* Review article

Cellulase Production by bacteria: A Review

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ABSTRACT

Cellulose is the most abundant and renewable 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, 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

1. INTRODUCTION

About 200 gigatons of CO_2 are fixed of earth every year and the equivalent amount of organic material has to be degraded approximately 30–% by plants and animals to 70–% by microorganisms (Gottschalk, 1988). On average, cellulose accounts as 50% of the dry weight of plant biomasson. Such plant biomass is the only foreseeable sustainable source of fuels and materials available to humanity. Agricultural residues are a great source of lignocellulosic biomass which is renewable, chiefly unexploited and inexpensive. These renewable resources are leaves, stems, and stalks from sources such as corn fibre, corn stover, sugarcane bagasse, rice straw, rice hulls, woody crops, and forest residues. Besides, there are multiple sources of lignocellulosic waste from industrial and agricultural processes, e.g., citrus peel waste, coconut biomass, sawdust, paper pulp, industrial waste, municipal cellulosic solid waste, and paper mill sludge. In addition, dedicated energy crops for biofuels could include perennial grasses such as Switchgrass and other forage feedstocks such as *Miscanthus*, Elephant grass, Bermuda grass, etc (Greene et al., 2004).

Approximately 70% of plant biomass is locked up in 5- and 6-carbon sugars. These sugars are found in lignocellulosic biomass, which is comprised of mainly cellulose (a homologous polymer of glucose linked by β 1.4 glycosidic bonds) hydrolysed by a complex enzyme system of microorganisms named as cellulase (exoglucanase, endoglucanase and β glucosidase etc.); less so, hemicelluloses (heterologous polymer of 5- and 6-carbon sugars consists of pentoses D-xylose, D-arabinose and hexoses D-mannose, D-glucose, D-galactose with sugar acids); and least of all lignin (a complex aromatic polymer). In hardwoods hemicellulose contains mainly xylans, while in softwood mainly glucomannans are present. Hydrolysis of hemicelluloses requires various types of enzymes. Briefly, xylan degradation requires endo-1-4,-β-xylanase, β-xylosidase, α-glucuronidase, α-L-arabinofuranosidase, as well as acetylxylan esterases. In glucomannan degradation β-mannanase and β-mannosidase are required to cleave the polymer backbone.

The limited nature of fossil fuels reserves which has been depleting at an alarming rate by civilized world. Burning of fossil fuels has also created a concern for unstable and uncertain petroleum sources, the rising cost of fuels and a concern with respect to global climate change. These concerns have shifted to utilize renewable resources for the production of a 'greener' energy replacement which can meet the high energy demand of the world. The Canadian renewable fuel standard has been raised and will contain 5% ethanol by 2010; the US Environmental Protection Agency raised their renewable fuel standard to 10.21% ethanol mixed fuels by 2009; while, the mandate for mixing ethanol in fuel for Brazil is 25% (set in 2007). Cellulases contribute to 8% of the worldwide industrial enzyme demands (Elba and Maria, 2007). The cellulase market is expected to expand dramatically when cellulases are used to hydrolyzed 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 (Zhang et al., 2006). In the period 2004 -2014 an increase of approximately 100-% in the use of cellulase as a speciality enzyme is expected (Costa et al., 2007). 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$5.40 per gallon of ethanol to approximately 20 cents per gallon of ethanol (Moreira, 2005), in which the two main strategies were (1) an economical improvement in production of cellulase to reduce US$ 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 (Knauf and Moniruzzaman, 2004).

In addition to this, the major indusrial 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(Hill et al., 2006). 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 (Lynd et al., 2005). Application of this enzyme in detergent, leather and paper industries demands identification of highly stable enzymes active at extreame pH and temperature. Some important applications of cellulases or cellulytic 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. Attemts 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.

2. CLASSIFICATION OF CELLULASE

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

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

Endoglucanases cut at random at internal amorphous sites in the cellulose polysaccharide chain, generating oligosaccharides of various lengths and consequently new chain ends. It is generally active against acid-swollen amorphous cellulose, soluble derivatives of cellulose such as CMC, cellooligosaccharides (Wood, 1989).

Table 1. Applications of cellulases or cellulolytic bacteria (Mandel, 1985)

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 andresidues	Sewage treatments

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

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

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

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

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

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

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

It catalyzes the reversible phosphorolytic cleavage of cellobiose. It was first discovered by Ayers (1959) in cells of *Ruminococcus flavefacience.*

$$\text{Cellobiose} + H_3PO_{4=} \text{ alfa} - D - \text{glucose 1-P} + \text{glucose}$$

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

It was found in cells of *Clostridium thermocellulam* (Sheth and Alexandr, 1969). It dose not act on cellobiose but catalyze the reversible phosphorylytic cleavage of cellodextrins ranging from cellotriose to cellohexose.

$$(1,4 - \beta\text{-D-glucosyl})_n + H_3PO_{4=} \; (1,4 - \beta\text{-D-glucosyl})_{n-1} + \text{alfa} - \text{D-Glucose-1-P}$$

2.7 Cellobiose epimerase (EC 5.1.3.11)

It was first reportedin cells of *Ruminococcus albus* (Tyler and Leatherwood, 1967). It catalyzes the following reaction:

$$\text{Cellobiose} = \text{4-O-}\ \beta\text{-D-glucosylmannose}$$

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 Endo-1, 4-beta-D-glucosidic linkages in cellulose, lichenin and cereal beta-D-glucans.	Endoglucanse, Endo-1,4- β –glucanse, Carboxymethyl cellulase, β -1,4-endoglucon hydrolase, Endocellulose	5, 6, 7, 8, 10, 12, 44, 51, 61, 74
ii)Exoglucanase or 1,4-β-D-glucan cellobiohydrolases (cellobiohydrolases)	E.C.3.2.1.91	Hydrolysis of 1,4-beta-D-glucosidiclinkages 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 cellooligosaccharide or from p-nitrphenyl- β-D-cellobioside	Ccellodextrinases	-
iv) β - Glucosidases or β-D-glucoside gluco-hydrolases	E.C.3.2.1.21 .	Hydrolysis of terminal non-reducing beta-D-glucose residueswith release of beta-D-glucose.	Gentobiase, Cellobiase, Amygdalase.	1, 3, 9
v) Cellobiose: orthophosphate alfa–D-glucosyl transferase	E.C. 2.4.1.49	It catalyzes the reversible phosphorolytic cleavage of cellobiose	Cellobiose phosphorylase	-
vi) 1,4-β-D-oligoglucan:orthophosphate alfa –D-glucosyl transferase	E.C. 2.4.1.20	It catalyzes the reversible phosphorolytic cleavage of celldextrins ranging from cellotriose to cellohexoses.	Cellodextrin phosphorylase	-
vii) Cellobiose 2-epimerase	EC 5.1.3.11	It catalyzes the cellobiose into 4-O- β-D-glucosylnannose.	Cellobiose 2-epimerase	-
viii) Complete Cellulase system	-	Catalyzes extensive hydrolysis of crystalline cellulose	Total cellulase	-

