CHAPTER II

LITERATURE REVIEW

Lignocellulose, a plant cell wall component, is one of the most abundant renewable terrestrial organic molecules in nature. It comprises of average 40% cellulose, 33% hemicellulose and 23% lignin by dry weight (Sa-pereira *et al.*, 2002).

Agricultural and Agro-industrial wastes are a plentiful and low cost lignocellulosic sources. Since cellulose is an unbranched linear polymer of glucose molecule with β-1, 4-linkes (Bok *et al.*, 1998) and hemicellulose is a short, branched-chain heteropolysaccharide of mixed pentosans and hexosans (Biesterveld *et al.*, 1994). Therefore, degradation of the cellulose and the hemicellulose results in a monomeric sugars which can be reserved as substrates for several industrial chemicals and fuels such as ethanol.

2.1 Cellulose

Cellulose is a linear homopolymer of β-1, 4-linked D-glucose residues. Naturally occurring cellulosic compounds are structurally heterogenous and have both amorphous and highly ordered crystalline regions. The degree of crystallinity varies with the source of the cellulose, and the more crystalline regions are resistant to enzymatic hydrolysis (Bok *et al.*, 1998). Figure 2.1 showed structure of the cellulose chain and the cellulose crystal.

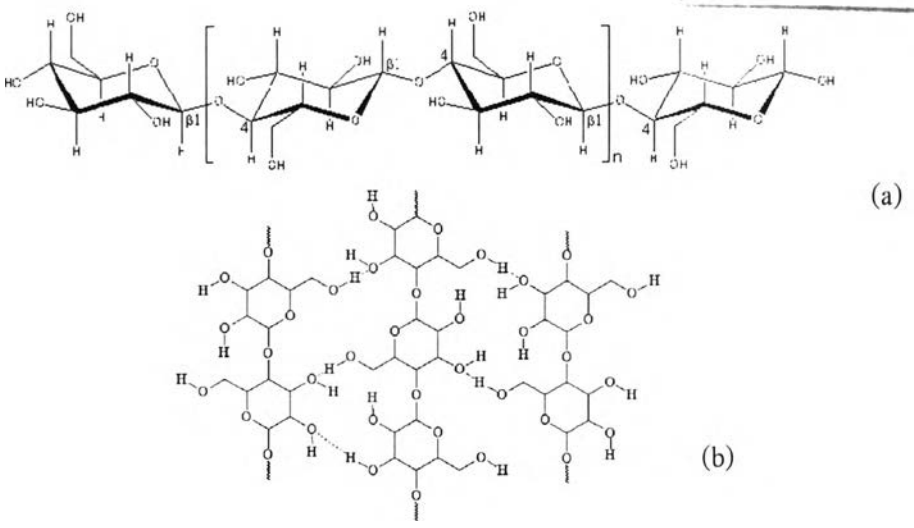

Fig. 2.1 Cellulose structure: cellulose chain (a), cellulose crystal showing hydrogen bonding within and between cellulose chain (b). (Harjunpaa, 1998).

The cellulose is hydrolysed by an enzymes which recognize β-1, 4-glycosidic linkages ; these enzymes are encoded by cellulase gene families (Béguin *et al.*, 1990).

2.2 Cellulase

Cellulase is a group of enzymes which hydrolyse β-1, 4-glycosidic linkages in cellulose. They consist of endo-β-glucanase (1,4-β-D-glucan-4-glucanohydrolase [E.C. 3.2.1.4]), exoglucanases (1,4-β-D-glucan cellobiohydrolase [E.C. 3.2.1.91]), glucan glucohydrolase (1,4-β-D-glucan glucohydrolase [E.C. 3.2.1.74]), and β-glucosidases (β-D-glucoside glucohydrolase [E.C. 3.2.1.21]). Endoglucanase randomly hydrolyze internal glycosidic linkages, which results in rapid decrease in polymer length and a gradual increase in the reducing sugar concentration. Exoglucanase hydrolyze cellulose chain by removing cellobiose either from the reducing ends or the nonreducing ends, which results in rapid release of reducing sugar but little change in polymer length. Glucose is produced primarily by the action of β-glucosidases on cellobiose and by the action of glucan glucohydrolase on cellooligomers (Bok *et al.*, 1998). β-glucosidases activity is under feed back inhibition of glucose which leads to an accumulation of cellobiose. The accumulation of cellobiose sequentially inhibits cellobiohydrolase (Harjunpaa, 1998). Cellulases and their sites of action on cellulose chain are shown in Figure 2.2.

