CHAPTER II
LITERATURE REVIEW

2.1 Lignocellulosic Biomass Materials

Lignocellulosic biomass comprising forestry, agricultural, and agro-industrial wastes are abundant, renewable, and inexpensive energy sources. Such wastes include a variety of materials such as sawdust, poplar trees, sugarcane bagasse, waste paper, brewer's spent grains, switchgrass, and straws, stems, stalks, leaves, husks, shells and peels from cereals like rice, wheat, corn, sorghum and barley, among others. Lignocellulose wastes are accumulated every year in large quantities, causing environmental problems. However, due to their chemical composition based on sugars and other compounds of interest, they could be utilized for the production of a number of value added products, such as ethanol, food additives, organic acids, enzymes, and others. Therefore, besides the environmental problems caused by their accumulation, the non-use of these materials constitutes a loss of potentially valuable sources (Mussatto and Teixeira, 2010). Figure 2.1 shows the way to produce bioethanol and these following aspects are considered as benefits of using lignocellulosic biomass: (Saxena *et al.*, 2009)

a) It is a renewable, potentially sustainable, and relatively environmentally friendly source of energy.
b) Increased use of biomass would extend the lifetime of diminishing crude oil supplies.
c) Lignocellulosic biomass fuels have negligible sulfur content and, therefore, do not contribute to sulfur dioxide emissions that cause acid rain.
d) The combustion of lignocellulosic biomass produces less ash than coal combustion and the ash produced can be used as a soil additive on farms, etc.
e) Lignocellulosic biomass is a domestic resource, which is not subject to world price fluctuations or the supply uncertainties as of imported fuels.

f) Lignocellulosic biomass provides a clean, renewable energy source that could improve our environment, economy, and energy securities.

g) Lignocellulosic biomass usage could be a way to prevent more carbon dioxide production in the atmosphere as it does not increase the atmospheric carbon dioxide level.

h) It has been reported that cellulosic ethanol and ethanol produced from other biomass resources have the potential to cut greenhouse gas emission by 86% (Kumar *et al.*, 2009).

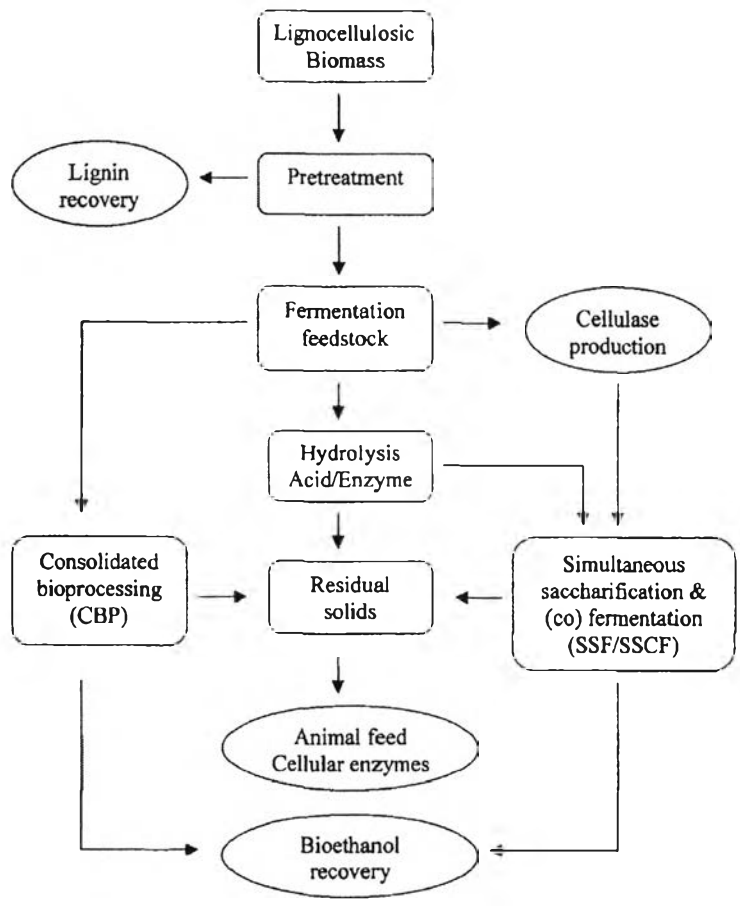

Figure 2.1 Integrated biomass processing scheme (Ward and Singh, 2002).

2.2 Chemical Structure

The chemical components of lignocellulosic feedstock can be divided into four major components. They are cellulose, hemicelluloses, lignin, and extractives. In general, the first three components have high molecular weights and contribute much mass, while the latter component is of small molecular size, and it is available in little quantity. Based on weight percentage, cellulose and hemicelluloses are higher in hardwoods compared to softwoods and wheat straw, while softwoods have higher lignin content (Ibrahim, 1998). Table 2.1 shows the composition of some lignocellulosic biomass. Basically, cellulose forms a skeleton, which is surrounded by hemicellulose and lignin (Figure 2.2).

Table 2.1 Chemical composition of various lignocellulosic biomass materials (Aita and Salvi, 2010)

Biomass	Cellulose	Hemicellulose	Lignin	Other*
	percent dry biomass			
Corn stover	36.4	21.4	17.2	25.0
Wheat straw	38.2	24.7	23.2	13.9
Rice straw	34.2	24.5	23.0	18.3
Miscanthus	31.0	24.4	17.6	27.0
Poplar sawdust	49.9	20.4	18.1	11.6
Sugarcane bagasse	40.2	21.5	24.2	14.1
Sorghum	44.5	27.7	22.0	5.8

*resin, fats, waxes, fatty acids and alcohols, terpentines, tannins and flavonoids

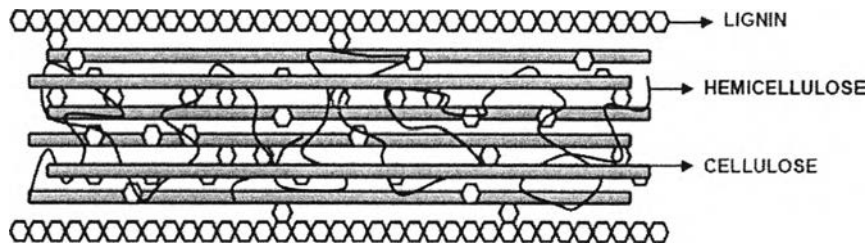

Figure 2.2 Representation of lignocellulosic structure showing cellulose, hemicellulose, and lignin fractions (Mussatto and Teixeira, 2010).

