CHAPTER II

LITERATURE REVIEW

Lignocellulosic biomass is the most abundant organic components which is renewable. It is composed of three major groups of polymers, cellulose, hemicellulose and lignin. Cellulose is a linear homopolymer of β-1, 4-linked D-glucose residues, hemicellulose is a heteropolymer of pentoses, hexoses, and sugar acids, and lignin is a complex polyphenolic polymer. Cellulose represents about 40% of the lignocellulosic biomass. The cellulose molecule is almost fully extended linear chain with a two-fold screw axis on which successive glucose residues are rotated 180^{o} relative to each other, and therefore the glycosidic oxygens point alternatively up and down. The structure of the cellulose chain is highly stabilized by intramolecular hydrogen bonds (Fig. 2.1)

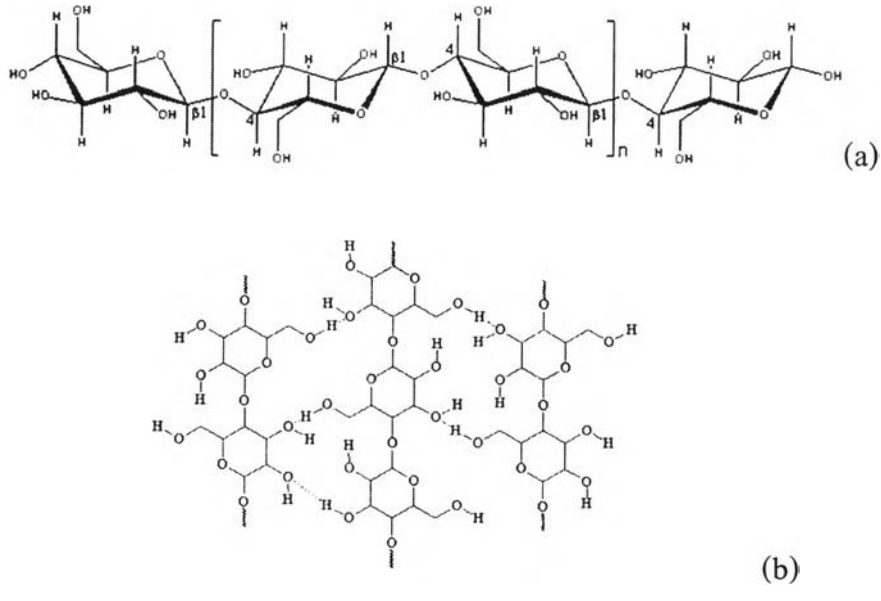

Fig. 2.1 Cellulose chain (a) and the hydrogen bonding within and between the chains in a cellulose crystal (b) (Harjunpaa, 1998)

1. Classification of cellulase

Cellulases are the group of enzymes which hydrolyse β-1,4-glycosidic linkages in cellulose. Since cellulose cannot penetrate into cells, cellulases are secreated outside the cells or bound to outer cell surfaces. Three different cellulolytic activities can be identified. Exoglucanases (1,4-β-D-glucan cellobiohydrolase, E.C. 3.2.1.91) hydrolyse cellulose from the free chain ends, producing mainly cellobiose as and end product. They are therefore called cellobiohydrolases. Endoglucanases (1,4-β-D-glucan-4-glucanohydrolase, E.C. 3.2.1.4) attack randomly internal linkages within the cellulose chain, creating free chain ends. β-Glucosidases (E.C. 3.2.1.21) are exoenzymes which hydrolyse small oligomers, mainly cellobiose to glucose (Fig. 2.2). This exoactivity is important because the accumulation of cellobiose strongly inhibits cellobiohydrolases (Beguin and Aubert, 1994; Harjunpaa, 1998).