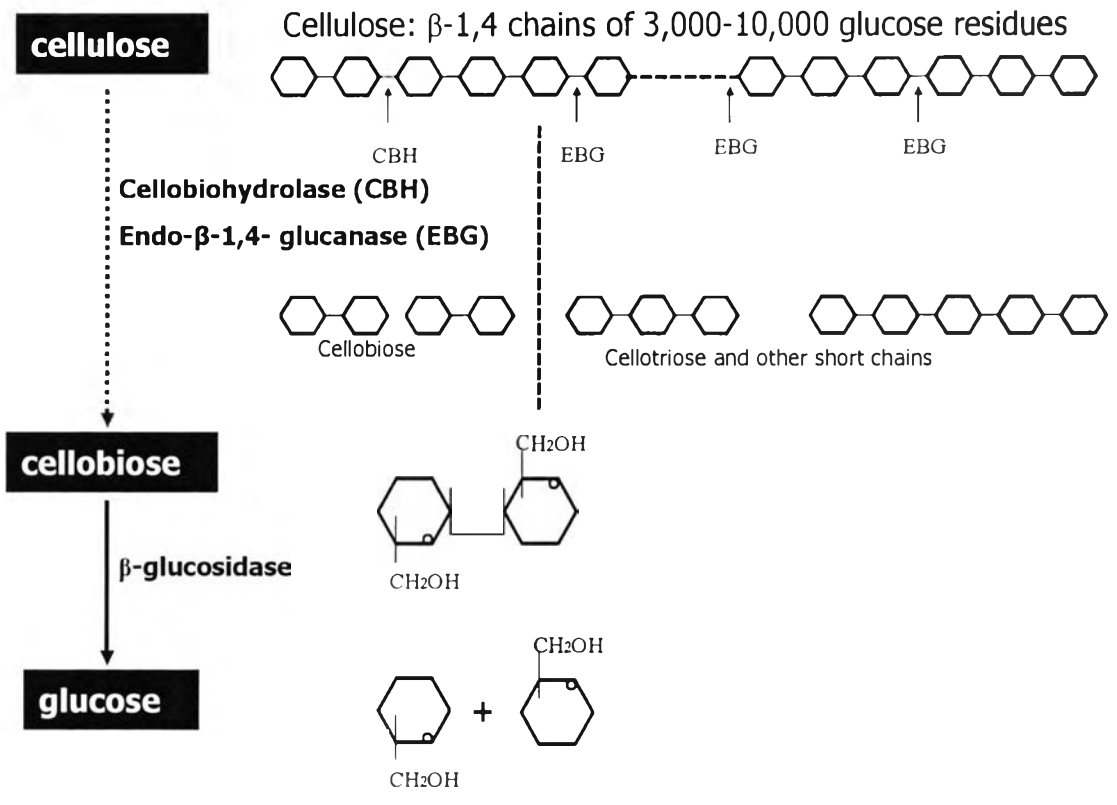

Fig. 2.2 Cellulases and their sites of action on cellulose chain (Deacon, 1997).

Cellulases are an environmental friendly means for cellulose utilization. They are used in many industrial processes such as food, paper, textile and ethanol fuel productions. Cellulases have been added to leftover fruit pulp after crushing to increase juice in a fruit juice industry (Bhat, 2000). Pretreatment of paper pulp by the cellulases improves mechanical properties of wood fiber which leads to better quality of paper (Muzariri *et al.*, 2001). Addition of the cellulases into detergents decreases a discoloration and fuzzing effects caused by numerous washing of textile in detergent industry (Haki and Rakshit, 2003). Hydrolysis of cellulose to glucose and fermentation of the cellulosic hydrolyzate was mostly in connection with ethanol fuel production. Several bacteria were found to produce cellulase.

2.3 Cellulase producing bacteria

Cellulase producing bacteria were isolated from various habitats and from both aerobic and anaerobic conditions as shown in Table 2.1 (Lynd *et al.*, 2002).

Table 2.1 Cellulase producing bacteria (Lynd *et al.*, 2002)

Conditions	Bacteria	Gram reaction	Representative species[a]
Aerobic	*Acidothermus*	+	*A. cellulolyticus*
	Bacillus	+	*B. brevis*, *B. pumilus*,
			B. agaradhaerans, *B. subtilis*
	Caldibacillus	+	*C. cellovorans*
	Cellulomonas[b]	+	*C. flavigena*, *C. uda*
	Cellvibrio	-	*C. flavus*, *C. gilvus*
	Cytophaga	-	*C. hutchinsonii*
	Erwinia	-	*C. carotovora*
	Micromonospora	+	*M. chalcae*
	Pseudomonas	-	*P. fluorescens var. cellulose*
	Sporocytophaga	-	*S. myxococcoides*
	Rhodothermus	-	*R. marinus*
	Streptomyces	+	*S. reticuli*
	Thermobifida	+	*T. fusca*
Anerobic	*Acetivibrio*	-	*A. cellulolyticus*
	Anaerocellum	+	*A. thermophilum*
	Butyrivibrio	+	*B. fibrisovens*
	Caldicellulosiruptor	-	*C. saccharolyticum*
Anaerobic	*Clostridium*	+	*C. thermoellum*,
			C. cellulolyticum
	Eubacterium	+	*E. cellulosovens*
	Fervidobacterium	-	*F. islandicum*
	Fibrobacter	-	*F. succinogenes*
	Halocella	-	*H. cellulolytica*
	Ruminococcus	+	*R. albus*, *R. flavefaciens*
	Spirochaeta	+	*S. thermophila*
	Thermogota	-	*T. neapolitana*

[a] Not all strains of the indicated species are cellulolytic.

[b] Most strains can also grow anaerobically.

Cellulose is digested by synergistic action of endoglucanase, cellobiohydrolase and β-glucosidase. Generally cellulolytic bacteria produce a variety of endoglucanases, but with few exceptions cellobiohydrolase is not a common feature of bacterial cellulase systems (Béguin *et al.*, 1990).

A distinction between cellulase of aerobic and anaerobic bacteria is that the former is secreted into culture filtrate, while the later cellulase resides in a large structure complex, cellulosome (Ferreira *et al.*, 1991).