2.2.1 Cellulose

Cellulose is the main structural constituent in plant cell walls and is found in an organized fibrous structure. The cellulose content of wood varies between species in the range of 40-50 %. Cellulose in biomass is present in both crystalline and amorphous forms. Crystalline cellulose comprises the major proportion of cellulose, whereas a small percentage of unorganized cellulose chains form amorphous cellulose. It is more susceptible to enzymatic degradation in its amorphous form. The structure of cellulose is a linear polymer chain linked together by β-(1,4)-glycosidic bonds with the formula $(C_6H_{10}O_5)_n$. Due to this linkage, cellobiose is found as the repeat unit for cellulose chains (Figure 2.3). The degree of polymerization (DP) (Equation 2.1) of native cellulose is in the range of 7,000-15,000 (Ibrahim, 1998; Kumar *et al.*, 2009).

$$DP = \frac{\text{Molecular weight of cellulose}}{\text{Molecular weight of one glucose unit}} \qquad (2.1)$$

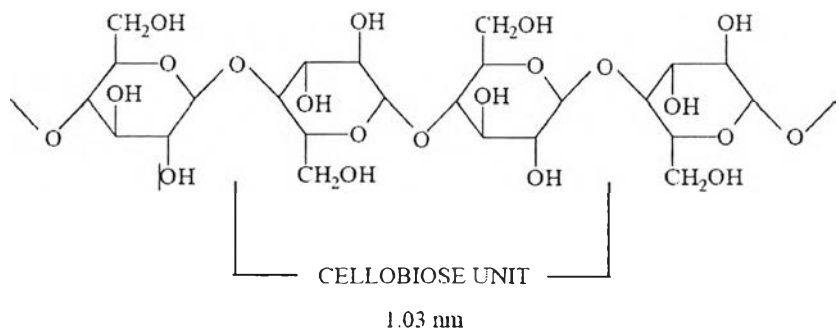

Figure 2.3 Schematic illustration of the cellulose chain (Balat *et al.*, 2009).

Glucose, also known as D-glucose, dextrose, or grape sugar, a simple sugar, can be used as the starting raw material in the production of a wide variety of chemicals and fuels. This is usually carried out with the help of microorganisms. For example, glucose can be easily fermented to ethanol. Another example is the conversion of glucose into solvents, such as acetone and butanol by *Clostridium acetobutylicum*. Because the volume of cellulose is so overwhelming, and the resource is renewable, the world will be likely to depend on it more heavily for food, fuel, chemical supplies, and raw materials in the future. It has the great potential of relieving the need for petroleum, whose supply is fast dwindling (Wang, 2009).

2.2.2 Hemicellulose and Starch

Unlike cellulose, hemicelluloses consist of different monosacharide units. In addition, the polymer chains of hemicelluloses have short branches and are amorphous. Because of the amorphous morphology, hemicelluloses are partially soluble in water. The backbone of the chains of hemicelluloses can be a homopolymer (generally consisting of single sugar repeat unit) or a heteropolymer (mixture of different sugars) with short branches linked by β-(1,4)-glycosidic bonds and occasionally β-(1,3)-glycosidic bonds. These monosaccharides include pentoses (xylose, rhamnose, and arabinose), hexoses (glucose, mannose, and galactose), and uronic acids (e.g., 4-*o*methylglucuronic, D-glucuronic, and D-galactouronic acids). Sugar components are listed in Figure 2.4. Among the most important sugar of the hemicelluloses component is xylose. In contrast to cellulose, the polymers present in hemicelluloses are easily hydrolyzable (Ibrahim, 1998; Kumar *et al.*, 2009). The main differences between cellulose and hemicellulose are shown in Table 2.2.

