International Journal of Recent Trends in Science And Technology, P-ISSN 2277-2812 E-ISSN 2249-8109
Special Issue, ACAEE: 2018 pp 17-25

Original Research Article

Cellulases for biofuel: A review

Ashiq Magrey[1*], Sanjay Sahay[2], Ragini Gothalwal[1]

[1]Department of Biotechnology, Barkatullah University, Bhopal, Madhya Pradesh, INDIA.
[2]NSCB Government P.G College, Biaora, Rajgarh, Madhya Pradesh, INDIA.
Email: ashiqmagrey@gmail.com

Abstract

Cellulases have wide applications in the biofuel industry. The three main components, namely endoglucanase, exoglucanase, and β-glucosidase effectively convert lignocellulosic biomass into fermentable sugar. The commercial production of cellulase is done using the submerged fermentation; however, it is costly and less economic for biofuels production. Moreover, microbial cellulase production process suffers from various bottlenecks. Because of the low cost, production of cellulase using solid-state fermentation by fungi is preferable. Cellulose is the main polymer in biomass and cellulases can hydrolyze it to cellobiose, which can be converted to glucose by β-glucosidase. Extensive research is being carried out to try to obtain cellulases with higher activity on pretreated biomass substrates by screening and sequencing new organisms, engineering cellulases with improved properties and by identifying proteins that can stimutate cellulases.. Despite extensive research on cellulases there are major gaps in our understanding of how they hydrolyze crystalline cellulose, act synergistically, and the role of carbohydrate binding modules. Therefore, the present review provides an overview on cellulase, main aspects of cellulase production and cellulase engineering for biofuels industry.

Key Words: Biofuels production, cellulase, commercial cellulase, fungal cellulase, solid-statefermentation, submerged fermentation,cellulase engineering.

[*]**Address for Correspondence:**
Ashiq Magrey, Department of Biotechnology, Barkatullah University, Bhopal, Madhya Pradesh, INDIA.
Email: ashiqmagrey@gmail.com

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Accessed Date: 26 March 2018

INTRODUCTION

Cellulase Enzymes: Enzymes are among the most important products obtained for human needs through Microbial sources. A large number of industrial processes in the areas of industrial, environmental and food biotechnology utilize enzymes at some stage or other. Current developments in biotechnology are yielding new applications for enzymes (Pandey *et al*., 1999). In the present techno- economic era, procurance of energy is one of the major problems which humanity is facing. All the waste cellulose is a source of food and is also a potential source of energy (Elder *et al.*, 1986). Mandels *et al.*, (1974) reported that the breakdown of cellulose into sugar can be achieved by acid hydrolysis as well as by enzymatic hydrolysis. But enzymatic hydrolysis is mostly preferred because it produces fewer by-products and proceeds under milder condition. Cellulases have been used and studied for most of the 20th century and are the most commercially important of all the enzyme families. The enzyme production is good by most of the fungi like *Aspergillus* and *Trichoderma* Sp. It has been reported that the structural complexity and rigidity of cellulosic substrates have given rise to a remarkable divergence in cellulose degradative enzymes. Microorganisms involved in degrading these complex structures have faced many evolutionary challenges, developing complex enzyme systems to handle these varying substrates. Organisms usually produce complex extracellular or membrane bound cellulolytic enzymes comprising a combination of activities (Mai *et al.*, 2004). Klyosov (1995) found that Cellulases are the group of hydrolytic enzymes that are capable of hydrolyzing insoluble cellulose to produce soluble oligosaccharides. Cellulases are modular enzymes that are composed of independently folding, structurally and functionally discrete units called domains. It has been reported that there have been a notable differences found in the cellulolytic enzymes isolated from various sources. The differences were mainly in their polypeptide

