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Production, characteristics and potential applications of the cellulolytic enzymes of thermophilic moulds

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ABSTRACT

Thermophilic moulds are capable of degrading plant organic residues at elevated temperatures by secreting a large array of hydrolases. These moulds produce cellulolytic enzymes in submerged as well as solid substrate fermentations. Cellulases from these moulds have been characterized, which display a high thermostability, catalytic activity and fast rate of reactions as compared to their mesophilic counterparts. Their optimal activities lie in the range of 50-70 °C and pH of 3.0-6.0 with molecular masses between 40 and 240 kDa. The genes encoding cellulolytic enzymes of the moulds have been cloned and expressed in both homologous and heterologous systems. Cellulolytic enzymes of thermophilic moulds have been shown to be efficient in plant biomass degradation. The applicability of cellulases in a number of biotechnological processes such as the production of biofuels, food and animal feeds, detergents and paper pulp processing has been demonstrated.

KEYWORDS: Thermophilic moulds, cellulases, lignocellulosics, solid state fermentation, submerged fermentation, biofuels, bioethanol

INTRODUCTION

Lignocellulosics are the most abundant biopolymers on Earth. Their hydrolysis into fermentable sugars, phenolics and sugar acids is necessary for using them as substrates in the production of industrially important value added products. Cellulases, hemicellulases and ligninases are the group of enzymes which carry out enzymatic hydrolysis of lignocelluloses in combination, and these enzymes collectively are known as 'lignocellulolytic enzymes'. Among lignocellulolytic enzymes, cellulases are the third most significant and key enzymes in the conversion of cellulose to simple sugars (Chinedu *et al.,* 2005). Cellulases are the group of enzymes which hydrolyze β-1,4- glycosidic bonds of intact cellulose and other cellooligosaccharides. For complete degradation of cellulose, three main enzymes [exoglucanase (EC 3.2.1.91), endoglucanase (EC 3.2.1.4), and β-glucosidase (EC 3.2.1.21)] act synergistically. Endoglucanases favourably cleave β-glucosidic bonds and hydrolyse the internal regions of the fibrils, exoglucanases (cellobiohydrolases) release cellobiose from terminal ends of chains, while the process of degradation is completed by β-glucosidases which hydrolyse cellobiose and other cellodextrins with a low degree of polymerizations to glucose (Szijarto *et al.,* 2004).

Among the estimated 3.0 million species of fungi extant on Earth (Hawksworth, 2012), more than 100,000 have so far been described, and among them, approximately 50 species are capable of growth in the temperature range of 40-60 ºC (Mouchacca, 2000). Thermophilic fungal species depict rather broad cardinal (minimum, maximum and optimum) temperatures which extend from 20 ºC to upwards of 50 ºC (Cooney and Emerson, 1964). A thermophilic mould grows well at 50 ºC and beyond, but considered not to grow below 20 ºC. The heat tolerant enzymes produced by thermophilic fungi are of immense interest in industrial bioprocesses.

Thermophilic fungi have evolved adaptations in response to varied environmental conditions like extreme pH, high salt concentrations and temperatures, which are hostile for most of the organisms, but allow them to survive and colonize various natural substrates. The search for industrially important enzymes such as lipases, cellulases, xylanases, amylases, and proteases led to the discovery of thermostable enzymes (Johri *et al.,* 1999; Singh 2016; Singh *et al.,* 2016). Thermostable enzymes have the advantages over these from mesophilic fungi because of their action at high temperatures leads to decrease in the viscosity of media and acceleration in the reaction rates, besides minimizing contamination risks (Fernandes *et al.,* 2008). Both biochemical and molecular (genomic) studies have confirmed the ability of these moulds in efficiently degrading a variety of lignocellulosic substrates (Singh *et al.,* 2016).

Myceliophthora thermophila (Moretti *et al.,* 2012) and *M. heterothallica* (Van Den Brink *et al.,* 2013) have a high potential in the bioconversion of lignocellulosic biomass into fermentable sugars. *Thermoascus aurantiacus* is another promising fungus which is capable of producing thermostable enzymes (McClendon *et al.,* 2012). Furthermore, the genomes of *M. thermophila* and *Thielavia terrestris* have been analysed recently (Berka *et al.,* 2011), which showed the evidence for their ability to degrade all the major polysaccharides present in lignocellulosic biomass. These studies and data reinforce and encourage the continued search for thermostable enzymes from thermophilic fungi. This review focuses on the production, characteristics and potential applications of cellulolytic enzymes of thermophilic moulds.

CELLULASES

Cellulases are a complex enzyme system that includes exo-1,4-β-D-glucanase, endo-1,4-β-D glucanase, and β-D-glucosidase (Maheshwari *et al.,* 2000). These are briefly described below:

ENDOGLUCANASE

Endoglucanase (endo-β-1, 4-D-glucanase or endo-β-1, 4-D-glucan-4- glucano-hydrolase) usually known as CMCase, randomly hydrolyses carboxymethyl cellulose (CMC) or

swollen cellulose resulting in the increase of concentration of reducing sugars by decreasing the length of polymer (Robson and Chambliss, 1989; Begum *et al.,* 2009). Cellodextrins, the intermediate products of cellulose hydrolysis, are also hydrolysed by this enzyme and converted into glucose and cellobiose (disaccharide), but it is not active on crystalline celluloses such as cotton or avicel.

EXOGLUCANASE

Exoglucanase (exo-β-1, 4-D glucanase or cellobiohydrolase) acts from the non-reducing end of chain of cellulose splitting-off the cellobiose units. Exoglucanases are also active on swollen cellulose and cellodextrins and partially degrade amorphous substrates, but they do not cause hydrolysis of soluble derivatives of cellulose such as CMC and hydroxyethyl cellulose. As a minor component, some cellulase systems also contain glucohydrolase (exo-1, 4-D-glucan-4-glucohydrolase) as a minor component (Joshi and Pandey, 1999).

β- GLUCOSIDASE

The complete hydrolysis of cellulose is accomplished by β-glucosidase (β-D-glucoside glucohydrolase) that cleaves glucose from the non-reducing end (i.e., with a free hydroxyl group at C-4) of cellooligosaccharides and cellobiose. Alkyl and aryl β-glucosides are also hydrolysed by these enzymes (Kubicek *et al.,* 1993).