3. SCREENING OF CELLULASE PRODUCING BACTERIA

Screening for bacterial cellulase activity in microbial isolates is typically performed on plates containing crystalline cellulose or microcrystalline cellulose such as Avicel in the agar at a final concentration of 0.1-0.5 %(w/v). After incubation of a suitable period, a zone of clearing surrounding the colonies will be indicated that cellulose producer (Kluepfel, 1988). In this semi-quantitative method

the diameter of the zone of clearing will reflect the cellulase activity of the bacterium in question. However, the most crystalline celluloses contain significant amounts of easily degraded region. The colonies of cellulolytic *Cytophaga* spp. did not shown any clearing zone (Schlegel and Schmidt, 1986). So the diameter of the clearing zone may not accurately reflect the true cellulase activity.

For a rapid screening of cellulase producing bacteria, after the incubation of the agar medium are containing 0.5% (W/V) carboxymethyl cellulose (CMC) as sole carbon source and flooded with 1% (W/V) Congo red (Teather and Wood, 1982). After 20 minutes, the dye is decanted and the plates are again flooded with 5M NaCl which is decanted after 20-30 minutes. Positive colonies are detected to be surrounded by a pale orange to clear zone against red background. The cellulolytic bacteria can be screened directly on such plate, but replica plating from master plate is preferred for isolation of active colonies as flooded reagent impairing isolation. Wood et al. (1988) have extended the use of congo red for screening of gene banks and characterization of isolated clones. Plant et al (1988) has reported a semi-quantitative assay for cellulase activity in bacteria by using cellulose-azure into the upper two layers of agar tubes. The dye released from the substrate is determined densitometrically.

Kasana et al. (2008) found that Gram's iodine for plate flooding in place of hexadecyltrimethyl ammonium bromide or Congo red, gave a more rapid and highly discernable result (Kasana et al., 2008). However, plate-screening methods using dyes are not quantitative or sensitive enough due to poor correlation between enzyme activity and halo size. This problem solved by the development of short cellooligosaccharides possessing modified reducing terminal with chromogenic/fluorogenic groups due to achievement of higher sensitivity and quantification. Several examples such as fluorescein, resorufin and 4-methylumbelliferone are well-established (Fia et al., 2005). But a major limitation of the incorporation of fluorescent substrates into agar plates is the tendency for hydrolysis products to diffuse widely and therefore these kinds of compounds are not as readily used. So, new substrates, 2-(2'-benzothiazolyl)-phenyl (BTP) cellooligosaccharides with degree of polymerization (D.P.) 2-4 (BTPG2-4) were synthesized for the screening of microbial cellulolytic activity in plate assays (Ivanen, 2009).

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

4. 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

199 of substrates. The majority of assays involve the accumulation of hydrolysis products, including
200 reducing sugars, total sugars, and chromophores are given in the Table 3.

201 **Table 3: The common colorimetric sugar assays**

Method		**Sample (ml)**	**Reagent (ml)**	**G amount (µg/sample)**	**G concn.**	**References**
Reducing Sugar Assay DNS	Micro	1- 3	3	20- 600	6.7- 600	Miller 1959
DNS	Micro	0.5	3	100- 2500	200- 5000	Ghosh 1987
Nelson-Somogyi	Micro	1- 5	2+2	1- 10	0.2- 10	Somogyi 1952
Nelson-Somogyi	Micro	2	2+2	10- 600	5- 300	Somogyi 1952
Nelson	Semi- Micro	2	2	5- 100	2.5- 50	Nelson 1944
Ferricyanide-1		1- 3	1+5	1- 9	0.3- 9	Park & Johnson 1949
Ferricyanide-2		1	0.25	0.18- 1.8	0.18- 1.8	Kidby & Davidson 1973
PAHBAH	Micro	0.5	1.5	0.5- 5	1- 10	Lever 1972
PAHBAH	Micro	0.01	3	5- 50	500- 5000	Lever 1972
BCA		0.5	0.5	0.2- 4.5	0.4 -9	Waffenschmidt & Janeicke 1987
Modified BCA		1	1	0.4 – 9	0.4 -9	Zhang & Lynd 2005 b
Total Sugar Assay Phenol-H_2SO_4		1	1+5	5- 100	10- 100	Dubois et al. 1956; Zhang & Lynd 2005 b
Anthrone-H_2SO_4		1	1+5	5- 100	10- 100	Roe 1955; Viles & Silverman 1949
Enzymatic Glucose Assay Glucose-HK/PGHD kit		0.01	1	2- 50	200- 5000	Sigma Kit
Glucose-HK/PGHD kit		0.2	0.5	2- 50	4 - 100	Zhang & Lynd 2004 a

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5. 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 cellulases, hemicellulases and often less complex than bacterial cellulase which are secreted to the medium for easy 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 novelcellulase 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 often expressed 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 acidiophilic and halophilic strains, which produce cellulolytic strains that are extremely resistant to environmental stresses. These strains can survive in the harsh conditions found in the bioconversion process and they often produce enzymes that are stable under extreme conditions which may be present in the bioconversion process. 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 ß-glucosidases, but not the complete system, are called “pseudocellulolytic”. Such pseudocellulolytic bacteria may have picked up the genes encoding these enzymes from true cellulolytis 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 – 4)

