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Microbial cellulases — Production, applications and challenges

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Microbial cellulases find applications in various industries and constitute a major group of the industrial enzymes. Recently, there is resurgence in utilization of biomass for fuel production employing cellulases and hence forth in obtaining better yields and novel activities. Improving the economics of such processes will involve cost reduction in cellulase production which may be achieved by better bioprocesses and genetic improvement of cellulase producers to yield more of the enzyme. The review discusses the current knowledge on cellulase production by microorganisms and the genetic controls exercised on it. It discusses the industrial applications of cellulases and the challenges in cellulase research especially in the direction of improving the process economics of enzyme production.

Keywords: Biofuel, Cellulase, Endoglucanase, β-Glucosidase, *Humicola*, Lignocellulose, *Trichoderma*

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Introduction

Cellulose is the most common organic polymer, representing about 1.5×10^{12} tons of the total annual biomass production through photosynthesis especially in the tropics, and is considered to be an almost inexhaustible source of raw material for different products[1]. It is the most abundant and renewable biopolymer on earth and the dominating waste material from agriculture[2]. A promising strategy for efficient utilization of this renewable resource is the microbial hydrolysis of lignocellulosic waste and fermentation of the resultant reducing sugars for production of desired metabolites or biofuel.

Cellulose is a crystalline polymer, an unusual feature among biopolymers. Cellulose chains in the crystals are stiffened by inter and intra chain hydrogen bonds and the adjacent sheets which overlie one another are held together by weak Van-der Waals forces. In nature, cellulose is present in a nearly pure state in a few instances whereas in most cases, the cellulose fibers are embedded in a matrix of other structural biopolymers, primarily hemicelluloses and lignin[3-4]. An important feature of this crystalline array is the relative impermeability of not only large molecules like enzymes but in some cases even small molecules like water. There are crystalline and amorphous regions, in the polymeric structure and in addition there exists several types of surface irregularities[5,6]. This heterogeneity makes the fibers capable of swelling when partially hydrated, with the result that the micro-pores and cavities become sufficiently large enough to allow penetration of larger molecules including enzymes. At the molecular level, cellulose is a linear polymer of glucose composed of anhydoglucose units coupled to each other by β-1-4 glycosidic bonds. The number of glucose units in the cellulose molecules varies and degree of polymerization ranges from 250 to well over 10,000 depending on the source and treatment method[7]. The nature of cellulosic substrate and its physical state are important factors in its enzymatic hydrolysis. Though lignocellulosic biomass is generally recalcitrant to microbial action, suitable pretreatments resulting in the disruption of lignin structure and increase accessibility of enzymes have been shown to increase the rate of its biodegradation[8].

Microbial degradation of lignocellulosic waste and the downstream products resulting from it is accomplished by a concerted action of several enzymes, the most prominent of which are the cellulases, which are produced by a number of microorganisms and comprise several different enzyme classifications. Cellulases hydrolyze cellulose (β-1,4-D-glucan linkages) and produce as primary products glucose, cellobiose and cello-oligosaccharides. There are three major types of cellulase enzymes [Cellobiohydrolase (CBH or 1,4-β-D-glucan cellobiohydrolase, EC 3.2.1.91), Endo-β-1,4-glucanase (EG or endo-1,4-β-D-glucan 4-

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glucanohydrolase, EC 3.2.14) and β-glucosidase (BG-EC 3.2.1.21)][9]. Enzymes within these classifications can be separated into individual components, such as microbial cellulase compositions may consist of one or more CBH components, one or more EG components and possibly β-glucosidases. The complete cellulase system comprising CBH, EG and BG components synergistically act to convert crystalline cellulose to glucose. The exo-cellobiohydrolases and the endoglucanases act togetherto hydrolyze cellulose to small cello-oligosaccharides. The oligosaccharides (mainly cellobiose) are subsequently hydrolyzed to glucose by a major β-glucosidase[10-11].

Cellulases are used in the textile industry[12-13], in detergents[14-15], pulp and paper industry[16], improving digestibility of animal feeds[17], in food industry[18], and the enzymes account for a significant share of the world enzyme market. The growing concerns about shortage of fossil fuels, the emission of green house gases and air pollution by incomplete combustion of fossil fuel has also resulted in an increased focus on production of bioethanol from lignocellulosics and especially the possibility to use cellulases and hemicellulases to perform enzymatic hydrolysis of the lignocellulosic material[19-20]. However, in production of bioethanol, the costs of the enzymes to be used for hydrolysis of the raw material need to be reduced and their efficiency increased in order to make the process economically feasible[21].

