

Cellulase Production by bacteria: A Review

Sangrila Sadhu¹ and Tushar Kanti Maiti ^{1*}

¹. Microbiology Laboratory, Department of Botany, The University of
Burdwan, Burdwan- 713104, WB, India

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ABSTRACT

Cellulose is an abundant natural biopolymer on earth and most dominating Agricultural waste. This cellulosic biomass is a renewable and abundant resource with great potential for bioconversion to value-added bioproducts. It can be degraded by cellulase produced by cellulolytic bacteria. This enzyme has various industrial applications and now considered as major group of industrial enzyme. The review discusses application of cellulase, classification of cellulase, quantification of cellulase, the types of cellulolytic bacteria and their screening. It describes the current knowledge of cellulase production by submerged fermentation and solid state fermentation, properties of cellulase and cloning and expression of cellulase gene. The biotechnological aspect of cellulase research and their future prospects are also discussed.

Key words: Cellulytic bacteria, bioconversion, cellulases, Endoglucanase, Exoglucanase, β -glucosidase, cellulosome

Corresponding author.

E mail tkmbu@yahoo.co.in

Phone: 91 (0342) 2659493 ®,

Mobile-(91)9434167047

Fax: (91) 0342 564452

27 1. INTRODUCTION

28 About 200 gigatons of CO₂ are fixed on earth every year and the equivalent amount of
29 organic material has to be degraded approximately 30 % by plants and animals to 70 % by
30 microorganisms [1]. On average, cellulose accounts as 50% of the dry weight of plant
31 biomass. Such plant biomass is the only foreseeable sustainable source of fuels and
32 materials available to humanity. Agricultural residues are a great source of lignocellulosic
33 biomass which is renewable, chiefly unexploited and inexpensive. These renewable
34 resources are leaves, stems, and stalks from sources such as corn fibre, corn stover,
35 sugarcane bagasse, rice straw, rice hulls, woody crops, and forest residues. Besides, there
36 are multiple sources of lignocellulosic waste from industrial and agricultural processes, e.g.,
37 citrus peel waste, coconut biomass, sawdust, paper pulp, industrial waste, municipal
38 cellulosic solid waste, and paper mill sludge. In addition, dedicated energy crops for biofuels
39 could include perennial grasses such as Switchgrass and other forage feedstocks such as
40 *Miscanthus*, Elephant grass, Bermuda grass, etc [2].

41 Approximately 70% of plant biomass is locked up in 5- and 6-carbon sugars. These sugars
42 are found in lignocellulosic biomass comprised of mainly cellulose (a homologous polymer of
43 glucose linked by β 1,4 glycosidic bonds) hydrolysed by a complex enzyme system named
44 as cellulase (exoglucanase, endoglucanase and β glucosidase etc.); lesser hemicelluloses
45 (heterologous polymer of 5- and 6-carbon sugars consists of pentoses D-xylose, D-
46 arabinose and hexoses D-mannose, D-glucose, D-galactose with sugar acids); and least of
47 all lignin (a complex aromatic polymer). In hardwoods hemicellulose contains mainly xylans,
48 while in softwood mainly glucomannans are present. Briefly, xylan degradation requires
49 endo-1,4- β -xylanase, β -xylosidase, α -glucuronidase, α -L-arabinofuranosidase, as well as
50 acetylxylan esterases. In glucomannan degradation β -mannanase and β -mannosidase are
51 required to cleave the polymer backbone.

52 The limited nature of fossil fuels reserves which has been depleting at an alarming rate by
53 civilized world. Burning of fossil fuels has also created a concern for unstable and uncertain
54 petroleum sources, the rising cost of fuels and a concern with respect to global climate
55 change. These concerns have shifted to utilize renewable resources for the production of a
56 'greener' energy replacement which can meet the high energy demand of the world. The
57 Canadian renewable fuel standard has been raised and will contain 5% ethanol by 2010; the
58 US Environmental Protection Agency raised their renewable fuel standard to 10.21% ethanol
59 mixed fuels by 2009; while, the mandate for mixing ethanol in fuel for Brazil is 25% (set in
60 2007). Cellulases contribute to 8% of the worldwide industrial enzyme demands [3]. The
61 cellulase market is expected to expand dramatically when cellulases are used to hydrolyzed
62 pretreated cellulosic material to sugars, which can be fermented to bioethanol and biobased

products on large scales. The cellulase market has been estimated in the United States to be as high as US \$ 400million per year [4]. In the period 2004 -2014 an increase of approximately 100 % in the use of cellulase as a speciality enzyme is expected [5]. The biotechnology companies Genencor International and Novozymes Biotech have reported the development of technology that has reduced the cellulase cost for the cellulose-to-ethanol process from US\$5.40 per gallon of ethanol to approximately 20 cents per gallon of ethanol [6], in which the two main strategies were (1) an economical improvement in production of cellulase to reduce US\$ per gram of enzyme by process and strain enhancement, e.g., cheaper medium from lactose to glucose and alternative inducer system and (2) an improvement in the cellulase enzyme performance to reduce grams of enzyme for achieving equivalent hydrolysis by cocktails and component improvement [7].

In addition to this, the major industrial application of cellulases are in textile industry for bio-polishing of fabrics and producing stonewashed look of denims, as well as in household laundry detergents for improving fabric softness and brightness [8]. Besides, they are used in animal feeds for improving the nutritional quality and digestibility, in processing of fruit juice and in baking, while de-inking of paper is yet another emerging application. A potential challenging area where cellulases would have a central role is the bioconversion of renewable cellulosic biomass to commodity chemicals [9]. Application of this enzyme in detergent, leather and paper industries demands identification of highly stable enzymes active at extreme pH and temperature. Some important applications of cellulases or cellulolytic bacteria are given in Table.1.