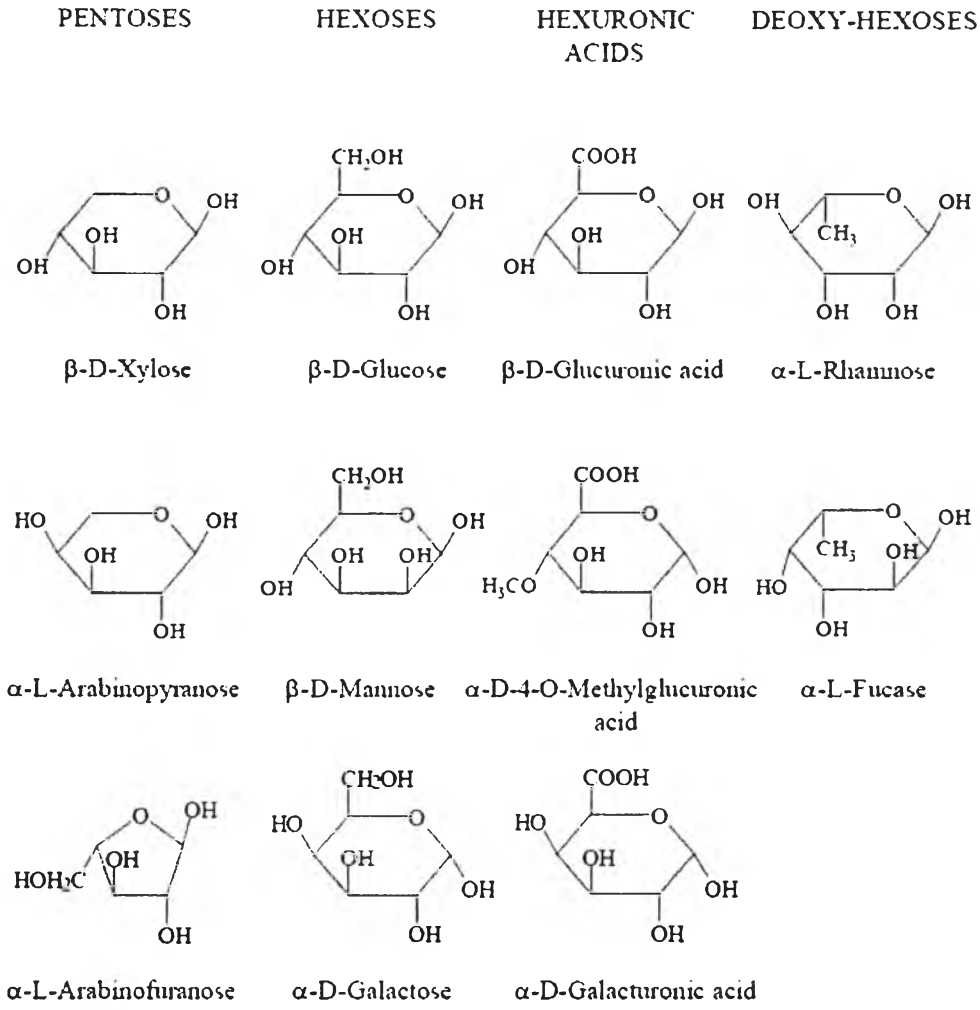

Figure 2.4 Schematic illustrations of sugar units of hemicelluloses (Balat *et al.*, 2009).

Table 2.2 Main differences between cellulose and hemicelluloses (http://www.abq.org.br)

Cellulose	Hemicelluloses
Homopolysaccharide composed of glucose units	Heteropolysaccharides composed of several units of pentoses and hexoses
High degree of polymerization (2,000 to 18,000)	Low degree of polymerization (50 to 300)
Produce fibrous arrangements	Do not produce fibrous arrangements
Presents crystalline and amorphous regions	Present only amorphous regions
Slowly hydrolyzed by diluted inorganic acid in high temperatures	Rapidly attacked by diluted inorganic acid in high temperatures
Is alkaline insoluble	Are alkaline soluble

Among the carbohydrates components, starch is the only structure that has linear and branched chains. The linear chain is known as amylose (Figure 2.5A). Their anhydroglucose units are linked by a-(1,4)-glycosidic bonds. In the case of branched chains, which is known as amylopectin (Figure 2.5B), the backbone is like amylose but it also has a-(1,6)-glycosidic bonds at the branch position (Ibrahim, 1998).

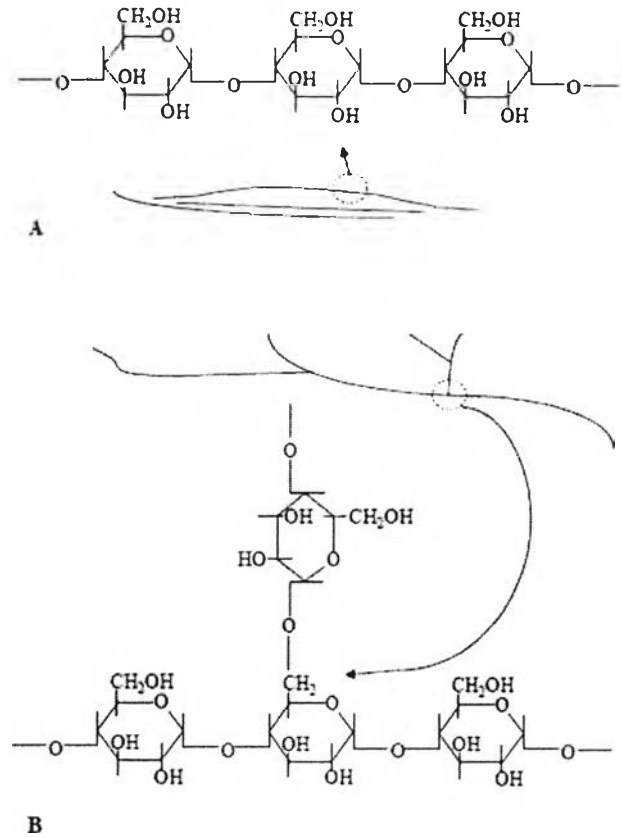

Figure 2.5 Schematic illustration of starch (A - Amylose chain, B - Amylopectin chain) (Ibrahim, 1998).

2.2.3 Lignin

Lignin is a complex, large molecular structure containing cross-linked polymers of phenolic monomers. Lignin gives mechanical strength to wood by gluing the fibers. It is present in the primary cell wall, imparting structural support, impermeability, and resistance against microbial attack (Ibrahim, 1998; Kumar *et al.*, 2009).

2.2.4 Extractives

Extractives are the organic substances which have low molecular weight and are soluble in neutral solvents. Resin, fats, waxes, fatty acids and alcohols, terpentines, tannins, and flavonoids are categorized as extractives. They only represent between 4-10 % of the total weight of dry wood, and the contents of extractives vary among wood species, geographical site, and season. Some extractives are toxic, and this is an advantage for the wood to resist attack by fungi and termites (Ibrahim, 1998).

2.3 Pretreatment

Many physicochemical structural and compositional factors hinder the hydrolysis of cellulose present in biomass to sugars and other organic compounds that can later be converted to fuels. For example, the presence of lignin in lignocelluloses leads to a protective barrier that prevents plant cell destruction by fungi and bacteria. For the conversion of biomass to fuel, the cellulose and hemicellulose must be broken down into their corresponding monomers (sugars), so that microorganisms can utilize them (Kumar *et al.*, 2009).

The goal of pretreatment is to make the cellulose accessible to hydrolysis for conversion to fuels such as removing lignin and hemicellulose, reducing the crystallinity of cellulose, and increase the porosity of lignocellulosic materials. Various pretreatment techniques, as shown in Table 2.3, change the physical and chemical structure of the lignocellulosic biomass and improve hydrolysis rates (Kumar *et al.*, 2009). Pretreatment can be the most expensive process in biomass to fuels conversion but it has great potential for improvements in efficiency and lowering of costs through further research and development (Chang *et al.*, 2011).

