieved a high delignification efficiency of 93.1% for P. tarda wood chips and 88.4% for sugar cane bagasse. Using hot aqueous 60% ethanol and 1.25% H_2SO_4 to pre-treat poplar chips at temperature 180 °C for 60 minutes before enzymatic hydrolysis, Pan et al. (2006) obtained 74% of the lignin removal, and 82% of the total cellulose converted to glucose. Additionally, in their earlier work, more than 90% of the cellulose in low lignin pulps (<18.4% residual lignin) was hydrolyzed to glucose in 48 h using an enzyme loading of 20 filter paper units (FPU)/g cellulose (Pan et al., 2005). The pulps performed well in both sequential and

simultaneous saccharification and fermentation trials, indicating an absence of metabolic inhibitors. Ethanol organosolv pretreatment (60% ethanol and 1.25% H_2SO_4) of beetle-killed lodgepole pine were also found very effective for increasing the enzymatic digestibility leading to 97% conversion of cellulose (Pan et al., 2007). In a study by Araque et al. (2007), organosolv pretreatment of woody biomass using acetone-water 1:1 (w/w) at pH 2.0 and 195 °C for 5 min also resulted in 71.8% total sugar yields in the enzymatic hydrolysis. In addition to remove lignin and hemi-cellulose, organosolv combined with sulfuric acid pretreatment could change the cellulose structure and markedly reduce the cellulose crystallinity, and hence greatly enhance the accessibility of cellulose to enzymes. nocellulosic materials. In a very recently study by Sannigrahi et al. (2010), Loblolly pine was pre-treated with 65% ethanol/water solution containing 1.1% sulfuric acid as catalyst at 170 °C for 1h. The enzymatic hydrolysis of the resulting substrate led to 70% conversion of the cellulose to glucose.

1.2.4 Biological pretreatment

Microorganisms can be used to treat the lignocellulosic materials for lignin removal and increase the accessibility of cellulose to enzymes. Biological pretreatment is a safe and environmental friendly method and has been increasingly advocated as an energy-saving process, as compared to the physical and chemical pretreatment (Okano et al., 2005). The microorganisms applied in this process can selectively degrade lignin and hemicelluloses. This is mainly due to the fact that cellulose maintains a high crystallinity and high degree of polymerization which is more resistance than the other parts of lignocellulosic materials to biological attack. Microorganisms, including brown-, white-,

and soft-rot fungi, have been successfully used to degrade lignin and hemicellulose in waste materials (Sun and Cheng, 2002; Kumar et al., 2009). Lignin degradation by white-rot fungi occurs through the action of lignin-degrading enzymes such as peroxidases and laccase (Lee et al., 2007). The activities of enzymes are regulated by carbon and nitrogen sources. Among the various fungi, white-rot fungi are the most effective ones for biological pretreatment of lignocellulosic materials. The white-rot fungus *P. chrysosporium* produces lignin-degrading enzymes (i.e. lignin peroxidases and manganese-dependent peroxidases) during secondary metabolism in response to carbon or nitrogen limitation (Boominathan and Reddy, 1992). Both enzymes have been found in the extracellular filtrates of many white-rot fungi for the degradation of wood cell walls (Kirk and Farrell, 1987; Waldner et al., 1988). Other enzymes, including polyphenol oxidases, laccases, H_2O_2 producing enzymes and quinone-reducing enzymes can also degrade lignin (Blanchette, 1991).

Hatakka et al. (1983) studied biological pretreatment of wheat straw by using 19 white-rot fungi and found that 35% of the straw was converted into reducing sugars by *Pleurotus ostreatus* in 5 weeks. Similar conversion was obtained in the pretreatment by *Phanerochaete sordida 37* and *Pycnoporus cinnabarinus 115* in 4 weeks. To prevent the loss of cellulose, a cellulase-less mutant of *Sporotrichum pulverulentum* was developed for the degradation of lignin in wood chips (Ander and Eriksson, 1977). Furthermore, Akin et al. (1995) reported that biodegradation of bermudagrass stems was improved by 29-32%, after 6 weeks, using *Ceriporiopsis subVermispora,* and by 63-77% using *Cyathus stercoreus*. Moreover, Lee et al. (2007) studied the effects of biological pretreatment on the Japanese red pine Pinus Densiflora. Of the three white-rot fungi

(*Ceriporia lacerata*, *Stereum hirsutum*, and *Polyporus brumalis)* tested, it was found that *S. hirsutum* degraded the lignin of the wood sample but not holocellulose (hemicellulose + cellulose) component. Extracelluar enzymes from *S. hirsutum* showed higher activity of ligninase and lower activity of cellulase than those from other white-rot fungi. In addition, Taniguchi et al. (2005) evaluated biological pretreatment of rice straw using four white-rot fungi (*Phanerochaete chrysosporium, Trametes versicolor, Ceriporiopsis subvermispora,* and *Pleurotus ostreatus*) on the basis of quantitative and structural changes in the components of the pretreated rice straw as well as susceptibility to enzymatic hydrolysis. Pretreatment with *P. ostreatus* resulted in selective degradation of the lignin and increased the susceptibility of rice straw to enzymatic hydrolysis.

In summary, biological pretreatment is a promising technology for delignification, considering the advantages of low energy cost and environmental friendness. The disadvantage of the biological pretreatment is the low kinetic and thus needs long time for reaction. Consequently, future studies in this area should focus on the improvement of kinetics and its selectivity to lignin.

1.3 Objective of this study

The primary goal of this study was to investigate various pretreatment methods for enzymatic hydrolysis of softwood biomass (pine sawdust) to fermentable sugars (glucose, xylose, etc.). Specific objectives included:

1.) Studying the feasibility of pretreatment of peat using H_2O_2 for delignification under alkaline condition.

2.) Pre-treating pine sawdust with various pretreatment methods including organosolv extraction, ultrasound and NaOH.

3.) Evaluating the glucose yield and total sugar yield from enzymatic hydrolysis of substrate pre-treated with different pretreatment methods.

1.4 Outline of this thesis

Chapter 1 provided an overview on different pretreatment methods for enzymatic hydrolysis of lignocellulosic materials, including physical, physicochemical, chemical and biological pretreatments. Chapter 2 describs the experimental materials and methods used in the thesis work. Chapter 3 presents detailed experimental results of delignification of peat using alkaline H_2O_2, and the glucose and total sugar yields in enzymatic hydrolysis of the pine sawdust after different pretreatment methods including organosolv extraction, organosolv + ultrasound, organosolv + NaOH, and organosolv + ultrasound + NaOH. Effects of various parameters (such as reaction time and enzyme doses) on the sugar yields in the enzymatic hydrolysis were also discussed. Chapter 4 summarizes the major conclusions from the present research and provided recommendations for future study.

Chapter Two

Experimental Materials and Methods

Described in this chapter are the experimental materials and methods used in the present study. The subsections are outlined in the following sequence: experimental materials; pretreatment of peat, pretreatment of pine sawdust, enzymatic hydrolysis of raw and pretreated pine sawdust, and characterization of pretreated peat hydrolyzates and solids, and pine sawdust before and after pretreatment.

2.1 Experimental Materials

The peat and pine sawdust samples were supplied from Peat Resources Ltd., Ontario, and a local lumber mill (Northern Wood Ltd), respectively. The peat sample and Pinus bank siana (Jack pine) sawdust were grounded with a Wiley mill and screened to particles of 20-mesh (~0.75 mm) for the experiments. The particles were dried in an oven at 105°C for 12 h before use. The results of proximate and ultimate analysis of peat and pine samples and the chemical compositions of the ash from peat and pine wood samples are summarized in Tables 1 and 2. The cellulose, hemicelluloses and lignin content were determined by the Analytical Laboratory of FP Innovations, Montreal, Canada. The samples were extracted with acetone to obtain the extractive-free test specimens. Carbohydrates were determined according to the TAPPI test method T249 cm – 85 and the acid-soluble and acid-insoluble lignin was determined according to the TAPPI test method T222 om – 88. The results are summarized in Table 3.

Table 1. Proximate and ultimate analyses of the peat sample and concentrations of major inorganic elements in the peat

Proximate analysis, wt% (d.b.[a])				Ultimate analysis, wt% (d.b.[a])				
Organic matters		Ash		C	H	N	S	O[b]
94.6		5.4		54.7	5.5	2.1	0.2	32.1
Major inorganic elements, wt% (d.b.[c])								
Na	K	Mg	Ca	P	Fe	S	Al	Si
<0.1	<0.1	0.1	0.8	0.1	0.6	0.1	0.3	0.1

[a] On a dry basis
[b] By difference
[c] Determined by ICP-AES

Table 2. Proximate and ultimate analysis results of the pine wood sample and chemical compositions of the ash from the wood sample

	Proximate analysis, wt%(d.b.[a])				Ultimate analysis, wt%(d.b.[a])				
	VM	FC	Ash		C	H	N	S	O[b]
Pine wood sample	81.52	18.31	0.17		53.3	6.2	0.1	0.1	40.3
	Major elements in the ash, ppmw(d.b.)[c]								
	Na	K	Mg	Ca	Mn	Fe	Zn	Al	Si
Ash from the sample	7	114	100	440	20	9	10	16	3

a On a dry basis
b By difference
c Determined by ICP-AES

Table 3. Carbohydrates and lignin contents (wt%)in peat and pine

Sample	Pine wood	Peat
Acid-insoluble (Klason) lignin	28.2	58.1
Acid-soluble lignin	0.22	2.13
Carbohydrates (by gas chromatography)		
Arabinan	1	0.3
Xylan	3.5	2.3
Mannan	11.4	2.2
Galactan	1.5	1.7
Glucan	44.7	15.2
Acetone extractives	5.95	5.79
Total lignin	28.4	60.2
Cellulose	40.2	14
Hemicelluloses	21.9	7.7

2.2 Experimental Methods

2.2.1 Pretreatment of peat

Peat samples were washed by rinsing with distilled water and dried in the oven for 12 hours before experiments. 1.0 g of dried peat sample was added into the H_2O_2 solution with various concentrations (w/v) in a sealed high pressure glass tube. The mixture was heated in a water bath shaker at a shaking speed of 100 rpm for a specific period of reaction time. For comparison, one sample was treated with dilute alkaline solution (NaOH solution) in the absence of H_2O_2 and kept the rest of the experimental conditions

the same. During the initial stage, oxygen evolution was active, and bubbles were released from the solution. The insoluble residue was collected by filtration and washed thoroughly with distilled water until the pH value was neutralized, and then dried in an oven in air at 60 °C. The supernatant was adjusted to pH 5.5 with 10% HCl and then concentrated by evaporation. Hemicelluloses were precipitated by pouring the concentrated supernatant into three volumes of ethanol. Solublized lignins were obtained from the corresponding supernatants after hemicelluloses' precipitation and then adjustment of pH of the filtration to 1.5~2.0 (Sun et al., 2000).

2.2.2 Pretreatment of pine sawdust

In order to enhance the efficiency of enzymatic hydrolysis of pine sawdust, pretreatment of pine sawdust was conducted using various methods, including organosolv extraction, organosolv extraction followed by ultrasonic treatment, organosolv extraction followed by NaOH treatment, and combined organosolv extraction, ultrasonic treatment and NaOH treatment in sequence, to remove lignin and/or hemicellulose and disrupt the structure of pine sawdust to increase the accessibility of cellulose to enzymes.

2.2.2.1 Organosolv extraction

Pine sawdust was milled to 20 meshes and was dried in the oven for 12 h before use. The experiment was carried out in a 1 L autoclave reactor under nitrogen atmosphere with temperature control, water cooling system and stirring speed control. The sample was weighted at 50 gram and mixed with solution of ethanol and distilled water at a ratio

1: 10 (w:v). Ethanol and distilled water was proportioned to 1:1 (v:v) in the solution. The reaction temperature was set at 190 °C and the stirring speed was controlled at 400 ± 20 rpm. The initial nitrogen pressure was 300 psi in the reactor and the maximum pressure during the reaction was 700 psi. After 4 hours' reaction, cooling water was charged into the jacket of the autoclave reactor to cool down the system to room temperature. The liquid and solids phase in the reactor was then separated by filtration. Liquid phase was used to recover hemicelluloses and lignin. Solid phase was considered to be cellulose rich residue and was divided into several groups for further treatment. The first group was further treated with ultrasound and NaOH in sequence and is marked the pine sample pretreated with organosolv+ultrasound+NaOH. The second group was further treated with ultrasound only and is marked as pine sample pretreated with organosolv+ultrasound. The third group was further treated with NaOH only and is marked as pine sample pretreated with organosolv+NaOH. The forth group was directly used in the following enzymatic hydrolysis process without any further treatment and is marked as pine samples pretreated with organosolv. For comparison, the raw (untreated) pine sawdust sample was used as a basis. Detailed experimental conditions of further treatments of oragnosolv extracted pine samples are described in the following sections.