The present review elucidated on bacterial cellulase production in both natural and technological context. Moreover, bacterial cellulase utilization from an integrative perspective and diversity of cellulolytic bacteria and enzyme systems are described. Attempts are made to discuss the mode of action of cellulase in bacterial system and molecular biology of their regulation. In addition, the review also addressed cloning and expression of cellulase genes in heterologous hosts and how these rare cellulases can help some of the major bottlenecks in the biofuel industry and how some unique bacterial strategies in biotechnology can help in biorefining.

99 **Table 1. Applications of cellulases or cellulolytic bacteria (Mandel, 1985) [10]**

	Application
1. Removal of cell walls, crude fibre	a) Release cell contents Flavors Oils Spices Polysaccharides(agar) Proteins(seeds, leaves)
	b) Improve rehydratability of dried vegetables Soup mixes
	c) Oil seed cakes Straws Barley Mesquite
	d) Production of plant protoplasts Genetic engineering (higher plants)
2. Production of glucose, soluble sugars	a) Animal feed Molasses(direct or by-product) Increase nutritive value (add sugar to high-fiber feed) Single-cell protein
	b) Industrial feedstock Glues, adhesives Solvents (ethanol, butanol, acetone.etc.)
	c) Raw material for fermentation industry Antibiotics Acetic acid, citric acid etc.
3. Production of lignin	Adhesives Resins Extenders Chemical raw materials
4. Miscellaneous food applications	a) Cell free protein High productivity High quality protein
	b) Addition of mycelia and extracellular protein Removal of crude fiber

	Conversion of fiber to sugar Removal of other unwanted compounds
	c)Protease production (e.g., meat tenderizer)
5.Decompositio of wastes and residues	Sewage treatments

100

101 2. CLASSIFICATION OF CELLULASE

102 Microorganisms produced extracellular cellulases that are either free or cell associated to
103 hydrolyze and metabolize insoluble cellulose. The biochemical analysis of cellulose systems
104 from aerobic and anaerobic bacteria and fungi has been comprehensively reviewed during
105 the past three decades. Following components of cellulase systems were classified based
106 on their mode of catalytic action (Table 2).

107 2.1 Endoglucanases or Endo-1, 4-β-D-glucan glucanohydrolases (EC 3.2.1.4)

108 Endoglucanases cut at random at internal amorphous sites in the cellulose polysaccharide
109 chain, generating oligosaccharides of various lengths and consequently new chain ends. It is
110 generally active against acid-swollen amorphous cellulose, soluble derivatives of cellulose
111 such as CMC, cellooligosaccharides [11].

112 2.2 Exoglucanase or 1, 4-β-D-glucan cellobiohydrolases (cellobiohydrolases) 113 (EC 3.2.1.91)

114 Exoglucanases act in a processive manner on the reducing or non-reducing ends of
115 cellulose polysaccharide chains, liberating either glucose (glucanohydrolases) or cellobiose
116 (cellobiohydrolase) as major products. These enzymes are active against crystalline
117 substrate such as Avicel, amorphous celluloses and cellooligosaccharides. However, they
118 are inactive against cellobiose or substituted soluble celluloses such as CMC.

119 2.3 Exoglucanases or 1, 4-β-D-oligoglucan cellobiohydrolases (also known as 120 cellodextrinases) (EC 3.2.1.74)

121 It catalyzes the removal of cellobiose from cellooligosaccharides or from p-nitrophenyl -β -D-
122 cellobioside butvinactive against amorphous cellulose or CMC.

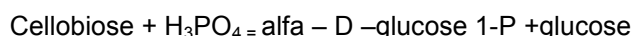
123 2.4 β - Glucosidases or β-D-glucoside glucohydrolases (EC 3.2.1.21)

124 β -Glucosidases hydrolyze soluble cellodextrins and cellobiose to glucose from non-reducing
125 end.It is inactive against crystalline or amorphous cellulose.

126 2.5 Cellobiose phosphorylase or Cellobiose: orthophosphate alfa-D-glucosyl 127 transferase (EC 2.4.1.20)

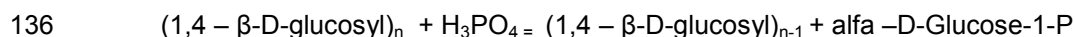
128 It catalyzes the reversible phosphorolytic cleavage of cellobiose. It was first discovered by
129 Ayers [12] in cells of *Ruminococcus flavefacience*.

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131 **2.6 Cellodextrin phosphorylase or 1,4-β-D-oligoglucan orthophosphate alfa –D-**
 132 **glucosyl transferase (EC 2.4.1.49)**

133 It was found in cells of *Clostridium thermocellum* [13]. It does not act on cellobiose but
 134 catalyze the reversible phosphorylytic cleavage of cellodextrins ranging from cellotriose to
 135 cellohexose.



137 **2.7 Cellobiose epimerase (EC 5.1.3.11)**

138 It was first reported in cells of *Ruminococcus albus* [14]. It catalyzes the following reaction:



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141 **3. SCREENING OF CELLULASE PRODUCING BACTERIA**

142 Screening for bacterial cellulase activity in microbial isolates is typically performed on plates
 143 containing crystalline cellulose or microcrystalline cellulose such as Avicel in the agar at a
 144 final concentration of 0.1-0.5 % (w/v). After incubation of a suitable period, a zone of clearing
 145 surrounding the colonies will be indicated that cellulose producer [15]. The colonies of
 146 cellulolytic *Cytophaga* spp. did not shown any clearing zone [16]. So the diameter of the
 147 clearing zone may not accurately reflect the true cellulase activity.

