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Bacterial cellulases

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Cellulolytic bacteria play an important role in the biosphere by recycling cellulose. The cellulase systems they produce usually comprise a number of distinct enzymes, which can have specific activities comparable to those of cellulolytic fungi. The cellulase systems of certain bacteria may be found as discrete multi-enzyme aggregates. Many cellulolytic bacteria utilize a multi-enzyme complex that binds to and synergistically hydrolyzes cellulose substrates. This review is an attempt to summarize the available literature concerning applications and cellulolytic enzymatic systems of the bacterial genera *Bacillus*, *Clostridium* and *Cellulomonas* sp. The composition of complex cellulases produced by these organisms were examined at the enzyme level.

Cellulose is the major carbohydrate synthesized by plants. It serves as a vast reservoir of glucose units linked by β-1, 4 glucosidic bonds. Release of the bound glucose units from cellulosic wastes by cleavage of β-glucosidic bonds, known as saccharification facilitates the utilization of glucose depot in cellulosic wastes for production of food, fuel, single cell protein, feed stock synthesis of chemicals and value - added products. Development of processes for conversion of low-value product in wastes/ effluents to high value product has become necessary and the saccharification process of cellulose waste relies on participation of cellulolytic organisms and their cellulase enzymes (Singh and Hayashi, 1994; Beguin and Aubert, 1994). This process has not yet reached to the level of commercialization due to many factors complexity of cellulose structure, production of cellulases in low amounts by cellulolytic organisms due to carbon gene repression, high cost of cellulase production and poor yields of glucose (Gregg and Saddler, 1996; Ruijter and Visser, 1997).

Cellulase enzymes are derived from cellulolytic microorganisms.Bacterial cellulases have demonstrated variable activities and modes of action on cellulose hydrolysis. The use of cellulases with higher specific activities and those which are not feedback inhibited by the products of hydrolysis will also aid in reducing the overall costs of cellulose bioconversion.

The cellulolytic enzyme system of bacteria seems to be quite complex. Two extracellular and one cell-bound endoglucanase components were identified in bacterium, *Pseudomonas fluorescens va*. Cellulose (Yamanae *et al.,* 1970). Two tightly substrate-bound (designated as CB1 and CB 2) components were observed with another bacterium, *Cellulomonas fimi* (Beguin *et al.,* 1992). CB 1 and 2 were identified as an endoglucanase and exoglucanase. The enzymology of cellulose degradation is documented in several recent reviews (Robson and Chambliss, 1984; Wood, 1992; Kudanga and Mwenje, 2005; Coughlan, 1992b; Felix and Ljungdahl, 1993; Gilbert and Hazelwood, 1993; Singh and Hayashi, 1994; Beguin and Aubert, 1994; Bayer *et al.*, 1994; Leschine, 1995).

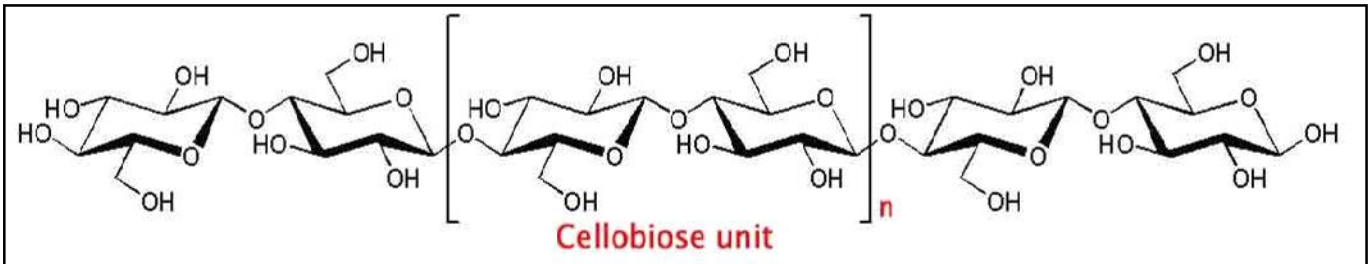


Fig. : Cellulose

Endoglucanases or Carboxy Methyl Cellulases (CMCases) (Endo-β-1,4-glucanase, EC 3.2.1.4) randomly