2.2.2.2 Organosolv extraction followed by ultrasonic treatment

The solid residue of Group 2 separated from liquid phase was weighted and placed in a 500mL beaker (the (w:v) ratio of solids to water was 5 g: 200 mL). The mixture was further treated with ultrasound (Fisher Scientific Ultrasonic Cleaner, 100W, 42KHz output) for 3 hours at room temperature. After that, the solid phase was separated from

the liquid phase by filtration. The collected solid (organosolv+ultrasonic treatment) was used for enzymatic hydrolysis. The purpose of ultrasonic treatment was to improve the accessibility of cellulose to enzymes.

2.2.2.3 Organosolv extraction followed by NaOH treatment

5 gram of solid residue of Group 3 separated from the liquid phase was placed in a 500mL beaker with the addition of 250 mL 1N NaOH. The NaOH treatment was carried out at 70 °C at a shaking speed of the water bath shaker at 100 rpm for 3 hours. After filtration, the solid residue was collected for enzymatic hydrolysis.

2.2.2.4 Organsolv extraction followed by ultrasonic and NaOH treatment

The solid residue of Group 1 from organosolv extraction was treated with ultrasound and NaOH in sequence at the same reaction conditions of ultrasound and NaOH solution treatment as described above. The solid was recovered after filtration and used in the enzymatic hydrolysis process.

2.2.3 Enzymatic hydrolysis

Pretreated pine samples (Groups 1, 2, 3, and 4) and raw pine sawdust were hydrolyzed using cellulase in 50mL flasks in a water bath shaker. The hydrolysis was performed in 25 mL 0.1M sodium citrate buffer solution in the flask. The pH of the buffer solution was 4.8. The temperature was set at 50 °C and the shaking speed at 100 rpm. The weight of substrate used was 0.10 gram in each individual enzymatic hydrolysis experiment to determine the effectiveness of different pretreatment methods (organosolv, organosolv+ultrasound, organosolv+NaOH, organosolv+ultrasound+NaOH), reaction time (0~72h), and enzyme doses (0~16FPU) on glucose yield, total sugar yield, and total

weight loss. After the reaction was completed, the liquid solution was separated from residual solids by filtration. The filtrate was stored at -20°C in a freezer before glucose and total sugar analyses. The weight of the residual solids after enzymatic hydrolysis was determined. Duplicate enzymatic hydrolysis experiments were performed under each tested condition.

2.3 Experimental analysis methods

2.3.1 Pretreatment efficiency

$$\text{Pretreatment efficiency (PE)} = 100\% \times \left(1 - {}^{\text{Mass of residual solid}}/_{\text{Mass of raw materials}}\right) \quad (2)$$

2.3.2 Delignification efficiency

$$\text{Delignification efficiency (DE)} = 100\% \times \left(1 - {}^{\text{Mass of residual lignin}}/_{\text{Mass of original lignin}}\right) \quad (3)$$

2.3.3 Reducing sugar (glucose) yield

$$\text{Reducing sugar (glucose) yield} = \frac{\text{Mass of reducing glucose from hydrolysis}}{\text{Mass ofcellulose + hemicellulose in substrate}} \times 100\% \quad (4)$$

2.3.4 Total sugar yield

$$\text{Total sugar yield} = \frac{\text{Mass of total sugar from hydrolysis}}{\text{Mass of cellulose + hemicellulose in substrate}} \times 100\% \quad (5)$$

2.3.5. Determination of glucose and total sugar concentration

Glucose concentration: The glucose concentration after enzymatic hydrolysis was determined using the dinitrosalicylic (DNS) acid method (Bailey, 1988). Glucose solution was used as standard. A standard glucose curve was prepared with each batch of measurements.

Total sugar concentration: The total sugar concentration after enzymatic hydrolysis was determined using the Anthrone reagent method (Dubois et al., 1956). Glucose was used as the standard. A fresh standard curve of glucose was prepared with each batch of measurements.

2.3.6. UV spectroscopy

Ultraviolet-visible spectroscopy or ultraviolet-visible spectrophotometer (UV-Vis or UV/Vis) refers to absorption spectroscopy in the UV-visible spectral region. It uses light in the visible and adjacent (near-UV and near-infrared (NIR)) ranges. The absorption in the visible range directly affects the perceived color of the chemicals involved. The UV measurements was employed to determine the characteristic absorption of the hydrolyzate solution of peat samples using a CARY-5E-UV-VIS-NIR spectrophotometer. The UV wavelength range was setting up from 240 to 380 nm. H_2O_2 was used as the the reference solution for the measurement. The solution from pretreatment of peat process was diluted properly before analysis.

2.3.7. FTIR spectroscopy

Fourier transform infrared spectroscopy (FTIR) is a measurement technique whereby spectra are collected based on measurements of the coherence of a radiative source, using time-domain or space-domain measurements of the electromagnetic radiation or other type of radiation. The characteristic peaks occurring on the spectra at different absorption intensities and wavenumbers represent characteristic function groups in the compound. The FTIR spectra of peat samples before and after H_2O_2 treatment were characterized using a BRUKER TENSOR37 equipment. The wavenumber used was in the range of 800 to 1800 cm^{-1} in order to investigate the characterization of the peat samples .

2.3.8. X-ray Diffraction

X-ray Diffraction is a method of determining the arrangement of component structure within a crystal, in which a beam of X-rays strikes a crystal and diffracts into many specific directions. From the angles and intensities of these diffracted beams, a spectrum can be given based on the crystallinity structure of the compound. In other words, XRD only reflects information of crystallinity structure. Crystallinity of the pine sawdust before and after pretreatment was determined by X-ray diffraction (XRD) using a diffractometer (Rigooku DMAX-RB) operated at 45 kV and 50 mA. The samples were scanned at 11°/min from $2\theta=10°$ to 30^{o}.

2.3.9. Scanning Electron Microscopy

The scanning electron microscope (SEM) is a type of electron microscope that images the sample surface by scanning it with a high-energy beam of electrons in a raster scan pattern. It was used to characterize the structure change in pine sawdust before and after pretreatment. The pine samples were milled to 20 mesh in particle size, mounted with gold before scanning. The images were taken by a JEOL5900LV SEM, Oxford INCA microanalysis full system with 4 "x5" analytical stage.

Chapter Three

Results and Discussion

This chapter includes three parts: 1.) delignification of peat; 2.) pretreatment of pine sawdust, and 3.) enzymatic hydrolysis of raw and pretreated pine sawdust. Details of each part are provided below.

3.1 Pre-treatment of Peat Using H_2O_2

3.1.1 Effect of reaction time on peat delignification

Figure 1 shows the pretreatment efficiency as a function of the different reaction times, evaluated by the percentage of weight loss of soluble biomass during the treatment. The results show that the PE increased with an increase in reaction time. The highest PE was about 44% at a reaction time of 18 h. The results also indicate that the PE was remarkably increased at the intital stage but less increase after 12h. Based on observations, the reaction was vigorous in the first couples of hours. The H_2O_2 was decomposed and realeased plenty of oxygen. The whole system was filled with oxygen bubbles and the subatrate was surrounded by them in the solution. After about 10h, less bubbles were present in the solution and the whole system intended to be stable and peaceful up to the required reaction time. H_2O_2 can easily decompose in alkaline solution and produce hydro-peroxide anion HOO- (Sun et al., 2002). This anion is a strong nucleophile that preferentially attacks ethylenic and carbonyl groups present in lignin. Then, the lignins can be disolved in alkaline solution. At the same time, H_2O_2 also can generate active radicals such as hydroxyl and superoxide radicals, which can cleave the

ether linkage between lignin and hemicellulose to cell wall (Sun et al., 2002; Sun and Cheng, 2002) in the delignification process. At the begining, the delignification was intense because both the lignin and H_2O_2 concentrations were relatively high. The oxygen released was also dramatic at this time because H_2O_2 itself is not stable in alkaline condition and can decompose to release oxygen. In some literatures, it is suggested that this could lead to another new method of pretreatment of biomass as wet oxidation method (Palonen et al., 2004; Martin et al, 2008; Klinke et al., 2002). It forms acids in hydrolysis process and oxidative reactions. As the reaction time went by, the lignin and H_2O_2 concentrations were lower than before and the reaction rate decreased.

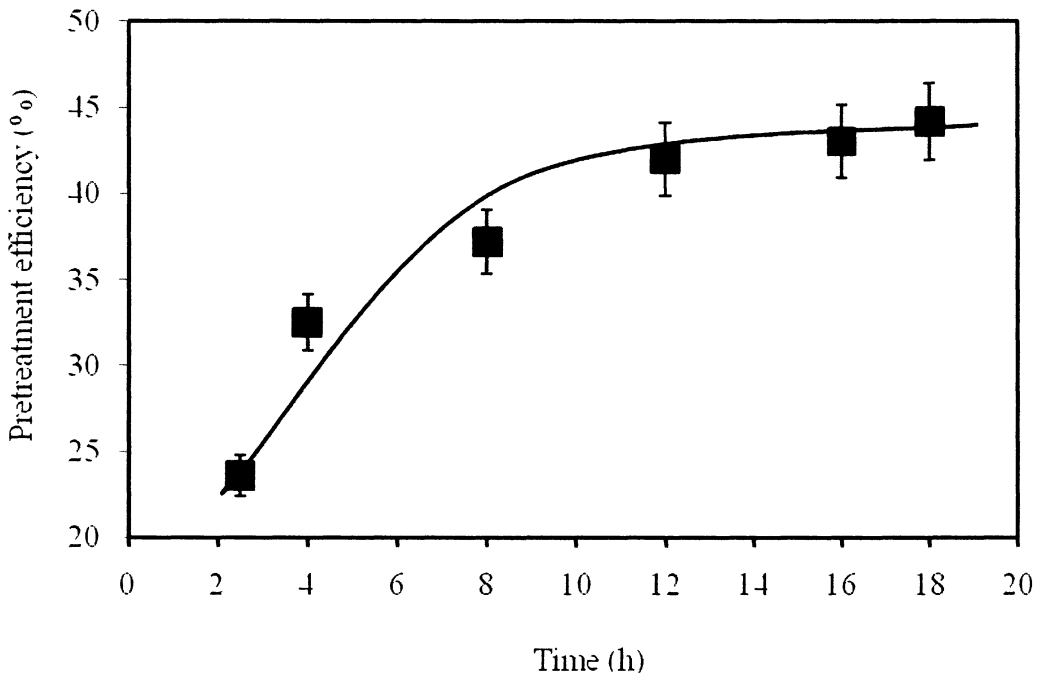


Figure 1. Effects of reaction time on pretreatment efficiency using 1.5 wt% H_2O_2 at pH 11.5 and 45°C

3.1.2 Effects of H_2O_2 concentration and reaction temperature on peat delignification

Figure 2 shows the effect of reaction temperature on the PE of soluble biomass in peat with various H_2O_2 concentrations. The maximum PE was about 43% and 47% at 35 and 45°C, respectively. The H_2O_2 concentration had significant effect on the PE. The PE increased significantly at the low range (≤0.5%) of H_2O_2 and less increase was observed at the higher H_2O_2 concentration range (1.0-2.1%) at 35 and 45°C. A higher H_2O_2 concentration provides more opportunities to generate active radicals and hydroperoxide anions, which accelerate the delignification process. On the other hand, these radicals and anions will interact with each other more frequently as the H_2O_2 concentration increases to produce more oxygen. This was observed during experiments. An increase in reaction temperature from 35 to 45°C resulted in a slight increment in the PE under various H_2O_2 concentrations. In general, the data curve showed that at 45°C, the reaction pattern was similar to the one at 35°C. The results showed that the PE was higher at 45°C, as compared to that at 35°C. The higher PE at 45°C could be due to the higher reaction rate at a higher temperature. However, a further increase in the reaction temperature to 50°C led to a decrease in the PE. From Figure 2, it is clear that a low PE of 14% was achieved at various H_2O_2 concentrations except for the 2.1% H_2O_2 concentration, at which a 30% of PE was obtained at 50°C. The lower PE at 50°C could be due to an increased decomposition rate of H_2O_2 at high temperatures. The stability of H_2O_2 decreases with an increase in temperature. If the temperature is higher than 90°C, more than 90% H_2O_2 will decompose (School of Aerospace, Tsinghua Space Center, Space Propulsion, http://www.tsinghua.edu.cn/docsn/lxx/mainpage/a/Web/index_files/page0007.htm;Schu

mb et al., 1955). Even at 60 °C, more than 50% of H_2O_2 will decompose. Therefore, 45°C seems to be the optimal reaction temperature that gives the maximum PE at various H_2O_2 concentrations.