2.4 Bacterial cellulase characteristics

In general, optimal temperature of bacterial cellulase activity ranged from 40-65 ° C. However, there are some reports on cellulolytic extreme thermophiles.

Sissons *et al* (1987) isolated extreme thermophilic cellulase producing bacteria from thermal-pool sites in New Zealand by avicel enrichment culture at 75 ° C. Five best isolates showed two distinct characteristics: 1) accumulation of reducing sugar in growth medium and giving free cellulase with avicellase activity, 2) no accumulation of reducing sugar in the medium and giving free cellulase with no avicellase activity. All of these 5 isolates were obligate anaerobic nonsporeforming rod which stained Gram negative, grew on pentose as well as hexose, and gave ethanol and acetate as major fermentation end products. The most active and stable cellulase producer was *Caldocellum saccharolyticum*. Its carboxymethyl cellulase activity was vary stable at 85 ° C and avicellase activity was relatively unaffected by Triton x-100, EDTA and dithiothreitol.

Bok *et al* (1998) purified 2 thermostable endocellulases, Cel A and Cel B, from *Thermotoga neapolitana*. The Cel A (29 kDa) had a broader optimal pH range (6.0-6.6) at 106 °C. The major end products, glucose and cellobiose, competitively inhibited Cel A and Cel B. Cel B preferentially cleaved larger cellooligomers, producing cellobiose as the end product. The enzymes was highly thermostable and had half life of 130 min at 106 ° C and 26 min at 110 ° C. Cel A and Cel B encoding gene were cloned in *Escherichia coli*. *Cel A* gene encode 257 amino acids protein, while *Cel B* gene encoded 274 amino acids protein. Both gene were inducible by cellobiose and repressed by glucose.

In nature where 80% of the biosphere and 90% of the marine environment have temperature below 5 ° C. Cellulose hydrolysis in these cold area are responsible by psychrophilic

and psychrotolerant microorganisms. Some obvious applications of psychrophilic cellulase are detergent additive in cold-wash, and stone-washing and biopolishing in textile industry. An advantage of using the psychrophilic cellulase in the above processes is rapidly and economically terminated by heating.

In 2003, Akila and Chandra isolated psychrotrophic *Clostridium* from a cold-adapted cattle manure biogas digester at 15 ° C. Growth temperature of the *Clostridium* ranged from 5-50 ° C with highest specific growth rate at 20 ° C. Extracellular hydrolytic enzymes produced were endoglucanase, β-glucosidase, filter paper cellulase, xylanase and β-xylosidase, all of which had maximal activity at 20 ° C.

Bacterial cellulase have a wide range of pH optima, acid to alkaline pH, while those of fungi are strictly in an acidic range (4.5-6.0). But study of bacterial cellulase lagged far behind that of fungal enzymes. This is due to the fact that most bacterial cellulase hydrolyze synthetic carboxymethyl cellulose but barely hydrolyze crystalline form of cellulose. However, alkaline bacterial cellulase had proved to be industrially useful as detergent additives. Since, the cellulase removed soil trapped in the amorphous region of cotton fibers by β-1, 4-glycosidic bond cleavage without damaging of cotton fabric (Hoshino and Ito, 1997). Serveral alkaline bacterial cellulases were reported: *Bacillus* sp. (Eudo *et al.*, 2001), *B. circulans* (Hakamada *et al.*, 2002), *B. agaradhaerans* (Rees *et al.*, 2003) and *Paenibacillus* sp. (Ogawa *et al.*, 2007). Their optimal pH ranged from 6-8.5.

Rees *et al* (2003) isolated novel cellulase gene by direct cloning of environmental genomic DNA. This is an approach to increase a genetic biodiversity pool of non cultivable microorganisms.

Singh *et al* (2004) isolated a novel strain of *Bacillus sphaericus* producing thermostable alkaline carboxymethyl cellulase (CMCase) from soil by using medium at pH 9.5. The purified CMCase was characterized and found to be multimeric protein.

2.5 Xylanase

Xylan, the most abundant hemicellulose, is a heteropolysaccharide with homopolymeric backbone chain of β-1, 4D-xylopyranose. The xylan backbone is 80% substituted with monomeric side-chains of arabinose or glucuronic acid linked to xylose, and also with oligomeric side chains containing arabinose, xylose, and sometimes galactose (Fig. 2.3). The frequency and composition of branches are depend upon xylan source. However, unsubstituted xylan has been isolated from guar seed husk, esparto grass and tobacco stalk (Saha, 2003).