Commercial production of cellulases has been tried by either solid or submerged culture including batch, fed batch, and continuous flow processes. Media used in cellulase fermentations contain cellulose in different degrees of purity[22-23], or as raw lignocellulosic substrates[24-26], which is especially true in the case of solid-state fermentation. Cellulases are inducible enzymes and the most problematic and expensive aspect of industrial cellulase production is providing the appropriate inducer for cellulases. Cellulase production on a commercial scale is induced by growing the fungus on solid cellulose or by culturing the organism in the presence of a disaccharide inducer such as lactose. However, on an industrial scale, both methods of induction result in high costs. Since the enzymes are inducible by cellulose, it is possible to use cellulose containing media for production but here again the process is controlled by the dynamics of induction and repression. At low concentrations of cellulose, glucose production may be too slow to meet the metabolic needs of active cell growth and function. On the other hand, cellulase synthesis can be halted by glucose repression when glucose generation is faster than consumption. Thus, expensive process control schemes are required to provide slow substrate addition and monitoring of glucose concentration[27]. Moreover, the slow continuous delivery of substrate can be difficult to achieve as a result of the solid nature of the cellulosic materials. The challenges in cellulase production involve developing suitable bioprocesses and media for cellulase fermentation, besides identification of cheaper substrates and inducers. Genetic modification of the cellulase producers to improve cellulase activity has gone a long way to give better producers with high enzyme titers[28-30], but still cellulase production economics needs further improvement for commercial production of ethanol from biomass.

Microorganisms producing Cellulases

Cellulolytic microbes are primarily carbohydrate degraders and are generally unable to use proteins or lipids as energy sources for growth[8]. Cellulolytic microbes notably the bacteria *Cellulomonas* and *Cytophaga* and most fungi can utilize a variety of other carbohydrates in addition to cellulose[31-32], while the anaerobic celluloytic species have a restricted carbohydrate range, limited to cellulose and or its hydrolytic products[33-34]. The ability to secrete large amounts of extracellular protein is characteristic of certain fungi and such strains are most suited for production of higher levels of extracellular cellulases. One of the most extensively studied fungi is *Trichoderma reesei*, which converts native as well as derived cellulose to glucose. Most commonly studied cellulolytic organisms include: Fungal species-*Trichoderma, Humicola, Penicillium, Aspergillus*; Bacteria-*Bacilli, Pseudomonads, Cellulomonas;* and Actinomycetes-*Streptomyces, Actinomucor,* and *Streptomyces*.

While several fungi can metabolize cellulose as an energy source, only few strains are capable of secreting a complex of cellulase enzymes, which could have practical application in the enzymatic hydrolysis of cellulose. Besides *T. reesei,* other fungi like *Humicola, Penicillium* and *Aspergillus* have the ability to yield high levels of extracellular cellulases[35-40]. Aerobic bacteria such as *Cellulomonas, Cellovibrio* and *Cytophaga* are capable of cellulose

Table 1 — Major microorganisms employed in cellulase production

Major group	Microorganism		Ref
	Genus	Species	
Fungi	*Aspergillus*	*A. niger*	40
		A. nidulans	43
		A. oryzae (recombinant)	44
	Fusarium	*F. solani*	46
		F. oxysporum	47
	Humicola	*H. insolens*	36
		H. grisea	42
	Melanocarpus	*M. albomyces*	48
	Penicillium	*P. brasilianum*	38
		P. occitanis	37
		P. decumbans	45
	Trichoderma	*T. reesei*	9
		T. longibrachiatum	41
		T. harzianum	18
Bacteria	*Acidothermus*	*A. cellulolyticus*	52
	Bacillus	*Bacillus sp*	49
		Bacillus subtilis	50
	Clostridium	*C. acetobutylicum*	54
		C. thremocellum	55
	Pseudomonas	*P. cellulosa*	51
	Rhodothermus	*R. marinus*	53
Actinomycetes	*Cellulomonas*	*C. fimi*	58
		C.bioazotea	32
		C.uda	59
	Streptomyces	*S. drozdowiczii*	60
		S. sp	61
		S. lividans	62
	Thermononospora	*T. fusca*	56
		T. curvata	57

degradation in pure cultures[8]. However, the microbes commercially exploited for cellulase preparations are mostly limited to *T. reesei, H. insolens, A. niger, Thermomonospora fusca, Bacillus sp*, and a few other organisms (Table 1).

