CHAPTER II
LITERATURE REVIEW

Lignocellulosic biomass offers another way to produce fuel ethanol. In order to produce ethanol from the lignocellulosic biomass, the collected biomass has to undergo pretreatment steps to isolate carbohydrate polymers, hydrolysis steps to obtain sugars, detoxification steps to reduce degradation products, and fermentation steps to produce bioethanol. **Figure 2.1** illustrates the general steps for ethanol production from lignocellulosic biomass.

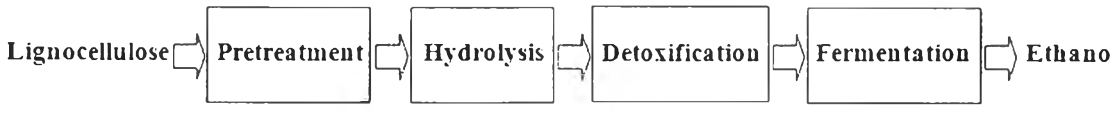

Figure 2.1 Ethanol production process from lignocellulosic biomass

2.1 The composition of lignocellulosic materials

Lignocellulosic materials consist mainly of cellulose, hemicelluloses, and lignin. Cellulose, hemicelluloses, and lignin exist in the cell wall of plants. Pectin, which is responsible for plant growth, cell wall extension, and cell binding, is also present in the cell wall (Willats *et al.*, 2001). Their composition may differ depending on the type of lignocellulosic materials. **Figure 2.2** shows the structure of plant cell wall (Cesarino *et al.*, 2012).

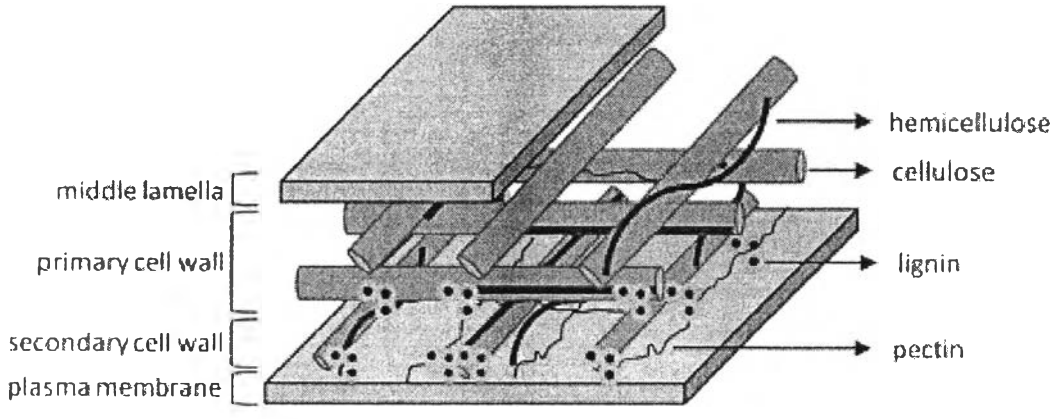

Figure 2.2 Detailed model of plant cell wall

Cellulose could make up to 35–50% of the biomass, while hemicellulose and lignin could make up to 20–35% and 10–25%, respectively. Some of the most common lignocellulosic materials include plants, agricultural remains, and wastes from the pulp and paper industry. **Table 2.1** shows the composition of some well known lignocellulosic materials (Sun *et al.*, 2002).

Table 2.1 Cellulose, hemicelluloses, and lignin contents in some lignocellulosic materials (Sun *et al.*, 2002)

Lignocellulosic materials	Composition (%), dry basis		
	Cellulose	Hemicellulose	Lignin
Hardwood stems	40–55	24–40	18–25
Softwood stems	45–50	25–35	25–35
Nut shells	25–30	25–30	30–40
Corn cobs	45	35	15
Grasses	25–40	35–50	10–30
Paper	85–99	0	0–15
Wheat straw	30	50	15
Leaves	15–20	80–85	0
Cotton seed hairs	80–95	5–20	0
Newspaper	40–55	25–40	18–30
Waste papers from chemical pulp	60–70	10–20	5–10
Primary wastewater solids	8–15	N/A	N/A
Solid cattle manure	1.6–4.7	1.4–3.3	2.7–5.7
Switchgrass	45	31.4	12
Swine waste	6	28	N/A

