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Progress in research on fungal cellulases for lignocellulose degradation

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Fungal cellulases offer advantages of a secreted enzyme complex and relative easiness and economy of producing enzyme. Considerable amount of work has been done on fungal cellulases, especially with resurgence of interest in biomass-ethanol and concept of bio-refineries. Significant information has also been gained on basic biology of organisms producing cellulases, and in process development for enzyme production and biomass saccharification. This review addresses developments in the field of fungal cellulases for lignocellulose degradation.

Keywords: Aspergillus, Bioethanol, Biomass, Biorefinery, Cellulase, Fungi, ²-Glucosidase, Trichoderma

Introduction

Bioconversion of lignocellulosic biomass (LB) can contribute significantly to the production of organic chemicals, majority of which (> 75%) are produced from primary base chemicals (ethylene, propylene, benzene, toluene and xylene). These compounds act as intermediates for synthesis of various polymers, resins and other chemicals[1]. Using LB as feedstock for a biorefinery, aromatic compounds can be produced from lignin, whereas low molecular weight aliphatic compounds can be produced from ethanol derived from cellulose and hemicellulose[2]. Cellulose, an almost inexhaustible raw material, is most abundant and ubiquitous biopolymer on earth. LB is also considered to be the only foreseeable source of energy[3]. LB is mainly composed[4] of (dry wt basis): cellulose, 40-60; hemicellulose, 20-40; and lignin, 10-25%. Most efficient method of biomass hydrolysis is through enzymatic saccharification[5] using cellulases and hemicellulases. Fungal cellulases (FCs) have proved to be a better candidate than other microbial cellulases, with their secreted free cellulase complexes comprising all three components of cellulase [endoglucanases, exoglucanases and cellobiases (²-glucosidases).

Cellulases are being commercially produced for biomass saccharification with all leading enzyme companies developing FCs. There is still a large gap between market price of enzyme and what would be economically feasible for a bio-refinery or bio-ethanol production facility, which uses LB as raw material. Sugar yield from a pretreated feedstock is largely dependent on the type of cellulases and their activities. These features will largely determine enzyme loading and duration of hydrolysis, which in turn determines overall process economics. Economics of ethanol from LB[6,7] shows that the cost of cellulase is a major contributor to production costs (40-49%) of the net production costs. Economical bioconversion requires an appropriate pretreated biomass and an effective cellulase system. FCs offer the advantage of highly efficient enzyme complexes, and relative easiness of production. Active research is going on worldwide in all aspects of cellulase enzyme technology including basic studies on fungal physiology and biochemistry with respect to biomass hydrolysis, cellulase gene regulation and expression, recombinant enzymes, protein engineering of cellulases, process development for cellulase production, development of enzyme cocktails, artificial cellulase complexes and fermentation.

Present paper reviews major research activities in FCs in perspective of their wider application in bio-ethanol industry and in bio-refineries.

Fungal Cellulases (FCs)

Cellulase Basics: Mode of Action and Synergism

Cellulases hydrolyze β-1, 4-D-glucan linkages in cellulose and produce glucose, cellobiose and cello-