PRODUCTION OF CELLULASES BY THERMOPHILIC MOULDS

Submerged fermentation (SmF): Submerged fermentation is the frequently used process for production of industrially important enzymes on a large scale. Generally, this process is carried out in the presence of liquid medium having soluble substrates which flow freely. The factors which makes it more attractive includes the facility of controlling parameters of the process, monitoring and downstream processing (Sukumaran *et al.,* 2005). The main drawback in this process is that it requires prolonged time for fermentation or gestation period with less productivity of enzyme (Singhania *et al.,* 2010). In order to attain superior productivity in SmF, various natural and synthetic carbon sources have been used. *Thermoascus aurantiacus* was first reported as the most active cellulase producer by Tong and Cole (1982) among several fungi tested. Its optimum temperature for growth was 45 °C, while maximum cellulase from filter paper was produced at 40 °C. For β-glucosidase and carboxymethylcellulase (CMCase) activity, optimum temperature was 70 °C but it was 65 °C for filter paper degrading activity (FPase). Maximum activity of β-glucosidase, and FPase and for CMCase was observed at pH 5.0 and 4.3, respectively. Bajaj *et al.* (2014) reported *Sporotrichum thermophile* LAR5 to efficiently utilize low-cost agricultural residues (wheat bran, maize bran, and rice husk) for cellulase production. Maximum production was observed in wheat bran (2000 U/l) followed by maize bran (1800 U/l) and rice husk (1600 U/l). The production of enzyme was enhanced substantially by the addition of peptone, mustard cake and soybean meal in medium. Cellulase was optimally active at 60 -70 ºC and pH 5.0. Busk and Lange (2013) studied the cellulolytic potential of sixteen thermophilic fungal strains on a medium containing microcrystalline cellulose as the only carbon source, and reported that *Thermomucor indicae-seudaticae* showed the highest optimal endoglucanase activity at pH 8.0. Matsakas *et al.* (2015) optimized growth conditions of *M. thermophila* for cellulase production in SmF. The optimum temperature, pH and agitation for cellulase activity were 65 ºC, pH 5.5 and 200, respectively. Among different carbon sources used for optimization, Brewer's spent grain supported highest enzyme production (0.11±0.01 FPU/ml) during fifth and sixth day of cultivation. Bala and Singh (2016) reported the production of endo-β-1,4-glucanase (CMCase), exo-β-1,4-glucanase (FPase) and β-glucosidase (BGL) using cane molasses medium and reported 36,420, 32,420 and 5,180 U L^{-1} production of CMCase, FPase and BGL, respectively. The optimal activity of these enzymes was reported at pH 5.0 and 60 ºC. The addition of Tween 80 further enhanced the production of the cellulolytic enzymes. Zambare *et al.* (2011) reported cellulase production from thermophilic consortium and maximum production (up to 367 U L^{-1} on prairie cord grass) was achieved at 60 ºC and pH 4.0. *M. thermophila* produced maximum cellulase at 45 ºC in presence of 1% solka/floc supplemented with urea after 2-4 days (Coutts and Smith, 1976). While Kawamori *et al*. (1987) reported that *T. aurantiacus* A-131when cultivated with 4 % alkali treated bagasse at 45°C in 4 days produced about 70 U ml^{-1} of CMCase and it had excellent thermostability as there was no loss of activity after exposure to 60 °C for 24 h. Grigorevski-Lima *et al.* (2009) reported the production of a thermostable and acid endoglucanase by *Aspergillus fumigatus* isolated from sugar cane bagasse. Highest levels of endoglucanase (365 U L^{-1}) was attained within 6 days of cultivation at 1 % sugarcane bagasse and 1.2 % corn steep liquor at pH 2.0 and 65 °C.

Solid State Fermentation (SSF): For the enhanced production of various enzymes, one of the important strategies employed in the industries is SSF. This process is performed in the absence of free water on the solid substrates which acts as solid support as well as source of nutrients for the growth of microorganisms. SSF is gaining more attention in the recent years, as it is an acceptable strategy for the reuse of the nutrient-rich agricultural residues with less energy input and facilitates bioconversion of agricultural residues to value-added products (Pandey, 2003). Because of low operating cost and capital investment, SSF is an attractive strategy to produce cellulases economically (Xia and Cen, 1999). The physico-chemical properties of substrates such as crystallinity, bed porosity and large surface area can affect the yield of cellulolytic enzymes by the moulds. In SSF, the growth and production of enzymes are influenced by the culture conditions such as pH, temperature, water activity and moisture content which are crucial factors. The major challenges in SSF are the generation of heat and presence of oxygen in the open space between substrate particles i.e., porosity and these challenges have to be addressed properly (Thibault *et al.,* 2000; Oostra *et al.,* 2001). The most commonly used agricultural residues used as substrates for cellulase production are brans and straws (wheat and rice), corn stover, sawdust, and sugarcane baggase. Moretti *et al.*

(2012) isolated thermotolerant and thermophilic fungal strains from soil, sugarcane piles and decaying organic matter on a medium having cardboard and corn straw as carbon source at 45 °C. *Myceliophthora thermophila* M.7.7 and *Aspergillus fumigatus* M.7.1 were identified as highest cellulase producers under SSF, and maximum enzyme activity was recorded at pH 5.0 and 60 ºC and 70 ºC, respectively. The endoglucanases from these fungi were stable at 40 to 65 °C and pH 4.0 to 9.0. *Aspergillus fumigatus* M.7.1 produced maximum β-glucosidase (40.4 U/g) and CMCase (40 to 47 U/g) when cultivated on the mixture of wheat bran and corn straw. *M. thermophila* M.7.7 produced 54 U/g of CMCase in a medium that contained wheat bran and sugarcane bagasse. Kilikian *et al.* (2014) reported the highest FPase (10.6 U/gdm) production by *M. thermophila* M77 using sugarcane bagasse and soybean bran. This activity was 4.4 times higher than that attained with wheat bran. Kaur *et al.* (2015) studied the production of cellulases from *Humicola fuscoatra* MTCC 1409 using rice straw as substrate and attained maximum production of cellobiase (9.30 U/g), CMCase (3.83 U/g) and FPase (1.6.7 U/g) at pH 6.0 and 45 ºC. *Myceliophthora thermophila* yielded high titres of cellulase (42 U/g DMB) using citrus pectin and wheat bran in 1:1 ratio after 4 days in SSF at pH 7.0 and 45 ºC (Kaur and Satyanarayana, 2004). Similarly, Pereira *et al.* (2015) reported that *M. thermophila* JCP 1-4 was the highest producer of endoglucanase (357.51 U g^{-1}), β-glucosidase (45.42 U g^{-1}). The highest endoglucanase (227.97 U g^{-1}), β-glucosidase (34.36 U g^{-1}) and FPase (0.34 FPU) production was recorded in 240, 192 and 96 h, respectively. β-Glucosidase produced by *Aspergillus fumigatus* P40M2 was stable at pH 3.0 - 5.5 and at 40-60 °C (Delabona *et al.,* 2013). Reetika *et al.* (2013) reported highest cellulolytic activity by DIA-4 strain at 45 °C in 72 h using a combination of wheat bran and sweet sorghum as substrate.

