Enzyme and Microbial Technology 46 (2010) 541–549

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Review

Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases

Reeta Rani Singhania [a], Rajeev K. Sukumaran [a], Anil Kumar Patel [b], Christian Larroche [b], Ashok Pandey [a,*]

[a] Biotechnology Division, National Institute for Interdisciplinary Science and Technology, CSIR, Trivandrum 695019, India
[b] Laboratoire de Genie Chimique et Biochimique, Universite Blasie Pascal, 24, avenue des Landais, BP 206, 63174 Aubiere Cedex, France

ARTICLE INFO

Article history:
Received 23 January 2010
Received in revised form 19 March 2010
Accepted 22 March 2010

Keywords:
Cellulases
Microbial
Solid-state fermentation
Submerged fermentation
Hydrolysis
Lignocellulose
Bioethanol

ABSTRACT

There is an increasing demand for cellulases in the market for various applications, among which the bioconversion of lignocellulosic biomass for ethanol production is the major one. Improvements in the titers as well as specific activities of cellulases are highly desired for its use in bioethanol production as well as in other applications. This review deals with developments in bioprocess technologies, solid-state and submerged fermentation as well as on the strategies adopted for improving cellulase production or properties, including engineering the genes or designing enzyme cocktails. It also gives a brief overview of commercially available cellulase preparations.

Contents

1. Introduction

Cellulases are enzymes which hydrolyse the β-1,4-glucosidic linkages of cellulose. They are present in 13 of the 82 glycoside hydrolase families identified by sequence analysis [1]. Cellulases hydrolyze the β-1,4-D-glucan linkages in cellulose and produce as primary products glucose, cellobiose and cello-oligosaccharides. This is the most extensively studied multiple enzyme complex comprising of endo-glucanases (EG), cellobiohydrolases (CBH) and β-glucosidases (BGL). Endo-glucanases produces nicks in the cellulose polymer exposing reducing and non-reducing ends, cellobiohydrolases acts upon these reducing and non-reducing ends to liberate cello-oligosaccharides and cellobiose units, and β-glucosidases cleaves the cellobiose to liberate glucose, thereby completing the hydrolysis [2]. The complete cellulase system comprising CBH, EG and BGL components thus acts synergistically to convert crystalline cellulose to glucose.

Cellulases are currently the third largest industrial enzyme worldwide, by dollar volume, because of their wide applications in cotton processing; paper recycling, in juice extraction, as deter-

* Corresponding author. Tel.: +91 471 251 52 79; fax: +91 471 249 17 12.
E-mail addresses: ashokpandey56@yahoo.co.in, pandey@niist.res.in (A. Pandey).

0141-0229/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2010.03.010

gent enzymes and animal feed additives. However, cellulases may become the largest volume industrial enzyme, if ethanol from lignocellulosic biomass through enzymatic route becomes a major transportation fuel.

There has been an increased interest worldwide for an alternate source of sustainable fuel due to the limited reserves of fossil fuel. Ethanol from lignocellulosic biomass appears promising, as the raw material is ubiquitous, abundant and could play a major role in green house gas reduction. It has been stated that 10% blend of bioethanol with gasoline would reduce the carbon dioxide emission by 3–6%, which makes bioethanol a cleaner fuel in addition to being a renewable alternative to petroleum [3]. Ethanol production from lignocellulosic biomass through the biological route seems very attractive and sustainable due to several reasons among which the renewable and ubiquitous nature of biomass and its non-competitiveness with food crops are the major ones. About 2.9×10^3 million tons of lignocellulosic residues are available from cereal crops and 3×10^3 million tons from pulse and oil seed crops. Also 5.4×10^2 million tons is produced annually from plantation crops worldwide [4]. Lignocellulosic biomass accounts for 50% of all the biomass available in the world asserting its potential as a feasible raw material for ethanol production. Bioconversion of cellulosic biomass employing cellulases to produce ethanol is foreseen as the most important application of this enzyme. The major limitation in developing a feasible lignocellulose-to-ethanol technology is the high cost and low titers of cellulase production.

There are a wide variety of microorganisms involved in cellulase production including aerobic and anaerobic bacteria [5–8]; white rot and soft rot fungi [9–11] and anaerobic fungi [12,13]. In filamentous fungi, actinomycetes and in aerobic bacteria, cellulases are mostly secreted as free molecules. Most of the cellulases exploited for industrial applications are from filamentous fungi such as *Trichoderma*, *Penicillium*, *Fusarium*, *Humicola*, *Phanerochaete*, etc., where a large number of cellulases are encountered [11,14–17]. Though the 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 [18]. These filamentous fungi produce cellulase complexes with all the three classes of enzymes at different proportion needed for the complete hydrolysis of cellulose. *Trichoderma reesei* is one among the most potent cellulase producers studied in detail. It produces two cellobiohydrolases (CBH I and CBH II) and the two endo-glucanases (EG1 and EG2), in a rough proportion of 60:20:10:10, which together can make up to 90% of the enzyme cocktail; while seven β-glucosidases-BGLI-BGLVII secreted by this fungus typically makes up less than 1% [19–21].

2. Cellulase market scenario

The demand for cellulases is consistently on the rise due to its diverse applications. There are several companies involved in cellulase production for textile detergent, paper industries and other industries. Globally, there are two major players known for cellulase production for biomass conversion—"Genencor" and "Novozyme". Both the companies has played a significant role in bringing down the cost of cellulase several folds by their active research and are continuing to bring down the cost by adopting novel technologies. Recently, Genencor has launched Accelerase®1500, a cellulase complex intended specifically for lignocellulosic biomass processing industries [22]. It is claimed to be more cost-effective and efficient for bioethanol industries than its predecessor—Accelerase®1000. Accelerase®1500 is produced with a genetically modified strain of *T. reesei*. This enzyme preparation is claimed to contain higher levels of β-glucosidase activity than all other commercial cellulases available today, so as to ensure almost complete conversion of cellobiose to glucose [22].

Genencor has also launched Accelerase® XY accessory xylanase enzyme complex that enhances both xylan (C5) and glucan (C6) conversion when blended with other Accelerase® enzyme products [22]. Similarly, Accelerase® XC is an accessory xylanase/cellulase enzyme complex that contains a broad profile of hemicellulase and cellulase activities and enhances both xylan (C5) and glucan (C6) conversion when blended with other Accelerase® enzyme products [22]. Also, Accelerase® BG is an accessory β-glucosidase enzyme that enhances glucan (C6) conversion when blended with cellulase products [22]. There are several potential cellulases which may prove to be effective for biomass hydrolysis when supplemented with β-glucosidase, indicating the importance of Accelerase® BG.

Novozymes also have a diverse range of cellulase preparations available based on application as Cellusoft®AP and Cellusoft®CR for bioblasting in textile mills, Carezyme® and Celluclean for laundry in detergent, Denimax® 601l for stonewash industry at low temperature as well as many others specific for particular application [23]. Novozyme also announced the availability of cellulase preparation specifically for biomass hydrolysis last year, though no information is available on the source of production as well as availability in the market. Amano Enzyme Inc. in Japan and MAP's India in India are another enzyme industry actively involved in cellulase production.

Though, most of the enzyme producing companies worldwide is involved in production and marketing of cellulases for diverse applications, there are very few of them who develop cellulases for biomass conversion, the most successful of them probably being Genencor and Novozyme.

Table 1 shows the major players marketing cellulases with different trademark and their source of origin, most of which may be genetically modified strains.

3. Cellulases for bioconversion

Microbial cellulases find applications in a variety of industries where cellulases of varying degrees of purity are desired. Though cellulases were initially investigated several decades back for the bioconversion of biomass, this later became unattractive and the other industrial applications of the enzyme as in animal feed, food, textiles and detergents and in the paper industry were predominantly pursued [24]. However, with the shortage of fossil fuels and the arising need to find alternative sources for renewable energy and fuels, there is a renewal of interest in the bioconversion of lignocellulosic biomass using cellulases and other enzymes.

