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Review

Thermostable cellulases: Current status and perspectives

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ABSTRACT

It is envisaged that the utilization of lignocellulosic biomass for ethanol production for transport sector, would make cellulases the most demanded industrial enzyme. The greatest potential of cellulolytic enzymes lies in ethanol production from biomass by enzymatic hydrolysis of cellulose but low thermostability and low titer of cellulase production resulting into high cost of the enzyme which is the major set-back. A number of research groups are working on cellulase to improve its thermostability so as to be able to perform hydrolysis at elevated temperatures which would eventually increase the efficiency of cellulose hydrolysis. The technologies developed from lignocellulosic biomass via cellulose hydrolysis promise environmental and economical sustainability in the long run along with non-dependence on nonrenewable energy source. This review deals with the important sources of thermostable cellulases, mechanism, its regulation, strategies to enhance the thermostability further with respect to its importance for biofuel applications.

1. Introduction

In recent years there has been remarkable apprehension about environmental safety which led to the green bioprocesses developments mainly to substitute most of the unsafe and inflated existing chemical processes. Hence, these initiatives raised the demand of enzymes in several industrial applications. Enzymes are biocatalyst and can be advantageous for industrial applications as they rarely require toxic metal ions for its function, hence creating the possibility to use more environmental friendly processing (Comfort et al., 2004).

Moreover, the requirement of enzymes that are more stable at elevated temperature, more active and substrate-specific enzymes are high in demand (Singh et al, 2017, Vaishnav et al, 2018). Thermostable enzymes offer robust catalyst alternatives as are able to withstand the often relatively harsh conditions of industrial processing. Use and development of molecular biology techniques, permitting genetic analysis and gene transfer for recombinant production, led to dramatically increased activities in the field of thermostable enzymes during the 1990's. This also stimulated isolation of a number of microbes from thermal environments in order to access enzymes that could significantly increase the window for enzymatic bioprocess operations.

At present time when the technology is moving at a fast pace towards biorefinery, the enzymes plays an utmost important role in bioconversion of biomass. There is a renewed interest in commercial utilization of lignocellulosic biomass which is considered to be the only foreseeable source of energy (Sukumaran et al., 2005, Lynd et al., 2002), and it has been projected based on a carbohydrate-based economy that the future of mankind would be highly dependent on utilization of these biomass. Environmental concerns on utilizing fossil fuels and its depletion led to the development on alternative and renewable sources of energy. Lignocellulosic biomass can be utilized to generate bioethanol via enzymatic route to be utilized as transportation fuel.

Cellulases are the second largest industrial enzyme by dollar volume which is going on increasing with the increased demand for various industrial applications such as detergent industry, textile industry, paper processing industry, animal feed industry and fruit juice industry. Though there are several potential industrial applications of cellulases, but the importance of lignocellulosic ethanol has brought cellulases in the main frontier (Sukumaran et al., 2005, Singhania et al., 2010). It is envisaged that cellulases may become the largest volume industrial enzyme if ethanol from lignocellulosic biomass through enzymatic route becomes a major transportation fuel. The commercial potential of using cellulases lies in its efficiency of converting lignocellulosic biomass into glucose through enzymatic hydrolysis which can be utilized to generate a number of value added products such as ethanol.

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Cellulose (β-1,4-linked glucose units) is the most abundant polysaccharide on the biosphere representing most promising raw material for bioconversion with the average annual production estimated at 4×10^9 tons (Yin et al., 2010). Only limited numbers of organisms have the ability to carry out its complete decomposition due to the recalcitrance of its glycosidic linkages (Lynd et al., 2002, Rastogi et al., 2010) requiring a complex enzyme cellulase, consisting of different components acting in a synergistic manner so as to hydrolyze cellulose completely.

2. Mode of action

Microbial cellulase systems can be regarded into complexed or non-complexed. Hydrolysis of insoluble cellulose mainly requires the extracellular cellulases secretion by the organism. The cellulase systems comprised of either extracellular or cell-linked enzymes and they are belonging to different classes or categorized based on their structural characteristics and mode of actions.

