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MICROBIAL CELLULASES: PRODUCTION, IMPROVEMENT APPROACHES, INDUSTRIAL APPLICATIONS AND FUTURE PERSPECTIVES

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ABSTRACT

Biotechnological conversion of cellulosic biomass is potentially sustainable approach to develop novel bioprocesses and products. Cellulose is an abundant natural biopolymer on earth and most dominating Agricultural waste. This cellulosic biomass is a renewable and abundant resource with great potential for bioconversion to value-added bioproducts. Microbial cellulases have become the focal biocatalysts due to their complex nature and wide spread industrial applications. Due to the complexity of enzyme system and immense industrial potential, cellulases have been a potential candidate for research by both the academic and industrial research groups. Nowadays, significant attentions have been devoted to the current knowledge of cellulase production and the challenges in cellulase research especially in the direction of improving the process economics of various industries.

Keywords: Biomass, cellulose, cellulase

INTRODUCTION

Cellulose abounds in nature as is the abundant natural product in the biosphere. It is the primary product of photosynthesis in terrestrial environments, and the most

abundant renewable bioresource produced in the biosphere i.e 100 billion dry tons/year [1, 2]. The production of bio-based products and bioenergy from less costly renewable lignocellulosic materials would bring benefits to the local economy, environment, and national energy security [3]. High costs of cellulases are one of the largest obstacles for commercialization of biomass biorefineries because a large amount of cellulase is consumed for biomass saccharification, for example, ~100 g enzymes per gallon cellulosic ethanol produced [4]. A number of bioconversion methods have been proposed and employed ranging from direct chemical methods like acid hydrolysis to biological methods such as application of cellulase enzymes. Acid hydrolysis of cellulosic materials though appear to be cheaper than cellulase hydrolysis but the former often requires high temperature and pressure; it is highly corrosive and leads to the accumulation of objectionable by-products thus leading to serious pollution. Enzymatic hydrolysis is an ecofriendly approach [5].

They are produced by fungi, bacteria, protozoans, plants, and animals. The catalytic modules of cellulases have been classified into numerous families based on their amino acid sequences and crystal structures [6]. Cellulases contain noncatalytic carbohydrate-binding modules (CBMs) and other functionally known or unknown modules, which may be located at the N- or C-terminus of a catalytic module. Cellulase is a synergistic enzyme and degradation of the cellulose to glucose or other oligosaccharide compounds requires a combined and cooperative action of atleast 3 enzymes namely an endo-1,4-β-glucanase (also referred to as Carboxymethyl Cellulase; EC 3.2.1.4), an exo-1,4-β-glucanase; EC 3.2.1.91 and a β-glucosidase; (EC 3.2.1.21). Cellulase systems exhibit higher collective activity than the sum of the activities of individual enzymes [7]. To hydrolyze and metabolize insoluble cellulose, the micro-organisms must secrete the cellulases (possibly except BG) that are either free or cell-surface-bound. Cellulases are also important in production of fermentable sugars and ethanol [8, 9]. Due to their vast applications and ever increasing demand, novel cellulases with better process suitability, high specificity and stability are being discovered from new lineages of cellulolytic organisms [10]. Now cellulases account for a significant share of the world's industrial enzyme market. The growing concerns about depletion of crude oil and the emissions of greenhouse gases have motivated the production of bio-ethanol from lignocellulose, especially through enzymatic hydrolysis of

lignocelluloses materials—sugar platform [11]. One of the primary challenges for process commercialization is the development of cost effective pretreatment technologies for lignocellulosic feed stocks [12]. Pretreatment is necessary to increase the accessibility of cellulose in lignocellulosic biomass to facilitate enzymatic hydrolysis because unlike traditional sources of fermentable sugars, such as starch and sucrose, the cellulose component of lignocelluloses is a structural polymer and is protected against enzymatic attack by surrounding matrix of lignin and hemicellulose [13]. However, costs of cellulase for hydro-lysis of pretreated lignocellulosic materials need to be reduced, and their catalytic efficiency should be further increased in order to make the process economically feasible. Engineering cellulolytic enzymes with improved catalytic efficiency and enhanced thermostability is important to commercialize lignocelluloses biorefinery. Individual cellulase can be enhanced by using either rational design or directed evolution. However, improvements in cellulase performance have been incremental, and no drastic activity enhancement has been reported to date. The further improvement on cellulase performance needs the better understanding of cellulose hydrolysis mechanisms as well as the relationship of cellulase molecular structure, function, and substrate characteristics.

SUB-UNITS OF CELLULASE AND THEIR MECHANISMS

Glycoside hydrolases cleave glucosidic bonds by using acid–base catalysis. The hydrolysis is performed by two catalytic residues of the enzyme: a general acid (proton donor) and a nucleophile/base [14]**.** Depending on the spatial position of these catalytic residues, hydrolysis occurs *via* retention or inversion of the anomeric configuration.