2.1.1 Cellulose

With more than 10^{11} tons estimated to be synthesized each year, cellulose is considered to be the most abundant biopolymer on earth (Brown, 2003). Cellulose is comprised of polymerized glucose monomers connected by β-1,4 linkage. The degree of polymerization of cellulose chains is about 500 to 25000. The arrangement is usually in

ordered or microcrystalline structures, which makes it quite challenging to hydrolyze under natural conditions (Malherbe *et al.*, 2002). It is commonly found in eukaryotic organisms such as plants and algae. However, cellulose could also be produced by prokaryotes such as bacteria and cyanobacteria (Brown, 2003). The chemical structure of cellulose is shown in **Figure 2.3**.

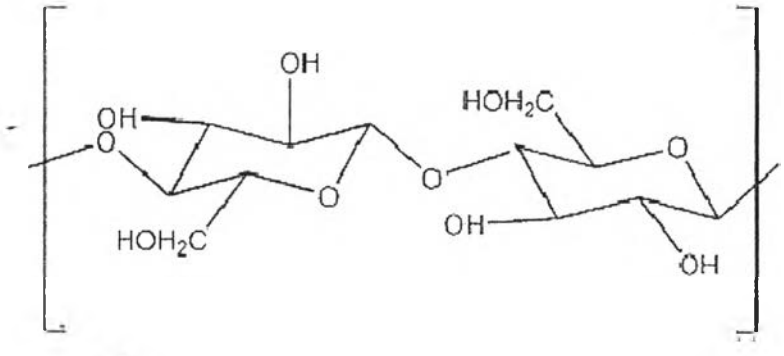

Figure 2.3 Structural formula of cellulose

2.1.2 Hemicellulose

Hemicellulose is the second most abundant polysaccharide found in nature. Unlike cellulose which is chemically homogenous, hemicellulose exists in various combinations of pentoses (β-D-xylose, α-L-arabinose), hexoses (β-D-mannose, β-D-glucose, α-D-galactose), and/or uronic acids (α-D-glucoronic, α-D-4-O-methylgalacturonic, and α-D-galacturonic acids) (Gírio *et al.*, 2010). Hemicellulose is less ordered than cellulose due to many branches with the degree of polymerization ranging from 100 to 200 (Malherbe *et al.*, 2002). Hemicelluloses of hardwoods consist primarily of glucoronoxylans (15–30%) while the hemicelluloses of softwoods possess galactoglucomannans as the dominant hemicelluloses (20–25%). The major types of polysaccharides present in grass hemicelluloses are glucuronoarabinoxylans (15–30%), xyloglucan (2–25%), and arabinoglucuronoxylan (5-10%) (Gírio *et al.*, 2010).

2.1.3 Lignin

Lignin is one of the complex macromolecules in the plant cell wall. It is responsible for plant structural support, impermeability, and resistance against microbial attack and oxidative stress (Mussatto *et al.*, 2010). Unlike cellulose, the structure of lignin varies between each type of lignocellulosic biomass. Lignin removal shows an improvement in biomass digestibility (Lee *et al.*, 2008).

2.2 Pretreatment of lignocellulosic biomass

The pretreatment of lignocellulosic biomass is essential in order to obtain the highest yield of sugar, and thus, bioethanol from the process. Once grass undergoes pretreatment steps, cellulose in the grass becomes more accessible to enzymes. Hydrolysis steps then can be completed more rapidly with higher yields. The pretreatment methods attempt to remove and/or partially depolymerize hemicelluloses, remove and/or modify lignin structure, increase surface area and porosity of biomass, and decrease cellulose crystallinity (Lee *et al.*, 2008). Suitable pretreatment steps may differ for each biomass depending on their properties. Several pretreatment options are available, namely utilizing concentrated acid, alkaline, diluted acid, sulfur oxide, hydrogen peroxide, steam explosion, ammonia fiber explosion, wet-oxidation, lime, liquid hot water, carbon dioxide explosion, and other organic solvents (Saha, 2003). Pretreatment is considered one of the most costly steps in the production of ethanol from biomass.