Cellulase Systems and the Control of Cellulase Gene Expression

Cellulase systems of microbes can be generally regarded as complexed[63-64] or non-complexed[65-67]. Utilization of insoluble cellulose requires the production of extracellular cellulases by the organism. The cellulase systems consist of either secreted or cell associated enzymes belonging to different classes categorized based on their mode of action and structural properties[68-69]. The three major type of cellulase activities recognized are: i) Endoglucanases/1-4-β-D-glucanohydrolases/EG-(EC 3.2.14); ii) Exoglucanases/1-4-β-D-glucan glucanohydrolases/ Cellobiohydrolase/ CBH-(EC 3.2.1.74); and iii) β-Glucosidases/BG/BGL/β-glucoside glucohydrolases-(EC 3.2.1.21). Endoglucanases cut at random at internal amorphous sites in the cellulose polysaccharide chain generating oligosaccarides and new chain ends. Exogulcanases act on the reducing and non reducing ends of the cellulose chains liberating glucose, cellobiose or cellooligosaccharides as major products. β-Glucosidases hydrolyze soluble cellodextrins and cellobiose to glucose.

Non-complexed cellulase systems from aerobic fungi and bacteria have components of cellulase system free and mostly secreted. Typical examples include cellulase system from *T. reesi*[70-71]. Fungus produces two exoglucanases-CBHI &CBHII, about eight endoglucanases-EGI-EGVIII, and seven β-glucosidases-BGI-BGVII[72]. Cellulase system of *H. insolens* is homologus to *T. reesei* and contains at least seven cellulases[36]. Aerobic bacteria like *Thermobifida* also produce all components of cellulolytic system including exo and endo glucanases [8]. Complexed cellulase systems (Cellulosomes) on the other hand are native to anaerobic bacteria. Cellulosomes are protuberances on the cell wall of the bacteria, which harbor stable enzyme complexes. The cellulolytic system of *Clostridia* has been studied in detail[64]. In *C. thermocellum*, the cellulosome consists of a non catalytic *cipA* protein[73] which has different catalytic modules responsible for exo and endo glucanase activities. Individual composition of the cellulosome varies with respect to the organism[8].

Cellulases are inducible enzymes and the regulation of cellulase production is finely controlled by activation and repression mechanisms. In *T. reesei*, genes are coordinately regulated[74]. The production of cellulolytic enzymes is induced only in presence of the substrate, and is repressed when easily utilizable sugars are available. Natural inducers of cellulase systems have been proposed as early as 1962[75], and the disaccharide sophorose is since then considered to be the most probable inducer of at least the *Trichoderma* cellulase system. It is proposed that the inducer is generated by the trans-glycosylation activity of basally expressed β-glucosidase[76,77]. Cellobiose, δ-cellobiose-1-5 lactone and other oxidized products of cellulose hydrolysis can also act as inducers of cellulose[8,78,79]. Lactose is another known inducer of cellulases and it is utilized in commercial production of the enzyme owing to economic considerations. Though the mechanism of

lactose induction is not fully understood, it is believed that the intracellular galactose-1-phosphate levels might control the signaling[80,81]. Glucose repression of cellulase system overrides its induction[74,82], and de-repression is believed to occur by an induction mechanism mediated by trans-glycosylation of glucose[83,84].

The promoter region of cellulases harbor binding sites for the *CREI* catabolite repressor protein as well as sites for the transcriptional activators including Activator of Cellulase Expression protein II (*ACE II*), besides CCAAT sequence, which binds general transcriptional activator complexes designated as '*HAP*' proteins[85]. *ACEII* binds to the promoters of *cbh1* in *T .reesei*, and is believed to control the expression of *cbh1, cbh2, egl1,* and *egl2*[86,87]. *Ace1* gene also produces a transcription factor similar to *ACEII* and has binding sites in *cbh1* promoter, but it acts as a repressor of cellulase gene expression[88,89]. Glucose repression of cellulase is supposed to be mediated through carbon catabolite repressor protein *CRE1* in *T.reesei*[90,91]. The promoter regions of *cbh1, cbh2, eg1* and *eg2* genes of *T. reesei* has *CRE1* binding sites indicating fine control of these genes by carbon catabolite repression[77]. A detailed review on the induction and catabolite repression of cellulases[92] gives better insight into molecular biology of cellulase gene regulation.

