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THESIS

CONVERSION OF SUGARCANE BAGASSE TO ETHANOL BY STEAM EXPLOSION PROCESS

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A Thesis Submitted in Partial Fulfillment of
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Due to high carbohydrate content (41.02% glucose and 34.60% xylose), sugarcane bagasse were often used as feedstock for ethanol production. The purpose of this work was to evaluate the effectiveness of two-step steam explosion pretreatment on sugarcane bagasse fractionation prior to the conversion into ethanol. The condition for the first steam explosion was optimized in order to remove most of hemicellulose as well as to alter bagasse structure. The severity factors, related to reaction temperature and residence time, were varied from 3.95 to 4.16. The best pretreatment was obtained at severity factor 3.96 when 53.55% of water insoluble fraction was recovered. This consisted of 56.51% cellulose, 6.54% xylose and 28.47% lignin, which after that was subjected to the second steam explosion. In this step, the solid residue was primarily impregnated in various concentrations of sulfuric acid (0.051, 0.102, 0.153 and 0.204 M). The 33.33% dry weight of soaking material was carried out in a steam explosion unit with severity factors of 3.64 and 3.94. In the production of ethanol, the substances for ethanol fermentation were compared. The solid from the first steam explosion was converted to ethanol through the SHF and SSF processes. The best ethanol yields from SHF and SSF processes were 10.70% and 11.89%, respectively. Liquid fraction from the second steam explosion pretreatment was fermented to ethanol. The best ethanol yield of 5.28% on bagasse basis was obtained. However, the SSF process was also applied on the mixture of liquid and solid fraction. This process provided the best ethanol yield as 11.73%. Thus, it could be concluded that the additional steam explosion pretreatment did not significantly improve the ethanol yield.

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Student's signature Thesis Advisor's signature

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Nanthaya Chumchuen

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LIST OF ABBREVIATIONS

DM	=	Dry matter
DP	=	Degree of polymerization
GC	=	Gas chromatography
HPLC	=	High performance liquid chromatography
SEM	=	Scanning electron microscopy
SHF	=	Separate hydrolysis and fermentation
SSF	=	Simultaneous saccharification and fermentation
TGA	=	Thermogravimetric analysis
WIS	=	Water insoluble fraction

CONVERSION OF SUGARCANE BAGASSE TO ETHANOL BY STEAM EXPLOSION PROCESS

INTRODUCTION

The increased concerns for the security of fossil fuel supply and environmental problem about global warming from the increased of greenhouse gases, mainly CO_2 which is gas from the combustion of fossil fuel, have resulted in an increasing worldwide interest in alternative non-petroleum–based sources of energy. As the transportation sector is practically entirely dependent on crude oil, and as it is responsible for half of the total CO_2 emission, therefore the market share of renewable biofuels, including fuel ethanol is increased. The use of fuel ethanol will significantly reduce net CO_2 emission once it replaces fossil fuels because the fermentation-derived ethanol is already a part of the global carbon cycle. However, to enhance the market position of the biofuel, the production cost should be reduced.

Nowadays, most of feedstocks for the ethanol production are sugarcane, corn and cassava root. However, lignocellulosic materials are considered as feedstock in the production of ethanol. Lignocellulosic materials are renewable, low cost and largely available, for example, agriculture residues such as bagasse, rice straw, corn cob and wood residues such as sawdust and wood chip. The main focus of this research is the utilization of sugarcane bagasse as feedstock for the ethanol production. Sugarcane bagasse is fibrous residue from sugar mill, which could be used as raw material in pulp and paper industry, particleboard industry or burning for energy. Sugarcane bagasse contains approximately 45-50% celluloses, 25-30% hemicellulose and 20-25% lignin. Cellulose in bagasse has to be hydrolyzed by acid or enzymes for yielding sugar and then sugar is further fermented to produce ethanol. Therefore, bagasse is one of an alternative feedstock in the ethanol production.

The steam explosion technology is a process using high temperature and high pressure to fractionate hemicellulose from biomass in a short period of time. The

leaving parts are cellulose and lignin, which can be separated later by dissolving in basic solution.

In this work, the condition of two-steps steam explosion pretreatment of sugarcane bagasse for ethanol production is optimized. The first steam explosion step is performed to remove hemicellulose from bagasse and the second pretreatment step is performed to hydrolyze the pulp from the first step to glucose. The main objectives of this study are :

1. Optimize the condition for the first steam explosion pretreatment step to remove hemicellulose from sugarcane bagasse.

2. Optimize the condition for the second steam explosion pretreatment step to hydrolyze cellulose in solid residue obtained from the first pretreatment step.

3. Compare the ethanol production using the liquid and slurry obtained from the second steam explosion pretreatment and the solid residue obtained from the first steam explosion pretreatment.

4. Compare the ethanol production between the separate hydrolysis and fermentation process (SHF) and the simultaneous saccharification and fermentation process (SSF).

LITERATURE REVIEW

1. Alternative fuel and fuel ethanol

In the past century, energy demand was increasing continuously due to the rapidly growing in the world population and many countries had development in industrialization (Sun and Cheng, 2002). The major energy demand had been supplied by the conventional energy sources such as coal, oil and natural gas. However, these energy resources were estimated to get depleted in the near future, include with the effective on environmental of fuel consumption had led to great attention in alternative energy resources such as solar, wind, thermal, hydroelectric and biomass (Saxena *et al*., 2007).

Biomass was being considered as an important energy resource over the world due to its large quantity, renewable and environment friendly. This organic matter is derived from growing plants including algae, trees and crops or from animal manure. Biomass was a carbon neutral resource that effectively stores solar energy in chemical bond (Saxena *et al*., 2007), thus it could be converted to energy source include solid, liquid and gaseous fuels through different conversion processes.

Ethanol or ethyl alcohol (molecular formation was C_2H_5OH) had been considered as a possible alternative fuel. Ethanol was colorless, limpid, volatile liquid which was flammable and toxic and had a burning taste. It boiled at 78.4 ºC and melted at -112.3 ºC, had a specific gravity of 0.7851 at 20 ºC and was soluble in water and most organic liquids. Ethanol could be blended with gasoline to gasohol for reducing the consumption of fossil fuel. Ethanol had also greater octane booster property thus it could be used as substitutable MTBE additive to improve the performance of gasoline by reducing the likelihood that engine knock problems would occur (Sanchez and Carlos, 2008). In addition, ethanol contain oxygen that was referred to as “oxygenate” so it could improves the fuel combustion process, thereby emission of CO and hydrocarbon compounds was reduced. Many countries had

researches for the addition of ethanol to gasoline, these fuel contain 10% and up to100 % ethanol by volume. Ethanol could be produced from sugar-based materials using biological technology. Sugarcane and corn are mainly used as feedstock for ethanol production in Brazil and USA (Sanchez and Carlos, 2008). Nevertheless, production of sugar or starch-based ethanol had created an increased demand for the feedstock, therefore leading to the lack in eatable crop and rising prices.

2. Bioethanol production

Ethanol could be produced from both chemical and biological processes. For chemical synthesis, ethanol was produced from the hydration reaction of ethylene using acid-catalysis. However, the feedstock of this process was petro-chemical so it could be depleted in the coming future. Biomass, the renewable material, was introduced to use as feedstock for converted to ethanol through biotechnology processes that was referred to as “bioethanol”.

Feedstock of bioethanol production was a variety of sugar-based biomass such as starch plants and sugar crops and cellulose-based material. The conversion process from biomass feedstock to ethanol consisted basically of two main steps as the conversion of polysaccharide to fermentable sugars and the fermentation of sugars to ethanol (Sun and Cheng, 2002). The conversion of polysaccharides to sugars depended on the breakdown process mainly as hydrolysis reaction. The catalysts of hydrolysis reaction mostly used were acid and enzyme. In the fermentation step, various microorganisms such as yeast and bacteria could convert fermentable sugars to ethanol by their metabolisms. Pretreatment of raw material before further to ethanol production process was also necessary for any biomass to facilitate the attack of enzyme or acid catalyst. Scheme of ethanol production from lignocellulosic biomass was shown in Figure 1.

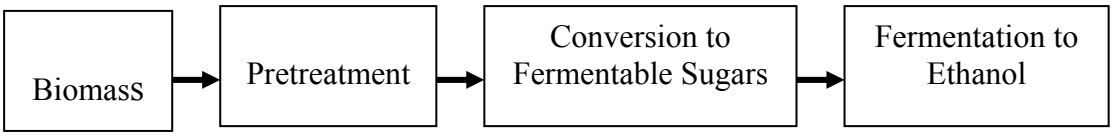

Figure 1 The process of ethanol production from biomass

3. Lignocellulosic biomass for ethanol production

Currently, bioethanol was mostly produced commercially from starch and sugars. Corn is the primary raw material for ethanol production in USA, while sugarcane is the main feedstock in Brazil. Moreover, wheat, barley and cassava are also used for the production of ethanol. However, these carbohydrate-based materials are too expensive and limited in the supply for commercial fuel production. Therefore, the largest potential feedstocks for ethanol production like lignocellulosic biomass are attended from many researchers. Lignocellulosic biomass is an attractive feedstock because of its availabl, low cost, large quantities and renewable. These materials include agricultural residues (e.g. corn stover and wheat straw), forestry wastes, wastepaper, yard waste and other components abundant and various industrial wastes. In the longer term, we would need to use woody (e.g. hybrid poplar) and herbaceous (e.g. switchgrass) crops to support large scale production of fuel (Wyman, 1996).

3.1 The compositions of lignocellulosic biomass

3.1.1 Cellulose

Cellulose, approximately 40-45%, was a major component of wood. Cellulose is a linear homopolysaccharide containing approximately 10^4 of β-D-glucose units which are linked by glucosidic linkages at C_1 and C_4 positions (Sjostrom 1993). Properties of cellulose in hardwood and softwood were not different. Nevertheless, it differs completely from starch that are repeating unit of glucose

linking by α-1, 4 linkages. Microfibrils, composed of cellulose bundles are highly crystalline structures supplemented with highly degree of polymerization (DP). Therefore, cellulose fibers are highly in a tensile strength, insoluble in most solvents and resistant to hydrolysis.

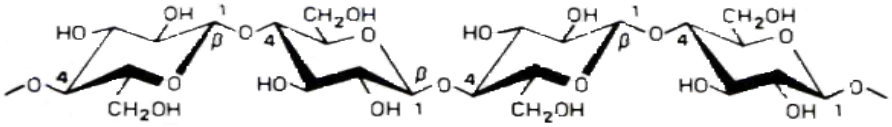

Figure 2 Structure of cellulose
Source: Sjostrom (1993)

3.1.2 Hemicellulose

Usually, lignocellulosic materials contain 20-30% hemicelluloses. Hemicellulose was located between the microfibrils or inter laminar spaces (Roehr, 2001). it was branched heteropolysaccharide which a DP only 200 (Sjostrom 1993). The sugar composition of hemicellulose was variable, generally consists of pentose (such as D-xylose and D-arabinose), hexose (such as D-glucose, D-mannose and D-galactose) and small amounts of sugar acid (Sjostrom, 1993). Hemicellulose was more easy to degrade than cellulose because it contains less ordered (amorphous) regions and low DP. The dominant component of hemicellulose from agricultural lignocellulosic materials was xylose (Hendriks *et al.*, 2009).

3.1.3 Lignin

Lignin was a phenolic macromolecule that is primarily formed by the free-radical polymerization of *p*-hydroxy cinnamyl alcohol unit with varying methoxyl content. Three of monomerric precursors were coniferyl alcohol, sinapyl alcohol and *p*-coumaryl alcohol. The units of precursors are joined by C-O-C (ether) and C-C linkages in three dimension structure. The ether linkage was dominant and the rest is of the carbon to carbon type (Sjostrom, 1993). The main role of lignin was

to give the plant structural support, impermeability and resistance against microbial attack and oxidative stress (Hendriks *et al.*, 2009).

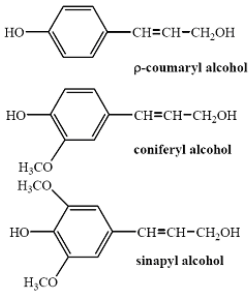

Figure 3 Structure of three precursors of lignin

Source: Sjostrom (1993)

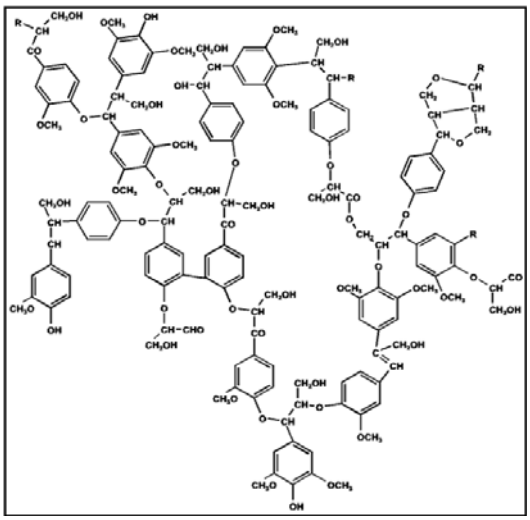

Figure 4 Structure of lignin

Source: Sjostrom (1993)

3.1.4 Extractive

Extractive was a minor fraction of wood. Nevertheless, it is a large variety of components. The extractive comprises an extraordinarily large number of individual compounds of both lipophilic and hydrophilic types such as

terpenoids and steroids, fats and waxes, and phenolic constituents. Various parts of the plants differ in their amount and types of extractive. For example, fats and waxes were in the ray parenchyma cells while phenolic extractives are present mainly in the bark. Due to that most of extractives were soluble in organic solvents, thus the quantitative determination was carried out by extraction with organic solvents. Extractive content was usually less than 10% in wood (Sjostrom 1993).

3.1.5 Ash

Ash was an inorganic component present in wood or plant at rather low amounts. This ash is originated mainly from a variety of metal salts, carbonates, silicates, oxalates and phosphates, deposited in cell walls and lumina (Sjostrom 1993).

3.2. Sugarcane Bagasse as feedstock for ethanol production

In recent years, there had been an increasing trend towards more efficient utilization of lignocellulosic agro-industrial residues, including sugarcane bagasse. Sugarcane bagasse was a fibrous residue after the sugarcane stalks were crushed to extract their juice. In some 80 developing countries, the sugar industrial currently produced some 1,100 million ton of sugar per years (Botha and Blottnitz, 2006). Per 1,000 kilograms of sugarcane provided 125 kilograms of bagasse, thus over 100 million tons of bagasse were produced annually throughout the world (Botha and Blottnitz, 2006). Chemically, about 40–50 % of dry residue was cellulose, much of which was in a crystalline structure. Another 25–35 % was hemicellulose, an amorphous polymer usually composed of xylose, arabinose, galactose, glucose, and mannose. The remainder was mostly lignin plus lesser amounts minerals, waxes and other compounds. Several processes and products had been reported that sugarcane bagasse was utilized as a raw material electricity for generation, pulp and paper production and products based on fermentation (Pandey *et al.,* 2000). One significant application of bagasse has been used as feedstock for the production of biofuel

(ethanol). The conversion of sugarcane bagasse into fermentable sugars was possible through thermal, chemical or enzymatic hydrolysis.

4. The pretreatment of lignocellulosic biomass

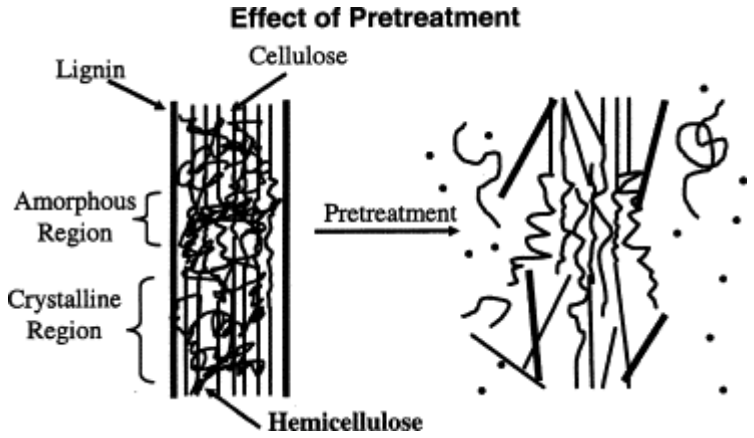

Figure 5 Schematic of goals of pretreatment on lignocellulosic biomass
Source: Mosier (1995)

The main purpose of pretreatment processes was to alter the structure of lignocellulosic materials to be more easy for conversion. The pretreatment must met the following requirements: first, to improve the formation of sugars by conversion processes, mainly as acid or enzyme hydrolysis, that require in accessible property of substrate. Accessibility of lignocellulosic materials was caused by high surface area, removal of lignin and/or hemicellulose as well as disordered structure of fiber. Moreover, low crystalinity of cellulose could also improve ability for hydrolysis. Next, the pretreatment must avoid or minimize loss of carbohydrate that was mainly effective from the degradation process. Sugar degradation could produce any byproducts (furfural, 5-hydroxy methyl furfural, levulinic acid) that were toxic for microorganisms in fermentation process. Unless degraded sugars, the formation of other byproducts (acetic acid, formic acid and phenolic compounds) inhibitory to the subsequent hydrolysis and fermentation processes were also avoided. Finally, overall process was to be cost-effective (Sun and Cheng 2002).

4.1 Pretreatment techniques

4.1.1 Physical pretreatments

Size reduction of lignocellulosic biomass was the process for improvement of digestibility. Size comminution by grinding, milling or chipping has been employed. The goal of size reduction was to reduce the crystallinity of the cellulose fibers in the biomass (Keshwani and Cheng, 2009). The size of material was usually 10–30 mm after chipping and 0.2–2 mm after milling or grinding (Sun and Cheng, 2002). The effects of size reduction of switchgrass achieved from ball milling were examined by Bridgeman *et al*. (2007). The results showed that extensive size reduction was undesirable as it caused significant carbohydrate losses which ultimately results in less reducing sugars and reduced ethanol yield. The energy requirements for size reduction depended on the final particle size and material characteristics (Cadoche and Lopez, 1989). Mani *et al.* (2004) examined the energy requirements for size reduction of switchgrass using a hammer mill, energy requirements increased linearly as particle size reduce and it was higher than that of corn stover, barley straw and wheat straw at the same moisture content.

Microwave pretreatment could disrupt the silicified waxy surface, breakdown the lignin-hemicellulose complex and partially remove silicon and lignin. Enhanced enzymatic saccharification of rice straw by microwave pretreatment was studied by Ma *et al.* (2009). The results showed that the maximal efficiencies of cellulose, hemicellulose and total saccharification were respectively increased by 30.6%, 43.3% and 30.3% under optimal condition. Microwave intensity, irradiation time and substrate concentration were factors governing.

4.1.2 Chemical pretreatments

Chemical pretreatments were popular methods to enhance the hydrolysis ability of lignocellulosic materials. Solubilization of hemicellulose and

lignin was a main goal of this method (Keshwani and Cheng, 2009). Acid hydrolysis was popular method that could be used for fractionation of materials. Moreover, ozonolysis, alkaline hydrolysis, oxidative delignification and organosolv process were also employed for pretreatment (Sun and Cheng, 2002).

Concentrate acids such as H_2SO_4 and HCl were powerful agents for treatment of lignocellulosic biomass. Due to that these chemicals are toxic and involve high costs in proceeding, therefore, leading to less employing. Dilute acid pretreatment had been studied extensively and was efficient for removal of hemicellulose but fails to effectively removed lignin (Keshwani and Cheng, 2009). Dilute acid pretreatment of switchgrass for bioethanol production was first examined by Wyman *et al*. (1992). The pretreatment was conducted at 140ºC for 1 h using sulfuric acid at low concentration (up to 0.5% v/v). Enzymatic hydrolysis of the resulting biomass yielded up to 70% conversion of cellulose into glucose over a five-day period. Wu and Lee (1997) used a two-stage dilute sulfuric acid pretreatment with an acid concentration of 0.0785% (w/w) to successfully remove 100% of hemicellulose from switchgrass.

Reactive oxygen species could remove both hemicellulose and lignin from lignocellulosic biomass. Lee *et al*. (2009) studied sugarcane bagasse oxidation using a combination of hypochlorite and peroxide. After pretreatment, cellulose was hydrolyzed 80-100% compared to 40% or less for hypochlorite treatment.

Ozone, a powerful oxidant, could be used to degrade lignin and hemicellulose in lignocellulosic biomass (Sun and Cheng, 2002). Ozonolysis was carried out at room temperature and was effective for lignin removal without the formation of toxic by-products. Silverstein *et al*. (2007) applied ozone pretreatment for conversion of cotton stalk to ethanol by sparging ozone gas through 10 % mixture of cotton stalks and water at 4ºC for 30, 60 and 90 min. Garcia-Cubero *et al.* (2009) pretreated wheat and rye straws with ozone to increase the enzyme hydrolysis. Lignin

content of biomass was reduced involving hemicellulose degradation and enzymatic hydrolysis yielding up to 88.6%. However, the large ozone requirement made the process expensive (Sun and Cheng, 2002).

Dilute alkali pretreatment using sodium hydroxide targeted intermolecular bonds between lignin and hemicellulose and improved the porosity of the biomass. Lime (calcium hydroxide) pretreatment of switchgrass was investigated by Chang *et al*. (1997). With a lime loading of 0.1 g g^{-1} of dry switchgrass, a pretreatment time of 2 h at 100 ºC, the study showed that a 72 h enzymatic hydrolysis of pretreated biomass yielded five times higher reducing sugars than untreated switchgrass.

In organosolv process, inorganic acid catalyzed in organic/inorganic solvent was used to break the internal lignin and hemicellulose bond (Sun and Cheng, 2002). The use of organic solvents such as methanol, ethanol, acetone and ethylene glycol had been studied. Catalyst was not necessary for satisfactory delignification at high temperature (Aziz and Sarkanen, 1989). Because of the solvent might be an inhibitor for enzyme and organism so removal of solvent from process was necessary (Sun and Cheng, 2002).

4.1.3 Physico-chemical pretreatment

Combination of physical and chemical methods was considered as the effective pretreatment. The best pretreatment method such as steam explosion, ammonia fiber explosion (AFEX) and liquid hot water (LHW) was introduced for lignocellulosic biomass pretreatment.

Steam explosion was the most commonly used method for pretreatment of lignocellulosic material. In steam explosion process, biomass was subjected to high-pressure saturated steam for a short time before a sudden drop in pressure causing an explosive decompression of the biomass. Typical conditions were

160–260°C and 0.69–4.83 MPa (Sun and Cheng, 2002). The process caused transformation of lignin and degradation of hemicellulose which improved the enzymatic hydrolysis of cellulose. However, the process produces inhibitory by-products that might impact down-stream processes.

AFEX was similar to steam explosion. The biomass was exposed to liquid ammonia at high temperature and pressure for a short period of time followed by a sudden drop in pressure. AFEX did not solubilize hemicellulose (Vlasenko *et al.,* 1997) but did require recovery of the ammonia for cost and environmental reasons (Sun and Cheng, 2002). Alizadeh *et al.* (2005) optimizd AFEX pretreatment of switchgrass by examining the impact of ammonia loading, moisture content and reactor temperature on the efficiency of enzymatic hydrolysis. The authors reported optimum pretreatment condition of 100 °C reactor temperature, ammonia loading of 1 g g^{-1} of biomass and a residence time of 5 min. These conditions yielded a 6-fold improvement in hydrolysis efficiency.