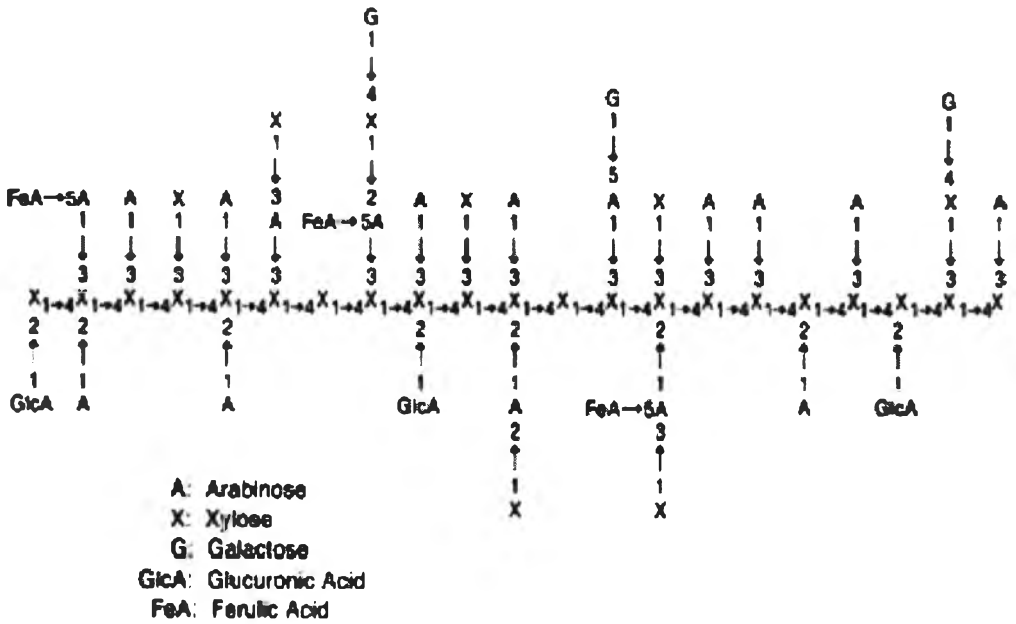

Fig. 2.3 Structure of corn fiber xylan (Saha, 2003)

Several hydrolytic enzymes with diverse specificity and mode of action are required to complete hydrolysis of xylan which is heterogeneity and complex structure. Xylan degrading enzymes are usually composed of the following hydrolytic enzymes : β-1,4-endoxylanase (1, 4-β-D-xylan xylohydrolase, E.C. 3.2.1.8), β-xylosidase (1, 4-β-D-xylan xylohydrolase, E.C. 3.2.1.37), α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, and phenolic acid (ferulic and *p*-coumaric acid) esterase (Fig. 2.4). These enzymes hydrolyze xylan cooperatively into its constituent sugars.

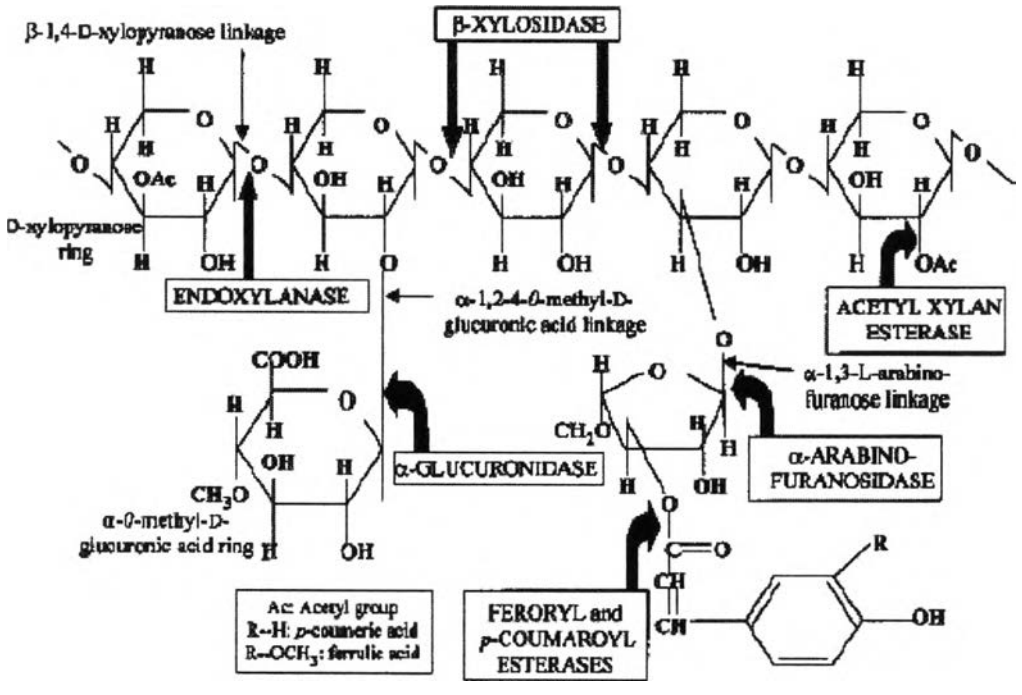

Fig. 2.4 Xylan degrading enzymes and their sites of action on xylan structure (Beg *et al.*, 2001)

Microorganisms including bacteria, actinomycetes, yeast and fungi produce xylan degrading enzymes as shown in Table 2.2.

