CHAPTER 1

INTRODUCTION

Palm oil is one of the major agro-industries in Southern Thailand. The palm oil industry has developed very fast in the last few years due to the use of palm oil as raw material for biodiesel production. The Thai government energy policy stated clearly to increase the national renewable energy (NRE) from 0.5% in 2002 to 8.0% in 2011 (Prasertsan and Sajjakulnukit, 2006). The continued increase of oil price in 2007-8 stimulated the increase production of ethanol from cassava and biodiesel from palm oil. To have adequate supply of raw material, the oil palm plantation has been planned to expand to 6.4×10^5 hectare (4×10^6 Rai) in 2011. This increased not only palm oil production but also its wastes. Conversion of these wastes to valuable products would be beneficial to both the industry and the country's economics.

At present there are more than 65 factories employing the standard oil extracting process (or the wet process) (Kasikorn Research Center, 2009). The standard palm oil milling process has a lot of wastes consisting of 20-28% empty fruit bunch (EFB), 11% palm pressed fiber (PPF) and 0.87 m^3/ton FFB of wastewater or palm oil mill effluent (POME) (Prasertsan and Prasertsan, 1996). Most of palm oil mill wastes are lignocellulosic materials that are biomass consisting of cellulose (34%), hemicellulose (26%) and lignin (28%) (Kaddami *et al.*, 2006). Hemicellulose and cellulose can be converted by chemical reagent to pentose and hexose, respectively, then further converted to furfural and hydroxymethylfurfural, respectively, which finally converted to levulinic acid. Furfural is important as it is used as a selective solvent for separating saturated (ethane, propane, and butane) from unsaturated (ethelene, propylene, naptha, and aromatic compounds such as benzene, toluene, and p-xylene) in petroleum refining, gas, oil, and diesel fuel (Mansilla *et al.*, 1998). On the other hand, furfural is an inhibitor of ethanol production from hemicellulose hydrolysis, furfural is therefore reduced and controlled (Gutierrez *et al.*, 2006). A minimum content of pentosans in lignocellulosic material is around 18-20%

but only one third of this amount can be converted to furfural (Jaeggle, 1975; Montane *et al.*, 1998).

In addition, hemicellulose can also be converted by chemical reagent or enzyme to monomeric sugars mostly as xylose, and then further converted by fermentation to bio-ethanol. PPF has been reported to its component, that consisting of cellulose, pentosan, and lignin (Aziz, *et al.*, 2002); hence, its composition indicated the potential to use PPF as one of the suitable raw materials for bio-ethanol production. Bio-ethanol can be mixed with gasoline in the suitable ratio such as E10 (E10 means 10 % (v/v) ethanol mixed with 90 % (v/v) gasoline). The use of biotechnological science overcomes the environmental potentials such as greenhouse effect relating to the combustion of fossil fuels in chemical process.

The biological process of ethanol fuel production utilizing lignocellulose as substrate requires: (1) delignification to liberate cellulose and hemicellulose from their complex with lignin, (2) depolymerization of the carbohydrate polymers (cellulose and hemicellulose) to produce free sugars, and (3) fermentation of mixed hexose and pentose sugars to produce ethanol (Lee, 1997). Bio-ethanol production by microorganisms from renewable biomass can reduce fossil fuel use and wastes from agro-industry can be appropriately utilized.

Review of literature

1. Standard palm oil milling process

Palm oil milling process can be classified into two types, dry process and wet process (or standard process), which is regardless the use of decanter (Prasertsan *et al.*, 1990). Both processes have many differences such as process details, yield percentage, oil properties (color, viscosity, and smell) and amount of waste including advantages and disadvantages of each process. Palm oil milling process is known to generate large quantity of liquid and solid wastes which are lignocellulosic materials.

The wet process can be sub-classified into two types, decanter using and no decanter usage, and was usually used in large factories (Prasertsan *et al.*,

1990). The wet process using decanter is shown in Fig. 1. Initial step, fresh bunches were heated by steam at 120-130°C pressure 40 pound/in^2 for 45 minutes. Heated bunches were fed to separator machine in order to separate seeds from bunches and then pericarps fibre were separated from nuts by digester machine. The pericarp fibre were fed in the screw press in order to extract palm oil and palm oil then was fed to decanter for separating palm oil from water and particles fibre including soil and dust. After that, palm oil was stored in the tank.

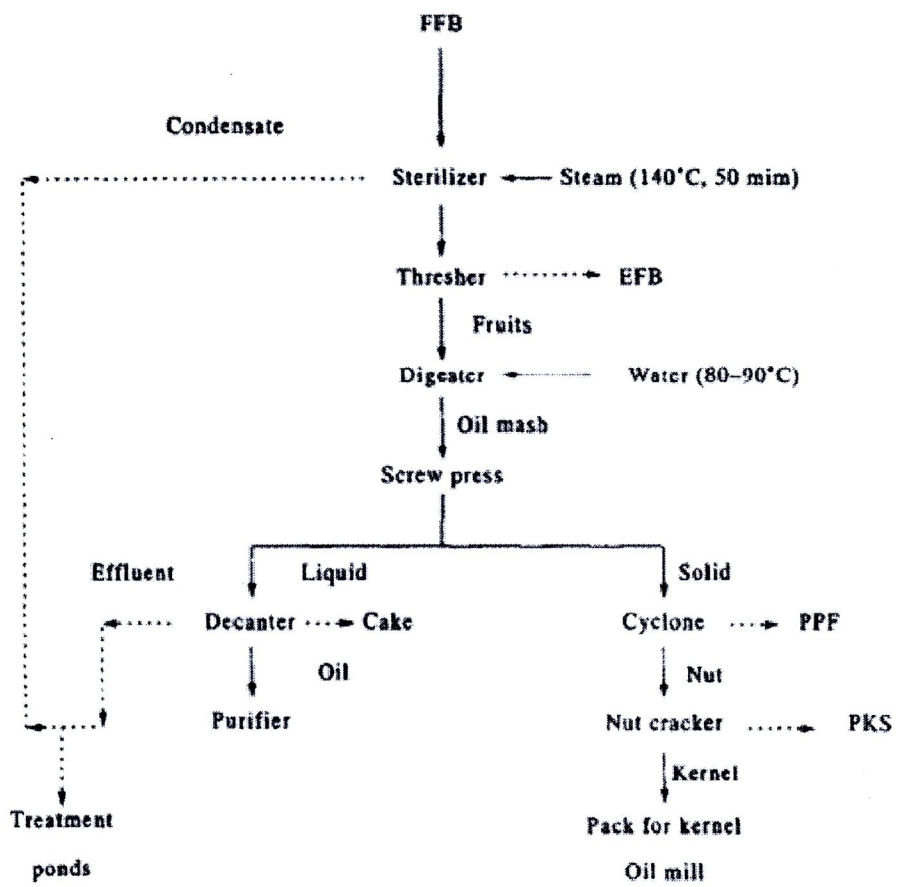


Figure 1. Schematic diagram of wet processing palm oil production using decanter (———) process; (·····) waste.

Source: Prasertsan and Prasertsan (1996)

2. Palm oil industry wastes and their utilization

Oil palm fruit consists of 68.3% pericarp and 31.7% nuts. The pericarp itself contains 23.3% pericarp fibre which has 6.4% (dry weight) holocellulose consisting of cellulose and hemicellulose (Kirdaldy and Sutanto, 1976). Due to the many components of oil palm fruit, the standard palm oil milling process have a lot of generating wastes consisting of 20-28% empty fruit bunch (EFB), 11-13% palm pressed fiber (PPF) and 0.87-1.50 m^3/ton FFB of wastewater or palm oil mill effluent (POME) (Prasertsan and Prasertsan, 1996; Sumathi *et al.*, 2008).

PPF has been utilized as fuel for boiler, fiber board production (Prasertsan and Prasertsan, 1996; Abdul Khalil *et al.*, 2007; Yin *et al.*, 2008) and mushroom cultivation (Ayodele and Okhuoya, 2007). In addition, huge quantities of biomass by-products are developed to produce value added products such as methane gas, bio-plastic, organic acids, bio-compost, plywood, activated carbon, and animal feedstock (Sumathi *et al.*, 2008). Even waste effluent, palm oil mill effluent (POME) has been converted to produce energy (Sumathi *et al.*, 2008). Nevertheless, it is still surplus and left unused in some palm oil mills.

An alternative approach of utilizing PPF for ethanol production was proposed in this study because bioethanol production is growing rapidly worldwide due to a substitute for fossil fuels in the transportation sector. The USA is the largest ethanol producer in the world, closely followed by Brazil. Both of them produce approximately 70% to the world's total production (Linde *et al.*, 2008). However, there is a material's difference for ethanol production in both countries; Brazil uses cane sugar while USA mainly utilizes starch from corn (Linde *et al.*, 2008).

2.1 Empty fruit bunch (EFB)

EFB is the major component solid waste of oil palm industry. It is rich in sugars, especially glucose (42% dry basis) and xylose (24% dry basis). EFB was used as a raw material for xylose production (Rahman *et al.*, 2006; Rahman *et al.*, 2007). Operational conditions were controlled at 120°C using various concentration of diluted sulfuric acid (2-6%) and reaction time (0-90 min). The optimal condition at reaction temperature of 120°C was 6% sulfuric acid for 15 min giving the maximum xylose of 29.4 g/l (Rahman *et al.*, 2006). Maximum concentration of xylose, 30.81 g/l

was achieved when reaction was carried out at 115°C for 60 min with 4%sulfuric acid concentration (Rahman *et al.*, 2007).

2.2 Palm pressed fiber (PPF)

Hemicellulose, a second major composition of PPF (Aziz *et al.*, 2002; Kelly-Yong *et al.*, 2007), could be converted to pentose sugars by using chemical (Abad *et al.*, 1997; Karimi *et al.*, 2006) or enzymes (Saha *et al.*, 2005; Karimi *et al.*, 2006). The pentose sugar, on the other hand, can be fermented to produce xylitol from xylose (Téllez-Luis *et al.*, 2002) and ethanol from xylose/arabinose (Limtong *et al.*, 2000; Sun and Cheng, 2005; Cheng *et al.*, 2007). Through chemical process, the pentose sugars can be converted directly to furfural and levulinic acid (Mansilla *et al.*, 1998). Furfural normally has been used in petroleum refining, gas, oil and diesel fuel as a selective solvent (Mansilla *et al.*, 1998) while levulinic acid could be used as plasticizer, textile, animal feed, fuel extender (methyltetrahydrofuran, MTHF), coating material and antifreeze (Bozell *et al.*, 2000). Furfural can be produced by either one-stage or two-stage process (Mansilla *et al.*, 1998; Punsuvon *et al.*, 2008). In one-stage process, pentosan is hydrolyzed into xylose and then dehydrated to furfural within the same reactor. This process gave low yield of furfural (0.7-3.3 wt %) (Mansilla *et al.*, 1998) and the residue solid can be used as a fuel. For two-stage process, hydrolysis and dehydration reaction process took places in two different reactors. The advantages of the two-stage process are higher furfural yield (3-15 wt %) than the one-stage process (Mansilla *et al.*, 1998). The residue solid can be utilized for production of cellulose, glucose and ethanol via fermentation (Punsuvon *et al.*, 2008).

2.3 Palm kernel shell (PKS)

PKS is the most hardly waste to decompose and usually used in the factory as firewood in boiler or disposed of by the land-fill method. Moreover, PKS has been successfully used to produce the activated carbon because of high carbon content (20.3%) and physically similar to the coconut shell. The demand of activated carbon in the future will be increased due to the stringent environmental control measurement (Prasertsan *et al.*, 1996).

2.4 Palm oil mill effluent (POME)

POME is the mixed effluent generated from two major wastewaters; sterilizer and decantor during the palm oil extraction in the wet process (Prasertsan *et al.*, 1990). The compositions of POME are given in Table 1 (Suwansaard, 2010). However, the milling process produces a huge volume of POME. Disposal of these wastes is already an economic burden on communities and industries, so creating a marketable product from this waste would reduce the treatment cost. Recovery of energy from waste might reduce the cost of wastewater treatment, and contribute to reducing our dependence on fossil fuel. Hydrogen and energy production could mitigate these problems. Hydrogen production by microorganisms can be divided into two main categories: one involves the use of photosynthetic bacteria (Suwansaard *et al.*, 2009) and algae under light conditions and the other, anaerobic fermentative bacteria under dark conditions (O-Thong *et al.*, 2008).

Table 1. Characteristics of palm oil mill effluent (POME)

Compositions	Unit	POME from JK Import Export Co., Ltd.	POME from Trang Palm Oil Co., Ltd.
pH		4.50	4.2-4.5
Color		Brown	Brown
Biochemical oxygen demand (BOD)	mg l^{-1}	38,740	22,000-54,300
Chemical oxygen demand (COD)	mg l^{-1}	50,057	75,200-96,300
Total nitrogen	mg l^{-1}	460	830-920
Acetic acid	mM	76.43	nr
Propionic acid	mM	27.98	nr
Butyric acid	mM	24.71	nr

nr: no reported

Source: O-Thong et al. (2008); Suwansaard, (2010)

3. Characteristics of lignocellulosic materials

In general, lignocellulosic wastes contain 70-80% carbohydrates consisting of 40–55% cellulose, 15–35% lignin and 25–40% hemicellulose (dry basis) (Pushpamalar *et al.*, 2006) whereas oil palm biomass consists of 32-42% cellulose, 21-38% hemicellulose and 11-27% lignin (Fig. 2) (Hamelinck *et al.*, 2003; Aziz *et al.*, 2002; Kelly-Yong *et al.*, 2007; Gutiérrez *et al.*, 2009).

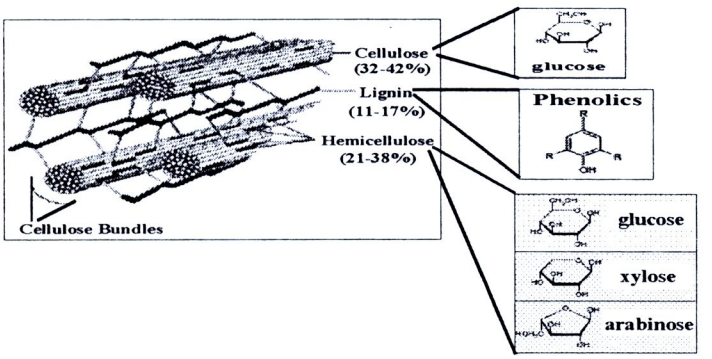


Figure 2. The composition of palm pressed fiber (PPF) and the final products.
Source: Modified from Hamelinck *et al.* (2003)

3.1 Cellulose

Cellulose with a molecular weight of about 100,000 is essentially a polymer with linear chains of glucopyranose units linked to each other by its β-1, 4 in the a configuration (Goyal *et al.*, 2006). However, the basic building block of cellulose is actually cellobiose, a dimer of two-glucose unit linked by β-(1-4) glycosidic bonds between C(4) of one sugar unit and the anomeric C(1) of the other (Fig. 3) (Ramos, 2003; Pushpamalar *et al.*, 2006). As glucose units are linked together into polymer chains, a molecule of water is lost, which makes the chemical formula $C_6H_{10}O_5$ for each monomer unit of "glucan".

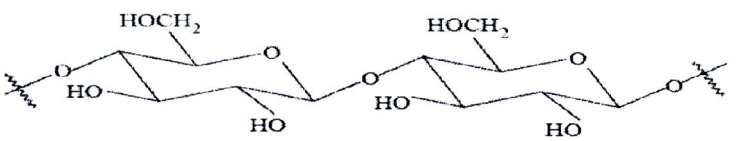


Figure 3. Fragment of a cellulose chain.
Source: Pushpamalar *et al.* (2006)

3.2 Hemicellulose

Hemicellulose, component of cell wall, is a complex mixture of several polysaccharides such as pentoses (xylose, rhamnose and arabinose), hexoses (glucose, mannose and galactose) and uronic acids (4-*O*-methyl-glucuronic and galacturonic acids) (Fig. 4). Its average molecular weight is of about 30,000, (Goyal *et al.*, 2006). In plant, hemicelluloses are normally connected to lignin (Ramos, 2003) which is a polymer of *p*-hydroxyphenylpropane units.