characteristics such as capacity to adsorb to cellulose, molecular weight, isoelectric points, carbohydrate content, catalytic activity, substrate specificity and amino acid composition and sequence. However, a cellulase system is an efficient hydrolysis of cellulose in a specific coordinated manner. It is a combination of three representative enzymes with or without cellulose binding domains (Lynd *et al.,* 2002). It has been seen that cellulases are grouped along with hemicellulases and other polysaccharide degrading enzymes such as glycosidehydrolases and with the other auxiliary enzymes including hemicelluloses hydrolases (Lynd *et al.,* 2002). But with the exponentially increasing genomic data, an alternative classification based on amino acid sequence similarities, of their catalytic domains has been suggested (Lynd *et al.,* 2002). While this system of classification was very effective, it was unable to easily accommodate enzymes that displayed both modes of catalysis, devising further classification towards more conclusive structural and mechanistic properties is yet required (Mai *et al*., 2004).Cellulases are composed of independently folding, structurally and functionally discrete units called domains or modules, making cellulases module has been reported by (Henrisat *et al*.,1998). Cellulases are inducible enzymes synthesized by a large diversity of microorganisms including both fungi and bacteria during their growth on cellulosic materials (Kubicek *et al*., 1993; Sanget *et al*., 2001). These microorganisms can be aerobic, anaerobic, mesophilic or thermophilic. Among them, the genera of *Clostridium*, *Cellulomonas*, *Thermomonospora*, *Trichoderma*, and *Aspergillus* are the most extensively studied cellulose producer (Khud *et al.,* 1999). Structurally fungal cellulases are simpler as compared to bacterial cellulase systems, cellulosomes (Bayer *et al.,* 1994). Fungal cellulases typically have two separate domains: a catalytic domain (CD) and a cellulose binding module (CBM), which is joined by a short polylinker region to the catalytic domain at the N-terminal. The CBM is comprised of approximately 35 amino acids, and the linker region is rich in serine and threonine. The main difference between cellulosomes and free cellulase enzyme is in the component of cellulosomes-cohesin containing scaffolding and dockerin containing enzyme. The free cellulase contains cellulose binding domains (CBMs), which are replaced by a dockerin in cellulosomal complex, and a single scaffolding-born CBM directs the entire cellulosomes complex to cellulosic biomass (Carval *et al.,* 2003; Bayer *et al.*,2004).

Basic research on cellulases: (Wilson 2009) reported that there has been extensive research on cellulases since the end of World War II, there are still some major gaps in our understanding of the mechanism by which they catalyze the hydrolysis of crystalline cellulose. One gap is information on the mechanism by which a cellulase binds a segment of a cellulose chain from a microfibril into its active site. This is probably the rate limit in gstep for crystalline cellulose degradation, so that understanding the mechanism of this step is very important for trying to engineer cellulases with higher activity on real cellulose substrates. Another gap is our understanding of how cellulosomes are able to efficiently catalyze the hydrolysis of cellulose, despite their large size that restricts their ability to access much of the cellulose surface area that is available to smaller free cellulases. A third gap is an understanding of the way in which certain free cellulose binding modules (CBM) stimulate cellulase hydrolysis (Wang *et al* 2008. , and Moser *et al*., 2008). It is possible that these domains modify the cellulose but exactly how is not known. Finally, while there are some plausible mechanisms for cellulase synergism, there is still much more to be learned about this important process (jeoh *et al*., 2006), particularly how mixtures of cellulases hydrolyze both crystalline and amorphous regions in bacterial cellulose while most individual enzymes only seem to degrade amorphous regions (chen *et al*., 2007).

Classification of cellulases: Cellulase is a complex enzyme system comprising of endo-1,4-β-D- glucanase (endoglucanase, EC 3.2.1.4), exo-1,4-β-D-glucanase (exoglucanase, EC 3.2.1.91) and β-D-glucosidase (β-D-glucoside glucanhydrolase, EC 3.2.1.21) (Joshi and panday, 1999).

Endoglucanase: Endoglucanase (endo-β-1,4-D-glucanase, endo-β-1,4-D-glucan-4- glucano-hydrolase) - often called as CMCase – hydrolyses carboxymethyl cellulose (CMC) or swollen cellulose in a random fashion. Accordingly, the length of the polymer decreases, resulting in the increase of reducing sugar concentration (Robson and chimbless, 1989 ; Begum *et al.,* 2009). Endoglucanase also acts on cellodextrins - the intermediate product of cellulose hydrolysis-and converts them to cellobiose (disaccharide) and glucose. These enzymes are inactive against crystalline celluloses such as cotton or avicel.

Exoglucanase: Exoglucanase (exo-β-1,4-D glucanase, cellobiohydrolase) degrades cellulose by splitting-off the cellobiose units from the non-reducing end of the chain. It is also active against swollen, partially degraded amorphous substrates and cellodextrins, but does not hydrolyze soluble derivatives of cellulose like carboxymethyl cellulose and hydroxyethyl cellulose. Some cellulase systems also contain glucohydrolase (exo-1,4-D-glucan-4-glucohydrolase) as a minor component (Joshi and panday, 1999)..