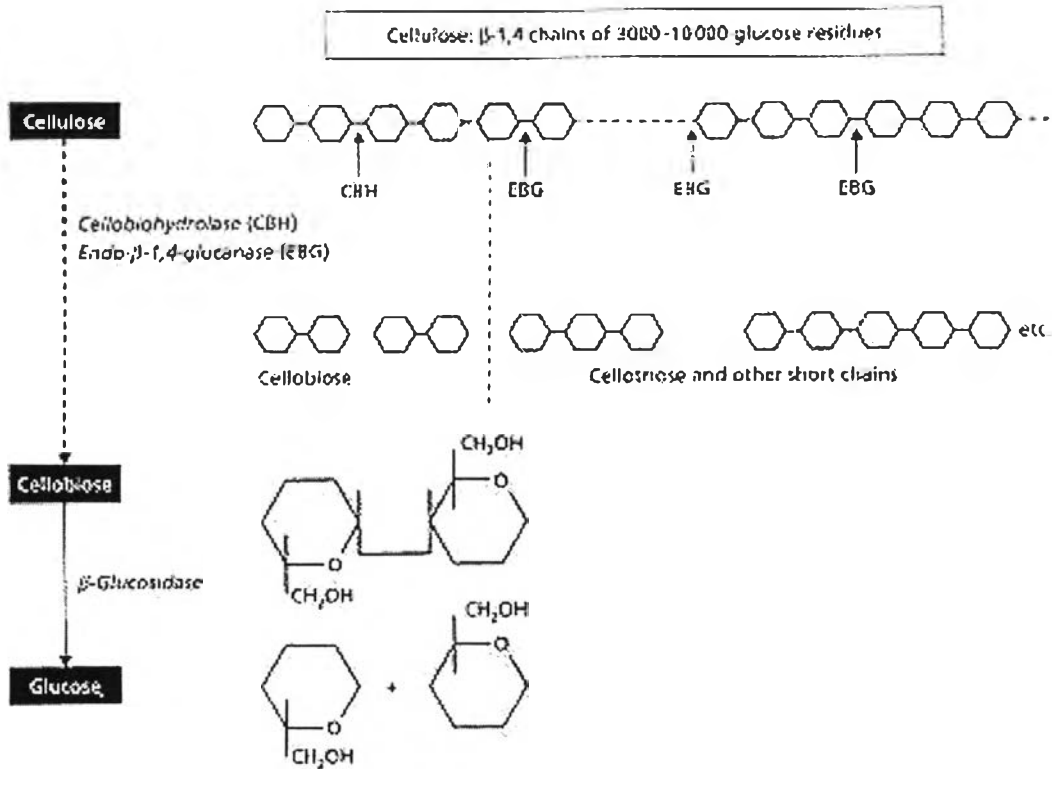

Fig. 2.2 Structure and enzymatic breakdown of cellulose (Deacon, 1997).

2. Cellulases from microorganisms

A wide variety of bacteria, fungi, yeasts, and actinomycetes are known to produce cellulose-degrading enzymes, Table 2.1 shows a list of cellulase producing bacteria and actinomycetes (Garrity, 2001).

Table 2.1 List of cellulolytic bacteria

Species	Growth temp.	Sources
Acetivibrio cellulolyticus	m	Sewage
Acetivibrio cellulosolvens	m	Sewage
Achromobacter Piechaudii	m	Soil
Acidothermus cellulolyticus	t	Acidic hot spring
Actinoplanes aurantiaca	m	Soil
Anaerocellum thermophilum	t	
Bacillus circulans	m	
Butyrivibrio fibrisolvens	m	Rumen
Caldibacillus cellulovorans	t	
Caldocellulosiruptor lactoaceticus	t	
Caldocellulosiruptor kristjanssonii	t	Hot spring
Caldocellulosiruptor saccharolyticus	t	Hot spring
Cellulomonas biazotea	m	
Cellulomonas cartae	m	
Cellulomonas cellasea	m	
Cellulomonas cellulans	m	Soil

Species	Growth Temp.	Sources
Cellulomonas fimi	m	Soil
Cellulomonas flavigena	m	Soil
Cellulomonas gelida	m	
Cellulomonas iranensis	m	Forest soil
Cellulomonas persica	m	Forest soil
Cellulomonas uda	m	Sewage
Cellvibrio fulvus	m	Soil
Cellvibrio Gilvus	m	Soil
Cellvibrio Mixtus	m	Soil
Clostridium acetobutylicum	m	Soil
Clostridium aldrichii	m	Wood fermenter
Clostridium celerescens	m	Manure
Clostridium cellobioparum	m	Rumen
Clostridium cellulofermentans	m	Manure
Clostridium cellulolyticum	m	Compost
Clostridium cellulosi	t	Manure
Clostridium cellulovorans	m	Wood fermenter
Clostridium chartatabidum	m	Rumen
Clostridium herbivorans	m	Pig intestine
Clostridium josui	t	Compost
Clostridium papyrosolvens	m	Paper mill
Clostridium sp. C7	m	

Species	Growth Temp.	Sources
Clostridium stercorarium	t	Compost
Clostridium thermocellum	t	Sewage, soil
Clostridium thermocopriae	t	Hot spring
Clostridium thermopapyrolyticum	t	Mud
Curtobacterium falcumfaciens	m	Soil
Cytophaga sp.	m	Soil
Eubacterium cellulolyticum	m	Rumen
Flavobacterium johnsoniae	m	Soil
Fibrobacter succinogenes	m	Rumen
Microbispora bispora	t	Soil
Micromonospora melonosporea	m	Compost
Myxobacter sp. AL-1	m	Soil
Pseudomonas fluorescens (cellulosa)	m	Plant pathogen
Pseudomonas Mendocina	m	Soil
Ruminococcus albus	m	Rumen
Ruminococcus flavefaciens	m	Rumen
Ruminococcus succinogenes	m	Rumen
Streptomyces alboguseolus	m	
Streptomyces aureofaciens	m	Compost
Streptomyces cellulolyticus	m	