Table 2.3 Summary of various processes used for the pretreatment of lignocellulosic biomass (Kumar *et al.*, 2009)

Pretreatment Process	Advantages	Limitations and disadvantages
Mechanical comminution	Reduces cellulose crystallinity	Power consumption usually higher than inherent biomass energy
Steam explosion	Causes hemicellulose degradation and lignin transformation; cost-effective	Destraction of a portion of the xylan fraction; incomplete disruption of the lignin-carbohydrate matrix; generation of compounds inhibitory to microorganisms
AFEX	Increase accessible surface area, removes lignin and hemicellulose to an extent; does not produce inhibitors for downstream processes	Not efficient for biomass with high lignin content
CO_2 explosion	Increase accessible surface area; cost-effective; does not cause formation of inhibitory compounds	Does not modify lignin or hemicelluloses
Ozonolysis	Reduces lignin content; does not produce toxic residues	Large amount of azone required; expensive
Acid hydrolysis	Hydrolyzes hemicellulose to xylose and other sugars; alters lignin structure	High cost; equipment corossion; formation of toxic substances
Alkaline hydrolysis	Removes hemicelluloses and lignin; increases accessible surface area	Long residence times required; irreversible salts formed and incorporated into biomass
Organosolv	Hydrolyzes lignin and hemicelluloses	Solvents need to be drained from the reactor, evaporated, condensed, and recycled; high cost
Pyrolysis	Produces gas and liquid products	High temperature; ash production
biological	Degrades lignin and hemicelluloses; low energy requirements	Rate of hydrolysis is very low

2.3.1 Steam Explosion

Steam explosion is also a method commonly used for hemicellulose hydrolysis. In this method, the biomass is heated using high-pressure saturated steam (0.69-4.83 MPa, 160-260 °C) for a short period (from seconds to few minutes). Steam condenses under high pressure, thereby wetting the material, and then the pressure is suddenly reduced, which makes the material undergo an explosive decompression. Limitations of steam explosion include an incomplete disruption of the lignin–carbohydrate matrix, and generation of compounds that may be inhibitory to microorganisms (Mussatto and Teixeira, 2010).

Sendelius (2005) compared and optimized various steam pretreatment conditions with respect to final ethanol yield, using sugarcane bagasse as feedstock. Steam pretreated sugarcane bagasse was enzymatically hydrolysed by a blend of Celluclast 1.5L and *Novozym* 188 cellulase mixtures. This was done to evaluate different pretreatment parameters (time, temperature and impregnating agent) with the intention of finding conditions, which generated high sugar yields. The pretreatment conditions assessed were: temperature: 180, 190 and 205 °C; time: 5 and 10 min; and impregnating agents: water, 2% SO_2 by weight of water in the bagasse and finally 0.25 g H_2SO_4 per 100 g dry matter. Hydrolysis was carried out for 72 h and temperature was kept at 40 °C, pH was initially 4.8. The most clearly tested pretreatment condition was: SO_2-impregnation with a temperature of 180 °C during 5 min, which gave an overall ethanol yield of 80%, based on theoretical value.

Zimbardi *et al.* (2007) investigated the synergistic effect of pre-impregnation by sulphuric acid and steam explosion. Sugar recovery by water extraction and cellulose digestibility by enzymes was considered. About 0.5 kg of corn stover was used in each experiment. The batch reactor was a 10 L chamber, in which about 1.5 kg of impregnated biomass was manually loaded. Typically, about 0.5 min has been required to attain and stabilize the set temperature. After 5 min of exposure to the saturated steam, the stover was exploded in a closed receiving chamber having a volume of 150 L, where the material remained 10–30 min. Nine conditions was tested for the

steam explosion treatment selecting the temperature of 180, 190, 200 °C and sulfuric acid loadings of 0, 1.5, 3 wt%. The 48 h hydrolysis yield of cellulose was higher than 90% when the stover was impregnated with 3 wt% acid. The glucose yield (by summing up the water extraction and enzymatic hydrolysis) reached the best value of 85% with 3 wt% acid loading and SE at 190 °C for 5 min.

Ruiz *et al.* (2008) studied effects of temperature on steam explosion pretreatment of sunflower stalks in order to optimize pretreatment temperature in the range 180–230 °C. Pretreatment of the raw material was carried out in a batch pilot unit based on Masonite technology and equipped with a 2 L reaction vessel designed to reach a maximum operating pressure of 4.12 MPa. The reactor was charged with 150 g (dry matter) of feedstock per batch. Saturated steam from the boiler was then allowed to enter the reactor and heat the raw material to the desired temperature (180, 190, 200, 210, 220 or 230 °C). The selected temperature was maintained for 5 min and then the reactor was suddenly depressurized. Enzymatic hydrolysis was performed in 0.05 M sodium citrate buffer (pH 4.8) at 50 °C on a rotary shaker (Certomat-R, B-Braun, Germany) at 150 rpm for 96 h and at 10% (w/v) pretreated material concentration. Samples were taken every 24 h for glucose concentration determination. From the results obtained in this work, it can be concluded that steam explosion pretreatment at 220 °C improves enzymatic hydrolysis yielded by four times compared to that from the unpretreated material (from 18 to 72%) and rendered a relatively concentrated glucose broth suitable for fermentation.

Soares *et al.* (2011) investigated the production of glucose and xylose from enzymatic hydrolysis of steam pretreated sugarcane bagasse. Different reaction conditions were studied to determine effects of the experimental conditions on the yield of the enzymatic reaction. The reaction was performed either without washing or with distilled water and 1% aqueous NaOH solution wash at room temperature (30 °C) with or without milling (i.e., 20 mesh or 6 mesh). A blend of cellulases, commercially named Celluclast 1.5L (endoglucanases and exoglucanases) and *Novozym* 188 (glucosidases), was used to perform the enzymatic hydrolysis of sugarcane bagasse. As expected, the glucose content increased with time. The xylose content in the reaction medium also increased with time, indicating that the enzymes promote the production of this sugar

from hemicellulose. Larger amounts of glucose, xylose, and cellobiose were obtained for larger amounts of enzymes. It was also found that the milling of sugarcane bagasse did not significantly influence the production of glucose by enzymatic hydrolysis. And from the results, they concluded that the highest amount of glucose can be obtained when unmilled sugarcane bagasse, which was washed with a 1% aqueous NaOH solution, was used as hydrolyzate for enzymatic hydrolysis step.