148 For a rapid screening of cellulase producing bacteria, after the incubation of the agar
 149 medium are containing 0.5% (W/V) carboxymethyl cellulose (CMC) as sole carbon source
 150 and flooded with 1% (W/V) Congo red [17]. After 20 minutes, the dye is decanted and the
 151 plates are again flooded with 5M NaCl which is decanted after 20-30 minutes. Positive
 152 colonies are detected to be surrounded by a pale orange to clear zone against red
 153 background. The cellulolytic bacteria can be screened directly on such plate, but replica
 154 plating from master plate is preferred for isolation of active colonies as flooded reagent
 155 impairing isolation. Plant et al. [18] has reported a semi-quantitative assay for cellulase
 156 activity in bacteria by using cellulose-azure into the upper two layers of agar tubes. The dye
 157 released from the substrate is determined densitometrically. Kasana et al. 2008 found that
 158 Gram's iodine for plate flooding in place of hexadecyltrimethyl ammonium bromide or Congo
 159 red, gave a more rapid and highly discernable result [19].

160 **Table 2. Bacterial cellulase enzyme system**

Enzyme	E. C. number	Reaction	Other Names	Family
i)Endo -1,4 β-D-glucan-glucanohydrolase	E. C. 3. 2. 1. 4	cut at random at internal amorphous sites of cellulose generating oligosaccharides of various lengths. It acts on	Endoglucanase, Endo-1,4- β – glucanase, Carboxymethyl	5, 6, 7, 8, 10, 12, 44, 51, 61,

		Endo-1, 4-beta-D-glucosidic linkages in cellulose, lichenin and cereal beta-D-glucans.	cellulase, β -1,4-endoglucan hydrolase, Endocellulose	74
ii) Exoglucanase or 1,4- β -D-glucan cellobiohydrolases (cellobiohydrolases)	E.C.3.2.1.91	Hydrolysis of 1,4-beta-D-glucosidic linkages in cellulose and cellotetraose, releasing cellobiose from the non-reducing ends of the chains	Exoglucanase, Exocellobiohydrolase, 1, 4- β -cellobiohydrolase.	5, 6, 7, 9, 10, 48,
iii) Exoglucanases or 1,4- β -D-oligoglucan cellobiohydrolases	EC 3.2.1.74	Removal of cellobiose from celooligosaccharide or from p-nitrophenyl- β -D-cellobioside	Cellobiohydrolases	-
iv) β - Glucosidases or β -D-glucoside glucosyl hydrolases	E.C.3.2.1.21	Hydrolysis of terminal non-reducing beta-D-glucose residues with release of beta-D-glucose.	Gentobiose, Cellobiose, Amygdalase.	1, 3, 9
v) Cellobiose: orthophosphate alpha-D-glucosyl transferase	E.C. 2.4.1.49	It catalyzes the reversible phosphorylative cleavage of cellobiose	Cellobiose phosphorylase	-
vi) 1,4- β -D-oligoglucan:orthophosphate alpha -D-glucosyl transferase	E.C. 2.4.1.20	It catalyzes the reversible phosphorylative cleavage of cellodextrins ranging from cellotriose to cellohexoses.	Cellodextrin phosphorylase	-
vii) Cellobiose 2-epimerase	EC 5.1.3.11	It catalyzes the conversion of cellobiose into 4-O- β -D-glucosylmannose.	Cellobiose 2-epimerase	-
viii) Complete Cellulase system	-	Catalyzes extensive hydrolysis of crystalline cellulose	Total cellulase	-

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162 However, plate-screening methods using dyes are not quantitative **method for the** poor
163 correlation between enzyme activity and halo size. This problem solved by the development
164 of short celooligosaccharides possessing modified reducing terminal with
165 chromogenic/fluorogenic groups e.g. fluorescein, resorufin and 4-methylumbelliferone for
166 higher sensitivity and quantification [20]. But a major limitation of the use of fluorescent
167 substrates into agar plates is the tendency for hydrolysis products to diffuse widely and
168 therefore are not as readily used such compounds. So, new substrates, 2-(2'-

169 benzothiazolyl)-phenyl (BTP) cellooligosaccharides were synthesized for the screening of
170 cellulolytic microorganisms in plate assays [21].

171 Researchers have now focused to cellulase genes from unculturable microorganisms in
172 extreme environments with hopes that the enzymes isolated will be novel and have specific
173 applications in the biorefining industry due to a higher resistance to harsh environmental
174 conditions. To identify novel cellulases from all species present, culturable and nonculturable
175 in a swift manner, a metagenomic clone library should be prepared and then functionally
176 screened.

177 **4. CELLULASE PRODUCTION USING THE SUBMERGED** 178 **FERMENTATION (SmF) AND SOLID STATE FERMENTATION (SSF) OR** 179 **CULTIVATION (SSC).**

180 Fermentation is the technique of biological conversion of complex substrates into simple
181 compounds by various microorganisms. It has been widely used for the production of
182 cellulase for their wide uses in industry. Over the years, fermentation techniques have
183 gained immense importance due to their economic and environmental advantages. Two
184 broad fermentation techniques have emerged as a result of this rapid development:
185 Submerged Fermentation (SmF) and Solid State Fermentation (SSF).

186 **4.1 Solid-State Fermentation (SSF) / Solid-State Dultivation (SSC)**

187 SSF utilizes solid substrates, like bran, bagasse, paddy straw, other agricultural waste and
188 paper pulp [22]. The main advantage of using these substrates is that nutrient-rich waste
189 materials can be easily recycled as cheaper substrates. SSF is best suited for fermentation
190 techniques involving fungi and microorganisms that require less moisture content. However,
191 it cannot be used in fermentation processes involving organisms that require high water
192 activity, such as bacteria [23].