Carbon dioxide explosion was not as effective as AFEX or steam explosion (Dale and Moreira, 1982). Dale and Moreira (1982) used this method to pretreat alfalfa (4 kg CO_2/kg fiber at the pressure of 5.62 MPa) and obtain 75% of the theoretical glucose released during 24 h of the enzymatic hydrolysis. The yields were relatively low compared to steam or ammonia explosion pretreatment, but high compared to the enzymatic hydrolysis without pretreatment. Zheng *et al*. (1998) compared CO_2 explosion with steam and ammonia explosion for pretreatment of recycled paper mix, sugarcane bagasse and repulping waste of recycled paper. They fould that CO_2 explosion was more cost-effective than ammonia explosion and did not cause the formation of inhibitory compounds that could occur in steam explosion.

Liquid hot water (LHW) was used instead of steam explosion. The objective of the liquid hot water was to solubilize mainly the hemicellulose to make the cellulose better accessible and to avoid the formation of inhibitors. To avoid the formation of inhibitors, the pH should be kept between 4 and 7 during the

pretreatment. Maintaining the pH between 4 and 7 minimizes the formation of monosaccharides, and therefore also the formation of degradation products that could further catalyze hydrolysis of the cellulosic material during pretreatment (Mosier *et al.,* 2005).

4.1.4 Biological pretreatments

Biological pretreatment was one of processes that had been utilized for lignocellulosic material pretreatment. Microorganisms such as fungi and bacteria were used for hemicellulose and lignin biodegradation. This pretreatment had low energy requirements and mild environmental condition. However, most of processes were too slow limiting application for industrial. White rot fungi were the most effective for biological pretreatment. Its could attack both cellulose and lignin while brown rots fungi mainly attacked only cellulose (Sanchez *et al.*, 2008). *Pleurotus ostreatus*, *Phanerochaete sordida* 37 and *Phycnoporus cinnabarinus* 155, were studied for conversion biomass material to sugars. *Sporotrichum pulverulentum* was proposed for lignin degradation as same as *Ceriporiopsis subvermispora* and *Cyathus stercoreus* (Sun and Cheng 2002).

4.2 Steam explosion pretreatment

Steam explosion technology was a method to defibrillate lignocellulose's materials that was studied about 60 years ago. The separation technique with steam explosion could be batch and continuous reactors. This technique could be applied in agriculture industry such as pulp and paper industry, cellulose industry and ethanol production industry. The steam explosion was a process that uses high temperature and high pressure of steam to fractionate hemicellulose from biomass in a short period of time. The leaving part was cellulose and lignin. Lignin could be separated later by dissolving in alkaline solution. The parameters controlling the steam explosion technique were reaction temperature (T) and retention time (t). The relationship between temperature and time had been

defined in one parameter as severity factor (R_0) (Ibrahim, 1998) as shown in the following equation:

$$R_0 = \log [t * \exp \{(T-100)/14.75)\}] \quad (1)$$

Where R_0 = Severity factor

T = Reaction temperature, °C

t = Retention time, min

The main component and basis in operation steam explosion machine was shown in Figure 6 with these details.

1 = Water tank
2 = Heater tank
3 = Valve
4 = Reactor
5 = Receiver tank
6 = Valve
7 = Tube to release pressure
8 = Ball value

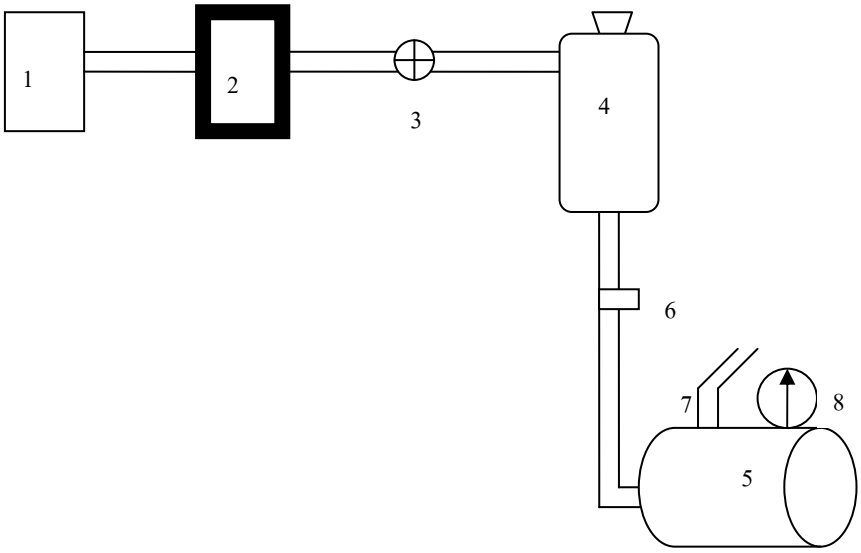

Figure 6 Main components of steam explosion machine

The basic operation of steam explosion was that water from machine 1 (water tank) was transferred to 2 (Heater tank) to produce steam. After that, steam was brought to valve 3 (Valve) which controled the steam pressure before releasing to 4 (Reactor) that was filled with chip. When open valve 3, steam entered the reactor 4. After that, the time was recorded for the explosion at constant pressure of steam until finish time. Thus, valve 6 connected between reactor 4 and receiver 5 (Receiver) was opened and the pulp was transferred into receiver 5. Then, pressure was released by open valve 7 until the pressure was zero on ball valve. After that, the receiver tank was opened and the pulp and solution were taken from the receiver tank.

Figure 7 showed the chemical mechanism of xylan during steam explosion. In the first reaction, high temperature and high pressure of steam change acetyl group in xylan molecule to acetic acid and the occurring acetic acid performs the hydrolysis and dehydration reactions to change xylan molecule to xylose, oligomer of xylose, furfural and 5-Hydroxymethylfurfural (HMF). In addition, the reaction broke down lignin molecule to small molecules such as phenolic compounds.

The possible mechanism occurred in steam explosion was summarized from equations 2 to 6 where the steam explosion pretreatment could produce value added products as shown in Figure 7.

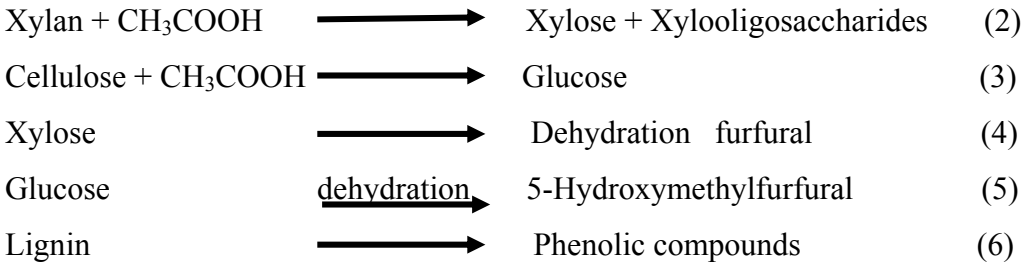

Figure 7 Possible mechanism reactions in steam explosion pretreatment

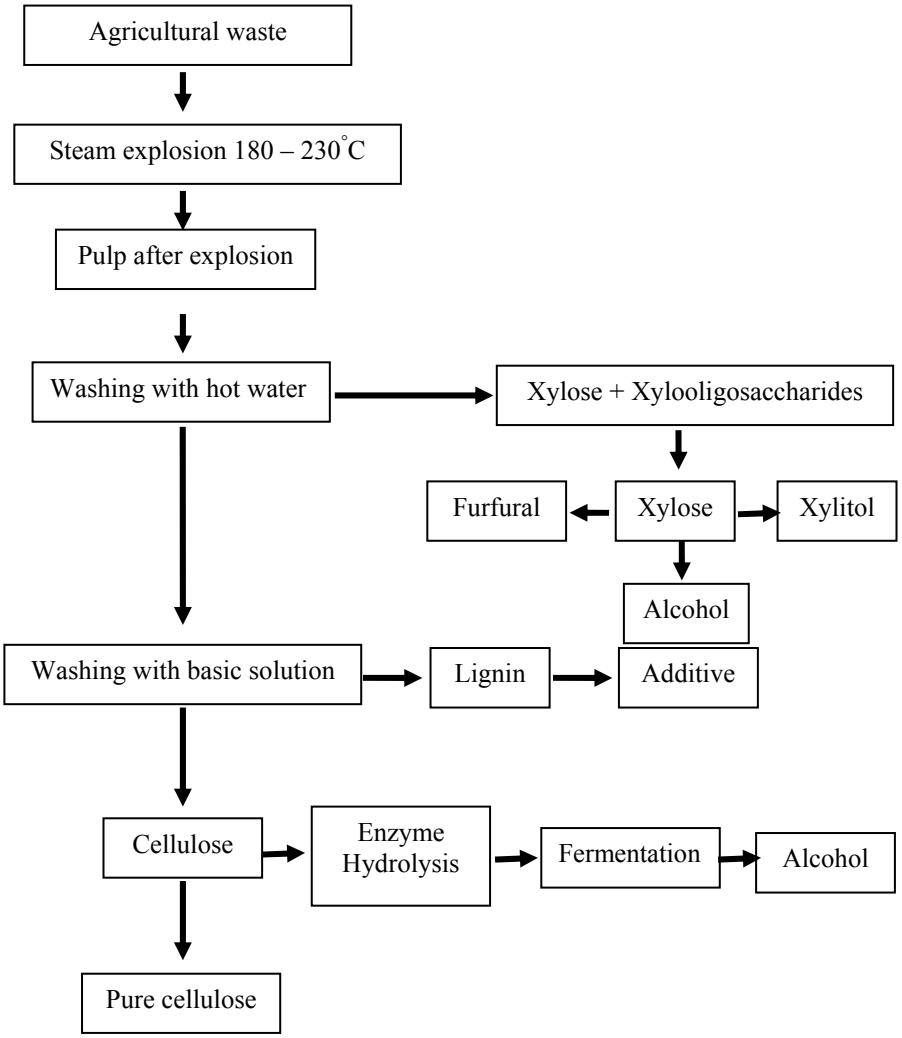

Figure 8 Applications of agricultural waste by steam explosion pretreatment machine

The application of steam explosion machine was used for pretreatment of wood or agricultural waste to separate cellulose, hemicellulose and lignin for producing value added product from each component as shown in Figure 8.

5. The conversion of lignocellulosic material to fermentable sugar

For ethanol production, the process involved hydrolyzing of cellulose and hemicellulose to their sugars, pentose- and hexose-sugars, for subsequent conversion to ethanol by fermentive process. Hydrolysis reaction of cellulose and hemicallulose

could used various catalysts, acid and enzyme catalyst were often employed in the digestion process. Fermentation was carbohydrate metabolism of microorganism in the absence of oxygen, any biochemical processes were appeared for conversion of sugars to ethanol. Aerobic yeast was often employed in ethanol production under proper condition and nutrient.

Achievement of glucose, main sugar for further to commercial ethanol fermentation, came from the digestion of cellulose using acid and enzymatic hydrolysis. The economic success of these processes would depend on their ability to obtain good sugar conversion, so difference in reaction of both processes were considered below.

5.1 Acid hydrolysis of cellulose

Acid hydrolysis had one potential method of converting cellulose to glucose. Acid catalyst was not specific for hydrolysis reaction so it can produce any byproducts during the process. The reaction process required high energy for the performance such as the reaction condition at high temperature and/or high pressure. The hydrolysis could be catalyzed by variety of acids such as sulfuric acid, hydrochloric acid and nitric acid. In reaction of hydrolysis, the linkages of cellulose, β-1, 4 glycosidase of cellulose, were broken down and then release products as glucose both mono- and oligomer forms. Sugar from hydrolysis may be in form of monomeric when high severe condition was used. However, reaction in excessive severity often degrades glucose to derivative form, 5-hydroxy-methylfurfural, of which was also converted to levulinic acid and others as shown in Figure 9. Both concentrate and dilute sulfuric acids were used in hydrolysis process, however, concentrate acid was much cheaper and overall yield of products were not significantly different, thus, most of researches intend to study dilute acid catalyzed hydrolysis.

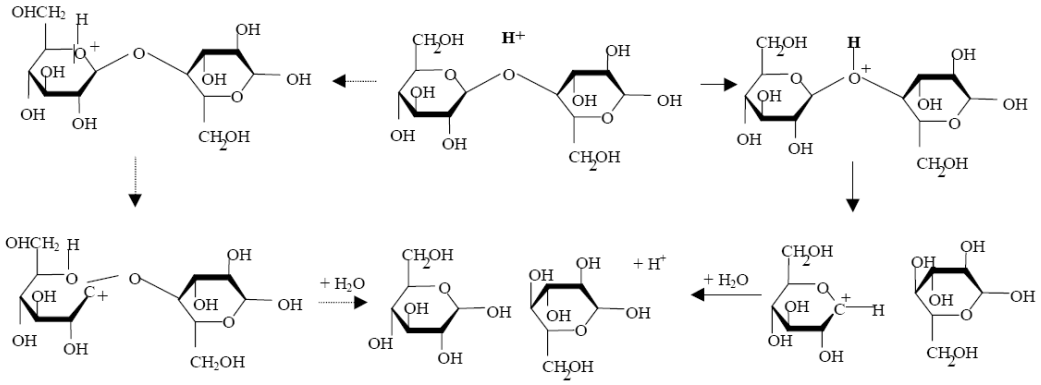

Figure 9 Mechanism of acid hydrolysis

5.2 Enzymatic hydrolysis

Enzymatic hydrolysis was high efficient technique for hydrolyzing cellulose. This process was received attention because it had 100 % selective for cellulose conversion, therefore, there was no degradation product (Sanchez *et al*., 2008). Additionally, enzymatic hydrolysis process did not affect the environment, and had low energy requirement for mild condition though a long reaction time. However, the cost of enzyme was prohibitive for deployment in enzymatic process. The enzymes were mostly obtained from a variety of microorganisms. Most of commercially came from *Trichoderma reesei* and small volume from *A. niger*. A mixture of enzymes using in hydrolysis reaction are compose of:

I. Cellulase

Cellulase was produced by fungi, bacteria, protozoans and other types of organisms such as plants and animals. The reactions of cellulase occur in two mechanisms that were endo-cellulase mechanism and exo-cellulase (or cellobiohydrolases, abbreviate CBH) mechanism. Endo-cellulase or endo-1,4-β-glucanase randomly hydrolyzes cellulose chains to produce a rapid decrease in chain length, broke internal bonds to disrupt the crystalline structure of cellulose and

exposed individual cellulose polysaccharide chains. Exo-cellulase cleaved 2-4 units from the ends of the exposed chains produced by endo-cellulase, resulting in the tetrasaccharides or disaccharides such as cellobiose. There were two main types of exo-cellulases - one type working processively from the reducing end, and one type working processively from the non-reducing end of cellulose.

II. β-glucosidase

β-glucosidase was a glucosidase enzyme which acts up on *β*-1,4 bonds linking two glucose or cellobiose. It catalyzes the hydrolysis of terminal non-reducing residues in *β*-D-glucosides with release of glucose**.**

The reaction mechanism of enzyme was shown in Figure 10.

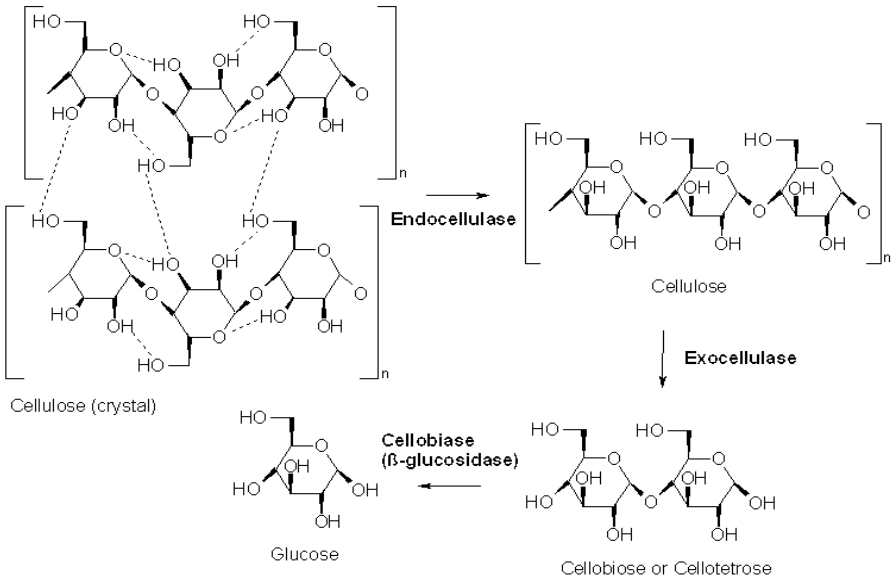

Figure 10 Mechanism of enzymatic hydrolysis

Table 1 Comparison of enzymatic and acid hydrolysis processes for cellulosic materials (Roehr, 2001)

Acid	Enzyme
1. Non-specific catalyst, therefore would delignify material as well as hydrolyzed cellulose 2. Decomposition of hemicellulose to inhibitory compounds (i.e.furfural) 3. Harsh reaction conditions necessary and therefore, increased costs for heat and corrosion-resistant equipment 4. High chemical costs required catalyst recovery and reuse 5. Rate of hydrolysis was high 6. Overall yield of glucose was low due to degradation	1. Specific macromolecular catalyst, therefore extensive physical and chemical pretreatment of material necessary to make cellulose available for degradation 2. Production of clear sugar syrup ready for subsequent anaerobic fermentation 3. Run under mild conditions (50ºC, atmospheric pressure, pH 4.8) 4. Cost to produce cellulases was the most expensive step in the process, therefore, recycle were necessary 5. Lower rate of hydrolysis 6. High glucose yield depending upon system and pretreatment

6. Fermentation

Fermentation, the metabolic activities of organisms, was used to synthesize ethanol. Ethanol producing microorganism, mainly yeast and bacteria, could convert sugars such as glucose ($C_6H_{12}O_6$) to metabolic waste products, ethanol (C_2H_5OH) and carbon dioxide (CO_2). The chemical equation below (equation (7)) summarizes the

ethanol fermentation, in which one mole of glucose was converted into two mole of ethanol and two mole of carbon dioxide :

$$C_6H_{12}O_6 \longrightarrow 2\ C_2H_5OH + 2\ CO_2 \quad (7)$$

Glucose Ethanol Carbon dioxide

Figure 11 Reaction of ethanol production from glucose
Source: Roehr (2001)

Therefore, on a weight basis, each gram of glucose could theoretically gave rise to 0.51 g of alcohol.

In general, important organism using in the fermentation of sugar to ethanol was yeasts. *Saccharomyces cerevisiae*, *Saccharomyces uvarum (carlsbergensis)*, *Schizosaccharomycees pombe* and *Kluyvero mycess* species are primary interesting yeast. Yeasts were capable to utilize a variety of substrates (such as glucose, galactose, mannose, fructose and xylose). The condition at temperatures of 28 – 35ºC and pH values of 3.5-6.0 was suitable to grow and efficiently ferment of yeast (Roehr, 2001). Yeasts carried out ethanol fermentation on sugars under anaerobic condition. However, a small concentration of oxygen was required for yeast growth as same as nutrients including carbon, oxygen, nitrogen hydrogen and small quantities of any elements and organic compounds.

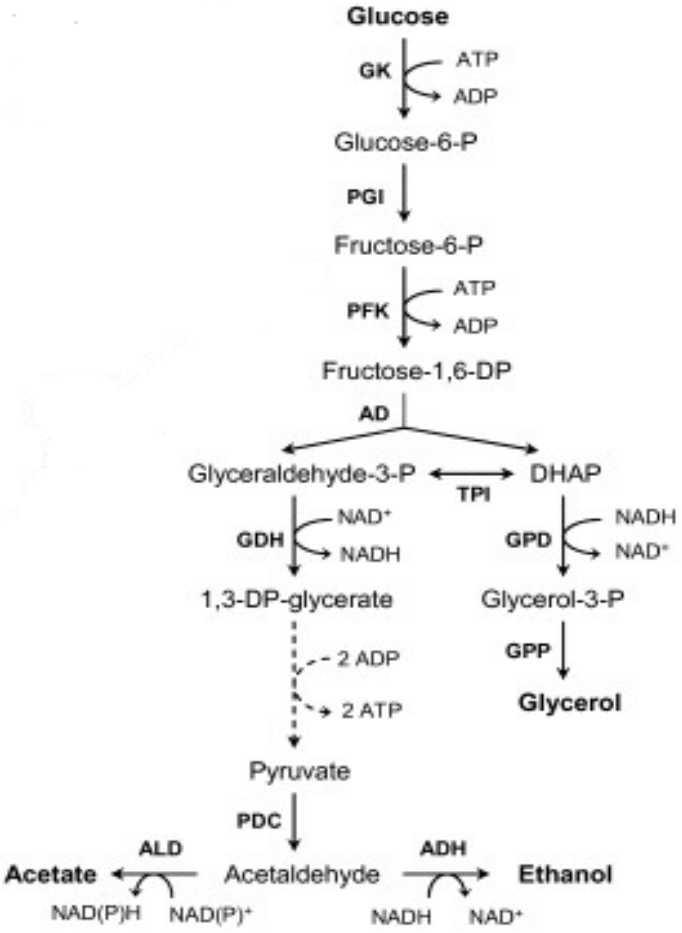

Figure 12 Mechanism of ethanol fermentation

7. Separate hydrolysis and fermentation processes

Hydrolysis of cellulosic materials performed separately from the fermentation step was known as separate hydrolysis and fermentation or SHF. Hydrolysis was used for production of sugar both acid and enzyme catalysis and then the subsequent fermentation was performed to convert the glucose rich solution to ethanol. The SSF process could be performed at optimal operating condition. The important factors to be taken into account for saccharification step were reaction time, temperature, pH, enzyme dosage and substrate loading (Sanchez *et al*., 2008).

8. Simultaneous saccharification and fermentation processes

Ethanol production combined both enzymatic hydrolysis stage and organism fermentation stage and was performed in the same reactor was called simultaneous saccharification and fermentation process or SSF. The SSF process could reduced cost of production because of time and energy which consumed in the performance were decreased. In addition, the reducing of glucose accumulation, fermentable hydrolysate, caused the increasing of ethanol yield. However, the presence of ethanol in fermented solution might inhibit the growing of the yeast.

In consequence of the process performed in the single stage, compatible conditions of both reactions were required. Temperature and pH of reaction could be suitable for the activation of cellulase enzyme, optimum temperatures ranging from 40-50°C and pH about 4.8, and the culture of microorganism, the growth condition of yeast was pH 5.5 at 30°C.

9. Experiment related with steam explosion pretreatment of lignocellulose to ethanol production

Nunes and Pourquie (1996) studied steam explosion pretreatment of eucalyptus wood under non acidic and acidic conditions for further enzymatic hydrolysis. The effects of this pretreatment were assessed. The results showed that during process, the pentose and hexose sugars were released either in monomeric or oligomeric forms, under acidic condition resulting in good solubilization. When hydrolyzed with cellulase enzyme, yield of substrates was the same in both conditions.

Moniruzzaman (1996) used steam explosion to pretreat rice straw. The result showed that high steam pressure for a short period of time of explosion effectively enhanced enzymatic saccharification and alcohol fermentation of rice straw.

Kaar *et al.* (1998) experimented steam explosion of sugarcane bagasse for ethanol production. In order to identify the optimum condition of the steam explosion, a range of operating temperatures at 188 - 243°C and residence times at 0.5-44 min were performed. The result showed that steam explosion pretreatment followed by enzymatic hydrolysis had high efficiency in converting monosaccharide sugar to ethanol.

Jeoh and Agblevor (2001) characterized and fermented steam exploded cotton gin waste. Steam explosion experiments was performed at severity from 2.02 to 4.96. Solubilization/degradation of material occurred during steam explosion. The pretreatment improved the enzyme hydrolysis of material from 42% to 67%. The highest yield of ethanol (83% of theoretical) was achieved for material treated at a severity of 3.56.

