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An Overview on Fungal Cellulases with an Industrial Perspective

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Abstract

Lignocellulose is the most abundant biopolymer available on earth. Hydrolysis of lignocellulose into fermentable sugars, sugar acids and phenolics is the pre-requisite for its successful exploitation as substrates for the large scale production of industrially significant value added products. Though remarkable collection of lignocellulolytic microorganisms has been brought to limelight, only a few especially fungi have been studied extensively as they secrete copious lignocellulolytic enzymes extracellularly. Enzymatic hydrolysis of lignocelluloses is carried out by a group of enzymes viz., cellulases, hemicellulases, ligninases in unison or individually, and are collectively known as lignocellulolytic enzymes. In this light of this background, followed by a short introduction on lignocellulose, lignocellulolytic enzymes and mainly focused on fungal cellulase, this review discusses recent approaches on the production of cellulase by submerged and solid-state fermentation strategies; current knowledge on the purification, characterisation of cellulase; and finally concluded with detailed industrial applications of fungal cellulase.

Keywords: Fungal cellulase; Lignocellulose; Production strategies; Purification; Characterisation; Lignocellulolytic enzymes

Introduction

As a consequence of industrialisation and rapid growth in population hike globally, utilisation of natural resources has been increased exponentially during the past few decades. Increasing concern regarding the environmental pollutions and the exhaustion of fossil fuels compelled us to exploit alternative renewable energy resources so as to meet the ever increasing energy requirements [1]. Now-a-days, the concept of waste-to-energy has become the primary focus of many industries with an economic perspective, and sustainable processes signifying the utilization of biomass judiciously-the most abundant organic renewable resource accounting for around 10-14% of the world's energy supply. The term biomass is the collective term denoting all the organic materials found on earth including terrestrial and aquatic plants and animals as well as the organic wastes [2]. Generally, the biomass encompasses: plant-based woody biomass (mainly lignocelluloses), plant-based non-woody biomass (starch, sugar and oils), and animal/human based biomass (animal fats and proteins, slurry/slaughter wastes, house hold wastes, etc.,). Among the plant-based woody biomass, lignocellulosic biomass is considered as a potential resource for renewable energy, which is normally used for land filling or simply burned off. Lignocellulose constitutes 60% of the plant cell wall, and is made up of three biopolymers of sugars and their derivatives, viz., lignin, hemicelluloses and cellulose. Annually, about 100 billion tons of plant dry material is generated in the world by photosynthetic activity. The surplus of lignocellulosic biomass is mainly treated as waste (though nothing is literally waste in this universe); hence, intensive research has been accomplished for the effective utilization of the lignocellulosic materials for the production of enzymes, biofuels, antioxidants, feeds, etc.,

Degradation of lignocellulosic biomass is carried out primarily by microbial intervention – i.e., utilize it as carbon and nutrient/energy source for their growth. They include species of bacteria (*Clostridium, Cellulomonas, Bacillus, Pseudomonas, Fibribacter, Ruminococcus, Butyrivibrio,* etc.,), fungi (*Aspergillus, Rhizopus, Trichoderma, Fusarium, Neurospora, Penicillium*, etc.,), and *actinomycetes (Thermomonospora, Thermoactinomyces,* etc.). Of them, fungi are the principal agents involved in the degradation of lignocelluloses. The fungal degradation of lignocellulose is mainly accomplished by producing two types of extracellular enzyme systems: hydrolytic and oxidative catalytic systems. The hydrolytic system produces hydrolases to degrade polysaccharides and the lignolytic system produces ligninases to degrade lignin and opens phenyl rings [3]. The bioconversion of lignocellulosic materials to various value added products mainly includes two processes; hydrolysis of lignocelluloses in to fermentable reducing sugars is carried out by a group of enzymes collectively known as lignocellulolytic enzymes, and subsequent fermentation of these sugars to various value added bio-products. Thus, this review mainly focused on the general aspects of lignocellulose, lignocelluolytic enzymes with special emphasis on fungal cellulases; moreover, characteristics, structure, application and production strategies of cellulase are discussed with appropriate illustrations [4].

