CHAPTER II

LITERATURE REVIEWS

2.1 Giunea grass

Guinea grass (*Panicum maximum*) is a tall (up to 2m) perennial grass, native of Africa. Its leaves are broad, flat, and long (Figure 1A). Guinea grass was introduced to Thailand as a forage plant because its leaves are soft and contain high protein level (13-21 % w/w). Flowering stalks (Figure 1B) of taller varieties can reach up to 3 to 4 m in height (Cameron and Lemcke, 2008). Purple guinea grass (*Panicum maximum* cv TD 53) grows well on a wide variety of soils and under light shade of trees and bushes. Therefore, it can grow all over Thailand and can be inserted in empty space of fully growing agricultural area, such as pararubber plantation ditches. Purple guinea grass is resistant to drought, but responds quickly to fertilizer and watering. It gives a very high yield (1.5 – 4.0 tons/rai) which lasts for 10 years or longer and also easy harvesting (Ria, 2001). This makes purple guinea grass as an ideal renewable substrate for lignocellulosic ethanol production.

Figure 1A Guinea grass (Department of Livestock Development, 2003 : online)

Figure 1B Flowering stock (Department of Livestock Development, 2003 : online)

2.2 Ethanol

Ethanol (ethyl alcohol, grain alcohol) is a clear, flammable, agreeable order and colorless liquid.Ethanol is a straight-chain alcohol, and its molecular formula is C_2H_5OH (Figure 2). The boiling point of ethanol is 78.3 $^{\circ}C$ (Shakhashiri, 2009). It is made of oxygen, hydrogen and carbon and is obtained from the fermentation of sugar. Ethanol is a renewable fuel because it is produced from biomass. Ethanol burns more cleanly and completely than gasoline or diesel fuel.

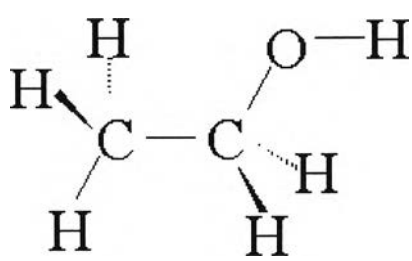


Figure 2 Chemical structure of ethanol. (Shakhashiri, 2009 : online)

2.2.1 Ethanol production

There are 2 major pathways for ethanol production.

2.2.1.1 Hydration of ethylene

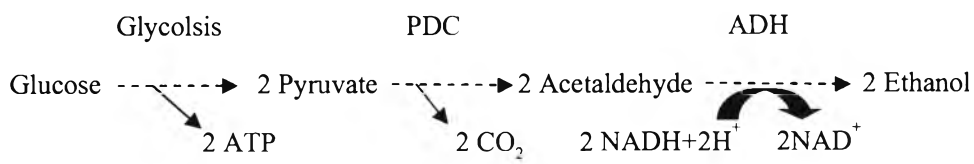
Ethanol is produced through the direct hydration of ethylene by reacting a mixture of ethylene and water in the vapor phase over a supported tungsten oxide (P_2O_5) catalyst followed chemical equation shown in Figure 3 (Wade, 1995).

$$H_2C{=}CH_2 + H_2O \xrightarrow[(P_2O_5)]{\text{100-300 atm, 300}^\circ\text{C}} CH_3\text{-}CH_2\text{-}OH$$

Figure 3 Hydration of ethylene pathway.

2.2.1.2. Alcoholic fermentation

Ethanol is produced by several microorganism including yeast and bacteria and fungi. These microorganisms convert glucose to ethanol via pathway as shown in Figure 4 (Walker, 1998).



PDC = Pyruvate decarboxylase

ADH = Alcohol dehydrogenase

Figure 4 Alcoholic fermentation pathways.

2.2.2 Raw material for ethanol production

Raw material for ethanol production can divide into 3 parts:

1. Starch such as corn, cassava, potato, wheat, etc. (Figure 5A, 5B, 5C)

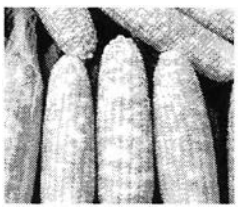


Figure 5A Corn

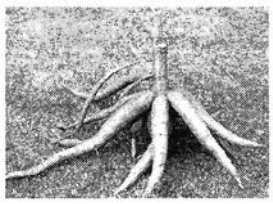


Figure 5B Cassava

Figure 5C Potato

(Department of Livestock Development, 2003 : online)

2. Sugar such as molasses, sugarcane, beetroot, etc. (Figure 6A, 6B, 6C)

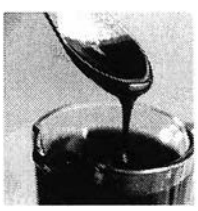


Figure 6A Molasses

Figure 6B Sugarcane

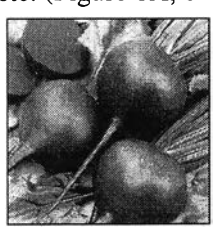


Figure 6C Beetroot

(Department of Livestock Development, 2003 : online)