2.3.2 Freeze Pretreatment

The advantages of a freeze pretreatment include a significantly lower environmental impact and less hazardous processed chemicals.

Chang *et al.* (2011) studied the effect of freeze pretreatment on enzymatic conversion of rice straw. The residue was pretreated at 20 °C for 2 h. Subsequently, the frozen rice straw was thawed at room temperature for 1 h. Then cellulase from *Aspergillus niger* (Sigma Chemical Co.) and xylanase from *Trichoderma viride* (Sigma Chemical Co.) were used for hydrolysis of untreated and pretreated rice straw. The result indicated that the freeze pretreatment was found to significantly increase the enzyme digestibility of rice straw from 48% to 84%. In addition, hydrolyzate analysis showed that the highest glucose yield obtained during the enzymatic hydrolysis step in the present study was 371.91 g/kg of dry rice straw, following the pretreatment. This can be concluded that freeze pretreatment was highly effective for enzymatic hydrolysis and low environmental impact.

2.4 Hydrolysis

Three major hydrolysis processes are typically used to produce a variety of sugars suitable for ethanol production: dilute acid, concentrated acid, and enzymatic hydrolysis. Hemicellulose can be readily hydrolyzed by dilute acids under moderate conditions, but much more extreme conditions are needed for cellulose hydrolysis. The presence of lignin and hemicellulose makes the accessibility of cellulase enzymes and acids to cellulose more difficult, thus reducing the efficiency of the hydrolysis process (Kumar *et al.*, 2009).

2.4.1 Dilute Acid Hydrolysis

In the dilute-acid process, the reaction is carried out at a high temperature and pressure, and because of low yields of glucose from cellulose in the hydrolysis step, the ethanol yield is low. Typically, this process uses high temperatures (160-230 °C), pressures (~10 atm) and the acid concentration is in the range of 2-5%. The acid hydrolysis process employs sulfuric and hydrochloric acid. This is a good alternative to selectively remove the hemicellulose fraction generating a solid residue basically composed by cellulose and lignin (cellulignin). However, it is a harsh process that leads to the formation of toxic degradation products, which can interfere with fermentation (Mussatto *et al.*, 2007; Kumar *et al.*, 2009; Lenihan *et al.*, 2010).

Lenihan *et al.* (2010) investigated the optimum conditions for acid hydrolysis of hemicellulosic biomass in the form potato peel; the chemical composition is shown in Table 2.4. The hydrolysis reaction was taken in a batch reactor using dilute phosphoric acid. Process parameters investigated included reactor temperature (from 135 °C to 200 °C) and acid concentration (from 2.5% (w/w) to 10% (w/w)). The results indicated that the optimum yield was obtained at 135 °C and 10% (w/w) acid concentration. 55.2 g sugar/100 g dry potato peel was produced after 8 min. Another result showed that temperature has a stronger relationship with the net rate of sugar production compared to the acid concentration. Therefore, it can be concluded that increasing the acid concentration is a more effective means of maximizing sugar yields than increasing the operating temperature. This work showed that the use of potato peel may be a feasible option as a feed material for the production of sugars for biofuel synthesis, due to its low cost and high sugar yields.

Table 2.4 Chemical composition of potato peel (Lenihan *et al.*, 2010)

Composition	Proportion
Cellulose	55.25%
Hemicellulose	11.71%
Lignin	14.24%
Moisture	10.00%
Ash	8.80%

2.4.2 Concentrated Acid Hydrolysis

The use of concentrated acid in the hydrolysis process can yield higher quantities of ethanol because of the approximately 100% conversion to glucose from cellulose. This process uses lower operating temperature (<50 °C) and longer retention times compared to the dilute-acid process, atmospheric pressures and 10-30% acid concentration (Kumar *et al.*, 2009).

In the United States, several related processes using H_2SO_4 have been developed, typically with 80–90% conversion of cellulose and hemicellulose into sugars. Nevertheless, the hazards of handling concentrated acids and the complexities of recycling them have limited the adoption of this technology (Binder and Raines, 2010).

2.4.3 Enzymatic Hydrolysis

Enzymes produced by a variety of microorganisms are also capable of breaking down lignocellulosic materials to sugars but require longer retention times. Lignocellulosic materials can similarly be enzymatically hydrolyzed under relatively mild conditions (50 °C and pH ~5), enabling effective cellulose breakdown without the formation of by products that would otherwise inhibit enzyme activity. This is the most common method of producing ethanol from lignocellulosic biomasses (Kumar *et al.*, 2009). There are several advantages of using this method such as milder conditions of pressure, temperature and pH, high specificity, elimination of hydroxymethyl furfural, amongst other toxic substances (lignin derivatives), low energy consumption, low material costs with construction of equipments, differently of those processes which utilize acid hydrolysis. However, the cost of operating is still high

(http://www.abq.org.br). Table 2.5 shows some types of enzymatic complexes that can be used to hydrolyze lignocellulosic materials.