Table 4. Properties of some Cellulase enzymes isolated from Anaerobic and Aerobic Cellulolytic bacteria

Name of the bacteria	Enzyme	Mol. Wt.	Optimum temp.(ºC)	Optimum pH	References
Aerobic					
Bacillus licheniformis 1	Endoglucanase	-	55	6.1	Dhillon et al. 1985
Bacillus sp (alkalophilic) 1139	Endoglucanase	92	-	9.0	Fukumori et al. 1985
Bacillus sp	Endoglucanase cel A	54	-	5.0-11.0	Sashihara et al. 1984

(alkalophilic) (cloned in E.coli) N-4	Endoglucanase cel B Endoglucanase cel C	46 100		5.0-11.0 9.0	Sashihara et al. 1984 Fukumori et al. 1989
Bacillus sp (neutrophilic) KSM-522	Endoglucanase	35	50	7.0-10.0	Kawai et al. 1988
Bacillus subtilis (cloned in B.megaterium)	Endoglucanase	33	60	5.5	Kim and Pack 1988
Bacillus subtilis DLG	Endoglucanase	35	55	4.8	Robson and Chambliss 1984
Cellulomonas uda	Exocellobiohydrolase	81	45-50	5.5-6.5	Nakamura and Kitamura 1988
Cellvibrio gilvus ATCC13127	Cellobiose phosphorylase	280	<40	7.6	Sasaki 1988
Microbispora bispora	Endoglucanase I Endoglucanase II Exoglucanase I Exoglucanase II ß-Glucosidase	44 57 75 95 -	- - - - -	5.5-7.2 5.5-7.2 5.9-7.2 5.9-7.2 6.0	Waldron et al. 1986; Yablonsky et al. 1988 Waldron et al. 1986
Thermomonospora fusca YX	Endoglucanase 1 Endoglucanase 2	94 46	74 58	6.0 6.0	Calza et al. 1985 Calza et al. 1985
Bacillus M-9	Endoglucanase	54	60	5.0	Bajaj et al. 2009
Bacillus amyoliguefaciens DL3	Endoglucanase	54	50	7.0	Lee et al. 2008
Bacillus sp. HSH-810	Endoglucanase	80	40-70	10.0	Kim et al. 2005
Thermomonospora sp.	Endoglucanase	38	50	5.0	George et al. 2001
Cellulomonas sp. YJ5	Endoglucanase	43.7	60	7.0	Yin et al. 2010
Pseudomanas flurescens	Endoglucanase	36	35	7.0	Bakare et al. 2005
Nocardiopsis sp. KNU	Endoglucanase	-	40	5.0	Saratale and Oh 2011
Bacillus subtilis YJ1	Cellulase	32.5	60	7.0	Xiao et al. 2010
Bacillus sp	Endoglucanase (*Ba* -	74.87	-	-	Zhang et al. 2007

(cloned in E.coli) AC-1	EGA)				
Anaerobic					
Acetivibrio cellulolyticus ATCC33288	Exoglucanase C1 Endoglucanase C2 Endoglucanase C3 ß-Glucosidase B1	38 33 10.4 81.0	- - - -	- - - -	Saddler and Khan 1981
Alcaligenes faecalis	ß-Glucosidase	100	-	-	Day and Withers 1986
Bacteroides cellulosolvens S-85	Endoglucanase EG1	65	39	6.4	McGavin and Forsberg 1988
Bacteroides succinogenes	Endoglucanase EG2	118	39	5.8	McGavin and Forsberg 1988
Clostridium josui	Endoglucanase	45	60	6.8	Fujino et al. 1989
Clostridium thermocopriae JT3-3	Endoglucanase	46	-	6.5	Jin and Toda 1988
Clostridium thermocellum LQRI	Endoglucanase I	94	62	5.2	Ng and Zeikus 1988
Ruminococcus albus SY3	Endoglucanase	30	-	-	Wood 1988b

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236 **6. MODE OF ACTION OF CELLULASE IN BACTERIAL SYSTEM**

237 Investigators have focused on four structures believed to be important in specific adhesion to
238 cellulose:1) large multicomponent complexes called cellulosomes (Morrison and Miron, 2000;); 2)
239 fimbriae or pili adhesions (Morrison and Miron, 2000); 3) Carbohydrate epitopes of bacterial
240 glycocalyx layer(Miron and Forsberg, 1999); and 4) enzyme binding domains (Mitsumori and Minato,
241 1997).

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6.1 Adhesion via Cellulosome like Complexes

Cellulosomes are large, stable, multienzyme complexes specialized in the adhesion to and degradation of cellulose that reside with protuberances visible on the cell surface. The cellulosome complex is composed of a central noncatalytic subunit (termed scaffoldin) which contains a cellulose binding domain (CBD) and a number of attachment sites (called cohesins), which serve to bind the enzymatic submits. The enzymatic submits contain a catalytic domain and a docking domain (called dockerin) the latter interacting with due of the cohesions on scaffoldinwith due of the cohesions on scaffoldin (Shoham et al., 1999). The most complex and best investigated cellulosome is that of the thermophilic bacterium *Clostridium thermocellum.*

6.2 Adhesion via Fimbriae or Pili

Fimbriae or pili, which have been implicated in bacterial adhesion which are surface appendages and 5 to 7 nm in width and 100 to 200 nm in length in gram-negative bacteria (Pell and Schofield, 1993). First, fimbriae were found on gram-negative bacteria, but they also are involved in adhesion of gram positive bacteria. As far has been learned about the role of fimbriae in adhesion, it has become clear that structural subunits of fimbriae are the actual adhesions. Some subunits in the gram-positive bacteria *Actinomyces viscosus* (Yeung and Cisar, 1990) and *S.sanguis* (Fenno et al., 1989) associated with the fimbriae have been identified. In E.coli, the carbohydrate blending sites of three types of fimbriae are in small (28 to 35 Kda) repeated subunits, most of which are in the lips of the fimbriae with a few additional sites along their length (Lindberg, 1987). In *R. albus* 8 a novel forms of cellulose-binding protein (cbpC 17.7 KDa) that belongs to the pil protein (CbpC 17.7 KDa being most similar to the type 4 fimbrial proteins of gram-negative, pathogenic bacteria (Larson et al., 1999).