Cellulases are hydrolyzing enzymes hydrolyze the β-1, 4-D-glucan bonds in cellulose to yield glucose, cellobiose and oligosaccharides. Several microorganisms are known as potential producers of cellulases. Cellulases have defined based on substrate specificity and structure in different enzyme classifications. However, mainly three types of cellulases are involved in the hydrolysis of native cellulose for example, cellobiohydrolase (CBH), endo-β-1, 4-glucanase (EG) and β-glucosidase (BGL) (Schulein, 1988). There are numerous enzymes within these classifications; however, the most studied fungus for cellulase production *Trichoderma reesei* produces two CBH components, eight EG components and seven β-glucosidases (Aro et al., 2005). This is the one which is most studied multiple-enzyme-complex comprising together endoglucanase (EG), cellobiohydrolase (CBH) and β-glucosidases (BGL). For effective and accomplished cellulose hydrolysis, synergistic action of these three cellulase components is essential. EG forms nicks in the cellulose polymer thus opening the reducing and non-reducing ends. Moreover, cellobiohydrolase (CBH) acts on these reducing and non-reducing ends to release cellobiose and cello-oligosaccharide units, whereas β-glucosidases (BGL) ultimately chops the cellobiose to free the glucose and completing the hydrolysis (Sukumaran et al., 2005). A complete cellulase system containing EG, CBH and BGL components, therefore acts synergistically to convert crystalline cellulose into glucose.

Most of the cellulases exhibit a specific two domain structure, (1) catalytic domain (CD) and (2) cellulose-binding domain (CBD) or carbohydrate binding module (CBM) which is usually connected through a peptide linker (Ohmiya et al., 1997, Sakka et al., 2000). The catalytic domain comprises the catalytic site whereas the CBDs domain contribute in binding to cellulose.

3. Thermostable cellulases for biofuel applications

Generally, the enzymatic hydrolysis of cellulose occurs at 40–50° which is usually regarded as slow rates of hydrolysis and are characterized with low yield of sugars with incomplete hydrolysis and are sensitive to microbial contamination. These limitations could be resolved by using thermostable enzymes produced by thermophilic/thermotolerant microorganism. Thermostable cellulases have the number of commercial applications, as the paper processing industries; which are always interested in such type of cellulases that can withstand higher temperatures. In addition, one of the most important applications of thermostable cellulase is in the bioconversion of cellulosic biomass into the fermentable sugars for biofuels production at elevated temperature, due to the fact that cellulose swells well at higher temperature thereby supporting the higher reaction rate which definitely would cause economic utilization of cellulase. Thus, thermophilic cellulases are desirable in such applications since their activity at higher temperatures offer several benefits such as reduced hydrolysis times (Viikari et al., 2007), decreased risk of contamination (Abdel Benat et al., 2010), facilitated recovery of volatile products such as ethanol (Taylor et al., 2009), and lower costs for cooling after thermal pretreatment (Turner et al., 2007, Yeoman et al., 2010). All above benefits result in increasing the economic utilization of cellulases; which is one of the major costs adding component to the bioethanol technology (Singhania et al., 2010, Vaishnav et al., 2018).

Thermostable enzymes from thermophillic microorganisms have attracted attention from researchers since last three decades; however, the interest in thermophiles and the way proteins are able to function at elevated temperatures actually started as early as in the 1960's (Brock and Freeze, 1969). Microorganisms that have optimal growth temperature as above 55 °C are regarded as thermophiles (Brock, 1986). It is dominated by bacterial species and only few eukaryotes are known to grow above this temperature, as some fungi grow in the temperature range 50–55 °C (Maheshwari et al., 2000). Further the microorganisms that can grow at and above 80 °C are known as hyperthermophiles (Kristjansson and Stetter, 1992). Hyperthermophilic species are dominated by archaea with few exceptions such as *Thermotoga* and *Aquifex* (Stetter, 1996).

3.1. Thermostable cellulase from bacteria

Cellulases are usually more stable than general enzymes in functioning at relatively high temperatures (Chang et al., 2016). Both fungi and bacterial both have been exploited for cellulase production. Till last few years, the emphasis was placed only on fungal cellulases because of its ability to secrete large amount of less complex cellulases extracellularly. It was cloned and produced via recombinant DNA technology in a rapidly growing bacterial host. However, recently the shift has been observed towards the bacterial cellulases, because of robust bacterial growth, its capability of survival in harsh conditions of bioconversion processes, stability and presence of multi-enzyme complexes which provides increased function and synergy.