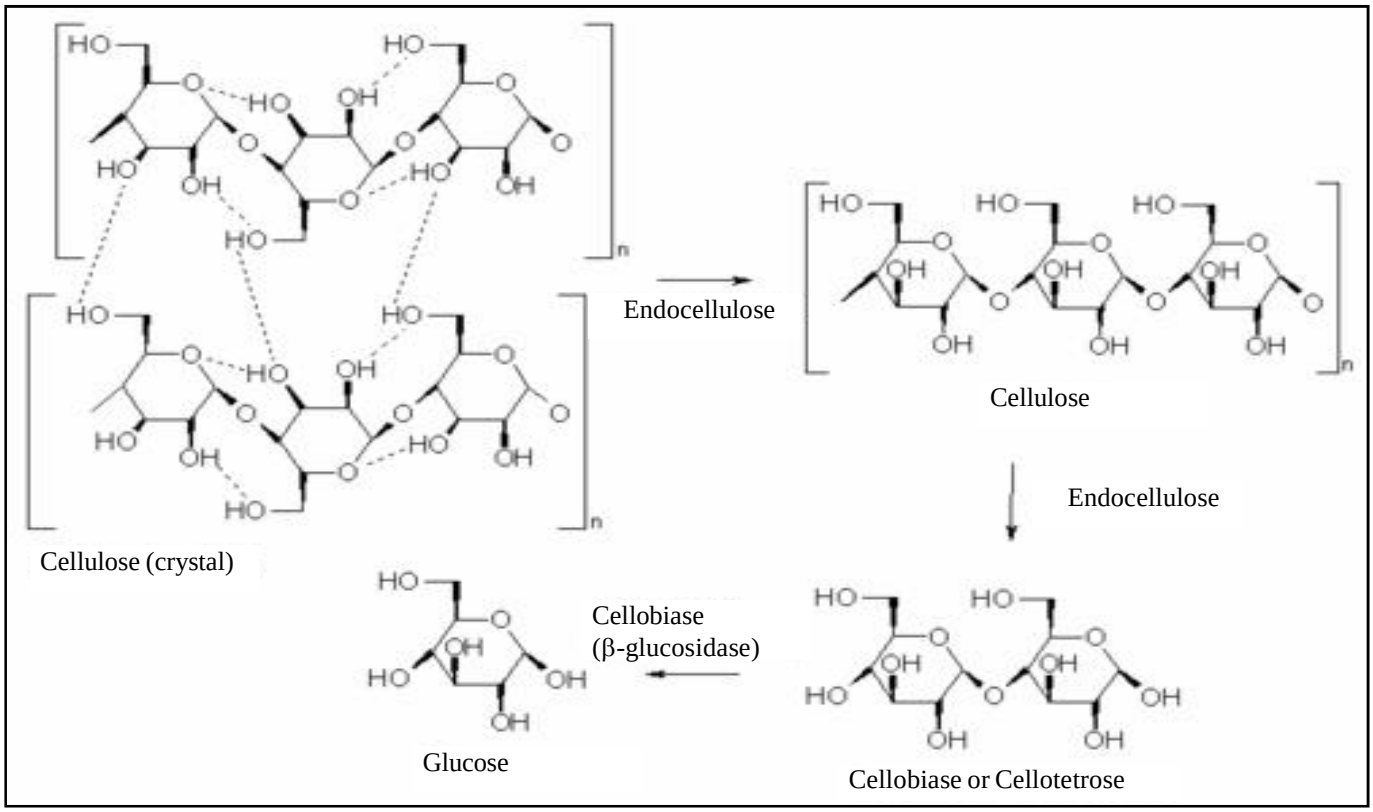
hydrolyse internal β-1, 4-glucosidic linkages within the cellulose chain resulting in the formation of gluco-oligosaccharides. Large number of reducing and non reducing ends are created in the oligosaccharides.

Exoglucanases or Cellobiohydrolases (Exo-1,4-β-glucanase EC 3.2.1.91) cleaves only external β-1, 4-glucosidic bonds from non-reducing end of cellulose and also oligosaccharides and split off glucose units. The distinction between exoglucanases and cellobiohydrolases is always not clear.

β-Glucosidases (β-D-glucoside glucohydrolase EC 3.2.1.21) hydrolyse cellobiose and low molecular weight dextrins to glucose.