The performance of cellulase mixtures in biomass conversion processes depends on several of its properties including stability, product inhibition, synergism among the different enzymes, productive binding to the cellulose, physical state as well as the composition of cellulosic biomass [25]. Cellulases are available in the market under different names or trademark for different applications which could also be tried for biomass hydrolysis. Nieves et al. [26] and Kabel et al. [27] analyzed and evaluated the potential of several commercial cellulases for biomass conversion. They performed the standard assays for different enzymes as Filter Paper Activity (FPU), CMCase, β-glucosidase and xylanase. It would not be feasible to predict the efficiency of cellulases for bioconversion on the basis of standard assays, as there are no clear relationships between cellulase activities on soluble substrates and those on insoluble substrates [28]. So the soluble substrates should not be used to predict the efficiency of cellulases for processing relevant solid substrates, such as plant cell walls. The choice of the enzyme preparation for a particular biomass would be more dependent on biomass characteristics rather than on standard enzyme activities measured [27]. Preparations having higher FPUs are desirable

Table 1
Commercial cellulases produced by companies and their sources.

Enzyme samples	Supplier	Source
Cellubrix (Celluclast)	Novozymes, Denmark	*T. longibrachiatum* and *A. niger*
Novozymes 188	Novozymes	*A. niger*
Cellulase 2000L	Rhodia-Danisco (Vinay, France)	*T. longibrachiatum*/*T. reesei*
Rohament CL	Rohm-AB Enzymes (Rajamaki, Finland)	*T. longibrachiatum*/*T. reesei*
Viscostar 150L	Dyadic (Jupiter, USA)	*T. longibrachiatum*/*T. reesei*
Multifect CL	Genencor Intl. (S. San Francisco, CA)	*T. reesei*
Bio-feed beta L	Novozymes	*T. longibrachiatum*/*T. reesei*
Energex L	Novozymes	*T. longibrachiatum*/*T. reesei*
Ultraflo L	Novozymes	*T. longibrachiatum*/*T. reesei*
Viscozyme L	Novozymes	*T. longibrachiatum*/*T. reesei*
Cellulyve	50L Lyven (Colombelles, France)	*T. longibrachiatum*/*T. reesei*
GC 440	Genencor-Danisco (Rochester, USA)	*T. longibrachiatum*/*T. reesei*
GC 880	Genencor	*T. longibrachiatum*/*T. reesei*
Spezyme CP	Genencor	*T. longibrachiatum*/*T. reesei*
GC 220	Genencor	*T. longibrachiatum*/*T. Reesei*
Accelerase®1500	Genencor	*T. Reesei*
Cellulase AP30K	Amano Enzyme	*A. niger*
Cellulase TRL	Solvay Enzymes (Elkhart, IN)	*T. reesei*/*T. Longibrachiatum*
Econase CE	Alko-EDC (New York, NY)	*T. reesei*/*T. Longibrachiatum*
Cellulase TAP106	Amano Enzyme (Troy, VA)	*T. viride*
Biocellulase TRI	Quest Intl. (Sarasota, FL)	*T. reesei*/*T. Longibrachiatum*
Biocellulase A	Quest Intl.	*A. niger*
Ultra-low microbial (ULM)	Iogen (Ottawa, Canada)	*T. reesei*/*T. Longibrachiatum*

for bioconversion, since filter paper is highly crystalline cellulose, the degradation of which depends on the combination of activities of EG and CBH, where the EG create new chain ends for the CBH to split off cellobiose which further get attacked by BGL to give glucose [29,30]. Preparations of cellulase from a single organism may not very efficient for hydrolysis of a particular feed stock. Though, the filamentous fungi are the major source of cellulases and hemicellulases and the mutant strains of *Trichoderma* including *T. reesei*, *T. viride* and *T. longibrachium* are the best known producers of the enzyme, it is also well known that these species of *Trichoderma* have a low level of β-glucosidase activity [31]. Cellulases for biomass conversion could be a blend or enzyme cocktail containing endo- and exo-cellulase, xylanase, β-glucosidase, pectinase, etc. which could vary for different biomass on the basis of their composition. The hydrolytic efficiency of a multi-enzyme complex for lignocellulose saccharification depends both on properties of individual enzymes and their ratio in the multi-enzyme cocktail [32]. The ideal cellulase complex must be highly active on the intended biomass feedstock, able to completely hydrolyze the biomass, operate well at mildly acidic pH, withstand process stress, and be cost-effective [33]. The success of any lignocellulosic ethanol project will depend on the ability to develop such cellulase systems. The key to developing cellulases those are effective towards a particular biomass feedstock is to artificially construct them either by enzyme assembly to form cocktails or to engineer the cellulase producers to express desired combination of cellulase enzymes [1]. Both these approaches have been tried with success. Enzyme cocktails have been developed by mixing *T. reesei* cellulase with other enzymes including xylanases, pectinases and β-glucosidases, and these cocktails were tried for hydrolysis of various feed stock [34,35]. One of the recent examples of cocktails developed, include the multi-enzyme complex developed based on highly active *Chrysosporium lucknowense* cellulases [32].

Characterization of cellulases has been achieved to a considerable extent by site-directed mutagenesis [28]. These studies as well as X-ray crystallography of the enzymes [28] have led to the identification and characterization of putative catalytic and binding residues, and the trapping of enzyme–substrate complexes. Nevertheless, understanding the mechanism of degradation of the natural substrate-crystalline cellulose remains a great challenge [2].

There are several challenges which have yet to be overcome, for example the recalcitrance of lignocellulosic biomass, which necessitates the pretreatment step to open up the fibers and decrease the crystallinity of cellulose, which again add to the cost of lignocellulosic–ethanol technology. Pretreatment methods also need to vary from biomass to biomass based on their compositional characteristic [36]. For developing an economically feasible technology, the use of cheaper raw material as a substrate for cellulase production could bring down the production costs [2]. Also eliminating the steps in downstream processing of the enzyme for bioconversion might help to bring down the cost of cellulases as would be other approaches like improving the specific activities, temperature and low pH tolerance as well as engineering the organism for improved production.

Most of the commercial cellulases available are produced from *T. reesei* and *Aspergillus niger* but *T. reesei* lack sufficient amount of β-glucosidase to perform a proper and complete hydrolysis [32]. Thus, the cellobiose accumulated due to an incomplete conversion caused by the limiting amounts of β-glucosidase inhibits exo- and endo-glucanases [2]. β-Glucosidases are also subject to product inhibition by the glucose beyond certain levels that vary between the different preparations and sources of the enzyme. One way to solve this issue is to add a glucose tolerant β-glucosidase to the reaction mixture containing other cellulase components and to employ this cocktail for biomass hydrolysis which would increase the efficiency of hydrolysis [2]. There are several reports available where an enzyme cocktail has been employed successfully for biomass conversion [28,37–39].

3.1. Desirable characteristics of the cellulase preparations for bioconversion

Lot of specific features, such as higher catalytic efficiency on insoluble cellulosic substrates, increased stability at elevated temperature and at a certain pH, and higher tolerances to end-product inhibition are desirable in cellulases for biomass conversion [40]. Acidic cellulases are usually more desirable for bioconversion since in situations where acidic pretreatment of biomass is employed or when the enzyme has to work in cocktail with *T. reesei* enzymes whose optimal activity is in the close to 5, a pH range of 4–6 is preferred. Most of the cellulases tried or recommended or for bioconversion perform better at pH range 4–6. Accelerase®1500 has

Table 2
List of the cellulase activity (FPAse), protein and properties of commercial enzymes on the basis of information available at the company site or otherwise on the basis of information available in the research articles.