3. Lignocellulose

   - Agro-industrial waste such as bagasses, paper pulp, etc. (Figure 7A)

   - Agricultural byproduct such as rice straw, corncob, etc. (Figure 7B, 7C)

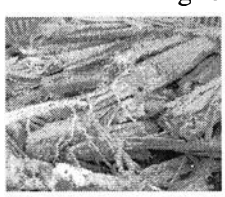


Figure 7A Bagasses

Figure 7B Rice straw

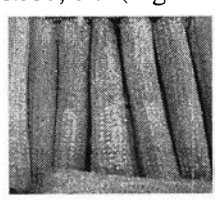


Figure 7C Corncob

(Department of Livestock Development, 2003 : online)

2.3 Lignocelluloses

Lignocellulose is a major component of plant cell walls (Figure 8), which is composed of (Taherzadeh and Karimi, 2007). It consists of 45% (w/w) cellulose, 30% (w/w) hemicellulose and 25% (w/w) lignin which form very complex structure through covalent bonding (Scalbert *et. al.*, 1985; Higuchi, 1990).

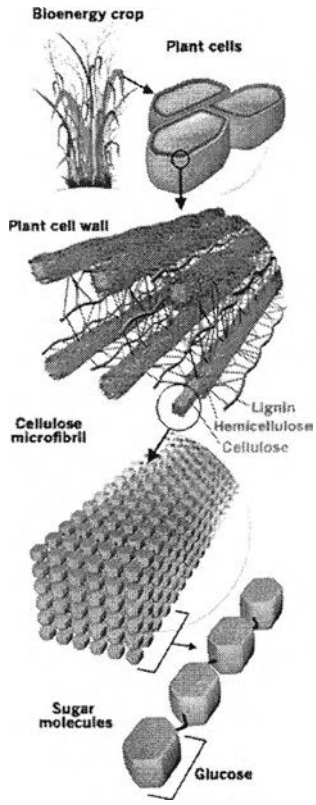


Figure 8 Lignocellulose in plant cell walls (Wyman, 2009 : online)

Cellulose is a linear homo-polysaccharide that consists of glucose (D-glucopyranose) units linked together by β-(1-4) glycosidic bonds (Figure 9). It is an organic compound with formula $(C_6H_{10}O_5)_n$ (Taherzadeh and Karimi, 2007).

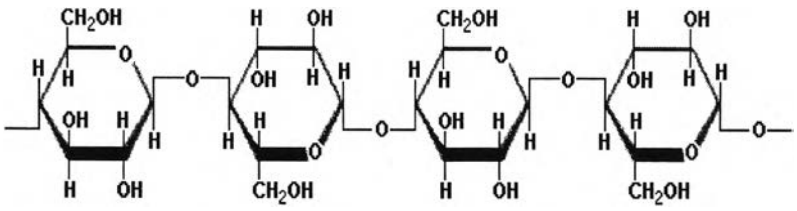


Figure 9 Cellulose structure (Zamora, 2005 : online)

Hemicellulose is hetero-polysaccharide whose chemical nature varies from tissue to tissue and from species to species. These polysaccharides are formed by a wide variety of building blocks including pentoses (*e.g.* xylose, rhamnose and arabinose), hexose(*e.g.* glucose, mannose and galactose) and uronic acids (*e.g.* 4-*O*-methyl-glucuronic and galacturonic acids) Hardwood hemicellulose contain mostly xylans, where as softwood hemicellulose contain mostly glucomannans. Xylans of many plant materials are heteropolysaccharides with homo polymeric backbone chains of 1,4-linked β- D - xylopyranose units (Figure10) (Saha *et. al.*, 2003). Softwood hemicellulose is more resistant to acid hydrolysis than hardwood hemicellulose.

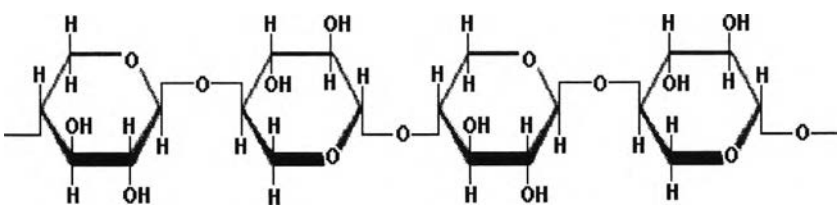


Figure 10 Homopolymeric backbone chain in xylan structure. (Zamora, 2005 : online)

Lignin is a phenolic macromolecule that is primarily formed by the free-radical polymerization of *p*-hydroxy cinnnamyl alcohol units with varying methoxyl contents. The chemical structure of lignin is very complicated and is based on three monomeric precursors:

coniferyl alcohol (Figure 11A), sinapyl alcohol (Figure 11B), and *p*-coumaryl alcohol (Figure 11C). The most important physical property of this organic macromolecule is its rigidity, which not only gives strength to the plant tissue but also prevent the collapse of the water-conducting elements (Taherzadeh and Karimi, 2007).