In general, two types of cellulase systems exists: one type consists of extracellular cellulases in filamentous fungi and in aerobic bacteria that act synergistically to degrade cellulose, while the second type is an enzyme complex called the "cellulosome," in anaerobic bacteria such as *Clostridium thermocellum* which consists of a non-enzymatic scaffolding protein associated with various enzymatic subunits that act synergistically to degrade cellulose and hemicelluloses (Mathew et al., 2008). Non-complexed cellulase systems are more common and are currently most exploited for industrial applications. However, the hydrolysis of cellulose by the bacteria (bacterial cellulase) has several features such as: more stability, increased specific activity and facilitated mass transfer (Viikari et al., 2007, Maki et al., 2009, Rastogi et al., 2010). Therefore, thermophilic cellulose-degrading bacteria have been isolated from various environments such as soil (Abdel-Fattah et al., 2007, Lee et al., 2008, Assareh et al., 2012), compost systems (Lu et al., 2005, Mayendea et al., 2006, Ng et al., 2009) and hot springs (Zhao et al., 2015; Potprommanee et al., 2017). Thermophilic bacteria belonging to the strains *Bacillus*, *Geobacillus*, *Caldibacillus*, *Acidothermus*, *Caldocellum* and *Clostridium* are known to produce thermostable cellulases as given in Table 1 (Rastogi et al., 2010, Zambare et al., 2011).

3.2. Thermostable cellulase from fungi

A number of thermophilic fungi have been isolated and studied in recent years such *Acremonium thermophilum*, *Chaetomium thermophilum*, *Humicola grisea*, *Humicola insolens*, *Melanocarpus albomyces*, *Talaromyces emersonii*, etc., and the cellulases produced from these fungi have been purified and characterized at both structural and functional level. Purified thermophilic fungal cellulases have been characterized in terms of their molecular weight, optimal pH, optimal temperature, thermostability, and glycosylation. The molecular weight of thermophilic fungal cellulase exhibits a wide range (30–250 kDa) with

Table 1
Showing different thermostable cellulases from various microorganisms.

Microorganisms	Name of enzyme	Thermostability at temperature	References
Bacillus	BsCel5A	70% activity at 75 °C for 30 min or even less	Santos et al. (2012)
Geobacillus sp. 70PC53	GsCelA	70% activity at 75 °C after 4 h	Ng et al. (2009)
Geobacillus sp. HTA426	CMCase	Stable at 50–70 °C for 5 h	Potprommanee et al. (2017)
Bacillus sp. SR22	Endoglucanase, Bc22Cel	30% of the activity retained at 80 °C at high salt molarity (1.5 M NaCl)	Dos Santos et al. (2018)
Acremonium hermophilum	Cel7a	Optimal pH 60 °C	Voutilainen et al. (2008)
Chaetomium thermophilum	Cel7a	Optimal pH 65 °C	Voutilainen et al. (2008)
Humicola grisea	Egl2	80% residual activity for 10 min at 75 °C	Takashima et al. (1999)
Chaetomium thermophilum	Cbh3	Half-life period 45 min at 70 °C	Li et al. (2009)
Humicola grisea	*egl3*	75% residual activity for10 min at 80 °C	Takashima et al. (1999)
Humicola grisea	*egl4*	75% residual activity for10 min at 80 °C	Takashima et al. (1999)
Humicola grisea var *thermoidea*	*Egl* and cbh1	Stable for 10 min at 60 °C and 55 °C respectively	Takashima et al. (1996)
Humicola insolens	*cbhII*	*T*1/2: 95 min at 63 °C	Heinzelman et al. (2009a,b)
Talaromyces emersonii	*cel3a*	*T*1/2: 62 min at 65 °C	Murray et al. (2004)
Talaromyces emersonii	*cel7*	*T*1/2: 68 min at 80 °C	Grassick et al. (2007)
Talaromyces emersonii	*cel7A*	*T*1/2: 30 min at 70 °C	Voutilainen et al. (2010)
Thermoascus aurantiacus	*Cbh1*	80% residual activity for 60 min at 65 °C	Hong et al. (2003a)
Thermoascus aurantiacus	*egl*	stable for 60 min at 70 °C	Hong et al. (2003b)
Thermoascus aurantiacus	*bgl1*	70% residual activity for 60 min at 60 °C	Hong et al. (2007)