Enzymes	Cellulase activity (FPAse/ml)	Protein (mg/ml)	Use pH	Temperature (°C)	Form available
Celluclast	56	122	5.0	50	Liquid
Novozymes 188	<5	168	5.0	50	Liquid
Cellulase 2000L	10	48	5.0	50	Liquid
Rohament CL	50	152	5.0	50	Liquid
Viscostar 150L	33	163	5.0	50	Liquid
Multifect CL	64	153	5.0	50	Liquid
Bio-feed beta L	<5	29	5.0	50	Liquid
Energex L	<5	222	4.5	50	Liquid
Ultraflo L	<5	32	5.0	50	Liquid
Viscozyme L	<5	29	5.0	50	Liquid
Cellulyve	24	105	5.0	50	Liquid
GC 440	<5	118	5.0	50	Liquid
GC 880	<5	162	5.0	50	Liquid
Spezyme CP	49	135	4.0	50	Liquid
GC 220	116	211	5.0	50	Liquid
Accelerase®1500	NA	NA	4.0–5.0	50–65	Liquid
Cellulase AP30K	0.17	6[a]	4.5	60	Powder
Cellulase TRL	95	167	4.5	50	Powder
Econase CE	40	95	5.0	50	Liquid
Cellulase TAP106	0.42	3.3[a]	5.0	50	Powder
Biocellulase TRI	68	279	4.5	50	Liquid
Biocellulase A	0.29	23[a]	5.0	55	Powder
Ultra-low microbial (ULM)	88	184	NA	NA	Liquid
Cazy GH5	2200[b]	2[c]	7.5	50	Liquid
Cazy GH 8	2400[b]	2[c]	7.0	60	Liquid

Filter paper units determined according to IUPAC method; pH 5.0 and 50 °C.
[a] Powder preparations were made to 2% w/v and equilibrated for 96 h in 50 mM acetate pH 4.8.
[b] One unit is defined as the amount of enzyme required to release 1 μmol of glucose-reducing-sugar equivalents per minute from barley β-glucan in 50 mM phosphate buffer, pH 7.0, at 60 °C, where reducing sugars are measured by the method of Miller. Protein has been determined by using the Pierce BSA assay.
[c] Protein determination method is not available.

an optimal pH of 4.6–5.0 but it gets inactivated below 4.0 or above pH 7.0. Usually 50 °C temperature is employed for hydrolysis by cellulases [39], even Accelerase®1500 works well at temperature ranging from 50 to 65 °C though at lower ends of the temperature range, the effective life time of enzyme is higher. Most of the filamentous fungi such as *T. reesei* and *A. niger* produce acidic cellulases, which are suitable for biomass hydrolysis. Some of the important features of commercially available cellulases with potential to be used in biomass conversion are listed in Table 2.

4. Advancement in research in bioprocess of cellulases

Cellulase production is a major area of research globally, and with the rejuvenated interest created due their applications in lignocellulose conversion, several investigators worldwide are working on some aspect of cellulase [2]. Production of low titers of cellulase has always been a major concern and thus several workers are trying to improve the production titers by adopting multifaceted approaches, which include the use of better bioprocess technologies, using cheaper or crude raw materials as substrates for enzyme production, bioengineering the microorganisms, etc. [2,19]. A significant portion of research tries to address the bioprocess improvement strategies for enhancing the yield and specific activities of cellulases. Sukumaran et al. [2] in their review has discussed the bioprocess technologies employed for cellulase production using diverse microorganisms and the future challenges. Majority of the reports on microbial production of cellulases utilizes the submerged fermentation technology (SmF) and the widely studied organism used in cellulase production—*T. reesei* has also been tested mostly in liquid media. However, in nature, the growth and cellulose utilization of aerobic microorganisms elaborating cellulases probably resembles solid-state fermentation than a liquid culture [41,42]. During last two decades solid-state fermentation has regained interest due to the high titers of enzyme production employing fungal cultures [43]. The lignocellulosic substrate type had the greatest impact on cellulase secretion. Some of the substrates significantly stimulated lignocellulolytic enzyme synthesis without supplementation of the culture medium with specific inducers [44]. Nevertheless, the advantages of better monitoring and handling are still associated with the submerged cultures [15].

Currently used bioprocesses and the status of cellulase production based on literature are provided in Table 3. A direct comparison of the cellulase yields and activities in these reports are impossible due to the differences in the methods of assay and the way the activities are being expressed despite the fact that there is an IUPAC approved method of assay for cellulase activity determination [45]. Moreover, there is no way of comparing cellulases produced by SSF and SmF directly.

4.1. Solid-state fermentation

Solid-state fermentation (SSF) is defined as the fermentation in absence or near absence of free water [46]. SSF for production of cellulases is rapidly gaining interest as a cost-effective technology as the microorganisms, especially fungal cultures produces comparatively high titers of cellulase due to the conditions of fermentation which shows similarity to the natural environment [47,48]. Filamentous fungi as *T. reesei*, *A. niger*, *Penicillium* sp., etc. have been employed for cellulase production using solid-state fermentation where a basal mineral salts medium was used for moistening the substrate. Chahal [49] had reported a higher yield of cellulases from *T. reesei* in SSF cultures compared to liquid cultures. Tengerdy [50] compared cellulase production in SmF and SSF systems and had indicated that there was about a 10-fold reduction in the production cost when SSF is employed for production. Solid-state cultures are strongly recommended as systems for producing cellulases at lower price than submerged cultures [51] as the product concentration remains quite higher thereby reducing the step in downstream processing, in turn reducing the cost of operation. Nigam and Singh [52] have reviewed the use of agricultural wastes as substrates for

Table 3
Types of bioprocesses and substrate involved for cellulase production.

Microorganism	Substrate	Method	Magnitude	Enzymes: activity	Reference (s)
Trichoderma citrinoviride	NA	SmF	Shake flask	FPAse 0.63 IU/ml, Endoglucanase 3.12 IU/ml, β-glucosidase 8.22 IU/ml, Cellobiase 1.94 IU/ml	[105]
Penicillium citrinum	Wheat bran	SSF	Shake flask	FPAse 1.72 IU/ml Endoglucanase 1.89 IU/ml	[54]
Acremonium cellulolytius (CF-2612)	Solka-folk	SmF	2-l jar fermenter	240.3 FPU/l/h (346.0 FPU/g carbohydrate)	[61]
Aspergillus niger A 20	Cellulose	SmF	Shake flask	Cellobiase 27.5 U/ml	[106]
Bacillus pumilus	CM cellulose/glycerol	SmF	SF	CMCase 1.9 U/ml, Cellobiase 1.2 U/ml	[107]
Bacillus sp KSM N252	Carboxymethyl cellulose	SmF	Shake flask	CMCase 0.17 U/mg protein	[108]
Chaetomium thermophilium CT2	Cellulose (sigma cell)	SmF	Shake flask	CMCase 2.7 IU/ml	[109]
Melnocarpus albomyces	Solka-Floc	SmF	700-l fermentor	Cellulase 1160 ECU/ml, Endoglucanase 3290 ECU/ml,	[110]
Mucor circinelloidens	Lactose	SmF	Shake flask	EGL 0.25 U/ml	[111]
Neurospora crassa	Wheat straw	SmF	Shake flask	FPAse 1.33 U/ml, CMCase 19.7 U/ml, BGL 0.58 U/ml	[112]
Penicillium janthinellum	Sugar cane bagasse	SmF	Shake flask	FPAse 0.55 U/ml, CMCase 21.5 U/ml, BGL 2.3 IU/ml	[113]
Phaenerocheate chrysosporium	Cellulose (Avicell)	SmF	100-l fermentor	Cellulase 29 mg/g cellulose	[69]
Rhodothermus marinus	CM cellulose	SmF	150-l fermentor	Endoglucanase 97.7 U/ml	[114]
Streptomyces sp T3-1	Carboxymethyl cellulose	SmF	50-l fermentor	CMCase 148 IU/ml, Avicellase 45 IU/ml, BGL 137 IU/ml	[115]
Streptomyces drozdowiczii	Wheat bran	SmF	Shake flask	CMCase 595 U/l	[116]
Thermotoga maritima	Xylose	SmF	Shake flask	Cellobiase 11 mU/ml, Avicellase 0.3 mU/ml Beta-glucosidase 30 mU/ml	[117]
Trichoderma reesei	Steam treated willow	SmF	22-l fermentor	FPAse 108 U/g cellulose	[71]
Trichoderma reesei RUT C30	Cellulose (Avicell)	SmF	Microbubble dispersion bioreactor	FPAse 1.8 U/ml	[118]
Trichoderma reesei RUT C30	Corrugated cardboard	SmF	30-l fermentor	FPAse 2.27 U/ml	[119]
Trichoderma reesei ZU-02	Corn Stover Residue	SmF	30-l fermentor	Cellulase 5.48 IU/ml, FPAse 0.25 U/ml	[120]
Trichoderma viridae	Sugar cane bagasse	SmF	Shake flask	FPAse 0.88 U/ml, CMCase 33.8 U/ml, BGL 0.33 U/ml	[113]
Penicillium occitanis	Paper pulp	SmF-fed-batch	20-l fermentor	FPAse 23 IU/ml, CMCase 21 IU/ml	[59]
Trichoderma reesei	Xylose/sorbose	SmF-continuous	Bioreactor	FPAse 0.69 U/ml/h	[63]
Aspergillus niger NRRL3	Wheat Bran/Corn cob	SSF	Flask	Cellobiase 215 IU/g, cellulose	[121]
Bacillus subtilis	Banana waste	SSF	Shake flask	FPAse 2.8 IU/gds, CMCase 9.6 IU/gds, Cellobiase 4.5 IU/gds	[122]
Bacillus subtilis	Soybean industry residue	SSF	Cylindrical bioreactor	FPAse 1.08 U/mg protein	[72]
Mixed culture: Trichoderma reesei, Aspergillus niger	Rice Chaff/Wheat Bran (9:1)	SSF	Flask	FPAse 5.64 IU/g	[123]
Penicillium decumbans	Wheat straw/bran (8:2)	SSF	SSF bioreactor	Fpase 20.4 IU/g	[124]
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	[125]
Trichoderma reesei ZU 02	Corn cob residue	SSF	Tray fermentor	FPAse 158 U/gds	[126]
Trichoderma reesei RUT C30	Wheat bran	SSF	Shake flask	3.8 U/gds FPU	[127]