β- glucosidase: β-glucosidase completes the process of hydrolysis of cellulose by cleaving cellobiose and

removing glucose from the non-reducing end (i.e., with a free hydroxyl group at C-4) of oligosaccharides. The enzyme also hydrolyzes alkyl and aryl β-glucosides (Kubicek *et al* 1993).

Breakdown of Cellulose by Cellulases: Hydrolysis of cellulose by the enzyme cellulase involves hydrolysis of the glycosidic bonds connecting the β-D-glucosyl residues of the cellulose. The general architecture of cellulases features two discrete globular domains: a catalytic domain, accountable for the hydrolysis reaction itself and a cellulose-binding domain, with no catalytic activity, nevertheless enhancing adsorption of the enzyme on to insoluble macromolecular cellulose. In the native enzymes the two domains are connected together by a linker peptide (Sukumaran *et al*., 2005). Mechanistically, cellulase is a family of 3 groups of enzymes, endo- (1,4)-β-D-glucanase (EC 3.2.1.4), exo-(1,4)- β-D-glucanase (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21). The exoglucanase (CBH) acts on the terminals of the cellulose chain and releases β-cellobiose as the end product; endoglucanase (EG) randomly attacks the internal O-glycosidic bonds producing glucan chains of different lengths and the β-glycosidases act specifically on the β-cellobiose disaccharides and produces glucose units. Although the mechanism of cellulose degradation by aerobic bacteria is similar to that of aerobic fungi but anaerobic bacteria operate on different system. Cellulosomes located on the cell surface mediate adherence of anaerobic cellulolytic bacterial cells to the substrate, which thereafter undergo a supramolecular reorganization, so that the cellulosomal subunits redistribute to interact with the various target substrates (Kuhad *et al*., 2011 and Sukumaran *et al*., 2005).

Lignocellulosic ethanol production: Most plant material is not sugar or starch, but contains cellulose, hemicellulose and lignin. The cell wall of plant consists of these three main substances. Hemicellulose and cellulose-glucose chains are stacked each other into crystalline fibrils, acting as a protector wall impenetrable to water or enzymes. Lignin, a more complex macromolecule, makes up of the rest (Schubert, 2006). Cellulose and hemicellulose can be converted into ethanol after converting them into sugar first, but lignin cannot. The process is more complicated than converting starch into sugar and then to ethanol. The first generation technologies for biofuel production were based on fermentation and distillation from sugar and starch rich crops. The second generation technology for biofuel is converting cellulose and hemicellulose from residues such as straw, forestry brash and dedicated energy crops to sugar, and then converted to biofuel by conventional fermentation and distillation. The conversion of cellulosic biomass (corn stove, wood chips) has a far higher potential for fuel production than any of the conventional biofuels. The challenge is biochemical plant lignins incclude the cellulose cell walls and they must be removed. Sugar in cellulosic biomass is locked up in forms of cellulose and hemicellulose. Current lignocellulosic ethanol technology are on three-stage process; in the first stage lignocellulosic materials are pretreated to lose the hemicelluloses-lignin bond and increase the accessibility of water or enzyme to cellulose, in the second stage cellulose is hydrolysed to glucose and in the final stage glucose is put to fermentation. There are many techniques for the pretreatment of lignocellulosic biomass such as steam explosion, dilute acid treatment, alkali treatment, ammonia fiber treatment etc. Among them dilute acid pretreatment is preferred since it removes a large fraction of the xylan and opens pores for subsequent enzymatic hydrolysis of the cellulose (Sannigrahi *et al*., 2011). For, hydrolysis of cellulose to glucose there are two techniques viz., steam explosion at high temperature/pressure and enzymatic one. Of these two techniques, enzymatic is given more focus as the other one is more energy intensive. The enzyme used for hydrolysis is cellulases which is a composite enzyme whose three main components together to bring about complete hydrolysis of cellulose as follows

a) Exo- β-1-4, glucanase: It acts on the non reducing end of the cellulose chain and successively removes single glucose units
b) Endo-β-1-4, glucanase: It randomly attacks the internal β-1-4, linkages
c) β-glucosidases or Cellobiases: It eventually breaks down cellobiose, the building unit of cellulose, to glucose.