Sharma *et al.* (2001) pretreated the sunflower stalks by steam explosion and sodium hydroxide, and then, enzymatic hydrolysis was carried out on treated material. Steam explosion at 1.05 kg/cm^3 for 1.5 h was found to be optimum condition. Maximum enzymatic hydrolysis of 57.8% was observed by treating 5% substrate with T. *reesei* Rut-C 30 cellulase at 50 °C, pH 5 for 72 h.

Soderstrorm *et al*. (2003) experimented two-step steam explosion of softwood by dilute H_2SO_4 impregnation for ethanol production. The first step of steam explosion was performed to remove hemicellulose from wood and the pulp obtained from the first step was hydrolyzed to glucose by acid at the second pretreatment step. The effect of pretreatment was assessed using both SHF and SSF. The ethanol yield of feedstock after two-step pretreatment followed by SHF was higher than that from one step pretreatment, however the SSF gave lower yield.

Emmel *et al*. (2003) investigated the fractionation of *Eucalyptus grandis* chips by dilute acid catalyzed steam explosion. The experiments were carried out under various pretreatment conditions (200-210 °C, 2-5 min) after impregnation of the wood

chips in 0.087 and 0.175% H_2SO_4. The best condition for hemicellulose recovery was the pretreatment with 0.175% H_2SO_4 at 210 °C for 2 min. Lower pretreatment temperatures at 200 °C were good enough to yield steam treated substrates that 90% cellulose conversion was obtained in 48 h using Celluclast 1.5L plus Novozym 188.

Ballesteros *et al.* (2004) reported that simultaneous saccharification and fermentation (SSF) process for ethanol production from various lignocellulosic woody (poplar and eucalyptus) and herbaceous (*Sorghum* sp. bagasse, wheat straw and *Brassica carinata* residue) materials using the thermotolerant yeast strain. Biomass samples were previously treated in a steam explosion pilot plant to provide biomass with increased cellulose content that relatived to untreated materials and to enhance cellulase enzyme accessibility. The SSF experiment was performed in laboratory condition at 42°C, 160 h. The result showed that eucalyptus wood, wheat straw, sweet sorghum gave ethanol content of 17.0, 18.0, 16.0 g/L, respectively, within 72 h of fermentation.

Cara *et al.* (2006) studied the conversion of olive tree into fermentable sugars by steam explosion pretreatment in temperature range 190-240°C and further submitted to delignification by alkaline peroxide. The result showed that steam pretreatment at 190°C followed by alkaline peroxide delignification and enzymatic hydrolysis led to a sugar yield, readily available for bioconversion into ethanol.

Ohgren *et al*. (2007) studied the ethanol fuel production from steam-pretreated corn stover using SSF at higher dry matter content. This study was performed on steam-pretreated corn stover at 5, 7.5 and 10% water-insoluble solids (WIS) with 2 g/L hexose fermenting *Saccharomyces cerevisiae*. The results showed that SSF at 10%WIS gave 74% of ethanol yield based on the glucose content in the raw material.

Ruiz *et al*. (2008) studied the steam explosion pretreatment prior to enzymatic hydrolysis of sunflower stalks. The stalks were subjected to steam explosion pretreatment in the temperatures ranging between 180°C and 230°C. The steam-

exploded pulp was further hydrolyzed by enzyme. The result showed that after 96 h of enzymatic reaction, a maximum hydrolysis yield of 72% was obtained after pretreatment at 220°C, corresponding to a glucose concentration of 43.7 g/L in hydrolysis media. With regard to the filtrate analysis, most of the hemicellulosic derived sugars released during the steam pretreatment were in the oligomeric form. The highest recovery was obtained at 210°C of pretreatment temperature. Moreover, the utilization of hemicellulosic-derived sugars as a fermentation substrate would improve the overall bioconversion of sunflower stalks into ethanol fuel.

MATERIALS AND METHODS

1. Materials

1. Acetic acid, CH_3COOH 99.5% (J.T. Baker, USA)
2. Barium hydroxide, $Ba(OH)_2$ (Merck, Germany)
3. Calcium hydroxide, $Ca(OH)_2$ (Merck, Germany)
4. Celluclast 1.5 L (Novozyme, Denmark)
5. Di-Ammonium hydrogen phosphate, $(NH_4)_2HPO_4$ (Carlo Erba, Italy)
6. D-Glucose, $C_6H_{12}O_6$ (Merck, Germany)
7. D-Xylose, $C_5H_{10}O_5$ (Fluka, Switzerland)
8. Ethyl alcohol, C_2H_5OH 99% (Merck, Germany)
9. Ethyl alcohol, C_2H_5OH 95% (Merck, Germany)
10. Furfural (J.T. Baker, USA.)
11. Folin-cioculteus phenol reagent (Fluka, Switzerland)
12. 5-hydroxy methyl furfural (Sigma, Denmark)
13. Magnesium sulphate, $MgSO_4.7H_2O$ (APS, Australia)
14. Novozyme 188 (Sigma, Denmark)
15. Sodium acetate, CH_3COONa (APS, Australia)
16. Sulfuric acid, H_2SO_4 72% (J.T. Baker, USA)
17. Sodium carbonate, Na_2CO_3 (Merck, Germany)
18. Sodium dihydrogen phosphate, NaH_2PO_4 (Carlo Erba, Italy)
19. Sodium hydroxide, NaOH (Fluka, Switzerland)
20. Sugarcane bagasse (Mitpol sugar mill, Supunburi, Thailand)
21. Toluene, $C_6H_5CH_3$ (J.T. Baker, USA)
22. Tannic acid (Sigma, Denmark)
23. Yeast extract (Himedia, India)
24. Yeast *Saccharomyces cerevisiae* TISTR 5339 (TISTR, Thailand)

2. Equipments

1. Autoclave (Dectra, USA)
2. Balance 2 digits (Mettler Toledo, PB3002, USA)
3. Balance 4 digits (Mettler Toledo, PAE200, USA)
4. Centrifuge (Tomy, Japan)
5. Gas Chromatography (GL-Science, Japan)
6. High Performance Liquid Chromatography (Shimdzu, Japan)
7. Hot air oven (Binder, Germany)
8. Hot plate (Barndstead Electromal, EME6 0250/CEB, UK)
9. Shaking water bath (Vision scientific, Korea)
10. Sieve 425 μm (D-42759, 40 meshes, Retsch, Germany)
11. Sieve 250 μm (60 mesh, Endocoris, England)
12. Steam explosion (Nitto Koatsu Company, Japan)
13. UV/Visible Spectroscopy (Jasco, V530, Japan)

3 Methods

3.1 Experimental design

The experimental procedure of this research was schematically shown in Figure 13. The pretreatment of sugarcane bagasse for ethanol production was investigated. The technique of steam explosion was introduced as a pretreatment method. This research purposed to study two steps of steam explosion pretreatment for generation of suitable material for ethanol production. The first steam explosion step was employed to eliminate hemicelluloses portion, undesired polysaccharides, from the bagasse and also to alter the structure of material to accessible property. The residue or water insoluble fraction after the first pretreatment was subjected to perform again in the second steam explosion step.

Slurry from the two-step steam explosion, a mixture of liquid and water insoluble fractions, was assayed for the potential ethanol production using SSF process. In addition, liquid fraction which mainly contained fermentable glucose was also fermented to ethanol.

The effects of the two-step pretreatment in ethanol yield were compared with the one-step pretreatment. The solid fraction from the first steam explosion was used as substrate to produce ethanol using both SSF process and SHF process.

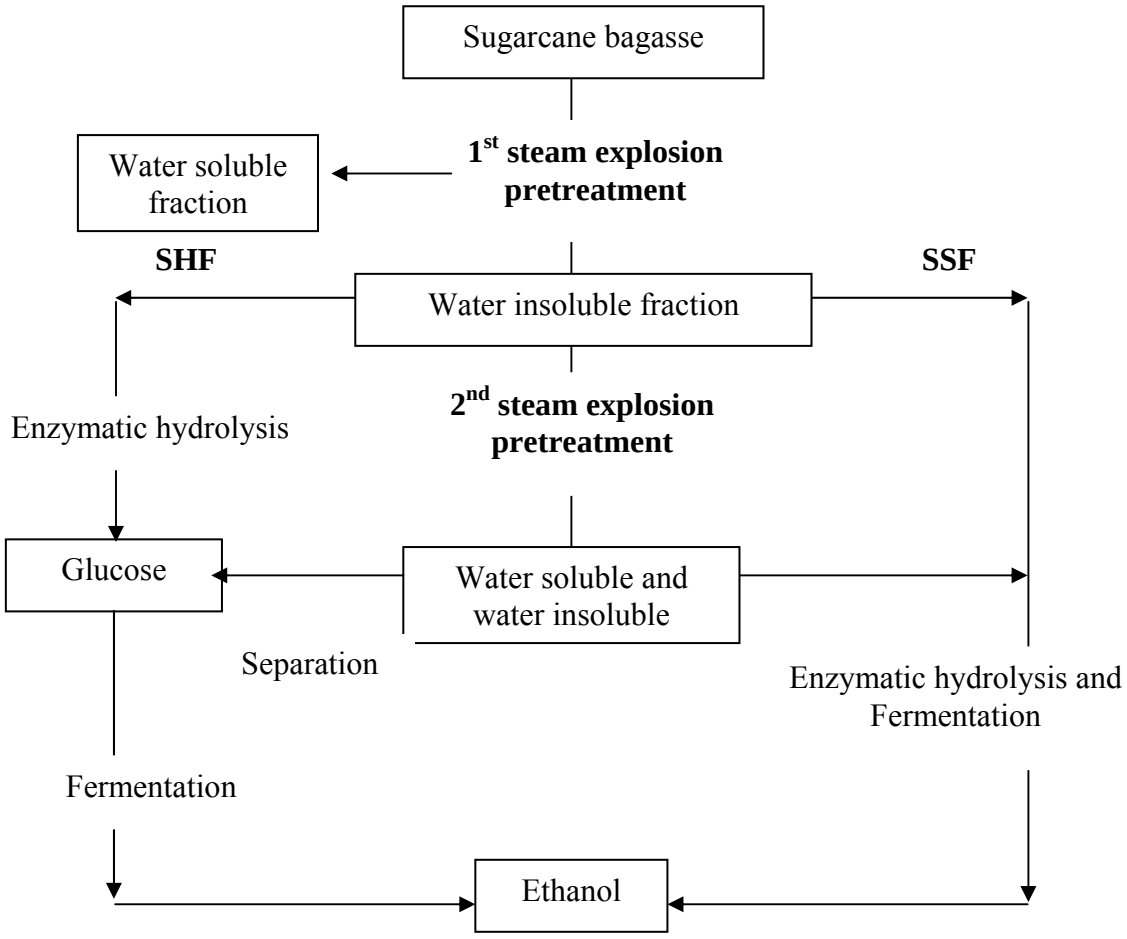

Figure 13 Experimental procedures for ethanol production from sugarcane bagasse

3.2 Sugarcane bagasse

Sugarcane bagasse used in the experiments was supplied by Mitpol Sugar Mill (Supunburi, Thailand). Four to six cm in length of bagasses was collected, air dried at room temperature and stored in plastic box until use. The chemical compositions, i.e. lignin, extractive, and ash of sugarcane bagasse were determined according to TAPPI test methods. Monosaccharides, composition of cellulose and hemicellulose, were measured by HPLC.

Figure 14 Sugarcane bagasse for experimental usage

3.3 Steam explosion pretreatment

3.3.1 The first steam explosion pretreatment

The steam explosion pretreatment of sugarcane bagasse was carried out in 2.5 L batch reactor of steam explosion apparatus (Nitto Koatsu Company, Japan). The 150 g of dried bagasse were loaded in the steam explosion reactor. Saturated steam from the boiler was then allowed to enter the reactor and heated the bagasse to desired temperatures (190, 200, 210 and 220°C). The temperature was maintained as a setting time (2, 3 and 4 min) and then the reactor was suddenly depressurized. After that, the steam exploded bagasse was recovered in

a receiver tank. The product was subjected to separation and analysis. Table 2 summarized the condition of the first steam explosion pretreatment.

Table 2 The condition of the first steam explosion pretreatment

Experiment	Residence time (mins)	Temperature (°C)	Pressure (bar)
1a	2	190	15.2
2a	3	190	15.0
3a	4	190	14.7
4a	2	200	17.2
5a	3	200	17.2
6a	4	200	17.2
7a	2	210	21.2
8a	3	213	21.1
9a	4	214	20.9
10a	2	220	23.0
11a	3	221	23.0
12a	4	221	23.0

In the separation step, the steam exploded products were separated into liquid and solid fractions using nylon bag filtration. The solid residue or water insoluble fraction (WIS) was subjected to remove any chemical byproducts and/or water soluble components by washing with 80°C distilled water for 30 min in to times and by cool water in one time. The washed WIS was air dried at room temperature for 3 days and weighed for calculation of solid recovery. The chemical compositions of solid residue were analyzed and the additional physical properties of these materials were also characterized for the possibility of ethanol production.

Figure 15 Steam explosion apparatus

In addition, the liquid fraction from the pretreatment was analyzed for glucose , xylose, and byproducts such as furfural, 5-hydroxy-methyl-furfural, acetic acid and phenolic compounds.

3.3.2 The second steam explosion pretreatment

The purpose of this section was to investigate the effect of second steam explosion pretreatment. The water insoluble fraction from the first pretreatment, in which a major part of hemicelluloses was removed and had a susceptible property to hydrolysis, was subjected to the steam explosion pretreatment again in the second step. In this step, the addition of acid catalyst was studied in order to increase the rate of hydrolysis. Dried solid residue loading was 100 g per batch. Before the second pretreatment, the sample was impregnated in dilute sulfuric acid at the concentrations of 0.051, 0.102, 0.153 and 0.204 M for 18 h with 1:15 (w/v) of solid and liquid ratio. When the impregnation time had elapsed, the excess liquid was removed by squeezing. The material that had a moisture content of 200% and amount

of acid of 1%, 2%, 3% and 4% was re-steam exploded at temperature 210°C for 2.5 and 5 min. Condition of the second steam explosion pretreatment was shown in Table 3.

Table 3 The condition of the second steam explosion pretreatment

Experiment	Concentration of sulfuric/material (% w/w)	Residence time (mins)	Temperature (°C)	Pressure (bar)
1b	1	2.5	210	18.5
2b	1	5	210	18.5
3b	2	2.5	210	18.5
4b	2	5	210	18.5
5b	3	2.5	210	18.5
6b	3	5	210	18.5
7b	4	2.5	210	18.5
8b	4	5	210	18.5

The product from receiver tank was volumetric adjusted to 6.5 L with water and a portion of this slurry was separated into the solid and liquid fractions for component analysis. The optimal condition which generated maximum glucose and minimum byproducts such as acetic acid, formic acid, furfural and 5-HMF was required. The liquid fraction was studied for ethanol production and the slurry was taken to study The ethanol production using SSF process.

3.4 Characterization of sugarcane bagasse and steam exploded sugarcane bagasse

3.4.1 Scanning electron microscope (SEM)

Morphological structures of the raw material and water insoluble fractions from one-step and two-step steam explosion pretreatment were characterized using a scanning electron microscope (SEM). The SEM image was used to investigate microfibril structure and surface topography in order to evaluate the effect of pretreatment on lignocellulosic materials. Prior to the scanning, the sample was coated with gold. The images were taken with enlargement of 25X, 100X and 200X.

3.4.2 Thermogravimetric analysis (TGA)

The analysis of thermogravimetric was used to investigate the degradation characteristic of the steam exploded and un-exploded sugarcane bagasse. The TGA curves indicated the heterogeneous property of materials. The 7 Series Thermal Analysis System (Perkin-Elmer, USA) was used to analyze the thermal stability of sample with a heating rate of 10°C/min in nitrogen environment.

3.5 Separate hydrolysis and fermentation

Separate hydrolysis and fermentation process (SHF) was performed for the ethanol production of water insoluble fraction from the first pretreatment step. In the conversion step of cellulose to monomeric sugars, the residues of bagasse were enzymatically hydrolyzed. Additionally, liquid fractions from the second step of pretreatment were also used as substrates for fermentation.

3.5.1 Hydrolysis of cellulose

For enzymatic hydrolysis, 4 g dry matter (DM) of pretreated sugarcane bagasse was placed in 500 mL Erlenmeyer flask and then diluted with 0.1 M acetate buffer solution (pH 4.8) to a final concentration of 2% by weight. Celluclast 1.5L, commercial cellulase enzyme solution with the activity of 74 FPU/mL, supplemented with Novozyme 188 (365 β-glucosidase IU/mL) were applied in the experiment with enzyme loading at 0.8 and 0.16 mL, respectively, to obtain 15

FPU cellulase and 15 IU β-glucosidase per one gram of substrate. The hydrolysis was carried out in duplicate with 150 rpm shaking at 50ºC for 72 h. The hydrolysate was taken after 72 h, filtered and analyzed by HPLC for monosaccharide compositions.

The slurry products from the second step of pretreatment were filtered to provide the liquid fraction which was glucose rich solution. Monosaccharides and byproducts were analyzed and neutralized by overliming (the addition of calcium hydroxide to pH 10 and then lower to pH 5 by sulfuric acid). The samples were kept freezing until used.

3.5.2 Fermentation of glucose

Fermentation was performed with a working volume of 50 mL in 100 mL Erlenmeyer flask. The hydrolysate solution were concentrated to over 62.5 g of glucose/L by evaporator under pressure and then adjusted to pH 5.5 with 20% (w/w) $Ca(OH)_2$ together with volumetric adjusted to glucose concentration of 62.5 g/L. The concentration of fermentable solution in volume of 40 mL was sterilized in autoclave for 20 min before used. Nutrients, consisting of 5 g/L $(NH_4)_2HPO_4$, 0.25 g/L $MgSO_4.7H_2O$, 1.2 g/L NaH_2PO_4 and 10 g/L yeast extract, were prepared and subjected to sterilization before used.

In order to produce cell mass of baker's yeast, *S. cerevisiae* was cultured in batch cultivation (50 g/L glucose, 0.5 g/L $(NH_4)_2HPO_4$, 0.025 g/L $MgSO_4.7H_2O$, 1.2 g/L NaH_2PO_4 and 1 g/L yeast extract). The culture were incubated at 30ºC for 24 h. After that, the cell were separated by centrifugation at 6000 rpm for 15 min, washed with sterile water, suspended in sterile water and adjusted to cell concentration of 50 g/L.

In fermentation process, nutrients and yeast solution were added to fermentation flask containing fermentable glucose. Working volume consisted of 50 g/L glucose, 0.5 g/L $(NH_4)_2HPO_4$, 0.025 g/L $MgSO_4.7H_2O$, 1.2 g/L NaH_2PO_4, 1

g/L yeast extract and 5 g/L yeast. The fermentation flasks were sealed with stopper connected with hypodermic needle for the removal of the released CO_2. The fermentation was performed at 30ºC for 72 h. Ethanol obtained from the fermentation process was analyzed by GC.

3.6 Simultaneous sacchaification and fermentation

The slurry from the two-step steam explosion pretreatment and the solid fraction of the one-step pretreatment were investigated as substrates for ethanol production. In the SSF process, hydrolysis and fermentation steps were simultaneously performed in the same reactor, thus, the conditions were adjusted to be suitable for the activity of both enzyme and microorganism.

For the water insoluble fraction from the first pretreatment, SSF experiment was carried out in a 250 mL Erlenmeyer flask containing 5% DM of material. Five grams of pretreated bagasse were placed in a flask, 80 mL of 0.1 M acetate buffer solution (pH 5) was added and then the fermentation flask was sterilized. One mL Celluclast 1.5L and 0.2 mL Novozyme 188 were loaded in the fermentation flask. Then, 10 mL nutrients and 10 mL yeast solution were added to a flask. The final concentration of fermentation solution was 50 g/L pretreated bagasse, 0.5 g/L $(NH_4)_2HPO_4$, 0.025 g/L $MgSO_4.7H_2O$, 1 g/L yeast extract and 5 g/L yeast. The fermentation flasks were sealed with stopper connected with a hypodermic needle for the removal of the released CO_2. The fermentation was performed at 37ºC for 72 h. Ethanol obtained from the fermentation process was analyzed by GC.

In ethanol production of the slurry from the two-step pretreatment, 325 mL of slurry were concentrated to the volume less than 80 mL. Its pH was adjusted to 5 by 20% (w/w) $Ca(OH)_2$ whereas total volume was maintained at lower than 80 mL. The fermentation flask was led to sterilization and reaction was performed as same as for the solid residue from the one-step pretreatment as described previously.

3.7 Analytical method

3.7.1 Preparation of solid sample for chemical analysis

The sugarcane bagasse and pretreated sugarcane bagasse were analyzed for their compositions before used. Samples were ground to a fine particle size in the grinding mill. The desired material was sized by sifting on a 40-mesh and 60-mesh screens. The materials which stayed on a 60-mesh screen were applied for a complete reaction with the reagent. The extractive compounds in sugarcane bagasse raw material were removed by solvent extraction. About 10 g of sample was extracted with 200 mL of toluene-ethanol (2:1) for 6 h in the Soxhlet apparatus. After that, the sample was transfered to suction in order to remove the excess solvent. The sample was returned to the Soxhlet apparatus and extracted again with 200 mL ethanol for 6 h. After removing the excess solvent, the samples were extracted in Erlenmeyer flask with boiling distilled water, heated for 1 h and then led to filtration and air-dried.

3.7.2 Moisture content

Two grams (± 0.001) of sample in a weighing bottle were dried for 2 h at 105ºC in the laboratory oven. The dried sample was cooled in a desiccator and weighed. The process was repeated until a constant weight was obtained. The moisture content was then calculated by dividing the moisture loss with the weight of dried sample and multiplying by 100.

3.7.3 Extractives compounds

The milled material (1-5 g) was extracted with toluene-ethanol (2:1) in a Soxhlet apparatus for minimum of 6-8 h. After that, solvent was removed by evaporation in the evaporator at temperature less than 60ºC, dried in an oven for 1 h and cooled in a dessiccator. This procedure was repeated until a constant weight was

obtained. The extractive content was calculated by dividing with the sample weight and multiplying by 100.

3.7.4 Ash content

The empty crucible was carefully cleaned and ignited in a muffle furnace at 525±25ºC for 30-60 min. After that, the crucible was cooled slightly to room temperature in a dessicator and weighed. The extractive free milled material was weighed at least 1 g and put into the crucible. The crucible was placed in muffle furnace at temperature about 100ºC, and the temperature was slowly raised to 525ºC with ignition time about 6 h. When the combustion was complete, the crucible was removed from the muffle furnace. Then, it was placed in a dessicator and cooled to room temperature. The crucible with ash was re-weighed and the ash content was calculated by dividing with the sample weight and multiply by 100.

3.7.5 Acid insoluble lignin content

The 1±0.1 g of extractive free milled material was weighed in the beaker and 15 mL of 72% H_2SO_4 was added into beaker at 2°C. The mixture was mixed and continuously hydrolyzed at 25^0C for 2 h. To ensure the complete reaction, the solution was poured into 3% H_2SO_4 and the final volume of 575 mL was made up with distilled water. The reaction was refluxed for 4 h. After that, the refluxed solution was allowed to stand overnight before it was filtered through a glass filter crucible No.4. The residue was washed with hot water and dried overnight at 100±5°C in oven. After that, it was placed in a dessicator for 1 h. The lignin content was calculated by dividing with the sample weight and multiplying by 100.