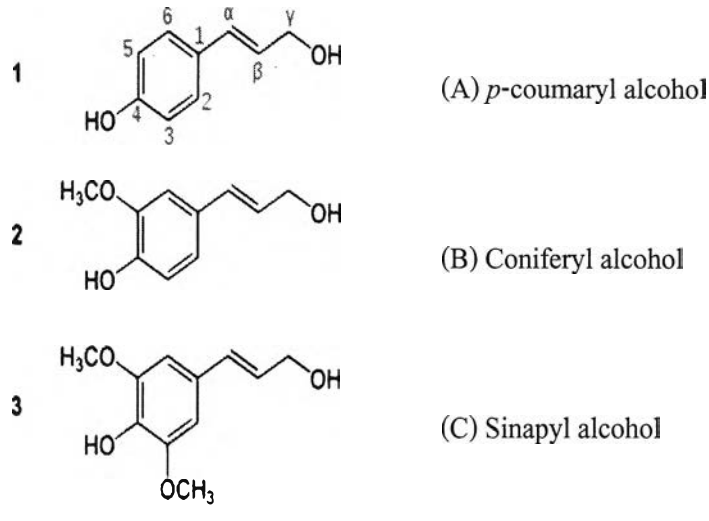


Figure 11 Three monomeric precursors of lignin : (A) *p*-coumaryl alcohol (B) coniferyl alcohol (C) sinapyl alcohol (Harborne, 1980 : online)

2.4 Lignocellulosic ethanol production

Lignocellulosic biomass is a cheap, renewable and abundantly available resource for ethanol production (Ho *et. al.*, 1998). Lignocellulose is a major component of plant cell wall and is composed of 3 major components: cellulose, hemicellulose and lignin. The cellulose can be hydrolyzed to fermentable sugar, glucose, by cellulase but these cellulose is hindered by hemicellulose and lignin. So, pretreatment process to increase the cellulose digestibility is necessary for lignocellulosic ethanol production (Zhao *et. al.*, 2009). Thompson *et. al.* (1992) and Chang and Holtzapple (2000) reported that cellulase susceptibility of cellulose in lignocellulose was depend on many factors such as cellulose crystallinity, polymerization degree, available surface area, pore size of cellulose and lignin content. Lignocellulosic ethanol production process is consisting of 3 main steps: 1) Pretreatment, break down lignocelluloses structure. 2) Enzymatic

hydrolysis, depolymerize cellulose to glucose by cellulase. 3) Fermentation, convert glucose to ethanol by microorganism (Margeot *et. al.*, 2009).

2.4.1 Pretreatment

Pretreatment is a process to increase enzymatic digestibility of cellulose in lignocellulose by removing of lignin and hemicellulose, reducing of cellulose crystallinity, and increasing of material porosity (Karimi *et. al.*, 2006). Bioconversion of lignocellulose to fermentable sugar, glucose, lignocellulose should undergo pretreatment. However, goal of the pretreatment should be effectively achieved without degradation or loss of carbohydrate, and without formation of inhibitory by-products for the subsequent hydrolysis and fermentation (Taherzadeh and Karimi, 2007).

The pretreatment methods may be classified into 3 major methods:

1) Biological pretreatment by using lignin-degradation microorganisms mostly basidiomycetes such as *Phanerochaete chrysosporium*, *Ceriporiopsis subvermispora*, *Phlebia subserialis* and *Pleurotus ostreatus* (Hatakka, 1994; Keller *et. al.*, 2000; Taniguchi *et. al.*, 2005). This method is safe and environmental friendly method for lignin removal but takes long time and is not cost effective (Yu *et. al.*, 2009).

Shi *et. al.* (2009) reported that fungal pretreatment of cotton stalk by *Phanerochaete chrysosporium* showed significant lignin and hemicellulose degradation compared with untreated stalk.

Hwang *et. al.* (2008) studied biological pretreatment of wood chips using four different white-rot fungi for 30 days. The result indicated that the glucose yield of pretreated wood by *Trametes versicolor* MrP 1 reached 45% by enzymatic hydrolysis.

Mtui *et. al.* (2009) studied fungal pretreatment of agricultural residues resulted to 45 - 75% and 65 - 80% holocellulose and lignin degradation, respectively.

2) Chemical pretreatment by using, for example sodium hydroxide, sulfuric acid, calcium hydroxide (lime), etc. to solubilize hemicellulose and lignin that cover cellulose make cellulose more accessible to the cellulase.