Three areas were focused upon in current research to bring down costs and increase productivity: developing energy crops dedicated to biofuel production, improving enzymes that deconstruct cellulosic biomass and optimising microbes for industrial scale conversion for biomass sugars into ethanol and other biofuel or bio products.

Cellulases for Biofuels Production: Depletion of fossil fuels and the increasing demand of alternate sources for renewable energy have developed a huge interest in cellulase production (Pandey *et al*., 2012). Cellulases have

potential application in biofuels production. Bioconversion of lignocellulosic substrate using cellulases and other enzymes are the thirst area for the commercialization of biofuels. The cellulase preparation in biomass conversion processes is based on number of its properties such as stability, product inhibition, synergism, and composition of lignocellulosic biomass etc. (Srivastava *et al*., 2015b). Commercial cellulases are

found in the market by different names for biomass hydrolysis. Nieves *et al*. (2009) have analyzed various commercial cellulases for the hydrolysis of biomass.These authors performed the standard enzymes assays of Filter Paper Activity (FPU), CM Case (EG), β-glucosidase (BGL) as well as xylanase. However, no clear relation between the enzymatic activities of cellulase on soluble and insoluble substrates could be found. Therefore, it is unpredictable to explain the efficiency of cellulase for effective bioconversion of cellulosic biomass. Nevertheless, cellulases having higher FPUs are desirable for effective conversion of biomass. Cellulase enzymes can be used to convert the cellulose portion of non food biomass, such as agricultural waste and energy crops, into fermentable sugars for subsequent conversion to renewable fuels and chemicals. Reprinted (adapted) with permission from (Payne *et al*., 2015).

ADVANCED DEVELOPMENT IN CELLULASES PRODUCTION PROCESSES

Production of cellulase is the thirst area of research around the world for cost-effective production of biofuels and, therefore,many researchers are consistently working in this field. Low production and high cost have always been a major constrain which are needed to be overcome by adopting the novel and versatile approaches. In this context, use of inexpensive raw materials as the substrates, use of genetically modified microorganisms, use of efficient crude thermostable/thermophilic enzyme are some of the key factors which can enhance the cellulase production, significantly (Srivastava *et al*., 2015a). Srivastava *et al*. (2015a) have discussed the use of thermostable enzyme using variousmicroorganisms for partial/complete hydrolysis of cellulose. These authors have systematically discussed the utility of thermostable cellulase over cellulase for effective hydrolysis. Ang *et al*. (2013) reported thermostable cellulase production from *Aspergillus fumigatus* SK1. These thermostable cellulose exhibited higher FPU activity with effective hydrolysis for shorter time period. It has also been reported that the thermostable enzyme can reduce the hydrolysis time (Dutta *et al*., 2014; Srivastava *et al*., 2015b). Among various advanced strategies for cellulase production, solid-state fermentation (SSF) is a potential and cost-effective approach (Pandey, 1994). The SSF is done without the presence of free water in large amount (Pandey *et al*., 2000) but in the presence of ample amount of moisture which provides support for the growth of fungi on lignocellulosic substrate (Singhania *et al*., 2009). Because of this, the cost of dewatering step during the downstream processing can be significantly reduced.Although, at the commercial scale cellulase production is carried out in the submerged fermentation, but due to the low yield and high cost this process is not economical. On the other hand, SSF has the efficiency to be up-scaled for greater volume production. Additionally, SSF is advantageous for higher enzymes concentration, higher productivity as well as low requirement of the sterility equipments (Holker *et al*., 2004). Besides, the crude enzyme components obtained from the SSF can be directly used for the hydrolysis of the lignocellulosic substrate. Further, it is expected that the production cost of SSF can be reduced by tenfold compared to the submerged fermentation (Raghavarao *et al*., 2003) due to the lower energy consumption (Holker *et al*., 2004) and suitable low cost lignocellulosic substrate (Singhania *et al*., 2010). SSF provides a suitable condition for filamentous fungi due to easy cultivation (Holker *et al*., 2004). Filamentous fungi such as *T. reesei*, *A. niger*, *A. fumigates* are well-known fungi for cellulase production under the SSF. Several studies have been reported on effective cellulase production under the solid-state fermentation (Ang *et al*., 2013; Srivastava *et al*., 2014; Chandra *et al*., 2010). Besides SSF, enhancement of cellulase production depends on selection of lignocellulosic materials used for SSF. Lignocellulosic biomass are found in huge quantity and considered as the potential substrates for biofules industries. These biomasses are obtained as waste products of agricultural practices, especially fromvarious agriculture based industries (Perez *et al*., 2002). Since these biomasses are natural and renewable resources of energy, they are the focal point of modern industries. Additionally, these lignocellulosic biomasses can effectively be converted into different value-added products such as cellulase production, sugar generation for bio-fuels production as well as cheap energy sources for microbial fermentation and enzyme production (Asgher *et al*., 2013; Iqbal *et al*., 2013). Thus, addition of suitable lignocellulosic substrate under the SSF can improve the cellulase production in greater extent. Apart from SSF and suitable substrate, cellulase production can be further enhanced by using the genetically modified organisms. Although, many filamentous fungi are able to produce cellulase but due to the lower yield, feasibility in the area of commercialization is not possible. Insertion of high cellulase producing gene into thermophilic organismcan improve the cellulase production, but very few information is available in the literatures. Therefore, a good understanding about the genetics of the organismis needed. Improvements in specific activities of cellulose can be possible via cellulase engineering which depend on rational design. The concept of artificial designing of cellulase seems to be more promising for cellulases having desired features. In addition with genetic modification, co-culture concept is advantageous for