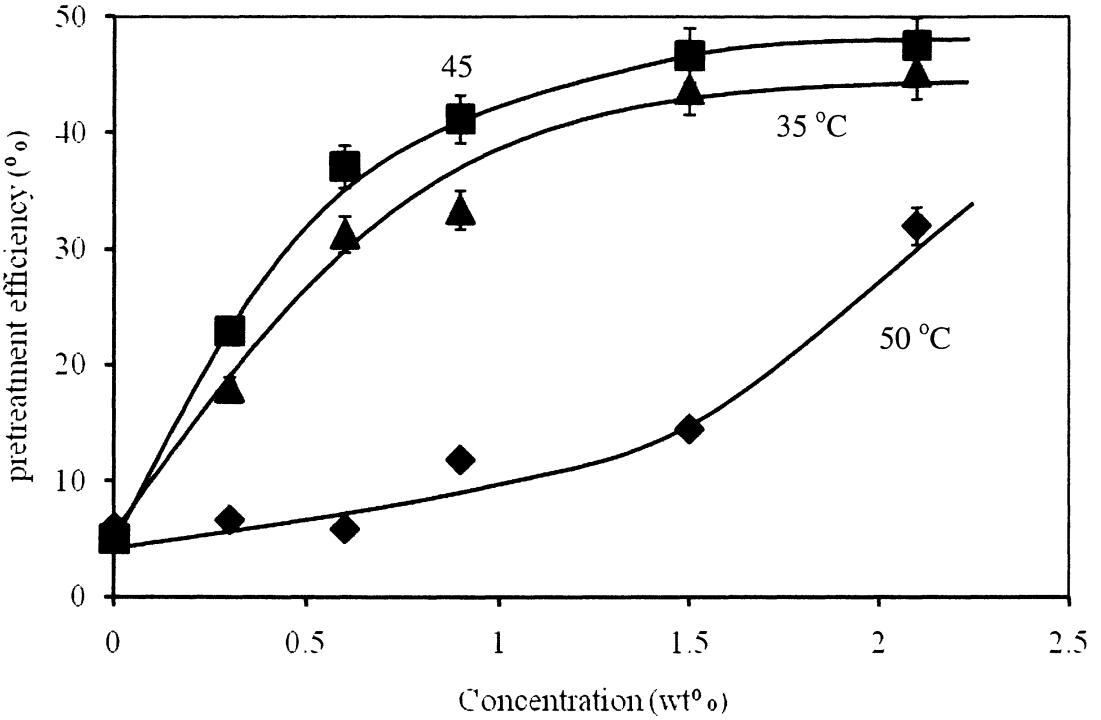


Figure 2. Effects of H_2O_2 concentration and reaction temperature on the PE at pH 11.5 for a reaction time of 12 h

3.1.3 UV spectroscopy

The filtrate collected from filtration of H_2O_2 treated peat solution was further studied by UV spectroscopy at a wide wavelength of 240-380 nm to identify the functional groups of compounds. The blank H_2O_2 solution was used as a reference. As shown in Figure 3, the four filtrates collected at different H_2O_2 concentrations show similar trend in the UV absorption spectra. All the curves exhibited the maximum absorption of typical lignin at about 278-280 nm. This suggests that pretreatment of peat

with H_2O_2 is effective for delignification. The peak originates from nonconjugated phenolic groups in lignin, which is consistent with the results reported by other researchers (Scalbert et al., 1986; Sun and Tomkinson, 2002). In addition, the absorption intensity increased as the H_2O_2 concentration increased from 0.6% to 2.1%. This is because the higher H_2O_2 concentration resulted in higher PE and delignification efficiency (DE). Correspondingly, the higher DE at a higher H_2O_2 concentration resulted in a higher concentration of soluble compounds in the filtrate. At higher H_2O_2 concentrations, more active radicals and hydroperoxide anions were generated and participated in the delignification process, and consequently, more fragments were cleaved out from lignin and dissolved in the solution, and the higher intensity represented in UV spectrum.

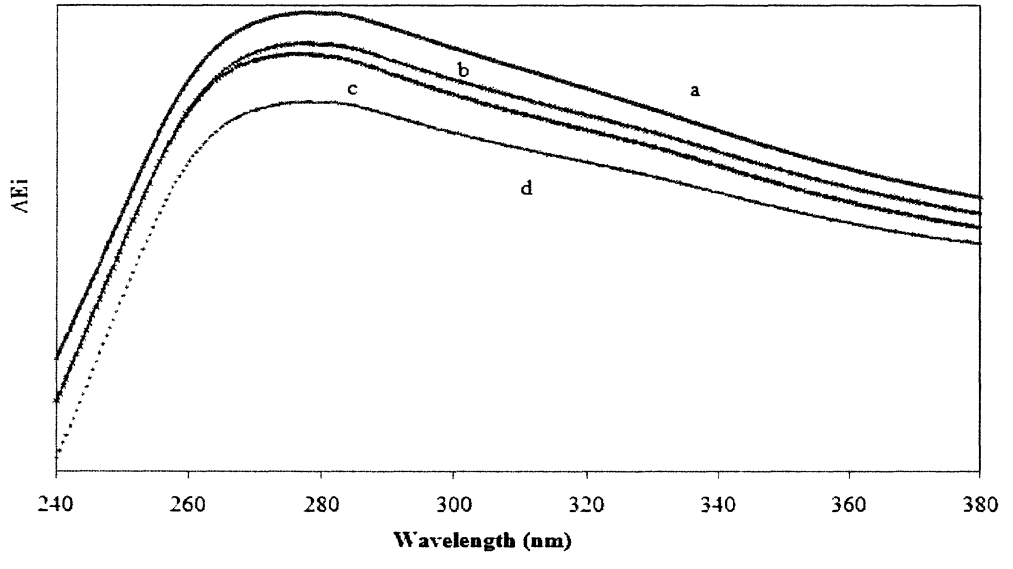


Figure 3. UV spectra of filtrates under different H_2O_2 concentrations of H_2O_2: a. 2.1% H_2O_2; b. 1.5% H_2O_2; c. 0.9% H_2O_2 and d.0.6% H_2O_2

3.1.4 FTIR spectroscopy

Fourier transform infrared spectroscopy (FTIR) was used to characterize the function groups of different solids samples: raw peat, residual peat after pretreatment, and solids precipitated from the filtrate with the addition of ethanol solution. As shown in Figure 4, the three FTIR spectra were similar but with different intensities. Sample c (raw peat) had the lowest intensity along the whole spectrum because the function groups in raw peat have very weak absorption potentials. All the compounds in the raw peat maintain their structures and characteristics well. They are relatively stable and difficult to absorb extra energy. It is clear from samples a (solids residue after pretreatment) and b (precipitated solids) that the original structure of raw peat was disordered after the pretreatment. And all related function groups and the one generated during the pretreatment are all much more exposure and easy to detection. The FTIR curves a and b showed similar pattern. Aromatic skeleton vibrations are assigned at 1598, 1511, and 1425 cm^{-1}. The bands at 1334 and 1270 cm^{-1} have been assigned to ring breathing with C-O stretching. The 1334 cm^{-1} band has been associated with syringyl units, and 1270 cm^{-1} band with guaiacyl units. The bands at 1129 and 1033 cm^{-1} indicate the aromatic C-H in-plain deformation for syringyl type and guaiacyl type, respectively. The information from the function groups on the spectra suggests that all three samples contain lignin with different content. The raw peat sample seems to have weak absorption signals on these functions because they are stick into the long polymer chain of lignin molecular or other cell walls or hemicelluloses bonding. Both samples a and b have relatively stronger and identified absorption peaks. The pretreatment with H_2O_2 degraded and cleaved the linkage between the components inside lignin and related to cell wall and hemicelluloses,

and dissolved partial lignin (sample b). However, there are still some lignins remaining in the solids residue (sample a). Therefore, the pretreatment did not cause any dramatic change on the structure of the compositions of lignin in the peat sample.

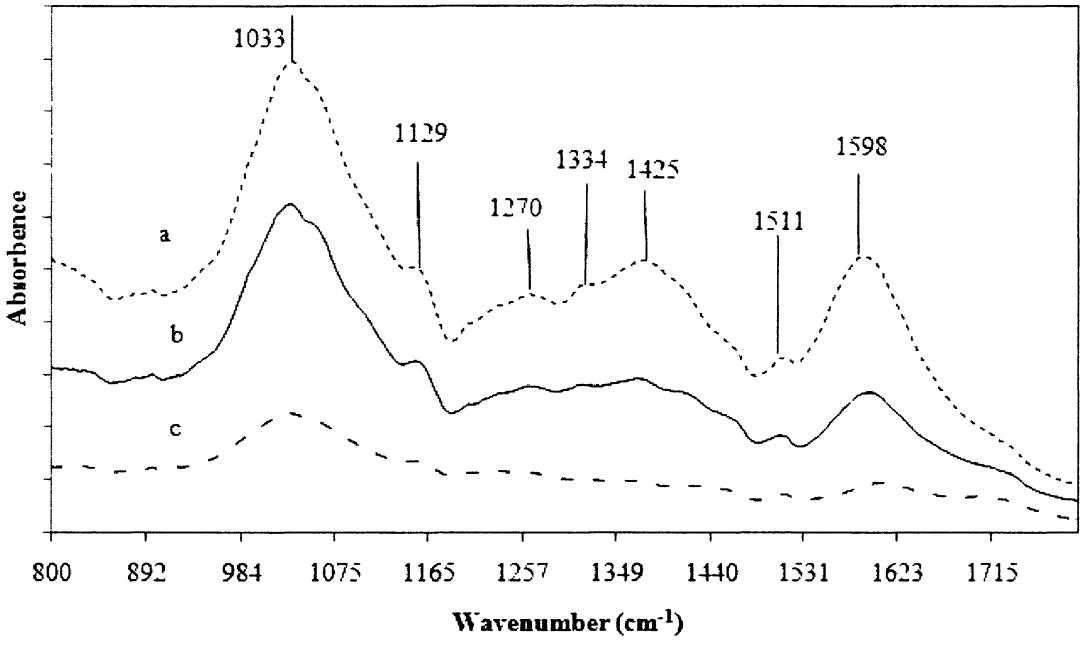


Figure 4. FTIR spectra of different peat sample: a. solids residue after filtration of pretreatment using 1.5 wt% H_2O_2 at pH 11.5, 45 °C, 12h; b. solids collected from the ethanol precipitation stage of supernatant of peat; c. raw peat sample.

3.2 Pre-treatment of Pine Sawdust

In this part, the results of white pine sawdust pretreatment with different methods: organosolv extraction, followed by ultrasonic and/or NaOH treatment, are reported. The organosolv extraction pretreatment was aiming to remove or cleave the linkages between lignin, hemicellulose and cellulose using organic solvent, loose the cellulose fibrils and decrease the degree of polymerization. Ultrasonic treatment was focusing on the crack of cell wall, dislocation of the secondary wall of the middle layer of cell wall, exposure and

fibrillation of the middle layer, mainly cellulose fibrils to enzymes under room temperature. NaOH treatment was further used to remove hemicellulose and residual lignin. The NaOH treatment is believed to be saponification of intermolecular ester bonds crosslinking hemicelluloses and other components such as lignin, also leading to increase in internal surface area and decrease in degree of polymerization.