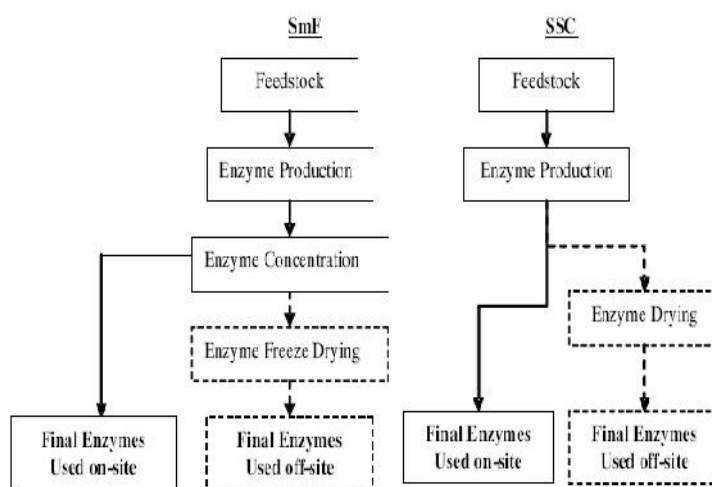
193 **4.2 Submerged Fermentation (SmF)/Liquid Fermentation (LF)**

194 SmF utilizes free flowing liquid substrates, such as molasses and broth [22]. This
195 fermentation technique is best suited for microorganisms such as bacteria that require high
196 moisture content. An additional advantage of this technique is that purification of products is
197 easier.

198 **4.3 A comparison between SmF and SSC method**

199 Cellulases are produced using the submerged fermentation (SmF) method traditionally, in
200 which the cultivation of microorganisms occurs in an aqueous solution containing nutrients.
201 An alternative to this traditional SmF method is the solid state cultivation (SSC) method,
202 which involves the growth of microorganisms on solid materials in the absence of free liquids
203 [24]. Since SSC involves relatively little liquid when compared with SmF, downstream

204 processing from SSC is theoretically simpler and less expensive (Figure -1 and Table 3.
 205 During the past ten years, a renewed interest in SSC has developed due, in part, to the
 206 recognition that many microorganisms, including genetically modified organisms (GMO),
 207 may produce their products more effectively by SSC [25]. SSC has three advantages viz. i)
 208 lower consumption of water and energy , ii) reduced waste stream and iii) more highly
 209 concentrated product [26]. Moreover, The biosynthesis of cellulases in SmF process is
 210 strongly affected by catabolic and end product repressions [27] and on the overcoming of
 211 these repressions to significant extent in solid state fermentation (SSF) system [28],
 212 therefore, are of economic importance. The amenability of SSF technique to use upto 20-
 213 30% substrate, in contrast to the maximum of 5% in SmF process, has been documented
 214 [29].
 215 The SSF is generally preferred as it offers many advantages such as two-three times higher
 216 enzyme production as well as protein rate, higher concentration of the product in the
 217 medium, direct use of air-dried fermented solids as source of enzyme which lead to
 218 elimination of expenses on downstream processing, employment of natural cellulosic wastes
 219 as substrate in contrast to the necessity of using pure cellulose in submerged fermentation
 220 (SmF) and the possibility of carrying out fermentation in non-aseptic conditions [30]. Some
 221 example of cellulase producing bacteria with their method of fermentation is given in Table 4.



Straight (dashed) lines represent on-site (off-site) enzyme production process

222

223 **Figure -1 Flow chart of enzyme production using the traditional SmF method**
 224 **compared to the SSC method. [26]**

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227 **Table 3: Comparison of characteristics for SmF and SSC methods**

Factor	SmF	SSC
Water	High volumes of water consumed and effluents discarded	Limited consumption of water and no effluent
Mechanical agitation	Good homogenization	Static conditions preferred
Scale up	Industrial equipment available	New design equipment needed
Energy	High energy consuming	Low energy consuming
Equipment Volume	High volumes and high costs	Low volumes and lost costs
Concentration	30-80g/l	100-300g/l

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229 **Table 4: Fermentative production of cellulase by bacteria**

Name of the bacteria	Temperature	Types of Substrates used	pH	Type	References
<i>Anoxybacillus flavithermus</i> EHP2	75 °C	CMC	7.5	SmF	[31]
<i>Anoxybacillus</i> sp. 527	70 °C	Crystalline cellulose	6.0	SmF	[32]
<i>Bacillus</i> sp.AC-1	70°C	CMC	4.5–6.5	SmF	[33]
<i>Bacillus</i> sp. LFC15	50°C		9–10	SmF	[34]
<i>Bacillus subtilis</i>	37°C	CMC	7.0	SmF	[35]
<i>Bacillus thuringiensis</i>	40°C	Soluble cellulose, CMC, Insoluble crystalline cellulose	4.0	SmF	[36]
<i>Bacillus</i> sp	50°C	Sugar Cane Bagasse	4.5-5.5	SSF	[37]
<i>Bacillus</i> sp. NZ	50°C	agricultural residues	9–10	SSF	[38]
<i>Bacillus</i> sp	-	Roundnut shell	-	SSF	[39]
<i>Bacillus Cereus</i>		Palm Kernel Cake		SSF	[40]
<i>Bacillus licheniformis</i> MVS1 <i>Bacillus</i> sp. MVS3	50-55	CMC, Filter paper	6.5 to 7.0	SSF	[41]
<i>Cellulomonas cellulans</i> MTCC 23	-	Paddy Straw	-	SmF	[42]
<i>Clostridium</i>		Cellulose and paper		SmF	[26]

<i>thermocellum</i>		pulp		and SSF	
<i>Cytophaga hutchinsonii</i> NCIM 2338	-	Paddy Straw	-	SmF	[42]
<i>Streptomyces</i> sp. BRC1 <i>Streptomyces</i> sp. BRC2	26°C	CMC	7.0-7.5	SmF	[43]
<i>Microbacterium</i> sp. MTCC 10047	37°C	CMC	7.0	SmF	[44]
<i>Bosea</i> sp. MTCC 10045	37°C	CMC	7.0	SmF	[45]

5. METHODS FOR QUANTIFICATION OF CELLULASES

All existing cellulase activity assays can be divided into three types: (1) the accumulation of products after hydrolysis, (2) the reduction in substrate quantity, and (3) the change in the physical properties of substrates. The majority of assays involve the accumulation of hydrolysis products, including reducing sugars, total sugars, and chromophores are given in the Table 5.