3.7.6 Total phenolic compounds

The standard curve of tannic acid was prepared from 1, 2, 3, 4 and 5 ppm. The 0.5, 1, 1.5, 2.0 and 2.5 mL of 100 ppm tannic acid were mixed with 1

mL of folin-ciocalteu phenol reagent in 50 mL volumetric flask. Then, 5 mL of 20% Na_2CO_3 were added to the flask and adjusted to 50 mL with water. The mixture was thoroughly mixed and maintained for 20 min. The absorbance was measured by UV-Vis spectrophotometer at 735 nm. For sample analysis, 1 mL of sample was used instead of standard tannic acid. The total phenolic compounds were presented in ppm unit.

3.7.7 Sugar content

3.7.7.1 From solid fraction

The monosaccharide content in solid residue was determined. The first step of hydrolysis was performed with 4 ml of 72% (w/w) H_2SO_4 at 30°C for 60 min. In the second step, the reaction mixture was performed in 3% (w/w) H_2SO_4 and subsequently autoclaved at 121°C for 1 h. The 10 mL of filtrate was pipetted to the beaker and pH was adjusted to 7 with saturated $Ba(OH)_2$ solution. The samples were centrifuged at 8500 rpm to separate solution from sludge. The solution was further filtered through 0.45 μm cellulose filter before injected onto the HPLC.

3.7.7.2 From liquid fraction

The sugar solution of liquid phase sample from pretreatment was determined for monomer content like the solid residues. For oligomeric analysis, the sample was hydrolyzed with 4% H_2SO_4 in the autoclave for 1 h before used. The 10 ml of sugar solution were pipetted to the beaker and pH was adjusted to 7 with $Ba(OH)_2$ solution. The samples were centrifuged at 8500 rpm to separate solution from solid. The solution was further filtered through 0.45 μm cellulose filter before injected into the HPLC.

Condition :

Equipment	:	High Performance Liquid Chromatography (Shimadzu, Japan)
Column	:	Aminex HPX-87C column(Bio- Rad)
Detector	:	Refractive index (RI) detector
Mobile phase	:	Deionized water
Oven	:	80 ºC
Flow rate	:	0.6 mL/min
Analysis method	:	External standard

3.7.8 Furfural and 5-hydroxy-methyl-furfural

The analysis of furfural and 5-hydroxymethyl furfural was performed on HPLC. Before injection, the solution was filtered through 0.45 μm cellulose filter, diluted to suitable concentration and subjected to HPLC.

Condition :

Equipment	:	High Performance Liquid Chromatography (Shimadzu, Japan)
Column	:	Aminex HPX-87H column(Bio- Rad)
Detector	:	UV detector, 280 nm
Mobile phase	:	0.005 M sulfuric acid
Oven	:	65ºC
Flow rate	:	0.6 mL/min
Analysis method	:	External standard

3.7.9 Acetic acid

The analysis of acetic acid was performed on HPLC. Before injection, the solution was filtered through 0.45 μm cellulose filter, diluted to suitable concentration and subjected to HPLC.

Condition :

Equipment	:	High Performance Liquid Chromatography (Shimadzu, Japan)
Column	:	Aminex HPX-87H column(Bio- Rad)
Detector	:	UV detector, 210 nm
Mobile phase	:	0.005 M sulfuric acid
Oven	:	65 ºC
Flow rate	:	0.6 mL/min
Analysis method	:	External standard

3.7.10 Ethanol analysis

1 µL of the solution was injected into GC. Before injection, the samples were centrifuged at 8500 rpm to separate solution from solid, filtered through 0.45 mm cellulose filter. 50 µL of sample solution were mixed with 50 µL of internal standard (n-propanol), diluted to 500 µL and then injected to the GC.

Condition :

Equipment	:	Gas Chromatography GL-4000 (GL-Sciences**,** Japan**)**
Column	:	GL-4000 (Chromosorb- 103 60/80) Glass column 3 m x 3 mm ID, 6.4 mm OD
Carrier gas	:	He
Flow rate	:	2 mL/min
Pressure	:	215 kPa
Detector	:	FID (Flame ionization detector) at 250°C
Oven	:	185°C
Injection	:	Standard and samples (1 µL) were injected to injection port at 250°C.

RESULTS AND DISCUSSION

1. Sugarcane bagasse

The chemical compositions of sugarcane bagasse were shown in Table 4. First, raw sugarcane bagasse was determined for the extractive content by toluene/ethanol extraction method to give the value of 3.70% on dried bagasse basis. After that, the extractive-free materials were analyzed for the chemical components. Glucose and xylose content, attributed to cellulose and hemicelluloses fraction presented in the material, respectively, were measured by HPLC technique. Sugarcane bagasse mainly consisted of 41.02% (w/w) glucose and 34.60% (w/w) xylose. Unless carbohydrate fraction, sugarcane bagasse was also composed of 20.83% (w/w) lignin, 1.11% (w/w) ashes and small amount of other compounds. Pendey *et al.* (2000) reported the main components of sugarcane bagasse as 50% cellulose, 25% hemicellulose and 25% lignin. The presence of high cellulose content (in glucose form) indicated the possibility of its used as feedstock for ethanol production. However, the remaining hemicellulose and lignin in bagasse were trouble because these were inhibitors and/or resistant to the conversion process. Therefore, the fractionation and alteration of sugarcane bagasse structure using the pretreatment process were required.

Table 4 The chemical compositions of sugarcane bagasse

Components	Percentage (w/w)*
Extractive in toluene/ethanol	3.70
Lignin	20.73
Ash	1.11
Glucose (cellulose)	41.02
Xylose (hemicellulose)	34.60

* based on the sugarcane bagasse weight

2. The steam explosion pretreatment

2.1 The first steam explosion pretreatment

The first steam explosion pretreatment of sugarcane bagasse resulted in water insoluble and liquid fraction as shown in Figure 16. This mixture was separated by filtration using nylon mesh cloth. The recovery and compositions of both solid and liquid fractions were expressed as percentages based on bagasse raw material. Additionally, the structural change of sugarcane bagasse after the pretreatment was characterized.

Figure 16 The product mixture derived from the first steam explosion of sugarcane bagasse

The first pretreatment of sugarcane bagasse was designed to perform at various temperatures, 190ºC, 200ºC, 210ºC and 220ºC, and retention times, 2, 3 and 4 min. Severity of reaction condition was expressed in terms of severity factor (R_0 or log R_0) when R_0 related to temperature (T) and retention time (t). The reaction ordinate concept was proposed by Overend and Chornet who reported it as a useful measure of reaction conditions during steam explosion processing (Kaar *et al*, 1998). The relationship between T and t was shown in the following equation :

$$R_0 = \int_0^t \exp\left[(T - 100/14.75)\right] dt$$

$$\log R_0 = \log \{t * \exp[(T - 100)/14{,}75]\}$$

when R_0 = Severity factor

T = Reaction temperature, ºC

t = Retention time, minute

Table 5 summarized the pretreatment conditions and severity factors ($\log R_0$) used in this study.

Table 5 The condition and severity factor of the first steam explosion pretreatment

Experiment	Retention time (mins)	Temperature ºC	$\log R_0$
1a	2	190	2.95
2a	3	190	3.13
3a	4	190	3.25
4a	2	200	3.25
5a	3	200	3.42
6a	4	200	3.55
7a	2	210	3.54
8a	3	213	3.80
9a	4	214	3.96
10a	2	220	3.83
11a	3	221	4.04
12a	4	221	4.16

During the first steam explosion pretreatment, some portion of the sugarcane bagasse was removed, therefore resulting in 68.77-53.19% solid recovery

as summarized in Figure 17. The result indicated the effect of severity condition on solid recovery. Higher severity factor resulted in lower solid content. This could be a consequence of the solubilization of bagasse during pretreatment. Low molecular extractives were extracted from the materials as same as that cellulose and hemicellulose were solubilized through the hydrolysis reaction (Varga *et al*., 2004). In addition, lignin fraction was also depolymerized by the influence of steam explosion. However, it did not seem to be significant due to that the breakdown reaction slowly occurred and the degraded products could then repolymerize. Loss of solid fraction was also considered due to the deposition of material in steam explosion reactor or during the separation and washing steps.

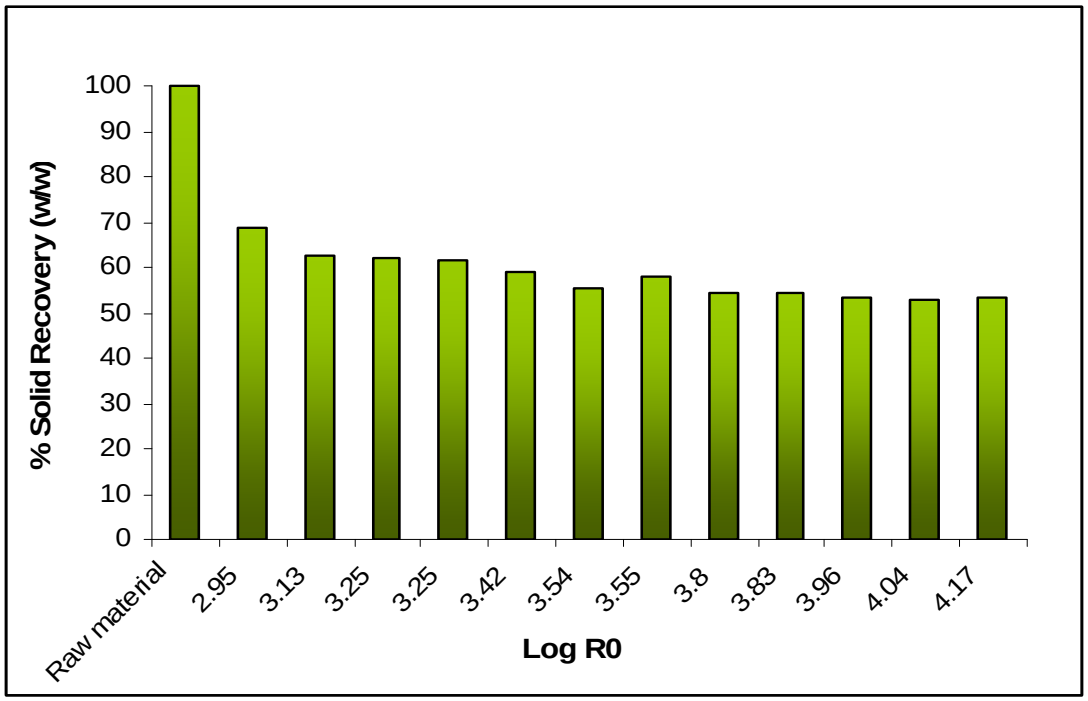

Figure 17 The solid recovery after the first steam explosion pretreatment

Figure 18 showed the photographs derived from steam-exploded sugarcane bagasse after the first pretreatment. Images A, B, C and D were the fiber materials from the pretreatment conditions with log R_0 3.25, 3.55, 3.96 and 4.16, respectively. These materials were subjected to chemical composition analysis.

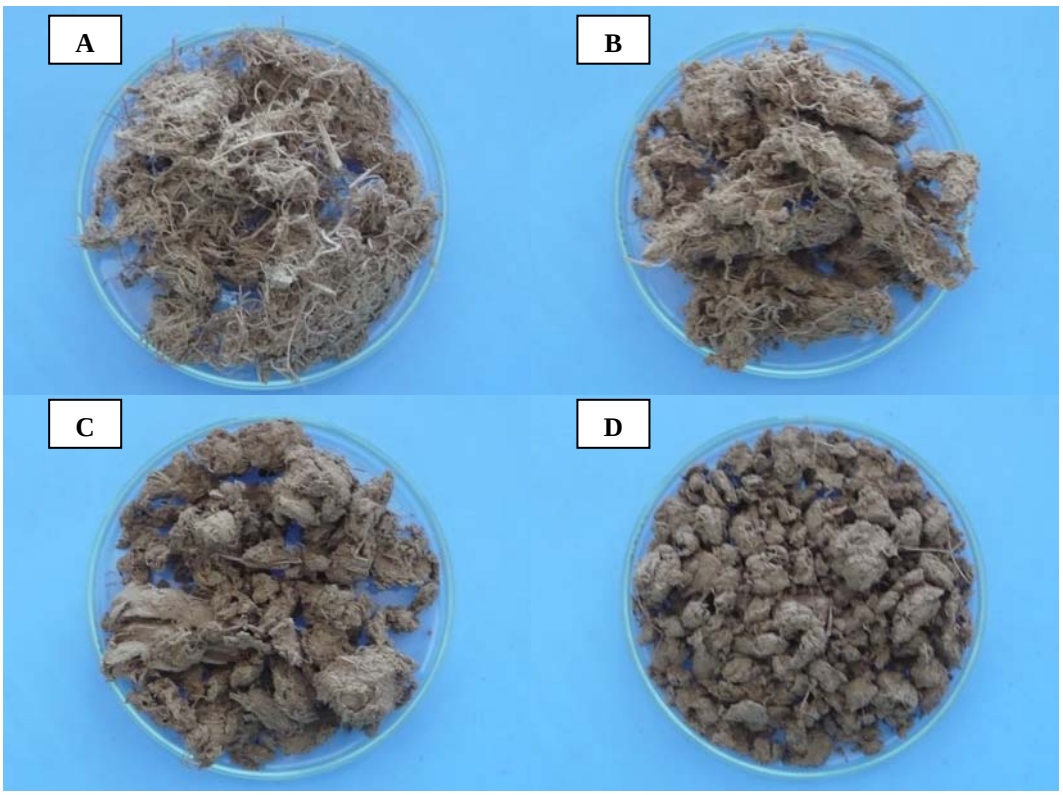

Figure 18 Water insoluble fractions of steam exploded sugarcane bagasse after the first steam explosion pretreatment. Images A, B, C and D were the fiber materials from the pretreatment conditions with log R_0 3.25, 3.55, 3.96 and 4.16, respectively

Figure 19 summarized the compositions of water insoluble fraction after pretreatment at various severity conditions. The data provided the amount of main components present in the fiber residues including cellulose (in glucose form), hemicellulose (in xylose form), lignin and inorganic component (ash). The contents of glucose were higher than xylose, and slightly increased (49.29% - 56.51%) with an increased severity condition. In contrast, xylose was greatly decreased from 19.49% to 6.54% with an increased severity condition. These results could be described by the difference in hydrolysis mechanism of cellulose and hemicellulose. Polymers of cellulose and hemicellulose were degraded from the bagasse material through hydrolysis reaction. This reaction mostly occurred in hemicellulose, due to its less

order structure and low molecular weight, thus resulting in the structure with easy hydrolysis.

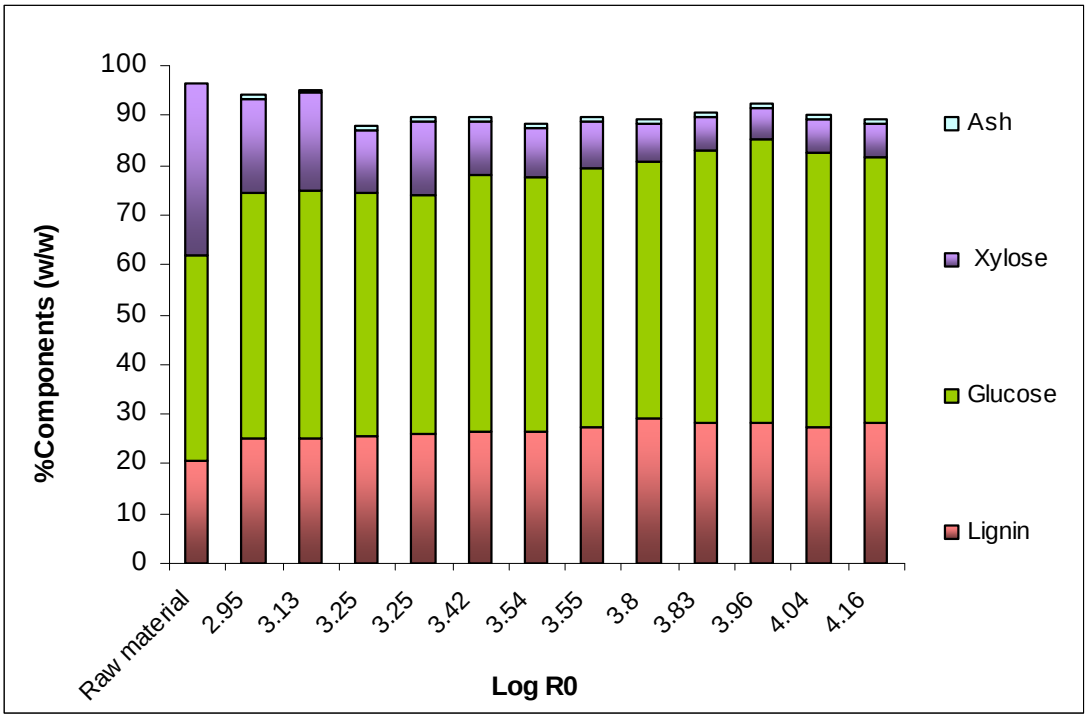

Figure 19 The chemical compositions of solid fraction after the first steam explosion pretreatment

Unlikely, cellulose contained high crystalline structure and high degree of polymerization in its structure, therefore, it was more resistant to the breakdown. For acid insoluble lignin, an increase in severity condition led to the increased lignin values. However, the 3-dimension rigid structure of lignin made it difficult to depolymerization. Moreover, the recondensation of these compounds also occurred, thus it seemed that lignin was not significantly solubilized during the pretreatment. The residues after pretreatment contained 24.92 to 28.95% remaining lignin.

The data shown in Figure 20 was the components recovery of pretreated sugarcane bagasse. In solid fraction, lignin recovery slightly reduced from the initial and varied from 17.16% to 14.46%. This data indicated that most lignin in sugarcane

bagasse was not solubilized during pretreatment. Likely, the content of glucose was not significantly affected under any condition. This could be because cellulose was hardly hydrolyzed. Removal of xylose that was greatly affected by the steam explosion pretreatment was obvious as it reduced from initial xylose 34.06 % to 13.20% with an increase in severity condition.

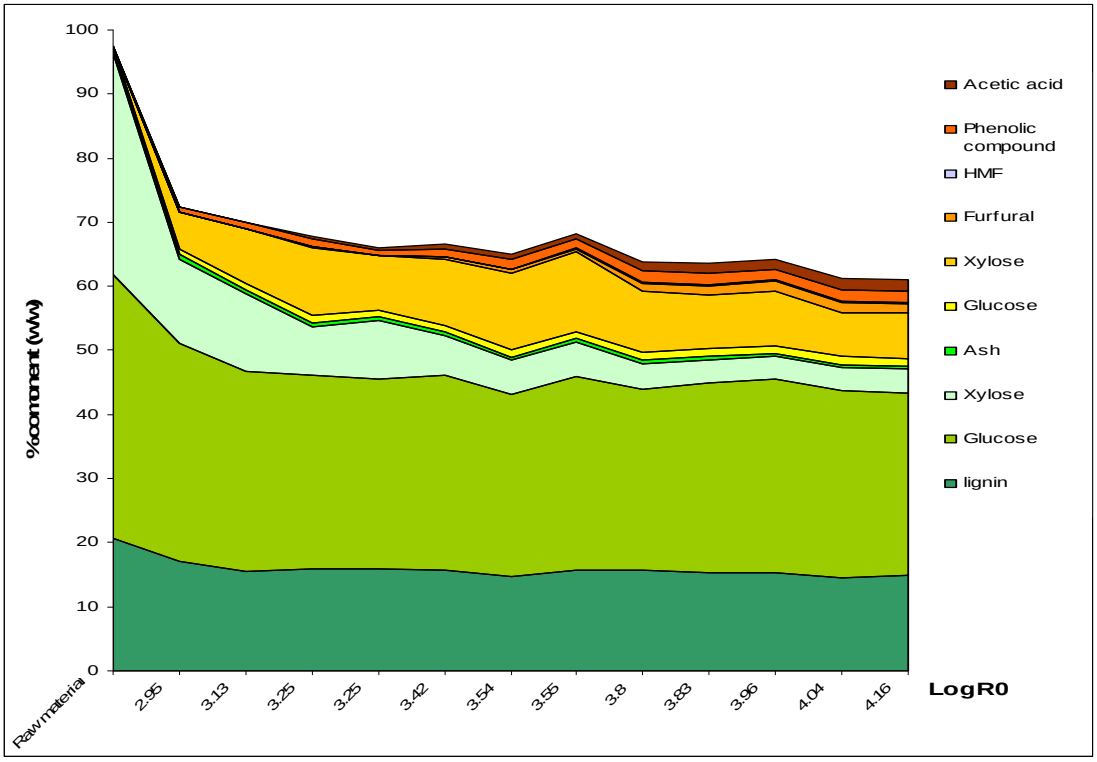

Figure 20 The components recovery of solid and water soluble fraction after the first steam explosion pretreatment

The compositions of liquid fraction after the first steam explosion pretreatment were shown in Figure 20. It consisted basically of sugar mixture, sugar degradation products, phenolic compounds and organic acids. The main sugars were xylose and glucose. Xylose, a dominant sugar in hemicellulose was highly present in both oligomeric and monomeric forms. Pretreatment under harsher condition resulted in higher total xylose solubilizing. The conditions with severity factor between 2.95 to 3.55 provided xylose in oligomeric form more than monomeric form. However, under the conditions with severity factor above 3.55, the oligomeric xylose was decreased.

The loss of xylose in various severe conditions could be decribed by the degradation of sugar, the severity condition of steam explosion was not only effective on the hydrolysis reaction but it also resulted in the degradation reaction. Higher severe condition resulted in higher conversion of oligomeric sugars to monomeric sugar. At the same time, monomeric sugars could be also degraded to degraded products such as furfural.

Beside the soluble sugars, various degraded products were also detected. Figure 20 showed the non-sugar compositions found in the liquid fraction such as acetic acid, furfural, HMF and phenolic compounds. The sugar degradation occurred through the mechanism of dehydration, the degradation product of xylose was furfural while degraded glucose was 5-hydroxy-methyl-furfural (HMF). Producing of furfural and HMF highly depended on the severity factor. When increased the severity factor, the content of furfural was increased (0% to 1.64%) like HMF (0.01% to 0.22%). These results contributed to the higher severity factor, the rate of dehydration reaction higher too.

Acetic acid was one of the main compound those was observed in the liquid fraction. Acetic acid was liberated from acetyl groups in hemicelluloses structure. It was usually used as an intra-catalyst for the reaction during steam explosion. In mild condition, amount of acetic acid increased with an increase of severity factor and it was rather stable under harsh condition. Small amount of lignin could be also solubilized to phenolic compounds. It was present in the liquid fraction ranging from 0.76 to 1.83% and varying with the severity factor. All of these byproducts showed to act as inhibitors for fermentation microorganisms (Larsson *et al*., 1999), thus, it was important to completely remove the liquid fraction from the solid residue before used as the material for the second steam explosion pretreatment.

The steam explosion pretreatment resulted in the structural change as well as the chemical composition change of sugarcane bagasse. The effects of pretreatment, hydrolysis and mechanical shearing on the structure of materials were characterized by various techniques. The surface morphology was characterized by

the Scanning Electron Microscope (SEM) whereas the thermal property was investigated using TGA photogram.