The pretreated pine sawdust samples are classified as four groups: Group1- organosolv extraction; Group 2- organosolv extraction + ultrasonic treatment; Group 3- organosolv extraction + NaOH treatment; and Group 4- organosolv extraction + ultrasonic + NaOH treatment. Pretreatment efficiency (PE) and delignification efficiency (DE) were determined after the pretreatment. The raw and pretreated pine sawdust samples were further characterized by using FTIR, XRD and SEM to identify changes in characteristic function groups, crystallinity structure and cell wall structure of pine sawdust before and after the treatments.

3.2.1 Pretreatment efficiency and delignification efficiency

The results of pretreatment efficiency (PE) and delignification efficiency (DE), after each pretreatment, are summarized in Table 4.

Table 4. Pretreatment efficiency and delignification efficiency of pine sawdust

	Group 1	Group 2	Group 3	Group4
Step 1	ethanol:water 1:1,190°C,700 psi, 4 hours	ethanol:water 1:1,190°C,700 psi, 4 hours	ethanol:water 1:1,190°C,700 psi,4 hours	ethanol:water 1:1,190°C,700 psi, 4 hours
Step 2	N/A	ultrasound, 100W,40KHz. 25°C,3 hours	NaOH,1mol/L , 70 °C,100 rpm,3 hours	ultrasound, 100 W,40KHz.25°C, 3hours
Step 3	N/A	N/A	N/A	NaOH,1mol/L,70 °C,100 rpm,3hours
PE	51.40%±2%	53.3%±1%	57.70%±1.1%	61.10%±1%
DE	76.50%±3%	77.20%±2.6%	81.5%±3%	86.4%±3%

- N/A Not applied
- Steps indicated different pretreatment methods taken

For organosolv extraction (Group 1), the PE and DE were 51 40% and 76.5%, respectively The results indicated that organosolv extraction had a significant effect on delignification The results are comparable or slightly higher than that of previous studies (Pan et al , 2006; Sannigrahi, et al., 2010) The slightly higher PE and higher DE were caused mainly by the fact that, under high temperature and pressure, organic reagents can act as scavenger to cleave the bonding of different function groups in lignin and between lignin and carbohydrates to make huge molecule of lignin form many small fragments This process also leads to the formation of acetic acid or other acids or byproducts from the acetyl groups in hemicelluloses under severe conditions. These acids can act as catalysts accelerating the rapture of lignin-carbohydrate complex (Duff and Murray,

1996). At the same time, water acts like a medium that accommodates these products to be dissolved in. At the end of the reaction, it produces two phases. Cellulose can be recovered in the solid phase, while part of hemicelluloses and lignin fractions can be obtained after extraction or distillation of the liquid phase. However, there are still some hemicellulose and lignin remaining in the cellulose rich solid. Pasquini et al. (2005) found that both temperature and pressure can affect PE and DE. An increase in temperature and pressure resulted in an increase in the PE and DE. The DE in the order of 93.1% for P. tarda wood chips and 88.4% for sugar cane bagasse were achieved when 16.0MPa and 190 °C were employed in the treatment reaction. The lower DE (76%) from the present study could be due to the low pressure (700 psi or4.8MPa) used, as compared to that of Pasquini et al. (2005).

For Group 2 (organosolv extraction followed by ultrasonic treatment), the PE (53.30%) and PE (77.2%) were further improved. The results suggest that ultrasonic treatment can affect PE and DE. This could be caused by the decreased particle size of pine sawdust after ultrasonic treatment. A decrease in particle size would lead to an increase in specific surface area and thus release hemicellulose and lignin. From the literature, it was found that ultrasonic treatment was widely used to enhance the extraction of hemicellulose in alkaline solutions by introducing violent cavitation. Moreover, mechanical impacts produced by the collapse of cavitation bubbles can disrupt the hydrophobic protein matrix surrounding the starch granules and the amylase-lipid complex, like shock wave propagation and microjet formation in the vicinity of a liquid-solid interface (Satish D. et al., 2009). The ultrasonic treated pine sawdust possessed less firmness and accounted 3~5% weight loss. The slightly increase in PE (from 51.4% to

53.30%) was due to the fact that distilled water rather than alkaline solution was used as liquid phase. Thus less extractives from mainly hemicellulose and partial cellulose could be dissolved in water. The comparable DE (from 76.5% to 77.2%) suggests that ultrasonic treatment was not very effective in removing lignin. This is true as ultrasound is mainly used as a pretreatment method for processing lignocellulosic materials to enhance the extraction of hemicellulose in alkaline solutions.

For Group 3 (organosolv extraction followed by NaOH treatment), the PE (57.70%) and DE (81.5%) were significantly improved, implying the high efficiency of NaOH treatment in extraction of hemicellulose and lignin. When the the organosolv extracted solid was added into NaOH solution, its color turned to dark brown The mixture was much finer and more viscous than it was ever before treatment. This could be explained by the fact that NaOH treatment of lignocellulosic materials can cause swelling, which leads to an increase in internal surface area, a decrease in the degree of polymerization, a degree in crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure (Kumar et al., 2009; Taherzaheh and Karimi, 2008). Thus, NaOH treatment resulted in an increase in PE and DE. This is consistent with the findings of the literature in that NaOH treatment mainly leads to the dissolution of hemicellulose and lignin, partial of cellulose too (Carvalheiro et al., 2008).

It is not surprising to see that organosolv extraction followed by ultrasonic and NaOH treatment in sequence (Group 4) led to the highest PE (61.1%) and highest DE (86.4%), as shown in Table 2. This is due to the fact that the combined pretreatment (Group 4) utilized all the advantages of the individual (organosolv extraction, ultrasonic treatment, and NaOH treatment) treatment.

3.2.2 Microstructure Change of Cell Wall via SEM

Effect of pretreatment (organosolv extraction, ultrasonic treatment, and NaOH treatment) on the microstructure of pine sawdust was studied using scanning electron microscopy (SEM). A comparison on the changes in microstructure of pine sawdust before and after pretreatment is shown in Figure 5 A, B, C and D.

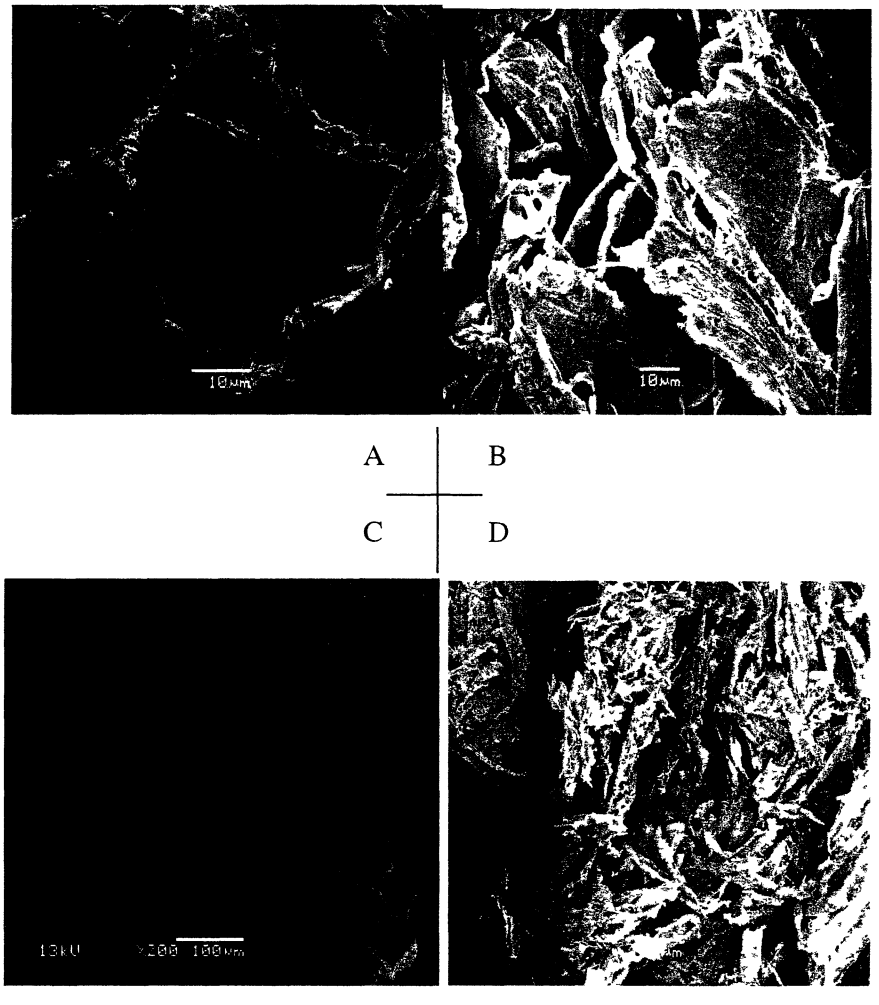


Figure 5. Microstructure changing of pine sawdust via SEM: A. single cell before treatment; B. single cell after the organosolv extraction treatment; C. pine wood structure before treatment; D. pine wood structure after the organosolv extraction treatment

The SEM pictures clearly show that the microstructure of pine sawdust changed after different pretreatments. In the raw pine sawdust, the cell wall structure was well maintained. For each cell, a rectangular structure was firmly maintained. This could be due to the fact that the cell wall is quite resistant to the force or other kind of materials from outside. And this pattern was extended to the whole system cell by cell to form the unique protective structure of lignocellulosic materials. It is believed that cell wall maintains its structure with the assistance of lignin and hemicelluloses. The pretreatments led to a significant swelling of the raw pine sawdust and caused disorder of the cell structure. After pretreatment, the cell wall seems to be devastated. The original cell wall was twisted and disordered. Significant morphological changes occurred. The raw pine sawdust sample exhibited rigid and highly ordered fibrils. The fibers of pine sawdust samples after pretreatment appeared to be distorted and twisted. The microfibrils were also isolated from the initial connected structure and fully exposed to outside, thus increasing the external surface area and the porosity. Furthermore, it was found that pine sample after pretreatment was much softer than the untreated one.

3.2.3 X-ray diffraction spectrum

Figure 6 shows the XRD spectra of pine sawdust before and after each pretreatment. It is clear that all the spectra present a similar trend. There are three peaks occurred at 14.6, 16.5 and 22.4 degree with different absorb intensities, respectively. These peaks correspond to cellulose, as there are normally three peaks at 2-theta of about 14.6, 16.5 and 22.4 deg., corresponding to the diffraction of cellulose's crystalline planes of (1$\bar{1}$0), (110) and (200) (Xu and Etcheverry, 2008). While hemicellulose and lignin have

amorphous structure so there is no any reflection on their structural features in the XRD spectra. However, for lignocellulosic biomass (with impure cellulose), the first two peaks normally overlap and exhibit a broad one at around 15-16 degree as shown in Figure 5. These curves and peaks indicate that the samples- before and after the pretreatment- contain cellulose, and the cellulose did not have any dramatic structure changing or disorder because the shapes of the peaks were similar to each other. Furthermore, the position of the peaks occurred at the same range of the angles.