Species	Growth Temp.	Sources
Streptomyces flavogriseus	m	Soil
Streptomyces lividans	m	
Sporocytophaga Myxococcoides	m	Soil
Streptomyces nitrosporeus	m	
Streptomyces olivochromogenes		
Streptomyces reticuli	m	Soil
Streptomyces rochei	m	Termite gut
Streptomyces thermovulgaris		
Streptomyces viridosporus		
Thermoactinomyces sp. *YX*	t	
Thermobifida Alba	m	
Thermobifida cellulolytica	t	Compost
Thermobifida fusca	t	Soil
Thermomonospora curvata	t	
Xanthomonas sp.	m	Brack water

m, mesophilic; t, thermophilic (growth optimum above 50 °C).

2. Cellulase-producing bacteria and their activities

Cellulase-producing bacteria were reported in aerobic and anaerobic genera as shown in Table 2.2. The aerobic strains in *Bacillus subtilis* produced cellulase in stationary phase of growth. The addition of glucose and cellobiose to a culture in this phase had no apparent effect on enzyme production. Maximum cellulase activity was observed at pH 4.8. No cellobiase activity was detected (Robson *et al.*, 1984). *Bacillus subtilis* strains produced maximum CMCase in a liquid medium containing 0.2% D (+) raffinose as inducer, and 0.5% each of yeast extract, casamino acids and proteose peptone at 50 $^{\circ}$ C at an initial pH of 6.0. CMCase activity was detected at early log phase of growth and reached the maximum level at early stationary phase of growth which occurred at the 10th hour of the cultivation. The optimal temperature for CMCase activity was 65°C and the enzyme was highly stable up to 60° C (Chan and Au, 1987).

A neutral cellulase (BSC) from *Bacillus subtilis* and an alkaline cellulase (NK1) from alkalophilic *Bacillus* sp. N-4 showed significant amino acid sequence homology. Despite the high homology, the pH-activity profiles of the two enzymes for carboxymethyl cellulose (CMC) hydrolysis were quite different; BSC showed a sharp optimum pH at 6, whereas NK1 showed its full activity in a broad range, from pH 6 to 10.5 (Hitomi, 1994).

Several strains of *Bacillus* species including *B. brevis, B. firmus, B. polymyxa, B. pumilus, B. subtilis, B. circulans* were reported as cellulase producing bacteria (Priest, 1977; Hakamada *et al.*, 2002) . The studies of *Bacillus* cellulase lagged far behind that of fungal enzymes. This was due to the fact that most *Bacillus* cellulase hydrolyze synthesic carboxymethyl cellulose (CMC) but barely hydrolyse crystalline forms of cellulose. Ito (1997) isolated alkaline cellulase producing *Bacillus* and showed for the first time that this bacterial cellulase was an effective additive to

laundry detergents. The cellulases removed soil trapped in the amorphous region of cotton fibers by cleavage of β–1,4– glucoside bond without damaging of cotton fabries because the enzymes have no activity toward crystalline cellulose (Hoshino and Ito, 1997). Alkaline cellulase of *Bacillus* sp. (Eudo *et al.,* 2001), *B. circulans* (Hakamada *et al.*, 2002) and *Paenibacillus* sp. (Ogawa *et al.,* 2007; Logan *et al.,* 2004) were .epc.ted. Their optimal pH was in the range of 6.0 – 8.5 .

Cellulomonas flavigena strains demonstrated that optimum growth at 45°C and pH 7.3. No growth was observed above 55° C. The optimum cellulolytic enzyme assay temperature was 40° C and pH 7.0 (Mohammad *et al.*, 1984).

Strain of *Clostridium* sp. produced extracellular cellulase during exponential growth, but the enzyme was not free in the growth medium until approximately 30 % of the cellulose was hydrolyzed. Their cellulase synthesis was repressed by glucose and cellobiose. Optimum pH of the enzyme was 6.5 (Lee *et al.*, 1975).

Alkalothermophilic actinomycete, *Thermomonospora* strain produced 23 IU/ml carboxymethyl cellulase. The enzyme exhibited optimum activity at pH 5 and temperature 50°C. The CMCase showed pH stability in the range 7–10. The enzyme retained 100% activity at 50°C for 72 h and had half-lives of 7 and 3 h at 60°C and 70°C, respectively (George *et al.*, 2001). *Thermomonospora curvata* isolated from municipal; refuse compost produced maximum cellulase at pH 5.0, 65 ° C (Stutzenberger, 1971).