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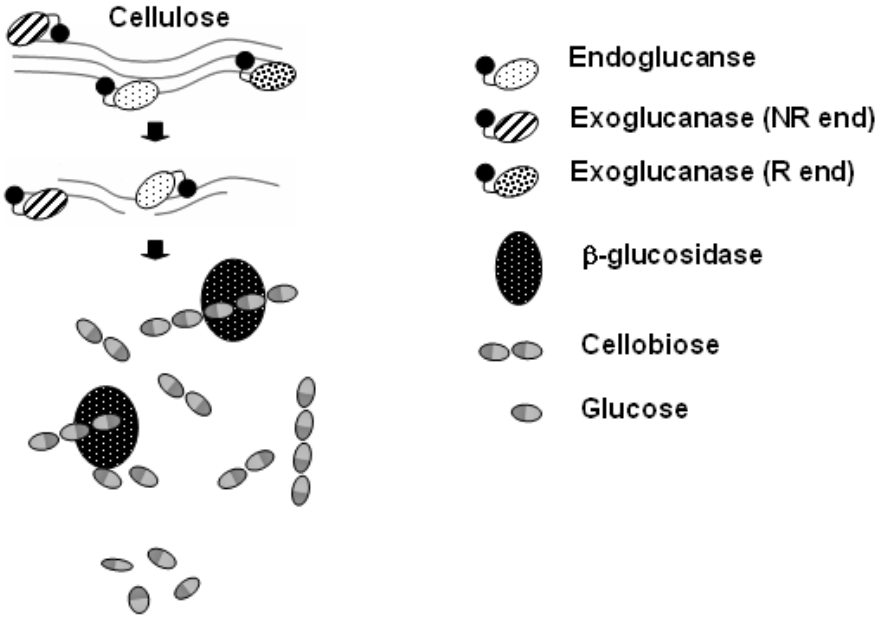

Fig. 1—Mechanism of cellulase action

oligosaccharides. Cellulases are produced by a number of microorganisms and comprise several different enzyme classifications. Three major types of cellulase enzymes[8] [cellobiohydrolase (CBH), endo-β-1, 4-glucanase (EG) and β-glucosidase (BGL)] are involved in hydrolysis of cellulose. There are multiple enzymes within these classifications. For example, most studied fungus for cellulase production,

Trichoderma reesei[9], produces 2 CBH, 8 EG and 7 BGL. EGs produce nicks in cellulose polymer exposing reducing and non-reducing ends, CBH acts upon these reducing and non-reducing ends to liberate cello-oligosaccharides and cellobiose units, and BGL cleaves cellobiose to liberate glucose completing hydrolysis (Fig. 1). Complete cellulase system comprising CBH, EG and BGL components thus acts synergistically to convert crystalline cellulose to glucose[10,11] Majority of cellulases have a characteristic two-domain structure[12,13] with a catalytic domain (CD) and a cellulose binding domain [CBD; also called carbohydrate binding module (CBM)) connected through a linker peptide]. Core domain or catalytic domain contains catalytic site whereas CBDs help in binding of enzyme to cellulose.

Degradation of native cellulase requires different levels of cooperation between cellulases. Such synergisms exist between endo and exoglucanases (exo/endo synergism) and between exoglucanases. In first type, EC action creates free ends, on which exoglucanases act, and in second one, exoglucanases cooperate by acting on reducing and non-reducing ends to bring about effective cellulose degradation[14]. Individual enzymes are not able to degrade cellulose completely while mixtures of enzymes enhance efficiency of saccharification. Supplementation of heterogeneous BGL is believed to enhance hydrolytic potential of FLs synergistically, though there are contradictory reports[15].

Cellulase Systems of Fungi

Components of cellulase system were initially classified based on their mode of action but are now classified based on their structural properties[16-18]. Cellulases are one of the largest groups in structural classification of glycosyl hydrolases. Cellulases and hemicellulases make up 15 of 70 identified glycosyl hydrolase families and some families are still divided to subfamilies. This classification is based on variability of catalytic domains and does not consider variability in cellulose binding domains. Cellulolytic enzyme systems are extensively studied in a wide variety of microorganisms, complexed or non-complexed[5], including aerobic and anaerobic bacteria[19], white rot and soft rot fungi[20] and anaerobic fungi[21]. In filamentous fungi, actinomycetes and in aerobic bacteria, cellulases are mostly secreted as free molecules. Cellulases in certain anaerobic cellulolytic bacteria like *Clostridium*