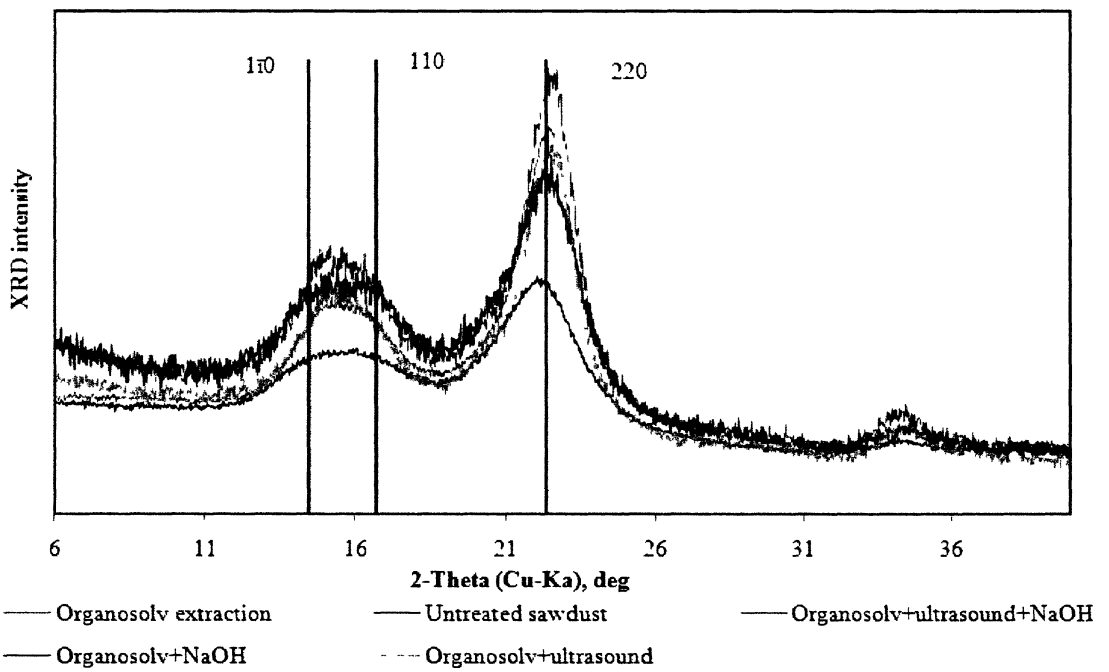


Figure 6. XRD spectra of pine sawdust samples after various treatments

The intensity of these curves is different. The ones treated with different pretreatment methods showed much more intensive signal at the same angle range than that of the raw pine sawdust. This could be due to the change in the structure and cellulose content in the raw and pretreated samples. In the raw pine sawdust, the cellulose was originally in the

inner side of the cell wall, covered by lignin and hemicelluloses. The outside hemicellulose and lignin layers make the cellulose less exposure to X-ray. In addition, cellulose content was relatively low in the raw pine sawdust. Consequently, the cellulose peaks were lower with weaker signals in the raw pine saw dust. After the pretreatments, the cell wall was twisted and lignin and hemicelluloses were partially removed as observed using SEM. It is easy for X-ray to get through the cell wall and reach the cellulose. Also at this time, the relative content of cellulose obviously increased compared to the one before pretreatment. As a result, the cellulose peaks were shaper and higher with more intensive signals in the pretreated pine sawdust. The results are consistent with the findings of previous studies (Xu and Etcheverry, 2008; Kim et al., 2003; Sreenivasan et al., 1996). The intensity of the cellulose peaks in pre-treated saw dust samples increased as an increase in the PE and DE. This is probably not surprising, as the cellulose content in the pretreated sawdust samples increased with an increase in the PE and DE.

3.2.4 FTIR spectroscopy

The functional groups of the raw, pretreated sawdust samples, pure cellulose and pure lignin were characterized using the FTIR technique. The results are shown in Figure 7. In Figure 7, 5 types of pine sawdust samples, one pure cellulose and one pure lignin samples were characterized from wavenumber 700 to 1750 cm^{-1} and showed many different absorption peaks. The types of vibrations at each absorption peak are summarized in Table 2. The pretreated sawdust samples had extremely similar peak patterns of the pure cellulose in the whole wavenumber range. This is because the cellulose content was higher in pretreated sawdust samples, as compared to the raw pine

sawdust. The absorption band 1217-1350 cm^{-1} is due to the stretching vibrations of C-O bands in cellulose. The 1036-1050 cm^{-1} wide absorption band was related to the C-O bonds and is associated with polysaccharides in cellulose. Second, taking cellulose, solid residue and raw pine sawdust materials into account, raw pine sawdust materials clearly had weaker absorption intensity at absorption bands 900-1200 cm^{-1}. This could be explained by the relative low cellulose content in the raw pine sawdust. In addition, the cellulose in raw pine sawdust was covered by the hemicelluloses and lignin layers, which makes it difficult to detect all the characteristic function groups. The absorption bands at 1275 cm^{-1} and 1516 to 1700 cm^{-1} corresponds to the functional groups of lignin. The absorption band at 1275 cm^{-1} is related to vibrations of guaicyl rings and the absorption bands at 1516 to 1700 cm^{-1} are caused by aromatic ring vibrations. The pure lignin and raw pine sawdust materials possessed stronger signal at these absorption band, due to the higher content of lignin. The pretreated pine sawdust had a weaker signal at these absorption bands, because the majority of lignin was removed by the pretreatment. It is not surprising that pure cellulose does not reveal any signal in this region because it contains no lignin. The results are consistent with the findings of previous study (Kim et al., 2003). At absorption band 1711cm^{-1}, it revealed the information related to carbonyl absorption in hemicelluloses. The raw and pretreated pine sawdust samples all showed signal at 1711cm^{-1}. This could also indicate that pretreatment did remove the majority of hemicelluloses from the raw materials. This conclusion is consistent with the findings of Sreenivasan et al.(1996).

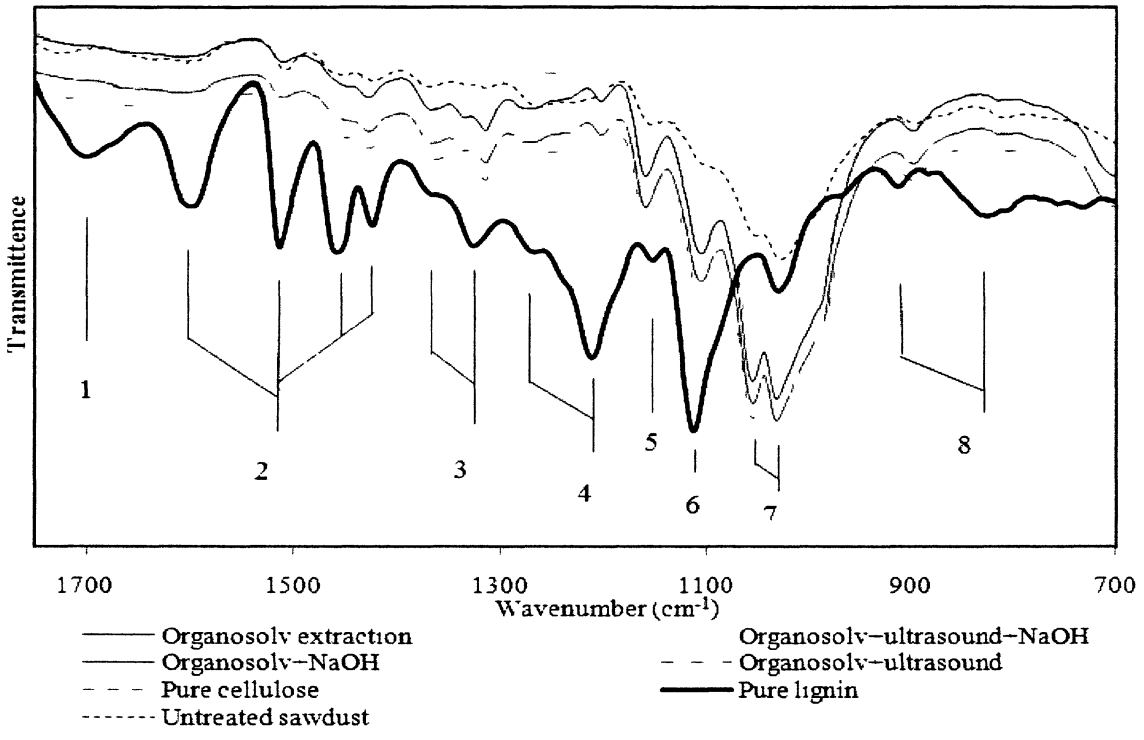


Figure 7. FTIR spectra of pine sawdust samples before and after different treatments

In summary, the FTIR spectra indicated that pretreatment was efficient in removing the majority of lignin, and part of the hemicellulose from raw pine sawdust and thus increased the cellulose content in solids residues.

Table 5 Peak assignment for the FTIR spectra

	Absorption band location (cm^{-1})	Type of vibration
1	1711	Stretching vibrations of C=O bonds at β location and in COOH group
2	1610	Aromatic ring vibrations
	1516	
	1450-1464	
3	1333-1350	Vibrations of syringyl rings and stretching vibrations of C-O bands
4	1217-1275	Vibrations of guaicyl rings and stretching vibrations of C-O bands
5	1150	Deformation vibrations of C-H bonds in guaicyl rings
6	1125	Deformation vibrations of C-H bonds in syryngyl rings
7	1036-1050	Deformation vibrations of C-H bonds in the aromatic rings and deformation vibrations of C-O bonds in primary alcohols
8	925	Deformation vibrations of C-H bonds in associated to aromatic rings
	821	

3.3 Enzymatic Hydrolysis of Pre-treated and Raw Pine Sawdust

In this part, the raw and pretreated pine sawdust samples were hydrolyzed using enzymes to produce sugar for biofermentation. Effects of reaction time and enzyme dose on the glucose yield, total sugar yield and weight loss of the raw and pretreated pine sawdust were systematically studied. The results of enzymatic hydrolysis are presented in the following sequences: 1.) effect of reaction time on the glucose and total sugar yields, and total weight loss; 2.) effect of enzyme dose on the glucose and total sugar yields, and total weight loss.

3.3.1 Effect of reaction time on glucose yield

Figure 8 shows the effect of reaction time on the glucose yield under two different enzyme doses. The general trends observed in Figure 8A (7.67 FPU) are the same as that observed in Figure 8 B (11.76 FPU). In the first 12 hours, the glucose yield increased significantly with time for most of the cases. After that, the glucose yiel flatted or only slightly increased with time (12 to 48 hours). This could be explained by the fact that the enzymatic hydrolysis rate, especially the initial hydrolysis rate, strongly depends on the initial extent of enzyme adsorption and the effectiveness of the adsorbed enzymes to promote the hydrolysis (Fan and Lee, 2004). At the beginning of the hydrolysis, there was ideally a maximum number of the active binding site on the surface of substrate and enzymes could be fully absorbed onto the area. At this time, the hydrolysis rate could be considered as the fastest. After certain period of time, the process of enzymes adsorption and desorption reached at a saturation point. Additionally, the production of cellobiose and glucose, which have been considered as inhibition for enzymatic hydrolysis, might be accumulated and inhibited enzymatic hydrolysis. As shown in Figure 8, there are significant differences in the glucose yield among pine sawdust samples pretreated using different methods. The raw pine sawdust (untreated) only had 5~6% glucose yield while pine sawdust treated with organo+ultrasound+NaOH contributed to nearly 16~18% glucose yield (Figure 8B), which is more than three times of that from the raw pine sawdust sample. The order of glucose yield positively correlated to the order of pretreatment efficiency (PE) and delignification efficiency (DE), as shown in Figure 9. In another word, a higher PE and DE led to a higher glucose yield. The results are consistent with the findings of Palonen et al. (2004). The results suggest that pretreatment did play

an important role in enzymatic hydrolysis. However, the maximum glucose yield was lower than that found by Sannigrahi et al.(2010) and Palonen et al. (2004). This was mainly caused by the different pretreatment methods used. Sannigrahi et al. (2010) obtained a nearly 70% of sugar yield when 65% ethanol/water solution containing 1.1% sulfuric acid was used as pretreatment reagent. This is because organosolv pretreatment with ethanol at higher ethanol content and the addition of acid could lead to a higher pretreatment efficiency not only for lignin but also for hemicellulose. While Palonen et al. (2004) used wet oxidation and steam treatment as pretreatment for softwood, which achieved a higher PE and DE. The higher lignin and hemicellulose removal efficiency led to the higher sugar yield. In our study, hemicelluose removal efficiency was much lower than that found by Sannigrahi et al. This explains the lower glucose yield from our study as compared to that found by Sannigrahi et al. (2010).

From Figure 8, it is clear that the glucose yield increased with an increase in the enzyme dose (Figure 8A vs. Figure 8B) for all the cases studied. This is because an increase in enzyme dose would result in an increased enzymatic hydrolysis rate, as more enzymes could be binded to the active site of substrate for hydrolysis.