Endoglucanase

These catalyse random cleavage of internal bonds of the cellulose chain to shorter chains within the macromolecules [15]. Endoglucanase, or CMCase, randomly cut β-1,4-bonds of cellulose chains, generating new ends. Different endoglucanases are produced by archaea, bacteria, fungi, plants, and animals [16]. The catalytic modules of most endoglucanases have a cleft/grove-shaped active site which allows the endoglucanases to bind and cleave the cellulose chain to generate glucose, soluble cellodextrins or insoluble cellulose fragment [17].

Exoglucanase

These are the cellulases that attack the non-reducing ends, releasing cellobiose [15]**.** Exoglucanases can effectively work on micro-crystalline cellulose, presumably

peeling cellulose chains from the microcrystalline structure. CBH is the most-studied exoglucanase. Different CBHs are produced by many bacteria and fungi, with catalytic modules belonging to families 5, 6, 7, 9, 48, and 74 glycoside hydrolases. Their role is believed to be somewhat similar to that of the *Trichoderma* CBHI (Cel7A) [18].

β- Glucosidase

β- Glucosidases are only active on cello-oligosaccharides and cellobiose, and release glucose monomers units from the cellobiose [15]. The activity of BG on insoluble cellulose is negligible. BGs degrade cellobiose, which is a known inhibitor of CBH and endoglucanase. Different BGs are produced by various archaea, bacteria, fungi, plants, and animals, with different catalytic modules belonging to families 1, 3, and 9. BGs have a pocket-shaped active site, which allows them to bind the nonreducing glucose unit and clip glucose off from cellobiose or cellodextrin [19].

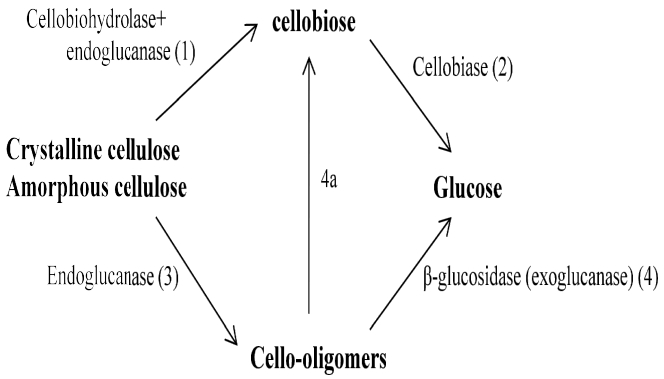

Fig.1 Mechanism of cellulose degradation by different subunits

CELLULASE PRODUCTION

Cellulase can be produced by either solid or submerged fermentations. But nearly all companies have chosen submerged fed-batch fermentation for producing low-cost cellulase because they can produce more than 100 g of crude cellulase (weight) per liter of broth. Most enzyme companies (e.g., Novozymes, Genencor, Iogen, etc.) produce commercial cellulases based on *Trichoderma* and *Aspergillus* or their derivative strains except Dyadic's *Chrysosporium lucknowense*. During the past years, Genencor and Novozymes claimed a 20- to 30-fold reduction in cellulase production costs to 20–30 cents per gallon of cellulosic ethanol [20]. It is estimated that current cellulase costs may range from 1.00 to 1.50 U.S. dollars per gallon of cellulosic ethanol. *Marinobacter* sp. (MSI032) isolated from the marine sponge Dendrilla nigra was optimized by Shanmughapriya *et al*. for the production of extracellular cellulolytic enzyme

(CMCase) by submerged fermentation [21].

A Comparison between SmF and SSF Method

Cellulases are produced using the submerged fermentation (SmF) method traditionally, in which the cultivation of microorganisms occurs in an aqueous solution containing nutrients. An alternative to this traditional SmF method is the solid state Fermentation (SSF) method, which involves the growth of microorganisms on solid materials in the absence of free liquids [22]**.** SSF has three advantages viz. i) lower consumption of water and energy, ii) reduced waste stream and iii) more highly concentrated product. Moreover, The biosynthesis of cellulases in SmF process is strongly affected by catabolic and end product repression and on the overcoming of these repressions to significant extent in solid state fermentation (SSF) system , therefore, are of economic importance. The amenability of SSF technique to use up to 20-30% substrate, in contrast to the maximum of 5% in SmF process, has been documented [23]. The SSF is generally preferred as it offers many advantages such as two-three times higher enzyme production as well as protein rate, higher concentration of the product in the medium, direct use of air-dried fermented solids as source of enzyme which lead to elimination of expenses on downstream processing, employment of natural cellulosic wastes as substrate in contrast to the necessity of using pure cellulose in submerged fermentation (SmF) and the possibility of carrying out fermentation in non-aseptic conditions.