Lignocelluloses

Lignocelluloses are the structural polysaccharides of plants that composed of cellulose (~50%), hemicellulose (~30%), and lignin (~20%), and are widely distributed among the vascular plants [5,6]. Cellulose and hemicellulose are polysaccharides composed of simple sugars; whereas, lignin is a complex network of aromatic alcohols. In general, hemicelluloses and lignin provide an amorphous matrix to which crystalline cellulose microfibrils are dispersed (Figures 1 and 2) [7,8]. Cellulose microfibrils are stabilised by intra- and inter-molecular hydrogen bonds, and are surrounded by hemicellulosic polymers. The cellulose-hemicellulose matrices are protected by lignin, an amorphous insoluble polymer which impedes the microbial attack on internal cellulosic structures [9].

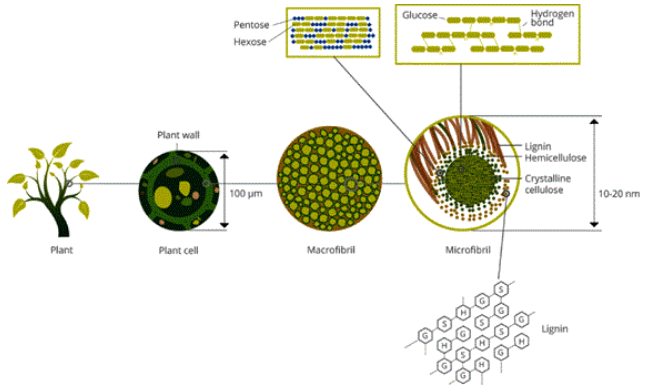

Figure 1: Structure of lignocelluloses (courtesy: Rubin, [7]; Streffer, [167]).

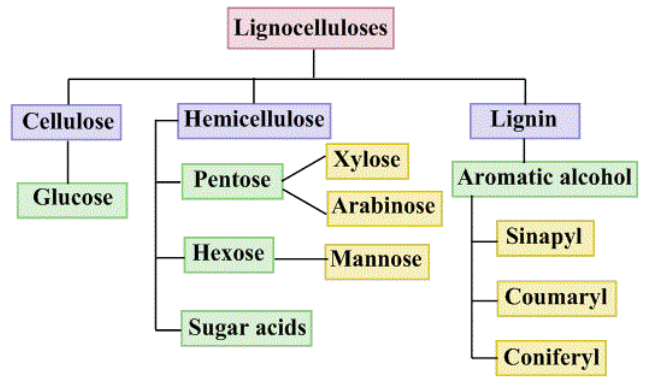

Figure 2: Schematic representation showing the contents present in the lignocelluloses.

Lignin

Lignin is the most complex polymer made up of phenylpropane units such as coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Figure 3) linked in a three-dimensional structure, which is difficult to degrade. Lignin component of soft woods contain more than 90% of coniferyl alcohol, and that of hardwood is composed of coniferyl and sinapyl alcohols in varying proportions [10]. They provide rigidity to the plant body, and play a critical role in liquid transport and prevention of microbial attack as well [11].

Hemicellulose

Hemicellulose-the second most copious part (15-35%) of lignocellulosic biomass - are heterogeneous polymers of sugar acids, pentoses (including arabinose and xylose), and hexoses (glucose, galactose, mannose) [9]. They are highly branched polymers and lack crystalinity. In nature, the composition of hemicellulose is uneven, which depends on the nature of plant source (Figure 4). Generally, in softwoods (e.g., gymnosperm) like spruce and pine, hemicellulosic part is composed mainly of mannan, especially glucomannan and galactoglucomannan; whereas, secondary walls of hardwood (e.g., angiosperm) and herbaceous plants embody chiefly of xylans [12].