carbohydrate contents ng between 2 and 50%. Usually, thermophilic fungal cellulase single polypeptides although it has been reported that some beta-glucosidases are dimeric (Mamma et al., 2004). Optimal pH and temperature are similar for the majority of the purified cellulases from thermophilic fungi which are quite similar to cellulases produced from mesophilic fungi too. Thermophilic fungal cellulases are active in the pH range 4.0–7.0 and exhibit maximum activity at 50–80 °C (Table 1). In addition, they exhibit remarkable thermal stability and are stable at 60 °C with longer half-lives at 70, 80, and 90 °C than those from other mesophilic fungi. The structural characteristics revealing the increased stability of thermophilic proteins have been studied more extensively in thermophilic bacteria and hyperthermophilic archaea (Pack and Yoo, 2004, Trivedi et al., 2006) in comparison to fungi. Hence, the understanding of the nature and mechanism of thermostability of proteins from thermophilic fungi is relatively poor in comparison with thermophilic proteins from thermophilic bacteria and hyperthermophilic archaea. Till date there is no common set of determinants for protein thermostability established so far and hence it has been proposed that there is more than one contributor responsible for protein thermostability. A recent analysis suggested that an increase in ion pairs on the protein surface and a stronger hydrophobic interior are the major factors supporting increased thermostability in proteins (Taylor and Vaisman, 2010). Hence, further characterization of amino acid residues and its role in thermostability is necessary for comprehensive understanding thermostability of cellulases in thermophilic fungi.

3.3. Thermostable cellulase from metagenome

The traditional approach used to identify and exploit these enzymes is culture-dependent, which greatly restricts the accessible diversity of thermophile-derived natural products. The relatively small percentage of microorganisms that can be readily cultured under laboratory conditions has led to the development of novel culture methods and the adoption of culture-independent methods (Ferrer et al., 2007, Lewin et al., 2013). However, the application of novel cultivation strategies is slow and will not enable cultivation of the extant diversity of microbial genomes from extreme environments, necessitating new approaches to exploit natural products from as-yet uncultured microbes for industrial application (Daniel, 2004). The use of a culture-independent metagenomics approach permits access to microbial genomes and their biologically active molecules through isolation of DNA from environmental microbes followed by direct sequencing or cloning DNA to generate a metagenomics library (Handelsman, 2004, Banik and Brady, 2010). The library can then be screened by both sequence-based and function-based methods for natural product discovery (Delmont et al., 2011, Milshteyn et al., 2014).

Even though several methodological biases have been recognized, a metagenomic approach has shown to be effective in discovery of enzymes with novel activities (Wilson and Piel, 2013; Milshteyn et al., 2014). The first published example of a functional metagenomic approach for enzyme discovery was the cloning of cellulases from "zoolibraries" (Healy et al., 1995). Hereafter, metagenomics approaches have been continuously used to discover many novel carbohydrate-active enzymes (CAZymes) from soil (Jiang et al., 2009; Nacke et al., 2012), cow rumens (Pope et al., 2010), sediments (Klippel et al.,2014), biochemical reactors (Mewis et al., 2013) and aquatic environments (Rebuffet et al., 2011, Martin et al., 2014). It has been hypothesized that carbohydrate-degrading enzymes would be encoded in the metagenomes of microorganisms populating oil reservoirs as it is given in the history that petroleum reserves are formed from phytoplankton and would have evolved enhanced thermal stability (Tissot and Welte, 1978). Identification of novel thermostable cellulases could be a vital step in improving economic feasibility of cellulosic biofuel production because processes such as simultaneous saccharification and fermentation (SSF) or separated hydrolysis and fermentation (SHF) are high temperature operations, which necessitates thermostable cellulase. Lewin et al. (2014) revealed that an oil reservoir microbial assemblage harbored novel metagenomic diversity and could be explored for thermostable cellulases and other CAZymes using a combination of function-and sequence-based methods, demonstrating the strength of hybrid screening approaches. A novel thermostable archaeal cellulases that are stable up to at least 80 °C was obtained. One of the cellulase candidates, was demonstrated to be a multi-module, thermostable archaeal enzyme with high activity on different cellulose substrates, producing cellobiose and glucose in a single enzyme reaction (Lewin et al., 2014). Jensen et al. (2018) explored a thermostable enzyme for cellulose processing from the metagenome of a thermostable microbial community derived from rice straw inoculated with compost and incubated at 55 °C. This led them to express and characterize a 45 kDa two domain thermostable bacterial cellulase comprised of a GH6 domain and a C-terminal CBM2domain. They presented the functional and structural characteristics of this enzyme, calledmgCel6A, and assess its potential for use in high-temperature industrial degradation of sulfite pulped lignocellulosic biomass (Norway spruce). Hence with the advent of novel techniques in metagenomic, this approach can be exploited to obtain a potent thermostable cellulase for industrial application.