NA = not available.
(Sukumaran et al. [2]).

cellulolytic enzyme production under SSF and strongly believe that with the appropriate technology, improved bioreactor design, and operation controls, SSF may become a competitive method for the production of cellulases. The review by Pandey et al. [53] on SSF for industrial enzyme production also describes the application of the technology for cellulase production. SSF can thus be considered as a future technology for commercial production of cellulases considering the low cost input and ability to utilize naturally available sources of cellulose as substrate. Cellulases produced in solid-state culture shows remarkable stability towards temperature, pH, metal ions, etc. Dutta et al. [54] claimed the cellulase produced by SSF employing *Penicillium citrini* showed tolerance to alkali for the first time. SSF proves to be efficient technology for cellulase production for bioconversion, since purity is not very stringent necessity for this application, and a concentrated enzyme preparation would serve the purpose. It has also been reported that the enzyme produced using the same biomass as to be used for bioconversion proves more efficient than the one produced on other cellulosic substrate [19].

Optimization of SSF conditions for production, when attempted may still improve the overall production economics and also make it an attractive technology for cellulase production. It offers many advantages over SmF, including high volumetric productivity, higher concentration of products, less effluent generation, and low catabolic repression which makes it a promising technology in near future [43].

4.2. Submerged fermentation

Submerged fermentation has been defined as fermentation in the presence of excess water. Almost all the large-scale enzyme producing facilities are using the proven technology of SmF due to better monitoring and ease of handling. Though bacteria and actinomycetes are also reported for cellulase production, the titers

are very low to make the technology economically feasible. Most of the commercial cellulases are produced by the filamentous fungi—*T. reesei* or *A. niger* under SmF [6,55]. Cellulase production in cultures is highly influenced by various parameters including the nature of the cellulosic substrate, pH of the medium, nutrient availability, inducer supplementation, fermentation temperature, etc., and a large-scale production of cellulases requires understanding and proper controlling of the growth and enzyme production capabilities of the producer. Cellulases produced by compost organisms such as the filamentous fungi—*Trichoderma*, *Penicillium*, *Aspergillus*, *Humicola*, etc., can perform at diverse ranges of pH and temperature. The media formulation for fermentation is of significant concern since no general composition can give the optimum growth and cellulase production. Also the media used are mostly specific for the organism concerned. In *T. reesei*, a basal medium after Mandels and Reese [56] or Mandel and Weber [57] has been most frequently used with or without modifications. However, several of these media are extremely complicated since many factors and their interactions can affect cellulase productivity [58].

Microbial cellulases are subject to induction and repression mechanisms and the process design and media formulation for cellulase production has to take care of these aspects. Among the known inducers of cellulase genes, lactose is considered to be the only economically feasible additive in industrial fermentation media [21]. Though majority of the processes described are batch processes, there have been attempts to produce cellulase in fed-batch with improved enzyme production [59–61], or continuous mode [62–64], which supposedly helps to override the repression caused by the accumulation of reducing sugar. Increased fermentation time with a low productivity has been the major technical limitation in fermentative production of cellulases since long. A two-stage continuous process for cellulase production could be employed in which the growth phase and production phase was separated by different pH and temperature optima. This has been described as early as 1979 [65]. 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 [66,67]. 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 the liquid cultures of cellulolytic microbes for production of the enzymes and natural cellulosic materials when used as the carbon source gave poor enzyme yields [68]. While using soluble substrates, the break down products may hamper cellulase synthesis by promoting catabolite repression due to accumulation of free sugars. The carbon sources in majority of the commercial cellulase fermentations are cellulosic biomass ranging from pure cellulose to straw, spent hulls of cereals and pulses, rice or wheat bran, bagasse, paper industry waste, dairy manure and various other lignocellulosic residues [60,69–73]. Increased production in fermenters may be achieved by a gradient feed of a suitable cellulose and maintenance of process conditions at their optimal [61]. Most of these are capable of inducing the cellulase system in fungi often at par with the known inducers or sometimes even better [15].

Cellulases produced by SSF add SmF have been listed in Table 3.

4.3. Morphological/growth relationship with cellulase production

Cellulase production in cultures is growth associated. Filamentous fungi have the tendency to grow adhered to surfaces and the influence of this type of growth on fungal physiology has not yet been thoroughly studied, particularly when related to productivity [74]. Under natural conditions, fungal contact with surfaces is required for nutrient uptake, enzyme secretion and hyphal apical growth. Filamentous fungi can grow to significant extent in the absence of free water [75]. Considering that submerged free floating fungal growth is not natural, growth on and within solid substrates is fundamentally related to cell adhesion. Growth morphology of filamentous fungi is an important parameter related to productivity of several industrial processes. Though favorable physiological aspects are shared with solid-state fermentation, fungal biofilms present better possibilities for process control and scale-up [74]. Importance of morphology in the productivity of fungal submerged processes, placing biofilms in a preferential category has been elucidated by Villena and Gutierrez-Correa [74]. Fungal biofilms are morphologically efficient systems for enzyme production. In several fungal species, principally those that adhere through adhesive production, adhesion can be considered as a metabolically active process [76,77] involving signaling and differential gene expression mechanisms [78]. A clear relationship between morphology and productivity has not yet been established [79]. Since, fungal morphology influences the productivity of fungal fermentations; it is of major importance to know the fungal behavior during culture for cellulase production. Fermentation medium component as well as physico-chemical factors could be responsible for variation in morphology. Even the addition of different buffer system with varied concentration; influence the fungal morphology, which shows a clear transition from clumped to pelleted forms in cultures [80]. Fungal growth as well as morphology can be strongly correlated to cellulase production.

5. Engineered/artificial cellulases

Though several filamentous fungi are capable of cellulase production, the yield of the enzyme and the levels of individual cellulase components are not often satisfactory for commercialization. Improvements in cellulase titers as well as the ability to tailor the ratios of endo- and exo-glucanases and β-glucosidase produced by organisms are highly desired for biomass conversion. Very relevant information related to cellulase gene regulation was revealed more than a decade ago as obvious from the publications. A recent publication on the study of *T. reesei* genome [81] revealed that despite being the best known producer of cellulases; the genome of the fungus contains fewer cellulases and hemicellulases than any other sequenced fungi. Authors could not find any deep insight into the highly efficient protein secretion machinery in the fungus. This work has tremendous implications in the understanding the genetics of this important organism, and also would enable improved production processes critical to reducing the cost of biomass conversion.