2.2.1 Physical Pretreatment

Physical pretreatment such as milling and grinding can increase external surface area of lignocellulosic biomass. In addition to milling and grinding, scientists also employ thermal pretreatment to enhance the production of sugar. Factors that affect thermal pretreatment are steam temperature, residence time, particle size, moisture

content, and catalyst concentration. Thermal pretreatment is one of the appropriate pretreatment steps to treat softwoods. The methods, such as steam explosion and microwave irradiation, can be utilized to improve enzymatic digestibility in softwoods. In contrast, hardwoods contain lower lignin and higher pentose sugars compared to softwoods; high temperature can cause thermal degradation of pentose sugars. Steam explosion pretreatment proves to be an effective physical pretreatment method to increase enzymatic digestibility of straws. The pretreatment results in the evaporation of water inside cells and the increase of specific surface area as straws explode. Some hemicelluloses decompose to acids which catalyze decomposition of hemicelluloses and lignin. The decomposition essentially releases cellulose.

Microwave is an effective treatment in improving enzymatic digestibility in rice straw because it changes the structure of cellulose and degrades lignin. The electromagnetic field of microwave is hypothesized to cause an explosion among particles which results in the disruption of crystal structures in lignocelluloses (Lee *et al.*, 2008). Moreover, Zhu lab reports that the combination of microwave and alkaline pretreatment could remove more lignin and hemicelluloses from rice straw with shorter pretreatment time compared to alkaline-only pretreatment (Zhu *et al.*, 2006). Thus, microwave-assisted alkali treatment is an efficient method to improve enzymatic digestibility.

2.2.2 Chemical Pretreatment

Agricultural residues, grasses, and softwoods show better diffusivities of sulfuric acid than hardwoods. Consequently, the pretreatment time can be shortened for the former lignocellulosic biomasses. The successful pretreatments for grass (*Miscanthus sinensis)* employ diluted ammonium hydroxide with low temperature steam by microwave (Boonmanumsin *et al.*, 2012). The study on the production of monomeric sugar from mission grass using diluted sodium hydroxide shows that the concentration of sodium hydroxide affects the removal of lignin as well as the degradation of sugars.

Thus, the optimization of diluted chemicals is required to maximize the production of monomeric sugars (Tatijarern *et al.*, 2013).

2.3 Hydrolysis

Most cellulose chains are crystalline. Therefore, harsh conditions are required to liberate glucose. However, extremely harsh conditions could potentially result in sugar degradation. Dilute acid hydrolysis is the most used technique in breaking down cellulose and hemicellulose. The adequate condition for cellulose and hemicellulose hydrolysis is 1-4% of diluted acids, under 120–160 °C (Mussatto *et al.*, 2010).

Researchers start using xylanase and cellulase to increase internal surface area of the biomass. The advantages of using biological treatments are their high specificity, low energy consumption, no chemical requirement, and gentle environmental conditions to avoid sugar degradation (Mussatto *et al.*, 2010). The enzyme, such as xylanases, result in the degradation of hemicellulose. The degraded hemicellulose improves the hydrolysis of lignocellulosic biomass by providing cellulases with more internal surface area. The hydrolysis steps, therefore, become more efficient.

2.3.1 Cellulase

The utilization of cellulase results in the hydrolysis of cellulose chains. Cellulase specifically hydrolyzes β-1,4-glycosidic linkages in cellulose. Three types of cellulase enzymes are endoglucanase (endo-1-4-β-glucanase), exoglucanase (cellobiohydrolase), and β-glucosidase. Endoglucanase randomly attacks in the amorphous region of cellulose, results in more surface area for successive attacks by exoglucanase. Exoglucanase can hydrolyze the highly crystalline part of a cellulose chain. It degrades both ends of cellulose chain into smallest repeating units of cellulose (also known as cellobioses). The glucose molecules in cellobioses are linked by β-1,4-glycosidic linkages. The

enzyme β-glucosidase is responsible for hydrolyzing the linkages in cellobioses. **Figure 2.4** illustrates a model of cellulases (Ratanakhanokchai *et al.*, 2013).