IMPROVEMENT APPROACHES FOR CELLULASE

Cellulase engineering contains three major directions: (1) the reconstitution of designer cellulosome or cellulase mixtures (cocktails) active on insoluble cellulosic substrates (2) directed evolution for each cellulase and (3) rational design for each cellulase, yielding an improved hydrolysis rate or better cellulose digestibility.

Designer Cellulosome

Designer cellulosomes include the construction of chimeric scaffoldins that contain divergent cohesins and matching dockerin-bearing enzymes. This arrangement allows researchers to control the composition and spatial arrangement of the resultant designer cellulosomes. In order to get more efficient designer cellulosomes, glucoside hydrolases have been incorporated into cellulosomes [24]. Cellulosomes with proximity synergy among cellulase components exhibit increased activity on crystalline cellulose. A designer cellulosome combines multiple

enzymes and forms a single macromolecular complex, which is useful for understanding cellulosome action and for biotechnological applications [25]. Bayer et al. have cellulosomized two exoglucanases produced by *T. fusca*: Cel6B and Cel48A. The recombinant fusion proteins tagged with dockerins have shown to bind efficiently and specifically to their matching cohesins.

Directed Evolution

Directed evolution is a robust protein-engineering tool independent of knowledge of the protein structure and of the interaction between enzymes and the substrate [26]. The greatest challenge of this method is developing tools to correctly evaluate the performance of the enzyme mutants generated [18].

Selection and Screening. Selection is always preferred over screening because it has several orders of magnitude higher efficiencies than does screening [18]. Selection requires a phenotypic link between the target gene and its encoding product that confers selective advantage to its producer. Selection on solid media in Petri dishes is commonly used because a number of mutants can be identified conveniently by visual inspection of growth. But selection power may be weak for secretory enzymes because of cross-feeding of the soluble products**.** Screening can be divided into two categories: (1) facilitated screening, which distinguishes mutants on the basis of distinct phenotypes, such as chromosphere release or halo-forming and (2) random screening, which picks mutants randomly [27]. Nearly all directed-evolution examples for endoglucanases have been reported by using facilitated screening on solid plates containing CMC followed by Congo Red staining [28].

Rational Design

Rational design requires detailed knowledge of protein structure, of the structural causes of biological catalysis or structural-based molecular modeling and of the ideal structure–function relationship. Heterogeneous cellulose hydrolysis is a very complicated process [18], which makes rational design of cellulase difficult. Site-directed mutagenesis has been applied to investigation of cellulase mechanisms and enhancement in enzyme properties. Using a SCHEMA structure-guided recombination method, more thermostable CBH hybrids have been obtained [29]. Beside site-directed mutagenesis, the insertion or domain deletion has been adopted to alter the cellulase performance [30]. Despite continuing efforts to enhance noncomplexed cellulase performances, there is no general rule for improving cellulase activity on solid cellulase

substrates. Therefore, rational design for cellulase remains on a trial-and-test stage.

INDUSTRIAL APPLICATIONS OF CELLULASES

Pulp and Paper Industry: Interest in the application of cellulases in the pulp and paper industry has increased considerably during the last decade [31]. The mechanical pulping processes such as refining and grinding of the woody raw material lead to pulps with high content of fines, bulk, and stiffness [32]. While endoglucanases have the ability to decrease the pulp viscosity with a lower degree of hydrolysis, cellulases have also been reported to enhance the bleachability of softwood kraft pulp producing a final brightness score comparable to that of xylanase treatment [33].

Textile Industry: Cellulases are the most successful enzymes used in textile wet processing, especially finishing of cellulose-based textiles [34] Cellulases have been successfully used for the biostoning of jeans and biopolishing of cotton and other cellulosic fabrics [35]. During the biostoning process, cellulases act on the cotton fabric and break off the small fiber ends on the yarn surface, thereby loosening the dye, which is easily removed by mechanical abrasion in the wash cycle [36]. While the biopolishing is usually carried out during the wet processing stages, which include desizing, scouring, bleaching, dyeing, and finishing [37]. Cellulase preparations rich in endoglucanases are best suited for biopolishing enhancing fabric look, feel, and color without needing any chemical coating of fibers [38]. The action of cellulases removes short fibers, surface fuzziness, creates a smooth and glossy appearance, and improves color brightness, hydrophilicity and moisture absorbance, and environmentally friendly process.