T. reesei and other filamentous fungi produce non-complexed cellulases. Cellulase engineering for non-complexed cellulase systems contains three major research directions: (1) rational design for each cellulase, based on knowledge of the cellulase structure and the catalytic mechanism [1,82]; (2) expression cassette and directed evolution for each cellulase, in which the improved enzymes or ones with new properties were selected after random mutagenesis and/or molecular recombination [55,83–85]; and (3) the reconstitution of cellulase cocktails active on insoluble cellulosic substrates, yielding an improved hydrolysis rate or higher cellulose digestibility [39,86–89]. Improvements in specific cellulase activities for non-complexed cellulase mixtures can be implemented through cellulase engineering based on rational design or directed evolution for each component of cellulase, as well as its reconstitution [90].

Artificial cellulase designing appears to open a new avenue to this field and seems to be more promising for creating cellulases with desired features. Highly thermostable class II cellobiohydrolase (CBH II) chimeras were developed by structure guided recombination of three fungal class II cellobiohydrolase (CBH II)

[25]. Increasing the thermostability and improvement in activity of CBH II were two major goals. Optimized mixtures of cellulase for biomass conversion could be formulated once inventories of stable cellulases are available.

Site-directed mutagenesis have tremendous opportunities for introducing desired characteristics to the enzymes acting on soluble substrates, but cellulases with its complex insoluble and heterogeneous substrate–cellulose is relatively difficult to manipulate [1] Among the several reasons for this challenge is the unexplained dynamic interaction between the cellulose binding domain (CBD) and the catalytic domain [25]. Still there are several excellent reviews which summarize numerous studies using site-directed mutagenesis for investigating cellulase mechanisms and improving enzyme properties [1,82].

Baker et al. reported a 20% improvement in the activity on microcrystalline cellulose of a modified endoglucanase Cel5A from *Acidothermus cellulolyticus* [91]. The Cel5A endoglucanase, whose high-resolution crystallographic structure has been available, was subjected to a series of mutations designed to alter the chemistry of the product-leaving side of the active site cleft. A mutant (Y245G) was shown to increase *Ki* of cellobiose by 15-fold, by substitution of a non-aromatic residue at site 245. However, today there are no general rules for site-directed mutagenesis strategies for improving cellulase activity on solid cellulose substrates and it still remains in a trial-and-test process [28].

Potent cellulase genes from filamentous fungi such as, *Trichoderma* and *Aspergillus* can be isolated, cloned and expressed in fungal hosts to get better combination or synergism. The cellobiohydrolase I (CBH I) promoter of *T. reesei* is a highly efficient known promoter with unusually high rate of expression under cellulase induction conditions and has been used to drive the expression of β-glucosidase [92] and endoglucanase [93] thereby improving the cellulase profile of the host strain. The promoter has also been used to drive the expression of various homologous and heterologous proteins in *Trichoderma* [94,95]. Glucose repression of cellulase genes has been addressed by using a truncated CBH I promoter lacking binding sites for the carbon catabolite repressor CRE1 [96]. Another major strategy employed for improving cellulase production in presence of glucose is to use promoters that are insensitive to glucose repression. Nakari-Setala and Pentilla [97] had used the promoters of transcription elongation factors *1α* and *tef1*, and that of an unidentified cDNA (*cDNA1*) for driving the 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 the promoter to abolish repression can dramatically improve production of the cloned protein.

The cellulase system of *T. reesei* as well as of several other fungi is limited by the relatively lesser amount of β-glucosidase and its feed back inhibition by glucose. β-Glucosidase which is insensitive or at least tolerant to glucose and cellobiose is highly desired for the conversion of cellulosic biomass to glucose as cellulase systems of several other fungi are limited by the relatively lesser amount of β-glucosidase and its feed back inhibition by glucose [15]. Research on this line has yielded potential β-glucosidases from different microorganisms like *Candida peltata* [98], *Aspergillus oryzae* [99], and *A. niger* [15,100]. One of the major approaches taken towards improving the cellulase complex for biomass hydrolysis is to increase the copy number of β-glucosidase gene and thus the amount of the BGL enzyme in the cellulase mixture produced by *T. reesei* [101] while other is to alter the cellulase profile of *T. reesei* by introducing glucose tolerant BGL gene into the fungus [92].

Preparations of cellulase from a single organism may not be highly efficient for hydrolysis of different feed stock (see Section 3).

Another interesting idea is the use of artificial cellulosomes generated by engineering cellulosome bearing bacteria to express heterologous cellulases. Chimeric cellulosomes have been described for degradation of cellulosic substrates either by incorporating bacterial [102,103] or fungal [104] cellulases in cellulosomes by genetic engineering. The artificial cellulase complexes displayed enhanced activities compared to the corresponding free systems at least in the case of the bacterial enzymes [102,103]. The benefits of developing heterologous cellulase expression systems in rapidly growing bacteria include substantial enhancement of enzyme stability and specific activity, the potential for greater cell densities using fed-batch cultures, a dramatic reduction in cell-growth time, and the potential for protein overproduction [104]. The enhancement in activity was proposed to be resultant of the additional synergy induced by enzyme proximity within the complex and the effect of the cellulose binding module offered by the chimeric scaffold in that anchors the whole complex at substrate surface [104].

Above approaches discussed could be useful for developing cellulases for various specific applications, most importantly for bioconversion.

6. Conclusions

Development of improved cellulases for bioconversion seems to help materialize the dream of developing eco-friendly lignocellulosic ethanol to a reality. Improvement in cellulases has been achieved partly by developments in production technology such as, adopting cheaper bioprocess technology, employing cheaper substrate and employing engineered organisms and partly by developments of artificial/engineered cellulases and cocktails of enzyme. The leading enzyme companies claim and also have brought down the price of cellulases significantly. Although the commercial lignocellulosic ethanol production has just began in some parts of the world, still continuous research is needed to improve varied aspects on cellulase production (such as cost, specific activity and substrate specificity) to achieve better techno-economic feasibility. Artificial/engineered cellulases and enzyme cocktails rich in glucose tolerant β-glucosidase has been proved successful for increasing the rate or efficiency of hydrolysis of biomass so as to prove the technology economically feasible. But, the fact cannot be denied that despite several efforts, cellulase for bioconversion though available in the market is not easily accessible. It signifies the long way till to go.

Acknowledgement

Authors are thankful to the TIFAC, New Delhi for providing the financial support for setting up the Centre for Biofules at NIIST; one of the authors (RRS) is grateful to the CSIR, New Delhi for the award of Senior Research Fellowship to her.

References

[1] Schulein M. Protein engineering of cellulases. Biochim Biophys Acta-Protein Struct Mol Enzymol 2000;1543(2):239–52.
[2] Sukumaran RK, Singhania RR, Pandey A. Microbial cellulases—production, applications and challenges. J Sci Ind Res 2005;64:832–44.
[3] Novozyme company, website http://www.novozymes.com/en/MainStructure/ProductsAndSolutions/Fuel+ethanol/Fuel+ethanol.htm.
[4] FAOSTAT, 2006. FAO statistical databases, http://faostat.fao.org/.
[5] Gilkes NR, Henrissat B, Kilburn DG, Miller Jr RC, Warren RA. Microbiol Rev 1991;55:303–15.
[6] Kumar B, Trivedi P, Mishra AK, Pandey A, Palni LMS. Microbial diversity of soil from two hot springs in Uttaranchal, Himalaya. Microbiol Res 2004;159(2):141–6.
[7] Thirumale S, Swaroopa Rani D, Nand K. Control of cellulase formation by trehalose in *Clostridium papyrosolvens* CFR-703. Proc Biochem 2001;37:241–5.
[8] Chung-Yi W, Yi-Ru H, Ng C-C, Chan H, Lin H-T, Wen-Sheng T, Shyu YT. Purification and characterization of a novel halostable cellulase from *Salinivibrio* sp. strain NTU-05. Enzyme Microb Technol 2009;44(6–7):373–9.