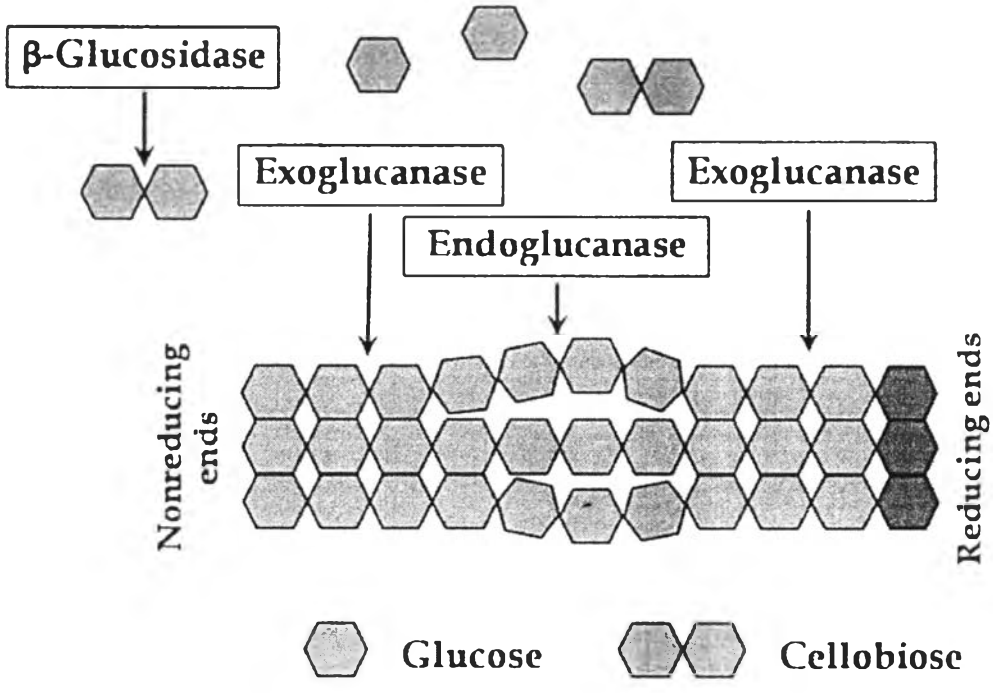

Figure 2.4 A model of cellulases: endoglucanases (endo-1-4-β-glucanase), exoglucanase (cellobiohydrolase), and β-glucosidase

2.3.2 Hemicellulase

Hemicellulose is mostly made of chains of xylose sugars (xylan). The enzyme responsible for the degradation of xylan is called xylanase. Two common xylanases are endoxylanase (endo-1,4-β-xylanase), and 1,4-β-xylosidase. Endoxylanase hydrolyzes xylan to form oligosaccharides of xylose sugars. The enzyme 1,4-β-xylosidase then breaks down the oligosaccharides to form individual xylose sugars. Furthermore, Xylanases require accessory enzymes including α-L-arabinofuranosidase, α-4-O-methyl-D-glucuronidase, acetyl xylan esterase, ferulic acid esterase, and p-coumaric acid

esterase in order to hydrolyze wood xylans efficiently **(Figure 2.5)** (Ratanakhanokchai *et al.*, 2013).