thermocellum are organized into high molecular weight complexes called cellulosomes[22], where enzyme systems are called complexed. Cellulosomes are found as protuberances on cell wall and are stable enzyme complexes capable of binding cellulose and bringing its degradation. Much of what is known about cellulosomes has come though studies on anaerobic bacterium – *Clostridium thermocellum*[22]. Cellulase-hemicellulase complex of *C. thermocellum* contains up to 26 polypeptides with at least 12 endo and exo cellulases, 3 xylanases, lichenase and a non-catalytic cellulosome integrating protein (CipA) or scaffoldin. Enzymes bind through dockerin moieties onto complementary receptors on scaffoldin called cohesins[21]. Type of activities and number of catalytic domains may be different in other anaerobic bacteria with complexed cellulolytic systems, but basic architecture of cellulosome is almost conserved. Cellulosomes in anaerobic fungi have a catalytic unit subunit linked with 2-3 copies of conserved, 40 amino acid cysteine rich, non-catalytic docking domain (NCDD) by a serine threonine rich linker. NCDD bears no homology with bacterial dockerins, but on the contrary, have similar size and number of polypeptides. Enzymes associated with fungal cellulosomes are modular and are from t genera *Neocallimastix*, *Orpinomyces*, and *Piromyces*[23]. However, molecular arrangement of fungal cellulosomes remains unknown.

Non complexed cellulase systems are more common and are presently most exploited class for industrial applications. These are mainly found in aerobic fungi, and are largely described based on *Trichoderma, Penicillum, Fusarium, Humicola, Phanerochaete* etc, where a large number of cellulases are encountered. Of these, cellulase system of *T. reesei* consists of 2 CBHs (CBHI & CBHII), 8 EGs (EGI-EGVIII), and 7 BGLs (BGI-BGVII)[9]. Among several other fungi that are capable of cellulose degradation, *Humicola, Aspergilli, Penicillium, Neurospora, Chaetomium* and *Fusarium* have been studied in detail. *H. insolens* cellulolytic system is homologous to *T reesei*, as it also contains 2 CBHs (CBH and CBH), 5 EGs (EG, EG, EG, EGV and EGV)[24] but lacks CBM in EG as well as EG. *H. grisea* produces a thermo-stable endoglucanase (Cel12A) enzyme with sequence similarity to *T reesei* Cel12A[25]. Species of *Aspergilli* are known to produce all three enzyme activities of cellulase complex[26-28] and exhibit strong hydrolytic activity towards cellulose but are major producers of BGLs at comparatively high level than *T reesei* and some of these BGLs are also glucose tolerant[29,30]. *T reesei* BGLs is subject to product inhibition and through it is sufficient to support growth on cellulosic material; it is often supplemented with *Aspergillus* BGLs for biomass saccharification at industrial level[31]. Existence of 2 EGs and 1 BGLs is also reported from *A. oryzae*[32]. *P. chrysosporium*, a white rot fungus, produces a complex array of cellulases, hemicellulases and ligninase capable of lignocellulose degradation[33].It produces a cellulase system with 1 CBHII and 6 CBHI-like homologues, of which CBHI-4 is major cellobiohydrolase[34]. A 28 kDa EG28, lacking a CBM, is also reported in the fungus[35]. Synergism between EG28 and cellobiohydrolases was demonstrated, and suggested that EG28 is homologous to EGIII of *T. reesei* and *H. insolens*. A detailed review of non complexed cellulases may be found elsewhere[36].

High titers of cellulase production are also reported in species of *Penicillium*[37,38] and there are reports on strain improvement for increased cellulase production by mutation[39]. Five different cellulases were reported from *P brasilianum*[40].