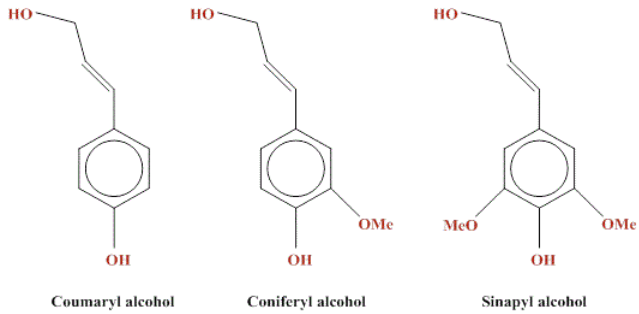

Figure 3: Three phenylpropane monomers in lignin.

Cellulose

About 50% of the CO2 fixed photo-synthetically is stored in the form of cellulose in plants [13]. Cellulose is a high molecular weight (MW) homopolymer constituting (1,4)-D-glucopyranose units joined by β-1,4 linkages with the repeating units of the disaccharide (the cellobiose) (Figure 5). The cellulose chains interact with each other via numerous cross linkages such as hydrogen bonds and van der Waal's interactions to form bundles which aggregate to form microfibrils with of 5-15 nm diameters [5,6].

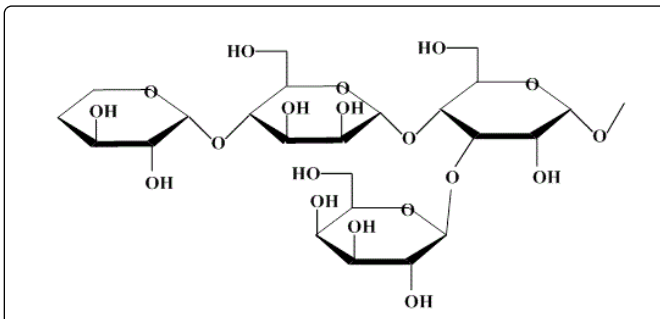

Figure 4: Structure of hemicelluloses.

Each chain of cellulose contributes to the microfibril, as if a small thread contributes in making a rope. Generally, the cellulose chains are arranged parallely in the microfibrils as a highly ordered form, resulting in crystalline structures; while, the less ordered structures contribute to the amorphous regions [14]. The rigidity of cellulose is a direct consequence of its structure. Thus, even though cellulose is a homo-polysaccharide, its structure is heterogeneous; for instance, filter paper is considered as highly crystalline cellulose, therefore, IUPAC has recommended the hydrolysis of filter paper as a standard measurement for total cellulolytic activity [15]. The most prevalent component in lignocelluloses is cellulose.

Lignocellulolytic enzymes

Even though a large chunk of lignocelluloses are formed annually, its bulkiness on earth does not accumulate due to the swift action of microorganisms on it. They efficiently degrade these organic materials so as to provide themselves with carbon and energy source for their growth, and allow the recycling of carbon back into the ecosystem [16]. Although a large number of microbes can thrive on lignocelluloses, a

few of them produce the complete battery of enzymes necessary for the breakdown of lignocelluloses into simpler molecules for further aerobic or anaerobic catabolism. In nature, a wide variety of bacteria and fungi are evolved to produce lignocellulolytic enzymes as a part of ecological recycling.

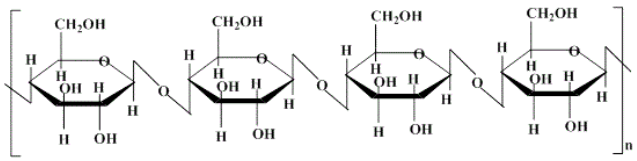

Figure 5: Structure of cellulose consisting of cellobiose as repeating units.

The advent of biotechnology and bioprocess technology has boosted up the effective utilization of lignocelluloses for the production of these valuable lignocellulolytic enzymes. Fungi are mainly exploited for the production of lignocellulolytic enzymes on large scale for use in industry, as they grow attached to the solid substrates with limited moisture content, i.e., natural solid-state fermentation (SSF) [17].

Degradation of lignocelluloses is generally carried out by a complex array of enzymes including ligninases, hemicellulases and cellulases (Figure 6).