- Acid pretreatment

Hsu (1996) reported that sulfuric acid pretreatment had been most extensively used because it was inexpensive and effective.

Zheng (2009) reported that acid pretreatment method was derived from the concentrated acid hydrolysis such as concentrated H_2SO_4 and HCl hydrolysis, which was powerful and effective for cellulose hydrolysis but concentrated acid is toxic, corrosive, hazardous and requires reactors that need expensive construction material resistant to corrosion.

From the report of Zheng (2009) resulted in dilute acid pretreatment has received numerous research interests. It had been successfully developed for pretreatment of lignocellulosic biomass.

Karimi *et. al.* (2006) reported that dilute acid pretreatment method gave high reaction rate and significantly improved cellulose hydrolysis. Depending on the substrate and the conditions used, up to 95% of the hemicellulosic sugar could be recovered by dilute-acid hydrolysis from the lignocellulosic feedstock.

Taherzadeh and Karimi (2007) reported that dilute acid pretreatment was an economical method in ethanol production from lignocellulose because this method gave high xylan to xylose conversion yields.

Grohmann et. al. (1968) and Torget and Hsu (1994) reported that the major advantage of dilute acid pretreatment over steam-explosion was significantly higher xylose yield. When using batch dilute sulfuric acid pretreatment process, xylose yield was showed to approach 80% -90% of theoretical value.

Taherzadeh and Karimi (2007) reported that different chemical inhibitors (furfural, hydroxymethylfurfural and some volatile compounds from hemicellolose) might be produced during the acid pretreatment which reduced cellulase activity.

- Alkali pretreatmemt

Gossett *et. al.* (1982) reported that alkali pretreatment could solubilize both hemicellulose and some lignin, and the solubilized hemicellulose can further convert to volatile compound such as furfural which inhibited downstream fermentation process while lignin could inhibit fermentation process, too.

Hsu (1996) reported that the effectiveness of alkaline pretreatment depended on substrate and treatment conditions. Generally, alkaline pretreatment was more effective on agricultural residues and herbaceous crops than wood material.

Chang *et. al.* (2001) reported that lime was an effective pretreatment agent for herbaceous biomass (switchgrass, bagasse, wheat straw and corn stover), and oxidative lime (lime+oxygen) was effective pretreatment agent for woody biomass.

Gandi *et. al.* (1997) and Rabelo *et. al.* (2009) reported that lime (calcium hydroxide) pretreatment produced low fermentation inhibitors, provided low-cost alternative for lignin solubilization. Approximately 33% of lignin and 100% of acetyl groups were removed. The action of lime is slower than other pretreatments lime is an inexpensive reagent, safe and can be easily recovered

Kim and Holtzapple (2005) used lime to pretreat corn stover revealed that obtained maximum lignin removal of 87.5% at 55 $^{\circ}$C for 4 weeks with aeration.

Playne (1984) reported that using lime pretreatment at ambient conditions for up to 192 h can be enhanced the enzyme digestibility of the sugarcane bagasse from 20% to 72%. He also concluded that lime would be the choice chemical based on the cost of chemicals.

3) Physical pretreatment by cutting, milling including heating, irradiation (gamma-ray, electron-beam and microwave) and autohydrolysis. This method reduces substrate particle size (increases an available surface area), decreases cellulose crystallinity and degree of polymerization. Disadvantage of the physical pretreatment is a high energy requirement.

Yang *et. al.* (2008) reported that gamma radiation can breakdown of the structure of powder of 140 mesh wheat straw leading to weight loss and glucose yield of 13.40% at 500 kGy.

Zhu *et. al.* (2005a, 2005b, 2006) reported that microwave irradiation (up to 700 W) at various exposure times resulted to weight loss due to degradation of cellulose, hemicellulose and lignin.

Negro *et. al.* (2003) and Ballesteros *et. al.* (2008) reported that autohydrolysis or uncatalyzed steam-explosion was method which used only steam water. In this method, biomass particles are rapidly heated by high-pressure saturated steam for a period time to promote the

hemicellulose hydrolysis. The key factors for uncatalyzed steam- explosion are treatment time, temperature, particle size and moisture content.

In 2003, Ramos reported that the best pretreatment is a combination of physical and chemical method (physiochemical method) because the method has the highest efficacy for fractionating wood into its 3 major components and for enhancing the susceptibility of cellulose to enzymatic attack.

Physiochemical pretreatment was a method which combined chemical and physical treatment systems resulted in dissolving hemicellulose, alteration of lignin structure and providing an improved accessibility of the cellulose for hydrolytic enzymes (Hendriks and Zeeman, 2009). The mechanism of this method were delignification, decrease of the cellulose crystallinity and degree of polymerization and partial or complete hydrolysis of hemicellulose (Taherzadeh and Karimi, 2007).

