CHAPTER II
LITERATURE REVIEW

2.1 Cellulose

Cellulose is a polymerized form of glucose molecules with β-1,4-linkage, as shown in Figure 2.1, which provides the primary structural component for plants. Cellulose is formed by both intra- and inter-molecular hydrogen bonds, and it consists of composite forms of highly crystallized microfibrils among amorphous matrices. The difference in the glucose linkage between starch and cellulose makes it impossible for the starch digesting enzymes, e.g. alpha-amylase, to break down cellulose. Many properties of cellulose depend on its chain length or degree of polymerization and the number of glucose units that make up one polymer molecule (Zhang *et al.*, 2006).

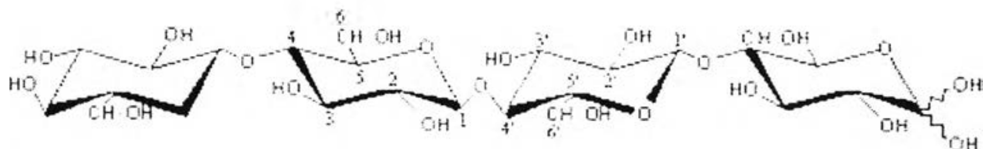

Figure 2.1 Schematic representation of a cellulose chain (Pérez and Mackie, 2001).

2.2 Glucose

Glucose 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 alleviating the need for petroleum, whose supply is fast dwindling (Wang, 2009).

2.3 Cellulose Pretreatment

Pretreatment is a necessary step in bioconversion of cellulosic materials to fuels and chemicals. After the biomass is shredded, chipped, ground, milled, or otherwise pulverized, it undergoes a pretreatment to separate the cellulose from the hemicellulose and the lignin. The primary purpose of pretreatment is to make the cellulosic biomass accessible and reactive to allow high rates and yields on enzymatic hydrolysis. To date, a number of pretreatment methods have been proposed and investigated, including dilute acid pretreatment, steam explosion, hydrothermal processes, organic solvent pretreatment, ammonia fiber explosion, and strong alkali pretreatment. Interesting claims have been made with regard to the effectiveness of these pretreatment methods. The economic and environmental constraints, however, limit the applicability of these known methods, Werner (2006).

2.3.1 Steam Explosion

This process first exposes the biomass to high-pressure steam for a short period of time and then quickly decompresses it to atmospheric pressure. The depressurization causes the rapid expansion of water from steam, and the biomass explodes into a pulp. Some of the useful fibers may be lost in the explosion stage if the temperatures are too high, but the process can be catalyzed by chemical inputs, like sulfur dioxide or ammonia.

2.3.2 Hydrothermal Pretreatment

The hydrothermal process adds the biomass to water and then raises the temperature and pressure.

2.3.3 Dilute Acid Pretreatment

By using dilute sulfuric acid at a high temperature and pressure hydrolyzes the hemicellulose, the lignin remains, but the cellulose is more accessible. There are three different processing technologies used in the dilute acid pretreatment: countercurrent processing, two-temperature processing, and pressurized hot washing.

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2.3.3.1 Countercurrent Processing

There is a tradeoff between temperature and acid concentration: at lower temperatures, a more concentrated acid must be used, and sulfuric acid is fairly expensive; at higher temperatures, a more dilute acid may be used, but higher temperatures can result in the degradation of the hemicellulosic sugars. A countercurrent reactor allows the dissolved sugars to flow out of the reaction chamber soon after they are hydrolyzed.

2.3.3.2 Two-Temperature Processing

Often one portion of the hemicellulose is easier to hydrolyze than the rest. The biomass can be pretreated in one chamber at a lower temperature to remove the more acquiescent fraction, and is then moved to another chamber at a higher temperature to deal with the rest. This protects the hydrolyzed sugars from unnecessary exposure to greater heat.

2.3.3.3 Pressurized Hot Washing

The pressurized hot washing is another method of National Renewable Energy Laborator (NREL) for cellulose conversion developed for removing the hydrolyzed hemicellulosic sugars from the cellulose following the pretreatment step. The liquid is squeezed out of the solid cellulose, and is then washed away by hot water. This process results in the removal of some lignin as well, making the cellulose even more accessible. As a result, the hydrolysis process becomes faster and more economically feasible.