4. Structure of thermostable cellulase

Modular structure is a common characteristic of non-complex cellulases. Typically, cello biohydrolases and endocellulases are composed of four regions as signal peptide that mediates secretion, a cellulose-binding domain (CBD) for anchorage to the substrate, a hinge region (linker) rich in Ser, Thr and Pro residues, and a catalytic domain (CD) responsible for the hydrolysis of the substrate. The mature proteins are *O*- and *N*-glycosylated in the hinge region and the CDs, respectively. *T. emersonii* CBHII is characterized by a modular structure (Murray et al., 2003) whereas CBH1 from the same fungus consists solely of a catalytic domain (Grassick et al., 2004). Similarly, *Chaetomium thermophilum* CBH1 and CBH2 consist of a typical CBD, a linker, and a catalytic domain. In contrast, CBH3 only comprises a catalytic domain and lacks a CBD and a hinge region (Li et al., 2009). Thus, variations between cellulases within the same mechanistic class have been observed. Fungal CBDs are composed of less than 40 amino acid residues which interact with cellulose through a flat or platform-like hydrophobic binding site formed by three conserved aromatic residues. The binding site is thought to be complementary to the flat surfaces presented by cellulose crystals (Hashimoto, 2006, Shoseyov et al., 2006). The (110) faces of the cellulose crystalline micro-fibrils have been proposed as the putative CBD binding site (Dagel et al., 2011). This arrangement enables the gluco-pyranoside rings of cellulose to be fully exposed and available for hydrophobic interactions. For the efficient hydrolysis of crystalline cellulose by these enzymes, the tight binding to cellulose mediated by the CBD is necessary which was demonstrated by deletion of the CBDs from *T. reesei* Cel7A and Cel6A and *H. grisea*CBH1 greatly reducing enzymatic activity toward crystalline cellulose (Takashima et al., 1998). Substitution of the three conserved aromatic residues (W494, W520, and, Y521) in *H. grisea* CBH1 CBD with other amino acids (G, F or W) has demonstrated the importance of these residues in the interdependency of high activity of *H. grisea* CBH1 on crystalline cellulose and high cellulose-binding ability (Takashima et al., 2007). Several thermophilic fungal cellulases have been studied for its 3D structures. Li et al. (2011) has reviewed different 3D structures of thermophilic cellulases belonging to different glycosyl hydrolase classes such as 5, 6, 7, 12 and 45.

5. Strategies for improved thermostability

Even though the thermostable cellulases are available, the prospective to increase their thermostability further would be advantageous for industrial applications. Mutation is an accepted strategy for changes to be made at genetic level. However, the screening of large number of mutants is tedious, it is still most accepted method for improvement. Improvement of *M. albomyces* Cel7B has been pursued by error-prone PCR, and 49 positive mutant clones were screened from 14,600 random clones by arobotic high-throughput thermostability screening method (Voutilainen et al., 2007). Two positive thermostable mutants, Ala30Thr and Ser290Thr, showed improvements in unfolding temperatures (*T*m) by 1.5 and 3.5 °C, respectively. In addition, the optimum temperature on a soluble substrate for the Ala30Thr mutant was improved by 5 °C. The amino acid alterations are located in the β-strands furthest away from the active site tunnel of the Cel7B enzyme, which could improve protein packing. Hence, random mutagenesis was employed as successful strategy for improving thermostability, however as mentioned earlier too, the screening is a challenge. Recently, Cel7A cellobiohydrolase from the thermophilic fungus *T. emersonii* was engineered using rational mutagenesis to improve its thermostability and activity (Voutilainen et al., 2010). Additional disulfide bridges were introduced into the catalytic module of Cel7A. Three mutants demonstrated improved thermostability reflected by an improvement in avicel hydrolysis efficiency at 75 °C. Structural analysis of *H. grisea* Cel12A, a thermostable endoglucanase, has revealed three unusual free cysteines in the enzyme: Cys175, Cys206, and Cys216. Subsequently, the following Cel12A mutants were constructed by site-directed mutagenesis: Cys175Gly, Cys206Pro, and Cys216Val. It was found that the three free cysteines play a significant role in modulating the stability of the enzyme (Sandgren et al., 2005). More specifically, mutation of Cys206 to Pro and Cys216 to Val caused a reduction in the *T*m of 9.1 and 5.5 °C, respectively, compared to the wild-type enzyme. Moreover, when the free Cys175was mutated to a Gly, the *T*m of the enzyme was increased by 1.3 °C. It has been reported that endoglucanases are characterized by variations in amino acid compositions resulting in fold-specific thermostability (Yennamalli et al., 2011) thus providing new strategies for improvement of thermostability.