Table 5: The common colorimetric sugar assays (modified from Zhang et al. 2006) [4]

Method		Sample (mL)	Reagent (mL)	Glucose amount (µg/sample)	Glucose concn. (mg/L)	References
Reducing Sugar Assay	Micro	1- 3	3	20- 600	6.7- 600	[46]
DNS						
DNS	Micro	0.5	3	100- 2500	200- 5000	[47]
Nelson-Somogyi	Micro	1- 5	2+2	1- 10	0.2- 10	[48]
Nelson-Somogyi	Micro	2	2+2	10- 600	5- 300	[48]
Nelson	Semi- Micro	2	2	5- 100	2.5- 50	[49]
Ferricyanide-1		1- 3	1+5	1- 9	0.3- 9	[50]
Ferricyanide-2		1	0.25	0.18- 1.8	0.18- 1.8	[51]
PAHBAH	Micro	0.5	1.5	0.5- 5	1- 10	[52]
PAHBAH	Micro	0.01	3	5- 50	500- 5000	[52]
BCA		0.5	0.5	0.2- 4.5	0.4 -9	[53]
Modified BCA		1	1	0.4 – 9	0.4 -9	[54]
Total Sugar Assay		1	1+5	5- 100	10- 100	[55, 54]
Phenol-H ₂ SO ₄						
Anthrone-H ₂ SO ₄		1	1+5	5- 100	10- 100	[56, 57]
Enzymatic Glucose Assay		0.01	1	2- 50	200- 5000	Sigma Kit
Glucose-HK/PGHD kit						
Glucose-HK/PGHD kit		0.2	0.5	2- 50	4 - 100	[58]

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6. CELLULASE PRODUCING BACTERIA AND THEIR CHARACTERIZATION

Both fungi and bacteria have been exploited for their abilities to produce a wide variety of cellulases and hemicellulases. Most emphasis has been placed on the use of fungi because of their capability to produce copious amounts of **cellulytic enzymes** and often less complex than bacterial cellulase and easy for extraction and purification. It can therefore be more readily cloned and produced via recombination in a rapidly growing bacterial host. However, the isolation and characterization of novel cellulase from bacteria are now becoming widely exploited. There are several reasons for these shifts viz. i) bacteria often have a higher growth rate than fungi allowing for higher recombinant production of enzymes, ii) bacterial cellulases are often more complex and are in multi-enzyme complexes providing increased function and synergy iii) bacteria inhabit a wide variety of environmental and industrial niches like thermophilic or psychrophilic, alkaliphilic or acidiphilic and halophilic strains, which produce cellulolytic strains that are extremely resistant to environmental stresses. These strains can survive and produce cellulytic enzymes in the harsh conditions which are found to stable under extreme conditions and which may be used in the bioconversion process [59]. This may increase rates of enzymatic hydrolysis, fermentation, and, product recovery. Researchers are now focusing on utilizing, and improving these enzymes for use in the biofuel and bioproduct industries.

Many bacteria can grow on cellulose and many produce enzymes that catalyze the degradation of soluble derivatives of cellulose or the amorphous regions of crystalline cellulose. However few bacteria synthesize the complete enzyme system that can result in extensive hydrolysis of the crystalline material found in nature. These few bacteria should be called “true cellulolytic” bacteria and those bacteria that produce some endoglucanases and β -glucosidases, but not the complete system, are called “pseudocellulolytic” [60]. Such pseudocellulolytic bacteria may have picked up the genes encoding these enzymes from true cellulolytic species by horizontal transfer.

There are different types of bacteria isolated from different **environment** produced cellulase. Some of the important bacteria and the characteristic features of their cellulase component are given below (Table 6)

274 **Table 6. Properties of some Cellulase enzymes isolated from Anaerobic and Aerobic**
275 **Cellulolytic bacteria (modified from Frank et al. 1992) [60]**