6.3 Adhesion via Carbohydrates epitopes of bacterial glycocalyx

From electron microscopy observations, most of the evidence about adhesion via carbohydrate epitopes has been found (Cheng and Costerton, 1980). Several studies reported that the slime layer surrounding *Ruminococcus albus* and *Ruminococcus flavefaciens* has composed of glycoproteins (Carbohydrate residues) were involved in adhesions of the bacteria (Cheng and Costerton, 1980). If glycocalyx carbohydrate was removed by periodate oxidation with the protease and dextranase treatment, the adhesion of *R.albus* and *E.succinogenes* to cellulose has been decreased (Pell and Schofield, 1993). More direct evidence for the role of carbohydrate in adhesion was given recently in *Fibrobacter* species (Miron and Forsberg, 1999).

6.4 Adhesion via cellulose- Binding Domains of cellulolytic enzymes

Examination of cellulase structure in some organisms has revealed two functional domains, the active catalytic domain that is responsible for the hydrolytic cleavage of the glycosidic bonds and the binding domain that binds the bacterial enzymes to its substrate. Because of the conserved aromatic residues, it thought that CBD attached to cellulose either by hydrogen bonding or hydrophobic

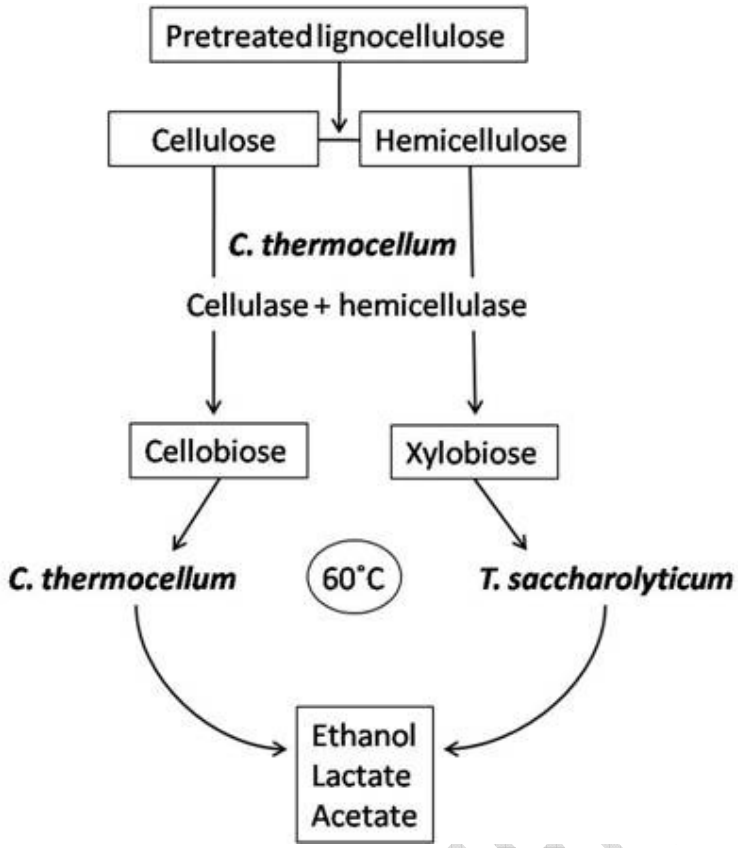
interaction. It has been shown that bacteria lacking these domains were less adherent and in some cases, less able to digest crystalled cellulose (Tomme et al., 1995). Distinct binding domains have been identified in *F.Succinogenes*, including the CBD of endoglunase 2 (EG2) and EGF (McGavin and Forsberg, 1989). Karita et al (Karita et al., 1997) cloned a gene egvI from *R.albus* F-40 and found that the enzyme contained a distinct CBD.

7. CO CULTURE

Bacterial co-cultures can offer a means to improve hydrolysis of cellulose as well as enhance product utilization and thus increase desirable fermentation products. *Clostriium thermocellum* has gained special interest for co-culture with organisms capable of fermenting pentose sugars to ethanol because *C. thermocellum* can only ferement hexose sugars. Hence *C. thermocellum* has been co-cultivated with other anaerobic thermophilic clostridia or close relatives such as *Clostridium thermosacccharolyicum* (now classified as *Thermoanaerobacterium saccharolyticum*) (Venkateswaren and Demain, 1986), *Clostridium thermohydrosulfuricum* (Saddler and Chan, 1985), *Thermoanaerobacter ethanolicus* (Weigel and Ljungdahl, 1979) and *Thermoanaerobium brockii* (Lamed and Zeikus, 1980). These organisms can share a syntrophic relationsip with *C.thermocellum* which exploits its cellulases and hemicellulases to hydrolyze cellulose to cellobiose and cellodextrans, and hemicelluloses to mainly xylobiose, arabinoxylans and xylooligosaccharides. *C.thermocellum* will then convert cellulose breakdown products to ethanol while the latter strains will utilize hemicellulose hydrolysis products to produce ethanol; this avoids the competition for substrates between species and maximizes product formation **(Figure 1**). The current challenge with this type of co-culture application is the increased production of by-products such as acetate and lactate which decrease ethanol production by showing the growth rate of cells (Herrero et al., 1985).

Developing bacterial co-cultures can be a tedious task. To establish a stable co-culture, media and growth requirements, such as temperature, atmosphere and carbon source, must be fine-tuned to permit equal growth of each strain. Stable co-cultures may not only depend on the media and growth requirements of each strain, but may also be controlled more specifically by metabolic interactions(i.e. syntrophic relationships or alternatively competition for substrates) and other interactions (i.e. growth promoting or growth inhibiting such as antibiotics) (Kato et al., 2008).

The alternative of bacterial co-culture would be to engineer one microorganism to complete an entire task from start to finish itself. In the case of *C.thermocellum*, this would mean metabolically engineering this strain to ferment pentose sugars in addition to hexose sugars. This is a difficult task as far as molecular engineering goes in clostridia due the recalcitrance of clostridia to genetic manipulation. Co-cultivation has advantage because it reduces the number of exogenous elements produced by a single bacterial population and therefore reduces the chance of metabolic imbalance for host cells. Additionally, division of labor will simplify the optimization of each reaction path way (Brenner et al., 2008). Although bacterial co-culture is not an uncommon concept, its use in the bioconversion of lignocellulosic biomass is still premature and offers great potential.



