CHAPTER TWO

LITERATURE REVIEW

2.1 Pretreatment of Lignocellulosic Biomass

Lignocellulosic biomass has a high cellulose and hemicellulose content. For instance, rice straw has 32-47% of cellulose, 19-27% of hemicelluloses, and 5-24% of lignin (Garotte *et al*., 2002). Hemicellulose contains mainly pentose, in which xylose is the most important sugar (14.8-20.2%) (Roberto *et al.,* 2003). Rice straw is composed of cellulose and hemicelluloses that are densely packed by layers of lignin. Such an architecture inhibits enzymatic hydrolysis. Therefore, it is necessary to pre-treat the biomass before hydrolysis by the action of enzymes (Lynd *et al*., 1996). Pretreatment was aimed to decrease the crystallinity of cellulose, to increase the total surface area of biomass, to break the lignin seal and to remove hemicellulose, thereby increasing the yield of glucose. Pretreatment has been considered the most expensive step in the production of bioethanol from biomass as it uses a lot of chemicals and energy (Mosier *et al*., 2005).

Xyloglucan (Figure 1.5) is the major hemicellulose found in plants. Xyloglucan has a backbone similar to cellulose consisting of β-1,4-D-glucopyranose residues, to which α-D-xylopyranose residues attach to the C-6 position (FRY, 1989). Generally 60-75% of glucose residues are branched with xylose. Xyloglucan is strongly associated with cellulose microfibrils by hydrogen bonding, hence providing a strong microstructure that protects the plant cells from bursting due to the osmotic pressure. During plant growth,

xyloglucan degrades to form smaller oligosaccharides and loosen the cellulose-xyloglucan network thus allowing cell expansion. Hydrogen bondings between the cellulose and xyloglucan are strong, making the extraction of xyloglucan difficult (Ebringerova *et al*., 2005). The common method used to extract the xyloglucan is alkaline extraction (Pauly *et al.*, 1999). Three types of xyloglucan have been identified in the cellulose-xyloglucan network. The first type can be accessed easily and is susceptible to enzymatic hydrolysis. They form loops, dead-ends and connecting adjacent cellulose microfibrils. The second type is associated with the surface of cellulose microfibrils that can be removed by alkaline pretreatment. The third type is the xyloglucan that is entrapped in a non crystalline region of cellulose microfibrils that is most resistant to hydrolysis. The solubilization of xyloglucan coating layer is important to increase the efficiency of hydrolysis making cellulose more accessible to cellulase enzymes (Vincken *et al*., 1994).

Lignocellulosic biomass can be pretreated by chemical method by utilizing chemicals such as acids and alkalis or by biological method by using enzymes produced in microorganisms. In this section, the different types of pretreatment are discussed.

2.1.1 Pretreatment by Phosphoric Acid- Acetone

Phosphoric acid-acetone was used to pre-treat the lignocellulosic material. Results showed that pretreatment by phosphoric acid-acetone can effectively increase the yield of bioethanol. As an example, Bermudagrass was pretreated at 50°C for 60min with different phosphoric acid concentrations and the pretreated samples were hydrolysed by cellulase for 24h. The enzymatic digestibility is slightly higher compared with the untreated sample

(43.1% vs 39.6%). Raising the concentration of phosphoric acid further increases the digestibility to 53.6% (Zhang *et al*., 2006). The lignocellulosic structure cannot be broken down with a phosphoric acid below 73% concentration. The digestibility can achieve 97.3% with concentrated phosphoric acid pretreatment. The enzymatic hydrolysis profiles show that cellulase readily attack the more accessible samples pretreated with concentrated phosphoric acid-acetone. The sample was hydrolysed up to 90% at 2h and up to 96% at 24h. Pretreatment can break down the crystalline cellulose to amorphous cellulose and remove hemicellulose and lignin (Kim and Mazza, 2008). Sometimes, dilute phosphoric acid (2-6%) was used for pretreatment because sodium phosphate was formed after neutralization of hydrolysate with sodium hydroxide. The usage of dilute phosphoric acid requires a high temperature or high pressure for a long time and requires a lot of energy supply. Compared to the dilute acid, concentrated phosphoric acid pretreatment lowers the capital cost for equipment and unit operation cost. (Li *et al*., 2009).