Table 2.5 Accepted and systematic names of enzymatic complexes capable of hydrolyzing lignocellulosic materials (Graminha *et al.*, 2008)

Cellulases
Cellulase EC 3.2.1.4 (1,4-(1,3;1,4)-β-D-glucan 4-glucano hydrolase)
Cellulose 1,4-β-cellobiosidase EC 3.2.1.91 (1,4-β-D-glucan cellobiohydrolase)
Glucan 1,4-β-glucosidase EC 3.2.1.74 (1,4-β-D-glucan glucohydrolase)
β-Glucosidase EC 3.2.1.21 (β-D-glucosidase glucohydrolase)
Hemicellulases
Endo-1,4-β-xylanase EC 3.2.1.8 (1,4-β-D-xylan xylanohydrolase)
Xylan-1,4-β-xylosidase EC 3.2.1.37 (1,4-β-D-xylan xylohydrolase)
α-N-Arabinofuranosidase EC 3.2.1.55 (α-L-arabinofuranosidase arabinofuranohydrolase)
Acetylesterase EC 3.1.1.6 (acetic-ester acetylhydrolase)
Ligninases
Laccase EC 1.10.3.2 (benzenediol:oxigen oxidoreductase)
Manganese peroxides EC 1.11.1.13 (Mn(II):hydrogen-peroxide oxidoreductase) oxidoreductase)
Lignin peroxidase EC 1.11.1.14 (1,2-bis(3,4-dimethoxy phenyl) propane-1,3-diol:hydrogen-peroxide oxidoreductase)
Pectinases
Pectinasterase EC 3.1.1.11 (pectin pectylhydrolase)
Pectin lyase
Pectate lyase EC 4.2.2.2 ((1→4)-α-D-galacturonan lyase)
Pectate disaccharidelyase EC 4.2.2.9 (1→4)-α-D-galacturonan reducing-end-disacchasride-lyase)
Polygalacturonase
Polyhalacturonase EC 3.2..1.15 (poly(1,4-α-D-galacturonide) glycanohydrolase)
Galacturan 1,4-α-galacturonidase EC 3.2.1.67 (poly(1,4-α-D-galacturonide) galacturonohydrolase)

For the basic mechanism, enzymatic hydrolysis of cellulose occurs by a complex system of reactions involving several steps. These steps are (1) transfer of enzymes from the bulk aqueous phase to the surface of the cellulose particles, (2) adsorption of the enzymes and formation of enzyme-substrate complexes (ES), (3) hydrolysis of cellulose, (4) transfer of the cellodextrins, glucose and cellobiose, from the surface of the cellulosic particles to the bulk aqueous phase, and (5) hydrolysis of cellodextrins and cellobiose into glucose in the aqueous phase. Adsorption of enzymes

and the formation of enzyme-substrate complexes are considered to be critical steps in the enzymatic hydrolysis of cellulose. These steps are influenced by the structural features of cellulose, the mode of interaction between the cellulases and the cellulose fiber, the nature of the cellulases employed and the enzymes' susceptibility to product inhibition (Walker and Wilson, 1991).

Rivers (1988) studied the effects of substrate composition, cellulose crystallinity, and particle size on the yields of enzymatic hydrolysis for two agricultural crop wastes, bagasse and rice straw. *Trichoderma reesei* QM 9414G was used to produce a full complement cellulase system consisting of endoglucanase, cellobiohydrolase, and cellobiase activities to hydrolyze cellulose. The absolute change in the crystallinity of rice straw following pretreatment did, however, appear to correlate with conversions to either glucose or ethanol. Substrate particle size was determined and found not to be a major factor in enzymatic hydrolysis within the range studied. However, modification of the lignocellulose matrix from its native state by caustic pretreatment resulted in the most significant increases in enzymatic hydrolysis reinforcing the concept that the nature of the native lignocellulose matrix is a major limiting factor in enzymatic hydrolysis of bagasse and rice straw. The data indicate that each individual lignocellulosic substrate requires a specific pretreatment in order to achieve maximum enzymatic hydrolysis.

Chen *et al.* (2007) investigated the enzymatic hydrolysis of corncob. The cellulosic residue was pretreated by 1% H_2SO_4 at 108 °C for 3 h and further hydrolyzed by cellulose from *Trichoderma reesei* ZU-02 and the hydrolysis yield was 67.5%. Poor cellobiase activity in *T. reesei* cellulase limited the conversion of cellobiose to glucose, and the accumulation of cellobiose caused severe feedback inhibition to the activities of β-1,4-endoglucanase and β-1,4-exoglucanase in cellulose system. Adding cellobiase from *Aspergillus niger* ZU-07 greatly reduced the inhibitory effect caused by cellobiose, and the hydrolysis yield was improved to 83.9% with enhanced cellobiase activity of 6.5 CBU/g substrate.

Mussatto *et al.* (2007) studied the effect of hemicellulose and lignin on cellulose conversion to glucose by using the enzymatic hydrolysis method. The raw materials were brewer's spent grain (BSG) in three different forms: original (untreated),

pretreated by dilute acid (cellulignin), and pretreated by a sequence of dilute acid and dilute alkali (cellulose pulp). The hydrolysis was carried out using a commercial cellulose concentrate (Celluclast 1.5L) in an enzyme/substrate ratio of 45 FPU/g, 2% (w/v) substrate concentration, 45 °C for 96 h. According to the results, the cellulose hydrolysis was affected by the presence of hemicellulose and/or lignin in the sample. The cellulose conversion ratio (defined as glucose yield + cellobiose yield) from cellulignin was 3.5 times higher than that from untreated sample, whereas from cellulose pulp such value was 4-times higher, correspondent to 91.8% (glucose yield of 85.6%). It can be concluded that the lower the hemicellulose and lignin contents in the BSG sample, the better the performance of cellulose enzymatic hydrolysis into glucose. Nevertheless, it was not necessary to promote a complete removal of hemicellulose and lignin to achieve high cellulose conversion ratio during the enzymatic hydrolysis of BSG, since for cellulignin this value corresponded to 78.1%.

2.5 Cellulase Enzymes

Cellulase refers to a class of enzymes produced chiefly by fungi, bacteria, and protozoans that catalyze the hydrolysis of cellulose. However, there are also cellulases produced by plants and animals (Kumar *et al.*, 2009). Its production provides a catalyst for cellulose hydrolysis to glucose, to be used for eventual production of ethanol. The production of cellulase enzymes with a batch growth of the fungus *Trichoderma reesei* is dependent on many factors, as the *Trichoderma* cellulase system is of a complex nature (Velkovska *et al.*, 1997).

Due to the difficulties in determination of cellulase activities, an IUPAC commission has recommended several tests for the measurement of cellulolytic enzymes. Esterbauer *et al.* (1991) investigated the overall cellulose activity and indicated that one international filter paper unit (FPU) is defined as that amount of enzyme which forms 1 μmol (=0.18 mg) glucose/min under the standard conditions. It is important to note that cellulases behave differently to other enzymes and show no linear relationship between rate (glucose formed per unit time) and enzyme concentration.