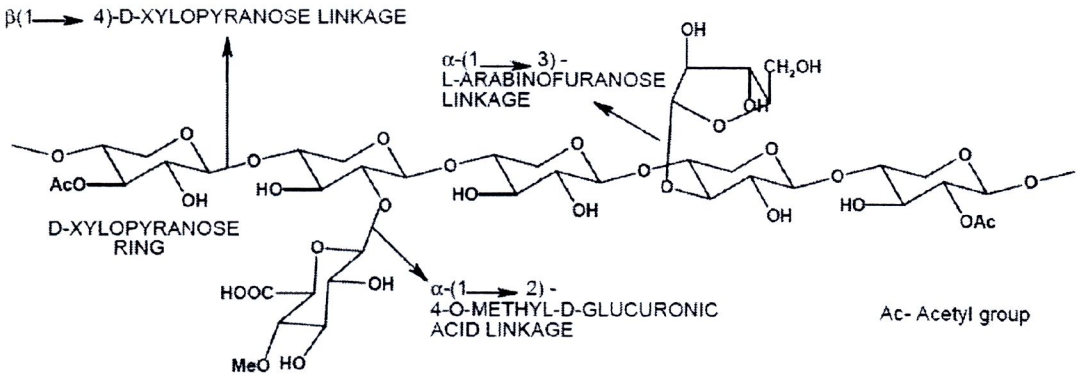


Figure 4. Fragment of a hemicellulose chain.
Source: Lachke (2002)

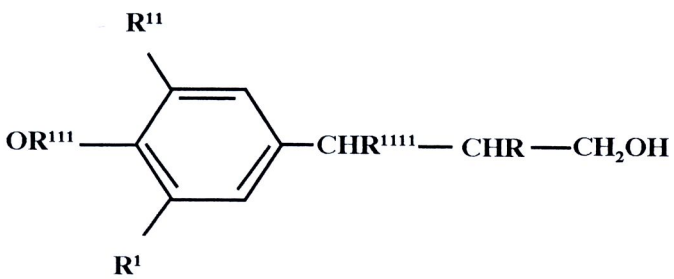
3.3 Lignin

Lignin, component of cell wall, is a mononuclear aromatic polymer of phenylpropane units linked in a three dimensional structure (Fig. 5) (Lee, 1997) also found in the cell wall. Due to the near position of hemicellulose and lignin in the cell wall, adjacent to each other, both these compounds can form a complex termed as lignocellulose (Goyal *et al.*, 2006). The principal structure elements of lignin have been clarified. Biosynthetically, lignin releases from lignocellulosic materials via formation of three precursor alcohols (Lee, 1997):

(1) *p*-hydroxycinnamyl (coumaryl) alcohol, which gives rise to *p*-hydroxyphenyl units in the polymer

(2) 4-hydroxy-3-methoxycinnamyl (coniferyl) alcohol, the guaiacyl units

(3) 4-hydroxy-3,5-dimethoxy-cinnamyl (sinapyl) alcohol, the syringyl units



R = another phenyl propane unit
R^{111} = H or R
R^{1111} = OH or R

Guaiacyl: $R^1 = OCH_3$, $R^{11} = H$
Syringyl: $R^1 = R^{11} = OCH_3$
p-hydroxyphenyl: $R^1 = R^{11} = H$

Figure 5. The elementary phenylpropane building blocks of various lignin.
Source: Lee (1997)

The chemical bonds linked between lignin and hemicellulose are ester, ether and glycosidic bonds. The ether linkages are more common and stronger than ester bonds between lignin and carbohydrates. These units and bonds, therefore, it makes lignins extremely resistant to chemical and enzymatic degradation. However, chemical degradation can be achieved by alkali and some catalysts hydrolysis. Biological degradation of lignin can be achieved by white rot fungi (Ohkuma *et al.*, 2001).

4. Lignocellulosic pretreatment

It is evident that the importance of lignocellulosic biomass as a feedstock for ethanol production. Lignocellulosic complex is the most abundant biopolymer in the Earth. It is considered that lignocellulosic biomass comprises about 50% of world biomass. Its annual production was estimated in 10–50 billion ton (Claassen *et al.*, 1999). In general, prospective lignocellulosic materials for fuel ethanol production can be divided into six main groups: crop residues (cane bagasse, corn stover, wheat straw, rice straw, rice hulls, barley straw, sweet sorghum bagasse, olive stones and pulp), hardwood (aspen, poplar), softwood (pine, spruce), cellulose

wastes (newsprint, waste office paper, recycled paper sludge), herbaceous biomass (alfalfa hay, switchgrass, reed canary grass, coastal bermudagrass, thimothy grass), and municipal solid wastes (MSW). The composition of most of these materials can be found elsewhere (Sun and Cheng, 2002; Sánchez *et al.*, 2008).

Lignocellulosic materials are difficultly converted to sugars by directly biological methods because a lignin network covers the layer of cell walls. Therefore, various physical, chemical and biological pretreatments have been developed in order to degrade or remove the lignin in the biomass (Asada *et al.*, 2005).

4.1 Physical methods

4.1.1 Mechanical comminution

The objective of the mechanical pretreatment is a reduction of particle size and cristallinity of lignocellulosic in order to increase the specific surface and reduce the degree of polymerization. This can be produced by a combination of chipping, grinding or milling depending on the final particle size of the material (10–30 mm after chipping and 0.2–2 mm after milling or grinding) (Sun and Cheng, 2002). Different milling processes (ball milling, two-roll milling, hammer milling, colloid milling and vibro energy milling) can be used to improve the enzymatic hydrolysis of lignocelullosic materials (Taherzadeh and Karimi, 2008). The energy requirements of mechanical comminution of lignocellulosic materials depend on the final particle size and biomass characteristics. Although mechanical pretreatment methods increase cellulose reactivity towards enzymatic hydrolysis, they are unattractive due to their high energy and capital costs (Ghosh and Ghose, 2003; Sánchez *et al.*, 2008).

4.1.2 Pyrolysis

Pyrolysis has also been tested as a physical method for pretreatment of lignocellulosic biomass since cellulose rapidly decomposes when is treated at high temperatures (>300°C) (Sánchez *et al.*, 2008).

4.1.3 Extrusion

Extrusion process is a novel and promising physical pretreatment method for biomass conversion to ethanol production. In extrusion, the materials are

subjected to heating, mixing and shearing, resulting in physical and chemical modifications during the passage through the extruder. Screw speed and barrel temperature are believed to disrupt the lignocellulose structure causing defibrillation, fibrillation and shortening of the fibers, and, in the end, increasing accessibility of carbohydrates to enzymatic attack. The different bioreactor parameters must be taken into account to achieve the highest efficiency in the process. In recent studies application of enzymes during extrusion process is being considered as a promising technology for ethanol production (Alvira *et al.*, 2010).

4.2 Physical-chemical methods

4.2.1 Steam explosion

Steam explosion is a process for separation of cellulose, hemicellulose and lignin using high temperature (more than 200°C) and high pressure (1.5 Mpa) for a short time (1-10 min.). The hydrolysis of glycosidic linkages in hemicelluloses and the β-*O*-4 ether bonds in lignin are cleaved by acetic acid formed at high temperature from acetyl groups present in hemicelluloses (autohydrolysis) (Sun *et al.*, 2005; Asada *et al.*, 2005; Chen *et al.*, 2005). Therefore, during steam explosion, significant amounts of hemicelluloses are partially hydrolysed and some lignin is depolymerised. Sugars and phenolic compounds are soluble in water whereas the exploded solid residue is cellulose. Some of the possible end products of steam-exploded wood are ethanol, xylitol, lactic acid and furfural or furfural derivatives (Sun *et al.*, 2005). After treating with steam explosion, hemicelluloses are recovered from the exploded fiber by three times of washing with water at 90°C for 15 min at a fiber to water ratio of 1/20 w/w. The washed solution is concentrated under vacuum at 60°C in an evaporator (Montane *et al.*, 1998). Lignin removal from wheat straw has been studied by using a two-stage process based on steam explosion pre-treatment and followed by hydrogen peroxide in alkaline condition post-treatment gave the maximum lignin removal yield (92.3-99.4%) when the steam explosion pre-treatment was performed at 220°C, 22 atm for 3 min with a solid to liquid ratio of 2:1 (Sun *et al.*, 2005). Steam explosion pretreatment of bagasse has also been studied. The effective removal of lignin under 206°C for 4 min was 50.7% (Punsuvon *et al.*, 2008).

4.2.2 Liquid hot water (LHW)

One of the most promising methods is the pretreatment in the neutralization process with liquid hot water (LHW) or thermohydrolysis that does not employ any catalysts (Sánchez *et al.*, 2008; Pérez *et al.*, 2008). LHW pretreatment, in which pressure is utilized to maintain water in the liquid state at elevated temperatures, has been reported to have the potential to remove most hemicellulose while minimizing cellulose hydrolysis and sugar degradation reactions. For example, it has been shown to remove up to 80% of the hemicellulose and to enhance the enzymatic digestibility of pretreated material in herbaceous feedstocks, such as corn fiber and sugarcane bagasse (Párez *et al.*, 2008). Two fractions are obtained after filtration of the slurry generated in LHW pretreatment: one cellulose-enriched (water-insoluble solids fraction) and another one rich in hemicellulose-derived sugars (HDS, liquid fraction or prehydrolyzate).

Laser et al. (2002) mention that under optimal conditions, this method is comparable to dilute acid pretreatment but without addition of acids or production of neutralization wastes. In addition, this technology presents elevated recovery rates of pentoses and does not generate inhibitors (Sánchez *et al.*, 2008). Nevertheless, solid load is much less than for steam explosion, which is usually greater than 50%. Wheat straw was also studied by using LHW. The optimal conditions were 188°C and 40 min, leading to HDS recovery yield of 43.6% of HDS content in raw material and enzymatic hydrolysis (EH) yield of 79.8% of theoretical was obtained (Párez *et al.*, 2008).

4.2.3 Microwave hydrolysis

Microwave-based pretreatment can be considered a physicochemical process since both thermal and non-thermal effects are often involved (Alvira *et al.*, 2010). Pretreatments were carried out by immersing the biomass in dilute chemical reagents and exposing the slurry to microwave radiation for residence times ranging from 5 to 20 min (Keshwani, 2009). Preliminary experiments identified alkalis as suitable chemical reagents for microwave-based pretreatment. An evaluation of different alkalis identified sodium hydroxide as the most effective alkali reagent (Alvira *et al.*, 2010). Wheat bran pretreatment was achieved by microwave giving

high yields of glucose and xylose when performed under 170°C with no sulfuric acid for various reaction time (20, 30 and 40 min). In additionally, pretreatment temperatures below 170°C have been shown to yield low concentration of furfural and HMF (Palmarola-Adrados *et al.*, 2005). The advantages of this pretreatment are not only to decrease amount of sample required and the vessel also provides a close system with a constant amount of material throughout the process (Palmarola-Adrados *et al.*, 2005).

4.2.4 Ammonia fiber explosion (AFEX)

In the AFEX process, biomass is treated with liquid anhydrous ammonia at temperatures between 60 and 100°C and high pressure for a variable period of time (Alvira *et al.*, 2010). The pressure is then released, resulting in a rapid expansion of the ammonia gas that causes swelling and physical disruption of biomass fibers and partial decrystallization of cellulose. While some other pretreatments such as steam explosion produce slurry that can be separated in a solid and a liquid fractions, AFEX produces only a pretreated solid material. AFEX has been reported to decrease cellulose crystallinity and disrupt lignin–carbohydrates linkages (Laureano-Pérez *et al.*, 2005). During the pretreatment only a small amount of the solid material is solubilized; little hemicellulose and lignin is removed (Wyman *et al.*, 2005a). Deacetylation of hemicellulose is also observed. AFEX removes the least acetyl groups from certain lignocellulosic materials (Kumar *et al.*, 2009b). Digestibility of biomass is increased after AFEX pretreatment (Galbe and Zacchi, 2007) and therefore the enzymatic hydrolysis results in greater yields. Both cellulases and hemicellulases will be required in hydrolysis process due to the considerable remaining hemicellulose in the pretreated material (Alvira *et al.*, 2010).

Ammonia recovery and recycle is feasible despite of its high volatility (Teymouri *et al.*, 2005) but the associated complexity and costs of ammonia recovery may be significant regarding commercial potential of the AFEX pretreatment (Mosier *et al.*, 2005b). No formation of inhibitors for the downstream biological processes is one of the main advantages of the ammonia pretreatment, even though some phenolic fragments of lignin and other cell wall extractives may remain on the cellulosic surface (Alvira *et al.*, 2010). The AFEX pretreatment is more effective on agricultural

residues and herbaceous crops, with limited effectiveness demonstrated on woody biomass and other high lignin feedstocks (Wyman *et al.*, 2005a). There have been reported recent strategies to optimize the conditions in the AFEX pretreatment in studies using different materials (Teymouri *et al.*, 2005). At optimal conditions AFEX can achieve more than 90% conversion of cellulose and hemicellulose to fermentable sugars for a broad variety of lignocellulosic materials. In fact, despite of little removal of lignin or hemicellulose in the AFEX process, enzymatic digestion at low enzyme loadings results very high comparing other pretreatment alternatives (Wyman *et al.*, 2005b). This may suggest that ammonia affects lignin and possibly hemicellulose differently than other chemicals, reducing the ability of lignin to adsorb enzyme and/or to make its access to cellulose more difficult (Alvira *et al.*, 2010). Barley hull was pretreated by using aqueous ammonia, to be converted into sugars. The best pretreatment conditions were 75°C for 48 h with 15 wt% aqueous ammonia and 1:12 of solid to liquid ratio resulting in saccharification yield of 83 % glucan and 63 % xylan with decrease of 50-66% of the original lignin (Kim *et al.*, 2008).

4.2.5 CO_2 explosion

Carbon dioxide explosion is also used for lignocellulosic biomass pretreatment. The method is based on the utilization of CO_2 as a supercritical fluid, which refers to a fluid that is in a gaseous form but is compressed at temperatures above its critical point to a liquid like density. Supercritical pretreatment conditions can effectively remove lignin increasing substrate digestibility (Alvira *et al.*, 2010). Addition of co-solvents such ethanol can improve delignification. Supercritical carbon dioxide (SC-CO_2) has been mostly used as an extraction solvent but it is being considered for non-extractive purposes due to its many advantages (Schacht *et al.*, 2008). In aqueous solution CO_2 forms carbonic acid, which favors the polymers hydrolysis. CO_2 molecules are comparable in size to water and ammonia and they can penetrate in the same way the small pores of lignocellulose. This mechanism is facilitated by high pressure. After the explosive release of CO_2 pressure, disruption of cellulose and hemicellulose structure is observed and consequently accessible surface area of the substrate to enzymatic attack increases. Operation at low temperatures compared to other methods prevents monosaccharides degradation, but in comparison

to steam and ammonia explosion sugar yields obtained are lower. Nevertheless a comparison of different pretreatment methods on several substrates showed that CO_2 explosion was more cost-effective than ammonia explosion and formation of inhibitors was lower compared to steam explosion (Zheng *et al.*, 1998).

4.2.6 Irradiation pretreatment

Ionizing irradiation can modify and disrupt the structure of lignocellulose and can be an effective method of pretreatment of lignocellulosic biomass for sugar production (Chunping *et al.*, 2008). The irradiation degradation of various lignocellulosic materials for increasing sugar yield has been reported, such as bagasse (Han *et al.*, 1981), rice straw, chaff, sawdust (Kumakura and Kaetsu, 1979, 1984a), corn stalk, peanut husk (Chosdu *et al.*, 1993), oil palm empty fruit bunch (Matsuhashi *et al.*, 1995). The radiationinduced reactions in the macromolecules of the cellulose materials are known to be initiated through rapid localization of the absorbed energy within the molecules to produce long- and short-lived radicals which caused the secondary degradation of materials through chemical reactions such as chain scission, cross-linking, and so forth (Khan *et al.*, 2006). The efficiency of these two types of reactions depends mainly on the polymer structure and radiation dose (Charlesby, 1981).

4.2.7 Ultrasound pretreatment

The effect of ultrasound on lignocellulosic biomass have been employed for extracting hemicelluloses, cellulose and lignin but less research has been addressed to study the susceptibility of lignocellulosic materials to hydrolysis (Alvira *et al.*, 2010). In spite of the minor research on ultrasound pretreatment from lignocellulose, some researchers have also shown that saccharification of cellulose is enhanced efficiently by ultrasonic pretreatment (Yachmenev *et al.*, 2009). Higher enzymatic hydrolysis yields after ultrasound pretreatment could be explained because cavitation effects caused by introduction of ultrasound field into the enzyme processing solution greatly enhance the transport of enzyme macromolecules toward the substrate surface (Alvira *et al.*, 2010). Furthermore, mechanical impacts, produced by the collapse of cavitation bubbles, provide an important benefit of opening up the surface of solid substrates to the action of enzymes, in addition, the maximum effects

of cavitation occur at 50°C, which is the optimum temperature for many enzymes (Yachmenev *et al.*, 2009).

4.3 Chemical methods

Chemical processes are an efficient method for breaking the large complex structure of biomass to the small molecules including separation of their components (cellulose, hemicellulose and lignin). These methods have to control various parameters such as temperature, reaction time and chemical concentration, etc because of the vigor reaction.