These enzymes act in a synergistic or cooperative manner. The endoglucanase opens up linear cellulose molecules, producing reducing and non reducing ends that, in turn, can be attacked by exoglucanase. Exoglucanases, in turn, act to remove molecular strand of cellulose and expose more internal sites for endoglucanase binding. Activities of both of these enzymes, in particular, CBH are inhibited by cellobiose. The cleavage of cellobiose to glucose by β-glucosidase greatly reduces this inhibition and allows continued cellulolytic activity. This classical synergism between exoglucanase and endoglucanase has been extended to encompass a number of cellulolytic fungi (Henrissat *et al.*, 1985; Wood, 1992; Wood and McCrae, 1986; Wood *et al.*, 1994; Wood *et al.*, 1995). Even synergistic interactions (exo-exosynergism) between isozymic forms of exoglucanase (CBH I and CBH II) occurred in solubilising crystalline cellulose (Fagerstam and Pettersson, 1980; Henrissat *et al.*, 1985).

In contrast to cellulase systems of aerobic bacteria, cellulases of most anaerobic bacteria are organized into large, multiprotein complexes (Felix and Ljungdahl, 1993; Gilbert and Hazelwood *et al.*, 1993; Leschine, 1995). The most thoroughly investigated cellulase complex that of *Clostridium thermocellum*, was termed cellulosome by Lamed and Bayer (Lamed and Bayer, 1991). On the cell surface, these multiprotein, multifunctional enzyme appear as polycellulosomal aggregate and promote adherence of the bacterium to cellulose. Cellulosomes of *C. thermocellum* strains comprise 14-26 polypeptide subunits (Kohring *et al.*, 1990). The largest subunit, cellulosome integrating protein Cip A has cellulose binding domain and nine internal repeated docking sequence that bind catalytic subunits (Leschine, 1995). Cellulosome catalyzes multiple, nearly simultaneous cuttings of glucan chain (Felix and Ljungdahl, 1993). Such multiprotein cellulase complexes are produced by many diverse anaerobes Fibrobacter (Haigler and Weimer, 1991).



It should be noted, however, the cellulolytic bacteria also exist among the *Streptomyces* (Ishaque and Kluepfel, 1980; Kluepfel *et al.*, 1980; VanZyl, 1985; Mackenzie *et al.*, 1984; MacKenzie *et al.*, 1984), *Pseudomonas* (Wolff *et al.*, 1986; Lejeune *et al.*, 1988; Schell, 1987;Yamane, *et al.*, 1970), *Cellovibrio* (Wynne and Pemberton, 1986; Oberkotter and Rosenberg, 1978) and Microspora (Yablonsky *et al.*, 1988; Fogarty and Griffin, 1972). We have focused on the production of endo and exo-β-1, 4-glucanases and the measurements of specific activities of purified enzymes in standard assay systems where these have been reported. However, even measurement of specific activity on a widely used substrate such as CMC may vary according to the degree of substitution and the over all chain length of the substrate.

Bacillus species:

Members of the *Bacillaceae* are gram positive, endospore-forming bacteria which are known to secrete a wide variety of enzymes, including cellulases, amylases, proteases, β-glucanases and xylanases. *Bacillus* sp. produce several cellulases in which most of the enzyme is extracellular and a small amount is cell bound. The regulation of endo-β-l, 4-glucanase production in *Bacillus* sp. appears to vary from species to species and even from strain to strain.

The study of *Bacillus* cellulases has been greatly aided by the ease of genetic manipulation in the genus and because the genomes of several species have been relatively well characterized. These studies have also been facilitated by the fact that the cellulolytic enzymes of atleast for the anaerobic cellulolytic bacteria examined to date, crystalline cellulose degradation seems to occur by a different mechanism than in cellulolytic fungal systems. A variety of species secrete cellulases, including strains of *B.subtilis* (Robson and Chambliss, 1984; Koide *et al.*, 1986), *B.polymyxa* (Thayer, 1978), B.cereus (Dhillon *et al.*, 2000) and *B.licheniformis* (Thayer, 1978). Most of the better characterized bacterial cellulolytic systems were first studied at a biochemical level and are now being studied at the molecular level.