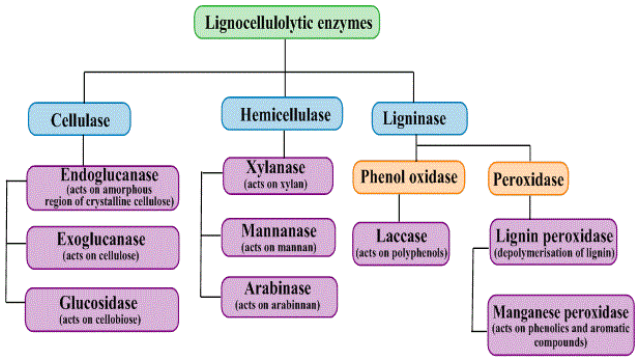

Figure 6: Schematic representation of lignocellulolytic enzymes.

Ligninases/lignases

Ligninases break down lignin into low MW compounds that are assimilated by other microorganisms. Generally, ligninases are of two types: (a) phenol oxidases or laccases and (b) peroxidases (lignin peroxidase and manganese peroxidase). Laccases (EC 1.10.3.2) are copper containing glycoproteins, which may be monomeric, dimeric or tetrameric in nature. The MW of the monomer varies from 50 to 100 kDa. Laccases are involved in the degradation of lignin via oxidation of phenolic compounds to yield phenoxy radicals and quinines [18]. *Aspergillus nidulans, Phanerochaete chrysosporium, Lentinula edodes, Phellinus ribis, Pleurotus pulmonarius* are the known producers of laccase [19]. Peroxidases are included in the family of oxidoreductases that catalyse the depolymerisation of lignin utilising H_2O_2. Lignin peroxidase (EC 1.11.1.14) is a heme protein possessing high redox potential with low optimum pH, nearly 3 [19]. Lignin peroxidase is less specific towards its substrates and oxidises a wide range of phenolic, aromatic, non-phenolic and organic substrates. Usually, the MW of Lignin peroxidase isoenzymes ranges from 38 to 46 kDa [20]. In contrast, manganese peroxidases (EC 1.11.1.13) utilise Mn^{2+} as electron donor and oxidises phenolic structures to phenoxyl radicals; the MW of manganese peroxidise ranges from 38 to 62.5 kDa [21].

Hemicellulase

Hemicellulases are glycoside hydrolases or carbohydrate esterases represented by xylanases (EC 3.2.1.8), β-mannanases (EC 3.2.1.78), arabinofuranosidases (EC 3.2.1.55), and β-xylosidases (EC 3.2.1.37). Xylan constitutes around 70% of hemicelluloses, and is hydrolyzed by xylanases to oligomers, which are further degraded to xylose by β-xylosidases. Moreover, other hemicellulases like mannanase and arabinase are also required for the complete degradation of hemicelluloses, which depends on its chemical composition [1,9].

Cellulases

The third but the most significant group of lignocellulolytic enzyme is cellulases, the key enzymes for the conversion of cellulose into simple sugars [22]. Cellulase is a family of enzymes hyrolysing β-1,4-glycosidic bonds of intact cellulose and other related cello-oligosaccharide derivatives. Synergistic action of three principal types of the enzymes, viz., endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) is required to accomplish the degradation of intact hydrogen-bond-ordered cellulose. Endoglucanases preferentially hydrolyze the amorphous (internal) regions of the fibrils randomly by cleaving β-glucosidic bonds; cellobiohydrolases are exoglucanases releasing cellobiose, from the termini of the chains, while β-glucosidases complete the degradation process by hydrolyzing cellobiose and other cellodextrins with a low degree of polymerization to glucose units (Figure 7) [23]. Among the lignocellulytic enzymes, cellulases found significant potential for industrial applications, especially in sectors of foods, chemicals, detergents, cosmetics, pulp and paper, etc. [17].

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) [13].

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 [24,25]. 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.