2.1.2 Pretreatment by Dilute Sulfuric Acid

Other results show that the lignin composition in raw materials will greatly affect enzymatic saccharification and affect ethanol yield (Jørgensen *et al.,* 2007). The enzymatic hydrolysis is inversely proportional to the concentration of sulfuric acid used in the pretreatment. It is suggested that a higher concentration of acid will increase the inhibition of the enzyme, thereby dilute acid pretreatment is preferred (Palonen *et al*., 2004). Dilute acid pretreatment is regarded as the most efficient pretreatment because most of the glucan in hemicellulose remains in the biomass after pretreatment and the glucan can be converted

into glucose to yield bioethanol in the proceeding production steps. An accessible total surface area of the solid residue which is the cellulose is important because the cellulase enzyme must collide with the cellulose before it can hydrolyse the cellulose according to the Collision Theory (Thompson *et al*., 1992). The rate of reaction increases with total surface area, hence, pretreatment becomes a very important step to increase the total surface exposed to the cellulase. The amorphous portion of the cellulose can be attacked by cellulase more readily compared to crystalline cellulose (Mansfield *et al.,* 1999). The lignin polymer is considered as an enzyme adsorbent because it reduces the concentration of cellulase for hydrolysis and slows down the rate of reaction, hence needs to be removed (Vinzant and Ehrman, 1997). Therefore, pretreatment plays two important roles in the production of bioethanol; firstly, it increases the total surface area of cellulose, secondly, it removes lignin in the lignocellulosic biomass. (Guo *et al*., 2009).

2.1.3 Pretreatment by Alkali-Peracetic Acid

In Zhao's study, the use of NaOH-peracetic acid pretreatment under mild conditions greatly increased the digestibility of sugarcane bagasse. More than 90% of reducing sugars can be obtained from the enzymatic hydrolysis of sugarcane bagasse that has been pretreated by 10% NaOH in a 3:1 liquid-to-solid ratio at 90°C for 1.5h and followed by delignification with 10% peracetic acid (PAA) at 75°C for 2.5h (Zhao *et al*., 2009). The use of alkali-peracetic acid has advantages over acid and alkali pretreatment because it could be conducted under milder conditions with less carbohydrate being degraded in the process. In the first stage, the biomass swelled and was partially

delignified by NaOH and subsequently, further delignified by PAA. Pretreatment is important to remove the hemicellulose and lignin in the biomass, to decrease the crystallinity of cellulose and to increase the porosity of the material thereby increasing the accessibility of cellulose towards enzymatic hydrolysis (Martin *et al*., 2007; Sun and Cheng, 2002; Mosier *et al*., 2005).

2.1.4 Pretreatment by Dilute Alkali

Pretreatment of lignocellulosic materials is important to liberate cellulose from its lignin seal and reduce the crystallinity of cellulose before the hydrolysis process. Alkaline pretreatment disrupt the ester bond linking lignin (Figure 1.7) and xylan (Figure 1.4) and therefore the lignin can be removed (Chen and Dixon., 2007). It was reported that xylan-rich cell walls that contain a large amount of lignin prevent enzymatic hydrolysis (Jeoh *et al*., 2007). Alkali pretreatment has an advantage over acid pretreatment because less degradation of sugar occurs during the pretreatment (Carrillo *et al*., 2005).

In McIntosh and Vancov's study, the alkaline pretreatment was carried out at a lower temperature, pressure and residence time. Agents for alkaline pretreatment such as sodium hydroxide, ammonia and lime are not costly (Varga *et al*., 2002) and the pretreated biomass requires less enzyme usage for hydrolysis. Hence, the cost of production of bioethanol can be reduced (Sendich *et al*., 2008).