SCHEMA uses protein structure data to generate new purpose-specific sequences that minimize structure disruption when they are recombined in chimeric proteins. Chang et al. (2016) have shown the probable mechanism of thermostability by comparing two cellulases each from *Bacillus* and *Geobacillus* bacteria. These cellulases are characterized by unique thermostability and are potentially useful in the biofuel and animal feed industries. The group has identified a cellulase, GsCelA, from thermophilic *Geobacillus* sp. 70PC53, which is much more thermostable than its *Bacillus* homolog, BsCel5A. Thus, these two cellulases provide a pair of structures ideal for investigating the mechanism regarding how these cellulases can retain activity at high temperature. Chang et al. (2016), have applied the SCHEMA non-contiguous recombination algorithm as a novel tool, which assigns protein sequences into blocks for domain swapping in a way that lessens structural disruption, to generate a set of chimeric proteins derived from the recombination of GsCelA and BsCel5A. Analyzing the activity and thermostability of this designed library set, which requires only a limited number of chimeras by SCHEMA calculations, revealed that one of the blocks may contribute to the higher thermostability of GsCelA. Hence, the thermostable chimeric cellulase containing this block when tested against swollen avicel showed significantly higher activity (22–43%) and higher thermostability compared to the parental enzymes. With further structural determinations and mutagenesis analyses, a 310 helix was identified as being responsible for the improved thermostability of this block. It was also found that in the presence of ionic calcium and crown ether (CR), the chimeric cellulase retained 40% residual activity even after heat treatment at 90 °C. Thus combining crystal structure determinations and structure-guided SCHEMA recombination, the mechanism responsible for the high thermostability enzymes could be determined and a novel recombinant enzyme with significantly higher activity could be generated. SCHEMA has been employed to create thermostable fungal cellulases (Heinzelman et al., 2009a,b). The high resolution of *H. insolens* CBHII as a template for SCHEMA yielded a collection of highly thermostable CBHII chimeras. Using the computer-generated sequences, a total of 31 new cellulase genes were synthesized and expressed in *Saccharomyces cerevisiae*; each of these cellulases was found to be more stable than the most stable parent cellulase from *H. insolens*, as measured either by half-life of inactivation at 63 °C or by *T*1/2. These findings demonstrated the value of using structure-guided recombination to discover important sequence-function relationships for efficient generation of highly stable cellulases Chang et al. (2016).

6. Mechanism of thermostability

In *Sulfolobus solfataricus* a small DNA binding protein, Sso7d, not only imparts thermostability to the DNA but also promotes the annealing of complementary strands above the melting point and the ATPase-dependent rescue of the aggregated proteins (Ciaramella et al., 2002). Thermophiles are reported to have a zigzag structure of surface layer proteins which are thermostable and resist denaturation and proteolysis as well (Kumar and Nussinov, 2001). Chaperones are produced by these organisms which help to refold the proteins to their native form and restore their functions (Laksanalamai and Robb, 2004; Singh et al., 2010). Besides the above strategies, thermophilic bacteria,

actinomycetes and archaea tolerate high temperatures by increased electrostatic, hydrophobic and disulfide interactions in their proteins (Ladenstein and Ren, 2006, Pebone et al., 2008). Certain thermophilic enzymes are stabilized by certain conformational changes (Fitter, 2003). However, certain metals, inorganic salts and substrate molecules are also reported to impart the thermostability (Vieille and Zeikus, 2001). Based on the thermal behavior of these enzymes, the Equilibrium Model has been described to reveal the effect of temperature on enzyme activity by reversible active-inactive transition states (Daniel et al., 2008). Due to the increasing demand of highly thermostable industrial enzymes, certain computational algorithms and bioinformatic tools have been designed, which can predict protein rigidity and stability. Protein stabilization can be carried out by site-directed mutagenesis, and gene shuffling (Hayashi et al., 2001). The GsCelA enzyme belongs to a particular group of *Geobacillus*. The recombinant GsCelA expressed in *E. coli* exhibited ten-fold greater specific activity than the commercially available endo-glucanase from *Trichoderma reesei* and uniquely retained its activity after long-term heating and low pH treatments (Ng et al., 2009). The amino acid sequence of GsCelA indicates it is a member of the glycoside hydrolase GH5 family of cellulases but shares only 53.1% similarity with other members in this group (Ng et al., 2009). In contrast to its full-length sequence, the catalytic core of GsCelA has 60% homology with that of BsCel5A from *Bacillus subtilis* 168. BsCel5A, another cellulase belonging to the GH5 enzymes, is the major endoglucanase in *Bacillus*. BsCel5A from different *Bacillus subtilis* strains have been cloned and characterized for their application in biofuel production (Meng et al., 2014, Santos et al., 2012). BsCel5A is also a thermostable enzyme, though it is not as tolerant at high temperatures as GsCelA, retaining 70% of its optimal activity after incubation at 75 °C for 30 min or less. A TIM-barrel (α/β)8 catalytic domain and a β-sheet cellulose binding module (CBM3) were shown to be present in the cellulase BsCel5A (Santos et al., 2012).