Table 2.1 Advantages and disadvantages of pretreatment

Pretreatment	Advantages	Disadvantages
Mechanical comminution[a]	Improve the digestibility of biomass	Require exorbitant amount of energy
Steam explosion[b] (autohydrolysis)	1. Low energy requirement compared to mechanical comminution 2. No recycling or environmental costs	1. Formation of inhibitory compounds 2. Destruction of xylan fraction 3. Incomplete disruption of the lignin-carbohydrate matrix
Ammonia fiber explosion[b] (AFEX)	1. Significantly improve saccharification rates of various herbaceous crops 2. Not produce inhibitors for downstream biological process 3. Not require small particle size for Efficacy	Not very effective for biomass with high lignin content
CO_2 explosion[a]	1. More cost effective than ammonia fiber explosion 2. No formation of inhibitory Compounds	Low yield compared to steam or ammonia explosion
Ozonolysis[a]	1. Effectively remove lignin 2. Not produce toxic residues for the downstream process 3. Carry out at room temperature and pressure	Large amount of ozone required, making the process expensive
Acid hydrolysis[b]	1. Achieve high xylan-to-xylose conversion yields (less severe conditions) 2. Significant improve cellulose Hydrolysis	1. Higher cost than some physico-chemical pretreatment 2. Need neutralization of pH
Alkaline hydrolysis[b]	1. Decrease degree of polymerization and crystallinity 2. Separation of structural linkages between lignin and carbohydrates 3. Disruption of lignin structure	No effect for soft woods with lignin content greater than 26%
Biological pretreatment[a]	1. Low energy requirement 2. Mild environmental conditions	Very low hydrolysis rate

[a] Kumar *et al.*, (2009). [b] Dimian and Bildea (2008)

2.4 Ionic Liquids

Ionic liquids (ILs) are liquids that contain essentially only ions. This term is commonly used for simple molten salts, whose melting point is relatively low (below 100°C). They are primarily made of cations and, in particular, salts that are liquid at room temperature, so-called room-temperature ionic liquids, or RTILs. Inorganic salts, such as sodium halogens, are solids with a melting point well above 500°C (Berthod *et al.*, 2008). The novelty of the new non-molecular class of IL solvents is the low melting temperature of these salts. ILs are suggested for the dissolution of cellulose. Table 2.2 illustrates details about solubility of cellulose in some ionic liquids.

Table 2.2 Solubility of cellulose in some ionic liquids (Novoselov *et al.*, 2007)

Ionic liquid	Solubility
1-Butyl-3-methylimidazolium chloride	Soluble [c]
1-Allyl-3-methylimidazolium chloride	Soluble
1-Allyl-3-butylimidazolium chloride	Soluble
1,3-Diallylimidazolium chloride	Soluble
1-Butyl-2,3-diethylimidazolium chloride	Dissolves slowly
1-Allyl-3-propargylimidazolium chloride	Dissolves slowly
1-Butyl-2,3-dimethylimidazolium thiocyanate	Insoluble
1-Butyl-3-methylimidazolium saccharinate	Insoluble
1-Butyl-3-methylimidazolium tosylate	Insoluble
1-Butyl-3-methylimidazolium hydrogensulfate	Insoluble
1-Allyl-3-methylimidazolium dicyanamide	Insoluble
1-Allyl-3-butylimidazolium dicyanamide	Insoluble
1-Allyloxy-3-methylimidazolium dicyanamide	Insoluble
1-Allyloxy-3-methylimidazolium chloride	Insoluble

[c] Cellulose is considered as soluble if its concentration in the given solvent of 3% and over can be attained.

Furthermore, Table 2.3 compares melting points of selected chlorides. On the whole, it is shown that 1-buthyl-3-methylimidazolium chloride ([BMIM]Cl) not only has low melting point but also can dissolve cellulose.

Table 2.3 Melting points of selected chlorides

Salt	Melting point (°C)
Sodium chloride	803
Potassium chloride	772
1,3-Dimethylinudazolium chloride	125
1-Ethyl-3-methylimidazolium chloride	85
1-Buthyl-3-methylimidazolium chloride	65

For 1-butyl-3-methylimidazolium and 1-allyl-3-methylimidazolium chlorides, ≥10% of cellulose can be dissolved (Novoselov *et al.*, 2007).

Swatloki *et al.* (2002) investigated the dissolution of cellulose in different types of ILs and experiment conditions. They found that [BMIM]Cl was one of effective ILs, which is able to use for dissolution cellulose pulp, as shown in Figure 2.2. The cellulose pulp can be dissolved in [BMIM]Cl to 10 wt% at 100°C and up to 25 wt% when using microwave as the heating source. From this result, they speculated that the high chloride concentration and activity in [BMIM]Cl is highly effective in breaking the intra- and interhydrogen bond of cellulose chains.