Regulation of Cellulase Gene Expression in Fungi:

***Trichoderma reesei* Cellulase System as a Model**

T. reesei cellulases are inducible enzymes and regulation of cellulase production is finely controlled by activation and repression mechanisms[41] and genes are found to be coordinately regulated[42]. Cellulase production in *T reesei* is Sophorose and is proposed as inducer of at least *Trichoderma* cellulase system and is thought to be generated by trans-glycosylation activity of a basally expressed BGL[43,44]. Cellobiose, δ-cellobiose-1-5 lactone and other oxidized products of cellulose hydrolysis can also act as inducers of cellulose[5]. Lactose, another known inducer of cellulase, is utilized in commercial production of enzyme due to economic reasons. Though mechanism of lactose induction is not fully understood, it is now known that lack of galactose mutarotase activity is crucial for cellulase induction in fungus[45].

Glucose repression of cellulase system overrides its induction[42,46] and de-repression is believed to occur by an induction mechanism mediated by trans-glycosylation of glucose[47,48]. Analyses of promoters of cellobiohydrolase I and II has shown binding sites of at least three transcription activators (ACEI, ACEII and HAP 2/3/5) and one carbon catabolite repressor (CREI).

Molecular mechanism of gene induction in presence of cellulose is still unclear. Expression of cellobiohydrolases and at least two endoglucanases (egl1 and egl2) are believed to be controlled by ACEII binding to their promoters[49,50]. ACEI is believed to act as a repressor of cellulase gene expression[51,52]. HAP 2/3/4 binding to promoter of cellobiohydrolase I is evidenced by the presence of its binding sequence in promoter region[53]. Glucose repression of cellulase is supposed to be mediated through carbon catabolite repressor protein *CRE1*[54,55], and promoter regions of *cbh1, cbh2, eg1* and *eg2* genes have *CRE1* binding sites indicating fine control of these genes by carbon catabolite repression[43]. Suto & Tomita[56] has given a detailed review on induction and catabolite repression of cellulases

Engineered Cellulolytic Fungi and Artificial Cellulase Complexes

Though there are several fungi capable of cellulase production, enzyme yield and levels of individual cellulase components are not often satisfactory for commercial biomass saccharification. Improvements in cellulase titers as well as ability to tailor ratios of endo and exo glucanases and BGL produced by organisms are highly desired. Filamentous fungi possess an efficient secretion system that is capable of glycosylating proteins and have higher specific growth rates than plant, insect or mammalian cells. Though filamentous growth form causes difficulties in mass transfer compared to yeast or bacterial growth, efficient technologies have been developed for antibiotic, organic acid and native enzyme production from filamentous fungi[57]. Expression cassettes, site directed mutagenesis and antisense technology have been successfully employed in engineering of fungi for cellulase production. Potent cellulase genes from different filamentous fungi can be isolated, cloned and expressed in fungal hosts (especially cellulase producers like *Trichoderma* and *Aspergillus*) to get better combination or synergism. Even classical approaches of inducing genetic variation like random mutagenesis have yielded strains of *T. reesei* with significant improvement in cellulase production[58]. Major approach towards engineering organisms for cellulase production have been the use of modern molecular biology techniques, especially for construction of genetically modified fungi with improved cellulase profiles.

CBHI promoter of *T reesei* is a highly efficient promoter with unusually high rate of expression under cellulase induction conditions and this promoter has been used to drive expression of BGL[59] and EGs[60], thereby improving cellulase profile of host strain. Authors had suggested feasibility of using such expression constructs in several filamentous fungi including *Trichoderma, Aspergillus, Penicillium, Humicola, Fusarium, Verticillium, Neurospora, Pleurotus* etc.[61]. Promoter has also used to drive expression of homologous and heterologous proteins in *Trichoderma*[62,63]. CBH I and CBH II promoters from *T. longibrachium* has also been used successfully for expression of cellulases in this fungus[64].

Glucose repression of cellulase genes has been addressed by using a truncated CBH I promoter lacking binding sites for carbon catabolite repressor CRE1[65]. Another major strategy employed for improving cellulase production in presence of glucose is to use promoters that are insensitive to glucose repression. Nakari-Setala & Pentilla[70] used promoters of transcription elongation factors *1α* and *tef1*, and that of an unidentified cDNA (*cDNA1*) for driving expression of endoglucanase and cellobiohydrolase in *T. reesei* with the result of de-repression of these enzymes. These studies indicate that proper engineering of sequences to obtain expression of proteins from *cbh1* promoter and manipulations of promoter to abolish repression can dramatically improve production of cloned protein.