Figure 21 The photograph of sugarcane bagasse prior to the pretreatment

Changes in the morphological structures of sugarcane bagasse before and after steam explosion process were observed with SEM image. Figure 22 was sugarcane bagasse prior to the pretreatment. Most of the nontreated sugarcane bagasse were 3-5 cm in length, cellulose microfibril was associated with hemicellulose and surrounded by lignin seal. After the pretreatment, most of hemicellulose and part of lignin and cellulose were removed (Alemdar and Sain, 2007) from sugarcane bagasse as supported by the data of the chemical changes in previous topic. Fibers of steam exploded sugarcane bagasse appeared to be distorted, the microfibrils were separated from the initial connected structure and fully exposed. This cause owed to increase the external surface area and the porosity to enhance the conversion of cellulose to glucose (Xu *et al.*, 2007). The severity of steam explosion was effective to increase the accessible surface. Figure 21 showed the SEM images of sugarcane bagasse and steam-exploded sugarcane bagasse. The image A was nontreated sugarcane bagasse, cellulose fibril was mostly covered with cementing components. When it was pretreated by mild conditions of steam explosion ($logR_0$ 2.95 and $logR_0$ 3.25), the breakdown of fibril and the removal of lignin and hemicellulose occurred at lower rate. This result contributed to low surface area and porosity. The use of more severe

condition ($logR_0$ 3.54, $logR_0$ 3.96 and $logR_0$ 4.16) improved the removal of sealing region. The fiber was broken down and disordered much more than that in milder pretreatment. The pretreatment at $logR_0$ 3.96 and more severe condition provided the material with less in fiber and order. All results had led to that higher severity factor made higher accessible surface area, and thus further to higher hydrolysis rate.

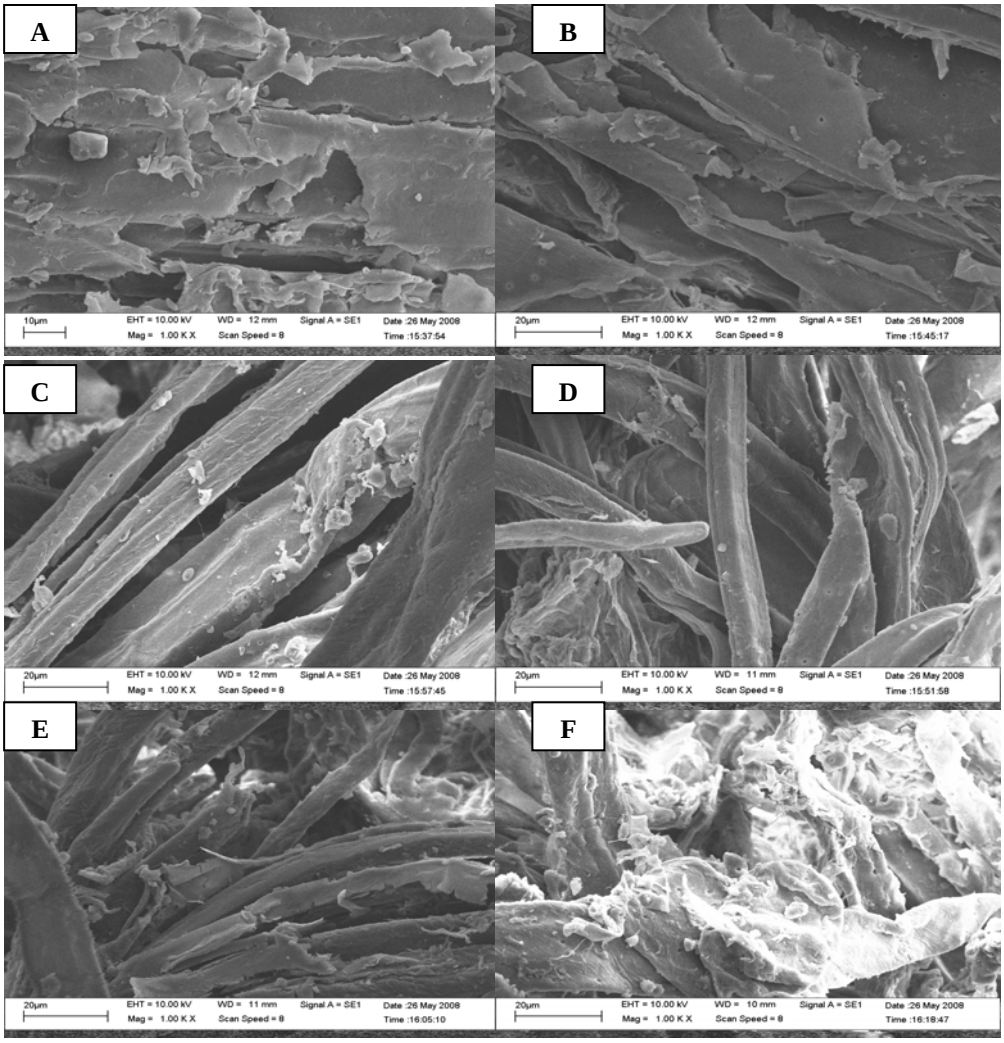

Figure 22 The SEM images of sugarcane bagasse and steam-exploded sugarcane bagasse. The images A, B, C, D, E and F were nontreated sugarcane bagasse, treated sugarcane bagasse at $logR_0$ 2.95, $logR_0$ 3.25, $logR_0$ 3.54, $logR_0$ 3.96 and $logR_0$ 4.16, respectively

Thermal analysis was performed for characterization of heterogeneous organic material. The thermal degradability was affected by the chemical composition of the material, different components resulted in different thermal behaviors (Negro *et al*, 2003). Figure 23 illustrated typical TGA curves of sugarcane bagasse (A) and steam explosion residues obtained from the conditions at $logR_0$ 3.25 (B), $logR_0$ 3.96 (C) and $logR_0$ 4.16 (D), respectively. The decomposition of sugarcane bagasse took place in several steps. The losses of moisture and extractive first occurred and then hemicellulose and cellulose started to decompose at 250ºC and 300ºC, respectively. Lignin was decomposed at temperatures ranging from 330ºC to 520ºC. The residue from the pretreatment at $logR_0$ 3.25 had similar TGA curve with the untreated material, due to that it contained similar components. Under high severe condition of pretreatment, the remaining components were cellulose and lignin. TGA curves indicated two main steps of decomposition, the first was cellulose and the next was lignin. A shift of decomposition temperature of cellulose and lignin could be described by the increase in order structure of cellulose and the formation of more thermo-labile lignin after pretreatment (Negro *et al*, 2003). This indicated that the steam-exploded material had higher thermal stability than untreated raw materials.

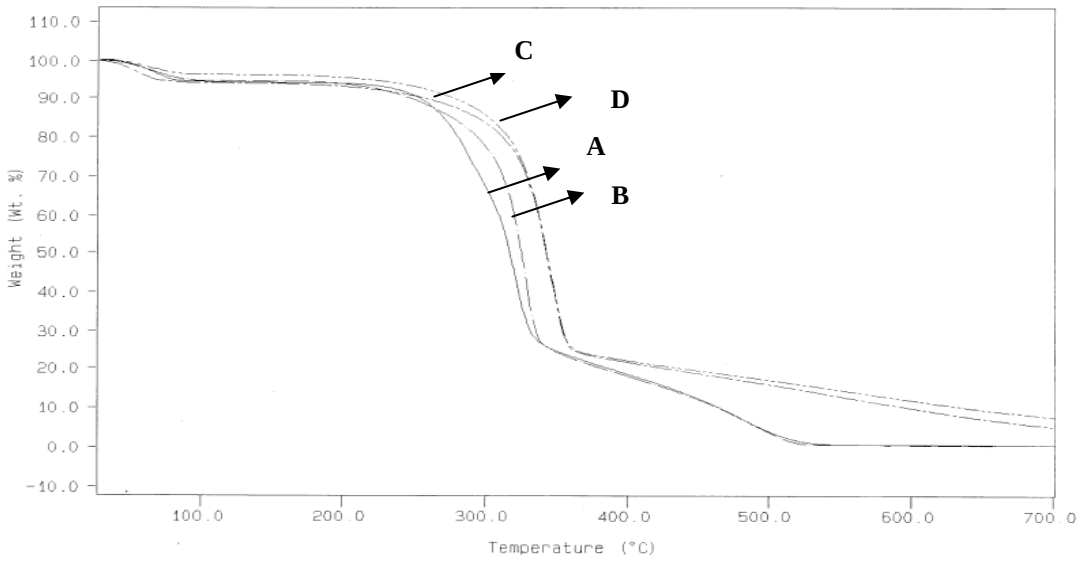

Figure 23 The TGA curve of sugarcane bagasse and pretreated sugarcane bagasse

2.2 The second steam explosion pretreatment

The water insoluble material containing least amount of hemicellulose (214ºC, 4 min) from the first pretreatment was subjected to the second steam explosion step. For this study, dilute acid catalyst was added to aid the hydrolysis reaction.

Twelve batches of steam explosion at optimum condition (the first pretreatment) were performed in order to obtain sufficient material for the second step of steam explosion. After that, the combined residues from all 12 batches were impregnated in dilute sulfuric acid and subjected to the second step of steam explosion.

Figure 24 The products from the second steam explosion pretreatment

Products from the second steam explosion pretreatment were in the slurry form. The fibrous was not obvious, however, it appeared as the powder. Solid fraction was separated from the hydrolysate by filtration through filter paper no.1. Two phases of the products were analyzed for the remaining solid and chemical compositions analysis. The hydrolysate was further studied for ethanol production. A portion of slurry product was not separated and was also used as raw material in the ethanol production.

Table 6 The condition and severity factor of the second steam explosion pretreatment

Experiment	Temperature (ºC)	Retention time (mins)	Log R_0	% H_2SO_4 (w/w)
1b	210	2.5	3.64	1
2b	210	5	3.94	1
3b	210	2.5	3.64	2
4b	210	5	3.94	2
5b	210	2.5	3.64	3
6b	210	5	3.94	3
7b	210	2.5	3.64	4
8b	210	5	3.94	4

The second steam explosion pretreatment was performed at the temperature 210ºC with the residence times at 2.5 and 5 min. Table 6 shows the conditions and severity of the second steam explosion. The severity factors ($\log R_0$) were 3.64 and 3.94 for the experiments performed at 210ºC for 2.5 and 5 min, respectively. The severity of the second pretreatment was affected not only by the temperature and residence time, but also by the amount of sulfuric acid catalyst. This study investigated the influence of sulfuric acid concentration (1%, 2%, 3% and 4% of dry weight of raw material) on the steam explosion reaction.

The remaining solid after the second steam explosion of pretreatment varied with various pretreatment conditions as shown in Figure 25. The solid content decreased with an increase in severity factor and acid addition. Weight loss of the material after pretreatment mainly owed to the solubilizing of cellulose. Cellulose was mostly released from the material because its structure was preliminarily altered by the first steam explosion pretreatment, thus, it made easy to the hydrolysis reaction.

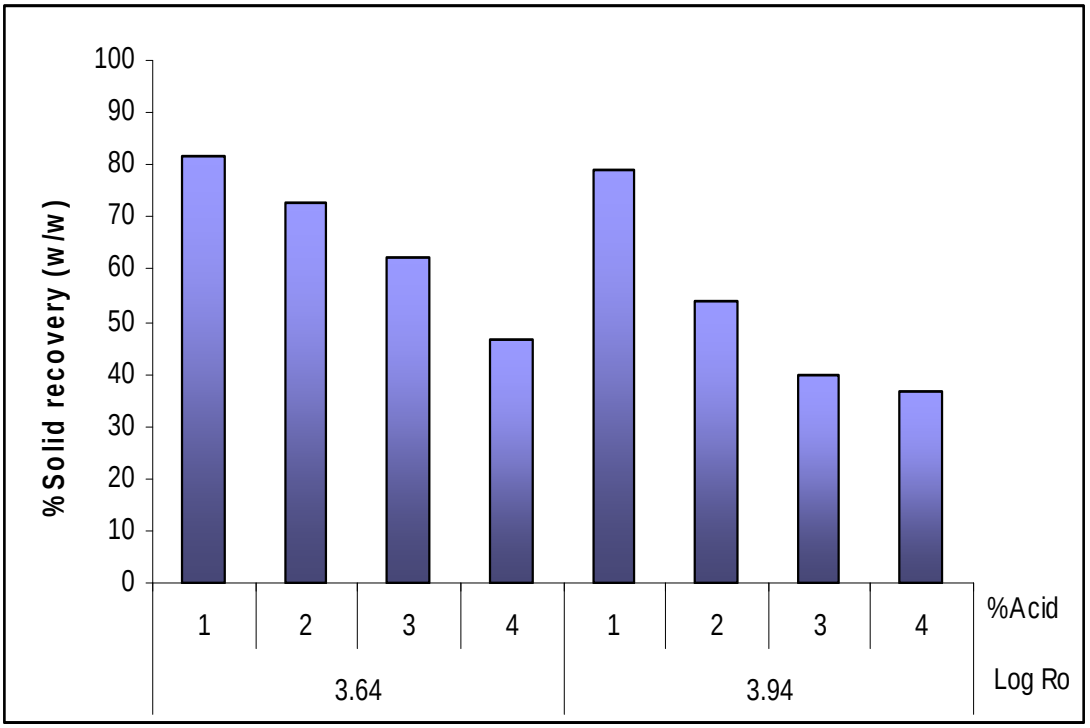

Figure 25 The solid recovery after the second steam explosion pretreatment

Dilute acid catalyzed the steam explosion and therefore greatly resulted in the solubilization of cellulose. This occurred through the hydrolysis reaction which the rate of reaction was increased with an increase in the severity factor. The sugar components present in the hydrolysate were shown in Figure 26. Glucose was detected as a dominant component varying from 9.83% to 25.80% based on the first pretreated material. An increase in severity factor from 3.64 to 3.94 gave rise to the soluble glucose. However, when the acid catalyst was applied at high amount, 3% and 4% (w/w) H_2SO_4, glucose was reduced via the degradation to HMF and others. Highest yield of glucose released from the second steam explosion pretreatment of sugarcane bagasse was 25.80% of the residues from the first pretreatment or 13.82% of sugarcane bagasse. These results indicated that a majority portion of cellulose was not hydrolyzed to glucose but it probably remained in solid residue. High glucose remaining in the material led to the interest in the ethanol production of mixture products.

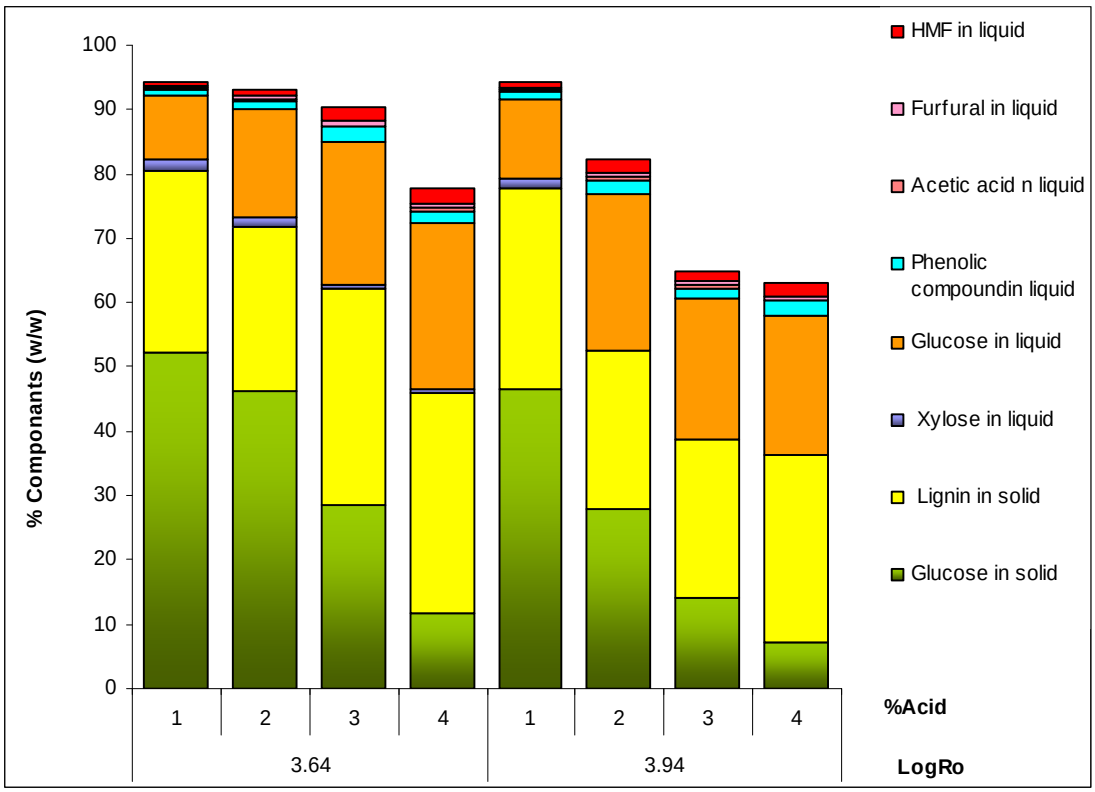

Figure 26 The components recovery after the second steam explosion pretreatment

Unless glucose, the liquid fraction was also analyzed for byproducts. Phenolic compounds, acetic acid, furfural and HMF were detected as shown in Figure 26. Acetic acid and furfural were found at low content. They were considered to be derived from acethyl group and xylose, respectively, in hemicellulose region that remained a little content in the raw material. Lignin was slightly degraded to phenolic compounds, giving the content varying from 0.86% to 2.57%. HMF was the main byproduct in the hydrolysate, it increased when the severity factor and the amount of catalyst were increased. From glucose solubilizing, HMF was found at lower amount than glucose degradation due to that the HMF could be also degraded to levulinic acid and other compounds. In addition, high amount of sulfuric acid that added to the material prior to the pretreatment was detected in the liquid. This acid was greatly toxic for the hydrolysis as well as the fermentation, thus, it should be reduced or eliminated from the hydrolysate prior to further application

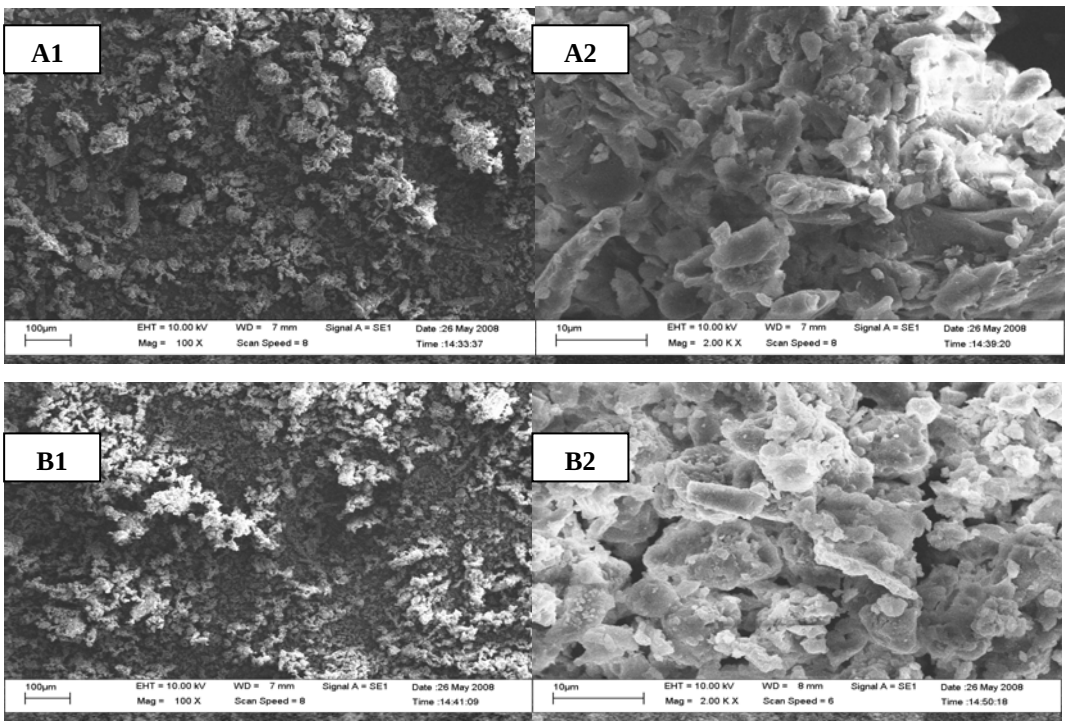

Figure 27 The SEM images of the second steam exploded sugarcane bagasse. The images A1 and A2 were solid fractions from the pretreatment at $logR_0$ 3.64 with 1% acid and the images B1 and B2 were solid fractions from the pretreatment at $logR_0$ 3.94 with 3% acid, respectively

The water insoluble residue from the second steam explosion mainly consisted of lignin and cellulose. Figure 26 showed the contents of lignin and glucose remaining in the pretreated material. In the residue, glucose decreased with increased severity factor and acid catalyst while lignin was increased. When compared with the material before the second steam explosion pretreatment and untreated sugarcane bagasse, lignin was not significantly varied, which was contrast with glucose that reduced with increased severity factor and acid catalyst. These results followed the difficultly to break down of lignin and the easy to hydrolyze cellulose.

The water insoluble faction from the second steam explosion pretreatment was in the form of fiberless material. Figure 27 showed the SEM images of solid fraction from the pretreatment at $logR_0$ 3.64 with 1% acid (image A) and

$logR_0$ 3.94 with 3% acid (image B). The effect of second steam explosion pretreatment had greater result in the morphological structure of sugarcane bagasse. Strong complex structure of lignocellulosic materials were destroyed, the fibrils were mostly degraded since cellulose chains were removed. Pretreatment at lower condition (A) led to lower cellulose hydrolysis, thus, the residue contained both cellulose and lignin. Image A1 showed that the material was fine powder and image A2 contained high surface area and porosity of material. At higher condition (B), most of cellulose was removed, then the amplificatory image of lignin rich material was smaller particle and had higher surface area.

3. The separate hydrolysis and fermentation process (SHF)

The ethanol production of pretreated sugarcane bagasse was studied through separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). Generally, the SHF process was performed with enzymatic hydrolysis process followed by yeast's fermentation. However, in this study, glucose for fermentation process was derived through the acid catalyzed steam explosion in the second step. However, for the comparison with general pathway, the residues from the first step of pretreatment were also passed to the enzymatic hydrolysis processes for further examination in the ethanol yield like the two-step pretreated hydrolysate.

3.1 Enzymatic hydrolysis of the residue after the first steam explosion of sugarcane bagasse

The effect of enzymatic hydrolysis on the structure of pretreated sugarcane bagasse was evaluated. Yield of glucose achieved from the enzyme processing on the first pretreated material was presented in Figure 28. For untreated sugarcane bagasse, glucose present in cellulose fibrous was converted to monomeric glucose with the percentage of conversion as small as 9.55. This indicated that most of cellulose was not hydrolyzed by enzyme due to the close-fisted structure. In

contrast, the enzymatic conversion of cellulose increased when pretreated sugarcane bagasse was applied in the reaction. Glucose yield was less resulted from the glucose because the amount of glucose present in each residue was nearly content. However, these yields were greatly influenced from the altered structural characteristic of the pretreated material. The structure of sugarcane bagasse was modified by steam explosion to improve the property to appropriate for hydrolysis. Figure 29 showed the effect of pretreated sugarcane bagasse characteristic on the rate of enzymatic hydrolysis. Most of dissolved glucose (about 75%) was released within the first 24 h of enzymatic hydrolysis and continued to increase with increased time of hydrolysis (Cara *et al*., 2008). Untreated sugarcane bagasse ($logR_0$ 0) were hardly hydrolyzed because it had the inhibitive structure for hydrolysis. The other residues derived from the pretreatment at $logR_0$ 2.95, 3.25, 3.55 and 3.80 were more hydrolyzed to glucose and increasing of the severity factor resulted in an increase in the digestibility of material (Guo *et al*., 2008).