316

317 **Figure 1. Simplified process of *C. thermocellum* and *T. saccharolyticum used in* co-culture for**
318 **ethanol production**. *C. thermocellum* produces the cellulases and hemicellulases for hydrolysis of
319 lignocelluloses to sugars such as cellobiose and xylobiose. In addition, *C. thermocellum* can utilize
320 hexose sugars derived from celluloses to produce ethanol. While, the hemicelluloses derived
321 pentoses can be utilized by *T. saccharolyticum*. *T. saccharolyticum* also contributes to cellobiose
322 reduction and is a good ethanol producer (modified from Demain et al.)

323

324 **8. CLONING AND EXPRESSION OF CELLULASE GENES IN HETEROLOGOUS HOSTS**

325 In attempts to clone cellulase genes and express those in bacterial hosts have been undertaken and
326 a review of some of these developments has been presented by Pasternak and Glick (1987).
327 Forsberg et al. (1986) have reviewed the characteristics and cloning of bacterial cellulases,
328 particularly from the rumen anaerobe *Bacteriodes succinogenes.* The most important of these are i)
329 The strategies of cloing cellulase genes from eukaryotic fungal hosts cannot rely on direct expression
330 in a prokaryotic cell because of the differences in the translation mechanism in the two groups, (ii)
331 since the eukaryotic genomes are much larger than those of prokaryotes, a genomic clone bank from
332 a eukaryote needs to be constructed with piece of DNA which are 20-40 kb long. A vector like pBR
333 322 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 pursuant to cellulose conversion via CBP begins with non cellulolytic microorganisms having excellent product formation properties and 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 gene 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* subsp *cellulose* was introduced into *Z.mobilis* (Lejueune et al.,1988). This recombinant strain, however, produced the heterologous endoglucanase intracellularly throughout the growth phase independent of the glucose concentration in the medium (Lejueune et al., 1988). 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 (Yoon et al., 1988).

In contrast to the *P. fluorescens* and *B. subtilis* genes, the endoglucanase gene (Cel Z) of *Erwinia chrysanthemi* was efficiently expressed in *Z.mobilis* (Brestic Goachet et al., 1989). 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* (Okamoto et al., 1994). 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* (Zhou and Ingram, 2001). Initially the expression of Cel Y in *E.coli* was poor was due to promoter construction (Guiseppi, 1991). However, by using a surrogate promoter from *Z.mobilis*, the expression of cel Z in *E.Coli* was increased sixfold.

10. CELLULASE BIOTECHNOLOGY: THE FUTURE

The use of lignocellulosics for the production of ethanol or other chemical feedstocks is one of the most difficult tasks encountered in the history of biotechnology. The methodological basis for studying microbial cellulose utilization is considered relative to quantification of cells and enzymes in the cultures. Quantitative description of cellulose hydrolysis is addressed with respect to adsorption of cellulase enzymes, rates of enzymatic hydrolysis, bioenergetics of microbial cellulose utilization and

contrasting features compared to soluble substrate kinetics. A biological perspective on processing cellulosic biomass is presented, including features of pretreated substrates and alternative process configurations. Organism development is considered for "Consolidated bioprocessing" (CBP)–, in which the production of cellulolytic enzymes, hydrolysis of biomass and fermentation of resulting sugars to desired products occur in one step. Two organism developmental stratigies for CBP are examined: 1) improve product yield and tolerance in microorganisms able to utilize cellulose or (ii) express a heterologous system for cellulose hydrolysis and utilization in microorganisms that exhibit high product yield and tolerance.

REFERENCES

1. Ayers WA (1959). Phosphorolysis and synthesis of cellobiose by cell extracts from *Ruminococcus flavefaciens*. Biochem Biophys Res Comm, 140, 219-229.

   Bacillus strain KSM - 522 that produces alkaline carboxymethyl cellulose. Agric Biol Chem, 52, 1425-1432.

2. Bajaj, B.K., Pangotra, H., Wani, A.M., Sharma, P., Sharma, A. (2009). Partial purification and characterization of a highly thermostable and pH stable endoglucanase from a newly isolated *Bacillus* strain M-9. Indian J Chem Technol, 16, 382-387.
3. Bakare, M.K., Adewale, I.O., Ajayi, A., Shonukan, O.O. (2005). Purufication and characterization of cellulase from the wild-type and two improved mutants of *Pseudomonas fluorescens.* Afri J Biotechnol, 4, 898-904.
4. Brenner, K., Lingchong, Y., Arnold, F.H. (2008). Engineering microbial consortia: a new frontier in synthetic biology. Trends Biotechnol, 66(9), 483-489.
5. Brestic-Goachet, N., Gunasekaran, P., Cami, B., Baratti, J. C. **(**1989). Transfer and expression of an *Erwinia chrysanthemi* cellulase gene in *Zymomonas mobilis*. J Gen Microbiol, 135, 893–902.
6. Calza, R.E., Irwin, D.C., Wilson, D.B. (1985) Purification and characterization of two ß-1,4-endoglucanases from Thermomonospora fusca. Biochemistry, 24, 7797-7804.
7. Cheng, K.J., Costerton, J. W. (1980). Adhesive bacteria – Their role in the digestion of plant material, urea and ephithelial cells. Pages 225-250 in Digestive Physiology and Metabolism in Ruminants. Ruckebusch, Y., Thivend, P. (Eds.), MTP press Ltd., Lancaster, England.