[9] Tanaka H, Koike K, Itakura S, Enoki A. Degradation of wood and enzyme production by *Ceriporiopsis subvermispora*. Enzyme Microb Technol 2009;45(5):384–90.
[10] Shrestha P, Khanal SK, Pomettoiii AL, Van Leeuwen J. Enzyme production by wood-rot and soft-rot fungi cultivated on corn fiber followed by simultaneous saccharification and fermentation. J Agric Food Chem 2009;57(10):4156–61.
[11] Lo C-M, Zhang Q, Callow NV, Ju L-K. Cellulase production by continuous culture of *Trichoderma reesei* Rut C30 using acid hydrolysate prepared to retain more oligosaccharides for induction. Bioresour Technol 2010;101(2):717–23.
[12] Dashtban M, Schraft H, Qin W. Fungal bioconversion of lignocellulosic residues; opportunities and perspectives. Int J Biol Sci 2009;5(6):578–95.
[13] Ljungdahl LG. The cellulase/hemicellulase system of the anaerobic fungus Orpinomyces PC-2 and aspects of its applied use. Ann NY Acad Sci 2008;1125:308–21.
[14] de Siqueira FG, de Siqueira EG, Jaramillo PMD, Silveira MHL, Andreaus J, Couto FA, Batista LR, Filho EXF. The potential of agro-industrial residues for production of holocellulase from filamentous fungi. Int Biodeter Biodegr 2010;64(1):20–6.
[15] Mathew GM, Sukumaran RK, Singhania RR, Ashok P. Progress in research on fungal cellulases for lignocellulose degradation. J Sci Ind Res 2008;67(11):898–907.
[16] Javed MM, Khan TS, Haq I-U. Sugar cane bagasse pretreatment: an attempt to enhance the production potential of cellulases by humicola insolens TAS-13. Electron J Environ Agric Food Chem 2007;6(8):2290–6.
[17] Bak JS, Ko JK, Choi I-G, Park Y-C, Seo J-H, Kim KH. Fungal pretreatment of lignocellulose by *Phanerochaete chrysosporium* to produce ethanol from rice straw. Biotechnol Bioeng 2009;104(3):471–82.
[18] Wiebe MG. Stable production of recombinant proteins in filamentous fungi—problems and improvements. Mycologist 2003;17:140–4.
[19] Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 2002;66:506.
[20] Herpoë l-Gimbert I, Margeot A, Dolla A, Jan G, Mollé D, Lignon S, Mathis H, Sigoillot JC, Monot F, Asther M. Comparative secretome analyses of two *Trichoderma reesei* RUT-C30 and CL847 hypersecretory strains. Biotechnol Biofuels 2008;1:18.
[21] Aro N, Pakula T, Penttilä M. Transcriptional regulation of plant cell wall degradation by filamentous fungi. FEMS Microbiol Rev 2005, doi:10.1016/j.femsre.2004.11.006.
[22] Genencor website: dated 01/14/2010 http://www.genencor.com/wps/wcm/connect/genencor/genencor/products_and_services/business_development/biorefineries/products/accellerase_product_line_en.htm.
[23] http://www.bioenergy.novozymes.com/cellulosic-biofuel/01/14/2010.
[24] Bhat MK. Cellulases and related enzymes in biotechnology. Biotechnol Adv 2000;18:355–83.
[25] Heinelman PC, Snow D, Wu I, Nguyen C, Villalobos A, Govindarajan S, Minshull J, Arnold FH. A family of thermostable fungal cellulases created by structure-guided recombination. PNAS 2009;106(14):5610–5.
[26] Nieves RA, Ehrman CI, Adney WS, Elander RT, Himmel ME. Technical communication: survey and analysis of commercial cellulase preparations suitable for biomass conversion to ethanol. World J Microb Biot 2009;14:301–4.
[27] Kabel MA, van der Maarel MJEC, Klip G, Voragen AGJ, Schols HA. Standard assays do not predict the efficiency of commercial cellulase preparations towards plant materials. Biotechnol Bioeng 2005;93:56–63.
[28] Zhang Y-H, Himmel EM, Mielenz JR. Outlook for cellulase improvement: screening and selection strategies. Biotechnol Adv 2006;24:452–81.
[29] Teeri TT. Crystalline cellulose degradation: new insight into the function of cellobiohydrolases. Trends Biotechnol 1997;15:160–7.
[30] Vincken J-P, Beldman G, Voragen AGJ. The effect of xyloglucans on the degradation of cell wall embedded cellulose by the combined action of cellobiohydrolase and endoglucanases from *Trichoderma viride*. Plant Physiol 1994;104:99–107.
[31] Duff SJB, Murray WD. Bioconversion of forest products industry waste cellulosics to fuel ethanol: a review. Bioresour Technol 1996;55:1–33.
[32] Gusakov AV, Salanovich TN, Antonov AI, Ustinov BB, Okunev ON, Burlingame R, Emalfarb M, Baez M, Sinitsyn AP. Design of highly efficient cellulase mixtures for enzymatic hydrolysis of cellulose. Biotechnol Bioeng 2007;97:1028–38.
[33] Knauf M, Moniruzzaman M. Lignocellulosic biomass processing: a perspective. Int Sugar J 2004;106:147–50.
[34] Berlin A, Maximenco V, Gilkes N, Saddler J. Optimization of enzyme complexes for lignocellulose hydrolysis. Biotechnol Bioeng 2007;97:287–96.
[35] Xin Z, Yinbo Q, Peiji G. Acceleration of ethanol production from paper mill waste fiber by supplementation with β-glucosidase. Enzyme Microb Technol 1993:1562–5.
[36] Hendriks ATWM, Zeeman G. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour Technol 2009;100:10–8.
[37] Park J-Y, Seyama T, Shiroma R, Ike M, Srichuwong S, Nagata K, Yumiko A-S, Tokuyasu K. Efficient recovery of glucose and fructose via enzymatic saccharification of rice straw with soft carbohydrates. Biosci Biotech Biochem 2009;73(5):1072–7.
[38] Singh S, Simmons BA, Vogel KP. Visualization of biomass solubilization and cellulose regeneration during ionic liquid pretreatment of switchgrass. Biotechnol Bioeng 2009;104(1):68–75.
[39] Sukumaran RK, Singhania RR, Mathew GM, Pandey A. Cellulase production using biomass feed stock and its application in lignocellulose saccharification for bioethanol production. Renew Energ 2009;34(2):421–4.