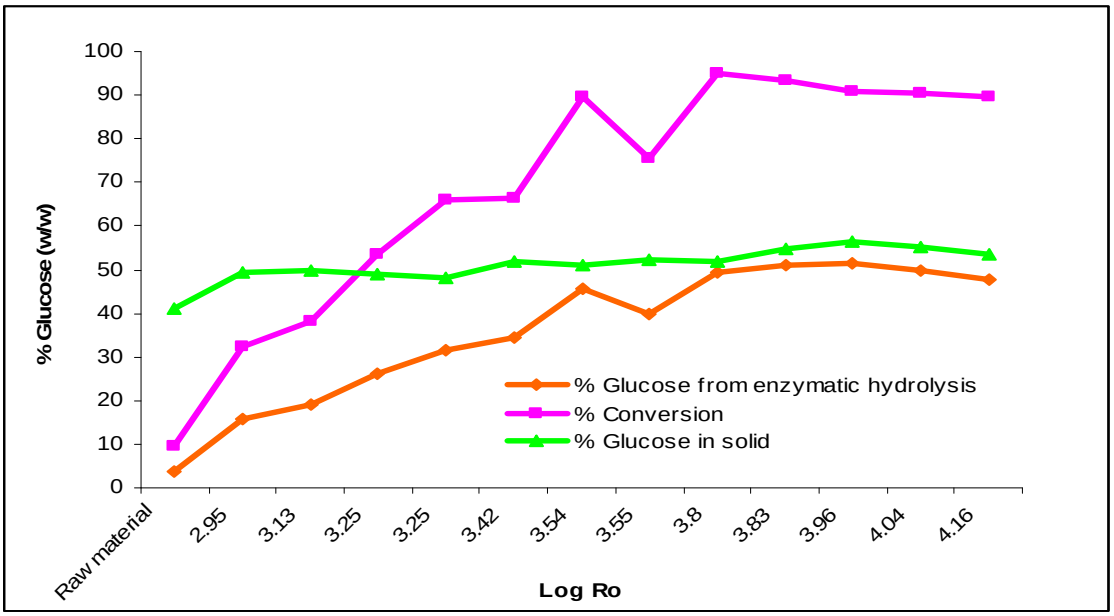

Figure 28 Yield of glucose (based on the solid residue weight) and cellulose conversion from enzymatic hydrolysis of the residues after the first steam explosion pretreatment

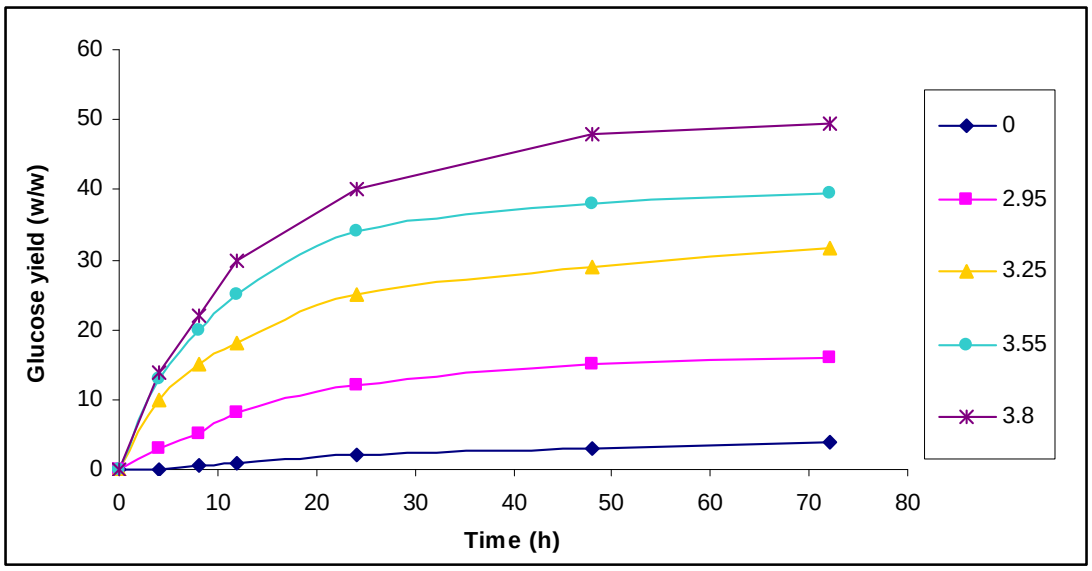

Figure 29 The effect of enzymatic hydrolysis on glucose yield

The hydrolysate from the enzymatic hydrolysis was subjected to ethanol fermentation process. Figure 30 showed the glucose yield, the conversion percentage and ethanol yield from the SHF process. The 10.98% to 27.69% of glucose was released from the enzymatic hydrolysis. This sugar could be converted to ethanol by the fermentation of baker's yeast. Similar conversion of glucose to ethanol was observed for all experimented condition. This might be explained using the control of the fermentation condition. Additionally, the enzymatic hydrolysis process did not produce toxic degradation products (HMF, furfural, organic acid), therefore leading to slight difference in glucose to ethanol conversion. Thus, the yield of ethanol mostly depended on the efficiency for enzymatic digestibility of the material. It increased when the severity of steam explosion was increased. Interestingly, high severity condition ($logR_0$ 4.04 and 4.16) resulted in lower ethanol yield.

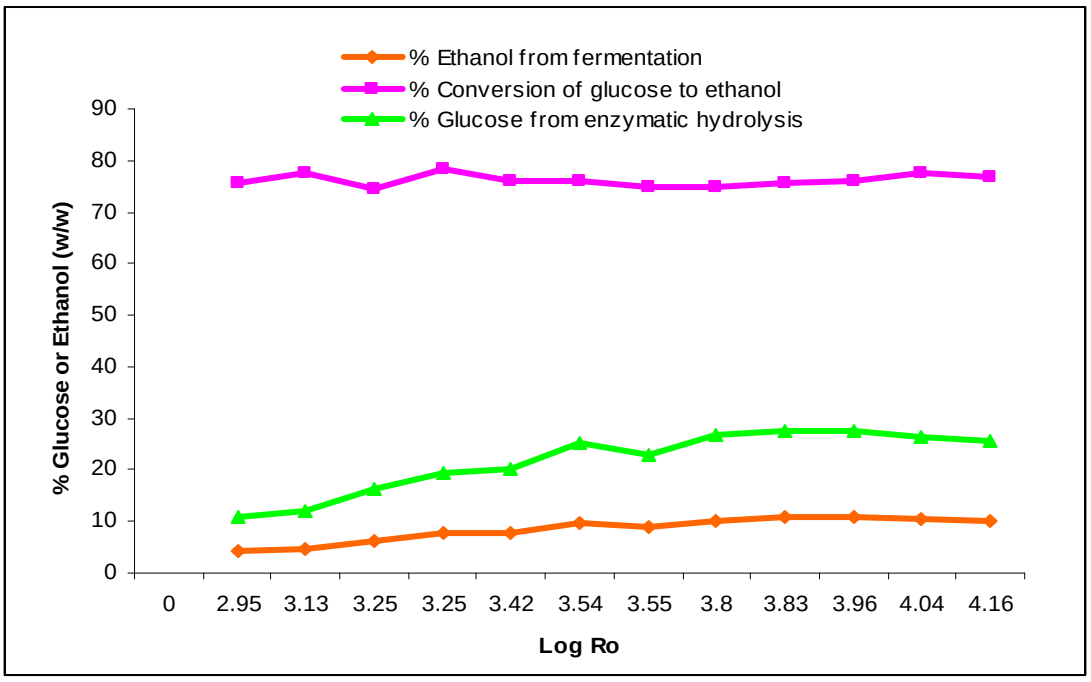

Figure 30 Yield of glucose and ethanol from the fermentation of hydrolysate (based on the sugarcane bagasse weight) obtained from enzymatic hydrolysis of solid residues after the first steam explosion pretreatment

3.2 The second steam explosion followed by fermentation

The hydrolysates containing inhibitor compounds (sulfuric acid, weak organic acids and others) were passed through the toxicfication process by overliming. Due to that the parameters in the fermentation process from all materials were controlled to similarity, the glucose to ethanol conversion was not significantly different. Ethanol yield, therefore, greatly depended on the yield of glucose obtained from the pretreatment as shown in Figure 31.

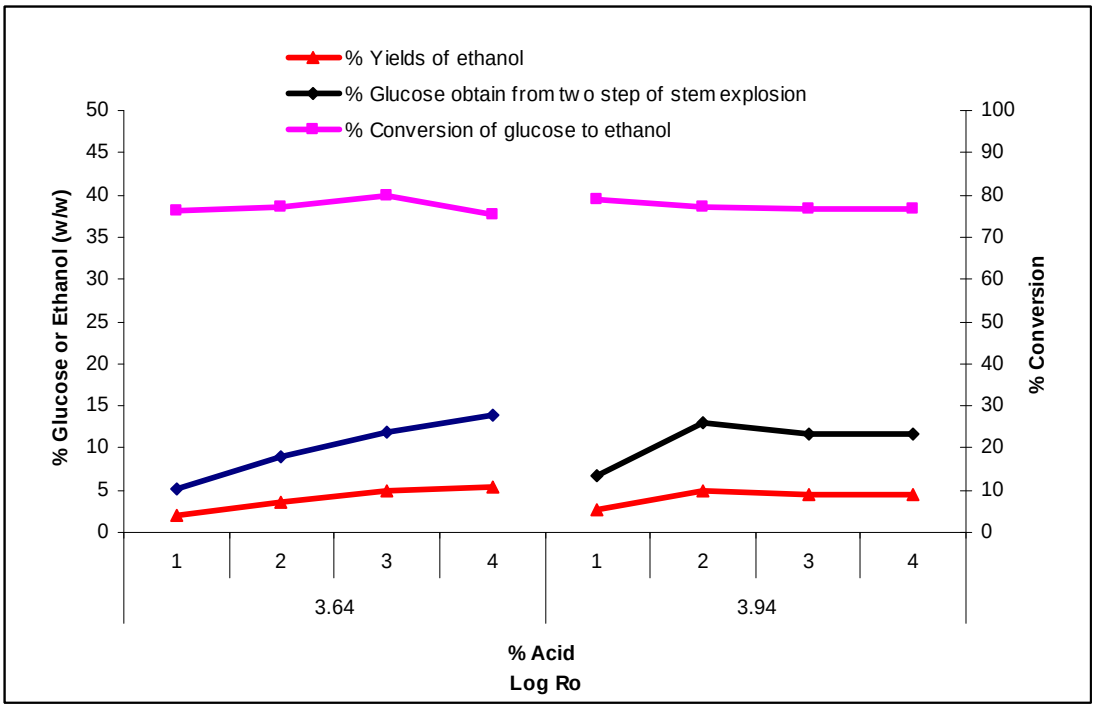

Figure 31 Yield of glucose and ethanol from the fermentation of hydrolysate (based on the sugarcane bagasse weight) obtained from the second steam explosion pretreatment

The comparison of ethanol yield derived from acid catalyzed steam explosion and enzymatic hydrolysis was considered. The results showed better ethanol yield from the enzymatic hydrolysis followed by fermentation (10.70%) than that obtained from the second steam explosion and fermentation (5.28%) by 50%. This could be explained by that a portion of cellulose was not hydrolyzed by the second steam explosion pretreatment.

4. The simultaneous saccharification and fermentation (SSF)

The SSF experiment was carried out on the material from the first pretreatment and slurry from the second steam explosion pretreatment. Yield of ethanol depended on the potentiality for hydrolysis of the substrate as well as the efficiency of fermentation by baker's yeast. The condition used in SSF experiment

was not optimal for both enzyme and yeast processes, thus this might be the cause for reduction of working efficiency of process. However, combining both processes resulted in a lower cost and reduced the risk of contamination (Wyman *et al*., 1992). This also included the reducing of end-product inhibitor (glucose) for the enzymatic process (Ballesteros *et al*., 2008) and the un-excessive of substrate for yeast's fermentation, therefore, leading to high yield of ethanol.

4.1 Simultaneous saccharification and fermentation of the slurry after the second steam explosion of sugarcane bagasse

The slurry from the second steam explosion of sugarcane bagasse was assessed for the ethanol yield using SSF. During SSF process, glucose released from the second steam pretreatment was fermented to ethanol whereas cellulose remained in solid fraction was simultaneously hydrolyzed to glucose and subjected to ethanol fermentation. The solid fraction, fine grain and high surface area, was more accessible to the enzyme attack. The fermentation of glucose from the second pretreatment and the enzymatic hydrolysis provided the ethanol yield and conversion percentage as presented in Figure 32. The yield of ethanol after SSF varied from 5.35% to 11.3% with conversion percentages varying from 68.04 to 71.31 that was not significantly different.

4.2 Simultaneous saccharification and fermentation of the residue after the first steam explosion of sugarcane bagasse

SSF process was performed on the residue material from the first pretreatment of sugarcane bagasse in comparison with that from the second pretreated slurry. The result was shown in Figure 33. Yield of ethanol varied with the severity condition, the values increased from 4.72% to 11.89% when the severity factor was increased. This result could be described by the modified structure of the residues. More severe condition resulted in more surface area, and then leading to more conversion of cellulose to glucose and also to higher ethanol yield.

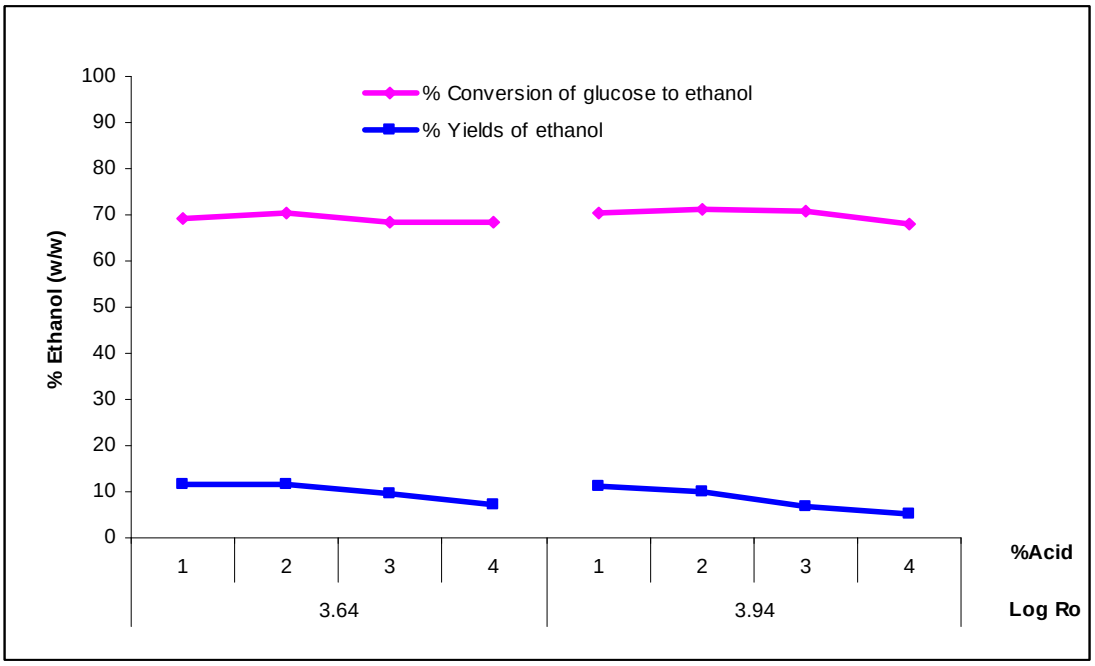

Figure 32 Yield of ethanol from the fermentation of hydrolysate and solid residue (based on the sugarcane bagasse weight) obtained from the second steam explosion pretreatment

In the first pretreatment at $logR_0$ 3.96, maximum yield of ethanol from the SSF process of slurry after the second pretreatment (11.73%) was higher than that from the process of the material from the first pretreatment (10.73%). This could be because that during the second pretreatment, a portion of cellulose was hydrolyzed to glucose by acid catalysis, thus when hydrolysis again with enzyme, the conversion of cellulose was probably higher than that from the first. However, SSF yield of the residues from the first pretreatment was highest yield as 11.89% at $logR_0$ 3.80.

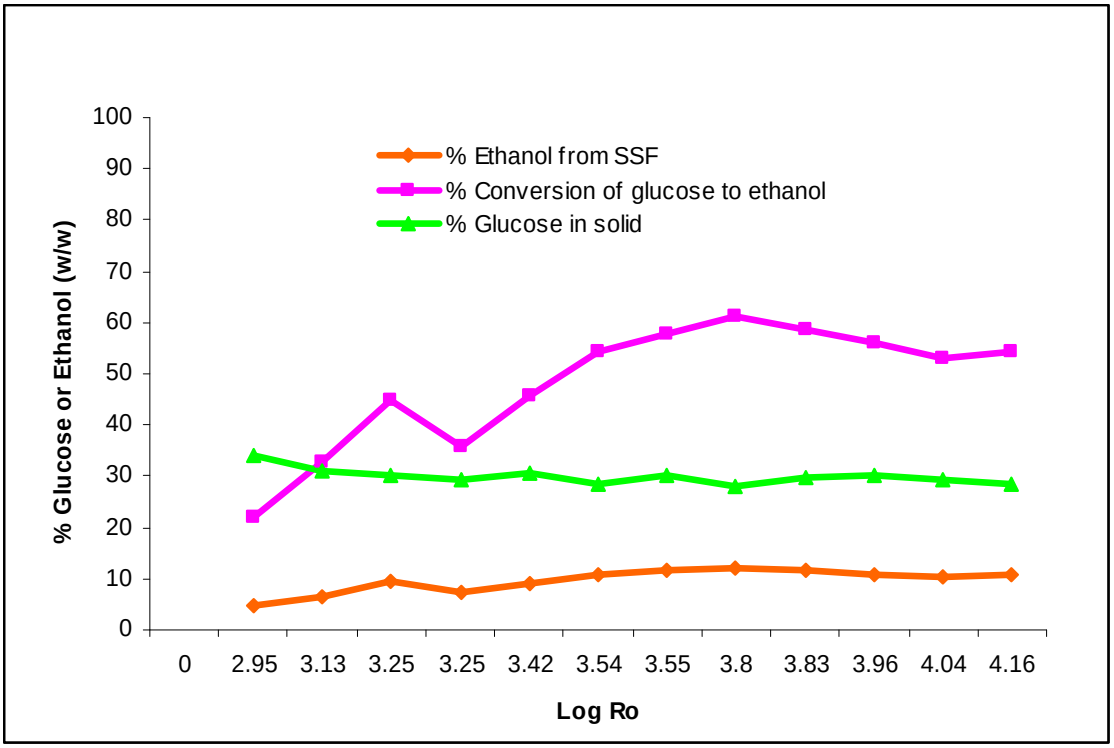

Figure 33 Yield of ethanol from the fermentation of solid residue (based on the sugarcane bagasse weight) from the first steam explosion pretreatment

CONCLUSIONS

Sugarcane bagasse, an abundant residue from sugar industry, predominantly contains 41.02 % (w/w) glucose (cellulose), 34.60 % (w/w) xylose (hemicellulose) and 20.83 % (w/w) lignin. High content in glucose could be attributed to the potential of sugarcane bagasse as substrate for ethanol production. The conversion process from sugarcane bagasse to ethanol consists basically of two steps, the conversion of cellulose to glucose and the conversion of glucose to ethanol. However, the solubilizing of glucose from sugarcane bagasse is usuually inhibited by the tough complex network structure of the materials, thus the pretreatment prior to the conversion process was required. Steam explosion was recognized as the pretreatment of lignocellulosic materials like sugarcane bagasse. This study applies the steam explosion as the two steps of pretreatment, the first to remove hemicellulose as well as to alter the structure while the second to convert cellulose to glucose.

In the first step of steam explosion, various severity conditions were investigated. The experiments were performed under the conditions with severity factors ($\log R_0$) ranging from 2.95 to 4.16. Products from the pretreatment were separated into solid and liquid fractions. The solid recovery after processing varied from 68.77 % to 53.05 % with severity factor increasing. The chemical compositions analysis of the residues showed glucose and lignin as the main components while small amount of xylose was detected. When increased the severity factor, remaining xylose contents were reduced from 34.60% to 3.50% (on bagasse basis). Unlikely, the solubilizing of glucose and lignin hardly occurred because their structures were resistant to the hydrolysis. The residues from each experiment contained various glucose contents from 49.29 % to 56.51 %. Unless the steam explosion was effective on the chemical changes of sugarcane bagasse, structure of this material was also altered. The structures of residues were modified to improve the accessible surface area which then resulting in the hydrolysis yield. More severity factor led to more surface area on the residue structure.

The least-hemicellulose containing residues from the first pretrearment at $logR_0$ 3.96 were subjected to the second steam explosion. In this step, dilute sulfuric acid was added to catalyze the hydrolysis reaction. The hydrolysates after this second pretreatment were examined for sugars and byproducts. Based on the residues weight after the first pretreatment, 9.83-25.80 % of glucose was detected in the hydrolysate. High dissolved glucose was obtained from the steam explosion at high severity factor and high amount of acid. Maximum glucose yield (25.80 %) was obtained from the second pretreatment at $logR_0$ 3.64 with 4 % acid. However, during the pretreatment, byproducts such as HMF, furfural, acetic acid and phenolic compounds, were produced. These compounds were the inhibitors for yeast's fermentation, therefore, they were eliminated by the overliming method prior to further fermentation.

Additionally, the water insoluble fraction from the first step of pretreatment was also evaluated for the glucose yield derived from an enzymatic hydrolysis. More accessible surface area greatly resulted in the rate of conversion to soluble glucose with the conversion percentage of 32.39 % to 95.09 % providing the yield of glucose as 15.97 % to 51.46 %. The advantage of the hydrolysis using enzyme catalyst was that the process did not produce toxic byproducts.

Two alternative ways of ethanol production were investigated, SHF and SSF processes. The hydrolysates derived from the first steam explosion and the second steam explosion were used as substrates. For SHF process, ethanol yield of hydrolysate from the second steam explosion varied from 2.03 % to 5.28 % with slight difference from the theoretical yield (75.25 % to 79.88 %). As same as the enzymatic hydrolysate, yield of ethanol were varied from 4.24 % to 10.70 %. This yield was according to the glucose yield obtained from enzyme hydrolysis which was affected from steam explosion. Yield of ethanol from the second pretreatment was less than that from the enzymatic pathway by about 50 % due to that a portion of cellulose also remained in the solid fraction after the steam explosion pretreatment.

The ethanol production using SSF process was investigated and the ethanol yield was compared with that from the SHF. The substrates used in this experiment were slurry from the second pretreatment and the solid residue after the first steam explosion. The slurry from the second pretreatment was converted to ethanol 11.73-5.34 % with the conversion about 68.04 to 71.31 %. The best ethanol yield was obtained from $\log R_0$ 3.64 with 2 % acid. This value was higher than that from the SHF of only hydrolysate because the glucose present in the solid fraction was also converted to ethanol. Moreover, the ethanol from the SSF of the solid residues after the first steam explosion varied from 4.72 % to 11.89 % with the cellulose conversion about 21.91 % to 61.03 %. The best yield of this work (11.89 %) was not different with the ethanol yield of the second pretreated sugarcane bagasse.

To this end, it could be concluded that the steam explosion pretreatment improved the ethanol yield from sugarcane bagasse. However, the first steam explosion was enough as a pretreatment technique. The effectiveness of the first pretreatment led to the highest ethanol yield 11.89 % on bagasse basis when SSF process was applied. Likely, the second steam explosion pretreatment also led to as high ethanol yield as 11.73 % when SSF process was performed.

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APPENDICES

Appendix A

Method of analysis

APPENDIX method A1 Analysis of Moisture Content

1. Weigh a sample approximately 2 ±0.1g (A) in a tarred weighing bottle.

2. Dry for 2 hours in an oven at 100^0C, cool in desiccators, replace stopper and open the stopper momentarily to equalize the air pressure and weigh the sample.

3. Return bottle to the oven for 1 hours, repeat the cooling and weighing as above for successive hourly periods until constant weight (B) was reached.