8. Costa, R.B., Silva, M.V.A., Freitas, F.C., Leita˜o, V.S.F., Lacerda, P.S.B. , Ferrara, M.A., Bon, E.P.S. (2008). Mercado e Perspectivas de Uso de Enzimas Industriais e Especiais no Brasil. In: Bon EPS, Ferrara MA, Corvo ML, Vermelho AB, Paiva CLA, Alencastro RB, Coelho RRR (org) Enzimas em Biotecnologia, Produc¸a˜o, Aplicac ¸o˜es e Mercados, edition no.1st; Intercieˆncia, Rio de Janeiro, pp. 463–488.
9. Day, A.G., Withers, S.G. (1986). The purification and characterization of a ß-glucosidase from *Alcaligenes faecalis*. Biobhem Cell Biol, 64, 914-922.
10. Demain, A.L. (2005). Newcomb M, Wu JHD. Cellulase, Clostridia, and Ethanol. Microbiol Mole Biol Rev, **69**(1), 124-154.
11. Dubois, B., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F. (1956) Colormetic method for determination of arrears and relative substances. Anal Chem, 28, 350-6.A
12. Elba, P.S. Bon, Maria, A.F. Bioethanol production via enzymatic hydrolysis of cellulosic biomass. Published in 'The role of agricultural biotechnologies for production of bioenergy in developing countries an FAO seminar held in Rome on 12 October 2007. http:// www.fao.org/biotech/seminaroct 2007.htm
13. Fenno, J.C., LeBlanc, D.J., Fives-Taylor, P. (1989). Nucleotide sequence analysis of a type I fimbrial gene *of Streptococcus sanguis* FW213. Infec Immun, 57, 3527-3533.
14. Fia, G., Giovani, G., Rosi, I. (2005). Study of β-glucosidase production by wine-related yeasts during alcoholic fermentation. A new rapid fluorimetric method to determine enzymatic activity. J Appl Microbiol, 99, 509-517.
15. Forsberg, C.W., Taylor, K., Crosby, B. Thomas, D.Y. (1986). In: Biotechnology and renewable energy Moo-Young, M., Hasnain, S. Lamptey, J. (Eds.) Elsevier Appl Sci Publishers London, 101.
16. Fujino, T., Sukhumavashi, T., Sasaki, T., Ohmiya, K., Shimizu, S. (1989). Purification and properties of an endo-1, 4- ß-glucanase from *Clostridium josui*. I Bacteriol 171, 4076-4079.
17. Fukumori F., Kudo, T., Sashihara, N., Nagata, Y., Ito, K., Horikoshi, K. (1989). The third cellulase of alkalophilic *Bacillus* sp. Strain N-4. Evolutionary relationships within the cel gene family. Gene, 76, 289-298.
18. Fukumori, F., Kudo, T., Horikoshi, K. (1985). Purification and properties of cellulase from alkalophilic *Bacillus* sp. No. 1139. J Gen Microbiol, 131, 3339-3346.

19. George, P.S., Ahmad, A., Rao, M.B. (2001). Studies on carboxymethyl cellulase produced by an alkalothermophilic actinomycete. Bioresource Technol, 77, 171-175.
20. Ghose, T.K. (1987) Measurement of cellulose activities. Pure Appl Chem, 59, 257-68.
21. Gottschalk, G. (1988). In Proc. FEMS Symp.Biochemistry and Genetics of Cellulose Degradation. Aubert, J.P., Beguin, P., Millet, J. (Eds), Academic Press London p.3.
22. Greene et al. (2004) Growing energy. How biofuels can help end America's oil dependence. Nat Res Def Council Rep, 1-86.
23. Guiseppi, A., Aymeric, J. L., Cami, B., Barras, F., Creuzet, N. **(**1991). Sequence analysis of the cellulase-encoding *celY* gene of *Erwinia chrysanthemi*: a possible case of interspecies gene transfer. Gene, 106, 109–114.
24. Herrero, A.A., Gomez, R.F., Roberts, M.F. (1985). 31P NMR studies of *Clostridium thermocellum*. Mechanism of end product inhibition by ethanol. J Biol Chem, 260(12), 7442-7451.
25. Hill, J., Nelson, E., Tilman, D., Polasky, S., Tiffany, D. (2006)**.** Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. Proc Natl Acad Sci USA, 314, 1598-1600.
26. Ivanen, D.R., Rongjina, N.L., Shishlyannikov, S.M., Litviakova, G.I., Isaeva-Ivanova, L.S., Shabalin, K.A., Kulminskaya, A.A. (2009). Novel precipitated fluorescent substrates for the screening of cellulolytic microorganisms. J Microbiol Meth, 76(3), 295-300.
27. Jin, F., Toda, K. (1988). Isolation of new anaerobic, thermophillic and cellulolytic bacteria JT strains and their cellulase production. J Ferment Technol, 66, 389-396.
28. Karita, S., Sakka, K., Ohmiya, K. (1997). Cellulosomes and cellulose complexes of anaerobic microbes: their structure models and function Pages 47-57 in rumen Microbes and digestive Physiology in Ruminants. Onodera, R., Itabashi, H., Ushida, K., Yano, H., Sasaki, Y. (Eds.) Japan Scientific Societies Press, Tokyo.
29. Kasana, R.C., Salawan, R., Dhar, H., Dutt, S., Gulati, A. (2008). A rapid and easy method for the detection of microbial celluloses on agar plates using gram's iodine. Curr Microbiol, 57(5), 53-507.
30. Kato, S., Haruta, S., Cui, Z.J., Ishii, M., Igarashi, Y. (2008). Network relationships of bacteria in a stable mixed culture. Microb Ecol, 56(3), 403-11.
31. Kawai, S., Okoshi, H., Ozaki, K., Shikata, S., Ara, K., Ito, S. (1988). Neutrophilic