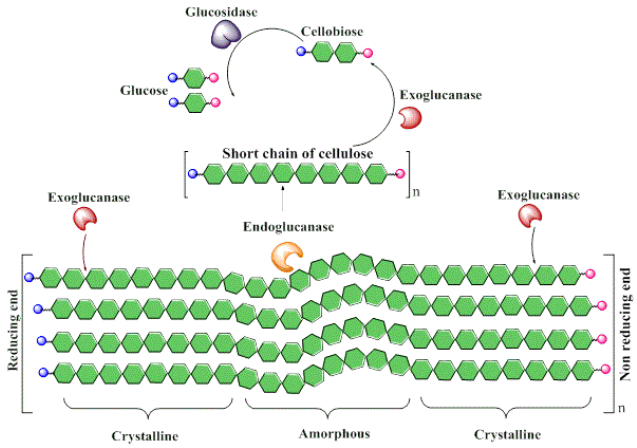

Figure 7: Diagramatic representation of the enzymatic hydrolysis of cellulose by the synergistic action of cellulases.

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 [13].

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 [26].

Fungal cellulases

The microbial hydrolysis of insoluble cellulose requires the action of multiple cellulases (endoglucanases, exoglucanases and β-glucosidases) in a synergistic manner, so that the complex polymer is converted to simple sugars. Among the microorganisms, fungi in particular are dynamic cellulose decomposers, and possibly responsible for 80% of the cellulose breakdown on earth [27]. This is particularly true in forest ecosystem, where fungi are the principal agents decomposing cellulose and lignin [28]. The cellulose decomposing fungi include members of the ascomycota, basidiomycota, and deuteromycota, as well as some chytrids that occur in the rumen of some animals. Efficient cellulolytic fungi are represented by the species of *Aspergillus, Penicillium, Chaetomium, Trichoderma, Fusarium, Stachybotrys, Cladosporium, Alternaria, Acremonium, Ceratocystis, Myrothecium, Humicola,* etc. [29,30].

Moreover, the aerobic fungal cellulases are usually preferred by the industry, because they are extracellular, adaptive in nature and usually secreted in large quantities during growth. This is in sharp contrast to many bacterial as well as anaerobic fungal cellulases which exist as tight multi-enzyme complexes; often membrane bound as cellosomes, from which it is difficult to recover individual active enzyme species; hence, economically less important [31]. Production of cellulolytic enzyme from aerobic fungi is wide spread; among them, species of *Aspergillus, Trichoderma, Penicillium* and *Sclerotium* are found as highly cellulolytic, and are mainly considered for commercial exploitation [32,33].

Structurally, fungal cellulases are simple and modular enzyme with functionally distinct modules or domains [34]. Some of them possess two domains, catalytic domain and carbohydrate binding domains connected by a serine- and threonine-rich polylinker with varying chain length and structure (Figure 8) [35]. The carbohydrate binding modules vary in size ranging from 4 to 20 kDa, and are rich in aromatic and often polar amino acid residues that immobilize the substrate during catalysis. The active site of the catalytic domain may be topologically tunnel, cleft or pocket in shape allowing the hydrolysis of the substrate efficiently [36].

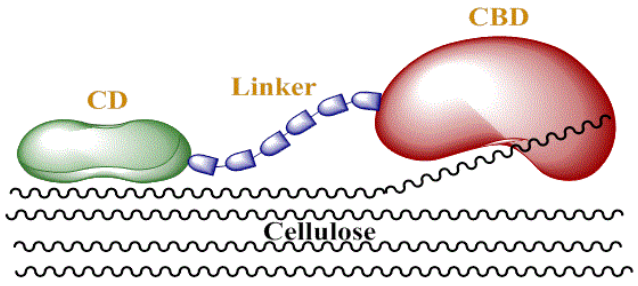

Figure 8: General structure of cellulase consisting of catalytic domain (CD) and carbohydrate binding domain (CBD) joined via a linker peptide.