Bioethanol Industry. Enzymatic saccharification of lignocellulosic materials such as sugarcane bagasse, corncob, rice straw, switch grass, saw dust, and forest residues by cellulases for biofuel production is perhaps the most popular application currently being investigated [34]. Bioconversion of lignocellulosic materials into useful and higher value products normally requires multistep processes. These processes include; pretreatment, hydrolysis of the polymers to produce readily metabolizable molecules (e.g., hexose and pentose sugars), bioconversion of these smaller molecules to support microbial growth and/or produce chemical products, and the separation and purification of the desired products [39]. Strategies for recycling and reusage of the enzymes may also be used to reduce

enzymatic hydrolysis costs. The recovery of enzymes is largely influenced by adsorption of the enzymes onto the substrate, especially to lignin and enzyme inactivation [40].

Wine and Brewery Industry. Microbial glucanases and related polysaccharides play important roles in fermentation processes to produce alcoholic beverages including beers and wines [34]. These enzymes can improve both quality and yields of the fermented products. Glucanases are added either during mashing or primary fermentation to hydrolyze glucan, reduce the viscosity of wort, and improve the filterability [41]. In wine production, enzymes such as pectinases, glucanases, and hemicellulases play an important role by improving color extraction, skin maceration, must clarification, filtration, and finally the wine quality and stability [38]. *β*- Glucosidases can improve the aroma of wines by modifying glycosylated precursors [42]. The main benefits of using these enzymes during wine making include better maceration, improved color extraction, easy clarification, easy filtration, improved wine quality, and improved stability [43]. Beer brewing is based on the action of enzymes activated during malting and fermentation. Malting of barley depends on seed germination, which initiates the biosynthesis and activation of *α*- and *β*-amylases, carboxypeptidase, and *β*- glucanase that hydrolyze the seed reserves [42].

Agricultural Industries: Various enzyme preparations consisting of different combinations of cellulases, hemi-cellulases, and pectinases have potential applications in agriculture for enhancing growth of crops and controlling plant diseases [44]. Plant or fungal protoplasts produced using microbial hydrolases can be used to produce hybrid strains with desirable properties. Cellulases and related enzymes from certain fungi are capable of degrading the cell wall of plant pathogens in controlling the plant disease [45]. Fungal *β*-glucanases are capable of controlling diseases by degrading cell walls of plant pathogens. Many cellulolytic fungi including *Trichoderma* sp., *Geocladium* sp., *Chaetomium* sp., and *Penicillium* sp. are known to play a key role in agriculture by facilitating enhanced seed germination, rapid plant growth and flowering, improved root system and increased crop yields [46]. The exoglucanase promoters of *Trichoderma* have been used for the expression of chymosin and other proteins: glucoamylase, lignin peroxidase, and laccase [47].

Food Processing Industry: Cellulases also have an important application as a part of macerating enzymes complex

(cellulases, xylanases, and pectinases) used for extraction and clarification of fruit and vegetable juices to increase the yield of juices [48]. The use of macerating enzymes increases both yield and process performance without additional capital investment [49].. The macerating enzymes are used to improve cloud stability and texture and decrease viscosity of the nectars and purees from tropical fruits such as mango, peach, papaya, plum, apricot, and pear [41]. Texture, flavor, and aroma properties of fruits and vegetables can be improved by reducing excessive bitterness of citrus fruits by infusion of enzymes such as pectinases and β-glucosidases [50]. Thus, the macerating enzymes, composed of mainly cellulase and pectinase, play a key role in food biotechnology and their demand will likely increase for extraction of juice from a wide range of fruits and vegetables [51]. Furthermore, infusion of pectinases and β-glucosidases has also shown to alter the texture, flavor, and other sensory properties such as aroma and volatile characteristics of fruits and vegetables [52].

PERSPECTIVES

Biomass saccharification is the largest technical and economic obstacle to biorefinery and biofuels production. To address this challenge, there are three different approaches or their combinations: (1) biomass pretreatment/fractionation and enzymatic hydrolysis, (2) cellulase engineering, and (3) CBP. Biomass pretreatment/fractionation must be further enhanced for better overall cost efficiency; for example, increasing substrate reactivity so as to decrease cellulase use, co-utilization of ligno-cellulose components (e.g., lignin, hemicellulose) for high-value products, recycling costly cellulase by readsorption of free enzymes or desorbed enzymes. Cellulase engineering must be focused on (1) increasing cellulase specific activity on pretreated biomass through enzyme cocktail or rational design or directed evolution, (2) increasing cellulase stability for cellulase recycling, and (3) decreasing enzyme production costs ($ per kilogram of dry protein). CBP microorganisms or consortium would simplify the whole process and increase productivity. Cellulases are being commercially produced by several industries globally and are widely being used in food, animal feed, fermentation, agriculture, pulp and paper, and textile applications. With modern biotechnology tools, especially in the area of microbial genetics, novel enzymes and new enzyme applications will become available for the various industries. Improvements in cellulase activities or imparting of desired features to enzymes by protein engineering

are probably other areas where cellulase research has to advance. In practice, the above three approaches would be integrated together for maximizing the re-turns from biorefinery and biofuels.

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