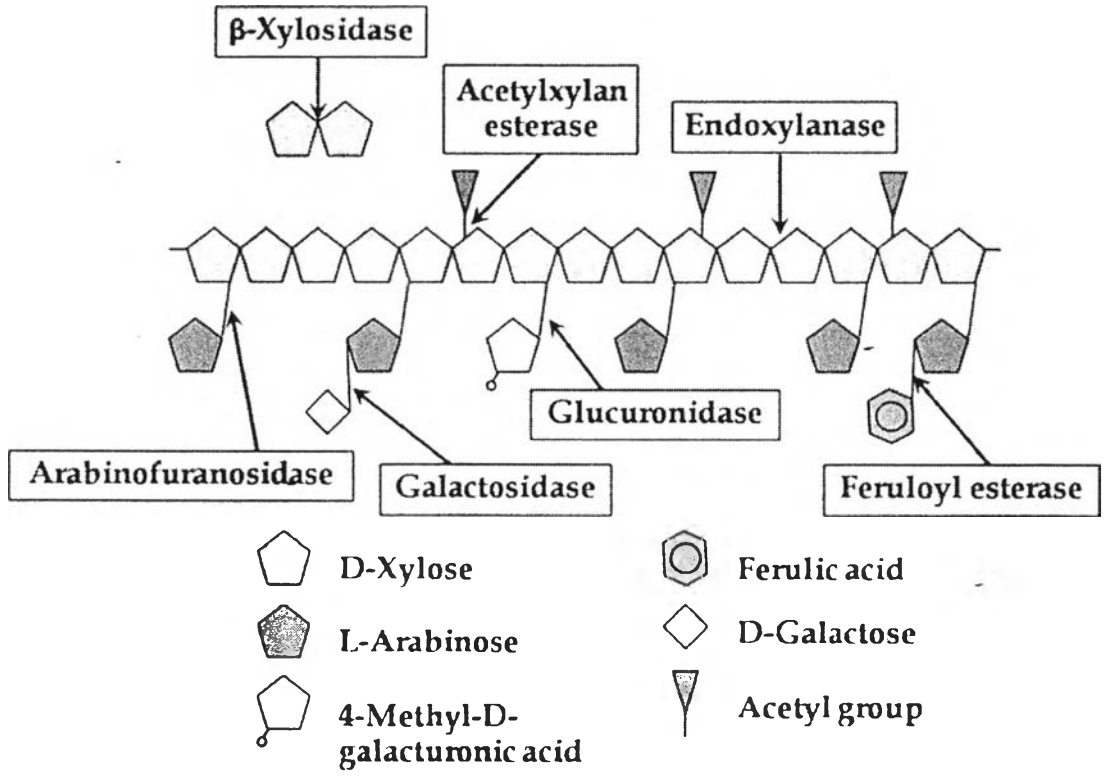

Figure 2.5 A model of xylanases and the accessory enzymes in wood xylans

2.4 Factors affecting hydrolysis

2.4.1 Physical factors

pH

A study of *Aspergillus niger* is observed and reported that the optimal pH for the highest cellulase production is at 5.5, while the pH of about 5.5-6.5 is the optimal pH for the production of β-glucosidase. The range in the pH depends on the pH of the medium. A study of *Neurospora crass* and *Humicola fuscoatra* can detect the presence of

cellulase when the pH is 7, and the enzyme concentration increases steadily when the pH is 7.5 (Kumar *et al.*, 2008).

Temperature

Temperature has a crucial importance in the hydrolysis process. Studies on various microorganisms (*Thielavia terrestris-225, Mycelieopthora fergussi-246C, Aspergillus wentii, Penicillum rubrum, Aspergillus niger)* indicated that the temperature for analyzing the activity of cellulase falls within 50°–65 °C, while the most favorable growth temperature for these microorganism is 25°–30 °C. Temperature beyond 65 °C results in rapid decrease in the enzyme activity. This is caused by the denaturation of the enzyme (Kumar *et al.*, 2008).

2.4.2 Chemical factors

Carbon source

The observation of *Aspergillus niger* shows that the production of cellulases increase when 12% of carbon is present. However, a further addition of carbon results in decrease of cellulase production; the lack of oxygen and other nutrients may be the cause. Kumar reports that bagasse shows an increase in saccharification when the carbon concentration is 4% w/v higher than normal. The same result is observed during the hydrolysis of rice straw and other paper wastes at the carbon concentration of 2.5–6.5% w/v (Kumar *et al.*, 2008).