4.3.1 Alkali pretreatment

The effect that some bases have on lignocellulosic biomass is the basis of alkaline pretreatments, which are effective depending on the lignin content of the biomass. Alkali pretreatments increase cellulose digestibility and they are more effective for lignin solubilization, exhibiting minor cellulose and hemicellulose solubilization than acid or hydrothermal processes (Carvalheiro *et al.*, 2008). Alkali pretreatment can be performed at room temperature and times ranging from seconds to days. It is described to cause less sugar degradation than acid pretreatment and it was shown to be more effective on agricultural residues than on wood materials (Kumar *et al.*, 2009). Nevertheless, possible loss of ethanol production due to the presence of toxic compounds during alkaline hydrolysis must be taken into consideration to optimize the pretreatment conditions (Alvira *et al.*, 2010).

Sodium, potassium, calcium and ammonium hydroxides are suitable alkaline pretreatments. NaOH causes swelling, increasing the internal surface of cellulose and decreasing the degree of polymerization and cristallinity, which provokes lignin structure disruption (Taherzadeh and Karimi, 2008). For example, wheat straw was pretreated with a 0.5 M NaOH solution for 6 h at 80°C in a thermostated 2 liters batch stirred reactor (300 rpm) using a solid–liquid ratio of 5% (w/v) and after that, it was cool down immediately (Ramos, 2003). NaOH has been reported to increase hardwood digestibility from 14% to 55% by reducing lignin content from 24–55% to 20% (Kumar *et al.*, 2009).

$Ca(OH)_2$, also known as lime, has been widely studied. Lime pretreatment removes amorphous substances such as lignin, which increases the crystallinity index (Alvira *et al.*, 2010). Lignin removal increases enzyme effectiveness by reducing non-productive adsorption sites for enzymes and by increasing cellulose accessibility (Kim and Holtzapple, 2006). Lime also removes acetyl groups from hemicellulose that is an obstacle of enzymes and enhancing cellulose digestibility (Mosier *et al.*, 2005a). Lime has been proven successfully at temperatures from 85–150°C and for 3–13 h with corn stover (Kim and Holtzapple, 2006) or poplar wood (Chang *et al.*, 2001). Pretreatment with lime has lower cost and less safety requirements compared to NaOH or KOH pretreatments and can be easily recovered from hydrolysate by reaction with CO_2 (Mosier *et al.*, 2005a).

Addition of an oxidant agent (oxygen/H_2O_2) to alkaline pretreatment ($NaOH/Ca(OH)_2$) can improve the performance by favoring lignin removal (Carvalheiro *et al.*, 2008). Ethanol yields of 0.33 g/g have been obtained in simultaneous saccharification and fermentation (SSF) processes with *Escherichia coli* FBR5 from wheat straw pretreated with alkali peroxide (Saha and Cotta, 2006). Furthemore, no furfural or HMF were detected in hydrolysates obtained with alkaline peroxide pretreatment which favours the fermentation step in an ethanol production process (Taherzadeh and Karimi, 2008).

4.3.2 Ozonolysis pretreatment

Ozone pretreatment is a process for reducing the lignin content of lignocellulosic materials. Ozone was used to degrade lignin and hemicellulose in many biomasses i.e. cotton straw, wheat straw, bagasse, pine, peanut, green hay and poplar sawdust. The degradation is mainly limited to lignin. Hemicellulose is slightly affected, but cellulose is not degraded (Kumar *et al.*, 2009). The pretreatment is usually performed at room temperature and normal pressure and does not lead to the formation of inhibitory compounds that can affect the subsequent hydrolysis and fermentation (Alvira *et al.*, 2010). After wheat straw pretreatment by ozone, the rate of enzymatic hydrolysis increased following 60% removal of the lignin. Enzymatic hydrolysis yield of poplar sawdust increased from 0% to 57% as the lignin decreased from 29% to 8% (Kumar *et al.*, 2009). Despite of some interesting results further

research has to be performed regarding ethanol production from lignocellulosic materials pretreated with ozone. An important drawback to consider is the large amounts of ozone needed, which can make the process economically unviable (Sun and Cheng, 2002).

4.3.3 Acid hydrolysis pretreatment

The main objective of the acid pretreatments is to solubilize the hemicellulosic fraction of the biomass and to make the cellulose more accessible to enzymes (Alvira *et al.*, 2010). Diluted acid pretreatment appears as more favorable method for industrial applications and have been studied for pretreating wide range of lignocellulosic biomass. Different types of reactors such as percolation, plug flow, shrinking-bed, batch and countercurrent reactors have been applied for pretreatment of lignocellulosic materials (Taherzadeh and Karimi, 2008). It can be performed at high temperature (e.g. 180°C) during a short period of time; or at lower temperature (e.g. 120°C) for longer retention time (30–90 min). It presents the advantage of solubilizing hemicellulose, mainly xylan, but also converting solubilized hemicellulose to fermentable sugars (Saha, 1999). Nevertheless, depending on the process temperature, some sugar degradation compounds such as furfural and hydroxyl methyl furfural (HMF) and aromatic lignin degradation compounds are detected (Rahman *et al.*, 2006; Rahman *et al.*, 2007), and affect the microorganism metabolism in the fermentation step (Saha *et al.*, 2005). Anyhow, this pretreatment generates lower degradation products than concentrated acid pretreatments. Concentrated acid are toxic, corrosive, hazardous and required the specific reactor that are resistant to corrosion, which results the pretreatment process very expensive. Moreover, the concentrated acid must be recovered after hydrolysis to make the process economically feasible (Sun and Cheng, 2002).

High hydrolysis yields have been reported when pretreating lignocellulosic materials with diluted H_2SO_4 which is the most studied acid. Hydrochloric acid, phosphoric acid and nitric acid have also been tested (Mosier *et al.*, 2005a). Saccharification yield as high as 74% was shown when wheat straw was subjected to 0.75% v/v of H_2SO_4 at 121°C for 1 h (Saha *et al.*, 2005). Olive tree biomass was pretreated with 1.4% H_2SO_4 at 210°C resulting in 76.5% of hydrolysis

yields (Cara *et al.*, 2008). Recently, ethanol yield as high as 0.47 g/g glucose was achieved in fermentation tests with cashew apple bagasse pretreated with diluted H_2SO_4 at 121°C for 15 min (Rocha *et al.*, 2009).

Organic acids such as fumaric or maleic acids are appearing as alternatives to enhance cellulose hydrolysis for ethanol production. In this context, both acids were compared with sulfuric acid in terms of hydrolysis yields from wheat straw and formation of sugar degradation compounds during pretreatment. Results showed that organic acids can pretreat wheat straw with high efficiency although fumaric acid was less effective than maleic acid. Furthermore, less amount of furfural was formed in the maleic and fumaric acid pretreatments than with sulfuric acid (Kootstra *et al.*, 2009).

4.3.4 Oxidative delignification pretreatment

Degradation of lignin could be catalyzed by the peroxidase enzyme with the presence of hydrogen peroxide (H_2O_2). The pretreatment of bagasse with H_2O_2 greatly enhanced its susceptibility to enzymatic hydrolysis. 50% of the lignin content and most of the hemicellulose were solubilized by 2% H_2O_2 at 30°C within 8 hours, and 95% efficiency of glucose production from cellulose was achieved in the subsequent saccharification by cellulase at 45°C for 24 hours (Azzam, 1986).

4.3.5 Organosolv pretreatment

In the organosolvation process, an organic or aqueous organic solvent mixture with inorganic acid catalysts (sulfuric acid, hydrochloric acid, oxalic acid, acetylsalicylic acid and salicylic acid) is used to break the internal lignin and hemicellulose bonds (Chum *et al.*, 1988). A high yield of xylose can usually be obtained with the addition of acid. However, this acid addition can be avoided for a satisfactory delignification by increasing process temperature (above 185°C) (Alvira *et al.*, 2010). The solvents commonly used in the process are methanol, ethanol, acetone, ethylene glycol, triethylene glycol and tetrahydrofurfuryl alcohol (Chum *et al.*, 1988). Pulps and its lignin content between 6.4% and 27.4% (w/w) have been prepared from mixed softwoods using a biorefining technology or lignol process, which is based on an aqueous ethanol organosolvation extraction (Pan *et al.*, 2005). This process uses a blend of ethanol and water in the ratio of 50:50 (w/w) at 200°C

with a pressure of 400 psi to extract most of the lignin content from wood chips or other lignocellulosic materials. All pulps were readily hydrolyzed and lignin was removed to less than 18.4%). More than 90% of the cellulose in low lignin pulps was hydrolyzed to glucose in 48 hours.

Comparing to other chemical pretreatments the main advantage of organosolv process is the recovery of relatively pure lignin as a by-product (Zhao *et al.*, 2009a). Removal of solvents from the system is necessary using appropriate extraction and separation techniques, e.g., evaporation and condensation, and they should be recycled to reduce operational costs. Solvents need to be separated because they might be inhibitory to enzymatic hydrolysis and fermentative microorganisms (Sun and Cheng, 2002). The high commercial price of solvents is another important factor to consider for industrial applications. For economic reasons, among all possible solvents, the low-molecular weight alcohols with lower boiling points such as ethanol and methanol are favored (Alvira *et al.*, 2010).

4.3.6 Wet oxidation (WO)

In wet oxidation, the lignocellulosic biomass is treated with water and high pressure oxygen or air at elevated temperatures (more than 120°C) (Talebnia *et al.*, 2010). Typical oxygen pressure range is 120–480 psi (Schmidt and Thomsen, 1998). WO is an effective pretreatment method for the fractionation of wheat straw into a solubilized hemicellulose fraction and a cellulose-rich solid fraction with high susceptibility to enzymatic hydrolysis. Combination of alkali and WO not only improves the rate of lignin oxidation (and in turn enzymatic hydrolysis) but also prevents formation of furfural and HMF (Bjerre *et al.*, 1996). Acids formed during initial reaction in WO due to solubilization of hemicellulose components catalyze the subsequent hydrolytic reactions through which hemicelluloses are broken down into lower molecular weight fragments that are soluble in water (Talebnia *et al.*, 2010). Lignin degradation is also significant especially at the higher temperatures because phenol-like compounds and carbon–carbon linkage are very reactive under wet oxidation conditions. Lignin is decomposed to CO_2, H_2O and carboxylic acids (Klinke *et al.*, 2002).

4.3.7 Ionic liquids (ILs) pretreatment

The use of ILs as solvents for pretreatment of cellulosic biomass has recently received much attention. ILs are salts, typically composed of large organic cations and small inorganic anions, which exist as liquids at relatively low temperatures; often at room temperature. Their solvent properties can be varied by adjusting the anion and the alkyl constituents of the cation. These interesting properties include chemical and thermal stability, non-flammability, low vapor pressures and a tendency to remain liquid in a wide range of temperatures (Hayes, 2009). Since no toxic or explosive gases are formed, ILs is called "green" solvents. Carbohydrates and lignin can be simultaneously dissolved in ILs with anion activity (e.g. the 1-butyl-3 methylimidazolium cation $[C4mim]^+$) because ILs form hydrogen bonds between the non-hydrated chloride ions of the IL and the sugar hydroxyl protons in a 1:1 stoichiometry. As a result, the intricate network of non-covalent interactions among biomass polymers of cellulose, hemicellulose, and lignin is effectively disrupted while minimizing formation of degradation products. However, most data showing the effectiveness of ILs has been developed using pure crystalline cellulose, and its applicability to a more complex combination of constituents in lignocellulosic biomass requires further studies (Alvira *et al.*, 2010). Nevertheless, the use of ILs has also been already demonstrated on some lignocellulosic feedstocks such as straw (Li *et al.*, 2009) or wood (Lee *et al.*, 2009). Toxicity to enzymes and fermentative microorganisms must be studied before ILs can be considered a real option for biomass pretreatment (Yang and Wyman, 2008; Zhao *et al.*, 2009b). Depending on the amount of ILs residues remaining, significative negative effect on cellulase activity may be observed. Thus, ILs residues removal would be required to prevent decrease of final sugars concentrations (Alvira *et al.*, 2010).

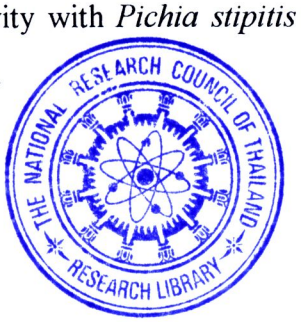
In a pretreatment study using 1-ethyl-3-methyl imidazolium diethyl phosphate, the yield of reducing sugars from wheat straw pretreated with this ionic liquid at 130°C for 30 min was 54.8% after being enzymatically hydrolyzed for 12 h (Li *et al.*, 2009). The fermentability of the hydrolysates obtained after enzymatic saccharification of the regenerated wheat straw was also evaluated. Results obtained

using *Saccharomyces cerevisiae* indicated that wheat straw pretreated by this IL did not bring any negative effect on the growth of *S. cerevisiae* (Li *et al.*, 2009).

4.4 Biological methods

Fungal pretreatment has been previously explored to upgrade lignocellulosic materials for feed and paper applications. Recently, this environmentally friendly approach has received renewed attention as a pretreatment method for enhancing enzymatic saccharification of lignocellulosic biomass in ethanol production processes (Alvira *et al.*, 2010). The potential method for removing lignin and releasing fermentable sugars is pretreatment followed by enzymatic and acidic hydrolysis. Lignin could be degraded by several fungal enzymes such as lignin peroxidase, Mn-dependent peroxidase, and laccase (mono-phenol oxidase) and its degradability depend on fungal strain, accessibility of lignin to enzyme, culture condition, and reactor design (Ohkuma *et al.*, 2001). Biological pretreatments employ microorganisms mainly brown, white and soft-rot fungi which degrade lignin and hemicellulose and very little of cellulose, more resistant than the other components (Sánchez, 2009). Lignin degradation by white-rot fungi is the most effective for biological pretreatment of lignocellulosic materials (Alvira *et al.*, 2010).

Several white-rot fungi such as *Phanerochaete chrysosporium*, *Ceriporia lacerata*, *Cyathus stercolerus*, *Ceriporiopsis subvermispora*, *Pycnoporus cinnarbarinus* and *Pleurotus ostreaus* have been examined on different lignocellulosic biomass showing high delignification efficiency (Kumar *et al.*, 2009; Shi *et al.*, 2008). Biological pretreatment by white-rot fungi has been combined with organosolv pretreatment in an ethanol production process by simultaneous saccharification and fermentation (SSF) from beech wood chips (Itoh *et al.*, 2003). Results from other recent studies have shown that fungal pretreatment of wheat straw for 10 days with a high lignin-degrading and low cellulose-degrading fungus (fungal isolate RCK-1) resulted in a reduction in acid loading for hydrolysis, an increase in the release of fermentable sugars and a reduction in the concentration of fermentation inhibitors. Ethanol yield and volumetric productivity with *Pichia stipitis* were 0.48 g/g and 0.54 g/l/h, respectively (Kuhar *et al.*, 2008).



The major sugars in enzyme hydrolysates are glucose and xylose released from cellulose and hemicellulose, respectively. The advantages of biological delignification may include higher product yields, fewer side reactions, less energy demand and less reactor resistance because of mild reaction condition. However, the main drawback to develop biological methods is the low hydrolysis rate obtained in most biological materials compared to other technologies (Sun and Cheng, 2002).

To move forward a cost-competitive biological pretreatment of lignocellulose, and improve the hydrolysis to eventually improve ethanol yields, there is a need to keep on studying and testing more basidiomycetes fungi for their ability to delignify the plant material quickly and efficiently (Alvira *et al.*, 2010).

5. Key factors for an effective pretreatment of lignocellulosic biomass

There are several key properties to take into consideration for low-cost and advanced pretreatment process (Yang and Wyman, 2008; Alvira *et al.*, 2010):

5.1 High yields for multiple crops, sites ages, harvesting times

Various pretreatments have been shown to be better suited for specific feedstocks. For example, alkaline-based pretreatment methods such as lime, ammonia fiber explosion (AFEX), and ammonia recycling percolation (ARP), can effectively reduce the lignin content of agricultural residues but are less satisfactory for processing recalcitrant substrate as softwoods. Acid based pretreatment processes have been shown to be effective on a wide range of lignocellulose substrate, but are relatively expensive.

5.2 Highly digestible pretreated solid

Cellulose from pretreatment should be highly digestible with yields higher than 90% in less than five and preferably less than 3 days with enzyme loading lower than 10 FPU/g cellulose (Yang and Wyman, 2008).

5.3 No significant sugars degradation

High yields close to 100% of fermentable cellulosic and hemicellulosic sugars should be achieved through pretreatment step (Alvira *et al.*, 2010).

5.4 Minimum amount of toxic compounds

The liquid hydrolyzate from pretreatment must be fermentable following a low-cost, high yield conditioning step. Harsh conditions during pretreatment lead to a partial hemicellulose degradation and generation of toxic compounds derived from sugar decomposition that could affect the proceeding hydrolysis and fermentation steps (Oliva *et al.*, 2003). Toxic compounds generated and their amounts depend on raw material and harshness of pretreatment. Degradation products from pretreatment of lignocellulose materials can be divided into the following classes: carboxylic acids, furan derivatives, and phenolic compounds. Main furan derivates are furfural and 5-hydroxymethylfurfural (HMF) derived from pentoses and hexoses degradation, respectively (Palmqvist and Hahn-Hägerdal, 2000b).Weak acids are mostly acetic and formic and levulinic acids Phenolic compounds include alcohols, aldehydes, ketones and acids (Klinke *et al.*, 2002).