PURIFICATION AND CHARACTERIZATION OF CELLULASES OF THERMOPHILIC MOULDS

Thermophilic fungi secrete cellulases and hemicellulases efficiently which are active under harsh conditions. Tansey and Brock (1972) found that thermophilic moulds *Sporotrichum thermophile*, *Thermoascus aurantiacus* and *Chaetomium thermophile* hydrolyzed cellulose two times faster than that of *Trichoderma viride*. Mandels (1975) reported thermophilic mould to hydrolyse polysaccharide fast, despite low cellulase titres in the culture filtrate. Purification and characterization of enzymes are considered as important steps. The catalysts produced on native lignocellulosic substrates are usually a mixture of varied compounds together with proteins. Purification of a significant macromolecule from such culture filtrates needs multistep treatments by the classical ways including precipitation, qualitative analysis and column chromatography (Maheshwari *et al*., 2000). Moloney *et al.* (1985) used Sephadex matrix for purification of proteins of different sizes by gel filtration chromatography. The techniques which are used for purification are totally dependent on the purification fold and yields of proteins. Cellulolytic enzymes from thermophilic moulds have been purified using salt/solvent precipitation followed by ion exchange and gel filtration (**Table 1**).

Recombinant cellobiohydrolase II from *Chaetomium thermophilum* was purified using ammonium sulphate fractionation and DEAE-Sepharose quick flow chromatography (Liu *et al*., 2005). Cellobiose dehydrogenases from thermophilic mould *M. thermophila* were purified, and the genes encoding cellobiose dehydrogenases were successfully cloned and characterised by Subramaniam *et al*. (1999). The purified enzyme showed optimal activity at 60˚C. The characterization of enzymes showed that molecular masses of enzymes were in the range of 91- 192 kDa and they were glycosylated. The activation energy was 26.3 kJ/mol along with acidic isoelectric points of 4.1 and 3.45 for enzymes I and II, respectively.

Cellulase is produced by *Humicola insolens* in wheat bran as a carbon source. After processing with the combinations of ion-exhange and gel filtration chromatography, pure endoglucanase, exoglucanase, and β-glucosidase were obtained in good amounts from the culture extract (Hayashida *et al*., 1988). *Talaromyces emersonii* produced multiple forms of cellulases (endoglucanase, exoglucanases, and β-glucosidases), which were purified by ion exchange chromatography and separated multiple forms of endoglucanases, which differed in carbohydrate content (28-51%), but had similar molecular masses (35 kDa by SDS-PAGE) with the pH optima between 5.5 - 5.8, temperature optima at 75-80˚C and iso-electric points (pI values) between 2.8 and 3.2 (Moloney *et al*., 1985). Endoglucanases were thought to be due to post- translational or post secretion alterations of one cistron product.

The combinations of various chromatographic techniques were used for purifications of β-glucosidase from *Melanocarpus* sp. using DEAE-Sepharose and PBE 94 columns (Kaur *et al*., 2007). The specific activity of purified enzyme was 10.04 μmol min^{-1} mg $protein^{-1}$ with 15.89 % yield. *Aspergillus oryzae* produced seven endoglucanases cloned from *Chateominm thermophile* (Davies *et al*., 1992; Schou *et al*., 1998). All the endoglucanases were purified by affinity chromatography. Similarly, Eriksen and Goksoyr (1976, 1977) purified different types of cellulases. All cellulases in combination were able to degrade cotton completely as compared to those when used separately. A cellulase from *Thermoascus aurantiacus* RBB-1 was purified by ammonium sulphate precipitation, activity and size exclusion chromatography (Dave *et al*., 2015). The recovery and purification fold were 13.3 % and 6.6, respectively with a molecular mass of 35 kDa. Optimum temperature for activity was 70˚C and stability was upto 80 ˚C for one h. Besides higher stability at 80˚C, the protein also showed half-life values of 192 and 144 h at 50 and 70 ˚C, respectively. Purified cellulase was optimally active at pH 4.0 with K_m and V_{max} values of 37 mg/ml and 82.6 U/min/mg, respectively with higher salt tolerance (Dave *et al*., 2015). A consortium of cellulases including β-glucosidase were purified form *T. aurantiacus* using gel filtration chromatography (Bio-gel P-60 matrix) followed by SDS-PAGE with molecular masses of 87 kDa for ☛glucosidase, 78 kDa for cellulase I, 49 kDa for