Phosphorus source

Fungi require phosphorus as sustenance for growth and metabolism. Furthermore, phosphorus is necessary in the construction of cell membrane. Although many sources of phosphorus such as potassium dihydrogen phosphate, tetra-sodium pyrophosphate, sodium β-glycerophosphate, and dipotassium hydrogen phosphate are tested, the

study shows that potassium dihydrogen phosphate is the best source of phosphorus in cellulase production process (Kumar *et al.*, 2008).

2.5 Detoxification

Hydrolysis process extracts numerous compounds including compounds from lignocellulosic structure, heavy metal ions, and lignin and sugar degradation products. Palmqvist reports some of the toxic compounds from the hydrolysis of spruce wood- **(Figure 2.6)** (Palmqvist *et al.*, 2000).

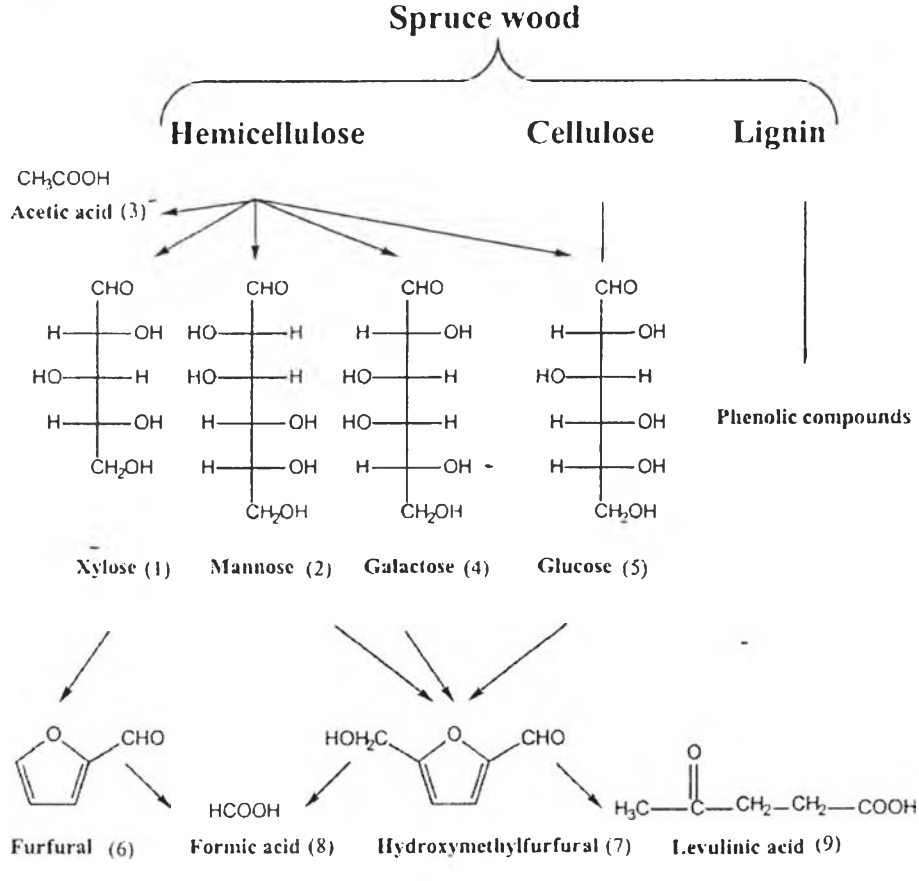

Figure 2.6 Sugar and lignin can further be degraded to form compounds that may decrease ethanol yield

In addition, dissolved oxygen concentration, and pH of the medium can be altered during the hydrolysis processes. All these variables can be connected to the toxicity, which could jeopardize the efficiency of the fermentation steps. Thus, lignocellulosic hydrolyzates should be subjected to a detoxification process in order to create a more habitable environment for the microorganisms to exist (Mussatto *et al.*, 2010).

Several types of detoxification methods through biological, physical, and chemical processes have been proposed (Mussatto *et al.*, 2010). The purpose of detoxification is to alter the toxic compounds into inactive compounds, or to reduce their concentration. Some factors including the degree of toxicity for the specific lignocellulosic biomass, and the tolerance of the microorganism have to be taken into consideration.