5.5 Biomass size reduction not required

Milling or grinding the raw material to small particle sizes before pretreatment is energy-intensive and costly technologies.

5.6 Operation in reasonable size and moderate cost reactors

Pretreatment reactors should be low in cost through minimizing their volume, employing appropriate materials of construction for highly corrosive chemical environments, and keeping operating pressures reasonable.

5.7 Non-production of solid-waste residues

The chemicals formed during hydrolyzate conditioning in preparation for subsequent steps should not present processing or disposal challenges.

5.8 Effectiveness at low moisture content

The use of raw materials at high dry matter content would reduce energy consumption during pretreatment.

5.9 Obtaining high sugar concentration

The concentration of sugars from the coupled operation of pretreatment and enzymatic hydrolysis should be above 10% to ensure an adequate ethanol concentration and to keep recovery and other downstream cost manageable.

5.10 Fermentation compatibility

The distribution of sugar recovery between pretreatment and subsequent enzymatic hydrolysis should be compatible with the choice of an organism able to ferment pentoses (arabinose and xylose) in hemicellulose.

5.11 Lignin recovery

Lignin and other constituents should be recovered to simplify downstream processing and for conversion into valuable co-products (Yang and Wyman, 2008).

5.12 Minimum heat and power requirements

Heat and power demands for pretreatment should be low and/or compatible with the thermally integrated process.

6. Detoxification of lignocellulosic hydrolyzates

During pretreatment and hydrolysis of lignocellulosic biomass, a great amount of compounds that can seriously inhibit the subsequent fermentation are formed in addition to fermentable sugars (Sánchez and Cardona, 2008). Inhibitory substances are generated as a result of the hydrolysis of the extractive components, organic and sugar acids esterified to hemicellulose (acetic, formic, glucuronic, galacturonic), and solubilized phenolic derivatives. In the same way, inhibitors are produced from the degradation products of soluble sugars (furfural, HMF) and lignin (cinnamaldehyde, *p*-hydroxybenzaldehyde, syringaldehyde), and as a consequence of corrosion (metal ions) (Lynd, 1996; Palmqvist and Hahn-Hägerdal, 2000b). For this reason and depending on the type of employed pretreatment and hydrolysis, detoxification of the streams that will undergo fermentation is required (Sánchez and Cardona, 2008).

Detoxification methods can be physical, chemical or biological. As pointed out by Palmqvist and Hahn-Hägerdal (2000a), these methods cannot be directly compared because they vary in the neutralization degree of the inhibitors. In addition, the fermenting microorganisms have different tolerances to the inhibitors. The main features of the detoxification methods employed for ethanol production from biomass and some examples are summarized in Table 2. Alkali treatment is

considered one of the best detoxification methods. By this method, furaldehydes and phenolic compounds are mainly removed leading to great improvement in fermentability, especially in the case of dilute-acid hydrolyzates (Persson *et al.*, 2002). Treatment with calcium hydroxide (overliming) or ammonia has shown better results than treatment with sodium or potassium hydroxide, but this difference has not been understood (Sánchez and Cardona, 2008). Martinez et al. (2001) performed the experimental optimization of the amount of added lime, which depends on the content of acids in each hydrolyzate. These authors developed a method for predicting the optimal addition dosage based on the titration of hydrolyzate with 2 N NaOH. Persson et al. (2002) indicate that the positive effects of alkali treatment cannot be completely explained by the removal of inhibitors and that this method could have possible stimulatory effects on fermenting microorganisms. Other very diverse detoxification methods have been proposed as: neutralization with lime followed by the addition of activated carbon and filtration for acetic acid removal; partial removal of acetic acid, furfural and soluble lignin by molecular sieves; vapor stripping for removal of volatile inhibitors (Olsson and Hahn-Hägerdal, 1996); and adsorption using activated carbon, diatomite, bentonite and zeolite after neutralization or overliming (Yu and Zhang, 2003). An alternative biological method for detoxification of dilute solutions resulting from biomass pretreated by pyrolysis has been proposed (Khiyami *et al.*, 2005). It is based on a bio-film reactor that uses a mixed culture of aerobic bacteria cells naturally immobilized on a plastic support. In this way, the bio-film associated cells are more resistant to the toxic substances released during the biomass pretreatment.

The presence of inhibitors directly influences on the course of ethanolic fermentation. In continuous or fed-batch fermentations, feed of the bioreactors is carried out with not very high flow rates allowing a low concentration of inhibitors in the broth. In continuous systems, inhibitors reduce the growth rate and, therefore, the process productivity that is directly linked to the dilution rate. In systems with cell retention (e.g. by cell recirculation using filtration, sedimentation or centrifugation), the increase of accumulables, including the inhibitors, makes the productivity to fall down imposing the need of implementing purge streams (Sánchez and Cardona, 2008). Taherzadeh (1999) developed a simple strategy for on-line

feedback control of fed-batch cultivation for in situ detoxification of spruce and birch hydrolyzates. Through this strategy, the same yeast cells converted the inhibitors and maintained their concentration at low levels without the need of any detoxification treatment. Thus, the maximal specific productivity of ethanol increased in more than 10 times (Nilsson *et al.*, 2001). Purwadi et al. (2007) employed a continuous cultivation system using a flocculating strain of *S. cerevisiae* to ferment a non-detoxified spruce hydrolyzate. Results obtained demonstrated that high-cell system with recycling of cells allow the in situ detoxification of the pretreated biomass at high dilution rates without the need of any detoxification method. It has shown the possibility of converting the hydrolyzates into ethanol in two hours (at dilution rates of 0.5 h^{-1}), which represents an important outcome in the cultivation of toxic pretreated lignocellulosic biomass. Most of the studies on the effect of toxic compounds on growth and ethanol production have been performed for *S. cerevisiae* and xylose-fermenting yeast (Sánchez and Cardona, 2008). Palmqvist et al. (1999) carried out extensive experiments for assessing the effect of acetic acid, furfural and *p*-hydroxybenzoic acid on growth and ethanol productivity of *S. cerevisiae* and *C. shehatae* through full factorial design.

One new approach to tackle the presence of inhibitors in biomass hydrolyzates is the development of inhibitor-tolerant strains of microorganisms by means of genetic modification and metabolic engineering. However, Belkacemi et al. (2002) point out that due to the synergistic interactions among inhibitors and lack of information about the mechanisms of these interactions, it is not clear against what inhibitor resistance is desired. In this way, intense efforts are being carried out for the identification of inhibitory substances, as well as the determination of their inhibition mechanisms. Palmqvist and Hahn-Hägerdal (2000b) have reviewed the main works carried out in this field applied to wood hydrolyzates. These authors emphasize that these studies will allow the minimization of inhibitors formation during pretreatment and hydrolysis, the prediction of hydrolyzates fermentability and the development of more efficient detoxification methods (Sánchez and Cardona, 2008).

7. Conversion of lignocellulosic materials to value added products

7.1 Hemicellulose (xylose) production

7.1.1 Diluted acid hydrolysis

Due to the fossil fuel crisis, bioconversion of lignocellulosic materials to chemicals and fuel are significantly interesting in recent decade as a low cost, renewable and widespread in nature (Rhaman *et al.*, 2006). These abundant lignocellulosic materials contain approximately 34% of cellulose, 26% of hemicellulose, and 28% of lignin (Kaddami *et al.*, 2006). Therefore, the utilizations of these invaluable material wastes to be valuable products i.e. furfural, ethanol, xylitol, and high grade paper by biochemical and chemical processes have been studied worldwide (Rhaman *et al.*, 2006). Autohydrolysis (Garrote *et al.*, 2001) and acid hydrolysis of various raw materials have been focused (Pessor *et al.*, 1997; Neureiter *et al.*, 2002; Rhaman *et al.*, 2006; Cheng *et al.*, 2007). Under autohydrolysis and controlled acid hydrolysis conditions, xylose is a mainly product from both processes because the lignin protective layer around the hemicellulose is weak under high temperature and pressure which allows the acid to hydrolyze the layer and amorphous xylan to form xylose (Rhaman *et al.*, 2006). On the other hand glucose could not been produced so much in the diluted acid hydrolysis because of the crystalline structure of cellulose (Rhaman *et al.*, 2006). Due to this problem, two-stage acid hydrolysis process can be constructed to produce xylose and glucose, respectively. Dilute acid at moderate temperatures, the first stage of acid hydrolysis, has established to be an efficiency of xylose production. In the second stage, more severe reaction conditions are engaged and glucose can be produced from cellulose hydrolysis (Pessor *et al.*, 1997).

Acid hydrolysis's advantages

1. To increase the enzymatic digestibility of the material (Palmarola-Adrados *et al.*, 2005; Linde *et al.*, 2008).

2. Acid recovery might not be required and there will be no significant losses of acid (Iranmahboob *et al.*, 2002).

3. The reaction time is faster than enzymatic hydrolysis (Garrote *et al.*, 2001).

4. This method can be used in many lignocellulosic waste materials.

Acid hydrolysis's disadvantages

By-products can be produced by dilute acid hydrolysis, i.e. acetic acid and furfural (Pessoa *et al.*, 1997; Rhaman *et al.*, 2006). Moreover, by-products (acetic acid and furfural) generated from dilute acid hydrolysis do not only reduce the yield on monomeric sugar but also act as the inhibitors in the fermentation (Pessoa *et al.*, 1997; Neureiter *et al.*, 2002). These inhibitors have affected on cell morphological change or ultimate death of the organism (Rhaman *et al.*, 2006).

Acetic acid has been produced from degradation of acetyl group, which contains in the hemicellulose structure (Garrote *et al.*, 2001) whereas xylose degradation is a cause of furfural production (Rhaman *et al.*, 2006). It was demonstrated that acid concentration is an important parameter for sugars production whereas temperature is mainly responsible for decomposition of sugars to various by-products (Neureiter *et al.*, 2002; Rhaman *et al.*, 2006). Moreover, temperature and reaction time are also affected on xylose decomposition (Delgenes *et al.*, 1996). Therefore, to keep the concentration of by-products in the hydrolysate at low level it is necessary to operate the hydrolysis reaction at less severe conditions (Rhaman *et al.*, 2006). Furaldehyde or furfural, acetate and hydroxymethylfuraldehyde (HMF) are main inhibitory compounds from acid hydrolysis of lignocellulosic materials. These toxic compounds have a negative affected on cell growth and ethanol production by yeast and bacteria (Delgenes *et al.*, 1996).

Table 2. Detoxification methods of streams resulting of pretreatment and hydrolysis of biomass to produce ethanol.

Methods	Procedure/agents	Samples	Stains	Results
Physical methods;				
Evaporation	Evaporation, separation of volatile and nonvolatile fractions and dilution of non-volatile fraction	Willow	*S. cerevisiae*	Reduction of acetic acid and phenolic compounds in non-volatile fraction.
		Aspen	*P. stipitis*	Roto-evaporation 93% yield of ref. fermn.; removal: 54% acetic acid, 100% furfural, 29% vanillin
Extraction	Organic solvents, 3:1 org. phase: aqueous phase volumetric ratio	Spruce	*S. cerevisiae*	Solv: diethyl ether (solv.); yield comparable to ref. fermn.; removal of acetic, formic and levulinic acids, furfural, HMF.
		Aspen	*P. stipitis*	Solv.: ethyl acetate; 93% yield of ref. fermn.; removal: 56% acetic acid, 100% furfural, 100% vanillin, 100% hydroxybenzoic acid.
		Pine	*S. cerevisiae*	Solv.: ethyl acetate; removal of low molecular phenolic compounds.
		Steam-exploded Poplar	*S. cerevisiae*	Solv.: ethyl acetate; EtOH yield (SSF): detoxified 0.51 g/g, undetox. 0 g/g; high degree of phenolic removal.
	Supercritical solvent in counter-current with the hydrolyzate, 20 MPa, 40°C; then, depressuri-zation	Dilute-acid spruce	*S. cerevisiae*	Solv.: supercritical CO_2; 98% yield of ref. fermn.; removal 93% furfural, 10% HMF.

Table 2. Detoxification methods of streams resulting of pretreatment and hydrolysis of biomass to produce ethanol **(cont.).**

Methods	Procedure/agents	Samples	Stains	Results
Physical methods;				
Adsorption	Activated carbon, 0.05–0.20 g/g glucose	Steam-exploded concentrated oak	*S. cerevisiae*	Detoxified with 140–170 g/l initial glucose was utilized; undetox. with 100 g/l initial glucose could not be completely utilized.
	Amberlite hydrophobic polymeric adsorbent XAD-4, 8% (w/v), 1.5 h, 25°C; regeneration with EtOH; then, neutraliz. with lime.	LHW-pretreated corn fiber Poplar	Recombinant *E. coli*	Reduction of furfural conc. from 1–5 to <0.01 g/l; 90% yield of theoretical; sugars are not adsorbed.
Chemical methods;				
Neutralization	$Ca(OH)_2$ or CaO, pH = 6, then membrane filtration or adsorption.	Acid hydrolysis of cotton waste pyrolysate	*S. cerevisiae*, *Pichia* sp.	Precipitation or removal of toxic compounds; 10% lower yield for *Pichia* sp.
		Steam-exploded Poplar	*S. cerevisiae*	EtOH yield (SSF): detoxified 0.86 g/g, undetox. 0 g/g.
Ionic exchange	Weak base resins Amberlyst A20, regenerated with NH_3.	Dilute-acid poplar	Recombinant *Z. mobilis*	Removal: 88% acetic acid, 100% H_2SO_4; 100% sugars recovery.
	Poly(4-vinyl pyridine)	Corn-stover	Recombinant *S. cerevisiae*	Sugars eluted earlier than all tested inhibitors; ferment. results were similar to that using pure sugars.

Table 2. Detoxification methods of streams resulting of pretreatment and hydrolysis of biomass to produce ethanol **(cont.).**

Methods	Procedure/agents	Samples	Stains	Results
Chemical methods; Alkaline detoxification (overliming)	$Ca(OH)_2$, pH = 9–10.5, then pH adjustment to 5.5–6.5 with H_2SO_4 or HCl	Dilute-acid of spruce	*S. cerevisiae*	Yield comparable to ref. fermn.; 20% removal of furfural and HMF.
		Steam-exploded bagasse	Recombinant *S. cerevisiae*	Removal of acid acetic, furfural and part of phenolic compounds.
		Rice hulls	Recombinant *E. coli*	39% reduction in fermentation time.
		Wheat straw	Recombinant *E. coli*	Reduction in fermn. time: SSF =18%, SHF = 67%.
		Dilute-acid bagasse hydrolysate	Recombinant *E. coli*	Removal: 51% furfural, 51% HMF, 41% phenolic compounds, 0% acetic acid; overliming at 60°C or 25°C, at high temperature.
Combined alkaline detoxification	KOH, pH = 10, then pH adjustment to 6,5 with HCl and addition of 1% sodium sulfite.	Bagasse hydrolysate	*P. stipitis*	Reduction of ketones and aldehydes, removal of volatile compounds when hydrolyzate is heated at 90°C.

Table 2. Detoxification methods of streams resulting of pretreatment and hydrolysis of biomass to produce ethanol **(cont.).**

Methods	Procedure/agents	Samples	Stains	Results
Biological methods;				
Enzymatic detoxification	Laccase (phenol oxidase) and lignin peroxidase from Trametes versicolor: 30°C, 12h	Willow	*S. cerevisiae*	2–3-fold increase of EtOH productivity compared to undetox.; laccase selectively removes phenolic low molecular weight compounds and phenolic acids.
		Steam-exploded bagasse	Recombinant *S. cerevisiae*	80% removal of phenolic compounds.
Microbial detoxification	*Trichoderma reesei*	Steam-exploded Willow	*S. cerevisiae*	3-fold increase of EtOH productivity compared to undetox.; 4-fold increase of yield; removal of acetic acid, furfural and benzoic acid derivatives.
	Immobilized to PCS mixed culture of *Pseudomonas putida* and *Streptomyces setonii* cells (biofilm reactor: PCS tubes attached to CSTR agitator shaft)	Diluted pyrolysate of corn stover		Detoxification of 10 and 25 vol.% of pyrolysate medium, and partially detoxification of 50 vol.% of pyrolysate medium.