Table 2.2 Xylanase producing microorganisms and their xylanase characteristics (Beg *et al.*, 2001)

Microorganism	Molecular Weight (kDa)	Optimum		Stability		pI	K_m (mg/ml)	V_{max} (μM/mine per mg)
		pH	Tempera-ture (°C)	pH	Tempera-ture (°C)			
Bacteria								
Acidobacterium capsulatum	41	5	65	3-8	20-50	7.3	3.5	403
Bacillus sp. W-1	2.5	6	65	4-10	40	8.5	4.5	-
Bacillus circulans WL-12	15	5.5-7	-	-	9.1	4	-	-
Bacillus stearothermophilus T-6	43	6.5	55	6.5-10	70	7.9	1.63	288
Bacillus sp. strain BP-23	32	5.5	50	9.5-11	55	9.3	-	-
Bacillus sp. strain BP-7	22-120	6	55	8-9	65	7-9	-	-
Bacillus polymyxa CECT 153	61	6.5	50	-	-	4.7	17.1	112
Bacillus sp. strain K-1	23	5.5	60	5-12	50-60	-	-	-
Bacillus sp. NG-27	-	7, 8.4	70	6-11	40-90	-	-	-
Bacillus sp. SPS-0	-	6	75	6-9	85	-	-	-
Bacillus sp. strain AR-009	23, 48	9-10	60-75	8-9	60-65	-	-	-
Bacillus sp. NCIM 59	15.8, 35	6	50-60	7	50	4, 8	1.58, 3.50	0.017, 0.742
Cellulomonas fimi	14-150	5-6.5	40-45	-	-	4.5-8.5	1.25-1.72	-
Cellulomonas sp. N.C.I.M. 2353	22, 33, 53	6.5	55	-	-	8	1.7, 1.5	380, 690
Micrococcus sp. AR-135	56	7.5-9	55	6.5-10	40	-	-	-
Staphylococcus sp. SG-13	60	7.5, 9.2	50	7.5-9.5	50	-	4	90
Thermoanaerobacterium sp. JW/SL-YS 485	24-180	6.2	80	-	-	4.37	3	-
Thermotoga maritime MSB8	40, 120	5.4, 6.2	92-105	-	-	5.6	1.1, 0.29	374, 4760
Fungi								
Acrophialophora nainiana	17	6	50	5	50	-	0.731, 0.343	-
Aspergillus niger	13.5-14.0	5.5	45	5-6	60	9	-	-
Aspergillus kawachii IFO 4308	26-35	2-5.5	50-60	1-10	30-60	3.5-6.7	-	-
Aspergillus nidulans	22-34	5.4	55	5.4	24-40	-	-	-
Aspergillus fischeri Fxn1	31	6	60	5-9.5	55	-	4.88	5.88
Aspergillus sojae	32.7, 35.5	5, 5.5	60, 50	5-8, 5-9	50, 35	3.5, 3.75	-	-

Table 2.2 (cont.) Xylanase producing microorganisms and their xylanase characteristics (Beg *et al.*, 2001)

Microorganism	Molecular Weight (kDa)	Optimum		Stability		pI	K_m (mg/ml)	V_{max} (μM/mine per mg)
		pH	Tempera-ture (°C)	pH	Tempera-ture (°C)			
Aspergillus sydowii MG 49	30	5.5	60	-	-	-	-	-
Cephalosporium sp.	30, 70	8	40	8-10	-	-	0.15	-
Fusarium oxysporum	20.8, 23.5	6	60, 55	7-10	30	-	9.5; 8.45, 8.7	0.41, 0.37
Geotrichum candidum	60-67	4	50	3-4.5	45	3.4	-	-
Paeciomyces varioti	20	4	50	-	-	5.2	49.5	-
Penicillium purpurogenum	33, 23	7, 3.5	60, 50	6-7.5, 4.5-7.5	40	8.6, 5.9	-	-
Thermomyces lanuginosus DSM 5826	25.5	7	60-70	5-9	60	4.1	7.3	-
Thermomyces lanuginosus -SSBP	23.6	6.5	70-75	5-12	60	3.8	3.26	6300
Trichoderma harziamum	20	5	50	-	40	-	0.58	0.106
Trichoderma reesei	20, 19	5-5.5, 4-4.5	45, 40	3-8.5, 2.5-8.5	-	9, 5.5	3-6.8, 14.8-22.3	-
Yeast								
Auriobasidium pullulans Y-2311-1	25	4.4	54	4.5	55	9.4	7.6	2650
Cryptococcus albidus	48	5	25	-	-	-	5.7, 5.3	-
Trichosporon cutaneum SL409	-	6.5	50	4.5-8.5	50	-	-	-
Actinomycete								
Streptomyces sp. EC10	32	7-8	60	-	-	6.8	3	-
Streptomyces sp. B-12-2	23.8-40.5	6-7	55-60	-	-	4.8-8.3	0.8-5.8	162-470
Streptomyces T7	20	4.5-5.5	60	5	37-50	7.8	10	7610
Streptomyces thermoviolaceus OPC-520	33, 54	7	60-70	-	-	4.2, 8	-	-
Streptomyces chattanoogensis CECT 3336	48	6	50	5-8	40-60	9	4, 0.3	78.2, 19.1
Streptomyces viridisporus T7A	59	7-8	65-70	5-9	70	10.2-10.5	-	-
Streptomyces sp. QG-11-3	-	8.6	60	5.4-9.2	50-75	-	1.2	
Thermomonospora curvata	15-36	6.8-7.8	75	-	-	4.2-8.4	1.4-2.5	

As shown in Table 2.2, most fungal xylanases are less thermostable than bacterial xylanases and have pH optima in acid range (4.5-5.5), while several bacterial xylanases showing alkaline pH optima have been reported.

Xylanases have significant current and potential uses for several industries (Beg *et al.*, 2001, Yang *et al.*, 1995, Kusakabe *et al.*, 1975) including

1) Paper and pulp (biobleaching of cellulosic pulp)
2) Textile (cellulose recovery from dissolving pulp)
3) Feed (pretreatment of forage crops to improve the digestibility of ruminants)
4) Chemicals (ethanol, xylitol, xylobiose, xylose *etc* from lignocellulosic biomasses)
5) Food (clarification of must and juices, xylooligosaccharide, food sweetener, production)
6) Waste treatment (saccharification of agricultural, industrial and municipal wastes).