cellulase production under the SSF. Improvement in cellulase production can be achieved via co-culture of different fungi in the single medium (Kalyani *et al*., 2013). Co-culture also has several advantages, e.g. higher productivity, adaptability and substrate utilization compared to pure culture (Holker *et al*., 2004). The enzymes system obtained via co-culturing of different fungi may interact with each other and forms a complete cellulase system. The performance of co-culturing fungi for cellulase production has also been reported by (Hu *et al*., 2011). These authors found that the lignocellulosic components were depolymerized to a greater extent whenthe fungi were co-cultured on lignocellulosic substrate during the SSF and showed better efficiency. In one of the other study by (Kalyani *et al*., 2013), β glucosidase activity was recovered by co-culturing of *Sistotrema brinkmannii* and *Agaricus arvensis*. In addition of various approaches to improve the cellulase production at the industrial scale, number of different co-factors such as addition of metal ions can also enhance the cellulase production, significantly (Srivastava *et al*., 2014).Recently, concept of nanomaterials has aroused as a new era in the revolution of renewable energy production. Recently, (Dutta *et al*., 2014) reported enhanced cellulase production in the presence of hydroxyapatite nanoparticles. In this study, an improved thermal stability of cellulose was achieved along with reducing sugars at the hydrolysis temperature of 80°C, when rice husk/rice straw was used as the substrates. In one of the very recent study, by (Srivastava *et al*., 2015b) an improved celluase production, thermal stability as well as sugar productivity in the presence of Fe3O4/alginate nanocomposite has been reported. In this study, a higher yield of cellulases using *A. fumigatus* AA001 under the SSF was achieved in the presence of Fe3O4/alginate nanocomposite. Further, cellulase production and its thermal stability were also improved in the presence of Fe3O4 nanoparticle and Fe3O4/ alginate nanocomposite. The results reported by the authors clearly exposed that nanoparticles can play an important role to improve the cellulase production as well as the entire bioconversion process. In addition, an improved cellulase production and thermal stability has also been reported in the presence of nickel cobaltite (NiCo2O4) nanoparticle under the SSF using the thermotolarent *A. fumigatus* NS (Class: Eurotiomycetes) (Srivastava *et al*., 2014). Thus, nano particles may be considered as one of the key factor to enhance the cellulase production in the near future. Apart from the aforementioned studies there are other literatures which have reported an improvement of cellulase production, thermal stability as well as its hydrolysis efficiency in the presence of nanoparticles (Ansari and Husain 2012; Shakeel and Qayyum 2012;Verma *et al*., 2013). Ansari and Husain (2012) have discussed the immobilization of cellulase enzyme in the presence of magnetic nanoparticles. These authors have proposed that the nanoparticle works as a carrier and improves not only the thermal stability but also the pH stability and tolerance, frominhibitor during the enzymatic hydrolysis. Verma *et al*. (2013) reported that the thermal stability of β- glucosidase enzyme was increased in presence of magnetic nanoparticles and exhibited half life of the same enzyme at 70°C. Although, nanoparticles have shown potential for improved cellulase production, thermal stability, and hydrolysis efficiency, their mechanism is not well understood; therefore, emphasize should be made on cellulase production using the nanomaterials.