Table 1 Purification of cellulolytic enzymes from thermophilic moulds

Fungi	Purification methods	Enzyme	Optimum pH	Optimum temperature (˚C)	MW (kDa)	Enzyme stability	References
Thermoascus aurantiacus	Cation exchange chromatography, gel filteration	β-glucosidase	3-9	70	85	At 4°C for several weeks	Tong *et al*., 1980
Chaetomium thermophilum CT2	Ammonium sulfate fraction, DEAE-Sepharose Fast flow chromatography	Recombinant cellobiohydrolase II	4	50	67	NR	Liu *et al*., 2005
Myceliophthora thermophila ATCC 48104	$(NH_4)_2SO_4$ precipitation and column chromatography on DEAE-Sephadex A-50, Sephadex G-200 and DEAE-Sephadex A-50	β-glucosidase	4.8	60	120	Purified enzyme in the absence of substrate was stable up to 60°C	Roy *et al*., 1991
M. thermophila D-14	Ammonium sulphate precipitation , ion-exchange chromatography	Endoglucanase	4.8	65	100	Stable for 60 min at temperatures up to 70 ˚C	Roy *et al*., 1990
Melanocarpus sp. MTCC 3922	Ammonium sulphate precipitation , ion-exchange chromatography	Endoglucanases (EG I and EG II)	6 and 5	50 and 70	40 and 50 kDa	NR	Kaur *et al*., 2007
Chaetomium thermophile var. dissitum	Ion-exchange chromatography, gel filtration	Endo- and exoglucanases	NR	NR	67 and 41	NR	Erisken and Goksoyr, 1977
Humicola insolens YH-8	Avicel adsorption, heat treatments and ammonium sulfate fractionation, Ion exchange chromatography	Avicelase, CMCase	5.3 for avicelase and 5.0 for CMCase	50	NR	Avicelase was stable after heating at 65°C for 5 min and CMCase retained 45~ of the original activity after heating at 95°C for 5 min	Hayasida and Yoshioka, 1980
Sporotrichum thermophile *S. thermophile* *ɔ. thermophile*	80% Ammonium sulphate precipitation , ion-exchange chromatography	β-glucosidase	5.4	65	240	at 50 "C for up to 6 h, but at 60 "C, 25 YO of the The effect of pH on enzyme activity was studied using original enzyme activity was lost after 2 h Stable at 50 °C for 6h, 25% activity lost at 60 °C after 2h	Bhat *et al*., 1993
Thermoascus aurantiacus RBB-1	ammonium sulfate precipitation, ion exchange and size exclusion chromatography	Endo-cellulase	4	70	35	Stability was up to 80 °C for 1 h	Dave *et al*., 2015
Allesheria terrestris	Ion exchange chromatography	Endo- and exoglucanases	NR	NR	69	Inactivated only by 20% at 65°C for 6 h in the absence of the substrate	Kvesitadze *et al*., 1997
Thermomyces lanuginosus-SSBP	ion exchange chromatography	β-D-glucosidase	6	65	200	30 min incubation at 50 ° C	Lin *et al*., 1999

cellulase II and 34 kDa for cellulase III. The enzymes contained varied carbohydrate contents of 33.0 to 5.5, 2.6 and 1.8 %, respectively. Cellulases exhibited the ability to degrade paper and yeast glucan in combination but solely cellulases I and III were active on CMC. The Km values of enzymes varied widely (Tong *et al*., 1980).

Melanocarpus sp. produced β-glucosidase which was active in narrow pH range with optimal activity at pH 5.0 and 6.0 (Bhat *et al*., 1993). It was stable for 240 min at 50 °C. Kaur *et al*., (2007) used isoelectric focussing gel technique for the purification of β-glucosidase of *Melanocarpus* sp. Purified product showed one form of enzyme with acidic pI value. Usually cellulases produced by thermophilic moulds were monomeric except β-glucosidase, which is dimeric (Maheshwari *et al*., 2000). *Allescheria terrestris* produced a high molecular (210-230 kDa) β-glucosidase consisting of six subunits which dissociated to monomeric forms at higher temperatures (Kvesitadze *et al*., 1997).

The endoglucanases with high molecular masses (30 to 100 kDa) from thermophilic moulds were more thermostable and mostly active at 55-80°C and pH 5.0-5.5 and high carbohydrate content up to 50 % (Li *et al*., 2011). Exoglucanase from thermophilic moulds were able to tolerate higher temperature than endoglucanase as they were mostly active in broad temperature range of 50 - 75 ºC with a molecular mass of 40 - 75 kDa. All exoglucanases are conjugated proteins. The pH and temperature optima for all pure cellulases from thermophilic moulds were similar (**Table 1**). The optimum pH for cellulases was in the range of 4.0-7.0 and 50-80 °C. The cellulases from thermophilic moulds were exceptionally thermostable and had a longer half life values at higher temperatures than those of mesophilic fungi. Glucosidase from *Thermomyces lanuginosus* was less active than β-glucosidase from *Melanocarpus sp.* MTCC 3922 at pH 5.0 and 60 ˚C (Lin *et al*., 1999; Kaur *et al*., 2007). The optimum pH and temperature were 5.0-6.0 and 50 ºC for β-glucosidases of most of the fungi (Bhat *et al*., 1993).

Cellulase of *Paecilomyces thermophila* was strongly inhibited by Hg^{2+}, but not affected by Zn^{2+}. Similarly, Zn^{2+} along with other metal ions such as sodium, potassium and calcium did not affect β-glucosidases of *Melanocarpus* sp. MTCC 3922, but the activity was repressed by $CuSO_4$ (Kaur *et al*., 2007). Kaur *et al*. (2007) purified two endoglucanases from *Melanocarpus* sp. MTCC 3922. The molecular masses of two glucanases and their pI were ~40 and 50 kDa, and ~4.0 and 3.6, respectively. Mercaptoethanol and SDS (denaturing agents) repressed one of the two endoglucnases (EG I) of *Melanocarpus* sp. MTCC 3922, the other (EG II) was not affected. Affinity for degrading avicel by EG I and EG II revealed the existence of polysaccharide binding domains (CBD), although EG II lacked CBD (Kaur *et al*., 2007). Roy *et al*. (1990) purified endoglucanase from *M. thermophila* D-14 using ammonium sulphate precipitation followed by ion chromatography using DEAE-Sephadex. The pure enzyme was optimally active at pH 4.8 and 65˚C. Metal ions such as

Hg^{2+}, Fe^{2+}, and Cu^{2+} positively affected the enzyme activity.

Extracellular β-glucosidase was purified from *M. thermophila* ATCC 48104 using ammonium sulphate precipitation and chromatographic techniques (Roy *et al*., 1991). The molecular mass was 120 kDa. Aldohexose acts as a competitive inhibitor. Enzyme activity was repressed by $HgCl_2$, $FeSO_4$ and EDTA. Recombinant β-1,4-glucanase from *Humicola insolens* Y1 was purified by SP- Sepharose ion exchange chromatography (Xu *et al*., 2015). The molecular mass was 33.5 kDa. Enzyme was thermostable at 60˚C for an h. Metal ions tested had no significant effect on the enzyme activity except CTAB and Cu^{2+}.