2.6 Degradation productions

2.6.1 Sugar degradation products

One of sugar degradation products that can inhibit cell growth rate is furfural. Furfural originates from a degradation of pentose sugars during hydrolysis. A study concludes that furfural with concentration lower than 0.5 g/L does not have a negative effect on cell growth, whereas the concentration higher than 2.0 g/L stunts the cell growth almost completely (99%) (Mussatto *et al.*, 2010).

During the hydrolysis process, a hexose sugar could degrade to form hydroxymethylfurfural. Hydroxymethylfurfural has similar inhibiting effect as furfural. High concentration of the compound causes microorganisms to stop growing (Mussatto *et al.*, 2010).

2.6.2 Lignin degradation products

Lignin degradation products are more toxic than furfural and hydroxymethylfurfural. Various compounds such as aromatic, polyaromatic, phenolic, and aldehydic are

released from lignin once the cellulosic biomass is subjected to undergo hydrolysis process. Phenolic compounds possess inhibitory effect on the fermentation. The compounds with low molecular weight are especially the most toxic (Ando *et al.*, 1986). Phenolic compounds destroy the integrity of cell membranes, which result in the failure of selective permeability of the membranes and enzyme matrices. Thus, the cell growth and sugar absorption are diminished (Mussatto *et al.*, 2010).

2.6.3 Compounds derived from lignocellulosic structure

During the hydrolytic process, the raw material extractives such as acidic resins, taninic, and terpene acids are released. Although they are not as toxic as the products from lignin degradation, these extractives show inhibitory effect at high concentration (Mussatto *et al.*, 2010).

2.6.4 Heavy metals ions

Heavy metal ions such as iron, chromium, nickel, and copper may come from corrosion of hydrolysis equipment. The ions can inhibit the microorganism's metabolic pathway. Microbial activity is slightly decreased under the presence of copper, nickel, chromium, and iron ions in the concentration lower than 4 mg/L (Mussatto *et al.*, 2010).

2.6.5 "Synergistic effect" of toxic compounds

The effect of toxic compounds also depends on other factors such as the microorganism's adaptation to the medium, the type of fermentation process, and the number of toxic compounds and their "synergistic effect". A study shows that ethanol production is slightly increased when a small concentration of acetic acid (up to 10 g/L) is present without furfural, and when a small concentration of furfural (up to 2 g/L) is present without acetic acid. However, when both compounds are present even at small concen-

tration, they inhibit the growth rate, cell mass yield, and ethanol yield. The toxicity of the hemicellulosic hydrolyzates is mostly due to the combination of many toxic compounds (Mussatto *et al.*, 2010)

2.7 Detoxification Methods

Different fermenting microorganisms possess varying degree of tolerance toward the degradation compounds. Furthermore, Palmqvist has claimed that even the same strains of microorganisms may have different tolerance toward the compounds (Palmqvist *et al.*, 2000). Thus, various detoxification methods need to be studied to carry out the suitable method for the microorganisms.

2.7.1 Biological detoxification

Biological detoxification utilizes enzymes such as peroxidase and laccase. Laccase has the ability to selectively remove phenolic monomers and phenolic acids by performing oxidative polymerization of the phenolic compounds (Palmqvist *et al.*, 2000). High molecular weight phenolic compounds are less toxic to the microorganisms than the low molecular weight compounds. The ethanol yield increases considerably with the utilization of laccase suggests that phenolic compounds plays a major role in the inhibition of ethanol production (Jönsson *et al.*, 1998).

A study on the fungus *Trichoderma reesei* shows that it is able to detoxify hemicellulose degradation products, which essentially increases the overall ethanol yield. The detoxification treatment with *T. reesei* removes acetic acid, furfural, and benzoic acid derivatives (Palmqvist *et al.*, 2000).