4. The moisture content could be calculated as follow:

$$\text{Percent of Moisture Content} = [(A-B)/A]100$$

Where A = weight of sample, g

B = Oven –dried weight of test specimen, g

APPENDIX method A2 Analysis of Ash Content

1. Carefully clean crucible and ignite in a muffle furnace at 525^0C for 30- 60 min. After ignition, cool slightly and then place in the desiccator, then cool to room temperature.

2. Transfer the test sample to the crucible, with the lid agar, gently carbonize the specimen in the crucible on the furnace at about 100^0C. Raise the temperature to 525^0C. Sample must be charred, not burned so that the temperature of the sample does not exceed 525^0C.

3. When the sample is completely combusted as indicated by the absence of black particles, remove the crucible from the furnace, replace the cover, and allow to cool, then place in the desiccators and cool to room temperature.

Calculation

$$\text{Percent of Ash Content} = (AX\ B)/\ 100$$

Where A = weight of ash, g

B = weight of test sample, g moisture - free

APPENDIX method A3 Analysis of Monosaccharide Content

Retention time value of monosaccharide

1. Prepare each of sugar standard and internal standard (inositol) at 100 mg/l.
2. The solution is filtered through 0.45 mm cellulose acetate filter (Millipore).
3. Inject 20µl of sugar standards and internal standard onto HPLC.

Response Factor (RF) for each of sugar standard

1. Prepare a series of glucose, xylose, arabinose, mannose and galactose standards in the range of 20-100 mg/l.
2. Prepare 1000 mg/l of internal standard.
3. Filter the sugar solutions though on 0.45 mm cellulose acetate filter before injection onto the HPLC.
4. Pipette 0.9 ml of standard glucose and 0.1 ml internal standard into vials and inject 20µl of sample onto the HPLC.
5. Calculate RF of each sugar standard and internal standard and then calculate the RF. Sample aliquots are filtered through a 0.45 µm poly(tetrafluoroethylene) filter prior to injection.
6. Prepare the sample for HPLC analysis by passing the decanted liquid through a 0.45 µm filter into an auto sampler vial. Seal and label the vial. Prepare each sample in duplicate if desired. If it is suspected that the sample concentrations may exceed the calibration range, dilute the samples as needed and record the dilution. The concentrations should be corrected for dilution after running. If necessary, neutralized samples may be stored in the refrigerator for three or four days. After this time, the samples should be considered compromised.

Response Factor calculation for each sugar standard and internal standard

$$RF = \frac{As \ X \ Ci}{Ai \ X \ Cs}$$

Where RF = Response Factor for each sugar standard

Cs = Concentration of sample, mg/l

Ci = Concentration of internal standard, mg/l

As = Peak area of sugar sample

Ai = Peak area of internal standard

Appendix B

Measurement of enzyme activities

APPENDIX method B1 Activity of Celluclast 1.5L

Substrate: Whatman No. 1 filter paper strip, 1.0 x 6.0 cm (= 50mg).

Method

1. Add 1.0 ml 0.05 M Na-citrate, pH 4.8, to a test tube of volume at least 25 ml.

2. Add 0.5 ml enzyme, diluted in citrate buffer. At least two dilutions must be made of each enzyme sample investigated. One dilution should release slightly more and one slightly less than 2.0 mg (absolute amount) of glucose (= reducing sugars as glucose) in the reaction conditions.

3. Temperate to 50°C, add one filter paper strip, mix (NB! it does not matter if a small part of the paper is above the liquid surface, but if the paper "winds" up the tube it must be pushed down again).

4. Incubate 50°C, 60 mm.

5. Add 3.0 ml DNS, mix. Transfer tube to a rack on the table.

6. Boil for exactly 5.0 mm in a vigorously boiling water bath containing sufficient water. All samples, enzyme blanks, glucose standards and the spectro zero should be boiled together. After boiling, transfer to a cold water bath.

7. Add 20 ml deionized or distilled water. Mix by completely inverting the tube several times so that the solution separates from the bottom of the tube at each inversion (NB. This is important!).

8. When the 'pulp' has settled well, i.e., after at least 20 mm, the color formed is measured against the spectro zero at 540 nm. If the paper pulp does not settle, it will do so after stirring with a glass rod. (The necessity for stirring can be seen after only a few minutes of settling time).

Spectro Zero

1.5 ml citrate buffer

3.0 ml DNS

5 mm boil, 20 ml H20, etc.

Spectro zero is used to set the spectrophotometer at zero absorbance

Enzyme blank

1.0 ml citrate buffer

0.5 ml enzyme

3.0 ml DNS

Boil, H20, etc.

Color measured against spectro zero and subtracted from the value of the appropriate reaction tube.

Standards

0.5 ml standard

1.2 ml citrate buffer

3.0 ml DNS

Boil, etc. and measured against spectro zero

Glucose Stock Solution

10 mg/ml anhydrous glucose

Aliquots of 5-10 ml can be stored frozen.

Remember to stir well after thawing

Dilutions:

1 ml + 0.5 ml buffer = 1:1.5 = 6.7 mg/ml

1 ml + 1 ml buffer = 1:2 = 5.0 mg/ml

1 ml + 2 ml buffer = 1:3 = 3.3 mg/ml

1 ml + 4 ml buffer = 1:5 = 2.0 mg/ml

Unit Calculation

1. Construct a linear glucose using the absolute of glucose (mg/0.5 ml) plotted against A_{540}.

2. Use this standard to translate the absorbance values of the sample (after subtraction of enzyme blank) into glucose (= mg glucose produced during the reaction) translate the dilutions used into enzyme concentration:

3. Estimate the concentration of enzyme which would have released exactly 2.0 mg of glucose by plotting glucose liberated against enzyme concentration on semi logarithmic graph paper.

Concentration = 1/dilution (volume of enzyme in dilution/ total volume of dilution)

4. Estimate the concentration of enzyme which would have released exactly 20 mg of glucose by plotting glucose liberated (2) against enzyme concentration (3) on semilogalithmic graph paper

5. Calculation FPU:

$$\text{FPU} = \frac{0.37}{\text{enzyme concentration to released 2.0 mg glucose}} \quad (\text{units ml}^{-1})$$

APPENDIX method B2 Activity of Novozyme 188

Substrate: 15.0 mM cellobiose in 0.05 M citrate buffer pH 4.8. Fresh cellobiose solution should be prepared daily

Method

1. Add 1 .0 ml of enzyme, diluted in citrate buffer, to a small test tube. At least two dilutionsmust be made of each enzyme sample investigated. One dilution should release slightly more and one slightly less than 1.0 mg (absolute amount) of glucose in the reaction conditions.

2. Temperate to 50°C.

3. Add 1.0 ml substrate solution, mix.

4. Incubate at 50°C for exactly 30 mm.

5. Terminate the reaction by immersing the tube in boiling water for exactly 5.0 mm.

6. Transfer the tube to a cold water bath and determine glucose produced using a standard procedure (e.g. using a kit based on the glucose oxidase reaction).

Cellobiose blank

1.0 ml cellobiose substrate solution

1.0 ml citrate buffer

30 mm, so°c

Boil 5.0 mm, cool.

Use in the GOD reaction and subtract absorbance from that of the sample. Note that a single cellobiose blank can be used for a whole series of activity determinations for which an enzyme blank is not necessary.

Enzyme blank

1.0 ml citrate buffer

1.0 ml enzyme dilution

30 mm, 50°C

Boil 5.0 mm, cool.

Use in the GOD reaction and subtract absorbance from that of the sample, along with the absorbance of the cellobiose blank. Enzyme blanks are necessary only when glucose is present in the enzyme preparation and/or when small dilutions are used.

Unit Calculation

1. Determine the glucose concentrations (mg/m1) in the cellobiase reaction mixtures obtained using at least two different enzyme dilutions.

2. Multiply by 2 to convert glucose concentrations into absolute amounts (mg).

3. Translate enzyme dilutions into concentrations:

$$\text{Concentration} = \frac{1}{\text{dilution}} \left(= \frac{\text{volume of enzyme sample in. dilution}}{\text{total volume of dilution}}\right)$$

4. Estimate the concentration of enzyme which would have released exactly 1 .0 mg of glucose by plotting glucose liberated (2) against enzyme concentrations (3) on semilogarithmic graph paper.

5. Calculate cellobiase activity:

$$CB = \frac{0.0926}{\text{Enzyme concentration to release 1.0 mg glucose}} \text{ Unit mL}^{-1}$$

The unit of cellobiase (CB) is based on the International Unit (IU)

1 IU = 1 μmol min^{-1} of substrate converted

= 2.0 μmol min^{-1} of glucose formed in the case of the CB reaction

Appendix C

Yeast cultivation

APPENDIX method C1 Microorganism and Inoculum culture

S. cerevisiae TISTR 5339 was obtained from Thailand Institute of Scientific and Technological Research (TISTR, Thailand). It was maintained on a medium containing 20.0 g/l glucose, 20.0 g/l peptone and 10.0 g/l yeast extract at 4^0C. The yeast was subcultured every month at 30^0C.

APPENDIX method C2 Aerobic batch cultivation

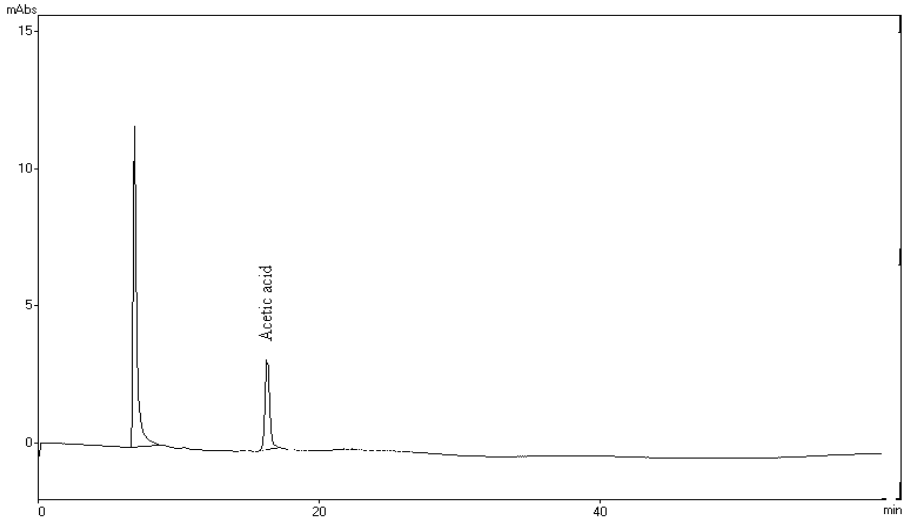
In order to produce cell mass, batch cultivation on glucose, with volume of 100 mL, was carry out at 30 °C and pH 5.0 for 24 h under sterile condition. The medium contain the following components: 50 g/L glucose, 0.5 g/L $(NH_4)_2HPO_4$, 0.025 g/L $MgSO_4.7H_2O$, 1.2 g/L Na_2HPO_4 and 1 g/L of yeast extract.

APPENDIX method C3 Cell harvest

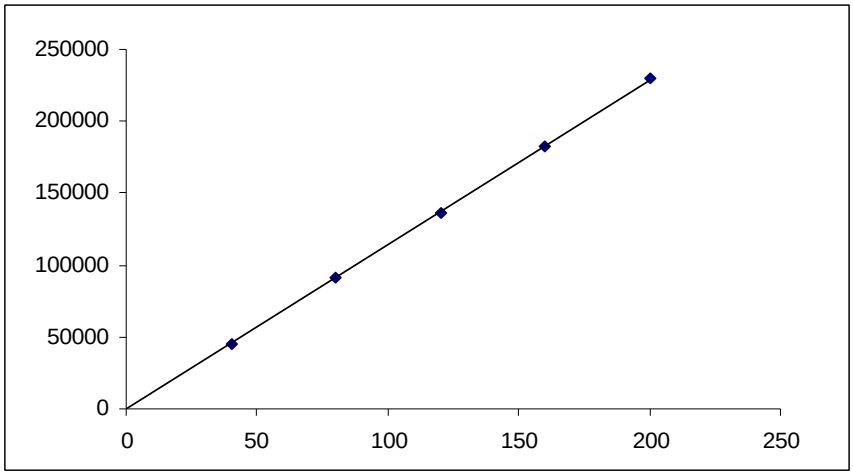
After 24 h, the cultivation liquid containing the yeast was transfer from the reactor into a sterile centrifuge bottle. The cultivation liquid was centrifuged. The supernatant was discarded and the pellets were transferred to added in order to obtain a cell suspension with a cell mass concentration of about 50 g DM/L