7. Regulation of thermostable cellulases

Regulation of cellulases in thermophilic fungi is quite similar to those of mesophilic fungi (Li et al., 2011) such as *Trichoderma reesei* in which the regulation has been studied in detail. On the other hand, the complexed cellulase systems for example cellulosomes are intrinsic to anaerobic bacteria. Cellulosomes usually bulges on the bacterial cell wall and abide stable enzyme complexes. Clostridial cellulolytic system has been thoroughly studied in the most comprehensively way in *C. thermocellum*. Cellulosome of *C. thermocellum* usually comprising a non-catalytic protein *cipA* with diverse catalytic modules and exhibit exo- and endo glucanase activities. The composition of the cellulosome differs with respect to microorganism. Fundamental understanding upon cellulase systems and their controlled regulation is vital for potential enzyme production and basic engineering strategies for best component combination to achieve maximum biomass hydrolysis.

Aerobic fungi and bacteria have mainly a non–complexed cellulase systems, in which all components of the cellulase system are excreted. Classic example of this type of cellulase system in reported in *Trichoderma reesei*. This fungus produces two exoglucanases marked as CBHI and CBHII; moreover, nearly eight endoglucanases named as EGI to EGVIII, and seven β-glucosidases-denoted as BGI to BGVII. In thermophilic fungi also multiple forms of cellulases are produced similar to mesophilic fungi (Maheshwari et al., 2000). *Humicola insolens* also known to have non-complexed cellulase system homologues to *T. reesei* and comprises as a minimum seven cellulases. *Thermobifida* bacterium also produces whole components of the cellulase system comprising exo- and endo-glucanases. For example, in *Humicola grisea* four CBH has been reported from family 7 whereas, in *Aspergillus niger*, a mesophilic fungus, two CBH are known. Observed multiplicity of cellulolytic enzymes (Soni and Soni, 2010) could be the result of genetic redundancy or may be the outcome of differential posttranslational and/or post-secretion processing (Maheshwari et al., 2000).

Cellulases are secreted as inducible enzymes thus the regulation of its production is exceptionally controlled by mechanisms of activation and repression. Cellulase genes of *T. reesei* are regulated extremely synchronized. Thermophilic fungi are also known to possess inducer/repressor system for cellulase regulation in which cellulases are induced by the presence of cellulose (Maheshwari et al., 2000). The secretion of cellulases are induced by the cellulose or other oligosaccharide products whereas it is suppressed with the enough availability of utilizable sugars (Sukumaran et al., 2005). Sophorose (disaccharide) is subsequently regarded to be the best possible inducer especially for the *Trichoderma* cellulase system, it was recommended that the inducer is formed via trans-glycosylation of basal expression of β-glucosidase. Likewise, oxidized products of cellulose hydrolysis such as cellobiose, δ-cellobiose-1-5 lactone etc. may also work as inducers of cellulase (Mandels et al., 1962). Another popular inducer utilized in commercial cellulase production is lactose, which is highly exploited as it is cheaper. However, mechanism of lactose for the cellulase induction is not fully understood, but it is anticipated that possibly levels of intracellular galactose-1-phosphate might be regulating the signaling for expression. Glucose mediated cellulase repression overrides the induction of cellulase system; however, de-repression might be carrying out via an induction mechanism occurred through glucose trans-glycosylation.