[40] Maki M, Leung KT, Qin W. The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. Int J Biol Sci 2009;5(5):500–16.
[41] Zhu JY, Pan XJ, Wang GS, Gleisner R. Sulfite pretreatment (SPORL) for robust enzymatic saccharification of spruce and red pine. Bioresour Technol 2009;100(8):2411–8.
[42] Hölker U, Höfer M, Lenz J. Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. Appl Microbiol Biotechnol 2004;64:175–86.
[43] Singhania RR, Patel AK, Soccol CR, Pandey A. Recent advances in solid-state fermentation. Biochem Eng J 2009;44(1):13–8.
[44] Elisashvili V, Kachlishvili E, Tsiklauri N, Metreveli E, Khardziani T, Agathos SN. Lignocellulose-degrading enzyme production by white-rot Basidiomycetes isolated from the forests of Georgia. World J Microb Biot 2009;25(2):331–9.
[45] Ghose TK. Measurement of cellulase activities. Pure Appl Chem 1987;59:257–68.
[46] Pandey A. Solid-state fermentation: an overview. In: Pandey A, editor. Solid state fermentation. New Delhi, India: Wiley Eastern Limited; 1994. p. 3–10.
[47] Cen P, Xia L. Production of cellulase by solid-state fermentation. Adv Biochem Eng Biotechnol 1999;65:69–92.
[48] Jha K, Khare SK, Gandhi AP. Solid-state Fermentation of soyhull for the production of cellulase. Bioresour Technol 1995;54:321–2.
[49] Chahal DS. Solid-state fermentation with *Trichoderma reesei* for cellulase production. Appl Environ Microbiol 1985;49:205–10.
[50] Tengerdy RP. Cellulase production by solid substrate fermentation. J Sci Ind Res 1996;55:313–6.
[51] Vintila T, Dragomirescu M, Jurcoane S, Vintila D, Caprita R, Maniu M. Production of cellulase by submerged and solid-state cultures and yeasts selection for conversion of lignocellulose to ethanol. Romanian Biotechnol Lett 2009;14:4275–81.
[52] Nigam P, Singh D. Processing of agricultural wastes in solid state fermentation for cellulolytic enzyme production. J Sci Ind Res 1996;55:457–67.
[53] Pandey A, Selvakumar P, Soccol CR, Nigam P. Solid state fermentation for the production of industrial enzymes. Curr Sci 1999;77:149–62.
[54] Dutta T, Sahoo R, Sengupta R, Ray SS, Bhattacharjee A, Ghosh S. Novel cellulases from an extremophilic filamentous fungi *Penicillium citrinum*: production and characterization. J Ind Microbiol Biotechnol 2008;35:275–82.
[55] Cherry JR, Fidantsef AL. Directed evolution of industrial enzymes: an update. Curr Opin Biotechnol 2003;14:438–43.
[56] Mandels M, Reese ET. Induction of cellulase in *Trichoderma viride* as influenced by carbon sources and metals. J Bacteriol 1957;73:269–78.
[57] Mandel M, Weber J. The production of cellulases. Adv Chem Ser 1969;95:391–413.
[58] Tholudur A, Ramirez WF, McMillan JD. Mathematical modeling and optimization of cellulase protein production using *Trichoderma reesei* RL-P37. Biotechnol Bioeng 1999;66:1–16.
[59] Belghith H, Ellouz-Chaabouni S, Gargouri A. Biostoning of denims by *Penicillium occitanis* (Pol6) cellulases. J Biotechnol 2001;89:257–62.
[60] Wen Z, Liao W, Chen S. Production of cellulase/b-glucosidase by the mixed fungi culture *Trichoderma reesei* and *Aspergillus phoenicis* on dairy manure. Process Biochem 2005, doi:10.1016/j.procbio.2005.03.044.
[61] Xu F, Yano S, Inoue H, Sawayama S. Strain improvement of *Acremonium cellulolyticus* for cellulase production by mutation. J Biosci Bioeng 2009;107(3):256–61.
[62] Ju LK, Afolabi OA. Wastepaper hydrolysate as soluble inducing substrate for cellulase production in continuous culture of *Trichoderma reesei*. Biotechnol Prog 1999:1591–7.
[63] Schafner DW, Toledo RT. Cellulase production in continuous culture by *Trichoderma reesei* on xylose-based media. Biotechnol Bioeng 1992;39:865–9.
[64] Bailey MJ, Tahtiharju J. Efficient cellulase production by *Trichoderma reesei* in continuous cultivation on lactose medium with a computer-controlled feeding strategy. Appl Microbiol Biotechnol 2003;62:156–62.
[65] Ryu D, Andereotti R, Mandels M, Gallo B, Reese ET. Studies on quantitative physiology of *Trichoderma reesei* with two-stage continuous culture for cellulose production. Biotechnol Bioeng 1979;21:1887–903.
[66] Fennington G, Neubauer D, Stutzenberger F. Cellulase biosynthesis in a catabolite repression-resistant mutant of *Thermomonospora curvata*. Appl Environ Microbiol 1984;47:201–4.
[67] Kawamori M, Morikawa Y, ShinSha Y, Takayama K, Takasawa S. Preparation of mutants resistant to catabolite repression. Agric Biol Chem 1985;49:2875–9.
[68] Tangnu KS, Blanch HW, Wilke CR. Enhanced production of cellulase, hemicellulase, and beta-glucosidase by *Trichoderma reesei* (Rut C-30). Biotechnol Bioeng 1981;23:1837–49.
[69] Szabo IJ, Johansson G, Pettersson G. Optimized cellulase production by *Phanerochaete chrysosporium*: control of catabolite repression by fed-batch cultivation. J Biotechnol 1996;48:221–30.
[70] Belghith H, Ellouz-Chaabouni S, Gargouri A. Biostoning of denims by *Penicillium occitanis* (Pol6) cellulases. J Biotechnol 1996;89:257–62.
[71] Reczey K, Szengyel ZS, Eklund R, Zacchi G. Cellulase production by *T. reesei*. Bioresour Technol 1996;57:25–30.
[72] Heck JX, Hertz PF, Ayub MAZ. Cellulase and xylanase production by isolated Amazon *bacillus* strains using soybean industrial residue based solid-state cultivation. Braz J Microbiol 2002;33:213–8.
[73] Romero MD, Aguado J, Gonzalez L, Ladero M. Cellulase production by *Neurospora crassa* on wheat straw. Enzyme Microb Technol 1999;25:244–50.