T reesei cellulase system as well as cellulase systems of several other fungi is limited by relatively lesser amount of BGL and its feed back inhibition by glucose. Enzyme is also inhibited by its own substrate, cellobiose[67]. Considering these, a BGL, which is insensitive or at least tolerant to glucose and cellobiose, is highly desired for conversion of cellulosic biomass to glucose[41]. Research on this line has yielded potential BGLs from different microorganisms like *Candida peltata*[68], *Aspergillus oryzae*[30] and *A. niger*[69]. One of the major approaches taken towards improving cellulase complex for biomass hydrolysis is to increase copy number of BGL gene and thus amount of BGL in cellulase mixture produced by *T. reesei*[70], while other is to alter cellulase profile of *T reesei* by introducing glucose tolerant BGL gene into fungus[59]. Similarly in another work, thermo-tolerant endoglucanase and BGL from thermophilic fungus *Thermoascus aurantiacus* were expressed in *Kluveromyces*[71].

Modification of cellulase properties to enhance efficiency or to impart desired features is another major

area of research. Studies[72] on protein engineering approaches adopted in cellulase modification apparently give basic information about cellulase molecular biology, which is crucial for designing of any strategy for genetic improvement of fungus for enhanced production of enzyme.

Preparations of cellulase from a single organism may not be highly efficient for hydrolysis of different feedstock. Hydrolytic efficiency of a multienzyme complex for lignocellulose saccharification depends both on properties of individual enzymes and their ratio in multienzyme cocktail[72]. Filamentous fungi are major source of cellulases and hemicellulases and mutant strains of *Trichoderma* (*T. reesei, T. viride* and *T. longibrachium*) are best-known producers of the enzyme. *Trichoderma* species have a low level of BGL activity[73] resulting in an inefficient biomass hydrolysis. Ideal cellulase complex must be highly active on intended biomass feedstock, able to completely hydrolyze biomass, operate well at mildly acidic pH, withstand process stress, and be cost effective[75,76]. The success of any lignocellulosic ethanol project will depend on the ability to develop such cellulase systems. Key to developing cellulases is to artificially construct them either by enzyme assembly to form cocktails or to engineer cellulase producers to express desired combination of cellulase enzymes. Enzyme cocktails have been developed by mixing *T reesei* cellulase with other enzymes (xylanases, pectinases and BGL), and these cocktails were tried to hydrolysis various feedstocks[77-79]. Recently developed cocktail include multienzyme complex developed based on highly active *Chrysosporium lucknowense* cellulases[73].

Artificial cellulosomes generated by engineering cellulosome bearing bacteria can be used to express heterologous cellulases. Chimeric cellulosomes have been described for degradation of cellulosic substrates either by incorporating bacterial[80,81] or fungal[82] cellulases in cellulosomes by genetic engineering. Artificial cellulase complexes displayed enhanced activities compared to corresponding free systems at least in the case of bacterial enzymes[80,81]. Enhancement in activity was proposed to be resultant of additional synergy induced by enzyme proximity within the complex and effect of cellulose binding module offered by chimeric scaffoldin that anchors the whole complex at substrate surface[82].