The alkaline pretreatment of Sorghum straw produced a dark brown solution consisting of soluble and insoluble materials. The recovery of the insoluble fractions revealed substantial loss of weight from the original starting materials. NaOH of higher

concentration resulted in a greater loss in mass. Temperature has the greatest impact on loss of mass, followed by alkalinity and residence time. The loss of lignin and hemicelluloses contributed to the loss of weight of biomass after exposure to NaOH. A higher concentration of NaOH resulted in a higher concentration of hemicellulose in the liquid fraction after pretreatment which means more hemicellulose is removed. Delignification can be improved by using more concentrated NaOH. Complete delignification was reported when 10% of NaOH were used. Alkaline treatment at 0.75% was ineffective to remove the lignin in biomass (McIntosh and Vancov, 2010). The lignin content has a strong negative correlation with the sugar released by enzymatic hydrolysis because lignin acts as a barrier that restricts cellulase from acting on cellulose (Chen and Dixon., 2007). The pretreated biomass yielded three times more sugar than the untreated biomass. This is due to the swelling and hydrolysis of lignin and hemicellulose by NaOH (Cheng *et al*., 2008; Varga *et al*., 2003).

2.1.5 Pretreatment by Microwave-Alkali

Agriculture residues such as rice straw and rice hulls were mainly managed by burning or incorporated into soils (Yu *et al*., 2009). Both of the methods applied have a bad impact on the environment. Burning rice straws will lead to significant air pollution that can cause global warming and respiratory diseases. The incorporation of agricultural residues into wet soil will enhance the emission of methane gas that leads to the greenhouse effect. Therefore, the use of agricultural waste such as rice straws and hulls avoids the problems stated above and also produce a clean fuel that does not produce

acidic gases such as sulphur dioxide that can lead to corrosion of buildings and lowering of the pH of rivers and lakes. Lignocellulosic biomass contain a complex matrix of polymer that is resistant to the liberation of monosaccharides. Hence, pretreatment has been given much focus in order to increase the digestibility by opening the complex matrix of polymers (Singh *et al*., 2010).

In Singh's study, the microwave-alkaline pretreatment was carried out in a laboratory microwave. Optimum conditions of pretreatment were: alkaline concentration 2.75%, irradiation time 22.50 min and substrate concentration 30 g/L, as optimized by box–behnken design (Singh *et al*., 2011). The lignocellulosic matrix is made up of cellulose and lignin bound by hemicellulose chains. Alkali pretreatment successfully reduced the lignin content because of the solubilization of lignin in the alkali and hence increase the accessibility of cellulose to microbial enzymes. As previously stated, removal of lignin helps to reduce the use of enzymes because it reduces binding of cellulase to lignin (Lu et al., 2002).

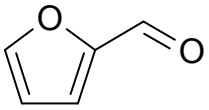
2.2 Hydrolysis of Lignocellulosic Biomass

Cellulose obtained after the pretreatment is hydrolysed to give glucose. The hydrolysis can be catalysed by biological catalysts such as enzymes, or by chemical catalysts such as concentrated or dilute acids or water. In this section, the different types of hydrolysis are discussed.

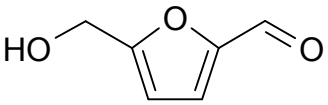
2.2.1 Hydrolysis by Dilute Sulfuric Acid

Based on the research studies, mild acid hydrolysis produces mainly xylose (5-C sugar) from xylan with the cellulose and lignin fractions remain unhydrolysed. Xylan has an amorphous structure that makes it more susceptible to mild acid hydrolysis (Rahman *et al*., 2007). Further more, xylose produced in the hydrolysis degraded rapidly to form furfural (Figure 2.1). Monosaccharides produced in the hydrolysis can be further reacted by acid to form unwanted products. Other by-products such as 5-hydroxymethyl furfural (HMF), acetate, hydroxybenzylaldehyde (HBA), siringaldehyde (SGA) and vanillin (Figure 2.1) that are toxic to the yeast in fermentation are formed as well (Rao *et al*., 2006).

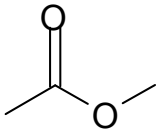
The figure below shows structures of some of the by-products formed in hydrolysis. They are toxic to the yeast used in fermentation and thus reduce the yield of ethanol.