The three major enzyme activities are (Mussatto and Teixeira, 2010):

a) Endocellulase (1,4-β-D-glucan-4-glucanhydrolase, endoglucanase, EG, EC 3.2.1.4), which attacks regions of low crystallinity in the cellulose fiber creating free chain ends.

b) Exocellulase (1,4-β-D-glucancellobiohydrolase, CBH, EC 3.2.1.91), which degrades the molecule further by removing cellobiose units from the free chain ends

c) β-glucosidase (β-D-glucosido-glucohydrolase, cellobiase, EC 3.2.1.21), which hydrolyzes cellobiose to produce glucose.

A synergistic action of the three enzyme activities is necessary for the hydrolysis of crystalline cellulose to glucose (Esterbauer *et al.*, 1991). A schematic representation of the cellulose enzymes over the cellulose structure is shown in Figure. 2.6.

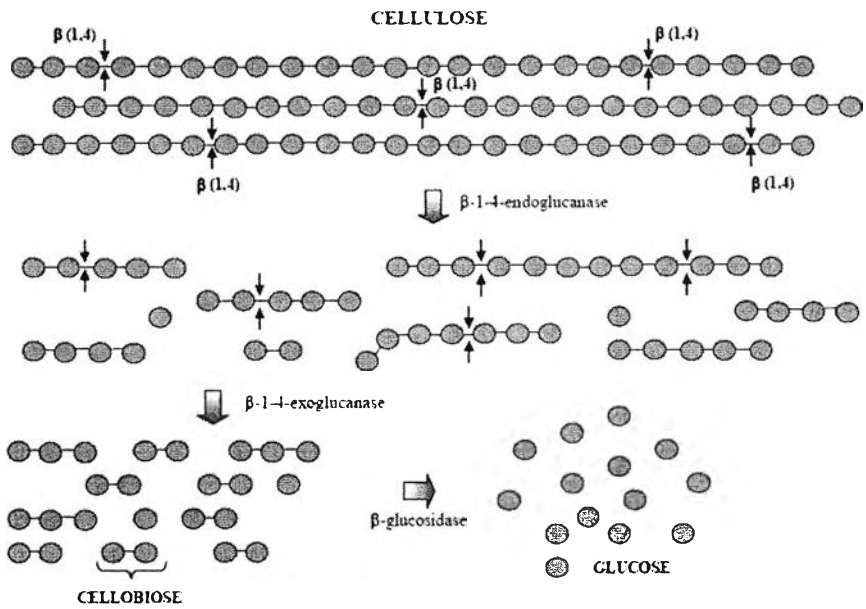

Figure 2.6 Schematic representation of the cellulase enzymes over the cellulose structure (Mussatto and Teixeira, 2010).

The hemicellulose and lignin removal causes extensive changes in the structure and accessibility of cellulose that becomes more accessible and more open to swelling on contact with cellulases. Therefore, the enzymatic hydrolysis of lignocellulosic materials is low prior to the hemicellulose and lignin removal. With the removal of these

two fractions, the cellulose crystallinity is reduced, and the material porosity and the accessible surface area are increased, favoring the enzymes attack (Mussatto *et al.*, 2007)

However, the high cost of cellulase is a major barrier to ethanol production from biomass mediated by enzymatic hydrolysis. Therefore, the key challenge in lignocellulose biotechnology is to develop a robust enzyme production system that can produce adequate amounts of highly efficient cellulase to make biomass depolymerization more rapid and less expensive (Ward and Singh, 2002). The ways to enhance enzyme formation can be achieved by screening for new organisms, mutation, development of efficient strains by genetic engineering, or by finding optimum conditions for their production (Haltrich *et al.*, 1994).

Haltrich *et al.* (1994) aimed to optimize a culture medium for the production of cellulase and to investigate the formation of different hemicellulolytic enzymes by a wild strain of *Sclerotium rolfsii.* This organism is known to form a complete cellulase system consisting of cellobiohydrolase, endoglucanase, and β-glucosidase activities, as well as several hemicellulases. The substrates included α-cellulose, beechwood xylan, wheat bran, wheat straw (0.5 mm, steam treated), corncob, corn stalk, corn leaves, green algae *(Ulva rifida),* and birchwood (steam treated). These raw materials were pretreated by steaming in an autoclave at 190 °C for 10 min and were ground in a mill to pass through a 0.25 or 0.5 mm screen. The results indicated that the amount of β-glucosidase in the enzyme preparation of *S. rolfsii* was sufficient to avoid accumulation of large quantities of cellobiose, which would strongly inhibit the action of cellulose and the addition of β-xylosidase could probably increase the total yield of xylose in the hydrolysis of lignocellulosic matter. Table 2.6 shows the results of analysis sugars formed from various substrates during an incubation period of 73 h.

Table 2.6 Formation of sugars as assayed by HPLC during enzymatic hydrolysis of different cellulosic and hemiceilulosic materials at 40 °C (Haltrich *et al.*, 1994)

Substrate	Sugar concentration (g/liter)				
	Glucose	Cellobiose	Xylose	Xylobiose	Arabinose
α-Cellulose	3.90	0.09	0.20	nd	0.14
Birchwood xylan	1.40	nd	4.10	11.80	nd
Wheat bran	2.60	nd	2.60	nd	0.85
Wheat straw	3.40	0.06	0.43	0.68	nd
Corn cobs	6.70	nd	0.60	0.14	0.22
Corn stalk	2.20	nd	0.70	nd	0.16
Corn leaves	2.40	nd	0.84	nd	0.18
Green algae	1.50	nd	0.19	nd	nd

Movagharnejad and Sohrabi (2003) studied the effect of enzyme to substrate ratio on product concentration by using rice pollards, sawdust, wood particles, and used papers as raw materials. The compositions of these cellulosic waste materials are summarized in Table 2.7. The process has been studied in enzymatic hydrolysis method at 50 °C by using Celluclast L/CCN 03056 and *Novozyme* 188 DCN/85-4 enzymes, where celluclast, mainly a mixture of different endo- and exo-cellulases, is a liquid cellulase preparation made by submerged fermentation of a selected strain of the fungus *T.reesei*. The results showed that the enzyme/substrate ratio was a sensitive factor in enzymatic hydrolysis of cellulosic materials. In systems with higher ratios of enzyme/substrate, the concentration of substrate played a major role in the progress of reaction, whereas, at lower ratios of enzyme/substrate, enzyme concentration was the key factor. In such systems, increasing the concentration of substrate may not lead to a rapid increase in product concentration.