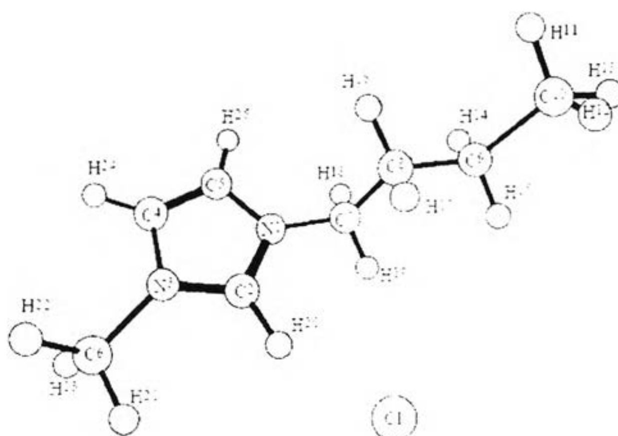

Figure 2.2 Molecule structure of 1-butyl-3-methylimidazolium chloride, [BMIM]Cl. (Novoselov *et al.*, 2007)

However, the cellulose solubility of [BMIM]Cl decreased as 1 wt% water (approximatly 0.5 mole fraction of H_2O) was added to the system. From the water addition, cellulose pulp was precipitated, and this was called "regenerated cellulose". The regenerated cellulose and initial dissolving pulp were characterized by scanning electron microscopic (SEM) and thermogravimetric analysis (TGA). SEM showed the change of cellulose morphology after it was dissolved in [BMIM]Cl, as shown in Figure 2.3. Figure 2.4 shows thermal decomposition profiles of dissolved pulp that was regenerated from [BMIM]Cl. The curve of initial cellulose sample showed rapid decomposition in temperature range from 350–360°C. The regenerated cellulose exhibited a lower onset temperature for decomposition.

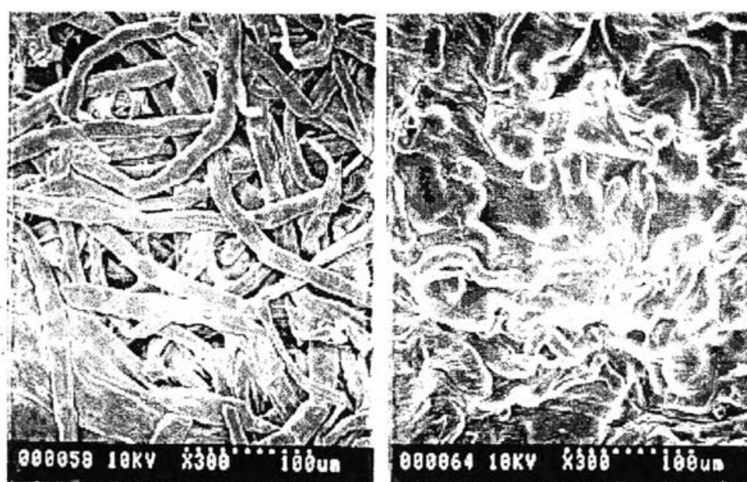

Figure 2.3 SEM micrographs of the initial dissolving pulp (left) and after dissolution in [BMIM]Cl and regeneration into water (right) (Swatloki *et al.*, 2002).

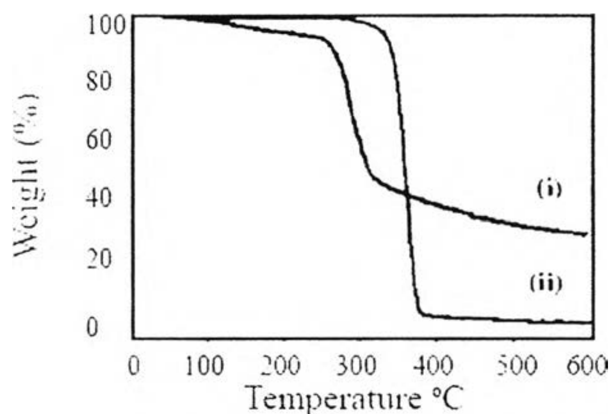

Figure 2.4 Thermal decomposition profiles of (i) regenerated cellulose and (ii) original dissolving pulp. Samples were heated in platinum sample containers under a nitrogen atmosphere at 10°C/min (Swatloki *et al.*, 2002).

Dadi *et al.* (2007) studied the application of ionic liquid in pretreatment of cellulose to alleviate recalcitrance to enzymatic hydrolysis. The XRD patterns showed the peaks of regenerated cellulose compared to the non-dissolved one at 5, 10, 15 and 30 wt%. The heights of the peak at $2\theta = 22.5°$, which indicated cellulose's crystallinity, was lower for regenerated cellulose at every percent weight than untreated cellulose, as shown in Figure 2.5.