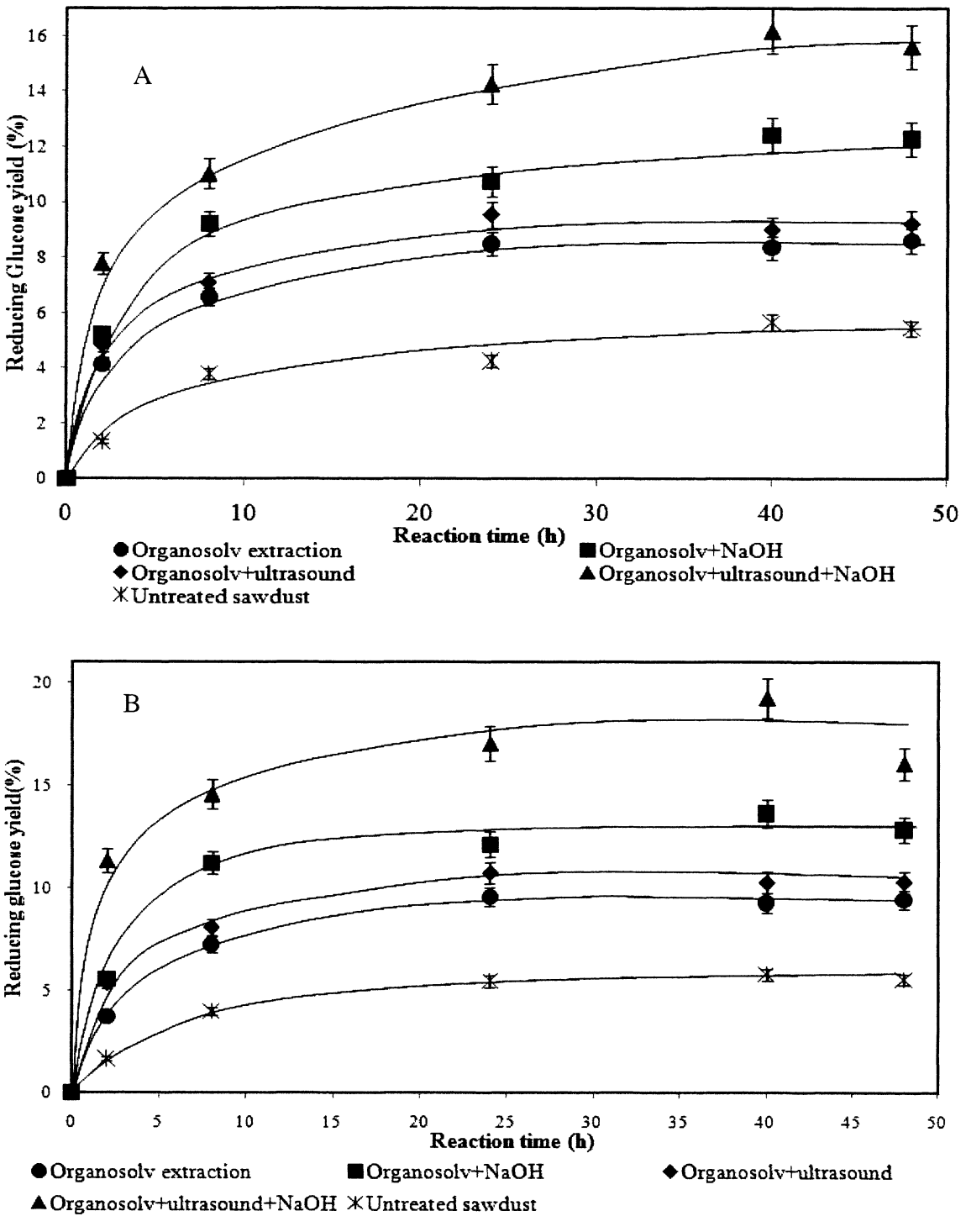


Figure 8. Glucose yield from various pretreated pine sawdust samples at 50 °C in sodium citrate solution, pH 4.8 and 100 rpm shaking speed: A. 7.67FPU; B. 11.76FPU

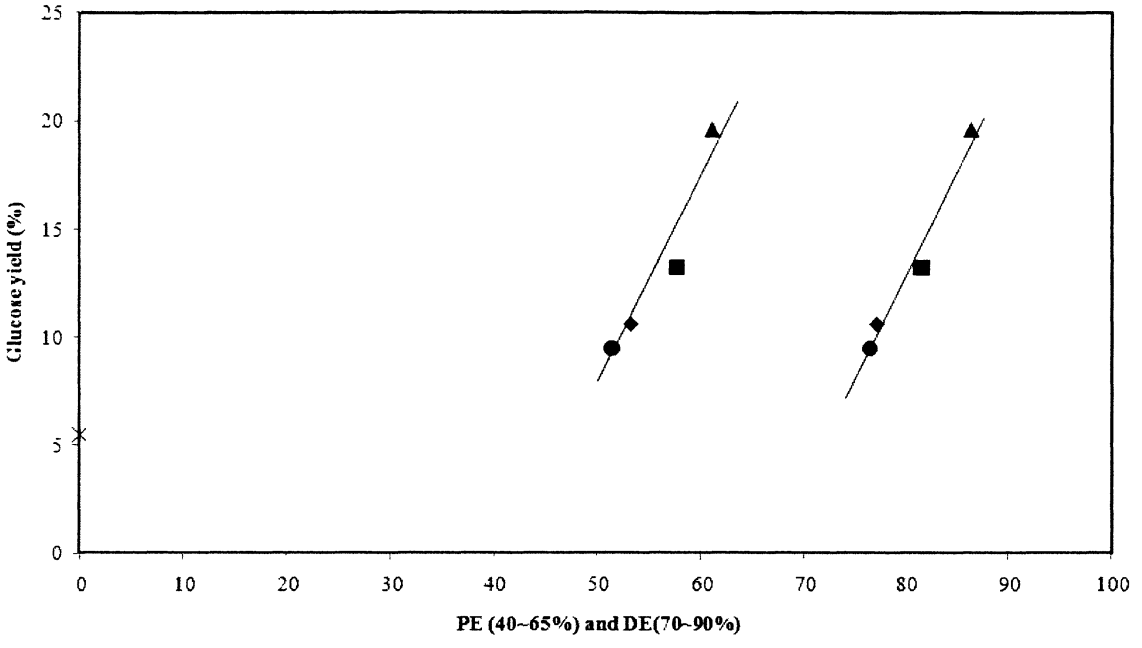


Figure 9. Effect of PE and DE on glucose yield: ● Organosolv extraction;

◆ Organosolv+ultrasound; ■ Organosolv+NaOH;

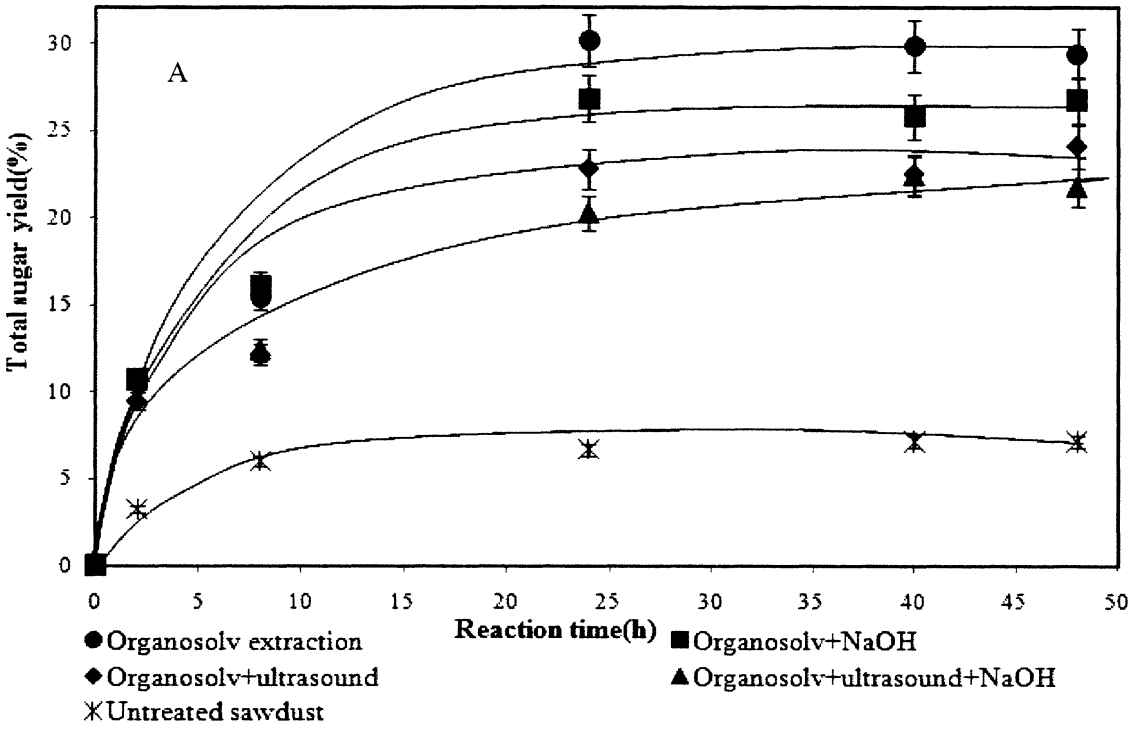
▲ Organosolv+ultrasound+NaOH ✱ Untreated sawdust

3.3.2. Effect of reaction time on total sugar yield

Figures 10 A and 10 B show the total sugar yield as the function of reaction time. Similar to the glucose yield, the total sugar yield increased with an incease in reaction time. In the initial 24 hours, the total sugar yield curves climbed up to about 27% and then reached at the maximum yield about 30% with the sample pretreated with organo+ultrasound+NaOH. In the next 24 hours, there was no significant increment occuring and the total sugar yield maitained at about 30% yield. The total sugar yield of other pretreated pine sawdust samples followed the same trend but with a lower total sugar yield. In contrast to the glucose yield, a higher PE and DE led to a lower total sugar yield (Figure 11). This was probably due to the fact that the higher removal efficiency of hemicellulose at a higher PE resulted in a lower content of hemicellulose in the solids

residues, which is a source for sugars other than glucose. Consequently, less sugars was produced during enzymatic hydrolysis at a higher PE.

The total sugar yield (Figure 10) was higher than the glucose yield (Figure 8). This is because the total sugar contains not only glucose but also other sugars.



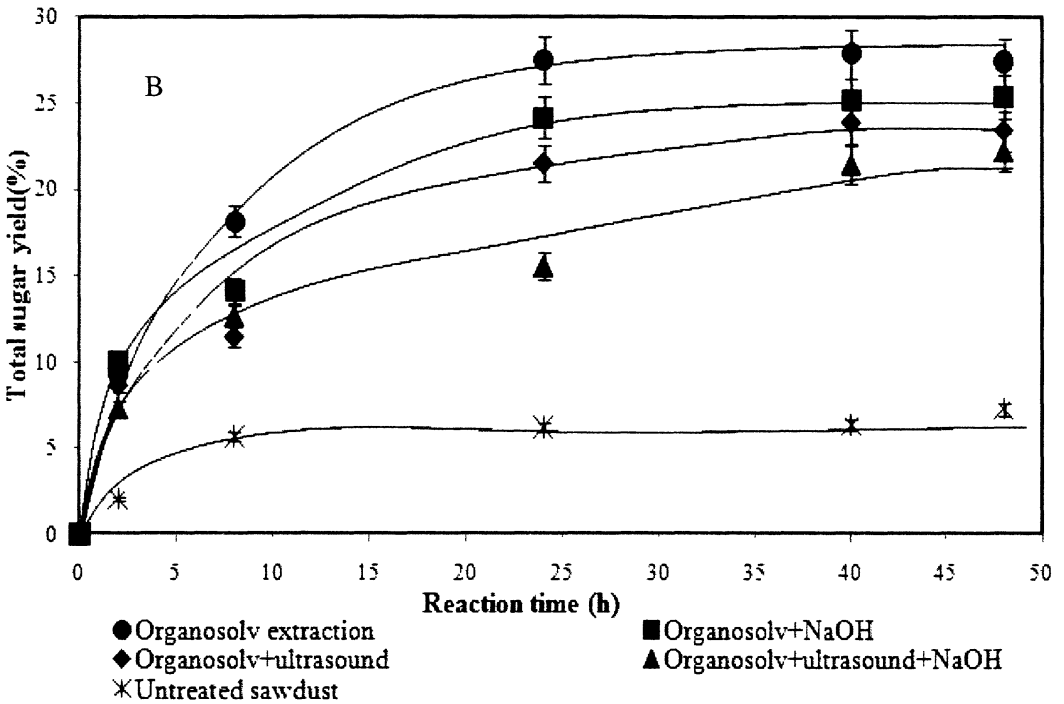


Figure 10. Total sugar yield from various pretreated pine sawdust samples at 50°C in sodium citrate buffer solution, pH 4.8, and 100 rpm shaking speed: A.; 11.76FPU; B. 7.67FPU.