CLONING AND EXPRESSION OF CELLULASES OF THERMOPHILIC MOULDS

For the commercial applications of enzymes, researchers used hyper-producing microorganisms. Cellulases of thermophilic moulds exhibited several desirable properties which make their applicability in the saccharification of lignocellulosic biomass for the production of biofuels and other high value-added products. However, in some cases, the yields of enzymes are low, therefore, over-expressing in a suitable host is the ideal solution to overcome this problem. Consequently thermophilic moulds have received attention in isolating and cloning cellulase encoding genes from several thermophilic fungi (Poças-Fonseca *et al*., 2000; Berquist *et al.,* 2004). Recombinant DNA techniques provide the means for producing enzymes from thermophiles in mesophiles. Recent advances in molecular biology and biotechnology facilitated gene cloning and over expression. The cellulase genes of thermophilic moulds were expressed in various mesophilic hosts such as bacteria like *E. coli*, yeasts like *Pichia pastoris* and filamentous fungi (**Table 2**). Microbial cellulases have been classified as 1, 3, 5, 6, 7, 8, 9, 10, 12, 16, 44, 45, 48, 51, and 61 types (Maheshwari *et al*., 2000). Cellulases from thermophilic moulds were mainly classified into 1, 3, 5, 6, 7, 12, and 45 types (Maheshwari *et al*., 2000). Mesophilic fungus *T. reesei* was used as a host for the expression of cellulase genes from *Melanocarpus albomyces* (Haakana *et al*., 2004) that resulted in improved cellulase activity as compared to parental strains. Cellulase encoding gene from *M. albomyces* was cloned, sequenced and finally expressed in *Trichoderma reesei* (Hakkana *et al*., 2004). Among two cellulase enzymes, Cel45A and Cel7A had molecular masses of 20 and 50 kDa, respectively, and the enzymes belong to different GH families 45 and 7 (Hakkana *et al*., 2004).

Pichia pastoris was used for the expression of cellobiohydrolase from *Chaetomium thermophilum* (Li *et al*., 2009). The pure recombinant enzyme displayed optimally active at pH 5.0 and 60˚C and it was active at higher temperature. Hong *et al*. (2007) used *Kluveromyces* as a cloning and expression host for endoglucanase of *Thermoascus aurantiacus*. Recombinant endoglucanase was purified by affinity technique with molecular mass of 35 kDa that belongs to GH5. Voutilainen *et al*. (2010) expressed the *T. emersonii* Cel7A cellobiohydrolase in *S. cerevisiae* and this expression system allowed the structure guided protein engineering by the introduction of further disulfide bridges within the catalytic domain of the enzyme. Mutant proteins consisted of more disulphide bridges at totally different positions. Mutant enzymes displayed improved T_m (from 78.5 to 84 ˚C) with higher half-lives at 70 ˚C than the wild. The mutants conjointly showed higher enzyme activity on Avicel at 75 ˚C. The cellulase gene from *H. insolens* was purified and cloned in *A. oryzae*. Structural study of the enzyme showed

Table 2 Biochemical properties of recombinant cellulases of thermophilic moulds

Fungi	Gene	Family	Expression vector	pI	Thermal stability	Optimum pH	Optimum temperature (˚C)	MW (KDa)	References
Melanocarpus albomyces	cel7b	7	*Trichoderma reesei*	4.23	NR	6-8	NR	50.0	Haakana *et al*., 2004
M. albomyces	cel45a	45	*T. reesei*	5.22	NR	6-8	NR	25.0	Haakana *et al*., 2004
M. albomyces	celL7b	7	*T. reesei*	4.15	NR	6-8	NR	44.8	Haakana *et al*., 2004
Chaetomium thermophilum	cel7a	7	*T. ressei*	NR	NR	4	65	54.6	Voutilainen *et al*., 2008
Talaromyces emersonii	cel7	7	*E.coli*	4.0	$T_{1/2}$: 68 min at 80°C	5	68	48.7	Grassick *et al*., 2004
T. emersonii	cel3a	3	*T. reesei*	NR	$T_{1/2}$: 62 min at 65 °C	NR	71.5	90.5	Murray *et al*., 2004
T. emersonii	cbh2	6A	*T. emersonii*	NR	NR	NR	NR	47.0	Murray *et al*., 2003
T. emersonii	cel7A	7	*Saccharomyces. cerevisiae*	NR	$T_{1/2}$: 30 min at 70°C	4-5	65	46.8	Voutilainen *et al*., 2010
Chaetomium thermophilum CT2	cbh2	NR	*Pichia pastoris* GS115	NR	50% of its original activity after 30 min at 70°C	4	50	67.0	Liu *et al*., 2005
C.thermophilum	cbh3	NR	*P. pastoris*	*5.2*	$T_{1/2}$: 10 min at 80°C	5	60	48.0	Li *et al*., 2009
Thermoascus aurantiacus	cbh1	7	*S. cerevisiae*	4.37	80% residual activity for 60 min at 65°C	6	65	48.7.0	Hong *et al*., 2003a
T. aurantiacus	eg1	5	*S. cerevisiae*	4.3	Stable for 60 min at 70°C	6	70	37.0	Hong *et al*., 2003b
T. aurantiacus IFO9748	eg1/ bgl1	3	*P. pastoris*	NR	70% activity after 1 h of incubation at 60°C	5	70	NR	Hong *et al*., 2007
T. aurantiacus	bgl1	3	*P. pastoris*	4.61	70% residual activity for 60 min at 60°C	5	70	93.5	Hong *et al*., 2007
T. aurantiacus	cel7a	7	*T. reesei*	4.44	NR	5	65	46.9	Voutilainen *et al*., 2008
T. aurantiacus	*cbh1*	7	*S. cerevisiae*	NR	NR	NR	65	NR	
Humicola insolens	cbhII	6	*S. cerevisiae*	NR	$T_{1/2}$: 95 min at 63°C	9	57	NR	Heinzelman *et al*., 2009
H. insolens	*Hicel6C*	6	*P. pastoris*	NR	After incubation at 60°C for 1 h, the enzyme retained greater than 90% of its initial activity	6.5	70	NR	Xu *et al*., 2015
Myceliophthora thermophila	eg7a	7	*P. pastoris* X-33	4.76	Retained more than 40 % at temperatures up to 80 °C for 8 h of incubation	5	60	65.0	Karnaouri *et al*., 2014
Talaromyces emersonii CBS394.64	TeEgl5A	5	*P. pastoris* GS115	NR	Enzyme remained more than 70 % of the maximal activity at 80–95 °C	4.5	NR	36.8	Wang *et al*., 2014