Research on Fermentation Technologies for Fungal Cellulase Production

A two stage continuous process for cellulase production has been described as early as 1979[83], in which growth and production phases were separated by different pH and temperature optima. Repression by glucose and cellobiose are known features of cellulase systems and several attempts have been directed towards development of mutants resistant to catabolite repression[1,84,85]. Cellulases of *T. reesei* are inducible enzymes and best activities were reported when grown in medium containing cellulose. Mostly, pure cellulose preparations like Solka-Floc and Avicell has been used in liquid cultures of cellulolytic microbes for production of enzymes and natural cellulosic materials when used as carbon source gave poor enzyme yields[86]. While using soluble substrates, break down products may hamper cellulase synthesis by promoting catabolite repression due to accumulation of free sugars. Increased production in fermenters may be achieved by a gradient feed of a suitable cellulose and maintenance of process conditions at their optimal. Cellulase production has been attempted on a wide range of substrates ranging from pure cellulose[87] to dairy manure[88] and traditionally agro-residues have been used frequently as carbon sources in cellulase fermentations. Most of these are capable of inducing cellulase system in fungi often at par with known inducers or sometimes even better.

Media formulation for fermentation is mostly specific for organism concerned and no general composition can give optimum growth and cellulase production. A basal medium after Mandal & Reese[89] has been most frequently used for cellulase production in fungi, especially in *T reesei*. Cellulase production in cultures is growth associated and is influenced by various factors (substrate used for enzyme production, pH of medium, fermentation temperature, aeration, inducers etc.), which alone or in interaction can affect cellulase productivity[90]. Among known inducers, lactose is most commonly used as medium additive due to its lower cost and availability[3]. Most frequently, media pH used for cellulase production by fungi is in acidic range, while optimal temperature reported was 25-30°C [91-95]. Cellulase production processes can be operated in batch, fed batch or continuous [96-100]. Fed batch or continuous mode in several cases can help to override repression caused by accumulation of reducing sugar. Major technical limitation in fermentative production of cellulases

remains e 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 enzyme production but also for bioconversion of LB employing cellulolytic microorganisms. Chahal[92] reported a higher yield of cellulases from *T. reesei* in SSF cultures compared to liquid cultures. Large-scale process employing SSF for commercial production of cellulase is reported in Japan using Koji technique, wherein *T. reesei* was cultivated on wheat bran[101]. Tengerdy[102] found that production cost in crude fermentation by SmF was about $20/kg, and it was only $0.2/kg by SSF, if *in situ* fermentation was used. Nigam & Singh[103] suggested that with appropriate technology, improved bioreactor design, and operation controls, SSF might become a competitive method for cellulase production. Reviews[104-105] are available on application of SSF technology for cellulase production. SSF can be proposed as a better technology for commercial production of cellulases considering low input cost and ability to utilize naturally available sources of cellulose as substrate.

Commercial Production of Fungal Cellulases for Cellulosic Ethanol: Research Progress

The demand for more stable, highly active and specific enzymes is growing rapidly and projected world market for industrial enzymes is rapidly growing at an annual rate of about 7.6% and is estimated to be $6 billion by 2012[106]. A majority of world's total supply of industrial enzymes is produced in Europe, USA and Japan[107]. Majority (75%) of industrial enzymes are hydrolases, followed by carbohydrolases. Biotechnology of cellulases and hemicellulases began in early 1980s, initially in animal feed industry followed by food applications[108,109]. The use of cellulases and hemicellulases has increased considerably, over the last two decades especially in textile, food, brewery and wine as well as in pulp and paper industries. Cellulases accounted for approx. 20% of world enzyme market in later half of last decade[62], and mostly the enzymes were sourced from fungi, *Trichoderma* and *Aspergillus*[101].

Though current applications of cellulases in food and textile industries generate millions of dollars, it is envisaged that utilization of LB for biofuel production will be major area where cellulases would be commercially exploited. Potential for ethanol production from biomass lies in enzymatic hydrolysis of cellulose using cellulolytic enzymes. However, cost of cellulases still is high to be used economically in bioconversion of biomass and major challenge for cellulosic ethanol is the cost reduction of enzymes. Large-scale applications of bioethanol in fuel blends will reduce CO_2 and other emissions from transport sector. Approx. 17 million tons of fuel ethanol is currently being produced from sugarcane and starch crop residues in Brazil, US and some EU countries combined at the cost of 0.5-0.7 $/l, which is about twice the price of gasoline. US and European market for bioethanol is projected to grow considerably in coming years due to the policies taken to substitute at least a fraction of fossil transport fuels by renewable biofuels. Lignocellulose to ethanol production technology have been extensively investigated in the US, Canada and some EU countries[110].