The endo-β-1,4-glucanases of *B.subtilis* strains generally exhibits thermal stabilities upto $50^0C \pm 5^0C$ although maximal activity is frequently observed at 60^0C. The optimal pH for cellulase activity is usually within the range pH 5.0-6.0 (Robson and Chambliss, 1984; Chan and Au, 1987; Park and Pack, 1986). The endo-acting nature of these glucanases has been confirmed in several studies (Chan and Au, 1987; Robson and Chambliss, 1984; Park and Pack, 1986). The endo-β-1, 4-glucanase produced by *B.subtilis* DLG is not inhibited by either glucose or cellobiose and is not able to significantly degrade crystalline cellulosic substrates. The specific activity of purified DLG endo-β-1,4-glucanase was determined to be 550 U TNP-CMCase mg^{-1} protein versus 300 U TNP-CMCase mg^{-1} protein for purified EGI of *T.reesei* QM9414. The endo-β-1,4-glucanases produced by alkalophilic *Bacillus* strains have pH optima of 9 and 10 for strain 1139 and N-4, respectively (Fukumori *et al.*, 1985; Horikoshi *et al.*, 1984). Both the endo-β-1,4-glucanases produced by strain N-4 and the single endoglucanase of strain 1139 are capable of hydrolyzing cellotetraose and cellotriose in addition to CMC and neither can degrade avicel significantly.

Cellulases of Bacillus strains:

Strains	Thermostability		Optimum pH	Specific activity (U/mg)
	Stability	Activity		
B.subtilis DLG	$50^0C \pm 5^0C$	60^0C	5.0 - 6.0	550
Bacillus N-4	53^0C	55^0C	9.0	440
Bacillus 1139	55^0C	60^0C	10.0	250

Clostridia species :

The *Clostridia* are gram positive, anaerobic, spore forming *Bacilli* with wide distribution in nature, being found in soil and in the intestinal tracts of humans and animals, among other places. Several *Clostridia*, including *Clostridium thermocellum*, *Clostridium cellulolyticum* and *Clostridium stercorarium* produce cellulases. *Clostridium thermocellum*, a thermophilic anaerobe, possesses one of the best characterized bacterial cellulase systems. It secretes a large number of highly active cellulolytic enzymes and can ferment cellulose directly to ethanol, acetic acid and lactic acid (Ng *et al.*, 1977). Characterization of the extracellular cellulases produced by this organism has been hampered by the tendency of cellulases to form a high molecular weight aggregate of 2.1 million daltons and ca.18 nm in diameter (Lamed *et al.*, 1983). This protein complex, termed as cellulosome, is resistant to disruption by a variety of reagents, including urea, non ionic detergents, guanidine hydrochloride and extremes in pH or ionic strength. However, sodium dodecyl sulfate (SDS) treatments above 70^0C disrupt the cellulosome and SDS-polyacrylamide gel electrophoresis (PAGE) has been employed to resolve the complex into at least 14-18 polypeptides, ranging in size from 48,000 - 2,10,000 daltons. Most of these proteins were characterized as having cellulase activity, with the notable exception of one strong antigenic polypeptide, the 2,10,000-dalton SI protein of strain YAS (which is equivalent to

the 2,50,000-dalton S_L protein of strain ATCC 27405) (Wu *et al.,* 1988).

To date, five distinct endo-β-1,4-glucanases of *C.thermocellum* and one from *C.stercorarium* have been purified and characterized. Ng and Zeikus (Ng and Zeikus, 1981) reported that an endo-β-1,4-glucanase from *C.thermocellum* displayed high activity towards CMC and cellooligosaccharides composed of 5,6 and 7 glucose residues, low activity on cellotetraose and avicel and no activity on cellotriose. It was isoelectric at pH 6.72 and exhibited maximal activity at pH 5.2.The molecular mass weight varied between 83,000 and 94,000 daltons depending on the method used to size the enzyme. Like wise an endo-ß-1,4-glucanase from a related organism, *C. stercorarium*, was reported to be in the range of 91,000 - 99,000 daltons (Creuzet and Frixon, 1983), it was isoelectric at pH 3.85 and thus is the most acidic endoglucanase from the *Clostridia* characterized to date. The enzyme was highly thermostable and showed optimal activity at pH 6.4. It is likely that the extracellular cellulase of *C.thermocellum* contains sulfhydryl groups which are essential for activity, since the ability of cellulase to degrade crystalline cellulose depended on the presence of both Ca^{+2} and a thiol reducing agent (Johnson *et al.*, 1982).