They also found that [BMIM]Cl IL-treated cellulose exhibited improved hydrolysis kinetics with optically transparent solutions formed after the first few hours of reaction, indicating relatively fast hydrolysis kinetics. With optimal IL-treatment conditions and enzyme loadings, initial rates of hydrolysis of IL-treated cellulose were two orders of magnitude higher than those observed with untreated cellulose. Among IL-treated cellulose preparations, the initial rates observed with samples containing only regenerated cellulose (RC) were higher than the initial rates for the samples that were mixtures of RC and partially crystalline cellulose (PCC). In spite of the observed differences in the initial rates and crystallinity index, all IL-treated cellulose preparations showed significantly higher glucose conversions compared with untreated cellulose; glucose conversion of about 80–85% was observed for IL-treated cellulose samples in 7 h of hydrolysis whereas it was only 20% for untreated cellulose. as shown in Figure 2.6.

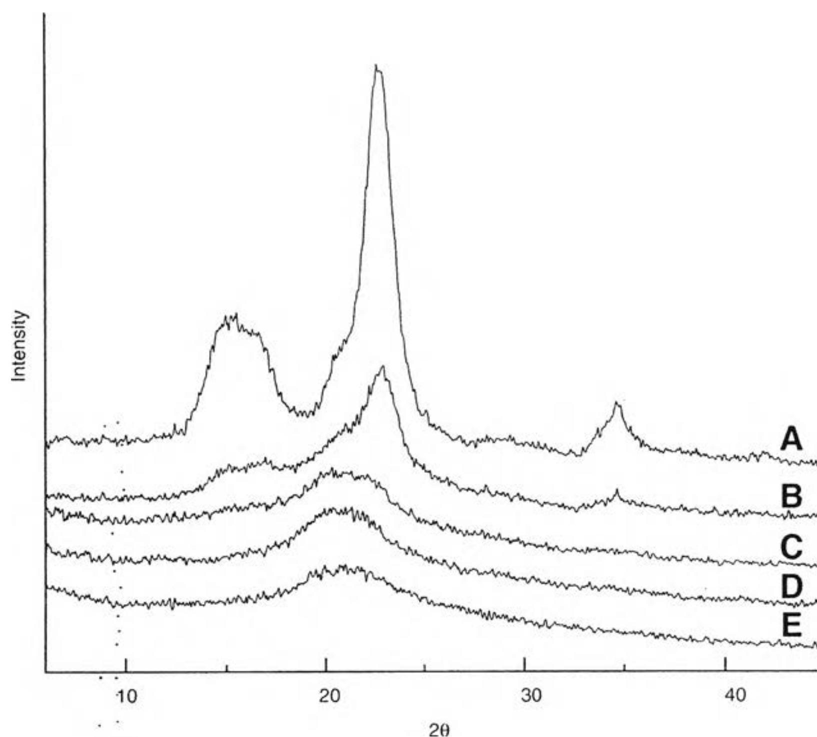

Figure 2.5 XRD patterns for IL-treated and untreated-cellulose. Untreated cellulose **(A)** exhibited a significantly greater degree of crystallinity than that of regenerated samples **(B–E)**. Cellulose samples were incubated in [BMIM]Cl at 120°C for 30 min and precipitated with deionized water. Samples B–E correspond to 30, 15, 10, and 5 wt% of cellulose (Dadi *et al.*, 2007).

Thus, it seems that it is not really necessary to completely eliminate the crystallinity of cellulose in order to achieve significant enhancement in hydrolysis rates; even with some residual crystallinity, most of the recalcitrance to hydrolysis can be mitigated. This offers the possibility to hydrolyze large amounts of cellulose rapidly using the proposed IL-pretreatment technique.