Genomic approaches: Genomic sequencing of cellulolytic organisms has been carried during the past decade and the genome sequences have provided important new information about how microorganisms degrade cellulose. The sequences of the aerobic microorganisms: *Hypocrea jecorina (Tricoderma reesei)*, *Phanerochaete chrysosporium*, and *Thermofida fusca*, all contain multiple cellulase genes, most of which encode a carbohydrate binding module (CBM), and several processive cellulase genes are present in each organism (Martinez *et al*., 2004 and Likidis *et al*., 2007). The genome sequences of Clostridium thermocellum, Ruminococcus albus and Ruminococcus flavifaciens all contain scaffoldin genes and multiple cellulases genes that encode docerin domains, consistent with the presence of cellulosomes in these anaerobic bacteria and several processive cellulase genes are found among the docerin encoding genes (Bayer *et al*., 2008). There are three cellulolytic microorganisms whose genomes do not contain known genes for processive cellulases or docerin domains or scaffoldins: *Cytophiga hutchinsonii* is an aerobic cellulolytic bacterium that is tightly bound to cellulose fibers during growth on cellulose (Xie *et al*., 2007), while *Fibrobacter succinogenes* is an anaerobic cellulolytic bacterium that also is tightly bound to cellulose fibers (Qi *et al*., 2007). From their genome sequences these organisms do not use either the free cellulase or the cellulosomal mechanism to degrade cellulose, so they must use a novel mechanism (Wilson *et al*., 2008). Finally *Postia plancenta* is an aerobic brown rot fungus that appears to produce hydrogen peroxide and Fe (II) ions that generate OH radicals that carry out cellulose depolymerisation (Martinez *et al*. ,2009). Further research is needed on each of these organisms to determine the detailed mechanisms that they use to completely metabolize cellulose. Metagenomics is also being used to try to identify new cellulases and a major study of DNA isolated from the microrganisms in termite

guts was reported recently (Warnecke *et al.* , 2007). About one hundred hydrolases related to cellulose degradation were identified including members of eight cellulose families; however, no members of families containing exocellulase genes were present. This might be due to the fact that termites chew up the biomass into very fine particles that may be easier to degrade then other forms of cellulose. It is interesting that screening of genes for cellulase activity, either from isolated organisms or from DNA libraries from various environmental samples has not identified any new cellase families in the past few years. One novel hydrolase containing both a glucanase and a xylanase was found in a library isolated from soil (Nam *et al*., 2009).

Non-cellulase protein stimulating cellulases: Several proteins have been identified that appear to modify cellulose and enhance its hydrolysis by cellulase. One is a class of plant proteins called expansions (Carey *et al.* , 2007). Another is a fungal protein with some homology to expansin called swollenin (Yao *et al.* , 2008). Recently an expansin like protein has been identified in *Bacillus subtilis* and its structure was determined. In another study, this protein was shown too stimulate corn stover hydrolysis by crude cellulase (Kerff *et al*,.2008 and Kim *et al*., 2009). Several organisms secrete proteins that only contain CBMs and two *T. fusca* proteins (E7, E8) have been purified and shown to stimulate low concentrations of cellulases (Moser *et al*., 2008). Finally there are the family 61 proteins mentioned earlier.

Modeling cellulase activity: Many attempts have been made to model the cellulase catalyzed hydrolysis of crystalline cellulose but we still do not know enough about this process to create a true mechanistic model. Peri *et al.* presented a detailed mechanistic model of amorphous cellulose hydrolysis by crude cellulase that fits their experimental results quite well (Peri *et al.* , 2007).