that enzyme had two domains, a chemical action domain and other one was cellulose binding domain which was covalently joined by a 33- amino acid sequences. Mackenzie *et al*. (1998) found that endoglucnasae was a polypeptide with β-sheets. Cel7A catalyst (cellobiohydrolases) of *Humicola grisea* showed more thermal stability than *H. jecorina* Cel7A on swollen polyose, and had higher ability to degrade pre-treated corn fodder at 65˚C (Momeni *et al*., 2014). The structure of endoglucnase from *H. insolens* and *T. reesei* was similar but their optimum pH values were different (Kleywegt *et al*., 1997), their pH optima were totally different at 7.5 and 4.5 (Schülein, 1997). The recombinant β-glucosidase was successfully expressed in brewer's yeast (Benoliel *et al*., 2010) that showed optimal activity at pH 6.0 and 40˚C. The molecular mass of the enzyme was 57 kDa. The enzyme showed positive activity against cellobiose and inhibited by metal ions such as Zn^{2+}, Al^{3+}, Cu^{2+}, Fe^{2}. Using p-nitrophenyl-β-D-glucopyranoside (pNPG) as the substrate, recombinant enzyme showed Vmax of 6.72 μmol min^{-1} mg^{-1} and Km of 0.16 mM (Benoliel *et al*., 2010). Cellobiose dehydrogenases (CDH) of *S. thermophile* and *H. insolens* (Canevascini *et al*., 1991; Coudray *et al*., 1982; Schou *et al*., 1998) were cloned and expressed. The recombinant enzymes had molecular masses of 92 and 95 kDa. The optimum pH and temperature for the activity of *H. insolens* was 7.5 and 60 ˚C. For that of *S. thermophile*, optimal pH was 4.0 (Schou *et al*., 1998; Subramaniam *et al*., 1999; Igarashi *et al*., 1999).

Structure and sequencing analysis of cellobiose dehydrogenase of *S. thermophile* showed that the enzyme had 3 different domains: catalytic domain with ketone end, a middle heme domain and cellulose binding domain of cellulase (Igarashi *et al*., 1999). Glycosylation was dependent on the strain and culture conditions (Bernstein *et al*., 1977). Notably, once a gene coding for cellulase from *T. emersonii* was expressed in *T. reesei*, enzyme contained large number of N- glycosylation cites in the enzyme active site (Basha and Palanivelu, 1998). Murray *et al*. (2004) isolated cel3a from *Talalaromyces emersonii* and expressed in *Trichoderma reesei* that showed high thermal stability ($T_{1/2}$ of 62 min at 65˚C). Enzyme had 71.5 ˚C as the optimal temperature with V_{max} of 512 1U/ mg and K_i of 0.254 mM. *Pichia pastoris* was used for the over expression of β-glucosidase from *Thermoascus aurnaticus* IFO9748 (Hong *et al*., 2007). The enzyme belongs to family GH3. Recombinant enzyme was highly thermostable and retained 70 % activity after 1 h exposure at 60˚C, but the optimum temperature for enzyme activity was 70 ˚C. Recombinant enzyme was also stable in a broad pH range from 3.0-8.0 with optimal activity at pH 5.0. Cellobiose was found as the best substrate for the enzyme (Hong *et al*., 2007). By using mathematical modelling, it was found that CBH II from *Humicola insolens* expressed in *S. cerevisiae* was an extremely stable cellulase with a relative molecular mass of 55 kDa (Heinzelman *et al*., 2009). A gene (TeEgl5A) from thermophilic mould *Talaromyces emersonii* encoding endo-1,4-β-glucanase was functionally expressed in *Pichia pastoris* (Wang *et al*., 2014). The recombinant enzyme exhibited optimal activity at 90˚C and pH 4.5 and was tolerant to a wide range of pH 1.0-10.0. The recombinant enzyme showed a grater resistance to metal ions and surfactants, and it was found that the enzyme displayed higher activity on substrates which had β-1,4-glycosidic bonds and β-1,3-glycosidic bonds such as laminarian, lichenan, CMC-Na, birchwood xylan and barley β-glucan. The enzyme displayed a higher efficiency in reducing medium viscosity than the commercial enzyme Ultraflo XL from novozyme (Wang *et al*., 2014).

Another cellulase enzyme gene form *Humicola insolens* Y1 (Hicel6C) was expressed in *P. pastoris* (Xu *et al*., 2015). The recombinant enzyme had optimal activity at neutral pH and 70˚C. The enzyme was different from those of thermophilic moulds, being alkali- tolerant retaining more than 98.0, 61.2 and 27.6 % of activity at pH 8.0, 9.0, and 10.0, respectively. By using β-glucan as a substrate, the enzyme had 1.29 mg/ml and 752 μmol/min/mg as K_m and V_{max} values, respectively. HiCel6C enzyme was specific to degrade β-1,4-glycosidic bonds and showed high enzyme activity using β-glucan, CMC-Na, lichenan as compared to laminarin (1, 3-β-glucan). It was also found that HiCel6C showed capacity to degrade the glycosidic linkages interior of cellooligosaccrides, thus a good endo-cleaving catalyst in nature (Xu *et al*., 2015). Cellobiohydrolase II (CBH II) of *Chaetomium thermophilum* CT2 was expressed in *P. pastoris* under the inducible AOXI promoter and produced 1.2 mg/ml of pure enzyme (Liu *et al*., 2005). The recombinant enzyme was purified using salt precipitation and ion exchange quick flow chromatography. The molecular mass determination was done by using SDS-PAGE and it was exactly the same as the native cellobiohydrolase II from *C. thermophilum* CT2 (67kDa). The enzyme was optimally active at 50˚C and pH 4.0 5.0. Enzyme was thermostable that retained 50% of the original activity for 30 min at 70˚C. The structure analysis of cellobiohydrolase IB of the fungus *T. emersonii* showed that this enzyme was a oversized glycoprotein with single domain and βsandwich structure that belongs to family GH7 (Grassick *et al*., 2004).