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Name of the bacteria	Enzyme	Mol. Wt.	Optimum temp.(°C)	Optimum pH	References
Aerobic					
<i>Bacillus licheniformis</i> 1	Endoglucanase	-	55	6.1	[61]
<i>Bacillus</i> sp (alkalophilic) 1139	Endoglucanase	92	-	9.0	[62]
<i>Bacillus</i> sp (alkalophilic) (cloned in E.coli) N-4	Endoglucanase cel A	54	-	5.0-11.0	[63]
	Endoglucanase cel B	46		5.0-11.0	[63]
	Endoglucanase cel C	100		9.0	[64]
<i>Bacillus</i> sp (neutrophilic) KSM-522	Endoglucanase	35	50	7.0-10.0	[65]
<i>Bacillus subtilis</i> (cloned in B.megaterium)	Endoglucanase	33	60	5.5	[66]
<i>Bacillus subtilis</i> DLG	Endoglucanase	35	55	4.8	[67]
<i>Cellulomonas uda</i>	Exocellobiohydrolase	81	45-50	5.5-6.5	[68]
<i>Cellvibrio gilvus</i> ATCC13127	Cellobiose phosphorylase	280	<40	7.6	[69]
<i>Microbispora bispora</i>	Endoglucanase I	44	-	5.5-7.2	[70]
	Endoglucanase II	57	-	5.5-7.2	[71]
	Exoglucanase I	75	-	5.9-7.2	
	Exoglucanase II	95	-	5.9-7.2	
	β-Glucosidase	-	-	6.0	[70]
<i>Thermomonospora fusca</i> YX	Endoglucanase 1	94	74	6.0	[72]
	Endoglucanase 2	46	58	6.0	[72]
<i>Bacillus</i> M-9	Endoglucanase	54	60	5.0	[73]
<i>Bacillus amyloliquefaciens</i> DL3	Endoglucanase	54	50	7.0	[74]
<i>Bacillus</i> sp. HSH-810	Endoglucanase	80	40-70	10.0	[75]
<i>Thermomonospora</i> sp.	Endoglucanase	38	50	5.0	[76]
<i>Cellulomonas</i> sp. YJ5	Endoglucanase	43.7	60	7.0	[77]
<i>Pseudomonas fluorescens</i>	Endoglucanase	36	35	7.0	[78]
<i>Nocardiopsis</i> sp. KNU	Endoglucanase	-	40	5.0	[79]
<i>Bacillus subtilis</i> YJ1	Cellulase	32.5	60	7.0	[80]
<i>Bacillus</i> sp (cloned in E.coli) AC-1	Endoglucanase (Ba - EGA)	74.87	-	-	[81]
<i>Cellulomonas</i> sp. ASN2.	Endoglucanase	-	60	7.5	[82]

<i>Bacillus coagulans</i> Co4	Endoglucanase	-	60	7.5	[83]
Anaerobic					
<i>Acetivibrio cellulolyticus</i> ATCC33288	Exoglucanase C1 Endoglucanase C2 Endoglucanase C3 β -Glucosidase B1	38 33 10.4 81.0	- - - -	- - - -	[84]
<i>Alcaligenes faecalis</i>	β -Glucosidase	100	-	-	[85]
<i>Bacteroides cellulosolvens</i> S-85	Endoglucanase EG1	65	39	6.4	[86]
<i>Bacteroides succinogenes</i>	Endoglucanase EG2	118	39	5.8	[86]
<i>Clostridium josui</i>	Endoglucanase	45	60	6.8	[87]
<i>Clostridium thermocopriae</i> JT3-3	Endoglucanase	46	-	6.5	[88]
<i>Clostridium thermocellum</i> LQRI	Endoglucanase I	94	62	5.2	[89]
<i>Ruminococcus albus</i> SY3	Endoglucanase	30	-	-	[90]

277

278 7. MODE OF ACTION OF CELLULASE IN BACTERIAL SYSTEM

279 Researchers have focused on four structures believed to be important in specific adhesion to
 280 cellulose viz. 1) large multicomponent complexes called cellulosomes [91]; 2) fimbriae or pili
 281 adhesions [91]; 3) Carbohydrate epitopes of bacterial glycocalyx layer [92]; and 4) enzyme
 282 binding domains [93].

283 7.1 Adhesion via Cellulosome like Complexes

284 Cellulosomes are large, stable, multi-enzyme complexes specialized in the adhesion to and
 285 degradation of cellulose that reside with protuberances visible on the cell surface. The
 286 cellulosome complex is composed of a central non-catalytic subunit (termed scaffoldin)
 287 which contains a cellulose binding domain (CBD) and a number of attachment sites (called
 288 cohesins) , which serve to bind the enzymatic submits. The enzymatic submits contain a
 289 catalytic domain and a docking domain (called dockerin) the latter interacting with due of the
 290 cohesions on scaffoldin [94]. The most complex and best investigated cellulosome is that of
 291 the thermophilic bacterium *Clostridium thermocellum*.

292 7.2 Adhesion via Fimbriae or Pili

293 Fimbriae or pili, which have been implicated in bacterial adhesion which are surface
 294 appendages and 5 to 7 nm in width and 100 to 200 nm in length in gram-negative bacteria
 295 [95]. As far has been learnt about the role of fimbriae in adhesion, it has become clear that
 296 structural subunits of fimbriae are the actual adhesions. Some subunits in the gram-positive

297 bacteria *Actinomyces viscosus* [96] and *S.sanguis* [97] associated with the fimbriae have
298 been identified. In *E.coli*, the carbohydrate binding sites of three types of fimbriae are in
299 small (28 to 35 Kda) repeated subunits, most of which are in the tips of the fimbriae with a
300 few additional sites along their length [98]. In *Ruminococcus albus*, a novel form of
301 cellulose-binding protein (cbpC 17.7 KDa) has been recognized that belongs to the pil
302 protein and most similar to the type 4 fimbrial proteins of gram-negative, pathogenic bacteria
303 [99].

304 **7.3 Adhesion via Carbohydrates epitopes of bacterial glycocalyx**

305 From electron microscopy observations, most of the evidence about adhesion via
306 carbohydrate epitopes has been found [100]. Several studies reported that the slime layer
307 surrounding *Ruminococcus albus* and *Ruminococcus flavefaciens* has composed of
308 glycoproteins (Carbohydrate residues) were involved in adhesions of the bacteria [100]. If
309 glycocalyx carbohydrate was removed by periodate oxidation with the protease and
310 dextranase treatment, the adhesion of *R.albus* and *Bacteroides succinogenes* to cellulose
311 has been decreased [95]. More direct evidence for the role of carbohydrate in adhesion was
312 given in *Fibrobacter* species [92].