Cellulase engineering: There are three main approaches that are being used to engineer cellulases with higher activity on crystalline cellulose: directed evolution, rational design, and increasing cellulase thermostability by either of the preceding methods, which can also lead to higher activity. Engineering more thermostable enzymes is relatively straightforward and there are some general approaches that can be applied to any enzyme for which a largenumber of related sequences are known, as is true for most cellulases (Heinzelman*et al*., 2009). A recent paper describes evolving *T. Reesei* Cel12A for enhanced thermostability while another evolved a family 5 endoglucanase with higher activity on CMC but it had no activity on crystalline cellulose (Nakazawa *et al.* , 2009 and Lin *et al*., 2009). At this time there are no published reports of engineered cellulases with major (greater than 1.5-fold) increases in activity on crystalline cellulose. Furthermore, to be useful in an industrial process the improved enzyme has to increase the activity of a synergistic mixture containing several cellulases and in several cases mutant enzymes with higher activity do not do this (Zhang *et al.* , 2000). At this time, it is not clear why this is happening but it has been shown for several exocellulases. Another surprising result is that an improved processive endocellulase catalytic domain, produced by combining two site directed mutations, that showed higher activity in synergistic mixtures then the wild type catalytic domain, did not show higher activity on crystalline cellulose then wild type intact enzyme when the missing domains were added back to form the intact mutant enzyme. This result seems surprising but it shows that activity on crystalline cellulose may involve interactions between the catalytic domain and the carbohydrate binding module (CBM) that go beyond the CBM simply anchoring the catalytic domain to the cellulose (Esteghlalian *et al.* , 2001). Directed evolution of cellulases with improved activity on crystalline cellulose requires that the mutant cellulases be screened on a crystalline substrate not on CMC as most mutations that increase CMCase activity decrease activity on crystalline cellulose. Furthermore, the native enzyme should be utilized, not the catalytic domain given the above result. Finally any improved enzymes need to tested in the appropriate synergistic mixture on the actual substrate for the final process in order to be certain that they will be useful. A problem with directed evolution is that it can only be used to screen potential single or with a massive screen potential double mutations, since the mutant library size required to include most possible larger multiple mutations is too large. Rational design does not have this limitation, but it does require a detail understanding of structure–functional relationships for cellulase crystalline cellulose activity that is still lacking. If we can gain a clear understanding of exactly how cellulases hydrolyze crystalline cellulose it should be possible to design enzymes with multiple changes that have higher activity on specific biomass substrates.

Designer cellulosomes: Another approach to engineering more active cellulose degrading enzymes is to create optimized cellulosomes by synthesizing hybrid scaffoldin molecules that contain cohesins with different binding specificity from different organisms. The exact composition and geometry of the enzymes in a cellulosome can be controlled by attaching the approbriate docerin domain to each enzyme in the cellulosome. In one experiment, the six *T. fusca* cellulases produced during growth on cellulose were modified by removing their family 2 CBM domain and replacing it with a docerin domain, thus converting a free cellulase

system into a cellulosomal system. There were a number of interesting findings from this approach but it did not produce a cellulosome with increased cellulase activity over the free cellulase system (Caspl *et al*., 2008). Another experiment involved adding CBM domains to two of the key cellulosomal enzymes. This inceased the activity of each enzyme when it was bound to an artificial scaffoldin containing one cohesin but various designer cellulosome containing the modified enzymes and other cellulases all had lower activity on crystalline cellulase than comparable designer cellulosomes containing the WT enzymes (Mingardon *et al*., 2007). These results do not invalidate the possibility of improving the activity of cellulosomes by the designer approach but we need to understand more about how the enzymes on cellulosomes interact to degrade crystalline cellulose before we can create better cellulosomes.

CONCLUDING REMARKS

This review focused on the application of cellulase in biofuels production and its industries. Specifically, the demand of cellulase is gradually increasing in biofuel industry and therefore, development of cost-effective methods to produce cellulase at large scale is needed. Although, it is challenging to develop the cost-effective technology and economy in biofuels industries, continuous efforts are being made in this field. The use of cheaper and waste material, thermotolraent/thermophilic organisms, thermostable/thermophilic enzyme, and addition of certain co-factor can enhance the cellulase and consequently the biofuels production. In addition, the genetic modification of cellulase producing microbes is another potential area to explore the higher amount of cellulase for biofuels production.

ACKNOWLEDGEMENT

The authors are thankful to the department of Biotechnology Barkatullah university Bhopal (M.P) and department of Botany Government NSCB P.G college Biora, Rajgarh, (M.P) whose comment and suggestion make this study more valuable.

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Source of Support: None Declared
Conflict of Interest: None Declared