Bioprocesses for Cellulase: Fermentation Production of Cellulolytic Enzymes

Majority of the reports on microbial production of cellulases utilizes submerged fermentation technology (SmF) and the widely studied organism used in cellulase production is *T. reesei*, which has also been tested mostly in liquid media. However, in nature, the growth and cellulose utilization of aerobic microorganisms elaborating cellulases probably resembles solid substrate fermentation than a liquid culture. Nevertheless, the advantages of better monitoring and handling are still associated with the submerged cultures.

Cellulase production in cultures is growth associated and is influenced by various factors and their interactions can affect cellulase productivity[93]. Among known inducers of cellulase genes, lactose is the only economically feasible additive in industrial fermentation media[72]. In *T. reesei*, a basal medium after Mandels & Reese[70] has been most frequently used with or without modifications. Carbon sources in majority of commercial cellulase fermentations are cellulosic biomass including straw, spent hulls of cereals and pulses, rice or wheat bran, bagasse, paper industry waste and various other lignocellulosic residues[13,26,50,94-98]. Though majority of the processes are batch processes, there has been attempts to produce cellulase in fed batch[13,99] or continuous[27,100,101] mode, which supposedly helps to override the repression caused by accumulation of reducing sugar. The major technical limitation in fermentative production of cellulases remains the increased fermentation times with a low productivity.

Solid-state fermentation (SSF) for production of cellulases is rapidly gaining interest as a cost effective technology, not only for production of the enzyme but also for the bioconversion of lignocellulosic biomass employing cellulolytic microorganisms[102-105]. Tengerdy[106] indicated that there was about a 10-fold reduction in the production cost in SSF than SmF. Pandey *et al*[107] on SSF for industrial enzyme production also describes the application of the technology for cellulase production. Though there are reports on SSF production of cellulases, the large scale commercial processes are still using the proven technology of SmF (Table 2).

Applications of Cellulases

Cellulases were initially investigated several decades back for the bioconversion of biomass which gave way to research in the industrial applications of the enzyme in animal feed, food, textiles and detergents and in the paper industry[123]. With the shortage of fossil fuels and the arising need to find alternative source for renewable energy and fuels, there is a renewal of interest in the bioconversion of lignocellulosic biomass using cellulases and other enzymes. In the other fields, however, the technologies and products using cellulases have reached the stage where these enzymes have become indispensable.

Textile Industry

Cellulases have become the third largest group of enzymes used in the industry since their introduction only since a decade[123]. They are used in the bio-stoning of denim garments for producing softness and the faded look of denim garments replacing the use of pumice stones which were traditionally employed in the industry[13,124-126]. They act on the cellulose fiber to release the indigo dye used for coloring the fabric,