461 *32.* Kidby, D.K., Davidson, D.J. (1973)**.** A convenient ferricyanide estimation of reducing sugars in
462 the nanomole range. Anal Biochem, 55, 321-325.
463 33. Kim, J.M., Pack, M.Y. (1988). Endo-ß-1, 4- glucanase encoded by *Bacillus subtilis* gene
464 cloned in *Bacillus megaterium*. Enzyme Microb Technol, 10, 347-351.
465 34. Kim, J.Y., Hur, S.H., Hong, J.H. (2005). Purification and characterization of an alkaline
466 cellulase from a newly isolated alkalophilic *Bacillus sp*. HSH-810. Biotechnol Lett, 27, 313-
467 316.
468 35. Kluepfel, D. (1988). Screening of prokaryotes for cellulose and hemicellulose-degrading
469 enzymres. Meth Enzymol, 160, 180-186.
470 36. Knauf, M., Moniruzzaman, M. (2004). Lignocellulosic biomass processing: a perspective. Int
471 Sugar J, 106, 147–50.
472 37. Lamed, R., Zeikus, J.G. (1980). Ethanol production by thermophilic bacteria: relationship
473 between fermentation product yields of and catabolic activities in *Clostridium thermocellum*
474 and *Thermoanaerobium brockii*. J Bacteriol, 144, 569-578.
475 38. Larson, M. A., Heng, N.C.K., Morrison, M. (1999). Identification of Phynyl-substituted acid
476 responsive operons in *Ruminococcus albus* using differential display RT-PCR. Pages 1-7 in
477 Proc. 99th Annu. General Mtg. Am. Soc. Microbiol., Washington, DC
478 39. Lee, Y.J., Kim, B.K., Lee, B.H., Jo, K.I., Lee, N.K., Chung, C.H., Lee, Y.C., Lee, J.W. (2008).
479 Purification and characterization of cellulase produced by *Bacillus amyoliquefaciens* DL-3
480 utilizing rice hull. Bioresource Technol, 99, 378-86.
481 40. Lejeune, A., Eveleigh, D.E., Colson**,** C. (1988). Expression of an endoglucanase gene of
482 *Pseudomonas fluorescens* var. *cellulosa* in *Zymomonas mobilis.* FEMS Microbiol. Lett, 49,
483 363–366.
484 41. Lever, M. (1972). A new reaction for colorimetric determination of carbohydrates. Analytical
485 Biochemistry, 47, 273-279.
486 42. Lindberg, F., Lund, B., Johansson, L., Normark, S. (1987). Localization of the receptor-
487 binding protein adhensin at the tip of the bacterial pilus. Nature, 328, 84-87.
488 43. Lynd, L.R., Van, Zyl W.H, McBride JE, Laser M. (2005). Consolidated bioprocessing of
489 cellulosic biomass: an update. Curr Opin Biotechnol, 16(5), 577-583.
490 44. Mandels M, 1985. Applications of cellulases. Biochem Soc. Trans, 13, 414-416

45. McGavin, M., Forsberg, C. W. (1989). Catalytic and substrate binding domains of endoglucanase 2 from Bacteroides succinogenes. J Bacteriol, 171, 3310-3315.

46. McGavin, M., Forsberg, C.W. (1988). Isolation and characterization of endoglucanases 1 and 2 from Bacteroides succinogenes S85. J Bacteriol, 170, 2914-2922.

47. Miller, G.I. (1959). Use of dimitrosaliecylic acid reagent for determination of reducing sugar. Anal Chem, 31, 426-8.

48. Miron, J., Forsberg, C. I. (1999). Characterisation of cellulose binding proteins which are involved in adhension mechanism of *Fibrobacter intestinalis* DR7. Appl Microbiol Biotechnol, 51, 491-497.

49. Mitsumori, M., Minato, H. (1997). Cellulose-binding proteins from rumen microorganisms. Page 47-57 *in* Rumen Microbes and digestive Physiology in Ruminants. Onodera, R., Itabashi, H., Ushida, K., Yano, H., Sasaki, Y. (Eds.) Japan Scientific Societies Press, Tokyo, Japan.

50. Moreira, N. (2005). Growing expectations: new technology could turn fuel into a bump crop. Sci News Online, 168(14), 209–24.

51. Morrison, M., Miron, J. (2000). Adhesion to cellulose by *Ruminococcus albus*: a combination of cellulosomes and and Pil-proteins? FEMS Microbiol. Letters, 185, 109-115.

52. Nakamura, K., Kitamura, K. (1988). Cellulases of *Cellulomonas uda*. Meth Enzymol, 160, 211-216.

53. Nelson, N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. J Biol Chem, 153, 375-380.

54. Ng, T.K., Zeikus, J.G. (1988). Endoglucanase from *Clostridium thermocellum.* Meth Enzymol, 160, 351- 355.

55. Okamoto, T., Yamano, S., Ikeaga, H., Nakmura, K. **(**1994). Cloning of the *Acetobacter xylinum* cellulase gene and its expression in *Escherichia coli* and *Zymomonas mobilis*. Appl Microbiol Biotechnol. 42:563–568.

56. Park, J.T., Johnson, M.J. (1949). A submicrodetermination of glucose. J Biol Chem, 181, 149–51.

57. Pasternak, J.J., Glick, B.R. (1987). Biomass conversion technology: Principles and practice Moo- Young (Ed) 139.

58. Pell, A. N., Schofield, P. (1993). Microbial adhesion and degradation of plant cell walls Pages 397-423 in Forage Cell Wall Structure and Digestibility. Hatfield, R. D., Jung, H. G., Ralph, J., Buxton, D. R., Mertens, D. R., Weimer, P. J. (Eds.) ASA-CSSA-SSSA, Madison, WI.
59. Plant, J.E., Attwell, R.W., Smith, C.A. (1988). A semiquantative assay for cellulolytic activity in microorganisms. J Microbiol Meth, 7, 259-264.
60. Robson, L.M., Chambliss, G.H. (1984). Characterization of the cellulolytic activity of a *Bacillus* isolate. Appl Environ Microbiol, 47: 1039-1046.
61. Roe, J.H. (1955). The determination of sugar in blood and spinal fluid with anthrone reagent. J Biol Chem, 212, 335–43.
62. Saddler, J.N., Chan, M.KH. (1985). Conversion of pretreated lignocellulosic substrates to ethanol by *Clostridium thermocellum* in mono- and coculture with *Clostridium thermosaccharolyticum* and *Clostridium thermohydrosulfuricum*. Can J Microbiol, 30, 212–220.
63. Saddler, J.N., Khan, A.W. (1981). Cellulolytic enzyme syatem of Acetivibrio cellulolyticus. Can J Microbiol, 27, 288-294.
64. Saratale, D.G., Oh, S.E. (2011). Production of thermotolerant and alkalotolerant cellulolytic enzymes by isolated *Nocardiopsis* sp. KNU. Biodegradation, 22, 905-919.
65. Sasaki, T. (1988). Cellobiose phosphorylase from *Cellvibrio gilvus*. Meth Enzymol, 160, 468-472.
66. Sashihara, N., Kudo, T., Horikoshi, K. (1984). Molecular cloning and expressionof cellulase genes of alkalophilic *Bacillus* sp. Strain N-4 in Escherichia coli. J Bacteriol, 158, 503-506.
67. Schlegel, H.G., Schmidt, K. (1986). General microbiology, 6th ed. (English translation by Kogut, M.), Cambridge University Press, Cambridge, UK.
68. Sheth K, Alexander JK (1969). Purification and properties of ß-1, 4-oligoglucan: orthophosphate glucosyl transferase from *Clostridium thermocellum*. J Biol Chem, 244, 457-464.
69. Shoham, Y., Lamed, R., Bayer, E. A. **(**1999). The cellulosome concept as an efficient microbial strategy for the degradation of insoluble polysaccharides. Trends Microbiol, 7, 275–281.
70. Somogyi, M. (1952). Notes on sugar determination. J Biol Chem, 195, 19-23.