Nujo–inositol was a suitable carbon source for growth and carboxymethylase activity (CMCase) of *Sinorhizobium fredii*. Optimum temperature and pH of *S. fredii* CMCase activity were 35° C and 7.0 , respectively (Chen *et al.*, 2004).

Endoglucanase activity of *Cellulomonas, Bacillus* and *Micrococcus* sp. strains isolated from coir retting effluents of estuarine were minimum at pH 5 and maximum at pH7. The enzyme activity was maximum at 40° C (Immanuel *et al.*, 2006).

Table 2.2 Major morphological and physiological features of cellulolytic bacteria (Lynd et al., 2002)

Relationship to oxygen	Genera	Representative species[a]	Gram reaction	Cell form	Resting state	Movement
Aerobic	*Acidothermus*	*A. cellulolyticus*	+	Rods		
	Bacillus	*B. brevis, B. pumilus, B. agaradhaerans, B. subtilis*	+	Rods	Endospore	Flagella
	Caldibacillus	*C. cellovorans*	+	Rods	Endospore	
	Cellulomonas[b]	*C. flavigena, C. uda*	+	Rods	None	Flagella
	Cellvibrio	*C. falvus, C. gilvus*	-	Curved rods	None	Flagella
	Cytophaga	*C. hutchinsonii*	-	Rods	None	Gliding
	Erwinia	*C. carotovora*	-	Rods	None	Flagella
	Micromonospora	*M. chalcae*	+	Filamentous rods	Spore[c]	Nonmotile
	Pseudomonas	*p. fluorescens var. cellulose*	-	Rods	None	Flagella
	Sporocytophaga	*S. myxococcoides*	-	Rods	Spore[c]	Gliding
	Rhodothermus	*R. marinus*		Rods		
	Streptomyces	*S. reticuli*	+	Filamentous rods	Spore[c]	Nonmotile
	Thermobifida	*T. fusca*	+	Filamentous rods	Spore[c]	Nonmotile

Table 2.2 (cont) Major morphological and physiological features of cellulolytic bacteria (Lynd et al., 2002)

Relationship to oxygen	Genera	Representative species[a]	Gram reaction	Cell form	Resting state	Movement
Anaerobic	*Acetivibrio*	*A. cellulolyticus*	-	Curved rods	None	Nonmotile
	Anaerocellum	*A. thermophilum*	+	Rods	None	Flagella
	Butyrivibrio	*B. fibrisolvens*	+	Curved rods	None	Flagella
	Caldicellulosiruptor	*C. saccharolyticum*	-	Rods	None	Flagella
	Clostridium	*C. thermoellum,*	+	Rods	Endospore	Flagella
		C. cellulolyticum				
	Eubacterium	*E. cellulosolvens*	+	Rods	None	Nonmotile
	Fervidobacterium	*F. islandicum*	-	Rods	None	Flagella
	Fibrobacter	*F. succinogenes*	-	Rods	None	Nonmotile
	Halocella	*H. cellulolytica*	-	Rods	None	Flagella
	Ruminococcus	*R. albus, R. flavefaciens*	+	Cocci	None	Nonmotile
	Spirochaeta	*S. Thermophila*	+	Spiral	None	
	Thermotoga	*T. neapolitana*	-	Rods		

[a] Not all strains of the indicated species are cellulolytic, and some less active or less studied celluloytic species within these genera are not listed.

[b] Most strains can also grow anaerobically.

[c] Unlike true endospores, these spores have only moderate resistance to environmental stress.

3. Industrial applications

Cellulases are an environmental friendly means for utilization of cellulose which is one of the most abundant organic molecules on the earth. The following is a list of processes that cellulases are used:

-Food Processing: The cellulase is used in juice preparation process, by adding to the leftover pulp after crushing. This method increases the yield of the juice.eventing further contamination because they can perform the juicing process at higher temperatures (Bhat, 2000).

-Textile Processes: In detergeants for keeping color brightness for longer. After numerous washings, clothes tend to have a faded and fuzzy look. Cellulases are added to detergents to decrease the discoloration and fuzzing effects caused by numerous washes. Thermophilic cellulases are added to detergents to create stone washed look in jeans (Haki and Rakshit, 2003; Vielle and Zeikus, 2001; Csiszar *et al.*, 2001).

-Paper Processing: Cellulase pretreatment of pulp improves the mechanical properties of the wood fiber, leading eventually to better paper quality (Muzariri *et al.*, 2001).

- Fuel Ethanol Production: Cellulases are used to saccharify cellulose in lignocellulosic biomass, such as agricultural and forestry residues to glucose. Then the glucose is converted to ethanol or other products (Dartmouth college, 2007).