Table 2 — Cellulase production –Bioprocesses and organisms employed

Microorganism	Substrate	Method	Magnitude	Enzymes - Activity	Ref (s)
Aspergillus niger A 20	Cellulose	SmF	Shake flask	Cellobiase -27.5 U/ml	108
A. niger NRRL3	Wheat bran/Corn cob	SSF	Flask	Cellobiase-215 IU/g cellulose	117
Bacillus pumilus	CMCellulose/Glycerol	SmF	SF	CMCase-1.9 U/ml, Cellobiase - 1.2U.ml	109
Bacillus sp KSM N252	Carboxymethyl cellulose	SmF	Shake flask	CMCase - 0.17 U/mg protein	110
B. subtilis	Soybean industry residue	SSF	Cylindrical bioreactor	FPAse -1.08U/mg protein	50
B. subtilis	Banana waste	SSF	Shake flask	FPAse - 2.8 IU/gds CMCase - 9.6 IU/gds Cellobiase - 4.5 IU/gds	118
Chaetomium thermophilium CT2	Cellulose (sigma cell)	SmF	Shake flask	CMCase -2.7 IU/ml	111
Melnocarpus albomyces	Solka floc	SmF	700L fermentor	Cellulase -1160 ECU/ml, Endoglucanase -3290 ECU/ml,	48
Mixed culture: T. reesei, A. niger	Rice chaff/ Wheat bran (9:1)	SSF	Flask	FPAse -5.64 IU/g	119
Mucor circinelloidens	Lactose	SmF	Shake flask	EGL - 0.25 U/ml	112
Neurospora crassa	Wheat straw	SmF	Shake flask	FPAse - 1.33 U/ml CMCase - 19.7 U/ml BGL - 0.58 U/ml	94
Penicillium decumbans	wheatstraw/bran (8:2)	SSF	SSF bioreactor	Fpase -20.4 IU/g	120
P. occitanis	Paper pulp	SmF-Fed batch	20L fermentor	FPAse - 23 IU/ml CMCase - 21 IU/ml	13
P. janthinellum	Sugar cane bagasee	SmF	Shake flask	FPAse -0.55U/ml, CMCase - 21.5 U/ml, BGL - 2.3I U/ml	97
Phaenerocheate chrysosporium	Cellulose (Avicell)	SmF	100L fermentor	Cellulase - 29mg/g cellulose	113
Rhodothermus marinus	CM cellulose	SmF	150L fermentor	Endoglucanase-97.7 U/ml	53
Steptomyces sp T3-1	Carboxymethyl cellulose	SmF	50L fermentor	CMCase - 148 IU/ml Avicellase- 45 Iu/ml BGL- 137 IU/ml	114
S. drodowiczii	Wheat bran	SmF	Shake flask	CMCase - 595 U/L	60
Thermoascus auranticus	Wheat straw	SSF	Perforated Drum Bioreactor	FPAse - 4.4 U/gds CBH -2.8 U/gds Endoglucanase - 987 U/gds BGL- 48.8 U/gds	121
Thermotoga maritima	Xylose	SmF	Shake flask	Cellobiase-11 mU/ml, Avicellase - 0.3 mU/ml, Beta Glucosidase-30mU/ml	115
Trichoderma reesei	Xylose /Sorbose	SmF-Continuous	Bioreactor	FPAse - 0.69 U/ml/h	100
T. reesei	Steam treated willow	SmF	22L fermentor	FPAse- 108 U/g cellulose	26
T. reesei RUT C30	Cellulose (Avicell)	SmF	Microbubble dispersion bioreactor	FPAse- 1.8U/ml	116
T. reesei RUT C30	Corrugated cardboard	SmF	30L fermentor	FPAse- 2.27 U/ml	95
T. reesei ZU 02	Corn cob residue	SSF	Tray fermentor	FPAse - 158 U/gDS	122
T. reesei ZU-02	Corn stover residue	SmF	30L fermentor	Cellulase - 5.48 IU/ml, FPAse - 0.25 U/ml	96
T. viridae	Sugar cane bagasee	SmF	Shake flask	FPAse - 0.88 U/ml, CMCase - 33.8 U/ml, BGL - 0.33 U/ml	97

producing the faded look of denim. *H. insolens* cellulase is most commonly employed in the biostoning, though use of acidic cellulase from *Trichoderma* along with proteases is found to be equally good[127.] Cellulases are utilized for digesting off the small fiber ends protruding from the fabric resulting in a better finish[127-128]. cellulases have also been used in softening[129], defibrillation[130], and in processes for providing localized variation in the color density of fibers[125,131].

Laundry and Detergents

Cellulases, in particular EGIII and CBH I, are commonly used in detergents for cleaning textiles. Several reports[28,132-133] disclose that EG III variants, in particular from *T. reesei*, are suitable for the use in detergents. *T. viride and T. harzianum* are also industrially utilized natural sources of cellulases, as *A. niger*[15]. Cellulase preparations, mainly from species of *Humicola* (*H. insolens* and *H. grisea* var. *thermoidea*) that are active under mild alkaline

conditions and at elevated temperatures, are commonly added in washing powders[134], and in detergents[135].