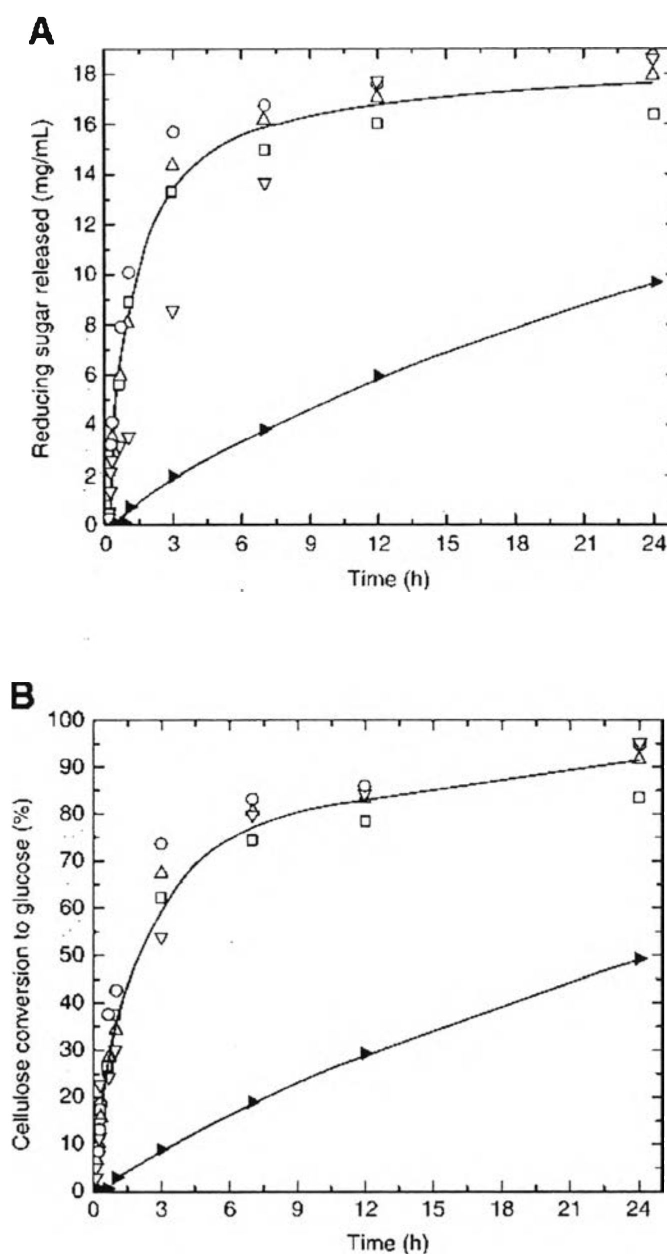

Figure 2.6 Cellulose samples of 5% (□), 10% (○), 15% (Δ), and 30% (∇) were incubated for 30 min in [BMIM]Cl at 120°C, and precipitated with deionized water. Hydrolysis rates of IL-incubated samples by *T. reesei* are compared with that of untreated Avicel (▲). **(A)** Total soluble sugars (measured using a DNSA assay) and **(B)** Percent cellulose conversion to glucose (measured by HPLC) (Dadi *et al.*, 2007).

Liu and Chen (2006) studied on enzymatic hydrolysis rates of wheat straw and steam-pretreated wheat straw that treated with [BMIM]Cl. They found that the hydrolysis rate of wheat straw treated with [BMIM]Cl could reach 70.37% and the steam-pretreated wheat straw treated with [BMIM]Cl could be completely hydrolyzed, while hydrolysis rates of the untreated wheat straw and the steam-pretreated wheat straw treated with water were 42.78% and 68.78%, respectively. Thus, the hydrolysis rates of materials were improved by treated with [BMIM]Cl.

Li *et al.* (2009) evaluated the effect of solvents using yield of reducing sugars from wheat straw regenerated from different solvents, as shown in Figure 2.7. It is so obvious that the yield of reducing sugars from regenerated wheat straw was higher than that of the water-treated one, due to its compatibility with both cellulose solubility and cellulase activity, and therefor [EMIM]DEP was chosen in their study.

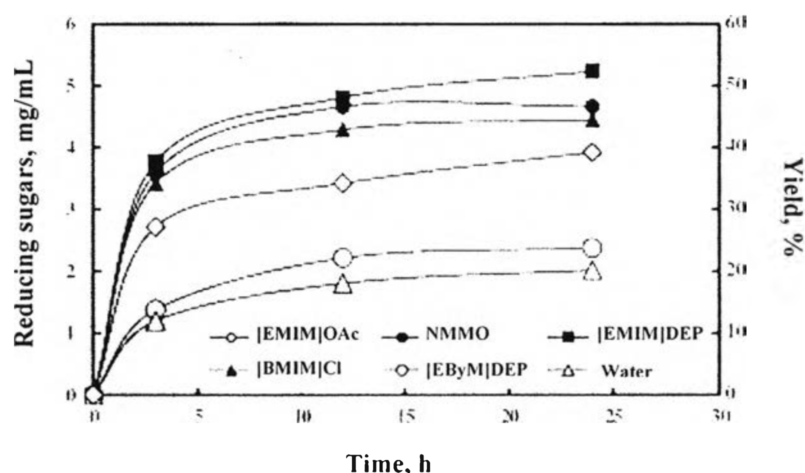

Figure 2.7 Selection of solvents for pretreatment of wheat straw. Pretreatment condition: the wheat straw samples were incubated for 1 h at 100 °C and regenerated using the anti-solvents water. Hydrolysis condition: regenerated and water-treated wheat straws (10 mg/mL) were hydrolyzed using a cellulase activity of 30 (FPU/g) substrate at 50 °C and pH 4.8 (Li *et al.*, 2009).