Current international players in the production of commercial cellulases include enzyme manufacturing giants Genencor and Novozymes. National Renewable Energy Laboratory (NREL) of USA have set their goals for reducing the cost of cellulases used in bioethanol production, for which projects were initiated in 2000 with Genencor Corporation and Novozymes as contract partners. Genencor in 2004 has achieved an estimated cellulase cost between $0.10-0.20 per gallon of ethanol in NREL's cost model[111]. The company had recently announced the launch of first ever commercial enzyme product for cellulose ethanol[112] and have recently announced a joint venture with DuPont to setup a cellulosic ethanol plant that will use corn stover and sugarcane bagasse as feedstock[113]. Similarly, collaborative subcontract between Novozymes and NREL has been able to reduce the cost of cellulases for biomass to ethanol to $0.10-0.18 /gal, which is almost 30 fold reduction from estimated cost in 2001[114]. Novozymes predicts that their enzymes will produce second generation bio-ethanol by 2010. The company also has announced setting up of an $80-100 million production facility in Nebraska for cellulase production[115].

Though enzyme majors Genencor and Novozymes are hoping of reducing enzyme cost for lignocellulosic ethanol production, still remains a long way to go in understanding mechanisms of cellulase gene regulation and structure to function relationships. One major step in this direction is an study on *T reesei* genome[116], which revealed that genome of fungus contains fewer cellulases and hemicellulases than any other sequenced fungi despite being the best known producer of cellulases.

Genes coding for enzymes acting on carbohydrate polymers are distributed in clusters and there are indications on existence of numerous biosynthetic pathways for secondary metabolite production. However, authors could not find any deep insight into highly efficient protein secretion machinery in fungus at least in initial analysis. This work has tremendous implications in understanding genetics of this important organism, which is used to produce cellulase enzymes, and other important proteins. Also, such knowledge will enable improved production processes critical to reducing the cost of biomass conversion.

Conclusions

After decades of research on lignocellulose utilization, it is now considered that enzyme based technologies for biomass conversion are most efficient, cost effective and environment friendly. Considerable progress has been made in cellulase enzyme research and enzyme preparations with significant cost advantages have been developed. It is speculated that even before the end of next decade, lignocellulosic ethanol will be a commercial reality. While enzyme majors like Genencor and Novozymes have proclaimed that their cellulase preparations for biomass saccharification are significantly reduced in cost to make biomass-ethanol a feasible option, it might still be few years from now when full-fledged commercial production of enzymes and bio-ethanol can commence. Apparently with increased number of plants for biomass conversion, demand for commercial cellulases will be further more and industries have to be prepared to meet these increasing demands. Wider applicability of existing cellulase preparations and the ones, which are being developed, for hydrolysis of more number of feedstocks may have to be demonstrated. Also needed is further understanding of microbial physiology and genetics of cellulase producers, wherein sequencing of *Trichoderma reesei* genome is a major step. Similar efforts will be needed in the case of other major cellulase producers also so that more information is built up on the molecular biology of cellulase producing fungi and their gene regulation. This information will be critical for future development of strains for cellulase production. While moving towards a carbohydrate based economy seems inevitable, other issues to be addressed are availability and sustainability of biomass for industry, possible scenario of monopolization etc. More research is also needed on distributed biomass conversion technologies and plants, which will be a more feasible option for developing and under-developed countries where cultivated land is dispersed. Distributed systems will offer the advantage of using locally available feedstock for bio-ethanol/bio-products at different geographic locations, as well as reduce on transportation cost of feedstock.

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