Food and Animal Feed

In food industry, cellulases are used in extraction and clarification of fruit and vegetable juices, production of fruit nectars and purees, and in the extraction of olive oil[18]. Glucanases are added to improve the malting of barley in beer manufacturing[136], and in wine industry, better maceration and color extraction is achieved by use of exogenous hemicellulases and glucanases[18]. Cellulases are also used in carotenoid extraction in the production of food coloring agents[137]. Enzyme preparations containing hemicellulase and pectinase in addition to cellulases are used to improve the nutritive quality of forages[138,139]. Improvements in feed digestibility and animal performance are reported with the use of cellulases in feed processing[17, 140]. Bedford *et al*[141] describes the feed additive use of *Trichoderma* cellulases in improving the feed conversion ratio and/or increasing the digestibility of a cereal-based feed.

Pulp and Paper Industry

In the pulp and paper industry, cellulases and hemicellulases have been employed for biomechanical pulping for modification of the coarse mechanical pulp and hand sheet strength properties[142,143], de-inking of recycled fibers[144] and for improving drainage and runnability of paper mills[145]. Cellulases are employed in the removing of inks, coating and toners from paper[146,147]. Bio-characterization of pulp fibers is another application where microbial cellulases are employed[148]. Cellulases are also used in preparation of easily biodegradable cardboard[149]. The enzyme is employed in the manufacture of soft paper including paper towels and sanitary paper[150,151], and preparations containing cellulases are used to remove adhered paper[152].

Biofuel

Perhaps the most important application currently being investigated actively is in the utilization of lignocellulosic wastes for the production of biofuel. The lignocellulosic residues represent the most abundant renewable resource available to mankind but their use is limited only due to lack of cost effective technologies. A potential application of cellulase is the conversion of cellulosic materials to glucose and other fermentable sugars, which in turn can be, used as microbial substrates for the production of single cell proteins or a variety of fermentation products like ethanol. Organisms with cellulase systems that are capable of converting biomass to alcohol directly are already reported[153-155]. But none of these systems described are effective alone to yield a commercially viable process. The strategy employed currently in bioethanol production from lignocellulosic residues is a multi-step process involving pre-treatment of the residue to remove lignin and hemicellulase fraction, cellulase treatment at 50°C to hydrolyze the cellulosic residue to generate fermentable sugars, and finally use of a fermentative microorganism to produce alcohol from the hydrolyzed cellulosic material[156]. The cellulase preparation needed for the bio-ethanol plant is prepared in the premises using same lignocellulosic residue as substrate, and the organism employed is almost always *T. ressei*. To develop efficient technologies for biofuel production, significant research have been directed towards the identification of efficient cellulase systems and process conditions, besides studies directed at the biochemical and genetic improvement of the existing organisms utilized in the process. The use of pure enzymes in the conversion of biomass to ethanol or to fermentation products is currently uneconomical due to the high cost of commercial cellulases. Effective strategies are yet to resolve and active research has to be taken up in this direction. Overall, cellulosic biomass is an attractive resource that can serve as substrate for the production of value added metabolites and cellulases as such.

Apart from these common applications, cellulases are also employed in formulations for removal of industrial slime[157], in research for generation of protoplast[158], and for generation of antibacterial chitooligosaccharides, which could be used in food preservation[159], immuno-modulation[160] and as a potent antitumor agent[161].

Future Perspectives – The Challenges in Cellulase Research

Lignocellulose is the potential source of biofuels, biofertilizers, animal feed and chemicals, besides being the raw material for paper industry. Exploitation of this renewable resource needs either chemical or biological treatment of the material, and

in the latter context cellulases have gained wide popularity over the past several decades. Research has shed light into the mechanisms of microbial cellulase production and has led to the development of technologies for production and applications of cellulose degrading enzymes. However, there is no single process, which is cost effective, and efficient in the conversion of the natural lignocellulosic materials for production of useful metabolites or biofuel. Use of the current commercial preparations of cellulase for bioconversion of lignocellulosic waste is economically not feasible.

The major goals for future cellulase research would be: (1) Reduction in the cost of cellulase production; and (2) Improving the performance of cellulases to make them more effective, so that less enzyme is needed[162]. The former task may include such measures as optimizing growth conditions or processes, whereas the latter require directed efforts in protein engineering and microbial genetics to improve the properties of the enzymes.