Sun and Cheng (2002) reported that the most successful physicochemical pretreatments include thermochemical treatments such as steam explosion, liquid hot water (LHW), ammonia fiber explosion (AFEX) and CO_2 explosion.

Mtui *et. al.* (2009) reported that addition of H_2SO_4 (or SO_2) or CO_2 in steam explosion of lignocellulosic waste can effectively improve enzymatic hydrolysis and decrease the production of inhibitory compounds.

Mosier *et. al.* (2005) reported that liquid hot water pretreatment of corn fiber at 160 $^{\circ}C$ and a pH above 4.0 can be dissolved 50% of the fiber within 20 min.

Teymouri *et. al.* (2005) reported that the AFEX pretreatment is an effective method. The rate and the extent of both glucose and xylose produced were substantially greater than the untreated sample.

McMillan (1994) reported that AFEX works only moderately and is not attractive for the biomass with high lignin content such as hardwood, softwood and newspaper. The yields of enzymatic hydrolysis of AFEX-pretreated newspaper (18%-30% lignin) and aspen chips (25% lignin) were reported as only 40% and below 50%, respectively.

2.4.2 Enzymatic hydrolysis

Enzymatic hydrolysis of cellulose to glucose is carried out by cellulase under mild conditions (e.g. pH 4.5-5.0 and temperature 40-50 °C). Cellulase is a group of enzyme (β-1,4-endoglucanase, β-1,4-exoglucanase and β-glucosidase (cellobiase)). Hydrolysis of natural cellulose to glucose depends on the synergism of the three enzymes (Chen *et. al.*, 2007. β-1,4-endoglucanase or 1,4-β-D-glucan-4-glucanohydrolase is more active against amorphous region of cellulose and it can also hydrolyze substituted cellulose, such as carboxymethylcellulose (CMC) (Himmel *et. al.*, 1996). β-1,4-exoglucanase or 1,4-β-D-glucan cellobiohydrolase is active on crystalline cellulose. The exoglucanase cleaves disaccharide (cellobiose) unit in cellulose chain either from nonreducing or reducing end, whereas the endoglucanase hydrolyses the cellulose chain internally.

β-Glucosidase cleaves cellobiose to glucose (Béguin, 1990). Cellulose hydrolyzes step is shown in Figure12.

Though, cellulase is produced by several microorganisms such as *Trichoderma*, *Aspergillus*, *Clostridium*, *Penicillium*, *Fusarium* and *Steptomyces*. Most of commercial cellulase is produced from *Trichoderma* sp. (Taherzadeh and Karimi, 2007). However, *Trichoderma* cellulase has low activity of β-glucosidase.

Several studies improved an activity of *Trichoderma* cellulase by supplementation with extra β-glucosidase (Taherzadeh and Karimi, 2007).

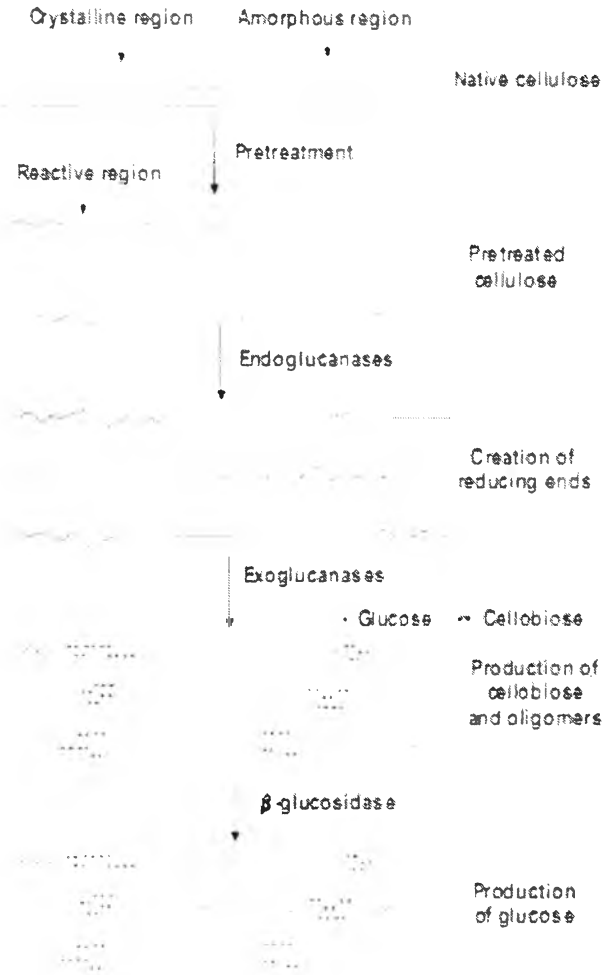


Figure 12 Schematic diagram showing hydrolysis cellulose by cellulolytic enzyme (Taherzadeh and Karimi, 2007)

Factors effects on enzymatic hydrolysis of lignocellulose are substrate concentration, pretreatment method, cellulase activity and hydrolysis conditions such as temperature, pH and mixing. High substrate concentration causes substrate inhibition resulting from problem in mixing and mass transfer (Taherzadeh and Karimi, 2007).