313 **7.4 Adhesion via cellulose- Binding Domains of cellulolytic enzymes**

314 Examination of cellulase structure in some organisms has revealed two functional domains,
315 the active catalytic domain that is responsible for the hydrolytic cleavage of the glycosidic
316 bonds and the binding domain that binds the bacterial enzymes to its substrate. Because of
317 the conserved aromatic residues, it was thought that CBD attached to cellulose either by
318 hydrogen bonding or hydrophobic interaction. It has been shown that bacteria lacking these
319 domains were less adherent and in some cases, less able to digest crystalline cellulose
320 [101]. Distinct binding domains have been identified in *Bacteroides succinogenes*, including
321 the CBD of endoglucanase 2 (EG2) [102]. Karita et al [103] cloned a gene *egvI* from *R.albus* F-
322 40 and found that the enzyme contained a distinct CBD.

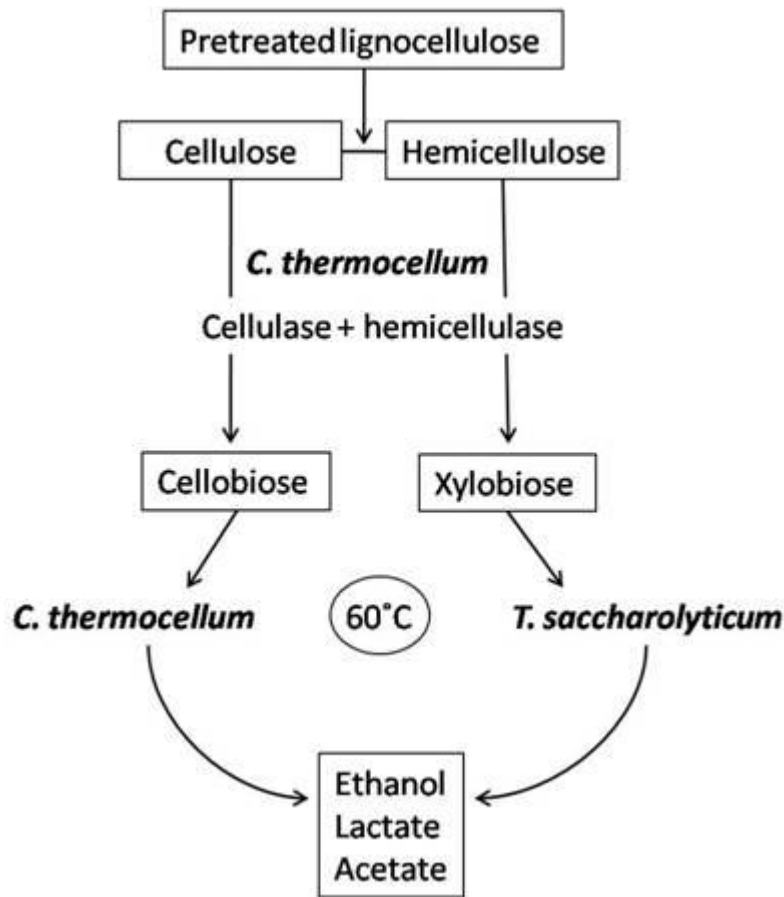
323 **8. CO-CULTURE**

324 Bacterial co-cultures can offer a means to improve hydrolysis of cellulose as well as
325 enhance product utilization and thus increase desirable fermentation products. *Clostridium*
326 *thermocellum* has gained special interest for co-culture with organisms capable of
327 fermenting pentose sugars to ethanol because *C. thermocellum* can only ferment hexose
328 sugars. Hence *C. thermocellum* has been co-cultivated with other anaerobic thermophilic
329 clostridia or close relatives such as *Clostridium thermosaccharolyticum* (now classified as
330 *Thermoanaerobacterium saccharolyticum*) [104], *Clostridium thermohydrosulfuricum* [105],
331 *Thermoanaerobacter ethanolicus* [106] and *Thermoanaerobium brockii* [107]. These
332 organisms can share a syntrophic relationship with *C.thermocellum* which exploits its

333 cellulases and hemicellulases to hydrolyze cellulose to cellobiose and cellodextrans, and
334 hemicelluloses to mainly xylobiose, arabinoxylans and xylooligosaccharides. *C.thermocellum*
335 will then convert cellulose breakdown products to ethanol while the latter strains will utilize
336 hemicellulose hydrolysis products to produce ethanol; this avoids the competition for
337 substrates between species and maximizes product formation (**Figure 2**). The challenge
338 with this type of co-culture application is the increased production of by-products such as
339 acetate and lactate which decrease ethanol production by slowing the growth rate of cells
340 [108].

341 Developing bacterial co- cultures can be a tedious task. To establish a stable co-
342 culture, media and growth requirements, such as temperature, atmosphere and carbon
343 source, must be synchronised to permit equal growth of each strain. Stable co-cultures may
344 not only depend on the media and growth requirements of each strain, but may also be
345 controlled more specifically by metabolic interactions(i.e. syntrophic relationships or
346 alternatively competition for substrates) and other interactions (i.e. growth promoting or
347 growth inhibiting such as antibiotics) [109].

348 The alternative of bacterial co-culture would be to engineer one microorganism to
349 complete an entire task from start to finish itself. In the case of *C.thermocellum*, this would
350 mean metabolically engineering this strain to ferment pentose sugars in addition to hexose
351 sugars. This is a difficult task as far as molecular engineering goes in clostridia due the
352 recalcitrance of clostridia to genetic manipulation. Co-cultivation has advantage because it
353 reduces the number of exogenous elements produced by a single bacterial population and
354 therefore reduces the chance of metabolic imbalance for host cells. Additionally, division of
355 labour will simplify the optimization of each reaction path way [110]. Although bacterial co-
356 culture is not an uncommon concept, its use in the bioconversion of lignocellulosic biomass
357 is still premature and offers great potential.