71. Teather, R.M., Wood, P.J. (1982). Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. Appl Environ Microbiol, 43, 777-780.

72. Tomme, P., Warren, R.A.J., Gikes, N.R. (1995). Cellulose hydrolysis by bacteria and fungi. Advances in Microbial Physiology, 37, 1-81.

73. Tyler TR, Leatherwood JM (1967). Epimerization of disaccharides by enzyme preparations from *Ruminococcus albus*. Arch. Biochem. Biophys, 119: 363-367.

74. Venkateswaren, S., Demain, A.L. (1986). The *Clostridium thermocellum- Clostridium thermosaccharolyticum* ethanol production process: nutritional studies and scale-down. Chem Eng Commun, 45, 53-60.

75. Viles, F.J., Silverman, L. (1949) Determination of starch and cellulose with anthrone. Anal Chem, 21, 950–3.

76. Waffenscfimidt, S., Janaccke, L. (1987). Assay of reducing sugars in the nanomole range with 2, 2-bicinchoninate. Anal Biochem, 165, 337–40.

77. Waldron, C.R., Jr., Becker- Vallone, C.A., Eveleigh, D.E. (1986). Isolation and characterization of a cellulolytic actinomycete *Microbispora bispora*. Appl Microbiol Biotechnol, 24, 477-486.

78. Weigel, J., Ljungdahl, L.G. (1979). Ethanol as fermentation product of extreme thermophilic, anaerobic bacteria. In: Dellweg, H, (Ed.). In Klassische Prozesse mit Neun Aussichten. Berlin, Germany: Verlag Versuchs und Lehranstalt fuer Spiritusfabrikation and Fermentation Technologie im insituut fuer Gaerungsgewerbe und Biotechnologie, pp.117-127.

79. Wood TM (1989). Mechanisms of cellulose degradation by enzymes from aerobic and anaerobic fungi, p. 17-35. In: MP Coughlan (ed.), Enzyme systems for lignocellulose degradation. Elsevier Applied Sceince, London.

80. Wood, T.M. (1988a). Preparation of crystalline, amorphous and dyed cellulase substrate. Meth Enzymol, 166, 19–45.

81. Wood, T.M. (1988b). Cellulases of Ruminococcus albus. Meth Enzymol, 160, 216-221.

82. Xiao, Z.R., Lin, H.H., Yin, L.J. (2010). Purification and characterization of a cellulase from Bacillus subtilis YJ1. J Marine Sci Technol, 18(3), 466-471.

580 83. Yablonsky, M.D., Bartley, T., Elliston, K.O., Kahrs, S.K., Shalita, Z.P., Eveleigh, D.E. (1988).
581 Characterization and cloning of the cellulase complex of *Microbispora bispora*, pp. 249-264.
582 In: Aubert, J.P., Béguin, P., Millet, J. (ed.), FEMSD Symposioum No. 43. Biochemistry and
583 genetics of cellulose degradation. Academic Ptress, New York.
584 84. Yeung, M., Cisar, J. O. (1990). Sequence homology between the subunits of two
585 immunologically and functionally distinct types of fimbriae of *Actinomyces spp.* J Bacteriol,
586 172, 2462-2468.
587 85. Yin, L.J., Huang, P.S., Lin, H.H. (2010). Isolation of cellulase-producing bacteria and
588 characterization of the cellulase from the isolated bacterium *Cellulomonas* Sp.YJ5. J Agric
589 Food Chem, 58, 9833–9837.
590 86. Yoon, K. H., Park, S. H., Pack, M. Y. **(**1988). Transfer of *Bacillus subtilis* endo-1, 4-glucanase
591 gene into *Zymomonas anaerobia*. Biotechnol. Lett, 10, :213–216.
592 87. Zhang, S., Yin, Q.Y., Li, Y. H., Ding, M., Xu, G. J. (2007). Molecular and biochemical
593 characterization of *Ba*- EGA, a cellulase secreted by *Bacillus* sp. AC-1 from Ampullaria
594 crosseans. Appl Microbiol Biotechnol, 75, 1327-1334.
595 88. Zhang, Y.H.P., Himmel, M.E., Mielenz, J.R. (2006). Outlook of cellulase improvement:
596 screening and selection strategies. Biotechnology advances, 24, 452-481.
597 89. Zhang, Y.H.P., Lynd, L.R. (2005b) Determination of the Number-average degree of
598 polymerization of cellodextrins and cellulose with application to enzymatic hydrolysis.
599 Biomacromolecules, 6, 1510-5.
600 90. Zhang, Y-H.P.**,** Lynd, L.R. (2004). Toward an aggregated understanding of enzymatic
601 hydrolysis of cellulose: Non-complexed cellulase systems. Biotechnology and Bioengineering,
602 88, 797-824.
603 91. Zhou, S., Ingram L. O. (2001). Simultaneous saccharification and fermentation of amorphous
604 cellulose to ethanol by recombinant *Klebsiella oxytoca* SZ21 without supplemental
605 cellulase. Biotechnol Lett. 23:1455– 1462.