Table 2.7 Composition of cellulosic waste materials (Movagharnejad and Sohrabi 2003)

Materials	Cellulose (%)	Lignin (%)	Hemicellulose (%)
Rice pollards	49.5	19.0	31.0
Sawdust	50.0	26.0	24.0
Used paper	66.8	7.5	25.7

Martins *et al.* (2008) compared the activity of different type of enzymes; *Penicillium echinulatum* (potential cellulase producer for bioconversion processes) and *Trichoderma reesei* cellulases (Celluclast 1.5L FG, *Novozymes*). The total cellulase activity was determined against Whatmann no.1 filter paper (Sigma–Aldrich, St. Louis, MO), using the DNS method and high performance liquid chromatography (HPLC; see the following section) to quantify total reducing sugars (RS) and soluble carbohydrates, respectively. Analysis of substrate hydrolysates demonstrated that *P. echinulatum* enzymes had higher β-glucosidase activity than Celluclast 1.5L FG, while the latter appeared to have greater cellobiohydrolase activity. Unlike Celluclast 1.5L FG, *P. echinulatum* cellulases had enough β-glucosidase activity to remove most of the cellobiose produced in hydrolysis experiments. However, Celluclast 1.5L FG became more powerful than *P. echinulatum* cellulases when supplemented with exogenous β-glucosidase activity (*Novozym* 188).

2.6 Enzyme from Termites

Schulz *et al.* (1986) studied the cellulase from the termite *Nasutitermes walker* and implied that *N. walker* consists of two enzymes. Each has broad specificity with predominantly one activity. One enzyme is an endo-α-l,4-glueanase (EC 3.2.1.4), which predominantly cleaves cellulose randomly to glucose, cellobiose and cellotriose. It hydrolyses cellotetraose to cellobiose but will not hydrolyse cellobiose or cellotriose. The second enzyme component is a β-l,4-glucosidase (EC 3.2.1.21) as its major activity is to hydrolyse cellobiose, cellotriose, and cellotetraose to glucose; it has some exoglucosidase activity as glucose is the only product produced from cellulose. They

concluded that it is now well established that higher termites produce cellulolytic enzymes.

Taechapoempol (2009) aimed to isolate cellulase-producing bacteria from Thai termites (lower termites: *Schedorhinotermes sp.*; and higher termites: *Microcerotermes sp.*) under various isolation conditions. All isolates were preliminarily screened for selecting effective cellulase-producing bacteria by using the Congo red-polysaccharide interaction technique. In this work, 47 cellulase-producing bacteria were isolated from Thai higher termites, *Microcerotermes sp.*, under three different isolation conditions. Only three effective isolates of A 002, M 015, and F 018 that possessed the highest HC value were tested comparatively for their enzymatic cellulase activities-Fpase, endoglucanase, and β-glucosidase at 37 °C and pH 7.2. The results showed that the isolate M 015 exhibited the highest endoglucanase activity whereas the isolate F 018 gave the highest FPase and β-glucosidase activities. Characteristics of isolates A 002, M 015, and F 018 by microbiological methods were shown in Table 2.8.

Table 2.8 Characteristics of isolates A 002, M 015, and F 018 by microbiological methods (Taechapoempol, 2009)

Isolate	Colonial appearance	Pigmentation	Cell shape	Gram's staining	Spore forming
A 002	Circular, flat, entire, rough, and membranous	Light brown cream	Rod	+	+
M 015	Spindle, raised, entire, glistering, and opaque	Light brown cream	Rod	+	+
F 018	Spindle, flat, filamentous, glistering, and opaque	Light brown cream	Rod	+	+

Eourarekullart (2011) investigated the effect of particle size of corncob, hydrolysis temperature, hydrolysis time, and strains of bacteria isolated from Thai higher termites on conversion of corncob to fermentable sugars. The studied parameters included the particle size of corncob (40 and 60 mesh), temperature (30 and 37 °C), and bacteria strain (A 002 and M 015). The results showed that maximum amount of glucose was 1.08 g/L when using these following conditions; the hydrolysis reaction with strain A 002 bacteria and 60 mesh size corncob at 37 °C.

2.7 Cassava Residue

Cassava is a major raw material used in many industries in Thailand (Srinorakutara *et al.*, 2006). Due to its efficient growth, year round availability, tolerance to extreme stress and its suitability to be incorporated into traditional low input farming systems, cassava is the third largest source of carbohydrates for human consumption in the world. It can be used to produce bioethanol. In situations, where water availability is not enough for the cultivation of sugar cane, cassava is the preferred feedstock for ethanol production (Ubalua, 2007).

Cassava residue, a waste from cassava starch plant, can also be utilized to produce ethanol due to its containing cellulose and hemi-cellulose at levels of 24.99 and 6.67 % (w/w), respectively. Use of cassava residue as a raw material in ethanol production not only reduces waste material created from the cassava starch industry, but also lowers the cost of ethanol production (Ubala, 2007; Srinorakutara *et al.*, 2006). The example of composition of cassava residue is shown in Table 2.9.

Table 2.9 The chemical composition of cassava waste (Srinorakutara *et al.*, 2006)

	Cassava waste %(w/w)		
	Sample 1	Sample 2	Sample 3
Moisture	78.16	79.5	82.74
Protein	1.82	2.03	2.31
Fat	0.09	0.2	0.16
Ash	1.61	2.38	2.05
Fiber	10.61	14.35	14.56
Starch	69.9	61.84	64.36