Moreover, they reported that anti-solvent, methanol, ethanol, and deionized water, can be used to regenerate wheat straw from [EMIM]DEP ionic liquid. Anti-solvent was added to wheat straw and ionic liquid mixture, after that the mixture was centrifuged and the supernatant was removed from the treated wheat straw. They

found that anti-solvent selection did not have the effect on the treated wheat straw, thus deionized water was selected as anti-solvent in terms of economical point, as shown Figure 2.8.

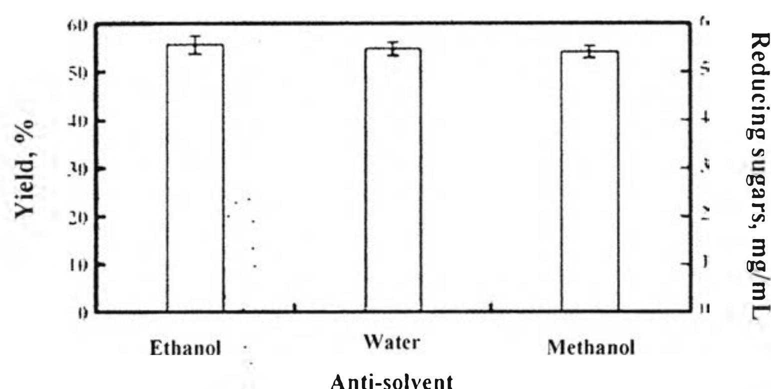

Figure 2.8 Effect of anti-solvent on the regeneration of wheat straw. Pretreatment condition: the wheat straw samples were incubated for 30 min at 130 °C and regenerated using the anti-solvents water, methanol, and ethanol, respectively. Hydrolysis condition: regenerated wheat straws (10 mg/mL) were hydrolyzed for 12 h using a cellulase activity of 30 FPU/g substrate at 50 °C and pH 4.8 (Li *et al.*, 2009).

2.5 Hydrolysis

2.5.1 Concentrated Acid Hydrolysis

Following the pretreatment step, cellulose is dried and then decrystallized through a reaction with concentrated sulfuric acid. The resulting gelatin is then diluted with water and heated to release sugars. In the past, the prohibitively high costs associated with large amounts of sulfuric acid have made the commercialization of concentrated acid hydrolysis a rarity. However, through separation from sugars by the use of either a membrane or a chromatographic column, much of the acid can be recycled, and these methods improve the process, becoming somewhat more economically viable, Werner (2006).

2.5.2 Dilute Acid Hydrolysis

This process operates on the same principle as the dilute acid pretreatment, except that the temperature of the reaction is raised to 215°C in order to break down cellulose. Again, there is a tradeoff between the acid concentration and temperature, and using a more dilute acid requires a higher temperature. To heat the large volume of liquid required for this process would be very expensive.

2.5.3 Enzymatic Hydrolysis

Enzymatic hydrolysis of cellulose is carried out by cellulase enzymes, instead of acid, to break down the cellulose after it has been pretreated. Furthermore, enzymatic degradation of cellulose to glucose is generally accomplished by synergistic action of three distinct classes of cellulase enzymes that are endo-1,4-β-glucanases, exo-1,4-β-D-glucanases, and β-D-glucosidases.

2.6 Cellulase

Mechanism for enzymatic cellulose hydrolysis involves synergistic actions by exoglucanase (EC 3.2.1.91), endoglucanase (EC 3.2.1.4), and β-glucosidase (EC 3.2.1.21). Exoglucanases processively cleave cellulose chains at the ends to release soluble cellobiose or glucose; endoglucanases hydrolyze accessible intramolecular β-1,4-glucosidic bonds of cellulose chains randomly to produce new chain ends; and β-glucosidases hydrolyze cellobiose to glucose in order to eliminate cellobiose inhibition. These three hydrolysis processes occur simultaneously, as shown in Figure 2.9. The enzymatic depolymerization step performed by endoglucanases and exoglucanases is the rate-limiting step for the whole cellulose hydrolysis process. Secondary hydrolysis that occurs in the liquid phase involves primarily the hydrolysis of cellobiose to glucose by β-glucosidases, although some β-glucosidases also hydrolyze longer cellodextrins (Zhang *et al.*, 2006).

During cellulose hydrolysis, the solid substrate characteristics vary, including (1) changes in the cellulose chain end number, resulting from generation by endoglucanases and consumption by exoglucanases and (2) changes in cellulose accessibility resulting from substrate consumption and cellulose fragmentation. The

combined actions of endoglucanases and exoglucanases modify the cellulose surface characteristics (topography) over time, resulting in significant changes in hydrolysis rates (Zhang *et al.*, 2006).