One of the most extensively studied aerobic fungi is *T. reesei*, which is capable of hydrolyzing native cellulose [37,38]. *T. reesei* possesses two genes encoding for exoglucanase, eight for endoglucanases and seven for glucosidases [39,40]. In the last few decades, thermophilic fungi have also been studied widely because of the fact that cellulose fibers bulge/swell up at higher temperatures, so that they become easily accessible for hydrolytic enzymes [41]. *Talaromyces emersonii* is a typical thermophilic fungus capable of producing cellulase, which was active even at 70°C and decomposes the intact cellulose [42]. Two strains of *Penicillium* were identified from subtropical soils with potentials for the production of cellulase [43]; *Chaetomium thermophilum, Sporotrichum thermophile, Talaromyces emersonii* and *Thermoascus aurantiacus* grew well and decomposed cellulose very rapidly, producing thermostable cellulases [41,44].

Cellulase production by fermentation

Submerged fermentation (SmF): The SmF - the most commonly used technology for the large scale production of enzymes - is generally carried out in the presence of free flowing liquid, in which soluble substrates are dispersed; however, compared to liquid medium, filamentous fungi like *Aspergillus, Penicillium and Trichoderma* normally produce large quantity of cellulase in solid medium. In fact, the easiness in controlling the process parameters, monitoring and downstream process make SmF more attractive [40]. In SmF, various factors such as pH, temperature, substrate concentration, inducer, medium composition, etc., influence the production of cellulase significantly. The main drawback in SmF is the requirement of long

fermentation time (i.e., gestation period) with less production [45]. Among microorganisms, bacteria are most commonly used for the production of enzymes by SmF [46,47]; but, some species of fungi such as *Aspergillus, Penicillium, Trichoderma,* etc. are also being cultivated under SmF for the production of cellulase. Table 1 shows major cellulase producing species/strains of fungi by utilizing different carbon sources.

Fungi	Substrate	Cellulase (U/mL)	Reference
A. heteromorphus	wheat straw	83	Singh et al.[67]
A. flavus BS1	CMC	2793	Sajith et al. [61]
A. fumigatus	wheat straw	321	Saqib et al. [136]
A. niger	CMC	1.6	Narasimha et al. [50]
A. niger	maize straw	102	Milala et al. [33]
A. niger	banana peel	12.4	Jadhav et al. [137]
A. niger	coir waste	3.3	Mrudula and Murugammal, [138]
A. niger	sawdust	3.9	Devi and Kumar [139]
A. niger MS82	grass	6.8	Sohail et al. [60]
P. fellutanum	lactose	81	Kathiresan and Manivannan, [140]
R. oryzae	water hyacinth	450	Karmakar and Ray [141]
T. harzianum	CMC	150	Rubeena et al. [142]
T. harzianum MTCC 8230	rice straw	1.7	Kocher et al. [143]
T. reesei RUTC30	sugarcane baggase	121	da Silva Delabona et al., [144]
T. reesei ZU-02	corn cob residue	5.5	Liming and Xueliang, [145]
T. viride	CMC	173	Neethu et al., [146]

Table 1: Production of cellulase by various fungi employing submerged fermentation.

The cellulolytic activities of fungi may vary depending upon the medium as well as the culture conditions. Hence, the formulation of suitable fermentation strategies is the key factor for deciding the efficiency of a fungus in terms of the production of cellulase. In order to obtain better productivity, various synthetic or natural carbon sources are used in SmF; for instance, Acharya et al. [48] studied the production of cellulase by *A. niger* on pretreated (by alkali) sawdust and the maximum cellulase activity was observed under optimized condition, i.e., pH between 4 and 4.5, 120 rpm, at 28°C and peptone as nitrogen source. Similarly, Karthikeyan et al. [49] investigated the production of cellulase by *Penicillium* strain K-P in liquid medium by supplementing various carbon and nitrogen sources at varying pH and temperature; the fungus showed the maximum cellulase activity in the presence of fructose, ammonium nitrate, pH 3.0 and 30°C on day 5. Production of cellulases was studied by *A. niger* in the presence of various carbon and nitrogen sources at varying pH; and found that the maximum production of cellulase was on Czapek-Dox medium supplemented with 1% CMC or sawdust at pH 5 [50]. From the aforesaid reports, it is clear that the production of cellulase mainly observed under acidic pH and 20-30°C; in addition to the ability of the fungi in utilizing the carbon and nitrogen sources present in the liquid medium.