Kaya *et. al.* (1995) studied about influence of surfactants, cationic, anionic and non-ionic surfactants, on the enzymatic hydrolysis of xylan and cellulose. The result showed that all surfactants had some effects on the activities of xylanase and cellulase. Surfactants could act as both accelerators and inhibitors for the enzymes. The enzyme activity improved with all nonionic surfactants studied.

Taherzadeh and Karimi (2007) reported that change of lignocellulose enzymatic hydrolysis by ionic and non-ionic surfactants due to modification of cellulose surface property.

Gupta *et. al.* (2009) found that non-ionic surfactant (Tween-80) was the most effective. Tween-80 protected the enzymes from thermal deactivation during the enzymatic hydrolysis was proposed. This may be the result of reduction in enzyme contact with the air-liquid interface due to surface activity of the surfactants.

The reduction in surface tension of the solution inhibits the non-productive attachment of the exoglucanase to the lignin surface and allows the saccharifying exoglucanase greater access to cellulose, which results in increase of sugar release (Hemmatinejad *et. al.*, 2002).

2.4.3 Fermentation

Lignocellulose is the largest source of hexose and pentose sugars. So, ethanol fermentation yield from lignocellulosic substrate requires the organism that can ferment both hexose sugars (glucose, mannose, and galactose) and pentose sugars (xylose and arabinose) in the presence of inhibitory compounds liberated from pretreatment step. (Margeot *et. al.*, 2009). *Saccharomyces cerevisiae*, which is preferred organism for fermentative ethanol production due to high ethanol efficacy and high tolerance to the inhibitory compounds but *S. cerevisiae* lacks of key enzymes in xylose-metabolising pathway (Zhao and Xia, 2009). *P. stipitis*, could ferment xylose to ethanol, had board substrate specificity and absolutely not required vitamin, was a potential organism for ethanolic-xylose fermentation (du Preez *et. al.*, 1986). *Candida shehatae* and *Pachysolen tannophilus* could also ferment xylose to ethanol efficiently (Abbi *et. al.*, 1996).

The lignocellulosic ethanol fermentation is divided into 2 methods: separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF).

- Separate Hydrolysis and Fermentation (SHF)

Separate hydrolysis and fermentation is a process of fermentation when enzymatic hydrolysis and fermentation are performed sequentially. The advantage of this process is possible to carry out the cellulase hydrolysis and fermentation at its optimum conditions. Soderstrom *et. al.* (2003); Wingre *et. al.* (2003); Saha *et. al.* (2005) and Olsson *et. al.* (2006) reported that the optimum temperature for cellulase activity is usually 45-50 ^{o}C depending on

cellulase-producing microorganisms, while the optimum fermentation temperature for ethanol-producing microorganisms is usually 30-36 °C.

Main drawback of the SHF process is an inhibition of cellulase activity by released sugars, mainly cellobiose and glucose. Philippidis and Smith (1995) reported that cellobiose concentration lower than 6 g/l reduced in cellulase activity by 60%. The inhibitory effect of glucose on cellulase activity is lower than that of cellobiose but it is a strong inhibitor for β-glucosidase. At 3 g/l of glucose, the activity of glucosidase was reduced by 75%.

Another problem of the SHF process is contamination because the hydrolysis process is too long and a dilute solution of sugars always has a risk of microbial contamination even at high temperature such as 45-50 °C. The enzyme can be the source of the contamination, because it is difficult to sterilize the cellulase due to its deactivation during autoclave.

- Simultaneous Saccharification and Fermentation (SSF)

Simultaneous Saccharification and Fermentation is a process that combines both enzymatic hydrolysis and fermentation in one step. In this process, glucose produced by hydrolyzing enzymes is consumed immediately by fermenting microorganism resulting in reduction of the cellulase inhibition. The advantages of the SSF process are reduction of reactor number and faster saccharification rate resulting in lower cost of process (Ghosh *et. al.*, 1982). The risk of contamination on the SSF process is lower than the SHF process because possibility of contamination is reduced in the presence of ethanol (Taherzadeh and Karimi, 2007).

Main drawback of the SSF process is the different between optimal conditions (mainly pH and temperature) for enzymatic saccharification and fermenta tion (Saha and Cotta, 2008). Philippidis *et. al.* (1993) reported that the optimal temperature for the SSF process was 37-38 °C, which was compromise between the best temperature for enzymatic saccharification (45-50 °C) and the best temperature for yeast fermentation (30 °C). There are many studies on screening and developing for thermotolerant yeast strains that perform well at 40 °C with high ethanol tolerance which expect to solve this problem of the SSF process (ref).