Optimization of growth conditions and processes has been attempted to a large extent in improving cellulase production. The section on fermentation production of cellulases describes many of these works basically dealing with empirical optimization of process variables to improve productivity. Many of the current commercial production technologies utilize submerged fermentation technology and employ hyper producing mutants[163]. In spite of several efforts directed at generating hyper producers by directed evolution, the cost of enzymes has remained high[164]. Alternative strategies thought of in cellulase production include mainly solid substrate fermentation on lignocellulosic biomass particularly by using host/substrate specific microorganisms. There are several reports on such use of filamentous fungi in production of optimal enzyme complex for the degradation of host lignocellulose[106,165-167].

Performance of enzyme complexes on lignocellulosic material is best when these complexes are prepared with the same lignocellulosic material as the host/substrate in fermentation[167,168]. Another strategy is to use mixed culture in the production of enzyme. Several reports have shown that mixed culture gives improved production and enzyme complexes with better hydrolytic activity[119,169,170]. Thus, SSF may be considered as a cost effective means for large scale production of cellulases which probably would be several fold cheaper compared to the current commercial preparations.

Cellulases are subject to regulation by various factors and some of the cis-acting promoter elements have been characterized[72]. Active research in this field has led to genetic improvement of cellulase production by various methods including over expressing cellulases from the *cbh1* promoter of *T. reesei* [41,171-173], and generation of desired variation in the cellulase production profile of organism[174,175]. The *cbh1* and *cbh2* promoters of *T. reesei* have also been exploited for expression of foreign proteins in *Trichoderma*[176-178]. Feedback inhibition of cellulase biosynthesis by the end products, glucose and cellobiose, generated by endogenous cellulolytic activity on the substrate is another major problem encountered in cellulase production. Cellobiose is an extremely potent inhibitor of the *CBH* and *EG* biosynthesis. *Trichoderma* and the other cellulase-producing microbes make very little β-glucosidase compared to other cellulolytic enzymes. The low amount of β-glucosidase results in a shortage of capacity to hydrolyze the cellobiose to glucose resulting in a feed back inhibition of enzyme production and in the case of biomass conversion applications in the inhibition of cellulases. This issue has been addressed by various means like addition of exogenous β-glucosidases to remove the cellobiose[179] and engineering β-glucosidase genes into the organism so that it is overproduced[175]. More and more research is oriented in genetic manipulations of cellulase producers for improving productivity. The developments in process design and medium formulations have come to an age and the future definitely requires controlled genetic interventions into the physiology of cellulase producers to improve production and thereby make the cellulase production process more cost effective. The major tasks ahead include overriding the feedback control by glucose and development of integrated bioprocesses for the production of cellulases.

Improvements in cellulase activities or imparting of desired features to enzymes by protein engineering are probably other areas where cellulase research has to advance. Active site modifications can be imparted through site directed mutagenesis and the mutant proteins can be used for understanding the mechanisms of action as well as for altering the substrate specificities or improving the activities. There are several reports of developments made in

this direction. Meinke *et al*[180] has generated a mutant enzyme with endoglucanase like features and improved activity by deleting C-terminal loop of *Clostridium fimi CELB*. Protein engineering has been successfully employed to improve the stability of a *Humicola* cellulase in presence of detergents[181], to improve the thermostability of an alkaline, mesophilic *endo*-1, 4-β-glucanase from alkaliphilic *Bacillus* sp[182] and for altering pH profile of cellobiohydrolase[183] and more recently endoglucanase[184] from *T. reesei*. Such modifications affecting the enzyme properties may be beneficial in improving the overall performance of cellulases and a better understanding of their mode of action, which will enable better utilization of enzymes in biomass conversion. More basic research is needed to make designer enzymes suited for specific applications.

Concluding Remarks

The biological aspects of processing of cellulosic biomass become the crux of future researches involving cellulases and cellulolytic microorganisms. The problems which warrants attention is not limited to cellulase production alone, but a concerted effort in understanding the basic physiology of cellulolytic microbes and the utilization of this knowledge coupled with engineering principles to achieve a better processing and utilization of this most abundant natural resource. The aspects open to consideration include technologies for pre-treatment of cellulosic materials for a better microbial attack, processes for cost effective production of cellulases, treatment of biomass for production of hydrolytic products, which can then serve as substrates for downstream fermentative production of valuable metabolites, organism development by metabolic engineering, and finally protein engineering to improve the properties of enzymes to increase their specific activities, process tolerance and stability.

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