[74] Villena GK, Gutierrez-Correa M. Morphological patterns of *Aspergillus niger* biofilms and pellets related to lignocellulolytic enzyme productivities. Lett Appl Microbiol 2007. ISSN 0266-8254 (doi:10.1111/j.1472-765X.2007.02183.x).
[75] Gao J, Weng H, Zhu D, Yuan M, Guan F, Xi Y. Production and characterization of cellulolytic enzymes from the thermoacidophillic fungal *Aspergillus terreus* M11 under solid-state cultivation of corn stover. Bioresour Technol 2009;99:7623–9.
[76] Osherov N, May GS. The molecular mechanisms of conidial germination. FEMS Microbiol Lett 2001;199:153–60.
[77] Tuckey SL, Talbot NJ. Surface attachment and pre-penetration stage development by plant pathogenic fungi. Annu Rev Phytopathol 2001;39:385–417.
[78] Gutiérrez-Correa M, Villena GK. Surface adhesion fermentation: a new fermentation category. Rev Peru Biol 2003;10:113–24.
[79] Grimm LH, Kelly S, Krull R, Hempel DC. Morphology and productivity of filamentous fungi. Appl Microbiol Biotechnol 2005;69:375–84.
[80] Ferreira Susana MP, Duarte AP, Queiroz JA, Domingues FC. Influence of buffer systems on *Trichoderma reesei* Rut C-30 morphology and cellulase production. Electron J Biotechnol 2009;12. ISSN: 0717-3458.
[81] Martinez D, Berka RM, Henrissat B, Saloheimo M, Arvas M, Baker SE, Chapman J, Chertkov O, Coutinho PM, Cullen D, Danchin EGJ, Grigoriev IV, Harris P, Jackson M, Kubicek CP, Han CS, Ho I, Larrondo LF, Lopez de Leon A, Magnuson JK, Merino S, Misra M, Nelson B, Putnam N, Robbertse B, Salamov AA, Schmoll M, Terry A, Thayer N, Westerholm-Parvinen A, Schoch CL, Yao J, Barbote R, Nelson MA, Detter C, Bruce D, Kuske CR, Xie G, Richardson P, Rokhsar DS, Lucas SM, Rubin EM, Dunn-Coleman N, Ward M, Brettin TS. Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). Nat Biotechnol 2008;26:553–60.
[82] Wilson DB. Studies of *Thermobifida fusca* plant cell wall degrading enzymes. Chem Rec 2004;4:72–82.
[83] Arnold FH. Combinatorial and computational challenges for biocatalyst design. Nature 2001;409:253–7.
[84] Hibbert EG, Baganz F, Hailes HC, Ward JM, Lye GJ, Woodley JM, Dalby PA. Directed evolution of biocatalytic processes. Biomol Eng 2005;22:11–9.
[85] Shoemaker HE, Mink D, Wubbolts MG. Dispelling the mythsbiocatalysis in industrial synthesis. Science 2003;299:1694–7.
[86] Boisset C, Petrequin C, Chanzy H, Henrissat B, Schulein M. Optimized mixtures of recombinant Humicola insolens cellulases for biodegradation of crystalline cellulose. Biotechnol Bioeng 2001;72:339–45.
[87] Himmel ME, Ruth MF, Wyman CE. Cellulase for commodity products from cellosic biomass. Curr Opin Biotechnol 1999;10:358–64.
[88] Kim E, Irwin DC, Walker LP, Wilson DB. Factorial optimization of a six-cellulase mixture. Biotechnol Bioeng 1998;58:494–501.
[89] Zhang Y-HP, Lynd LR. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems. Biotechnol Bioeng 2004;88:797–824.
[90] Zhang YHP, Himmel ME, Mielenz JR. Outlook for cellulase improvement: Screening and selection strategies. Biotechnol Adv 2006;24:452–81.
[91] Baker JO, McCarley JR, Lovett R, Yu CH, Adney WS, Rignall TR, et al. Catalytically enhanced endocellulase Cel5A from *Acidothermus cellulolyticus*. Appl Biochem Biotechnol 2005;121–124:129–48.
[92] White T, Hindle C. Genetic constructs and genetically modified microbes for enhanced production of beta-glucosidase, US Pat. 6015703 (to Iogen Corporation, Ottawa, CA) 18 January 2000.
[93] Watanabe M, Tatsuki M, Aoyagi K, Sumida N, Takeshi M. Regulatory sequence of cellulase cbh1 genes originating in *Trichoderma viride* and system for mass-producing proteins or peptides therewith, US Patent 6277596 (to Meiji Seika Kaisha Ltd., Tokyo, JP) 21 August 2001.
[94] Mantyla A, Paloheimo M, Suominen P. Industrial mutants and recombinant strains of *Trichoderma reesei*. In: Harman GE, Kubicek CP, editors. Trichoderma and Gliocladium, vol. 2. London, UK: Taylor & Francis Ltd.; 1998. p. 291–309.
[95] Penttila M. Heterologous protein production in Trichoderma. In: Harman GE, Kubicek CP, editors. Trichoderma and Gliocladium, vol. 2. London: Taylor & Francis; 1998. p. 367–82.
[96] Ilmen M, Onnela ML, Klemsdal S, Keränen S, Penttilä M. Functional analysis of the cellobiohydrolase I promoter of the filamentous fungus *Trichoderma reesei*. Mol Gen Genet 1996;253:303–14.
[97] Nakari-Setala T, Pentilla M. Production of *Trichoderma reesei* cellulases on glucose containing media. Appl Environ Microbiol 1995;61:3650–5.
[98] Saha BC, Bothast R. Production, purification, and characterization of a highly glucose-tolerant novel β-glucosidase from *Candida peltata*. Appl Environ Microbiol 1996;62:3165–70.
[99] Riou C, Salmon JM, Vallier MJ, Gunata Z, Barre P. Purification, characterization, and substrate specificity of a novel highly glucose-tolerant β-glucosidase from *Aspergillus oryzae*. Appl Environ Microbiol 1998;64:3607–14.
[100] Yan TR, Lin CL. Purification and characterization of a glucose tolerant beta-glucosidase from *Aspergillus niger* CCRC 31494. Biosci Biotechnol Bioeng 1997;61:965–70.
[101] Fowler T, Barnett CC, Shoemaker S. Improved saccharification of cellulose by cloning and amplification of the beta-glucosidase gene of *Trichoderma reesei*, Patent WO/1992/010581 A1 (to Genencor Int. Inc.) 25 June 1992.
[102] Fierobe HP, Bayer EA, Tardif C, Czjzek M, Mechaly A, Belaich A, Lamed R, Shoham Y, Belaich JP. Degradation of cellulose substrates by cellulosome chimeras. Substrate targeting versus proximity of enzyme components. J Biol Chem 2002;277:49621–30.
[103] Fierobe HP, Mingardon F, Mechaly A, Belaich A, Rincon MT, Pages S, Lamed R, Tardif C, Belaich JP, Bayer EA. Action of designer cellulosomes on homogeneous versus complex substrates: controlled incorporation of three distinct enzymes into a defined trifunctional scaffoldin. J Biol Chem 2005;280:16325–34.
[104] Mingardon F, Chanal A, Lopez-Contreras AM, Dray C, Bayer EA, Fierobe HP. Incorporation of fungal cellulases in bacterial minicellulosomes yields viable, synergistically acting cellulolytic complexes. Appl Environ Microbiol 2007;73:3822–32.
[105] Mahesh C, Alok K, Sangwan NS, Gaurav Shailendra S, Darokar Mahender P, Sangwan Rajinder S. Development of a mutant of *Trichoderma citrinoviride* for enhanced production of cellulases. Bioresour Technol 2009;100:1659–62.
[106] Abdel-Fattah AF, Osman MY, Abdel-Naby MA. Production an immobilization of cellobiase from *Aspergillus niger* A20. Chem Eng J 1997;68:189–96.
[107] Kotchoni OS, Shonukan OO, Gachomo WE. *Bacillus pumilus* BpCRI 6, a promising candidate for cellulase production under conditions of catabolite repression. Afr J Biotechnol 2003;2:140–6.
[108] Endo K, Hakamada Y, Takizawa S, Kubota H, Sumitomo N, Kobayashi T, Ito S. A novel alkaline endoglucanase from an alkaliphilic *Bacillus* isolate: enzymatic properties, and nucleotide and deduced amino acid sequences. Appl Microbiol Biotechnol 2001;57:109–16.
[109] Li DC, Lu M, Li YL, Lu J. Purification and characterization of an endocellulase from the thermophilic fungus *Chaetomium thermophilium* CT2. Enzyme Microb Technol 2003;33:932–7.
[110] Oinonen AM, Londesborough J, Joutsjoki V, Lantto R, Vehmaanperä J. Three cellulases from *Melanocarpus albomyces* for textile treatment at neutral pH. Enzyme Microb Technol 2004;34:332–41.
[111] Saha BC. Production, purification and properties of endoglucanase from a newly isolated strain of *Mucor circinelloides*. Process Biochem 2004;39:1871–6.
[112] Romero MD, Aguado J, Gonzalez L, Ladero M. Cellulase production by *Neurospora crassa* on wheat straw. Enzyme Microb Technol 1999;25:244–50.
[113] Adsul MG, Ghule JE, Singh R, Shaikh H, Bastawdea KB, Gokhale DV, Varma AJ. Polysaccharides from bagasse: applications in cellulase and xylanase production. Carbohydr Polym 2004;57:67–72.
[114] Hreggvidsson GO, KaisteE, Holst O, Eggertsson G, Palsdottir A, Kristjansson AJ. An extremely thermostable cellulase from the thermophilic eubacterium *Rhodothermus marinus*. Appl Environ Microbiol 1996;62:3047–9.
[115] Jang H, Chang K. Thermostable cellulases from *Streptomyces* sp. scale-up production in a 50-l fermenter. Biotechnol Lett 2005;27:239–42.
[116] Grigorevski de-Limaa AL, do-Nascimento RP, da-Silva Bon EP, Coelho RR. *Streptomyces drozdowiczii* cellulase production using agro-industrial by-products and its potential use in the detergent and textile industries. Enzyme Microb Technol 2005;37:272–7.
[117] Bronnenmeier K, Kern A, Liebl W, Staudenbauer WL. Purification of *Thermotoga maritima* enzymes for the degradation of cellulosic materials. Appl Environ Microbiol 1995;61:1399–407.
[118] Weber J, Agblevor FA. Microbubble fermentation of *Trichoderma reesei* for cellulase production. Process Biochem 2005;40:669–76.
[119] Szijarto N, Faigl Z, Réczey K, Mézesc M, Bersényi A. Cellulase fermentation on a novel substrate (waste cardboard) and subsequent utilization of home-produced cellulase and commercial amylase in a rabbit feeding trial. Ind Crops Prod 2004;20:49–57.
[120] Shen X, Xia L. Production and immobilization of cellobiase from *Aspergillus niger* ZU-07. Process Biochem 2004;39:1363–7.
[121] Tsao GT, Xia L, Cao N, Gong CS. Solid-state fermentation with *Aspergillus niger* for cellobiase production. Appl Biochem Biotechnol 2000;84–86:743–9.
[122] Krishna C. Production of bacterial cellulases by solid state bioprocessing of banana wastes. Bioresour Technol 1999;69:231–9.
[123] Yang YH, Wang BC, Wang QH, Xiang LJ, Duan CR. Research on solid-state fermentation on rice chaff with a microbial consortium. Colloids Surf B: Biointerf 2004;34:1–6.
[124] Fujian X, Hongzhang C, Zuohu L. Effect of periodically dynamic changes of air on cellulase production in solid-state fermentation. Enzyme Microb Technol 2002;30:45–8.
[125] Kalogeris E, Fountoukides G, Kekos D, Macris BJ. Design of a solid-state bioreactor for thermophilic microorganisms. Bioresour Technol 1999;67:313–5.
[126] Xia L, Cen P. Cellulase production by solid state fermentation on lignocellulosic waste from the xylose industry. Process Biochem 1999;34:909–12.
[127] Singhania RR, Sukumaran RK, Pandey A. Improved cellulase production by *Trichoderma reesei* RUT C30 under SSF through process optimization. Appl Biochem Biotechnol 2007;142:60–70.