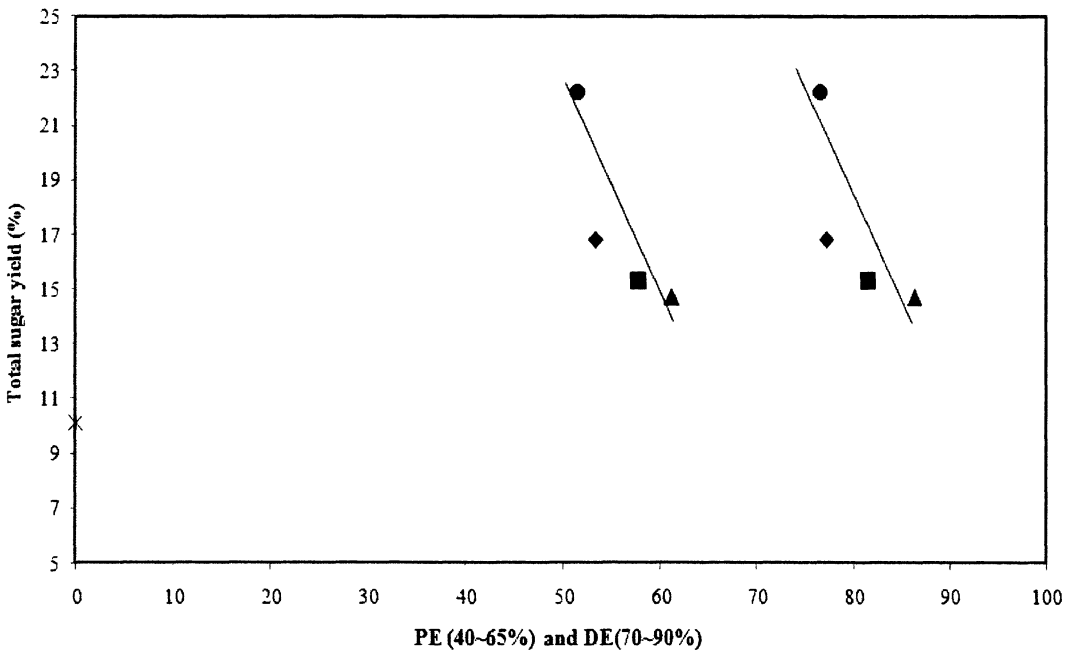


Figure 11. Effect of PE and DE on total sugar yield: ● Organo; ◆ Organo+ultrasound;

■ Organo+NaOH; ▲ Organo+ultrasound+NaOH ǂ Untreated sawdust

3.3.3 Effect of enzyme dose on glucose yield

Figure 12 shows the effect of enzyme dose on glucose yield. It is clear that the glucose yield was enhanced as the enzyme dose was increased. This is consistent with the observation of Figure 8. When the enzyme dose was incraesed to 7.78 or 11.67 FPU, the glucose yields attained the maximum values in most of the cases. After that, a further incraese in the enzyme dose led to a slight decraese in the glucose yield. The results are generally consistent with the findings in Figure 8 in that a higher PE and DE coresponded to a higher glucose yield. The highest glucose yield of 16~18% was obtained with organosolv+ultrasonic+NaOH treatment, while untreated pine only had 5~6% glucose yield at an enzyme loading of 11.67 FPU. Ultrasonic and NaOH treatment also enhanced enzymatic hydrolysis, as hown in Figure 12. The observed trends in Figure 12 could be explained by the following reasons. First, with an incraese in the PE and DE, the pretreated pine sawdust contained a higher content of cellulose and more binding sites and easier access of the binding sites to enzymes. Second, as enzyme loading was increased, more enzymes could be adsorbed, deadsorbed and readsorbed. In other word, more active binding sites were available for enzymatic hydrolysis. This is consistent with the findings of Nidetzky et al. (1994). They discovered that, during cellulose degradation by a *T. reesei* complex, the specific adsorption of each individual enzyme component was gradually increased (1994). Therefore the maximum glucose yield was increased up to about 16~18% with an increase in the enzyme dose to 11.76 FPU. However, when the enzyme dose was further increased, there was no more matched active binding sites available for extra enzymes. Furthermore, enzymes could lead to competitive inhibition to each other at higher concentrations. Consequently, glucose yield did not incraese

further. When glucose was produced in the reaction mixture at the beginning of hydrolysis, the rate and extent of hydrolysis decreased with an increase in glucose concentration. Also, in the presence of cellobiose the initial rate of glucose production was substantially decreased. Holtzapple et al. (1990) reported that all forms of the enzyme species (free, adsorbed and complexesed) in the process of cellulose hydrolysis were subject to inhibition. Consequently, there was an improvement in the glucose yield at a enzyme dose higher than 11.67 FPU.

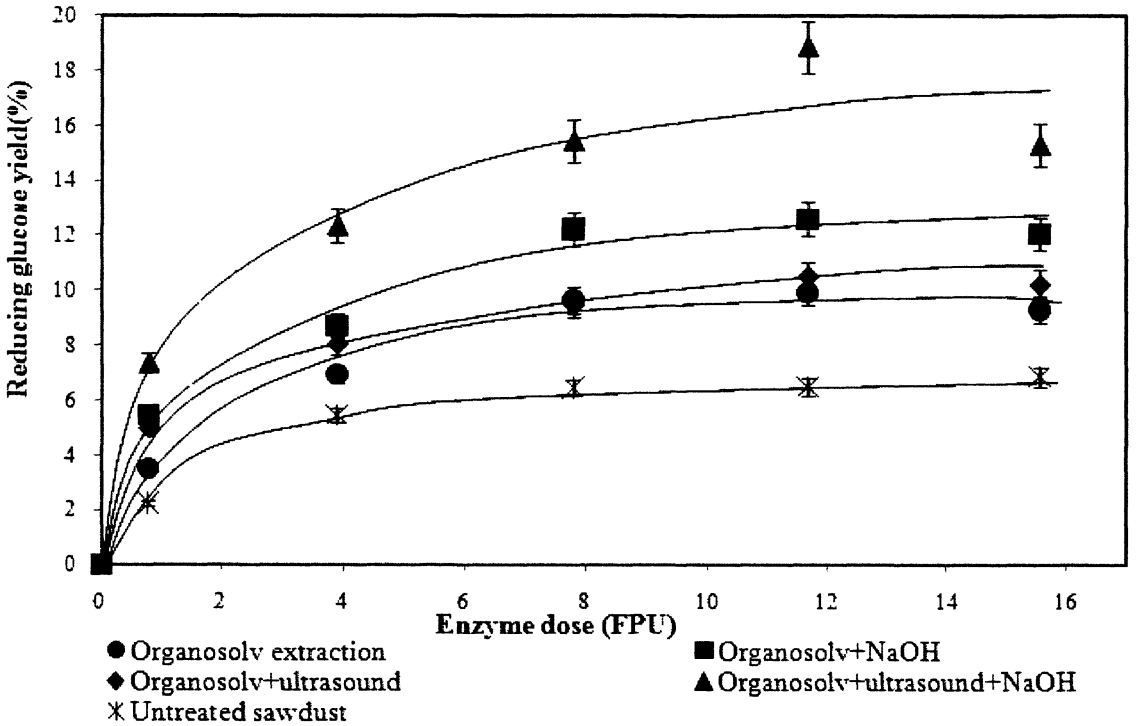


Figure 12. Glucose yield from various pretreated pine sawdust samples during enzymatic hydrolysis at 50 °C in sodium citrate buffer solution, pH 4.8, and 100 rpm shaking speed for 48 hours with various FPU doses of Cellulases and Novozymes

3.3.4. Effect of enzyme dose on total sugar yield

Figure 13 shows the total sugar yield as the function of enzyme doses. Basically, it has the similar trend as observed in Fugure 12. The enzymatic hydrolysis rate was increased with an increase in enzyme concentartion which led to an incraese in the total sugar yield. The maximum total sugar yield of 31% was achieved at an enzyme dose of 8 FPU. After that, the total sugar yield slightly decreased to a level 30% with a further increase in the enzyme dose. Untreated pine sawdust only had a 7~8% total sugar yield. There was almost 2 to 3 times increase in total sugar yield after pretreatment. Similar to the results shown in Figure 10, a lower PE and DE correlated to a higher total sugar yield.

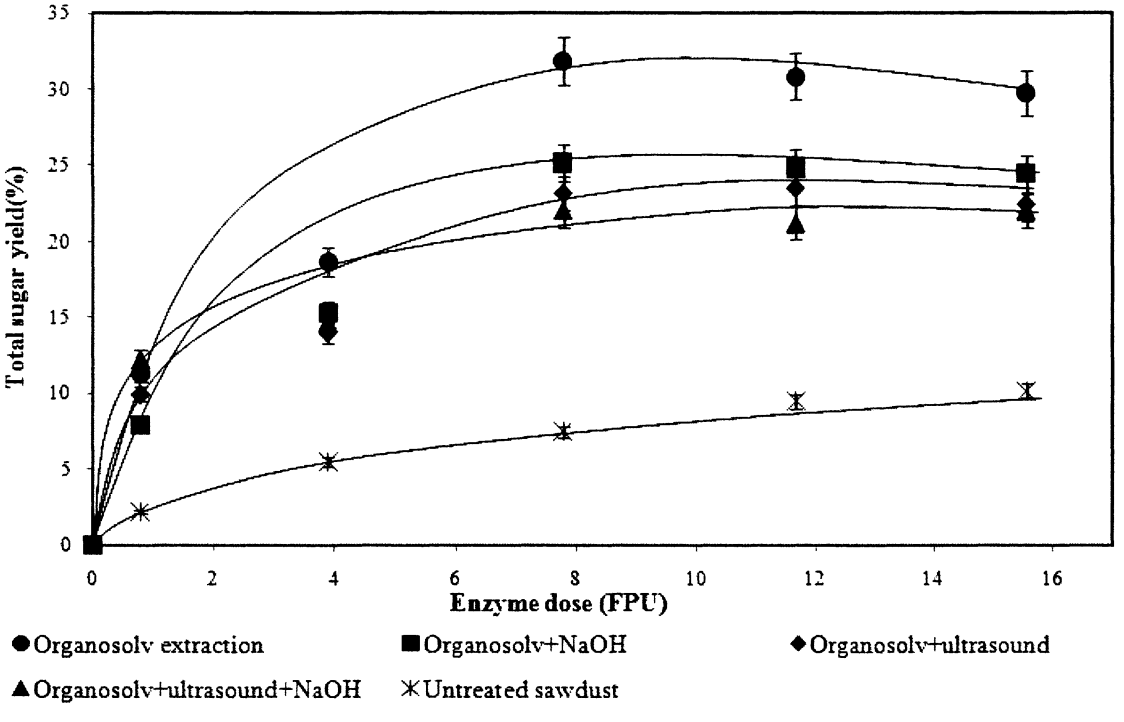


Figure 13. Total sugar yield from various pretreated pine sawdust samples during hydrolysis at 50 °C in sodium citrate buffer solution, pH 4.8, and 100 rpm shaking speed for 48 hours with various FPU doses of Cellulases and Novozymes

3.3.5. Effect of reaction time and enzyme dose on total weight loss

Figure 14 A and B and Figure 15 show the total weight loss of the raw and pretreated pine sawdust samples with experimental time and enzyme dose. It is clear that the total weight loss increased with an increase in reaction time (Figure 14) and enzyme dose (Figure 15). This suggests that more cellulose and hemicellulose were dissolved or hydrolyzed if the reaction time was longer or the enzyme dose was incraesed. As compared to the raw pine sample (5-20%), the pretreated pine samples had significantly higher total weight loss (45-60%). This is consistent with the observed glucose yield and total sugar yield, as the cellulose and hemicellulose in pretreated pine samples are easy access to enzyme for hydrolysis. However, the difference in total weight loss among the pretreated pine samples was not clear. Considering the experimental errors associated with the measurements, there were no significant difference among the oragnosolv extracted, organosolv + ultrosonic treated, and organosolv + NaOH treated pine samples in total weight loss. A general trend observed was that the organosolv + ultrsonic + NaOH treated pine sample had the highest total weight loss. This is consistent with the observed highest glucose yield and total sugar yield for that sample. A total weight loss of nearly 45-66% for the pretreated pine samples suggests that at least part of the hemicellulose were dissolved or hydrolyzed, as the cellulose content in the pretreated saw dust was only about 50%. It is well known that some side reactions would occur during enzymatic hydrolysis of carbohydrates under relatively low pH values, similar with the hydrolysis of hemicellulose (Stenberg et al., 1997; Ohgren et al., 2005; Soderstrom et al., 2002). The side reactions might trigger the hydrolysis of hemicellulose and cellulose at

pH 4.8 and form acids or other byproducts like HMF (hydroxymethylfurfural) dissolving in the solution.