Another problem of the SSF process is an inhibition of cellulase by produced ethanol. Wyman (1996) reported that 30g/l ethanol reduced cellulase activity by 25%. However, there has been less attention to cellulase inhibition of ethanol because practically it is

not possible to work with very high substrate concentration because of mixing problem and insufficiently mass transfer.

The SSF process gave higher reported ethanol yields and required lower amount of enzyme than the SHF process (Eklund and Zacchi, 1995; Karimi *et. al.*, 2006; McMillan *et. al.*, 1999; Sun and Cheng, 2002).

Ohgren *et. al.* (2007) compared an ethanol yield of steam-pretreated corn stover by fermented by the SHF and SSF method. They found that the SSF process gave 13% higher overall ethanol yields than the SHF process (72.4% versus 59.1% of the theoretical). The reduction of glucose inhibition in an enzymatic hydrolysis was found during the SSF process. So, the SSF process was concluded to be a better process configuration than the SHF process when the whole slurry was used.

Karimi *et. al.* (2006) and Abedinifar *et. al.* (2009) studied on ethanol production from duilute-acid pretreated rice straw by both the SHF and the SSF processes using *Mucor indicus*, *Rhizopus oryzae* and *Saccharomyces cerevisiae*. In case of the SHF process, *S. cerevisiae* had a great potential for ethanol fermentation followed by *M. indicus* and *R.oryzae*, respectively. While in the SSF process, *R. oryzae* had the best ethanol yield followed by *M. indicus*. Ethanol yield from the SHF process by *S. cerevisiae* was 0.39 g/g after 3 days of incubation. And ethanol yield from the SSF process by *R. oryzae* was 0.74 g/g after 2-3 days of incubation. From these result s indicated that the SSF process was better ethanol production process than the SHF process when performed with dilute-acid pretreated rice straw.

Softwood hemicellulose is mainly composed of mannose, which can be separated during the pretreatment and the fermentation in a separate bioreactor (Figure13) or possibly fermented together with the pretreated cellulose in SHF bioreactor. In case of ethanol production from hardwood and agricultural residues, the hemicellulose contains pentose which can be converted to ethanol in separate pentose-fermenting bioreactor (Figure 14) (Taherzadeh and Karimi, 2007).

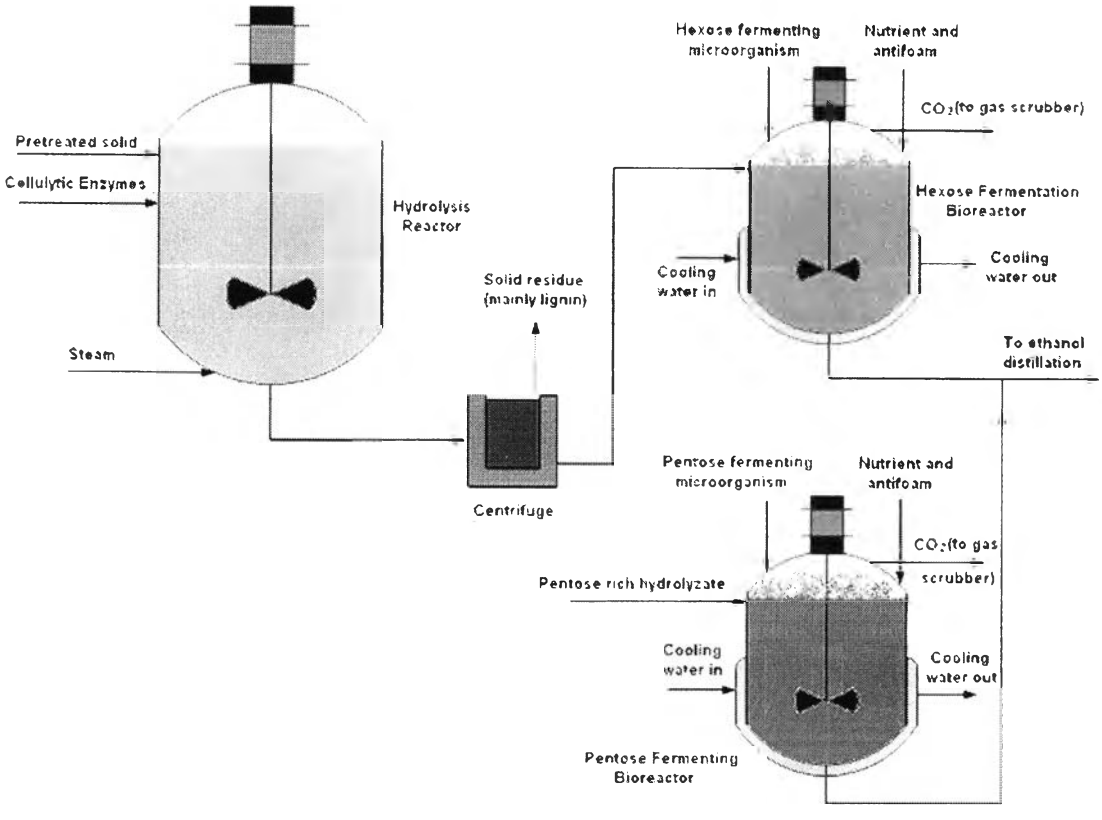


Figure 13 Process flow diagrams of separate enzymatic hydrolysis and fermentation (SHF) process. (Taherzadeh and Karimi, 2007).