Observations: Reference fermentation (ref. fermn.) refers to fermentation carried out in a glucose-based medium without inhibitors; undetox.– undetoxified; PCS – plastic composite support.
Source: Sánchez and Cardona, (2008)

7.1.2 Steam explosion

Steam explosion is a thermomechnochemical process. The breakdown of structural components is aided by heat in the form of steam (thermo), shear forces due to the expansion of moisture (mechano) and hydrolysis of glycosidic bonds (chemical) (Jeoh, 1998). By this pretreatment, the biomass is usually treated with high pressure at high temperature between 120°C and 240°C corresponding to the pressure between 5 and 34 bars and gave reaction time of for several seconds to a few minutes. During pretreatment, hemicellulose is solubilized in the liquid phase as oligomeric and monomeric sugars. The cellulose is in the solid phase then becomes more easily to the enzymatic hydrolysis (Galbe *et al.*, 2007).

Chen and Liu. (2006) studied on steam explosion for separating hemicellulose from chipped wheat straw which composed of 35.1% cellulose, 27.1% hemicellulose, 5.3% Klason lignin and 6.04% ash. After hydrolysis by steam explosion at 160-180°C, 1.5 MPa for 4.5 min and determination using HPLC, the ratio of monosaccharides to oligosaccharides was found to be 1:9 and the main component was xylose (85.9%) in content. The total recovery rate of hemicellulose was 80%.

7.1.3 Alkali method

Sun *et al.* (1996) studied NaOH concentration to hydrolyze wheat straw and produce hemicellulose. Solid residues were hydrolyzed 6 times to be hemicellulose. It was found that the condition of hydrolysis (alkali concentration, temperature and retention time) will be increased every steps in order to break down the complex structure of wheat straw. After 6 times of NaOH hydrolysis, 33.9% hemicellulose was obtained.

7.1.4 Combination of chemical or physical techniques for hemicellulose production

Hemicellulose production from wheat straw, in the first step ligonin content was obtained 11.2–12.3% of the total lignin due to the hydrolysis of substantial amounts of hemicelluloses during the steam pretreatment, even though small amounts of lignin present in the middle lamella were also degraded during the steam explosion. After that, 80.6–88.2% of the total lignin was obtained by alkaline

peroxide hydrolysis. Therefore, the two-stage treatment degraded 92.3–99.4% of the original lignin from wheat straw (Sun *et al.*, 2005). Moreover, hemicellulose production from olive stone by steam explosion combined with and without 0.1% sulfuric acid pretreatment were also studied (Fernandez-Bolanos, 2001). The maximum yield of the pentosan recovered in the water solution from steam explosion was 63% pentose via treated at 200°C for 2 min with 0.1% sulfuric acid or 215°C for 2 min without acid. This indicated that treated with acid can decrease level of temperature because acid is a promoter of hydrolysis.

Extraction of hemicellulose from EFB and sterilizer condensate was conducted using alkali treatment and solvent method. The optimal ratios of EFB to 12% KOH were found to be 1:50 (w/v) while extraction at 80°C for 20 min gave significantly higher hemicellulose concentration than other treatments. Addition of ethanol to precipitate the hemicellulose from the extracted solution in the ratio of 1:1 (v/v) gave the highest hemicellulose yield of 8.67 g/100 g EFB. For extraction of hemicellulose from sterilizer condensate, the optimal ratio of ethanol to the condensate was 2:1 (v/v) which gave a hemicellulose yield of 6.42 g/100 ml (Prasertsan and Oi, 2001).

Pretreatment of wood components by steam explosion at 180-230°C for 2-20 min gave the xylose yield of 10-20% and about 50% of the wood was obtained as solid residues in which the lignin and residual hemicelluloses might be removed by a subsequent alkali extraction (Shimizu, 1998). In addition, hemicellulose could also be produced from un-utilized bamboo, which provided 48 g holocellulose/100 g bamboo (Asada *et al.*, 2005). Hemicellulose could be also hydrolyzed from residual corrugated cardboard using acid hydrolysis; 1-3% sulfuric acid and heated at 130°C for 30-180 min. The suitable condition consisted of 1% sulfuric acid, heated at 130°C for 30 min and gave 14.1% w/w hemicellulose (Yanez *et al.*, 2004). Softwood chips (*pine* and *spruce*) (30x30x2 mm^3, moisture content of 20-30%) were treated at 175°C in combination with 4.5%SO_2 for 7.5 min. SO_2–catalyzed steam explosion was found to be an effective method to enhance percentages of hemicellulose (80-90%) into solution because of the partial conversion of SO_2 into sulfuric acid during the process (Shevchenko *et al.*, 2000). The

components of Eucalyptus wood were studied by various conditions of steam explosion. The optimal condition of C-6 sugar (hexose) and C-5 sugar (xylose) production was high temperature at 180°C, pressure at 19 bars for 6 min and then treated with cellulase (20 FPU/g substrate) at 50°C for 24 h, giving total sugar of 56.40 g/l (Nunes and Pourquie, 1996). Mulberry or *Morus alba Linn* hydrolyzed by steam explosion was achieved after heating at 190°C for 5 min. The retention time (min) of xylose was 15.019 min and its quantity was 82.242 mg/l (Punsuwan *et al.*, 2004).

The characteristic of exploded fibre of hemp or *Cannabis sativa*, which was an annual plant used in the pulp and paper industry, was studied by scanning electron microscopy (SEM). The original hemp's fibres were associated in the bundles containing 15-30 fibres while the exploded fibres were separated into packets containing 2-5 fibres. In addition, most of pectic substances at the surface of the bundles were removed after treated with steam explosion and water washing. These fibres were well separated (1-3 fibres) after using steam explosion in combination with a 2% sodium hydroxide extraction. The best hemp bundles (1-2 fibers) were achieved after steam explosion, water extraction, NaOH extraction and bleaching, respectively (Garcia-Jaldon *et al.*, 1998).

7.1.5 Xylanase hydrolysis

Xylanase (E.C. 3.2.1.8) is the name given to a class of enzymes which degrade the linear polysaccharide beta-1,4-xylan into xylose, thus breaking down hemicellulose, which is one of the major components of plant cell walls (Wulandari, 2009). Xylanase represents one of the largest groups of industrial enzymes with increasing market demands due to its applications in prebleaching of kraft pulps, bioconversion of agricultural residues, extraction of coffee and plant oils, improvement of the nutritional properties of agricultural silage and degumming of plant fibers, such as flax, sun hemp and ramie (Subramaniyan and Prema, 2002). A variety of microorganisms including bacteria, yeast, actinomycetes and filamentous fungi have been reported to produce xylanolytic enzymes. Each organism or strain has its own special conditions for maximum enzyme production and activity (Kapoor *et al.*, 2008). Complete depolymerization of xylan is accomplished by the synergistic

action of endo-xylanases and xylosidases along with arabinofuranosidases, ferulic acid esterases, uronidases, and other enzymes which, respectively, act on the xylan backbone, side chains and decorating units, producing fermentable xylooligomers and monomers (Collins *et al.*, 2005; Pastor *et al.*, 2007; Squina *et al.*, 2009). Microbial xylanases are the preferred catalysts for xylan hydrolysis due to their high specificity, mild reaction conditions, negligible substrate loss and side product generation (Chapla *et al.*, 2010).

Xylanases have commercial uses in various forms: (a) biobleaching agents in pulp and paper industry; (b) enhanced utilization of biomass in the biofuel industry; (c) production of xylitol (a low calorie sweetener); (d) foodstuff additive in bread, juice and wine manufacturing and (e) additive in animal feedstuff preparation (Kapoor *et al.*, 2008).

7.2 Glucose production

7.2.1 Concentrated acid hydrolysis

Concentrated acid have been used for decrystallization of cellulose followed by dilute acid hydrolysis to sugars. In order to save the chemical cost, separation of acid from sugars, acid recovery, and acid reconcentration are critical unit points. The concentrated acid disrupts the hydrogen bonding between cellulose chains, converting it to a completely amorphous state. Once the cellulose has been decrystallized, it forms a homogeneous gelatin with the acid. The cellulose is extremely susceptible to hydrolysis at this point. Therefore, dilution with water at modest temperatures provides complete and rapid hydrolysis to glucose, with little degradation (U.S. Department of Energy, 2008).

Concentrated acid have been reported by U.S. Department of Energy (2008). The first stage, materials were mixed with 70-77% (v/v) H_2SO_4 and then followed by adding water into the system to dilute the acid concentration to 20-30% H_2SO_4 for an hour at less than 50°C.

In addition, concentrated sulfuric acid had been used directly to produce glucose from α-cellulose (Xiang *et al.*, 2003). The α-cellulose form treated by concentrated sulfuric acid of 65% at high temperature (more than 200°C) can be

changed from fibrous form to gelatinous form. The results indicated that 65% H_2SO_4 pretreatment for 4 hours was successfully hydrolyzed α-cellulose around 95% after carrying out at 120°C, 4% H_2SO_4 for 90 min (Xiang *et al.*, 2003).

7.2.2 Cellulase hydrolysis

Cellulase is a class of enzymes produced chiefly by fungi, bacteria and protozoans that catalyze the cellulolysis. There are fives commonly types of cellulase based on the type of reaction catalyzed, consist of:

7.2.2.1 Endo-cellulase

It breaks internal bonds to disrupt the crystalline structure of cellulose and expose individual cellulose polysaccharide chains (Zhou *et al.*, 2009).

7.2.2.2 Exo-cellulase

It cleaves 2-4 units from the ends of the exposed chains produced by endocellulase, resulting in the tetrasaccharides or disaccharide in term of cellubiose. Two main types of exo-cellulase were identified based on the cleaved positions of cellulose; one type digests at the reducing end of cellulose while another type digests at the non-reducing end of cellulose (Zhou *et al.*, 2009).

7.2.2.3 Cellubiase or β-glucosidase

This cellulolytic enzyme digests disaccharides into individual monosaccharides (Zhou *et al.*, 2009).

7.2.2.4 Oxidative cellulase

It depolymerizes cellulose by radical reactions, as for instance cellulobiose dehydrogenase.

7.2.2.5 Cellulose phosphorylase

It also depolymerize cellulose using phosphates instead of water.

7.2.3 Cellulase production by microorganisms

The extracellular cellulolytic system of *Trichoderma reesei* is composed of 60–80% cellobiohydrolases or exogluconases, 20–36% of endogluconases and 1% of β-glucosidases, which all act synergistically in the conversion of cellulose into glucose (Ahamed and Vermette, 2008) whereas, the well studied fungus *T. reesei* can produce cellulases, including at least two cellobiohydrolases (EC 3.2.1.74), five endoglucanases (EC 3.2.1.4) and two β-

glucosidases (EC 3.2.1.21), which act synergistically during the conversion of cellulose to glucose (Zhang and Cai, 2008; Zhou *et al.*, 2009). *T. reesei* cellulase system is deficient in cellobiase, causing the accumulation of the disaccharide cellobiose, which produces repression and end product inhibition of the enzyme, both of which limit enzyme synthesis and activity (Ahamed and Vermette, 2008). The cellulase is an inducible enzyme system in which several carbon sources have been tested to find the best inducer such as cellulose and lactose whereas, glucose is an inhibitor in cellulase biosynthesis (Ahamed and Vermette, 2008).

Cellulase could be produced from several stains of bacteria and fungi. There were many research works reporting production of cellulase. For example, the cellulase production by *Bacillus* spp. isolated from compost giving the highest activity of 1.33 mg glucose released ml^{-1} min^{-1} at 70°C was studied (Mayende *et al.*, 2006). Ahamed and Vermette (2008) have reported that a mixture of lactose and lactobionic acid was added into the bioreactor as cellulase inducers. The use of a cellulose–yeast extract culture medium yielded the highest enzyme and cell production with a volumetric enzyme activity of 69.8 U/l/h, a filter paper activity of 5.02 U/ml, a CMCase activity of 4.2 U/ml, and a fungal biomass of 14.7 g/l. Zhang and Cai (2008) have reported that the production of reducing sugars and filter paper activity (FPA) could achieve 2.231 g/l and 12.92 U/ml, respectively, under enzymatic hydrolysis at 35°C, pH 4.5 for 96 h by broth of *Trichoderma reesei* ZM4-F3 in 2% NaOH pretreated rice straw as a substrate cultured for 36 h.

β-glucosidase could be produced by *Aspergillus phoenicis*. The β-glucosidase activity and FPA were 0.64 IU/ml and 1.54 FPU/ml, respectively, cultured in manure solid at 27 °C and pH 5.5, which is close to the optimal values for both fungi (Wen *et al.*, 2005). *Fusarium oxysporum VTT*-80134 has been found to produce insufficient amounts of enzymes to convert cellulose directly into ethanol. *F. oxyspoerum* produces sufficient activities of β-glucosidase to prevent severe product inhibition by cellobiose during the hydrolysis process (Panagiotou *et al.*, 2005). Fermentation performance by the fungus *F. oxysporum* under aerobic and anaerobic cultivation on cellulose was investigated. It was found that *F. oxysporum* grow with a maximum specific growth rate of 0.023 h^{-1} on cellulose under aerobic conditions

giving final endoglucanase, β- glucosidase and cellobiohydrolase activities of 55, 1.25 and 0.43 U/ml, respectively. Under anaerobic conditions it can produce ethanol with a yield of 0.35 g/g cellulose and significant amounts of acetic acid, with a yield of 0.2 g/g cellulose, as a by-product (Panagiotou *et al.*, 2005).

7.2.4 Factors affecting on enzymatic hydrolysis

The pretreatment is a necessary step to alter some structural characteristics of lignocellulose, increasing glucan and xylan accessibility to the enzymatic attack. These structural modifications of the lignocellulose are highly dependent on the type of pretreatment employed and have a great effect on the enzymatic hydrolysis (Kumar *et al.*, 2009) and subsequent steps. The choice of pretreatment technology for a particular raw material depends on several factors, some of them directly related to the enzymatic hydrolysis step such as sugar-release patterns and enzymes employed. Thus, the combination of the composition of the substrate, type of pretreatment, and dosage and efficiency of the enzymes used for the hydrolysis have a great influence on biomass digestibility; although the individual impacts of these factors on the enzymatic hydrolysis are still unclear (Alvira *et al.*, 2010).

Main factors that influence the enzymatic hydrolysis of cellulose in lignocellulosic feedstocks can be divided in two groups: enzyme-related and substrate-related factors, though many of them are interrelated during the hydrolysis process. Composition of the liquid fraction and solid process streams resulting from different pretreatment approaches can be widely different. These differences will have a great influence on the requirements for effective enzymatic saccharification in subsequent processing steps (Alvira *et al.*, 2010).

The reduction of pretreatment severity is sometimes required to reduce economic cost. Low severity factor results in less sugar-release and consequently higher amount and different types of enzymes will be required to achieve high sugar yields from both cellulose and hemicellulose fraction. In this context, development of hemicellulases and other accessory enzymes needed for complete degradation of lignocellulose components has become an important issue. Recent studies show the importance of new balanced enzymatic complexes containing optimal combinations

to effectively modify the complex structure of lignocellulosic materials (Garĉa-Aparicio *et al.*, 2007; Merino and Cherry, 2007; Alvira *et al.*, 2010).

Substrate-related factors limiting enzymatic hydrolysis are directly connected to the pretreatment employed. These factors are described separately below although their effect is normally interrelated (Alvira *et al.*, 2010).

7.3 Furfural production

Furfural is a liquid aldehide in hetero cyclic group, an organic solvent, and almond odor, less color or yellow when reacted with oxygen (auto-oxidation). It is dissolved in organic solvents but in inorganic solvents. Furfural is usually used as a solvent in many industries such as brewery, perfume, herbicide, insecticide and especially in petroleum fuel. The composition of diesel is almost large hydrocarbon, i.e. paraffin, olefin, naphthen and aromatic hydrocarbon. These compounds are the cause of uncompleted incineration, soot and smoke. Therefore, furfural is usually used as a solvent for dissolving these compounds in order to enhance the efficiency of incineration, to reduce soot and smoke. There are three solvent used in diesels; furfural, phenol and 1-methyl-2-pyrrolidone (MP). However, furfural is a favorite solvent due to cheaper, lower toxicity and simplifies.

Furfural and hydroxymethylfurfural (HMF) were produced from agricultural wastes such as corncobs, sugarcane bagasse, cottonseed hull and rice hull by acidic degradation process (Gutierrez *et al.*, 2006; Rahman *et al.*, 2006). The precursors of furfural were pentoses (mainly xylose), whereas hexoses (fructose, sucrose, and inulin) were the precursors of HMF production (Karimi *et al.*, 2006). The metabolism reaction of furfural production is given in Fig. 6. There were many parameters affecting on the efficiency of production such as acid concentration, temperature, and retention time. The optimal conditions were 6% sulfuric acid, 120°C and 15 min providing the highest furfural and xylose about 0.87 and 29.4 g/l, respectively (Rahman *et al.*, 2006).