2.6 Xylanase producing bacteria and their xylanase

Some recent reports on bacterial xylanase are listed below.

Kim *et al.* (2000) isolated cellulase free xylanase producing thermophilic actinomycetes from poultry faeces. The isolate B19 was assigned to the genus *Streptomyces* on the basis of chemotaxonomic and morphological criteria. Based upon almost complete 16S rRNA gene sequence, the isolate B19 was classified as *Streptomyces thermocoprophilus* sp. nov.

Sa-pereira *et al.* (2002) isolated xylanase producing *Bacillus subtilis* from hot-spring. Maximum xylanase produced was 12 units/ml when using oat spelt xylan, xylanase inducer, as carbon source at pH 6.0, 50 °C for 18 h. Under the optimized condition, no cellulase activity was detected. Xylanase production decreased as function of time but when trehalose was used as carbon source the xylanase production maintained constant for at least 80 hrs. Optimal pH and temperature for activity were 6.0 and 60 °C, respectively. The xylanase was completely stable at 60 °C for 3 hrs. DTT was found to enhance the activity about 2.5 fold.

Kopecny *et al.* (2003) reported novel xylanase producing *Pseudobutyrivibrio xylanivorans* sp. nov. (DSM 14809^{T}, ATCC BAA-455^{T}) isolated from rumen fluid of cow. It was Gram-negative, anaerobic, non sporeforming, curved rods, motile by means of single polar. DNA G+C content was 42.1 mol%.

Roy (2004) isolated xylanase producing *Bacillus* from soil in Bangladesh. The *Bacillus* produced maximum xylanase (55 units/ml) when grown in oat spelt xylan medium. High xylanase was also detected when oat spelt xylan was replace by wheat bran. Optimal pH and temperature of the xylanase were 6-7 and 50 $^{\circ}$C, respectively.

Roy and Uddin (2004) isolated xylanase producing, *Paenibacillus* from soil in Bangladesh. The *Paenibacillus* produced xylanase (MW 48 kDa) which had optimal pH and temperature at 7.0 and 55 $^{\circ}$C.

Virupakshi *et al.* (2005) isolated thermostable alkaline xylanase producing *Bacillus* from sugarcane molass. Xylanase production from various agricultural wastes (wheat bran, rice bran, sugarcane bagasse, ragi husk, gram bran, corncob) by solid-state fermentation was studied. Maximal xylanase was produced in rice bran moistened with mineral salt solution at 50 $^{\circ}$C for 72 h. Yeast extract, beef extract and xylan enhanced enzyme production, while glucose, lactose and fructose strongly repressed the enzyme production.

Sånchez *et al.* (2005) reported novel xylanase producing *Paenibacillus barcinonensis* sp. nov. (CECT 7022^{T}, DSM 15478^{T}) isolated from a rice field in Spain. It was Gram-positive, endospore forming. The strain was found to have MK-7 as predominant menaquinone and anteiso-$C_{15:0}$ as major fatty acid. It grew at 10-40 $^{\circ}$C and in the presence of lysozyme or 5% (w/v) NaCl.

Xu *et al.* (2005) isolated an alkali-tolerant xylanase producing *Pseudomonas* from an effluent of a pulp and paper mill in China. The isolate was consecutively mutagenized by UV radiation and NTG. Mutant selected, *Pseudomonas* WLUNO 24, produced maximum xylanase (1,245 units/ml) after incubating at 37 $^{\circ}$C for 24 hrs. The xylanase (25.4 kDa) was alkali-tolerant

(optimum pH 7.2-8.0) and cellulase free. It degraded xylan to xylo-oligosaccharides, 80% of which were xylobiose and xylotriose.

Ninawe *et al.* (2006) screened 20 soil samples collected from different locations in India for xylanase producing actinomycetes. Eighty-eight actinomycetes were isolated, and among these, 69 isolates were xylanase producing strains. Three isolates (SN32, SN77 and SN83) produced > 125 IU/ml xylanase. The isolates were identified as *Streptomyces cyaneus* (SN32), *S. tendae* (SN77) and *S. caclastis* (SN83).

Based upon primary sequence classification scheme described by Henrissat and Bairoch (1993), xylanase have been assigned to families 10 and 11 of the glycosyl hydrolases. The two families differ significantly in molecular mass, isoelectric points, substrate preferences, and oligoxylosidase generated as products. Members of family 10 have molecular mass of approximately 48 kDa, which member of family 11 are generally much smaller with molecular mass ranging from 19 to 31 kDa.

Hurlbert and Preston (2001) reported functional characteristics of novel xylanase (Xylanase A) produced by *Erwinia chrysanthemi* D1 isolated from corn. The xylanase A was found to have molecular mass of 42 kDa, isoelectric point of 8.8, and optimal pH and temperature of 6 and 35 $^{\circ}$C. The enzyme was still active at temperature higher than 40 $^{\circ}$C and pH of up to 9.0. It was most active on xylan substrates with low ratio of xylose to 4-o-methyl-D-glucuronic acid. Mode of action was unique with no internal cleavages of the xylan backbone between substituted xylose residues.