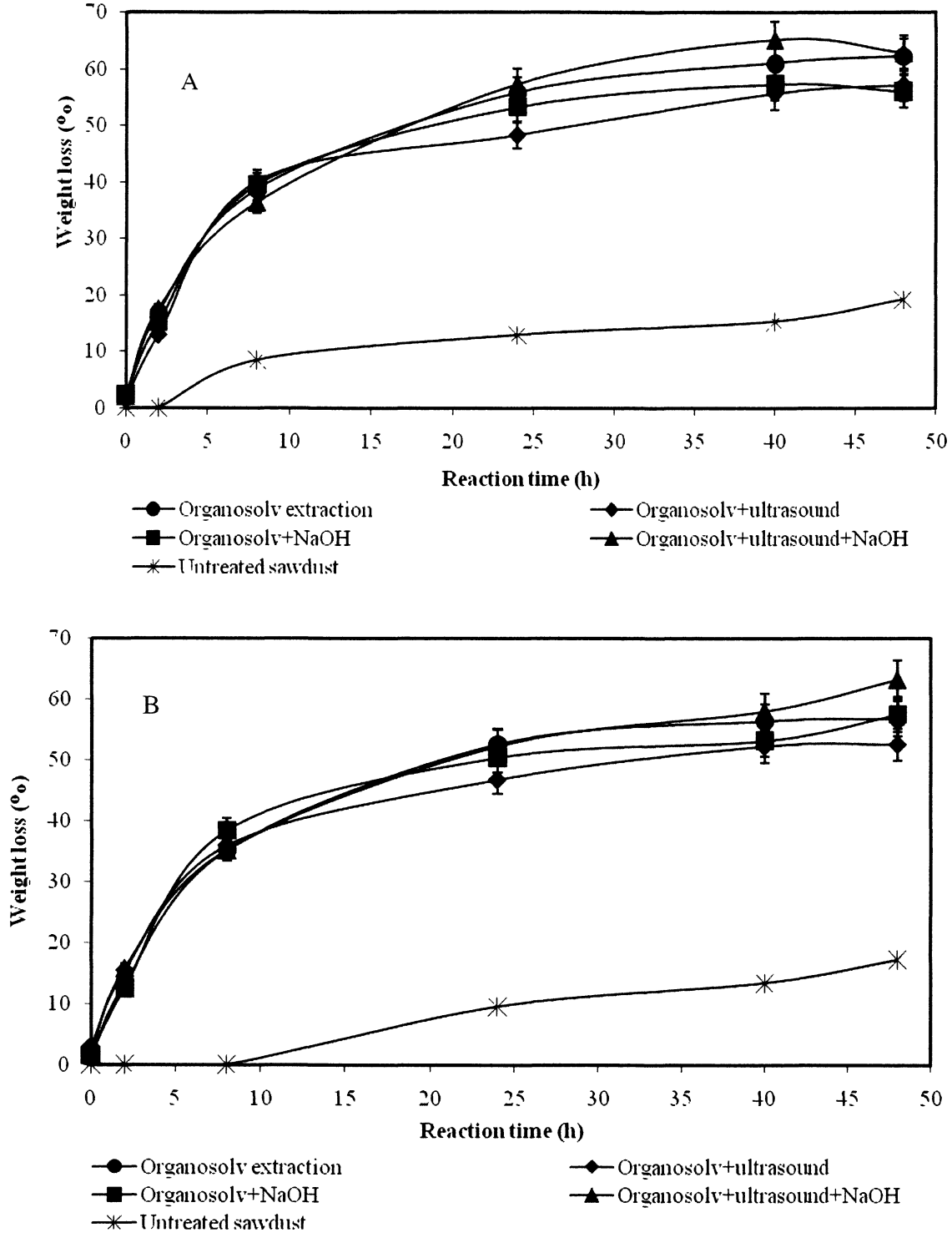


Figure 14. Total weight loss of various pretreated pine sawdust samples during hydrolysis at

50°C in sodium citrate buffer solution, pH 4.8 and 100 rpm shaking speed: A. 11.76FPU; B. 7.67FPU.

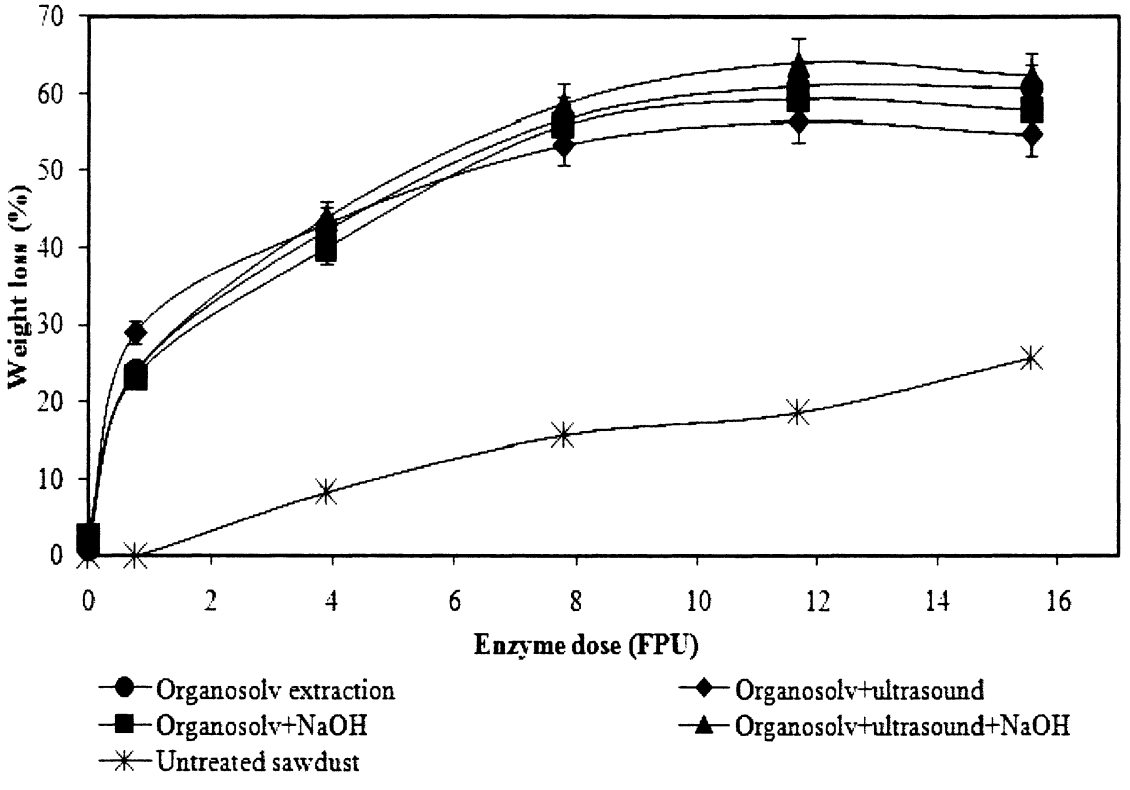


Figure 15. Total weight loss of various pretreated pine sawdust samples during hydrolysis at 50°C in sodium citrate buffer solution, pH 4.8, and 100 rpm shaking speed for 48 hours with various FPU doses of Cellulases and Novozymes

Chapter Four

Conclusions and Recommendations for Further Research

4.1 Conclusions

1) Pretreatment of peat with hydrogen peroxide under alkaline condition was studied for delignification in a batch reactor. Impact of reaction time, reaction temperature, and the concentration of hydrogen peroxide on lignin removal was systematically studied. Peat samples before and after pretreatment were characterized using Fourier transfer infrared spectroscopy (FTIR). The hydrolyzate solution was characterized using UV-spectrometer. The delignification efficiency increased with an increase in reaction time and H_2O_2 concentration. There was an optimal temperature (45°C) for denignification. The experimental results suggested the optimal reaction condition is 1.5% H_2O_2 at pH 11.5 under 45 °C for 12 h and about 45% biomass can be dissolved under optimal conditions. The results indicated that pretreatment had significant effects on delignification; however, the lignin's structure did not change after pretreatment

2) Pretreatment efficiency (PE) and delignification efficiency (DE) of pine samples treated with different pretreatment methods were systematically studied. SEM, FTIR, XRD analysis methods were used to examine the structural change before and after the pretreatment. All the pretreatment methods resulted in a significant removal of lignin and partial hemicellulose. The PE and DE were 51.40%±2% and 76.5%±3% for organosolv extracted pine; 53.3%±1% and 77.20%±2.6% for organosolv extracted +ultrasound treated pine; 57.7%±1.1% and 81.5%±3% for organosolv extracted +NaOH treated pine; 61.6%±1%and 86.4%±3% for organosolv extracted +ultrasound+NaOH

treated pine. Among all the methods used, organosolv+ultrasound+NaOH achieved the highest PE and DE, implying that the combination of these three methods could improve the removal of lignin and dissolution of hemicellulose. Through the observations from SEM figures, FTIR and XRD spectra, the structural features change of the components in cell wall were clearly demonstrated: the cell wall was disordered, twisted and exposed inner structure; the characteristic functional groups in lignin and hemicellulose were removed or reformed; and the pretreatment did not change the structure of cellulose and but increased its content in solids residues.

3) Enzymatic hydrolysis of pine samples treated with different pretreatment methods was systematically studied. The results show that pretreatment had a significant impact on the glucose yield and total sugar yield. The treatment with different methods increased the glucose and total sugar yield about two to three times under the optimal conditions. The maximum glucose yield and the maximum total sugar yield were 5.78% and 7.13%, for raw pine, 9.56% and 30.14% for organosolv extracted pine, 10.74% and 24.07% for organosolv extracted + ultrasound treated pine, 13.64% and 26.81% for organosolv extracted and NaOH treated pine, and 19.27% and 22.40% for organosolv extracted + ultrasound + NaOH treated pine, respectively. The PE and DE positively correlated to the glucose yield, while total sugar yield negatively correlated to the PE and DE.

4.2 Recommendations

1.) The stability of H_2O_2 in alkaline solution is a concern. Pretreatment of peat using H_2O_2 could be improved by adding stable catalyst, such as MgSO4 in order to avoid the decomposition of H_2O_2, especially under higher temperatures.

2.) When pine sample was treated with organosolv extraction, the proportional composition of water and ethanol needs to be further optimized. Many researchers indicated that higher ethanol content may result in higher pretreatment efficiency. In addition, the addition of acids benefits the hydrolysis of hemicellulose in this step, leaving more cellulosic solid. The recovery of hemicellulose after each pretreatment step is necessary too. It also can be used in enzymatic digestion process.

3.) It is necessary to study the mechanism of enzymatic hydrolysis to find the cause of why the higher enzyme loading and substrate concentration did not incraese the sugar yield as it was expected in the current case.

4.) More detailed HPLC work needs to be done to analyze the individual sugars and organic acids produced, in order to provide a more comprehensive picture on the products of enzymatic hydrolysis.

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