Rice straws were hydrolyzed by dilute sulfuric acid (0-1%) at high temperature (180-230°C) and pressure (1.5-2.0 MPa) in one and two stages, which could produce cellulose (82.3 g/kg of rice straw), hemicellulose (56.4 g/kg of rice

straw), furfural (8.5 g/kg of rice straw), and hydroxymethylfurfural (HMF) (12.7 g/kg of rice straw) (Karimi *et al.*, 2006). HMF could be converted to levulinic acid by hydrolysis at 195-215°C for 15-30 min. (Bozell *et al.*, 2000). Production of furfural by acid hydrolysis of olive stones was also reported (Montané *et al.*, 2002). The hydrolysis was conducted in dilute sulfuric acid (0.05-0.250 mol/l) at high temperature (220–240°C) and short reaction time (a few minutes at the most) and performed in a tubing-bomb reactor system. The maximum yield of 65% was achieved at the acid concentration of 0.250 mol/l at 240°C for 150 seconds. Pentosan content of 18.5% (dry basis) in olive stones produced furfural equivalent to 135 kg furfural/ton of dry olive stones.

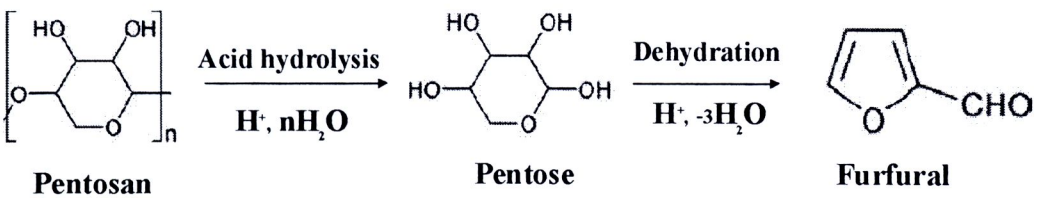


Figure 6. Reaction mechanism of acid hydrolysis of pentosan to furfural.
Source: Dias *et al.* (2005); Riansa-ngawong and Prasertsan (2010)

The market price of furfural was around 1700 US dollars/ton (Montané *et al.*, 2002). Furfural was mainly produced from lignocellulosic biomass by pentose dehydration (Gutierrez *et al.*, 2006; Karimi *et al.*, 2006). The capacities of furfural production were different depending on sources (dry biomass) such as 220 kg furfural/ton corncobs, 170 kg furfural/ton bagasse, 165 kg furfural/ton corn stalks, 160 kg furfural/ton sunflower hulls, 120 kg furfural/ton rice hulls, and 150-170 kg furfural/ton hardwoods (Montané *et al.*, 2002).

7.4 Ethanol production from biomass

The fuel ethanol can be obtained from energy crops and lignocellulosic biomass (Lee, 1997; Sánchez *et al.*, 1997). The complexity of the production process depends on the feedstock. In this way, the spectrum of designed and implemented technologies goes from the simple conversion of sugars by fermentation, to the multi-

stage conversion of lignocellulosic biomass into ethanol. The big diversity of technological alternatives requires the analysis of the global process along with the design and development of each one of the involved operations. Among the new research trends in this field, process integration has the key for reducing costs in ethanol industry and increasing bioethanol competitiveness related to gasoline (Sánchez and Cardona, 2008).

Table 3. Fuel ethanol programs in some countries.

Country	Feedstocks	Percentage of ethanol in gasoline blends, % (v/v)
Brazil	Sugar cane	24
USA	Corn	10
Cananda	Corn, wheat, barley	7.5-10
Colombia	Sugar cane	10
Spain	Wheat, barley	-
France	Sugar beet, wheat, corn	-
Sweden	Wheat	5
China	Corn, wheat	-
India	Sugar cane	5
Thailand	Cassava, sugar cane, rice	10

Source: Sánchez and Cardona, (2008)

7.4.1 Bio-ethanol as fuel

Ethanol (ethyl alcohol, bioethanol) is the most employed liquid biofuel either as a fuel or as a gasoline enhancer. Ethanol has some advantages when it is used as an oxygenate. Firstly, it has a higher oxygen content that implies a less amount of required additive. The increased percentage of oxygen allows a better oxidation of the gasoline hydrocarbons with the consequent reduction in the emission of CO and aromatic compounds. Related to methyl tertiary butyl ether (MTBE) which is a gasoline additive used as an oxygenate and to raise the octane number, ethanol has greater octane booster properties, it is not toxic, and does not contaminate water

sources. Nevertheless, ethanol production costs are higher than those of MTBE, gasoline mixed with alcohol conduces the electricity, and vapor pressure is higher that entails a greater volatilization, which can contribute to ozone and smog formation (Thomas and Kwong, 2001; Sánchez and Cardona, 2008). Many countries have implemented or are implementing programs for addition of ethanol to gasoline (Table 3). Fuel ethanol production has increased remarkably because many countries look for reducing oil imports, boosting rural economies and improving air quality.

The world ethyl alcohol production has reached about 51,000 mill liters, being the USA and Brazil the first producers (Table 4). In average, 73% of produced ethanol worldwide corresponds to fuel ethanol, 17% to beverage ethanol and 10% to industrial ethanol (Sánchez and Cardona, 2008).

Table 4. World production of ethyl alcohol (mill liters).

Country	2006
1. USA	18,376
2. Brazil	16,998
3. China	3,849
4. India	1,900
5. France	950
6. Germany	765
7. Russia	647
8. Canada	579
9. Spain	462
10. South Africa	386
11. Thailand	352
12. United Kingdom	280
13. Ukraine	269
14. Colombia	269
15. Poland	250
Total	51,056

7.4.2 Ethanol and its properties

Ethanol is the most common alcohol, which is produced from several sources i.e. starch (from corn and cassava), cellulose and hemicellulose (from lignocellulosic materials) through fermentation of these carbohydrates.

7.4.2.1 Physical properties

Ethanol is a volatile, colorless liquid that has a strong characteristic odor. It burns with a smokeless blue flame that is not always visible in normal light. The physical properties of ethanol stem primarily from the presence of its hydroxyl group and the shortness of its carbon chain. Ethanol's hydroxyl group is able to participate in hydrogen bonding, rendering it more viscous and less volatile than less polar organic compounds of similar molecular weight. Ethanol is a versatile solvent, miscible with water and with many organic solvents, including acetic acid, acetone, benzene, carbon tetrachloride, chloroform, diethyl ether, ethylene glycol, glycerol, nitromethane, pyridine, and toluene. It is also miscible with light aliphatic hydrocarbons, such as pentane and hexane, and with aliphatic chlorides such as trichloroethane and tetrachloroethylene.

7.4.2.2 Chemical properties

The chemical structure of ethanol is CH_3CH_2OH and has a density of 0.789 g/ml at 20°C with a molecular weight of 46.07 g/mol, a melting point of 144°C and a boiling point of 78°C. Ethanol is normally used to form blended gasoline fuels in concentration between 5-85% (Minteer, 2006).

7.4.3 Bio-ethanol production process from biomass

Biological process of bio-ethanol production utilizing lignocellulose as substrate requires: delignification, depolymerization, and fermentation (Lee, 1997).

(1). Deliginification

The potential method for removing lignin and releasing fermentable sugars is pretreatment followed by enzymatic and acidic hydrolysis. Lignin can be degraded by several fungal enzymes such as lignin peroxidase, Mn-dependent peroxidase, and laccase (mono-phenol oxidase) and its degradability depend on fungal strain, accessibility of lignin to enzyme, culture condition, and reactor design (Lee,

1997). The major sugars in enzyme hydrolysates are glucose and xylose released from cellulose and hemicellulose, respectively (Ahamed and Vermette, 2008; Zhang and Cai, 2008; Zhou *et al.*, 2008). The advantages of biological delignification may include higher product yields, fewer side reactions, less energy demand and less reactor resistance because of mild reaction condition (Lee, 1997).

Diluted acetic acid in combination with catalyst such as sodium chlorite is used to degradate lignin in biomass (Iiyama and Wallis, 1990). The residue is holocellulose containing cellulose and hemicellulose. Furthermore, steam explosion is also used to remove the lignin (Punsuvon *et al.*, 2008).

(2). Depolymerization

Hemicellulose was hydrolyzed to monosaccharides by many methods such as alkali hydrolysis, acid hydrolysis, enzyme hydrolysis as hemicellulase, and steam explosion. Hemicellulose could be hydrolyzed by diluted sulfuric acid and heated at 120°C for 30-60 min (Nigam, 2002). Acid and heat are the important parameters affecting on degradation of β 1,4 glycosidic linkages and β 1,6 glycosidic linkages of hemicellulose (Rhaman *et al.*, 2006). In the enzymatic system, hemicellulase can specifically break bond at glycosidic linkages. Finally, monomeric sugars are obtained.

Recently, some engineered bacteria that produce some enzymes required for the depolymerization of cellulose and efficiently fermentation all of the released sugars to ethanol have been developed (Nigam, 2002). For example, genetic engineering technique was used to improve *Zymomonas mobilis* 8b that both could use both glucose and xylose for ethanol production giving the ethanol yield of 0.42 g ethanol/g sugar (Mohagheghi *et al.*, 2004). Therefore, these recombinant microorganisms could be promising candidates to be applied to the direct ethanol fermentation.

(3). Fermentation

The ethanol fermentation process by fungi and bacteria has been well developed with glucose as a carbon and energy source. Xylose known as a hardly fermentable sugar by microorganisms is also compost mostly 50% in biomass (Nigam, 2002). The production of xylose can be done by enzymatic hydrolysis

(Lachke, 2002) and acid hydrolysis (Rhaman *et al.*, 2006). It was found that there are some of yeasts that are able to ferment xylose to ethanol such as *Candida shehatae* (Delgenes *et al.*, 1996), *Pachysolen tannophilus* (Bravo *et al.*, 1995) and *Pichia stipitis* (Abbi *et al.*, 1996; Lee, 1997; Nigam, 2002). To enhance the overall efficiency of biomass utilization, the fermentation process from both C-6 sugar and C-5 sugarneed to develop. The fermentation reactions of those sugars are represented by these following equations (Lachke, 2002).

Glucose; $C_6H_{12}O_6 \longrightarrow 2\ CH_3CH_2OH + 2\ CO_2$

Xylose; $3C_5H_{10}O_5 \longrightarrow 5\ CH_3CH_2OH + 5\ CO_2$

Arabinose; $3C_5H_{10}O_5 \longrightarrow 5\ CH_3CH_2OH + 5\ CO_2$

Ethanol yield is criteria to evaluate ethanol production. It is well known that 0.51 g ethanol is produced from 1 g glucose. However, the carbon flow in cells is also used for biomass production. Therefore, the theoretical ethanol yield is approximately 0.46-0.48 g ethanol/g glucose (Kopsahelis *et al.*, 2007).

7.4.3.1 Simultaneous saccharification and fermentation (SSF)

The avoidance of end products inhibition and thereby increasing the saccharification rate and the ethanol yield are one of the significant reasons for using SSF; however there are several additional potential advantages as the presence of ethanol in the culture medium causes the mixture to be less vulnerable to invasion by undesired microorganisms (Menon *et al.*, 2010). Moreover, the SSF process shows more attractive indexes than the separate hydrolysis and fermentation (SHF) as higher ethanol yields, less energetic consumption, decrease the number of vessels needed and thereby reduces the investment costs (Alkasrawi *et al.*, 2003; Menon *et al.*, 2010). In this case, the cellulases and microorganisms are added to the same process unit allowing that the glucose formed during the enzymatic hydrolysis of cellulose be immediately consumed by the microbial cells converting it into ethanol. Thus, the inhibition effect caused by the sugars over the cellulases is neutralized. However, the need of employing more dilute media to reach suitable rheological properties makes

that final product concentration be low. In addition, this process operates at non-optimal conditions for hydrolysis and requires higher enzyme dosage, which positively influences on substrate conversion, but negatively on process costs. Considering that enzymes account for an important part of production costs, it is necessary to find methods reducing the cellulases doses to be utilized. With this aim, addition of surfactants has been proposed (Alkasrawi *et al.*, 2003). The addition of the non-ionic surfactant Tween-20 to the steam exploded wood during a batch SSF using *S. cerevisiae* has some effects: 8% increase in ethanol yield, 50% reduction in cellulases dosage (from 44 FPU/g cellulose to 22 FPU/g cellulose), increase of enzyme activity at the end of the process, and decrease in the time required for reaching the highest ethanol concentration. It is postulated that the surfactant avoids or diminishes the non-useful adsorption of cellulases to the lignin (Sánchez and Cardona, 2008).

7.4.3.2 Separate hydrolysis and fermentation (SHF)

When sequential process is utilized, solid fraction of pretreated lignocellulosic material undergoes hydrolysis (saccharification). This fraction contains the cellulose in an accessible to acids or enzymes form. Once hydrolysis is completed, the resulting cellulose hydrolyzate is fermented and converted into ethanol. One of the main features of the SHF process is that each step can be performed at its optimal operating conditions. The most important factors to be taken into account for saccharification step are reaction time, temperature, pH, enzyme dosage and substrate load (Sánchez and Cardona, 2008).

By testing lignocellulosic material from sugar cane leaves, Hari Krishna et al. (1998) have found the best values, 65–70% cellulose conversion was achieved at 50°C and pH of 4.5. Although enzyme doses of 100 FPU/g cellulose caused almost a 100% hydrolysis, this amount of cellulases is not economically justifiable. Hence, 40 FPU/g cellulose dosage was proposed obtaining only 13% reduction in conversion. Regarding the substrate concentration, solids loads of 10% was defined as the most adequate considering arising mixing difficulties and accumulation of inhibitors in the reactioning medium (Sánchez and Cardona, 2008). Hydrolysis tests for steam-pretreated spruce also indicate the need of high enzyme

loadings of both cellulases and β-glucosidase to achieve cellulose conversions greater than 70% due to the less degradability of the softwood (Tengborg *et al.*, 2001).

Saha and Cotta (2006) obtained 96.7% yield of monomeric sugars using an enzymatic cocktail of cellulase, β-glucosidase and xylanase for saccharification of wheat straw pretreated by alkaline peroxide method. An ethanol concentration of 18.9 g/l and a yield of 0.46 g/g of available sugars were achieved in the subsequent fermentation using a recombinant *E. coli* strain capable of assimilating both hexoses and pentoses.

Nguyen et al. (1999) employed a mixed solids waste (construction lumber waste, almond tree prunings, wheat straw, office waste paper, and newsprint) for producing ethanol by SHF using yeasts. In this process, a recycling of enzymes was implemented through microfiltration and ultrafiltration achieving 90% cellulose hydrolysis at a net enzyme loading of 10 FPU/g cellulose.

7.4.4 Microbial key players in bio-ethanol production from lignocellulosic materials

Olsson and Hahn–Hägerdal (1996) presented a list of bacteria, yeasts, and filamentous fungi, producing ethanol from xylose. Among naturally occurring organisms, certain species of the yeasts *Candida*, *Pichia*, *Schizosaccharomyces*, *Kluyveromyces*, and *Pachysolen*, the filamentous fungi *Fusarium*, *Mucor*, *Monilia*, and *Paecilomyces*, and the bacteria *Clostridium*, *Bacillus*, *Bacteroides*, *Thermoanaerobacter*, and *Erwinia* produce ethanol. Among these microorganisms, *Candida shehatae*, *Pichia stipitis*, and *Fusarium oxysporum* resulted in high yields (>0.45 g ethanol/g xylose) and reasonable productivities (>0.17 g/l h). The characteristics required for an industrially suitable microorganism have been reported and are summarized in Table 5 (Dien *et al.*, 2003).

Table 5. Important characteristics for industrially ethanol production.

Characteristics	Requirement
Ethanol yield	>90% of theoretical
Ethanol tolerance	>40 g/l
Ethanol productivity	>1 g/l/h
Able to grow in undiluted hydrolysate	Resistance to inhibitors
Culture growth conditions retard contaminants	Acidic pH or higher temperature

Source: Dien *et al.* (2003)

(1) Bacteria

1.1 *Clostridium* sp.

The bioconversion of abundant and renewable cellulosic biomass into ethanol as an alternative to petroleum is gaining importance due to the realization of diminishing natural oil and gas resources. The single-step conversion of cellulosic biomass to ethanol by *Clostridium thermocellum* has advantages over the multiple-step process in which fungal cellulases and yeasts are used. However, the low ethanol tolerance (up to 1.5% v/v) and low ethanol yields (0.08-0.29 g/g) of the organism are the major limiting factors for its industrial exploitation. Furthermore, most of the studies have been carried out at low substrate concentrations using pure cellulosics. Therefore, it is necessary to conduct experiments with natural cellulosic materials using high ethanol yielding and ethanol tolerant *C. thermocellum* strains (Sudha Rani *et al.*, 1998).

Clostridium thermocellum produces an exocellular, multienzyme complex, termed cellulosome, which comprises numerous cellulases and hemicellulases. Searches for *C. thermocellum* genes involved in cellulose degradation were performed by several groups, resulting in the cloning of genes encoding 21 endoglucanases, 3 exoglucanases, 2 β-glucosidases and 4 xylanases (Guglielmi and Béguin, 1998). Thermophilic bacteria have a distinct advantage over conventional yeasts for ethanol production in their ability to use a variety of inexpensive biomass

feedstocks and their ability to withstand temperature extremes. Because these bacteria are inhibited by relatively low levels of ethanol, extractive fermentation using compressed solvents could prevent this toxicity and greatly enhance the economic viability of ethanol production by thermophilic organisms (Knutson *et al.*, 1999).