2.7.2 Physical detoxification

Roto-evaporation to near dryness is studied to be an effective method to remove acetic acid. The detoxification process could lower the concentration of acetic acid,

furfural, and vanillin by as much as 54%, 100%, and 29%, respectively (Wilson *et al.*, 1989).

Larger fermenting container for inoculation is shown to improve ethanol yield (Leonard *et al.*, 1945). Large inoculating container allows the inhibitors such as furfural and hydroxymethylfurfural (HMF) to spread out throughout the container, which reduces the overall concentration of the compounds.

2.7.3 Chemical detoxification

Overliming is one of the oldest techniques in the detoxification of lignocellulosic hydrolyzate (Palmqvist *et al.*, 2000). In the overliming process, calcium hydroxide or sodium hydroxide is used to increase the pH to 9-10. Then, the hydrolyzate is adjusted to pH 5.6-5.8 by sulfuric acid (Leonard *et al.*, 1945). The overliming process with calcium hydroxide results in higher ethanol yield than with sodium hydroxide. According to Palmqvist, overliming with calcium hydroxide causes the precipitation as well as removal of toxic compounds that are unstable at high pH (Palmqvist *et al.*, 2000).

Anion exchange is another method of detoxification processes. Although anion exchange at pH 10 could remove more than 80% of phenolic compounds and 70% of furfural and 5-HMF, the method causes a significant loss of fermentable sugar. Thus, the method is not a practical detoxification method (Larsson *et al.*, 1999, Palmqvist *et al.*, 2000).

One of the most efficient methods to remove inhibitors from lignocellulosic hydrolyzate is the combination of overliming, adding sulfite, and heating. The addition of sulfite after overliming is reported to reduce the fermentation time by a factor of three. Furthermore, heating results in the removal of volatile inhibitors (Palmqvist *et al.*, 2000).

2.8 Ethanol conversion process

The main sugars presented in these low-cost bioethanol feedstocks are glucose, xylose, and arabinose. Glucose obtained from lignocellulosic hydrolysis steps can be converted into ethanol through fermentation process. *Saccharomyces cerevisiae* and *Zymomonas mobilis* are the common yeasts used in the conversion of sugar to bioethanol. However, they cannot ferment xylose and arabinose. Other yeasts such as *Pachysolen tannophilus, Pichia stipitis,* and *Candida shehate* are used instead in fermenting xylose to ethanol. The conversion of xylose to ethanol still faces with restriction due to the fact that these yeasts have low ethanol tolerance, slow fermentation rate, sensitive to the rate of supplied oxygen, and responsive to inhibitors produced during the pretreatment. Other strains of yeasts can ferment arabinose to ethanol, but very low yield is obtained. Thus, no yeast has the ability to ferment all these sugars into ethanol (Saha, 2003).

Scientists then create recombinant organisms in order to enable the organisms to ferment both glucose and xylose. Using xylose reductase and xylitol dehydrogenase genes from *Pichia stipitis* and xylulokinase gene from *Saccharomyces cerevisiae*, scientists are able to develop a *Saccharomyces* strain with the ability to effectively ferment both glucose and xylose. However, since both sugars share the same transport system and the strain prefer glucose over xylose, simultaneous fermentation of glucose and xylose still face many difficulties (Lee *et al.*, 2008).

2.8.1 Submerged fermentation system

The most common fermentation processes is submerged fermentation system. Submerged fermentation system is a cultivation of microorganisms (bacteria, fungi, yeast, or algae) in the liquid medium. The system offers several advantages including the ease in making the medium, the simplicity in adjusting conditions (pH, dissolved oxygen, temperature, agitation, and nutrients) for the microorganism, and the uniformity of the growth of the microorganisms (Mussatto *et al.*, 2010).

2.8.2 Solid-state fermentation system

Moist solid materials are used as medium in solid-state fermentation system. Unlike the submerged fermentation system, solid-state fermentation system contains low moisture. The environment is appropriate for fungi because fungi can grow and spread their hyphae through the material. Solid-state fermentation system gives higher yield compared to its liquid counterparts. The main disadvantages of this process include heat transfer and culture homogeneity problems (Mussatto *et al.*, 2010).