APPLICATIONS OF CELLULASES OF THERMOPHILIC MOULDS

The enzyme industry has developed very rapidly in the last few decades. The total estimated market value of the enzyme industry was $ 5.0 billion in 2015, which is expected to increase to $ 6.3 billion in 2021 at a compound annual growth rate (CAGR) of 4.7% for 2016-2021 [http://www.bccresearch.com]. Cellulases (~ 15 %) also have a great impact on enzyme industry and it occupies third important enzyme in industrial applications. The other two important enzymes are amylase (~ 25%) and protease (~ 18 %). Microorganisms are the important sources of cellulases (Bhat, 2000; Kuhad *et al*., 2010). Cellulase production from thermophilic moulds received attention because of their utilization of all types of substrates mainly lignocellulosic biomass (Kaur and Satyanaryana, 2004; Kuhad *et al*., 2010). Now-a-days, energy production by using agricultural residues is gaining attention of many industries as it is a cheap and renewable. Use of inexhaustible energy source is encouraged because these sources can generate 10-14% world's energy supply. Apart from various chemicals and physical methods used for degradation of cellulosic biomass,

the use of enzymes of thermophilic microorganisms is being encouraged. The enzyme-based degradation of biomass finds applications in various industries (**Table 3**) [Bergquist *et al*., 2004; Bamforth, 2009; Kuhad *et al*., 2010].

Table 3. Potential biotechnological applications of cellulolytic enzymes of thermophilic moulds

Industry	Applications	References
Agriculture	Plant pathogen and disease control, enhanced seed germination, rapid plant growth and flowering, increased crop yields, reduced dependence on mineral fertilizers, preservation of high quality fodder.	Bhat, 2000; Sukumaran *et al*., 2005; Singh *et al*., 2007; Karmakar and Ray 2011
Biofuels	Bioethanol production, production of fuel oxygenates, bio-butanol production, H_2 production, conversion of cellulosic materials to ethanol, other solvents, organic acids, and lipids.	Sukumaran *et al*., 2005; Kuhad *et al* ., 2011
Detergent	Cellulase-based detergents, superior cleaning action without damaging fibers. Removal of rough protuberances in cotton fabrics, improved colour brightness and dirt removal, anti- redeposition of ink particles	Sukumaran *et al*., 2005; Singh *et al*., 2007; Karmakar and Ray 2011
Fermentation	Production of the wine, beer and liquor, activity of cellulase in composition with α-amylase and glucoamylase has been selected to achieve a higher ethanol yield in the distillate, by decreasing the concentrations of methanol, propanol, isobutanol and isoamyl & amyl alcohols concentration, improved malting and mashing; improved pressing and color extraction of grapes; improved aroma of wines; improved primary fermentation and quality of beer; improved viscosity and filterability of wort; improved must clarification in wine production; improved filtration rate and wine stability	Bhat, 2000; Sukumaran *et al*., 2005; Kuhad *et al*., 2011
Food and feed processes	Pretreatment of agricultural silage and grain feed, enhancement of the digestibility of grasses, improvement of yields in starch and protein extraction, decreased viscosity of the nectars from fruits, used in the improvement of the bakery products, improved feed digestion and absorption, increases the nutritive value of the feed, release of the antioxidants from fruit and vegetable pomace, clarification of fruit juices, improved texture, aroma and flavour, improved nutritional quality of animal feed of the fruit juices, improved texture and quality of bakery products, production of single cell protein	Rai *et al*., 2007; Karmakar and Ray, 2011
Textiles and Biopolymers synthesis	Mercerization of cotton, wool scouring, biostoning of jeans, biopolishing of textile fibers; pretreatment of bast fibers including jute, flax and ramie, softening of garments; improved stability of cellulosic fabrics; removal of excess dye from fabrics; restoration of colour brightness	Kaur et al., 2004; Kuhad et al., 2011
Agro and plants waste treatment	Application in biogas production unit, reduction of agricultural and municipal solid waste residues, production of the fermentable sugars for biofuels and useful products, reduce environmental production, bioconversion of lignocelluloses.	Karmakar and Ray, 2011; Kuhad et al., 2011

Food industry: Cellulases occupy a place in food industry. Cellulases are used as macerating enzymes along with xylanases and pectinases for the clarification and improved extraction of juices from fruits and vegetables. The stability of juices also improves when macerating enzymes are used for the extraction along with higher yields of juices (Kaur *et al*., 2004). Kaur *et al*. (2004) studied the effect of mixture of pectinase, cellulase, xylanase enzymes from *S. thermophile* on the yield of various fruit juices such as banana, grape and apple and found that the yields of juices increased as compared to when pectinase was used alone for extraction (Kaur *et al*., 2004). Cellulases, xylanases and pectinases are used in food biotechnology for improving the properties of fruits and vegetables (Bhat, 2000). The enzymes are also used to improve the texture of nectars and purees from tropical fruits (mango, pears, apples, apricot, papaya, peach and plum) [Sukumaran *et al*., 2005; Bhat, 2000]. Citrus fruit properties such as texture, flavour, and aroma of fruits were also improved by using enzymes such as pectinases and β-glucosidases (Rai *et al*., 2007). Use of cellulase enzymes decreased the viscosity of nectars and purees rapidly as compared to other methods (Grassin *et al*., 1996). In the present scenario, the use of cellulases in food industry is increasing along with other macerating enzymes as they can be used for all type of fruits and vegetables without any significant loss of yields and improvement in the digestion of animals (Dourado *et al*., 2002).