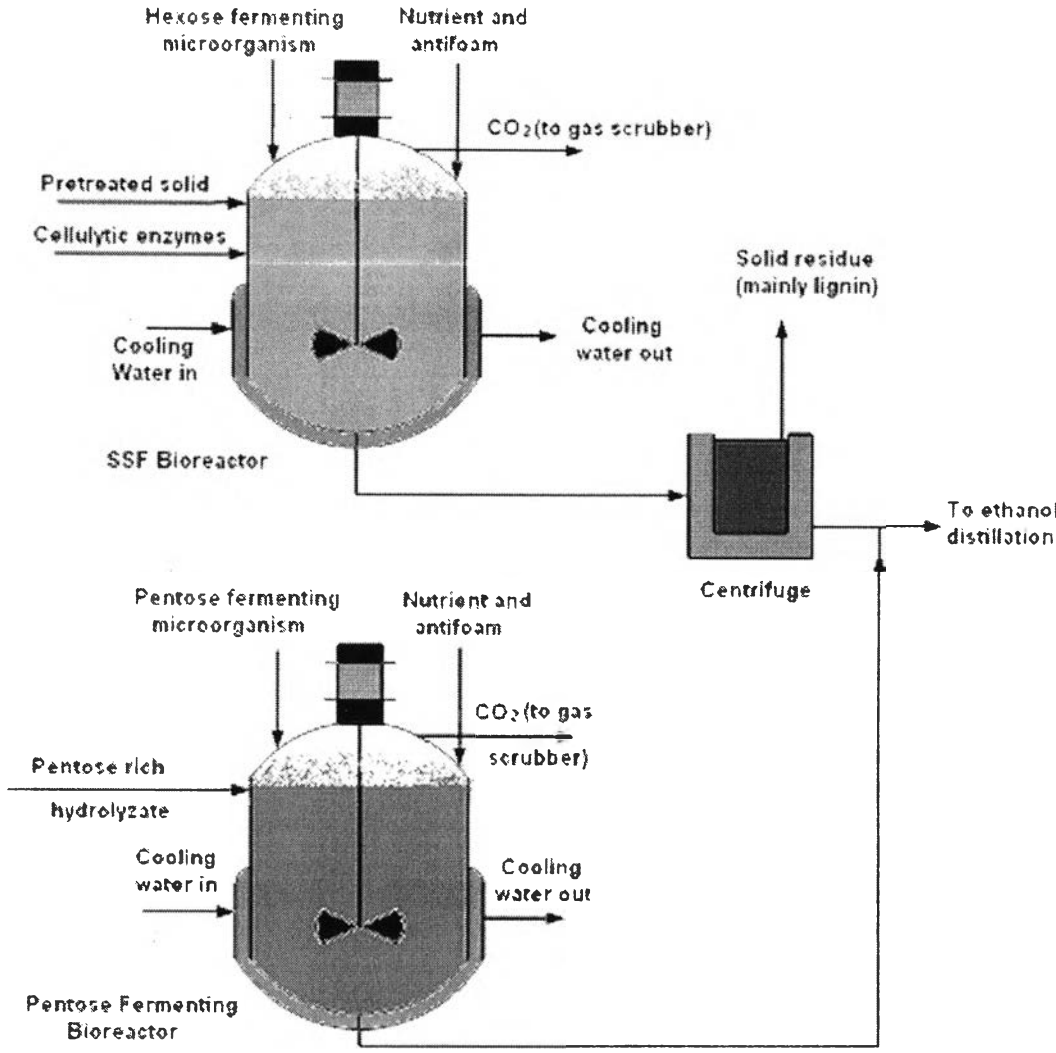


Figure 14 Process flow diagrams of simultaneous saccharification and fermentation (SSF) process (Taherzadeh and Karimi, 2007).

There are many studies on ethanol yield from pentose sugar fermentation using *Pichia stipitis* such as:

- Ethanol (0.39 g/g) was produced from D-xylose wood (du Preez *et. al.*, 1986).

- A 0.37 g/g of ethanol was produced from aspen wood (Delgenes *et. al.*, 1996).

- Kapoor *et. al.* (2008) reported an ethanol production of 0.36 g/g from *Prosopis juliflora.*

- Gupta *et. al.* (2009) obtained ethanol (0.39 g/g) from *Prosopis juliflora.*