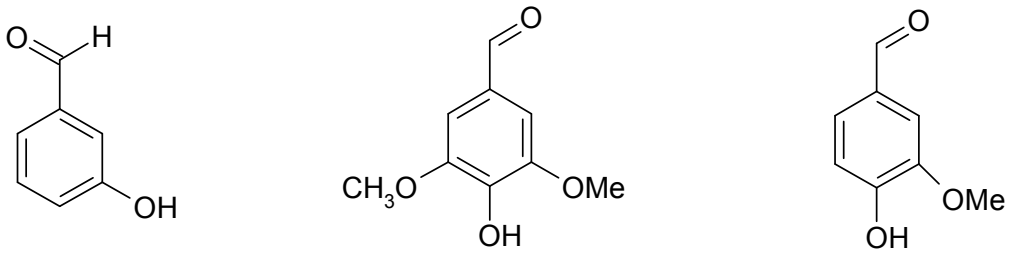
furfural



HMF



Acetate



HBA siringaldehyde vanillin

Figure 2.1: Structures of toxic by-products in acid hydrolysis

The hydrolysis of biomass was first done with 5% sulfuric acid. The pretreated biomass needs to be converted into glucose before it can be converted into ethanol by microorganisms such as yeast (El-zawawy *et al*., 2011). There are two types of acid catalysed hydrolysis which are dilute or concentrated acid hydrolysis. Dilute acids require operating temperatures between 200-240°C. Concentrated acids on the other hand are required in large quantities and therefore increase the cost of production. In El-zawawy's experiment, the pretreated biomass such as rice straw, banana leaves and corn cob were hydrolysed with 5% sulfuric acid for 2h. After the first hydrolysis, the glucose content was measured. As an alternative, acid hydrolysis can be done with 5% sulfuric acid under microwave radiation for 10min. Microwave-dilute acid hydrolysis yielded a higher percentage of glucose compared to the 2h hydrolysis by 5% sulfuric acid at high temperatures. However, the 2h hydrolysis gave a higher yield of ethanol although it gave a lower percentage of glucose. This can be explained by the formation of inhibitor by-products such as furans, organic acid and phenolic compounds under the effect of

microwave. These by-products are inhibitors for the fermentation, hence reduce the overall ethanol production (Sun and Cheng, 2002).

The most suitable concentration of sulfuric acid used is 5%. The optimum hydrolysis time for 5% sulfuric acid is 2h, giving highest fermentable glucose monomers from cellulose polymer (El-zawawy *et al*., 2011).

2.2.2 Hydrolysis by Concentrated Sulfuric Acid

Concentrated sulfuric acid provides a rapid conversion of cellulose into glucose and hemicellulose into 5-carbon sugars at a relatively low temperature compared to dilute acid. Hydrolysis by concentrated sulfuric acid minimizes sugar degradation and is more cost efficient and hence is more favorable than dilute acid. The biomass was soaked in 70% sulfuric acid at 40-50ºC for 2-4 h. Low temperatures minimize the degradation of sugar (Chandel *et al*., 2007). The solid residue is washed to recover more sugar. The solid residue was then dried and soaked in 30% sulfuric acid for another 50 min for further hydrolysis. The cost of production can be greatly reduced by recycling the acid. Recycling of the acid also helps to reduce the cost of waste management and reduce the negative impact on the environment (Jeffries and Jin, 2000).

2.2.3 Hydrolysis by a Mixture of Enzymes

Cellulase is a mixture of at least three enzymes namely endoglucanase (EC 3.2.1.4), exoglucanase or cellubiose hydrolase (CBH, EC3.2.1.91) and β-glucosidase (EC

3.2.1.21) that work together to hydrolyse cellulose such as cellulase produced from *Tricoderma reesei* (Van *et al*., 2007). The pretreated biomass in 100ml of sodium acetate buffer at pH 4.7 is sterilized at 121ºC for 20 min. The cellulase enzyme is then added to the cooled sterilsed mixture. The reaction is stopped by placing the sample in boiling water for 5 min. Other cellulase produced by microorganisms has been used as well, such as cellulase from *Penicillium janthinellium* (El-zawawy *et al*., 2011).

2.2.4 Hydrolysis by Water

During hydrolysis, some glucose may be lost by degradation to form hydroxymethylfurfural (HMF) (Aida *et al*., 2007). In order to maximize ethanol production, glucose degradation is not desired. Hydrolysis by liquid hot water (LHW) was done in two types of reactors, a batch autoclave and continuous flow apparatus. The results showed that a high solubility of biomass is possible to yield a high percentage of glucose. However, degradation of glucose and xylose into HMF and furfural respectively increases with the harshness of the hydrolysis condition (Rogalinski *et al.,* 2008).