Cellulases of Clostridium thermocellum:

Enzyme	Thermostability		pH		Specific activity (U/mg)	Size (Daltons) Deduced Measured
	Stability	Activity	Optimum	Isoelectric		
EGA	60^0C	75^0C	5.5 - 6.5	-	140	52,000 56,000
EGB	-	-	-	-	60	63,900 66,000
EGC	65^0C	-	6.0	6.2	27	- 40,000
EGD	-	60^0C	6.0	5.4	428	- 65,000

Cellulomonas species:

The cellulolytic system of the genus *Cellulomonas* has also been well studied. Members of this genus are gram variable, mesophilic, non spore forming rods which possess a genome of high G+C content (72%) (Stackebrandt and Kandler, 1974). These organisms are capable of using crystalline celluloses for growth and produce extracellular cellulases for the most part (Beguin *et al.,* 1977; Stoppock *et al.,* 1982). Cellulolytic species of *Cellulomonas* include *C. fimi, C. uda, C. flavigena* and *C. fermentans*. The cellulase system of *C. fimi* ATCC484 is composed of three or perhaps four enzymes. These enzymes appear to be stabilized by binding to cellulose fibers (Beguin and Eisen, 1978; Langsford *et al.,* 1984). Two appear to be endo-β-1,4-glucanases, while the third has exo-β-1,4-glucanase activity. *Cellulomonas* mutant strains that produced increased levels of cellulase and were resistant to glucose mediated repression of cellulase synthesis have been reported. Although both the *C. fimi* Eng A and Exg proteins can degrade CMC, they differ in their ability to degrade other substrates. Properties of enzymes are summarized in table, Both Eng A and Exg are glycoproteins, which probably accounts for the measured size being larger than the predicted size based on the size of their genes.

Cellulases of Cellulomonas fimi:

Enzyme	Size (Daltons)		Substrate hydrolysed
	Deduced	Measured	
EngA	51,800	58,000	CMC, lichenan
EngB	-	1,10,000	CMC
Eng	49,000	56,000	CMC, xylan

Applications of cellulases:

From a practical point of view, several processes involving cellulases can be considered. In the short term, applications requiring only partial hydrolysis are the most likely to become economically feasible, and some of them are already commercialized. In the food processing sector, crude cellulose (straw, wood shavings) is traditionally used to grow edible mushrooms, such as *Agaricus bisporus*, which is currently produced at a rate of 1 million tons/ year (Wood *et al.*, 1988). Cellulase preparations are already used to clear fruit juices from remaining pulp particles. Cellulases can also help in the extraction of oil and juice from seed and fruit pulp. Cellulases are used in a variety of applications in brewery and wine, textile, paper and pulp industries as well as in agriculture. These industrial enzymes have already reached a market of 1.6 billion $ U.S. In view of biotechnological importance,

microbial production of cellulases continues to be a subject of interest and to attract a great deal of attention from cross sections of scientists. The semi purified/purified enzymes had potential applications in food and pharmaceutical industries. This potential has stimulated the search for new microorganisms with better cellulolytic capabilities. Substantial efforts have been made by enzyme suppliers and industrial users to improve existing enzymes. The major long-term challenge for very large scale biotechnological applications remains the utilization as a source of glucose of the huge, renewable mass of cellulosic residues available every year. In addition, several cellulases can hydrolyse β-1,3-β-1,4-glucan, which is present in high amounts in low grade barley and hampers the filtration of beer. Recombinant yeasts producing β-1,3-β-1,4 glucanases are already available for the brewery industry (Penttila *et al.,* 1989). Cellulases are also expected to have an impact on the processing of animal feed. Treatment of silage with cellulases is expected to be beneficial for two reasons. Partial hydrolysis of the plant cell wall should speed up the release of sugars to be fermented by lactic acid bacteria. In addition, cellulases might improve the digestibility of silage.

Textile processing is another area where cellulases have been successfully put to use. Cellulases added to laundry powder shave off the microfibrils which tend to stick out of cotton fibers after several washing cycles. This significantly helps restore softness and colour brightness to cotton fabrics. Cellulases have also been used to remove excess dye from denim fabric in pre-faded blue jeans (biostoning).

Cellulase systems may also offer interesting spin-offs not directly related to cellulase activity proper. For example, CBDs could be used as tagging sequences for the affinity purification of recombinant proteins on a cheap cellulose matrix. The possibility was explored for the CBD of *C. fimi* CenA, which was fused by genetic engineering of *E.coli* alkaline phosphatase (Greenwood *et al.,* 1989) and to *Agrobacterium* sp. β-glucosidase (Ong *et al.,* 1989).

In conclusion, basic research has begun to unravel some of the unconventional solutions that cellulolytic microorganisms have evolved to degrade an unconventional substrate. One may hope that in a not too distant future, such knowledge will be helpful in the development of an increasing number of applications.

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