The promoter of typical cellulases mainly comprising binding sites not only for *CREI* catabolite repressor protein but also for transcriptional activators of cellulase expression proteins II (*ACE II*), also the CCAAT sequence which binds general transcriptional activator complexes denoted as '*HAP*' proteins. Moreover, *ACEII* binds to the *cbh1* promoters in *T. reesei*, hence it is assumed to regulate the *cbh1*, *cbh2*, *egl1*, and *egl2* expression. Likewise, *Ace1* gene also yields a transcription factor alike *ACEII* which has its binding sites at *cbh1* promoter; however, it performs as a cellulase repressor. Hypothetically, Glucose mediated repression of cellulase expression system in *T. reesei* is carrying out through *CRE1* catabolite repressor protein. Moreover, the promoter region of four genes in *T. reesei* such as *cbh1*, *cbh2*, *eg1* and *eg2* has *CRE1* binding sites demonstrating the controlled regulation of above genes via (carbon) catabolite repression. Just as *cre1* in *T. reesei* which is a regulatory gene sequence expressing a negatively affecting transcription factor inhibiting cellulase transcription in presence of glucose; CRE1 genes from two thermophilic fungi (*Talaromyces emersonii*, *Thermoascus aquaticus*) have also been reported. Though the full repertoire of transcription factors influencing cellulase gene expression in thermophilic fungi has not been described completely as in *T reesei*, but the potential regulatory element consensus sequences have been identified in the 5′ upstream regions of thermophilic fungal cellulase genes (Murray et al., 2003, Grassick et al., 2004, Collins et al., 2007, Pocas-Fonseca et al., 2000, Soni and Soni, 2010, Voutilainen et al., 2008).

Such information's offer better comprehension about biochemistry of cellulase for controlled gene regulation, however it is still uncertain how the related genes are being regulated and which transcriptional activator activate the promoter of cellulase. Though, considerable research in this area is being undertaken and anticipating that applied exploitation of the growing knowledge and interventions on genetics of cellulolytic microbes can improve cellulase production and related technology.

8. Heterologous expression

With the advent of genetic manipulation, it is possible to obtain thermostable enzymes from mesophilic host strain. The genes can be cloned and expressed into a host to give thermostable enzyme. Cellulases are glycosyl hydrolases classified into families 1, 3, 5, 6, 7, 8, 9, 10, 12, 16, 44, 45, 48, 51, and 61 (http://www.cazy.org/). Thermophilic fungal cellulases are found in families 1, 3, 5, 6, 7, 12, and 45 (Li et al., 2011). Based on literature, Li et al. (2011) gave a summary of

about 50 genes encoding thermostable fungal cellulases which were cloned, expressed and analyzed. Thus, the production technologies for thermostable cellulases would be similar to the existing one for mesophilic cellulase as the host are usually mesophilic fungi.

Cellulase genes from thermophilic fungi have been cloned and expressed successfully in host organisms such as *E. coli*, yeast and filamentous fungi. *T. reesei* was employed as host organism to express a gene encoding a beta-glucosidase of *T. emersonii* and as a result the recombinant secreted cellulase contained 17 potential N-glycosylation sites in its functionally active form (Murray et al., 2004). Majority of recombinant cellulase expressed in yeast and filamentous fungi are glycosylated (Takashima et al., 1999, Li et al., 2009). Importantly, the glycosylation of cellulases could contribute further to the improvement of their thermostability as it has been previously reported (Meldgaard and Svendsen, 1994). The extent of glycosylation depends on the culture condition and the type of strain as well (Mamma et al., 2004). However, extensive glycosylation in recombinant enzymes could lead to reduced activity and increased non-productive binding on cellulose (Jeoh et al., 2008). Hence, it is a trade-off and need to analyze the extent of glycosylation which is optimum for recombinant enzymes.

9. Conclusions and perspectives

Development of potential thermostable cellulases would play a major role in materializing the vision of eco-friendly lignocellulosic ethanol technology into a reality. Thermostability is the most desired trait of any industrial enzyme which allows fast rate of reaction at elevated temperature thereby decreasing the usage of enzyme. Although the commercial lignocellulosic ethanol production began in different parts of the world, still continuous research is desirable to improve thermostability of the cellulase and its production for improving cost, specific activity and substrate specificity to achieve better techno-economic feasibility. Thermostable cellulases from bacteria, fungal and even from metagenome would be useful. Successful attempts made towards bringing the desired changes in the protein appear promising for improving thermostability of cellulase significantly. Thermostable cellulase would allow to operate the process of hydrolysis at elevated temperature which would eventually led to increasing the rate or efficiency of hydrolysis of biomass resulting the economic feasibility of technology.

Thus, while remarkable developments have been made on cellulases research for improving technological potential and considerable successes have been claimed to be achieved, but the fact remains that still we do not have ‘efficient thermostable cellulases’ in the market for biomass hydrolysis. It signifies the need of long way to go on cellulases research.

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