In Rogalinski's method, liquid hot water experiments were carried out in a continuous-flow apparatus. The system pressure was set high (100bar) to maintain water as a liquid at 310°C. LHW experiments were also done in a batch autoclave. The sugars were identified by the refractive index detector. Results of experiments showed that solubilization of the substrate was 25%, sugar yield was less than 5% and degradation product was about 10%. A very interesting result showed that untreated sample gave a higher sugar yield, and the size of straw particles did not influence the sugar. From the

economic point of view, production of bioethanol by water can be very promising as it saves cost on pretreatment of biomass (Rogalinski *et al*., 2008).

2.3 Fermentation of Hydrolysate to Bioethanol

Lignocellulosic biomass is often hydrolysed and fermented by microorganisms to yield ethanol. Hydrolysis of the lignocellulosic material does not give glucose monomers only, other monomers such as xylose, mannose, galactose, arabinose, and oligosaccharides are also produced (Keshwani *et al*., 2009). In order to maximize the yield of ethanol, it is important that the chosen microorganisms are capable to convert the sugars other than glucose to ethanol. In theory, 0.51kg of ethanol and 0.49kg of carbon dioxide will be produced per 1kg of xylose and glucose (Howard *et al*., 2003).

The reaction can be represented by the following equations:-

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

(Equation 2.1)

$$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2$$

(Equation 2.2)

Most microorganisms use 6-carbon sugars such as glucose to produce ethanol in anaerobic respiration. *Saccharomyces cerevisiae* is one of the most promising yeasts that produces high yield of ethanol, it has high tolerance towards ethanol and other inhibitory products by hydrolysis and is easily available (Galbe and Zacchi, 2002; Tian *et al*., 2008).

Currently, the most promising ethanologenic bacteria that produce high yields of ethanol is the *Escherichia coli, Klebsiella oxytoca,* and *Zymomonas mobilis* (Keshwani *et al.,* 2009). Xylose fermenting microorganisms can be found among bacteria, fungi and yeast. They can be native or genetically engineered to carry out simultaneous saccharification and fermentation (Galbe *et al*., 2006). *Pichia stipitis, Candida shehatae* and *Candida parapsilosis* are examples of xylose-fermenting microorganisms (Katahira *et al*., 2006; Keshwani *et al.,* 2009).

In order to select a suitable yeast for the fermentation, some of the performance parameters need to be taken into consideration. An efficient yeast is capable of giving more than 90% bioetahnol yield, withstanding more 4% bioethanol tolerance, producing more than $1\ gl^{-1}h^{-1}$ bioethanol, using an inexpensive medium formulation, growing in a concentrated hydrolysate, resisting inhibitors (Dien *et al*., 2003).

Fermentation can be done in a closed culture system. The microorganism is added into the sugar containing solution to allow it to carry out fermentation. The fermenting solution is left undisturbed throughout the fermentation. Sometimes, acid or alkali can be added into the fermenting solution in order to control the pH, this is called batch process (Abtahi, 2008).

In the continuous process, the feed containing sugar is pumped continuously into a vessel containing the active yeast. In this process, the fermenter is operated in a continuous mode, whereby the cell densities of the microorganisms can be maintained at high level (Çaylak and Vardar, 1998). The continuous process gives a higher productivity of bioethanol than the batch process (Lawford, 1988).

2.4 Recovery of Ethanol by Distillation

Ethanol has a lower boiling point compared to water, thus enabling ethanol to be separated from ethanol-water mixture by fractional distillation. A large amount of energy is needed to recover ethanol from the liquid mixture with more than 80% water content as the ethanol content after fermentation is below 20%. The highest concentration of ethanol that can be achieved is 95.6% as ethanol and water form an azeotropic mixture. A beer column is capable of separating most of the ethanol from water and 37% of ethanol is obtained. The ethanol is then concentrated by a rectifying column to around 95% (Balat *et al.*, 2008).