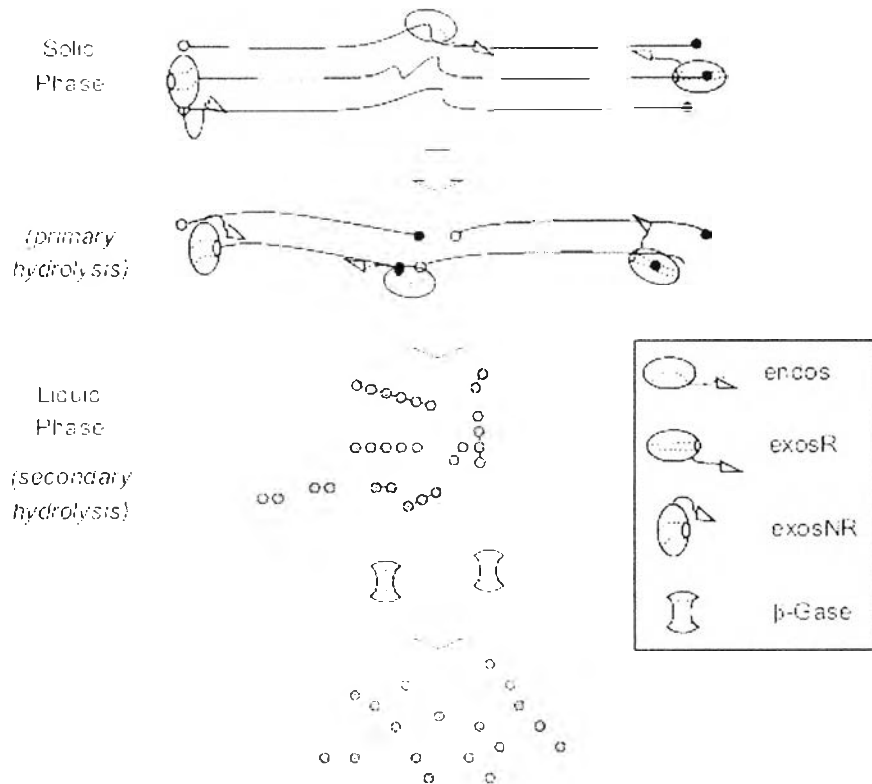

Figure 2.9 Mechanistic scheme of enzymatic cellulose hydrolysis by *Trichoderma*, non-complexed cellulase system (Zhang *et al.*, 2006).

Taechapoempol (2009) studied cellulase-producing bacteria, and effective isolates from Thai higher termites *Microcerotermes* sp. under different isolation conditions. Table 2.4 show preliminary identification of strain A 002, M 015, and F 018 by microbiological methods. He found that the most effective isolates from each isolation condition (aerobic, anaerobic, or anaerobic/aerobic), which had the highest HC value, were A 002, M 015, and F 018, respectively.

Table 2.4 Preliminary identification of strain A 002, M 015, and F 018 by microbiological methods (Taechapoempol, 2009)

Strain	**Colonial Appearance**	**Pigmentation**	**Cell shape**	**Gram's staining**	**Spore forming**	**Oxidase test**	**Catalase test**
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 green cream	Rod	+	+	-	+

Figure 2.10 shows the specific endoglucanase activity profiles of the three isolates incubated at 37°C. The result showed that M 015 had higher maximum specific activities than F 018 and A 002. The specific activity profile of strain M 015 reached the highest specific endoglucanase activity 1.098 U/mg protein at 16 h, where one unit (U) of cellulase activity is defined as the amount of enzyme capable of releasing 1 μmol of glucose equivalent per min under the assay conditions. The specific activity profile of F 018 was similar to that of M 015 and the maximum specific activity, 0.900 U/mg protein at 20 h. However, the specific activity profile of A 002 was different from those of the first two strains and reached the maximum value, 0.814 U/mg protein at 10 h.

The specific FPase activity profiles of all effective isolates at 37°C are shown in Figure 2.11. All isolates had similar profiles and reached maximum specific activity at 10 h. The specific FPase activities of F 018, M 015 and A 002 reached the maximum specific activity of 0.684, 0.532 and 0.626 U/mg protein, respectively, at this 10 h.

The specific activity profiles of β-glucosidase at 37°C of the three effective isolates are shown in Figure 2.12. The maximum values of each isolate were 0.620, 0.504, and 0.673 U/mg protein for A 002, M 015, and F 018, respectively (Taechapoempol, 2009).