Thermophilic and anaerobic *C. thermocellum* strains, SS21 and SS22, which produced 0.37 and 0.33g ethanol/g cellulose consumed, respectively, were recently obtained. The strains are tolerant to 4.0 and 5.0% (v/v) ethanol and on addition of ethanol at different culture ages, there was increase in ethanol tolerance up to 7.0 and 8.0% (v/v), respectively (Sudha Rani *et al.*, 1998).

1.2 *Zymomonas mobilis*

Zymomonas mobilis is an obligatorily fermentative Gram-negative bacterium that utilizes sucrose, glucose, and fructose by the Entner–Doudoroff (ED) pathway leading to the production of ethanol and CO_2 (Fig. 7) (Sprenger, 1996; Kang and Kang, 1998; Lee and Huang, 2000; Tao *et al.*, 2005). Morphology of *Z. mobilis* is shown in Fig. 8 (Davis *et al.*, 2006). Most *Z. mobilis* strains are capable of growth in the presence of up to 10% ethanol and of fermentation in media with up to 25% glucose (Kang and Kang, 1998).

Fermentation technologies utilizing strains of *Zymomonas mobilis*, in place of the traditional yeast, have been proposed by a number of authors for starch-based ethanol production, as they have been shown to ferment under fully anaerobic conditions with faster specific rates of glucose uptake and ethanol production as well as ethanol yields close to theoretical (Davis *et al.*, 2006). Ethanol productions from synthetic medium (Mohagheghi *et al.*, 2006) to liquid wastes such as agro-industry wastes (Ruanglek *et al.*, 2006) and hydrolysates such as wheat waste steam (Davis *et al.*, 2006), corn stover hydrolysate (Mohagheghi *et al.*, 2004) by *Z. mobilis* have been studied. Moreover, genetic engineering techniques were used as tools to mutant wild type strain of *Z. mobilis* for consuming of other sugars such as xylose (Mohagheghi *et al.*, 2004) and arabinose (Deanda *et al.*, 1996).

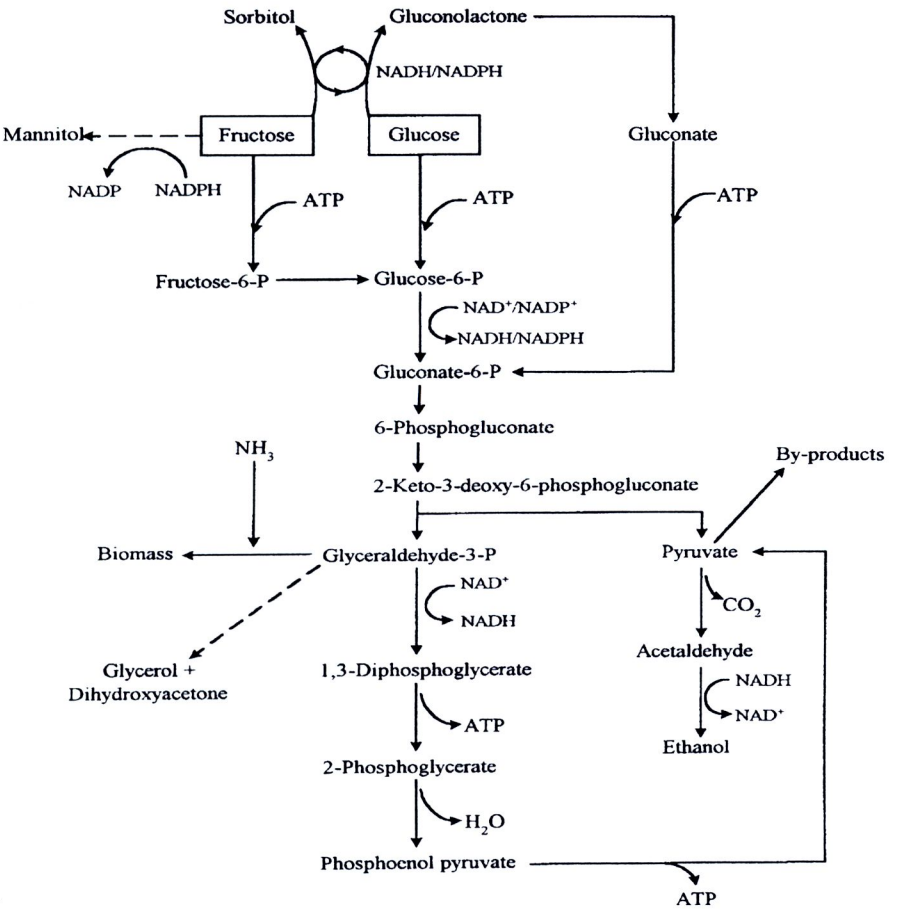


Figure 7. Schematic representation of glucose and fructose metabolism in *Z. mobilis*. ——▶common pathway, - - - -▶ exclusive fructose pathway.

Source: Lee and Huang (2000)

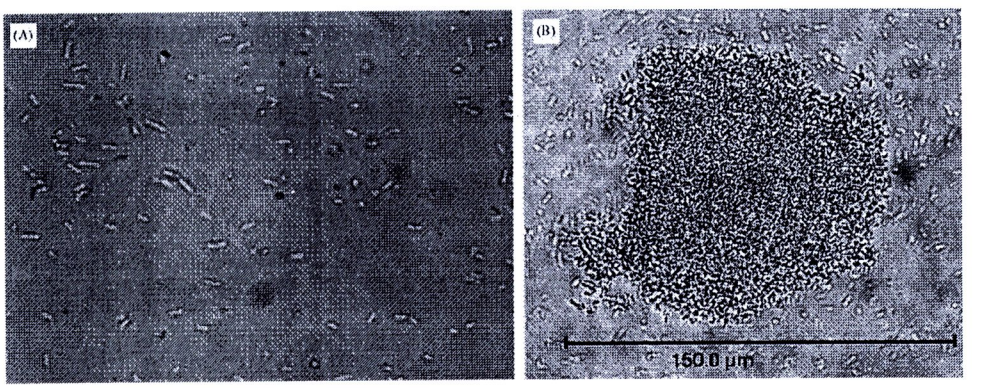


Figure 8. Photograph showing single cells of (A) *Z. mobilis* ZM4 and (B) its flocculent.

Source: Davis *et al.* (2006)

1.3 Enterobacter

Enterobacter aerogenes HU-101, isolated as a high-rate H_2 producer from methanogenic sludge, can convert various carbohydrates, such as sugars and sugar alcohols, to H_2, ethanol, 2,3-butanediol, lactate and acetate. *E. aerogenes* HU-101 mainly produces H_2 and ethanol with a minimal production of other by-products when glycerol was used as the substrate. Thus, the microorganism can be utilized for the high-yield production of H_2 and ethanol from biodiesel wastes containing glycerol (Ito *et al.*, 2005).

Ethanol production from glycerol-containing wastes discharged after a manufacturing process for biodiesel fuel (biodiesel wastes) using *Enterobacter aerogenes* HU-101 was studied (Ito *et al.*, 2005). The yield of ethanol decreased with an increase in the concentrations of biodiesel wastes and commercially available glycerol (pure glycerol). Furthermore, the rate of ethanol production from biodiesel wastes was much lower than those at the same concentration of pure glycerol, partially due to a high salt content in the wastes. In continuous culture with a packed-bed reactor using self-immobilized cells, the maximum rate of ethanol production from pure glycerol was 0.8 mol/mol-glycerol. However, using porous ceramics as a support material to fix cells in the reactor, the maximum ethanol production rate from biodiesel wastes reached 0.85 mol/mol-glycerol (Ito *et al.*, 2005).

(2) Yeast

Various yeasts are capable of fermenting D-xylose along with D-glucose. These are *Pachysolen tannophilus, Pichia stipitis,* and *Candida shehatae.* This has led to a growing interest in the use of lignocellulose residues for the industrial production of ethanol since the conversion of both the hemicellulose and cellulose fractions substantially increases the yield of ethanol (Sánchez *et al.*, 1997).

2.1 *Saccharomyces cerevisiae*

Baker's yeast (*Saccharomyces cerevisiae*) is the most commonly used microorganism for ethanol production due to its excellent characteristics of growing at high sugar concentrations and producing high yields of ethanol. However, it brings about two major problems for ethanol production from wood hydrolyzates. The first

one is due to the presence of toxic compounds in some of the hydrolyzates, which make the cells unable to grow (Taherzadeh *et al.*, 1997). This problem is usually tackled by detoxification. The second problem is related to its lack of capability to ferment xylose (Kötter and Ciriacy, 1993).

2.2 *Candida shehatae*

C. shehatae is one of a few yeasts, which can ferment both glucose and xylose to ethanol (Delgenes *et al.*, 1996). Moreover, mannose and galactose were also fermented by this yeast (Sreenath *et al.*, 2000). Bio-ethanol production from D-xylose of *C. shehatae* is via xylose pathway (Seiboth *et al.*, 2003), before passing through pentose phosphate pathway and glycolysis in order to produce ethanol (Fig. 9). Studies on the possible effects of the availability of oxygen on the metabolism of D-xylose by *C. shehatae* found that, in principle, an extra supply of oxygen was unnecessary, although ethanol production was indeed enhanced by added oxygen when using either D-xylose or D-glucose as the carbon source (Sánchez *et al.*, 1997). When the oxygen supply was restricted, some growth occurred, but no ethanol was produced. This indicates that for the efficient conversion of D-xylose into ethanol, the aeration rate should be higher than 0.02 v/v/min (Delgenes *et al.*, 1986). These publications suggested that an optimum aeration rate must be achieved in order to obtain maximum productivity and ethanol yield (Sánchez *et al.*, 1997). However, these explanations are contrast with Alexander et al. (1988). They demonstrated that *C. shehatae* exhibits three different types of metabolic behavior; (1) under fully aerobic conditions, in which oxygen is available in excess, respirative growth occurs without fermentation, (2) fermentation and respirative growth occur simultaneously under semi-aerobic conditions wherein growth is limited by the oxygen supply, (3) Under anaerobic conditions fermentation occurs without growth.

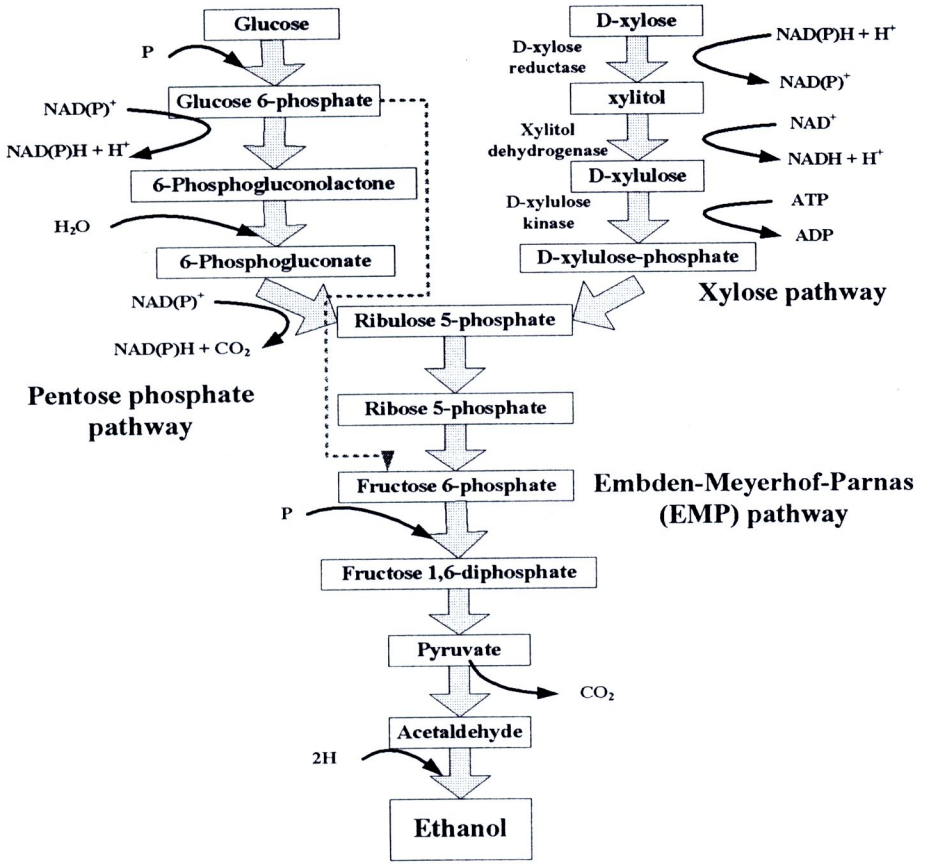


Figure 9. Diagram of ethanol production via xylose pathway, pentose phosphate pathway, and Embden-Meyerhof-Parnas (EMP) pathway.

Source: Modified from Seiboth *et al.* (2003)

Aerobic and anaerobic xylose metabolism may be limited by different factors. Aerobic xylose uptake in continuous culture appears to be transport-limited. Alternatively, aerobic xylose consumption could be affected by the levels of xylose reductase (XOR) or glucose6-phosphate dehydrogenase (GPD) (this enzyme provides the reductant necessary for NADPH-linked xylose reduction). Anaerobic metabolism proceeds at only a third the aerobic rate and may be limited by low levels of key enzymes such as NADH-linked XOR activity, xylitol dehydrogenase (XID) or alcohol dehydrogenase (ADH). NADH-linked XOR can relieve reductant imbalance that arises in cells under anoxic conditions (Bruinenberg *et al.*, 1984). Alcohol dehydrogenase is responsible for ethanol production and may be implicated in the lack of anaerobic fermentation by fully-aerobic cells (Alexander *et al.*, 1988).

With some xylose-metabolizing yeasts, their inability to produce ethanol anaerobically has been accounted for by an imbalance between NADH production and NADH consumption. The imbalance arises because xylose is first reduced to xylitol by an NADPH-linked aldehyde reductase (= xylose reductase or XOR, EC 1.1.1.21), and the resulting xylitol is converted to D-xylulose by an NAD^+ - linked xylitol dehydrogenase (Fig. 10). After phosphorylation, xylulose-5-phosphate is rearranged by non-oxidative reactions to yield hexose phosphate and triose phosphate. A portion of the hexose phosphate can be oxidized via glucose-6-phosphate dehydrogenase to yield NADPH for assimilation. Otherwise, metabolism continues through the glycolytic pathway to yield ethanol in a balanced fermentation (Fig. 10). Yeasts known to convert xylose to ethanol under anoxic conditions (i.e., ferment) also possess an XOR that is active with NADH as well as NADPH (Alexander *et al.*, 1988).

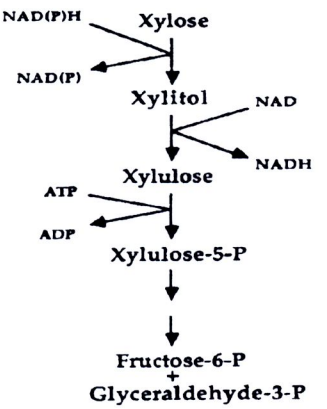


Figure 10. Initial steps in the metabolism of D-Xylose by yeasts.
Source: Alexander *et al.* (1988)

The most adequate pH for the growth of *C. shehatae* was between 3.5 and 4.5 (Du Preez *et al.*, 1984). Whereas Sreenath and Jeffries (2000) reported that the ethanol production rate from wood hydrolysate by *C. shehatae* Y-049 was optimum in the pH range of 5.5-6.0 giving ethanol yield of 0.41-0.46 g/g.

2.3 *Pachysolen tannophilus*

Pachysolen tannophilus can ferment both hexose and pentose sugars to ethanol. The optimal temperature for growth of this strain was at 30-32°C (Rorbuck *et al.*, 1995) while the optimal temperature for producing ethanol under anaerobic

condition was at 37°C (Converti *et al.*, 2001). The highest values for ethanol yield (0.39 g ethanol/g substrate) and the specific ethanol production rate (0.06 kg/kg.h) were obtained from fermentation at 30°C, pH 4.5 for 50 h (Sánchez *et al.*, 2004).

2.4 *Pichia stipitis*

Among the wild-type xylose-fermenting yeast strains for ethanol production, *Pichia stipitis* reportedly provides one of the best overall performances in terms of complete sugar utilization, minimal by-product formation, low sensitivity to temperature, and substrate concentration (Taherzadeh *et al.*, 2003). Furthermore, *P. stipitis* has no absolute vitamin requirement for xylose fermentation and is able to ferment a wide variety of sugars to ethanol (Taherzadeh *et al.*, 2003; Synowiecki and AL-Khateeb, 1997; Karimi *et al.*, 2006a). Ethanol yield between 0.24 and 0.47 g/g has been obtained by *P. stipitis* on different hydrolyzates (Taherzadeh *et al.*, 2003; Synowiecki and AL-Khateeb, 1997). However, *P. stipitis* has some limitations, among which the requirement of oxygen to maintain cell viability, xylose transport and ethanol productivity can be mentioned. While the yeast rapidly loses viability without sufficient oxygen, excess oxygen completely stops ethanol production and the cells respire the substrate to form biomass (Taherzadeh *et al.*, 2003). *P. stipitis* had a better performance in ethanol production at identical conditions with ethanol yield 0.38 g/g of the sugars within the hydrolyzate.