Appendix D

Chromatogram and calibration curve



APPENDIX Figure D1 HPLC chromatogram of standard solution of acetic acid



Acetic content

Correlation = 0.9999

Formulation : y = mx + b

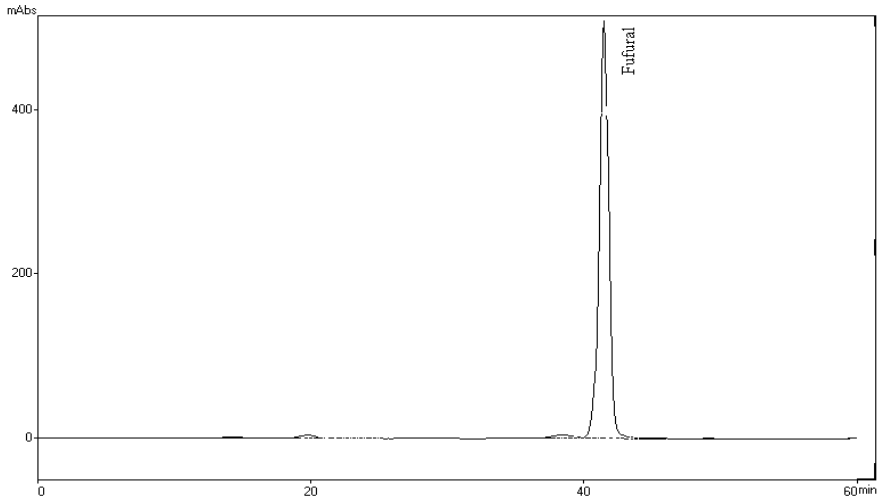
: m = 1143.5

: b = 0

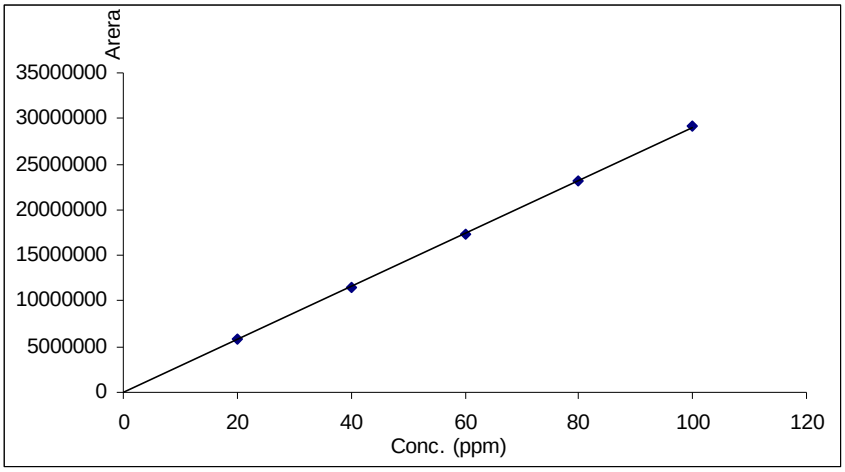
: x = Peak area

: y = Aceic acid

APPENDIX Figure D2 Calibration curve of standard solution of acetic acid



APPENDIX Figure D3 HPLC chromatogram of standard solution of furfural



Furfural content

Correlation = 1

Formulation : y = mx + b

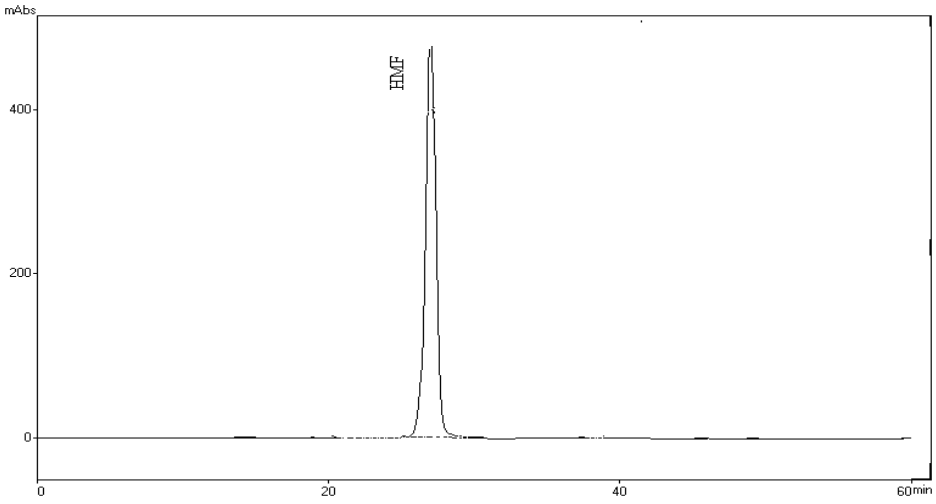
: m = 290703

: b = 0

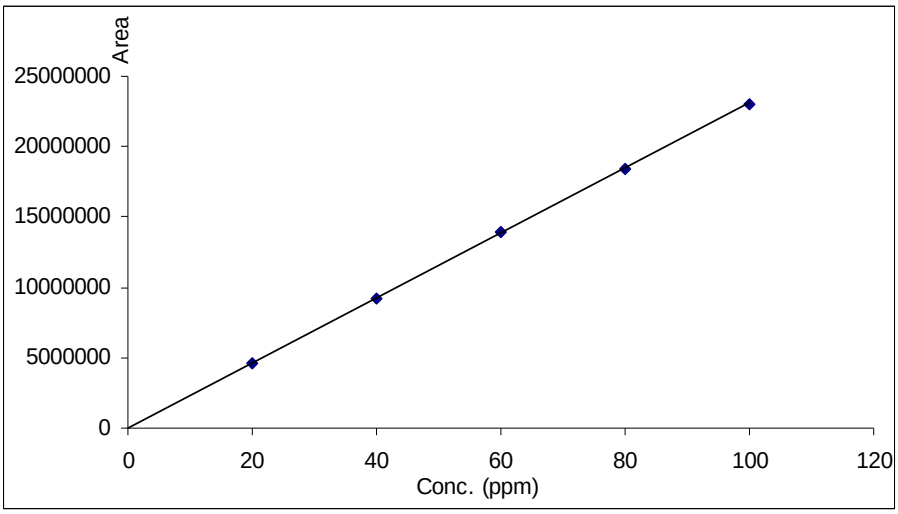
: x = Peak area

: y = Furfural

APPENDIX Figure D4 Calibration curve of standard solution of furfural



APPENDIX Figure D5 HPLC chromatogram of standard solution of HMH



HMF content

Correlation = 1

Formulation : y = mx + b

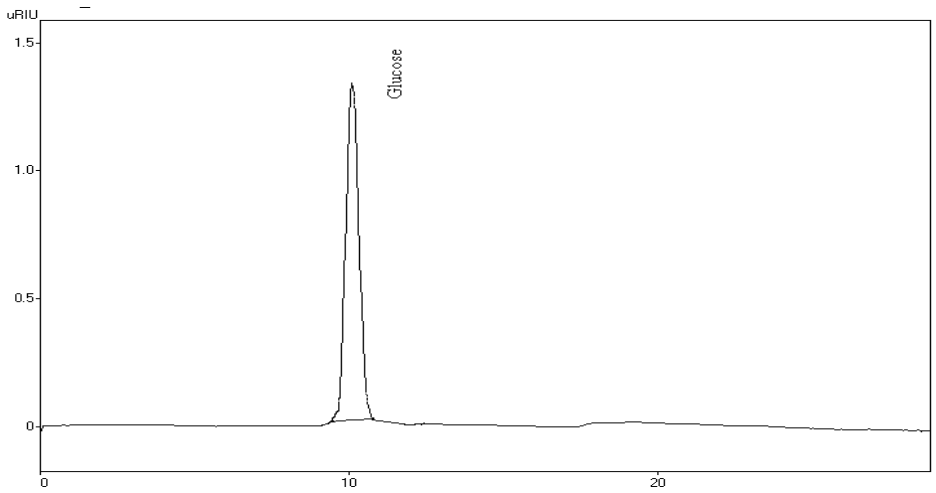
: m = 230783

: b = 0

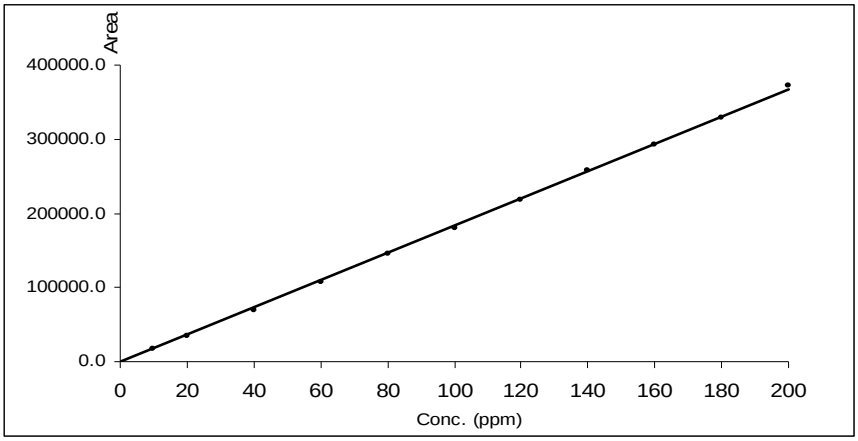
: x = Peak area

: y = HMF

APPENDIX Figure D6 Calibration curve of standard solution of HMF



APPENDIX Figure D7 HPLC chromatogram of standard solution of glucose



Glucose content

Correlation = 0.9989

Formulation : y = mx + b

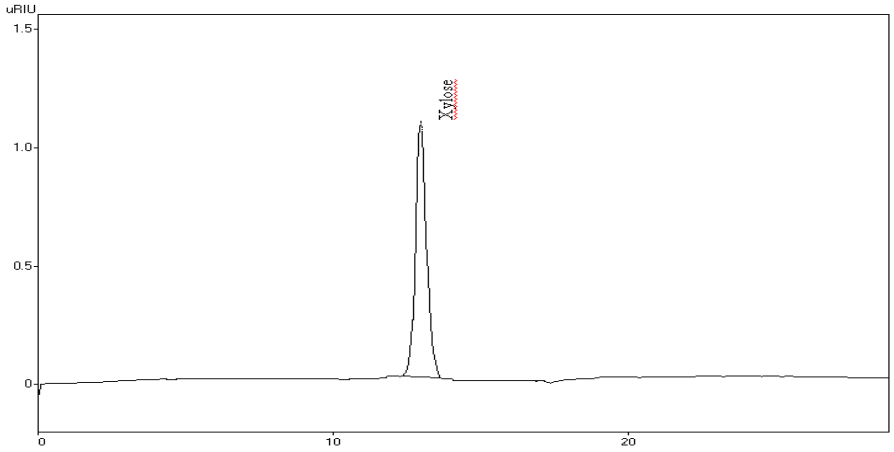
: m = 1831.9

: b = 0

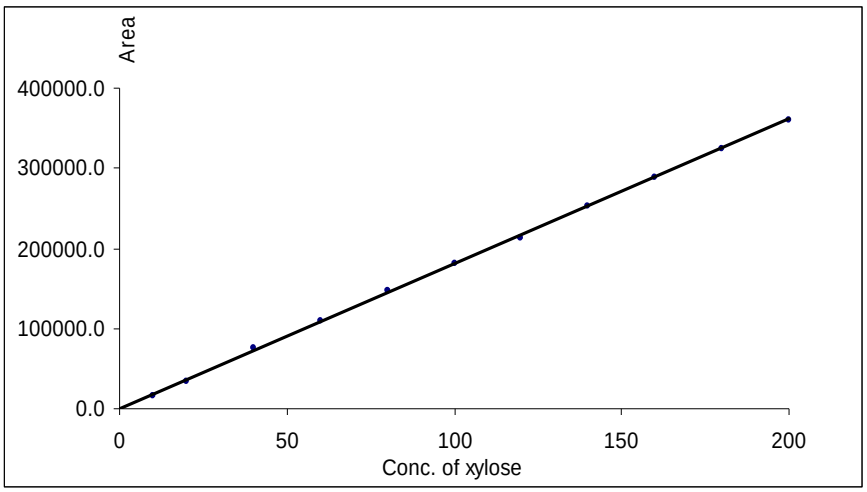
: x = Peak area

: y = glucose

APPENDIX Figure D8 Calibration curve of standard solution of glucose



APPENDIX Figure D9 Calibration curve of standard solution of xylose



Xylose content

Correlation = 0.9997

Formulation : y = mx + b

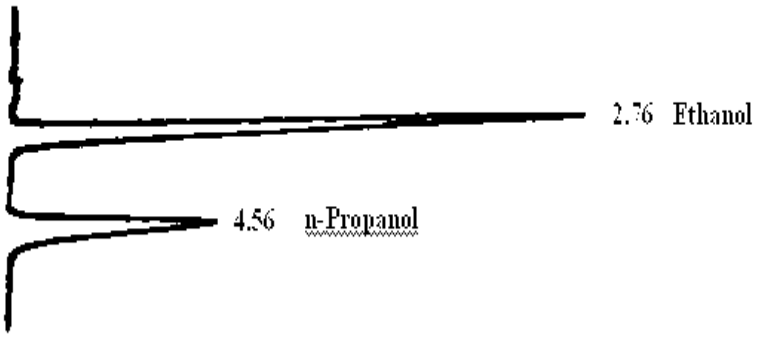
: m = 1806.6

: b = 0

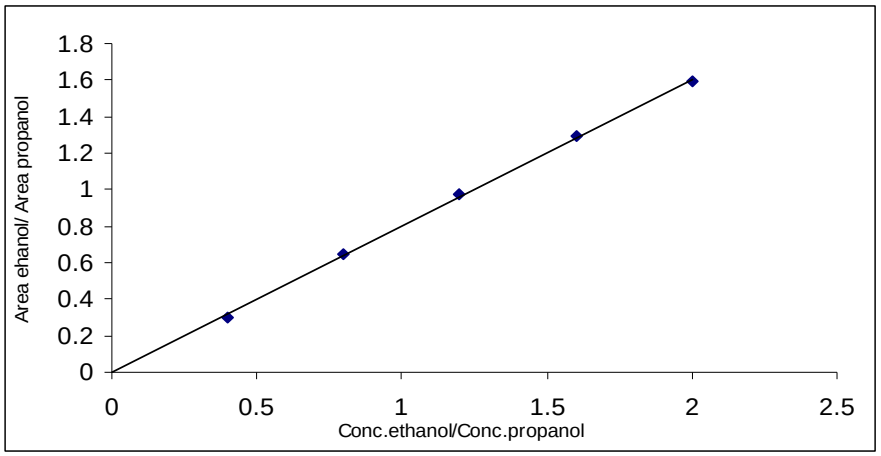
: x = Peak area

: y = Xylose

APPENDIX Figure D10 Calibration curve of standard solution of xylose



APPENDIX Figure D11 GC chromatogram of standard solution of ethanol



Ethanol content

Correlation = 0.9992

Formulation : $y = mx + b$

: $m = 0.803$

: $b = 0$

: x = Peak area of ethanol/ Peak area of propanol

: y = Ethanol/ Propanol

APPENDIX Figure D12 Calibration curve of standard solution of ethanol

Appendix E

Data

APPENDIX Table E1 The chemical composition of solid fraction after the first steam explosion pretreatment

log R_0	% lignin	% Glucose	% Xylose	% Ash
0	20.73	41.02	34.60	1.11
2.95	24.96	49.29	19.19	0.83
3.13	24.92	50.00	19.49	0.87
3.25	25.61	48.82	12.38	0.96
3.25	26.01	48.03	14.91	0.83
3.42	26.49	51.74	10.45	0.99
3.54	26.64	51.06	9.69	0.84
3.55	27.14	52.36	9.19	0.85
3.80	28.95	51.87	7.48	0.80
3.83	28.25	54.65	6.66	0.82
3.96	28.47	56.51	6.54	0.77
4.04	27.26	55.22	6.84	0.70
4.16	28.14	53.48	6.86	0.83

based on the solid after pretreatment weight

APPENDIX Table E2 The solid recovery after the first steam explosion pretreatment

log R_0	**Solid recovery (%w/w)**
2.95	68.77
3.13	62.38
3.25	61.90
3.25	61.41
3.42	59.05
3.54	55.43
3.55	57.85
3.80	54.30
3.83	54.25
3.96	53.55
4.04	53.05
4.17	53.19

based on the sugarcane bagasse weight

APPENDIX Table E3 The component of solid and water soluble fraction after the first steam explosion pretreatment

log R_0	Component in solid fraction				Component in water soluble fraction							
					Glucose		Xylose					
	lignin	Glucose	Xylose	Ash	monomeric	oligomeric	monomeric	oligomeric	Furfural	HMF	Phenolic compound	Acetic acid
0.00	20.73	41.02	34.60	1.11								
2.95	17.16	33.90	13.20	0.69	0.00	0.79	0.52	5.38	nd	0.01	0.74	0.03
3.13	15.55	31.19	12.16	0.52	0.01	0.99	0.69	7.89	nd	0.03	0.94	0.06
3.25	15.85	30.22	7.66	0.54	0.09	1.06	1.14	9.48	0.18	0.07	1.08	0.36
3.25	15.97	29.50	9.16	0.59	0.03	1.01	0.73	7.74	nd	0.03	0.91	0.25
3.42	15.64	30.55	6.17	0.49	0.09	0.91	1.57	8.83	0.31	0.03	1.14	0.87
3.54	14.77	28.30	5.37	0.55	0.12	1.03	2.66	9.26	0.51	0.12	1.47	0.82
3.55	15.70	30.29	5.32	0.49	0.12	0.99	3.77	8.66	0.52	0.10	1.39	0.76
3.80	15.72	28.17	4.06	0.46	0.21	1.04	5.40	4.22	1.25	0.16	1.65	1.40
3.83	15.33	29.65	3.61	0.43	0.24	0.98	5.37	3.09	1.37	0.18	1.80	1.55
3.96	15.25	30.26	3.50	0.44	0.25	0.96	5.37	3.29	1.52	0.19	1.53	1.60
4.04	14.46	29.29	3.63	0.41	0.32	0.93	5.51	1.28	1.64	0.21	1.83	1.73
4.16	14.97	28.45	3.65	0.37	0.31	1.00	5.33	1.69	1.53	0.22	1.79	1.76

based on the sugarcane bagasse weigh

APPENDIX Table E4 Percentage of solid recovery after the second steam explosion pretreatment

Severity pretreatment (log R_0, %Acid)	**% Solid recovery (w/w)***	**% Solid recovery (w/w)****
3.64, 1	81.87	43.84
3.94, 1	78.98	42.29
3.64, 2	72.60	38.88
3.94, 2	53.69	28.75
3.64, 3	62.40	33.42
3.94, 3	39.63	21.22
3.64, 4	46.60	24.95
3.94, 4	36.51	19.55

* based on the solid after first pretreatment weight

** base on the sugarcane bagasse weight

APPENDIX Table E5 The component of solid and water soluble fraction after the second steam explosion pretreatment

log R_0, %Acid	Component in solid fraction		Component in water soluble fraction					
	Glucose	Lignin	Xylose	Glucose	Phenolic compound	Acetic acid	Furfural	HMF
3.64, 1	52.21	28.21	1.99	9.83	0.86	0.4	0.25	0.43
3.94, 1	46.66	31.08	1.48	12.49	0.97	0.41	0.41	0.72
3.64, 2	46.13	25.58	1.62	16.87	1.02	0.4	0.43	1.03
3.94, 2	28.06	24.47	nd	24.32	2.16	0.5	0.67	2.18
3.64, 3	28.57	33.48	0.66	22.27	2.47	nd	0.69	2.21
3.94, 3	14.08	24.7	nd	21.85	1.55	0.48	0.57	1.7
3.64, 4	11.83	34.07	0.64	25.8	1.74	0.59	0.73	2.48
3.94, 4	7.13	29.09	nd	21.61	2.57	nd	0.67	1.86

based on the solid after first pretreatment weight

APPENDIX Table E6 Yield of glucose and cellulose conversion from enzymatic hydrolysis of the solid after the first steam explosion pretreatment

log R_0	**Glucose yield***	**% Conversion****
0	3.84	9.55
2.95	15.97	32.39
3.13	19.06	38.11
3.25	26.16	53.58
3.25	31.73	66.07
3.42	34.42	66.53
3.54	45.69	89.48
3.55	39.64	75.72
3.80	49.32	95.09
3.83	51.04	93.40
3.96	51.46	91.07
4.04	49.90	90.36
4.16	47.84	89.46

* based on the solid after first pretreatment weight

** based on the glucose weight (presented in the solid after first pretreatment)

APPENDIX Table E7 Yield of ethanol from the fermentation of hydrolysate obtained from the second steam explosion pretreatment

Log R_0, % acid	**% Glucose obtain from two step of steam explosion***	**% Conversion of glucose to ethanol****	**% Yields of ethanol***
3.64, 1	5.26	76.01	2.03
3.94, 1	6.69	78.81	2.68
3.64, 2	9.04	77.32	3.55
3.94, 2	13.03	76.94	4.96
3.64, 3	11.93	79.88	4.84
3.94, 3	11.70	76.71	4.56
3.64, 4	13.82	75.25	5.28
3.94, 4	11.57	76.84	4.52

* based on the sugarcane bagasse weight

** based on the theory

APPENDIX Table E8 Yield of ethanol from the fermentation of hydrolysate obtained from the enzymatic hydrolysis of the first steam explosion pretreated solid

Log R_0	% Glucose obtain from enzymatic hydrolysis*	% Conversion of glucose to ethanol**	% Yields of ethanol*
2.95	10.98	75.76	4.24
3.13	11.89	77.57	4.70
3.25	16.19	74.37	6.14
3.25	19.49	78.29	7.78
3.42	20.33	75.86	7.86
3.54	25.33	76.17	9.84
3.55	22.93	74.84	8.75
3.80	26.78	74.86	10.22
3.83	27.69	75.72	10.69
3.96	27.56	76.13	10.70
4.04	26.47	77.49	10.46
4.16	25.45	76.77	9.96

* based on the sugarcane bagasse weight

** based on the theory

APPENDIX Table E9 Yield of ethanol from SSF process of the slurry derived from the second steam explosion pretreatment

Log R_0, % acid	**% Conversion****	**% Yield of ethanol***
3.64, 1	69.07	11.70
3.94, 1	70.26	11.35
3.64, 2	70.57	11.73
3.94, 2	71.31	10.20
3.64, 3	68.29	9.48
3.94, 3	70.72	6.94
3.64, 4	68.53	7.04
3.94, 4	68.04	5.34

* based on the sugarcane bagasse weight

** based on the theory

APPENDIX Table E10 Yield of ethanol from SSF process of the solid derived from the first steam explosion pretreatment

Log R_0	**% Conversion****	**% Yield of ethanol***
2.95	21.91	4.72
3.13	32.77	6.50
3.25	44.85	9.29
3.25	35.67	7.35
3.42	45.49	9.15
3.54	54.28	10.76
3.55	57.75	11.77
3.80	61.03	11.89
3.83	58.77	11.44
3.96	56.09	10.57
4.04	52.96	10.47
4.16	54.51	10.66

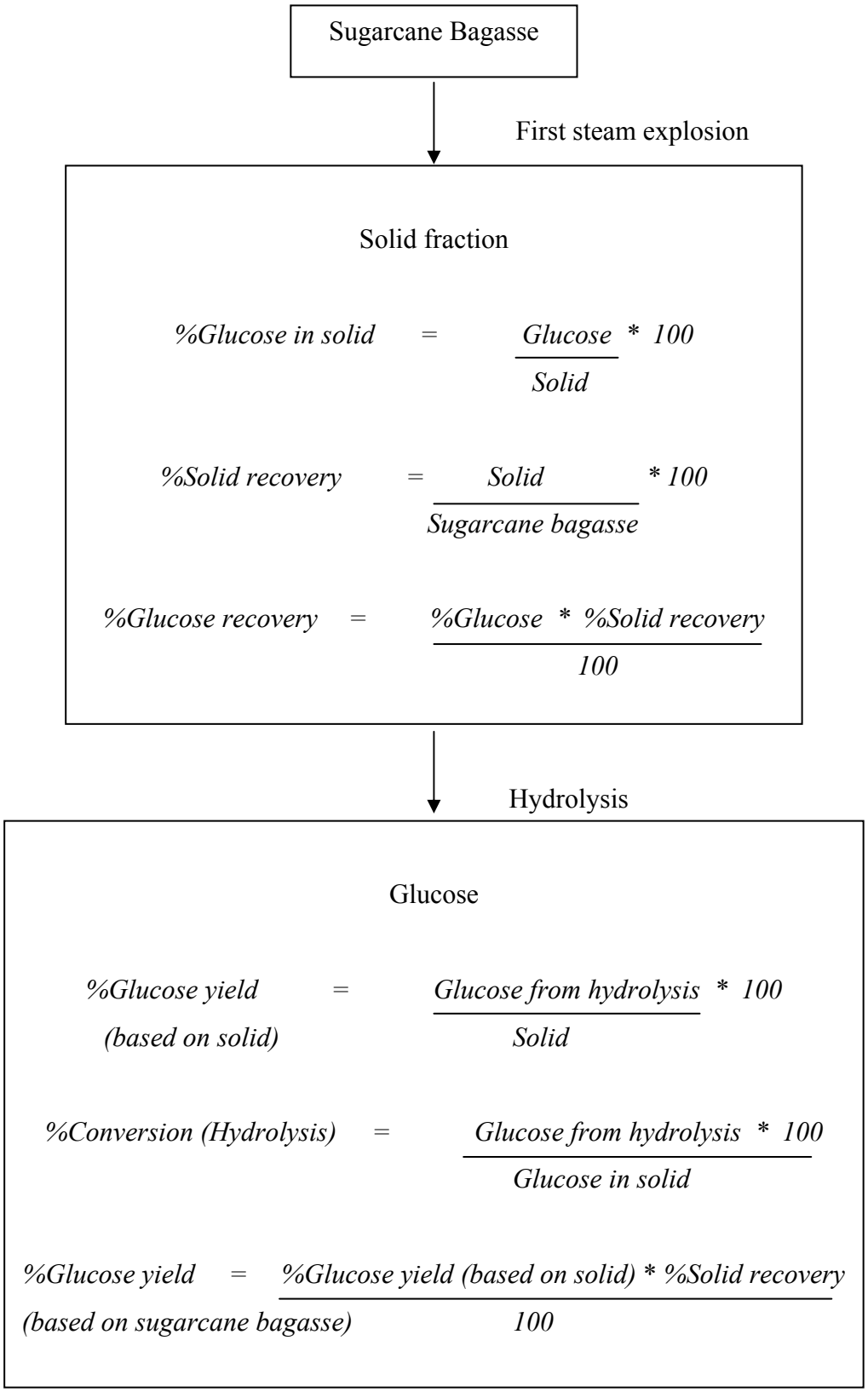
* based on the sugarcane bagasse weight

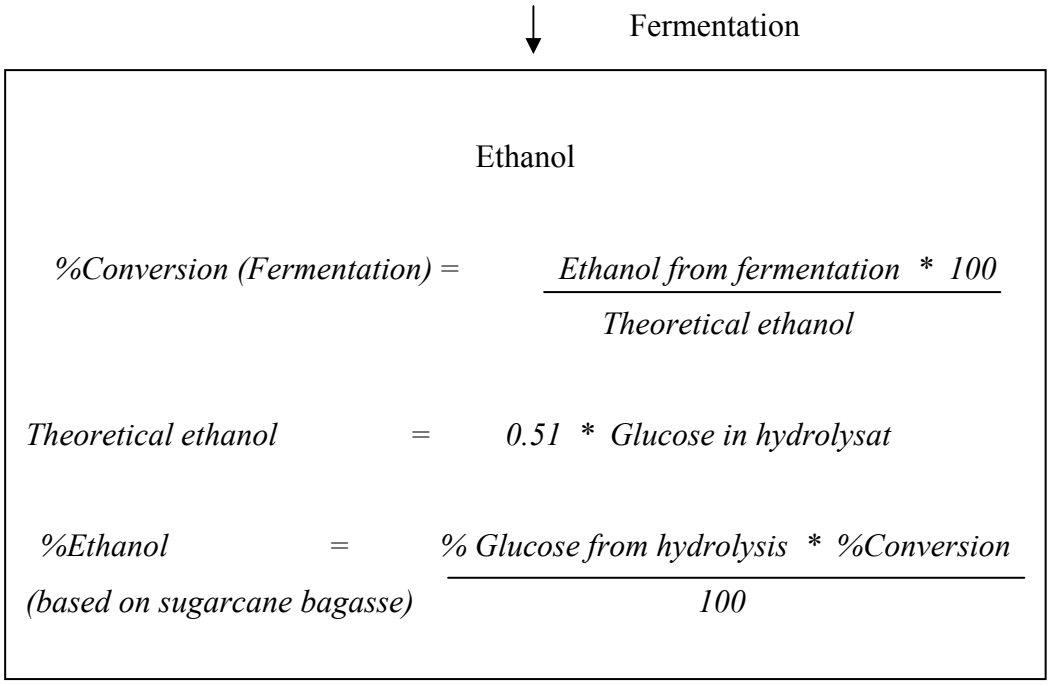
** based on the theory

Appendix F

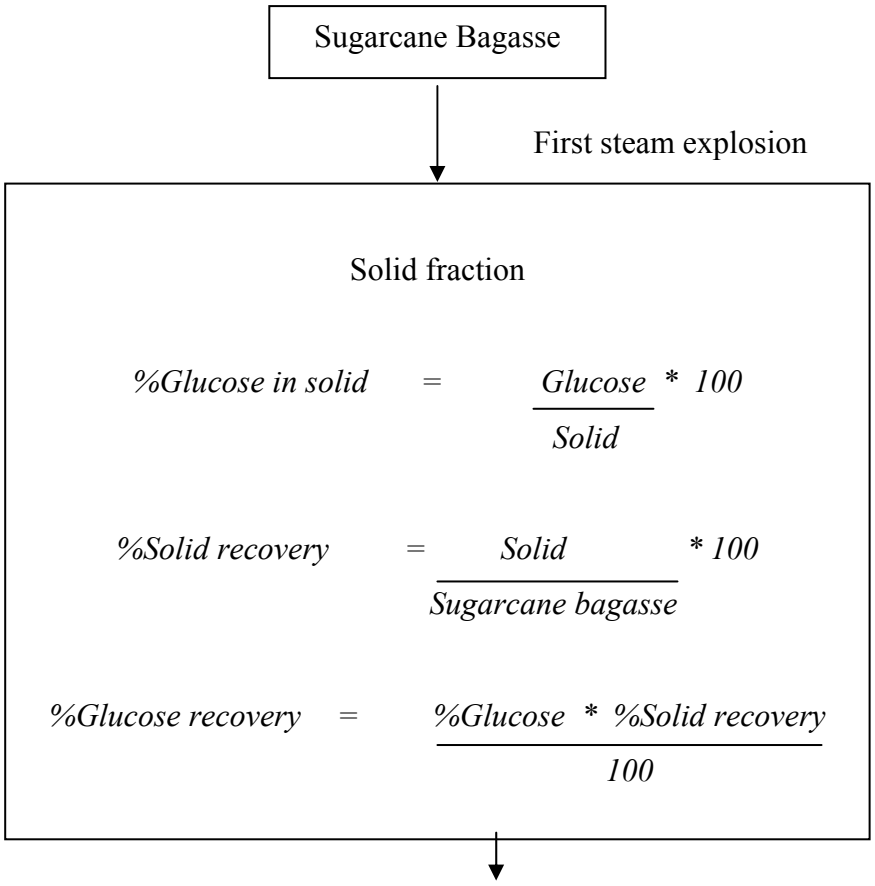
Data analysis

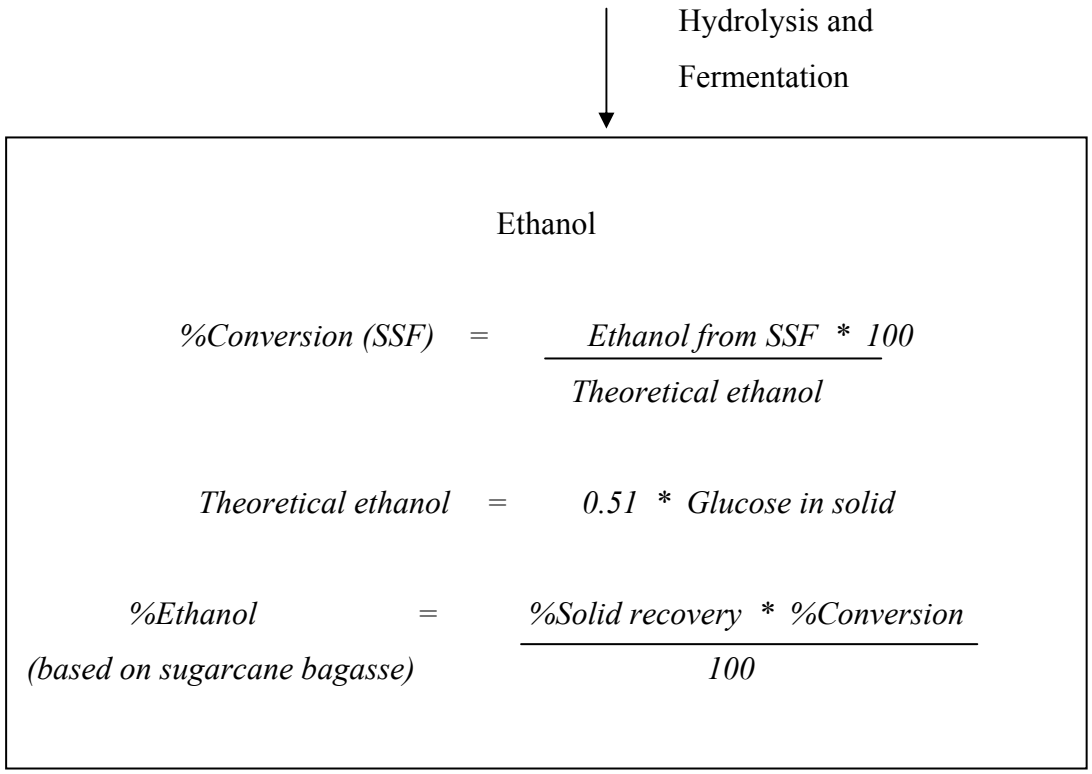
APPENDIX Figure F1 The analysis scheme of ethanol yield from SHF process





APPENDIX Figure F2 The analysis scheme of ethanol yield from SSF process




Hydrolysis and
Fermentation
Ethanol
%Conversion (SSF) = Ethanol from SSF * 100 / Theoretical ethanol
Theoretical ethanol = 0.51 * Glucose in solid
%Ethanol (based on sugarcane bagasse) = %Solid recovery * %Conversion / 100

CURRICULUM VITAE

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