Animal feed industry: The use of cellulases and hemicellulases in feed industry draw considerable attention of researchers as they have potential to improve the feed value of animal diets, thus, leading to improved efficiency of animals (Dhiman *et al*., 2002). Pre-treatment of agricultural residues, which are used as animal feeds, led to improvement in digestibility (Godfrey and West, 1996). The enzymes not only improved the feed value but also eliminated the anti-nutritional factors. Dietary fibre consists of various non-starch poylsaccharides such as inulin, lignin, dextrins, β-glucan, waxes and oligosaccharides (Dhiman *et al*., 2002). By the action of cellulases, anti-nutritional factors are removed leading to improvement in digestion and health of animals. Cellulases not only improve the silage production but also increase the digestibility of grasses. Cellulases can be used to improve the digestibility of silage production for cattle feeding making the availability of total digestible nutrients and energy with low water-soluble carbohydrates (Lewis *et al*., 1996). Enzyme cocktail comprising of cellulase, hemicellulase, and pectinase have been used in improving the nutritional quality of the forages (Lewis *et al*., 1996). Both cellulases and hemicellulases are responsible for dehulling of cereal grains, partial hydrolysis of lignocellulosic biomass, -glucans hydrolysis and better emulsification of feed materials and thus, improving the nutritional value of animal feeds (Cowan, 1996).

Biofuels: World faced a worldwide oil crisis in 1970s that led to increased attention on the use of cellulases in bio-fuel industry. The enzyme is used to degrade the cellulosic wastes into fermentable sugars, which are fermented to bio-ethanol. The purpose of this process is to reduce the burden of oil industry by reducing the import of oil. This will improve the quality of air by reducing the emission of gases in the air. Bioethanol is the most common and eco-friendly renewable fuel produced from lignocelluosic biomass by thermostable enzymes of thermophilic moulds by simultaneous liquefaction, saccharification and fermentation (Lynd *et al.,* 2005). For efficient bioconversion of lignocellulosic biomass, a strategy of efficient saccharification using cellulolytic enzymes is required. Thermostability is an important and desirable feature for cellulases as the saccharification of cellulose is faster at higher temperatures. Mesophilic *Trichoderma* has high endo and exoglucanase activities with lower β-glucosidase levels, therefore, it has limited utility in the hydrolysis of cellulose. Therefore, thermophiles could be suitable substitutes for this fungus. Thermophilic moulds such as *Sporotrichum thermophile* (Kaur *et al*., 2004; Berka *et al*., 2011; Singh 2016; Singh *et al.,* 2016)*, Thermoascus aurantiacus* (Gomes *et al*., 2000; Jain *et al*., 2015) *Scytalidium thermophillum* and *Thielavia terrestris* (Berka *et al*., 2011) have efficient enzymatic machinery for the hydrolysis of lignocellulosic materials. These microbes have, therefore, been proposed as good candidates for the conversion of lignocellulosic residues to sugars, therefore, have a great potential for applicability in bioethanol industries (Berka *et al*., 2011). The crude enzymes from *S. thermophile* BJAMDU5 resulted in the hydrolysis of waste tea cup paper and rice straw with a sugar yield of 578.12 and 421.79 mg/g substrate, respectively (Bala and Singh, 2016). *Thermoascus aurantiacus* and *Thielavia terrestris* were cultivated on various substrates for producing glycoside hydrolases (McClendon *et al*., (2012). Crude culture filtrates used in the saccharification of ionic liquid pre-treated switch grass (*Panicum virgatum*) revealed that *T. aurantiacus* enzymes released more sugars (glucose) than *T. terrestris* enzymes (McClendon *et al*., 2012).

Textiles industry: Cellulases are utilized in textile wet process, particularly in finishing of cellulose-based textiles,

with the goal of improved look (Hebeish and Abraham, 2007; Karmakar and Ray, 2011). Normally the thermophilic cellulases are utilized in the stone laundry of jeans to make them look light and in biopolishing of cotton (Kuhad *et al.,* 2011). These enzymes are also mixed with detergents for reducing the discolouration and fuzzing effects resulted by repeated washing (Kuhad *et al.*, 2011). Due to recurrent laundry, most cotton blended clothes tend to become flossy and less attractive, therefore, cellulases present in these detergents will take away these microfibrils and restore a sleek surface and original color to the clothes. Use of cellulases for treatment of raw cotton fibers i.e. non plain-woven materials improved the texture of fibres (Singh *et al.*, 2007). Use of cellulases together with lipase within the detergents could be a newer innovation in the field (Singh *et al.*, 2007). Cellulase preparations capable of modifying polyose fibrils will improve color brightness, feel, and dirt removal from the cotton mix clothes. The economic application of basic cellulases as a possible detergent additive is being actively pursued (Singh *et al*., 2007; Sukumaran *et al*., 2005). Nowadays, liquid detergents containing anionic or non-ionic wetting agent, acid or a soluble salt, protease, cellulase, and a mix of propanediol and boric acid or its by-product are used for improving the efficiency of cellulases. The cellulases are useful in the removal of rough protuberances in glossier and brighter-coloured materials (Karmakar and Ray, 2011).

Others: Various preparations consisting of mixtures of cellulases, hemicellulases, and pectinases have potential applications in agriculture for enhancing the growth of crops (Bhat, 2000). Historically straw incorporation is taken into account as a vital strategy to enhance soil quality and cut back dependence on mineral fertilizers (Ortiz Escobar and Hue, 2008, Tejada *et al.*, 2008). Microbial cellulases play a vital role in fermentation processes to supply alcoholic beverages together with beers and wines (Singh *et al.*, 2007). Macerating enzymes conjointly improve pressability, settling and juice yields of grapes used for wine fermentation. A variety of economic catalyst preparations are currently used in wine trade. Vital and consistent enhancements in grape pressability, subsidence rate, and total juice yield were achieved through a mixture of macerating enzymes. Such enhancements were noticeable solely with an accurate balance of pectinases, cellulases and hemicellulases. A variety of improved enzymes like cellulase and pectinase that might be exogenously added is expected to boost the productivity of the existing production processes in future (Bamforth, 2009).

CONCLUSIONS

Thermophilic moulds are known to secrete cellulolytic enzymes in submerged as well as solid state fermentations. Cellulases of thermophilic moulds have desirable properties, thus, better suited for industrial applications. Cellulases of thermophilic moulds have been cloned and expressed in different hosts and characterized. Cellulases have been utilized in saccharifying cellulosics, improving food and feed nutrition and other industries. Furthermore, the diversity of thermophilic moulds is less explored as compared to mesophiles. Further research efforts are therefore called for exploring the diversity of thermophilic moulds, studying structure - function aspects of cellulases and ameliorating catalytic activities of cellulases by site directed mutagenesis and directed evolution.

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