(3) Filamentous fungi

Although filamentous fungi such as *Rhizopus* sp. and *Mucor (please check) indicus* have been industrially used for a long time for several purposes, a number of process engineering problems are associated with these organisms due to their filamentous growth. Problems can appear in mixing, mass transfer, and heat transfer. Furthermore, attachment and growth on bioreactor walls, agitators, probes, and baffles cause heterogeneity within the bioreactor and problems in measurement of controlling parameters and cleaning of the bioreactor. Such potential problems might hinder industrial application for ethanol production from lignocellulose hydrolyzate (Karimi *et al.*, 2008). However, filamentous fungi have been used for ethanol production from several lignocellulosic hydrolysates because of their high tolerant to toxic compounds in hydrolysates (Karimi *et al.*, 2008).

7.4.5 Factors affecting on bio-ethanol production from lignocellulosic materials

7.4.5.1 Type of microorganisms

Most of microorganisms used in bio-ethanol production by fermentation are yeast and bacteria, which can convert rapidly mono-sugars to ethanol. For example, *Pachysolen tannophilus* (yeast) is able to convert glucose and xylose to ethanol (Bravo *et al.*, 1995), *Zymomonas mobilis* (bacteria) can use glucose, fructose or sucrose and convert to ethanol (Ahring *et al.*, 1996), *Pichia stipitis* is a specie of yeast that can convert xylose to ethanol, and *Candida shehatae* NCL-3501 is a good specie of yeast because it can convert both glucose and xylose rapidly to ethanol in the fermentation (Abbi *et al.*, 1996; Lee, 1997; Nigam, 2002). Recently, genetic engineering technique was used to improve some strains of yeast and bacteria *Zymomonas mobilis* 8b, that can use both glucose and xylose as substrates for ethanol production (Mohagheghi *et al.*, 2006). Ethanol productions of above microorganisms are performed in Table 6.

Table 6. Ethanol production from glucose and/or xylose by microorganisms.

Microorganism	Carbon source	Ethanol yield (g/g)	Ethanol productivity (g/l/h)	References
S. cerevisiae	Glu. and Xyl.	0.45	-	Laplace *et al.*, 1991
C. shehatae	Xyl.	0.48	0.19	Jeffries and Jin, 2000
	Glu. and Xyl.	0.37-0.47	-	Abbi, *et al.*, 1996
	Glu. and Xyl.	0.39	-	Laplace *et al.*, 1991
P. stipitis	Xyl.	0.45	0.34	Jeffries and Jin, 2000
	Xyl.	0.35-0.41	-	Nigam, 2002
	Glu. And Xyl.	0.44	-	Laplace *et al.*, 1991
P. tannophilus	Xyl.	0.24	0.13	Jeffries and Jin, 2000
	Glu. and Xyl.	0.34-0.39	-	Bravo *et al.*, 1995; Sanchez *et al.*, 2004
Z. mobilis	Glu. and Xyl.	0.42	-	Mohagheghi, *et al.*, 2006
	Glu. and Xyl.	0.43	-	Laplace *et al.*, 1991
	Sucr.	0.40	3.82	Lee and Huang, 1995

Remark: Xyl. = Xylose, Glu. = Glucose, Sucr. = Sucrose

7.4.5.2 Inhibitors

With acid hydrolysate and autohydrolysate, the maximum ethanol yields based on sugar consumption were 0.37 and 0.47 g/g with free cells, respectively (Abbi *et al.*, 1996). The lower ethanol production in acid hydrolysate compared to autohydrolysate may be due to the presence of inhibitory compounds such as furfural and phenolics, which are almost absent in autohydrolysates (Abbi *et al.*, 1996).

Acetic acid is one of the most prevalent. At the pH optimum for fermentation (5.5-6.0), acetic acid is largely undissociated. This permits diffusion into the cell cytoplasm, where it dissociates and decreases the intracellular pH. As a result, the proton gradient across the membrane cannot be maintained and the transport of various nutrients is impaired (Sreenath and Jeffries, 2000). Hence, in the presence of acetate, yeast fermentation of wood hydrolyzates is poor.

Several detoxification methods like neutralisation, overliming with calcium hydroxide, activated charcoal, ion exchange resins (Carvalheiro *et al.*, 2005) and enzymatic detoxification using laccase (Jönsson *et al.*, 1998) are known for removing various inhibitory compounds from lignocellulosic hydrolysates as described in Table 2.

Sugarcane bagasse hydrolysis with 2.5% (v/v) HCl yielded 30.29 g/l total reducing sugars along with various fermentation inhibitors such as furans, phenolics and acetic acid. The acid hydrolysate when treated with anion exchange resin brought about maximum reduction in furans (63.4%) and phenolics compounds (75.8%). Treatment of hydrolysate with activated charcoal caused 38.7% and 57.5% reduction in furans and total phenolics, respectively. Laccase reduced the most phenolics compounds (77.5%). Fermentation of these hydrolysates with *Candida shehatae* NCIM 3501 showed maximum ethanol yield (0.48 g/g) from ion exchange treated hydrolysate, followed by activated charcoal (0.42 g/g), laccase (0.37 g/g), overliming (0.30 g/g) and neutralized hydrolysate (0.22 g/g) (Chandel *et al.*, 2007).

7.4.5.3 Effect of pH

As far as the pH of the culture medium is concerned, it should be borne in mind that this variable affects cell growth and its influence may vary considerably

among yeast strains. The cell membranes are not completely permeable to hydrogen ions and so the intracellular pH and that of the culture medium may not be the same (Sánchez *et al.*, 1997). Apart from affecting cell membrane permeability, pH may also determine the solubility of some components of the medium: thus, a modification in the pH might also cause some micronutrient to precipitate and so become impossible to be assimilated. The ethanolic producing yeast and bacteria have the difference of pH for growth and fermentation. For example, the optimal pH for growth by *P. tannophilus* was 3.7 (Roebuck *et al.*, 1995), while the optimal pH from another researcher was 5.2 (Xu and Taylor, 1993). The optimal pH of *Zymomonas mobilis* was 4.93 (Bandaru *et al.*, 2006). The ethanol production from wood hydrolysate by *C. shehatae* Y-049 was optimum in the pH range of 5.5-6.0 giving ethanol yield of 0.41-0.46 g/g (Sreenath and Jeffries, 2000). Du Preez *et al.* (1986) report that the most adequate pH for the growth of *C. shehatae* was 3.5-4.5. Moreover, Sánchez *et al.* (1997) found that the best initial pH for ethanol production from xylose by *C. shehatae* in batch fermentation was 4.5. Under these conditions, the maximum specific growth rate (μ_{max}) was 0.329 h^{-1} and the specific ethanol production rate (qE) was 0.72 g/g/h and ethanol yield was 0.41 g/g.

7.4.5.4 Effect of oxygen

The dissolved oxygen tension (DOT) is particularly critical in attaining maximal ethanol production with xylose-fermenting yeasts. *P. stipitis* and *C. shehatae* require aeration for maximal ethanol production. Under anoxic conditions, the specific ethanol productivity of *P. stipitis* and *C. shehatae* decreased (Table 7), and especially in the case of *C. shehatae*, xylitol production increased (Jeffries and Jin, 2000).

Sánchez *et al.*, 1997 studied the effect of air supply on the production of ethanol from xylose using the yeast *C. shehatae* in a batch reactor. The aeration via the stirring vortex of the bioreactor was sufficient. Under these conditions, the maximum specific growth rate was 0.329 h^{-1}; overall biomass yield was 0.036 g/g; the specific uptake rate of xylose was 2.0 g/g/h; and the specific ethanol production rate was 0.72 g/g/h. The overall ethanol yield was 0.41 g/g.

In addition, rotary of the shaker should be effective enough to provide gentle mixing and surface aeration during the first period of the growth phase (Phisalaphong *et al.*, 2006). The oxygen requirement for ethanol production was considered, but it is apparent that oxygen plays various roles in the metabolism of xylose by eukaryotes. It is very important for a xylose-fermenting yeasts to possess an aldose reductase that is active with both NADH and NADPH in order to maintain redox balances during xylose assimilation (Verduyn *et al.*, 1985).

In the absence of aeration, ethanol accumulation is still continues, but at a much lower rate, and xylitol production increased (Jeffries and Jin, 2000). *C. shehatae* requires oxygen to maintain viability. Oxygen starvation induces cell death in *C. shehatae* when it is grown on xylose, but not when it is grown on glucose. Growth of *C. shehatae* was limited to one division or less when cells cultivated aerobically on either glucose or xylose are shifted from aerobic to anaerobic conditions. The cultivation of *P. stipitis* on glucose increases the activity of plasma membrane ATPase 3-folds in comparison to the activity obtained when cells are grown on xylose. These results indicated that plasma membrane ATPase activity, which is critical for transport, correlates with ethanol tolerance and the inhibitory effect of ethanol on growth. Plasma membrane ATPase is essential for maintaining the proton gradient that is responsible for uptake of nutrients. These yeasts require active electron transport for the synthesis of uracil, and hence can not make mRNA under anaerobic conditions (Jeffries and Jin, 2000).

Table 7. Performance of xylose-fermenting yeasts on aeration.

Strains	Substrate (g/l)	Condition	Ethanol yield (g/g)	Productivity (g/l/h)	Biomass yield (g/g)
P. stipitis CBS7126	Xylose (40)	Aerobic	0.18	0.17	0.39
		Facultative	0.47	0.20	0.05
		Anaerobic	0.40	0.02	0.03
	Glucose (40)	Aerobic	0.26	0.17	0.23
		Facultative	0.38	0.28	0.14
		Anaerobic	0.33	0.13	0.10
C. shehatae CBS2779	Xylose (40)	Aerobic	0.22	0.21	0.33
		Facultative	0.37	0.32	0.01
		Anaerobic	0.41	0.15	0.01
	Glucose (40)	Aerobic	0.33	0.35	0.21
		Facultative	0.42	0.51	0.03
		Anaerobic	0.44	0.29	0.02
P. tannophilus NRRL Y-2460	Xylose (40)	Aerobic	0.10	0.04	0.25
		Facultative	0.28	0.10	0.01
		Anaerobic	0.26	0.07	0.01
	Glucose (40)	Aerobic	0.31	0.38	0.14
		Facultative	0.43	0.49	0.06
		Anaerobic	0.42	0.18	0.04

Source: Jeffries and Jin (2000)

7.4.5.5 Effect of immobilized cell

Cell immobilization support for ethanol production has been classified into two types based on source: (i) synthetic supports such as gelatin, carrageen (Yu *et al.*, 2007), Ca-alginate (Behera *et al.* 2010), agar-agar (Behera *et al.* 2010), polyurethane (Fujii *et al.*, 1999) and ceramic beads or porous glass (Kourkoutas *et al.*, 2006), and (ii) natural supports such as chitosan (Fujii *et al.*, 1999), sawdust, wood chip, rice husk, rice straw, spent grain, delignified spent grain (Kopsahelis *et al.*, 2007), apple piece (Kourkoutas *et al.*, 2006), sorghum bagasse (Yu *et al.*, 2007) and watermelon pieces (Reddy *et al.*, 2008). The benefits of natural supports are wide spread in the nature, low cost, and ease to operate in bioprocess fermentation i.e. better operational stability, less contamination, protect cell from shear force, ease to separate cell in downstream process, less effect by inhibitory compounds and remain

cell viability for several cycles of operations (Chandel *et al.*, 2007; Reddy *et al.*, 2008; Behera *et al.*, 2010).

The immobilized yeast cells, *Debaromyces hansenii*, in Ca-alginate matrix produced ethanol with a yield of 0.46 g/g from hemicellulosic hydrolysates and were reused six times with 100% fermentation efficiency (Menon *et al.*, 2010).

7.4.5.6 Effect of temperature

Temperatures that provide for optimum biomass and ethanol productivities do not necessarily enable maximum ethanol accumulation. This implies that ethanol toxicity affects production. In *P. stipitis*, xylitol increases with high temperature. Maximum ethanol selectivity was achieved at 25-26°C (Jeffries and Jin, 2000). Numerous studies have shown that temperatures above 37°C are detrimental for ethanol production (Cazetta *et al.*, 2007). The deleterious effects of high temperature were considered to be due to the denaturation of ribosomes and enzymes and problems associated with the fluidity of membranes (Phisalaphong *et al.*, 2006). *P. tannophilus* can ferment both hexose and pentose sugars to ethanol. The optimal temperature for growth of this strain was at 30-32°C (Rorbuck *et al.*, 1995) while the optimal temperature for producing ethanol under anaerobic condition was at 37°C (Converti *et al.*, 2001). The highest values for ethanol yield (0.39 g ethanol/g substrate) and the specific ethanol production rate (0.06 g/g/h) were obtained from fermentation at 30°C, pH 4.5 for 50 h (Sánchez *et al.*, 2004).

7.4.5.7 Effect of carbon source and concentration

The maximum ethanol production attained by *P. stipitis* and *C. shehatae* doesn't affect by xylose utilization (Jeffries and Jin, 2000). The cell metabolism was strongly affected by the substrate and product concentrations, which could be classified into two types: limitation and inhibition (Phisalaphong *et al.*, 2006). However, the ethanol concentration resulting in growth inhibition depended on the sugar consumption. In the case of xylose, growth inhibition occurred at 30 g ethanol/l, but with glucose, cells continued to grow up to 34 g ethanol/l by *P. tannophilus* (Jeffries and Jin, 2000). The higher ethanol tolerance observed with glucose as a carbon source correlated with higher plasma membrane H^+-ATPase activity. The ethanol tolerance of *P. tannophilus* changes with the carbon source used

for growth. When cultivated on xylose as a sole carbon source, this yeast produces only 20 g/l of ethanol. However, *P. tannophilus* produces up to 55 g/l when cultivated on glucose. *P. tannophilus* also produces ethanol much more rapidly on glucose than on xylose (Jeffries and Jin, 2000).

Substrate concentration also affected on the ethanol production. The optimal D-xylose concentration for ethanol production by *P. tannophilus* was 25 g/l, which gave a maximum specific growth rate of 0.26 h^{-1}, biomass productivity of 0.023 g/l h, specific ethanol production rate of 0.065 g/g h and ethanol yield of 0.34 g/g (Bravo *et al.*, 1995).

7.4.5.8 Effect of glucose to xylose ratio

In the fermentation on mixture of glucose and xylose, glucose is utilized first. After glucose is used up (12 h), xylose is fermented (Zhao *et al.*, 2008). Ethanol concentration and biomass increased quickly at the beginning of fermentation according to the fast glucose consumption then increased slowly after glucose was exhausted. The maximum ethanol production by *P. tannophilus* were 5.80, 4.80 and 3.85 g/l appeared at the 36 h in fermentation on mixed glucose and xylose at 3:1, 1:1 and 1:3, respectively (Zhao *et al.*, 2008).

7.4.5.9 Type of fermentation

Fed-batch culture is a batch culture. It is fed continuously or sequentially with substrate without the removal of fermentation broth. It is widely used for the production of microbial biomass, ethanol, organic acids, antibiotics, vitamins, enzymes and other compounds. Fed-batch culture compared to the conventional batch culture has several advantages including very low concentration of residual sugars, higher dissolved oxygen in the medium, decreased fermentation time, higher productivity and reduced toxic effects of the medium components which are present at high concentrations (Roukas, 1996) as well as eliminating substrate inhibition (Ozmichi and Kargi, 2007).

When a portion of the fermentation broth is withdrawn at intervals and the residual part of the culture is used as an inoculum for the next fed-batch culture, the system is operated as a repeated fed-batch culture or semi-continuous culture. In addition to increased productivity, semi-continuous culture has the advantages which

are (i) it does not require new inocula for each consecutive fed-batch and (ii) the contamination of the medium is also lower than in the continuous culture. Thus semi-continuous culture is considered one of the most useful systems for economical ethanol production (Roukas, 1996).

OBJECTIVES

1. To extract holocellulose from palm pressed fiber (PPF) by alkaline extraction.
2. To produce hemicellulose from the extracted holocellulose by chemical process.
3. To optimize furfural production from hemicellulose using two-stage process.
4. To produce monomeric sugars from holocellulose and/or PPF and optimize the condition by chemical process.
5. To produce bio-ethanol from monomeric sugars by various microorganisms
6. To optimize bio-ethanol production from the selected source and microorganism.
7. To scale-up the ethanol production process by the selected strain.