358

359 **Figure 2.** Simplified process of *C. thermocellum* and *T. saccharolyticum* used in co-
 360 culture for ethanol production. *C. thermocellum* produces the cellulases and
 361 hemicellulases for hydrolysis of lignocelluloses to sugars such as cellobiose and xylobiose.
 362 In addition, *C. thermocellum* can utilize hexose sugars derived from celluloses to produce
 363 ethanol. While, the hemicelluloses derived pentoses can be utilized by *T.*
 364 *saccharolyticum*. *T. saccharolyticum* also contributes to cellobiose reduction and is a good
 365 ethanol producer (modified from Demain et al. 2005, [111])

366 9. CLONING AND EXPRESSION OF CELLULASE GENES IN 367 HETEROLOGOUS HOSTS

368 Cellulase genes cloning and expressed in bacterial hosts have been reviewed by Pasternak
 369 and Glick (1987) [112]. Forsberg et al. [113] have reviewed the characteristics and cloning of
 370 bacterial cellulases, particularly from the rumen anaerobe *Bacteriodes succinogenes*. The
 371 most important of these are i) The strategies of cloning cellulase genes from eukaryotic
 372 fungal hosts cannot rely on direct expression in a prokaryotic cell because of the differences
 373 in the translation mechanism in the two groups, (ii) since the eukaryotic genomes are much

larger than those of prokaryotes, a genomic clone bank from a eukaryotic cell needs to be constructed with piece of DNA which are 20-40 kb long. A vector like pBR 322 which does not replicate well with an insert greater than 10-15 Kb fails to give satisfactory results.

The recombinant cellulolytic strategy for organism's development for cellulose conversion via with non cellulolytic microorganisms, involves heterologous expression of a functional cellulase system. Such heterologous expression has been undertaken for a variety of purpose with a variety of microorganisms.

9.1 Heterologous cellulase expression in bacteria

9.1.1 *Zymomonas mobilis*

Several cellulase encoding genes have been cloned and expressed in *Z. mobilis* with various degrees of success. Using a broad host range, mobilizable plasmid vector, the endoglucanase gene (eglX) from *Pseudomonas fluorescens* sub sp cellulose was introduced into *Z.mobilis* [114]. This recombinant strain, however, produced the heterologous endoglucanase intracellularly throughout the growth phase independent of the glucose concentration in the medium [114]. Similarly, introduction of the *Bacillus subtilis* endoglucanase into *Z.mobilis* also resulted in poor expression and again no activity was obtained in the culture supernatant of the transformants [115].

In contrast to the *P. fluorescens* and *B. subtilis* genes, the endoglucanase gene (Cel Z) of *Erwinia chrysanthemi* was efficiently expressed in *Z.mobilis* [116]. The specific activity of the *Z.Mobilis* enzyme was comparable to that of the parent strain of *E.chrysanthemi*. Biosynthesis of Cel Z was reported to occur during the exponential growth phase of *Z.mobilis*. Approximately 35% of the enzyme was released into the medium in the absence of detectable cell lysis.

Another cellulase gene that has been successfully expressed in *Z.mobilis* was cloned from *Acetobactor xylinum* [117]. The CM Case gene from *A.xylinium* was efficiently expressed in *Z.mobilis* and about 75% of the enzyme activity was detected in the periplasmic space.

9.1.2 Enteric bacteria

Two *E.chrysanthemi* endoglucanases, encoded by celY and cel Z and the *A.xylinum* cellulase gene have been expressed in both *E.coli* as well as the related enteric bacterium *K.oxytoca* [118]. Initially the expression of Cel Y in *E.coli* was poor was due to promoter construction [119]. However, by using a surrogate promoter from *Z.mobilis*, the expression of cel Z in *E.coli* was increased six fold.

10. CELLULASE BIOTECHNOLOGY: THE FUTURE

The use of lignocellulosic materials for the production of ethanol or other chemical feedstocks is one of the most difficult tasks encountered in the history of biotechnology. The study of microbial cellulose utilization is by quantification of enzymes in the cultures,

410 purification and application of such enzyme is one of the important aspects of microbial
411 biotechnology. Quantitative description of cellulose hydrolysis is addressed with respect to
412 adsorption of cellulase enzymes, rates of enzymatic hydrolysis, bioenergetics of microbial
413 cellulose utilization and contrasting features compared to soluble substrate kinetics. A
414 biological perspective on processing cellulosic biomass is presented, including features of
415 pretreated substrates and alternative process configurations. Organism development is
416 considered for “Consolidated bioprocessing” (CBP) , in which the production of cellulolytic
417 enzymes, hydrolysis of biomass and fermentation of resulting sugars to desired products
418 occur in one step. Two organism developmental strategies for CBP are examined: 1)
419 improve product yield and tolerance in microorganisms able to utilize cellulose or (ii) express
420 a heterologous system for cellulose hydrolysis and utilization in microorganisms that exhibit
421 high product yield and tolerance.

422 **ACKNOWLEDGEMENTS**

423
424 Financial support for the first author provided by University Grant Commission through
425 Burdwan University is gratefully acknowledged.

426 **COMPETING INTERESTS**

427
428 Authors have declared that no competing interests exist.

429 **AUTHORS' CONTRIBUTIONS**

430
431
432 SS has performed acquisition of data from different paper and involved in drafting and
433 revising the manuscript. TKM has contributed drafting the manuscript, interpretation of data
434 and necessary correction of the manuscript. All authors read and approved the final
435 manuscript.

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