Usui *et al.* (2003) reported that xylanase (Xyn X) produced by *Aeromonas caviae* ME-1 was novel xylanase. Because the Xyn X exhibited extremely unusual product specificity compared to general endo-type xylanase. The Xyn X (38 kDa in family 10) was an intracellular enzyme which hydrolyzed xylan to xylobiose and xylotetrose. The enzyme was released from the cytoplasm to periplasm during osmotic down shock growth.

Among aerobic bacteria reported, member of the genus *Bacillus* and *Paenibacillus* are well known as cellulase and xylanase producing bacteria. While there were few reports on *Cohnella* because it was first classified in 2006 by Kämpfer *et al.*

2.7 Characteristics of *Bacillus*, *Paenibacillus* and *Cohnella*

Bacillus

Bacillus sp. is a genus of rod-shaped, Gram-positive bacteria and a member of the division Firmicutes. They are either obligate or facultative aerobes, and test positive for the enzyme catalase (Turnbull, 1996). Ubiquitous in nature, they include both free-living and pathogenic species. Under stressful environmental conditions, the cells produce oval endospores that can stay dormant for extended periods. These characteristics originally define the genus *Bacillus*, but not all such species are closely related, and many have been moved to other genera (Madigan and Martinko, 2005). It commonly found in soil. An easy way to isolate *Bacillus* is by placing non-sterile soil in a test tube with water, shaking, placing in melted Mannitol Salt Agar, and incubating at room temperature for at least a day. Colonies are usually large, spreading and irregularly-shaped. Under the microscope, the *Bacillus* appears as rods, and a substantial portion usually contains an oval endospore at one end, making it bulge. The cell wall of *Bacillus* is a rigid structure on the outside of the cell that forms the first barrier between the bacterium and the environment, and at the same time maintains cell shape and withstands the pressure generated by the cell's turgor. The cell wall is composed of peptidoglycan, teichoic and teichuronic acids. *B. subtilis* is the first bacterium for which the role of an actin-like cytoskeleton in cell shape determination and peptidoglycan synthesis is identified and for which the entire set of peptidoglycan synthesizing enzymes are localised (Scheffers, 2007). The organisms of these genera are characterized by the presence of DAP in the cell wall, by having major menaquinone (MK-7) , and by G+C contents of 37-47 mol% (Takeuchi and Hatano, 1998) (Table 2.3)

Table 2.3 Characteristics of *Bacillus* species (Venkateswaran *et al.*, 2003)

Test	*B. subtilis* IAM 1026^T	*B. pumilus* ATCC 7061^T	*B. circulans* ATCC 4513^T	*B. firmus* ATCC 14575^T
Enzyme production :				
β-Galactosidase	-	-	+	+
Arginine dihydrolase	-	-	-	-
Cytochrome oxidase	+	+	-	-
Acetoin production	-	+	-	-
Gelatin liquefication	+	-	-	-
Utilization of :				
Mannitol	-	-	+	+
Amygdalin	-	+	+	-
Fermentation of :				
Glycerol	+	+	+	+
Ribose	+	+	-	-
D-Xylose	-	+	+	-
Adonitol	+	-	-	-
Galactose	-	+	+	-
Rhamnose	-	-	-	-
Inositol	+	+	+	-
Sorbitol	+	-	+	-
N-Acetylglucosamine	-	+	+	+
Lactose	-	+	-	-
Melibiose	+	+	+	-
Melezitose	-	-	+	-
Raffinose	+	-	-	-
Strach	+	-	+	-
Glycogen	+	-	+	-
Gentiobiose	+	+	+	-
D-Turanose	+	+	+	+
D-Lyxose	-	-	+	-

Table 2.3 (cont.) Characteristics of *Bacillus* species (Venkateswaran *et al.*, 2003)

Test	*B. subtilis* IAM 1026^T	*B. pumilus* ATCC 7061^T	*B. circulans* ATCC 4513^T	*B. firmus* ATCC 14575^T
D-Tagalose	-	+	-	-
D-Arabitol	-	-	-	-
Gluconate	-	-	+	-
2-Ketogluconate	-	-	-	-

Paenibacillus

The genus *Paenibacillus* was defined in 1993 after an extensive comparative analysis of 16S rRNA gene sequences of 51 species of the genus *Bacillus* (Ash *et al.* 1991; 1993). Members of the genus *Paenibacillus* are aerobic or facultatively anaerobic organisms that produce ellipsoidal endospores in swollen sporangia and the cell wall show structure typical of Gram-positive bacteria, but sometimes stains are Gram-negative. *Paenibacillus* was reported as cellulase producing (Sánchez *et al.*, 2004) and as xylanase producing such as *Paenibacillus faviporus* (Velazquez, 2004), *Paenibacillus xylanilyticus* (Rivas *et al.*, 2005) and *Paenibacillus barcinonensis* (Sánchez *et al.*, 2005). The DNA G + C content ranges from 39 to 54 mol%. Anteiso-$C_{15:0}$ is the major cellular fatty acid (Shida *et al.*, 1997). *Paenibacillus cineris*, an aerobic endospore-forming bacteria, contains MK-7 as major menaquinone (Table 2.4).