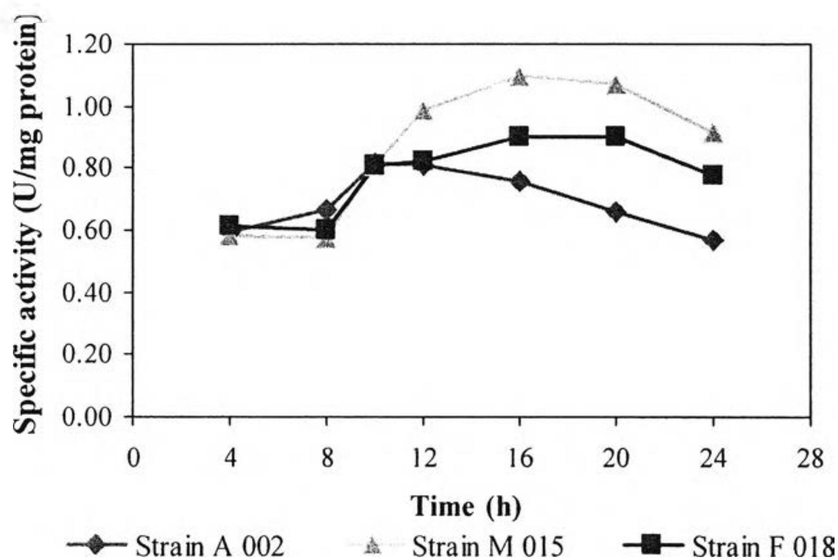

Figure 2.10 Comparison of specific endoglucanase activity of strain A 002, M 015, and F 018 at 37°C, and pH 7.2, and 24 h (Taechapoempol, 2009).

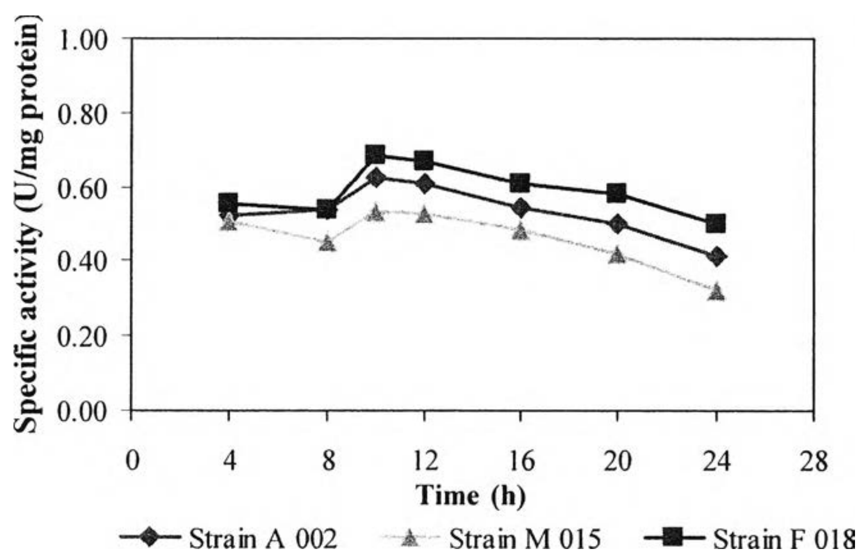

Figure 2.11 Comparison of specific Fpase activity between strain A 002, M 015, and F 018 at 37°C, pH 7.2, and 24 h (Taechapoempol, 2009).

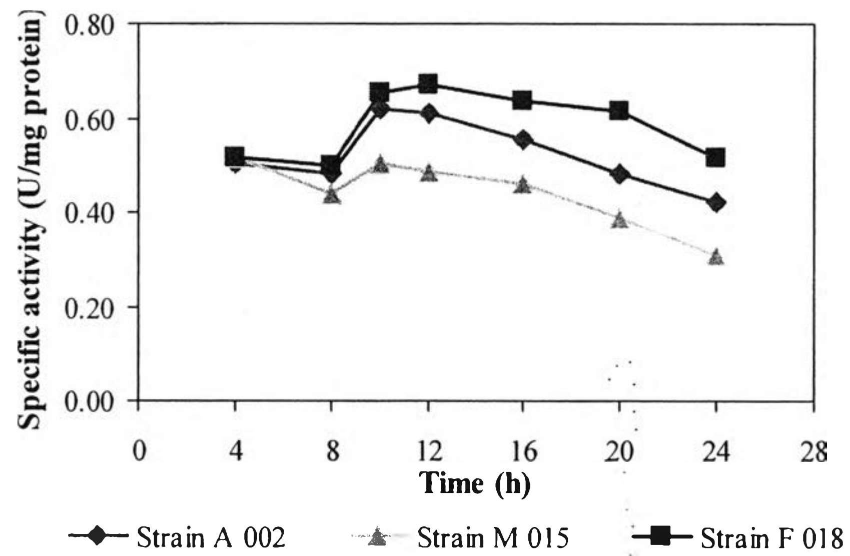

Figure 2.12 Comparison of specific β-glucosidase activity of strain A 002, M 015, and F 018 at 37°C, and 24 h (Taechapoempol, 2009).