Table 2.4 Characteristics of *Paenibacillus* species (Horn *et al.*, 2005)

Strains: 1, *P. ginsengisoli* Gsoil 1638^T ; 2, *P. anaericanus* KACC 11533^T ; 3, *P. nematophilus* DSM 13559^T ; 4, *P. chibensis* JCM 9905^T ; 5, *P. borealis* DSM 13118^T ; 6, *P. cooki* LMG 18419^T ; 7, *P. cineris* LMG 18439^T ; 8, *P. favisporus* LMG 20987^T ; 9, *P. rhizosphaerae* LMG 21955^T ; 10, *P. azotofixans* DSM 5976^T ; 11, *P. macquariensis* CIP 103269^T ; 12, *P. wynnii* LMG 22176^T ; 13, *P. antarcticus* LMG 22078^T ; 14, *P. polymyxa* DSM 36^T .

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Gram staining	+	-	-	+	-	-	-	+	+		-	-	+	+
Oxidase	+	+	-	-	-	+	+	+	+	-	-	-	+	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ONPG test	(+)	+	-		-	+	+	+	+	ND	-	+	-	+
Voges Proskauer test	+	-	+	+	-	+	-	-	+	+	-	-	-	+
Growth at/in														
3% (w/v) NaCl	+	-	-	ND	(+)	+	+	+	+	ND	+	-	+	+
10% (w/v) NaCl	(+)-	-	-	-	-	-	-	-	-	-	-	-	-	-
5°C	-	+	-	-	+	-	-	ND	-	-	+	+	+	+
10°C	-	+	+	+	+	-	-	ND	+	-	+	+	+	+
37°C	-	+	+	+	+	+	+	+	+	+	-	-	-	-
pH5.6	+	+	-	+	+	+	+	+	(+)	+	-	-	ND	+
Hydrolysis of														
Casein	-	-	-	-	+	(+)	(+)	-	-	-	-	-	-	+
Starch	-	+	+	+	-	ND	ND	+	ND	-	+	+	+	+
Gelatin	-	-	-	-	-	-	-	+	-	-	-	-	-	+
Esculin	-	+	+	ND	+	+	+	ND	ND	ND	+	+	-	+
Utilization of														
N-acetyl-D-glucosamine	-	+	ND	ND	+	ND	ND	ND	+	-	ND	ND	ND	-
L-arabinose	+	-	-	ND	+	ND	ND	-	ND	-	ND	ND	ND	+
D-melibiose	+	+	-	ND	V	ND	ND	ND	ND	+	ND	ND	ND	+
D-galactose	+	+	-	ND	+	ND	ND	ND	ND	+	ND	ND	ND	+
D-xylose	-	+	-	ND	+	ND	ND	+	ND	-	ND	ND	ND	+
Acetate	-	+	ND	-	-	ND	ND	ND	ND	+	ND	ND	ND	-
Acid production from														
D-mannitol	-	-	-	+	+	-	+	+	+	+	+	+	-	+
D-sorbitol	-	-		-	+	-	(+)	ND	ND	+	-	+	-	+
D-sucrose	-	-	+	+	+	+	+	+	+	+	+	+	+	+
D-melibiose	-	-	-	+	+	+	+	+	+	+	+	+	+	+
Amygdalin	-	-	+	ND	+	+	+	ND	+	+	+	+	+	+
L-arabinose	(+)	-	-	+	+	+	+	ND	+	-	-	v	+	+
DNA G+C (mol%)	50.7	42.6*	44	52.8	53.6	51.6	51.5	53	50.9	48-53	39	44.6	40.7	43-46

Symbols : +, positive; (+), weakly positive; -, negative; ND, not determined

Cohnella

Novel taxa that closely associated with the genera *Bacillus* or *Paenibacillus*. In particular, "*Paenibacillus hongkongensis*" was recently reclassified as *Cohnella hongkongensis* into the novel genus *Cohnella* that currently also includes *Cohnella thermotolerans* as the type species. Members of the genus *Cohnella* are spore-forming, aerobic, nonmotile, and thermotolerant organisms that contain MK-7 as the main menaquinone and iso-C16:0, anteiso-C15:0, and C16:0 as major fatty acids (Yoon *et al.*, 2007) (Table 2.5)

Table 2.5 Characteristics of *Cohnella* species (Kämpfer *et al.*, 2006)

Strains: 1, *Cohnella panacarvi* Gsoil 349^T ; 2, *Cohnella thermotolerans* DSM 17683^T; 3, *Cohnella hongkongensis* DSM17642^T.

Characteristic	1	2	3
Nitrate reduction	+	ND	+
Oxidase	+	+	ND
Catalase	(+)	ND	(+)
Assimilation of			
N-Acetyl-D-glucosamine	-	-	+
Gluconate	-	+	+
D-Melibiose	-	+	+
L-Rhamnose	-	(+)	+
D-Ribose	-	+	+
Salicin	+	-	-
Acid production from			
L-Arabinose	+	-	-
D-cellobiose	+	-	-
Dulcitol	+	-	-
D-Glucose	+	-	-
D-Maltose	+	-	-
D-mannose	+	-	-
D-Melibiose	+	-	-
Methyl α-D- glucoside	+	-	-
D-Lactose	+	-	-
D-Raffinose	+	-	-
Sucrose	+	-	-
D-Trehalose	+	-	-
D-Xylose	+	-	-
DNA G+C content (mol%)	53.4	59.0	47.6

Symbols: +, positive; -, negative; (+), weakly positive; ND, not determined.