Solid-state fermentation

The SSF is one of the important strategies employed in the industries for the enhanced production of various enzymes. SSF is carried out on the solid substrates in the presence of no free water (i.e., water is available in the bound form only), which acts as both solid support and source of nutrients. In the recent years, SSF is gaining more interest as a suitable strategy for the recycling of nutrient-rich wastes such as lignocelluloses. SSF facilitates not only the possibilities for the bioconversion of agro-residues to value-added products, but also it enables the efficient recycling of lignocellulosic materials with the expenditure of less energy [51]. Earlier days, SSF was considered as suitable only for the fermentation of food or food-associated products; but further studies showed several benefits of this technology; such as high enzyme yield at low cost, use of agricultural waste as substrate, and wider range of additional enzyme activities than found in SmF [52,53]. Thus, SSF is an attractive means to produce cellulase economically, because of its lower capital investment and operating cost [54]. Due to the discrete nature of SSF, the physico-chemical characteristics of substrate such as crystallinity, bed porosity and enormous surface area can influence the production of cellulolytic enzyme system in fungal cultures. In SSF, the operating conditions like temperature, pH and moisture content are vital factors influencing the microbial growth and production of cellulase. Availability of oxygen in the open space between substrate particles (i.e., porosity) and generation of heat are the major challenges in SSF, which have to be addressed properly [55,56].

Agricultural wastes such as brans of wheat and rice, corn stover, straws of wheat and rice, sugarcane baggase, sawdust, etc. are the most commonly used substrates for the production of cellulase. For instance, Liu et al. [57] investigated the production of cellulase on different lignocellulosic substrates such as straws of rice, wheat and cotton, corn stover and corncob using *A. fumigatus* Z5; of them, the corn comb supported the maximum production of endoglucanase. *P. echinulatum* 9A02S1 showed the maximum production of cellulolytic enzymes on the medium containing a mixture of pretreated sugarcane bagasse and wheat bran [58]. Dutta et al. [59] studied the production of cellulases from *P. citrinum* using brans of wheat and rice and rice straw as substrate; all these substrates supported the production of cellulases. *A. niger* MS82 efficiently utilized the lignocellulosic substrates like grass, bagasse and corncob with variable cellulase activities [60]. *A. flavus* BS1 competently utilized different lignocellulosic substrates and supported higher levels of cellulase activity [61]. *A. flavus* Linn NSPR 101 showed the production of cellulase on various natural substrates like bagasse, corncob and sawdust [62]. Thus, production of cellulase may vary between species of fungi; and from the above studies, it is evident that the wide range and higher enzyme activities are possible on waste lignocellulosic biomass as substrate. It is difficult to compare cellulolytic enzymes reported in literature; because different authors demonstrated the activity of these enzymes in different units. Nevertheless, Table 2 demonstrates the production of cellulase on various solid substrates by different species of fungi, and the activities are expressed in gram dry fermented substrates (gds).

Fungi	Substrate	Cellulase(U/gds)	Reference
A. flavus BS1	tapioca flour and sawdust	5408.5	Sajith et al., [14]
A. fumigatus	rice straw and wheat bran	14.7	Sherief et al., [147]
A. niger	wheat bran	3.2	Chandra et al., [148]
A. niger 38	wheat bran and wheat straw	14.8	Jecu, [149]
A. niger KK2	rice straw	130	Kang et al., [150]
A. niger MS82	grass	100	Sohail et al., [60]
A. terreus M11	corn stover	581	Gao et al., [151]
A. fumigatus Z5	corn stover	526.3	Liu et al., [57]
Aspergillus spp. MAM-F35	wheat straw	487	AboState et al., [152]
Fomitopsis sp. RCK2010	wheat bran	84	Deswal et al., [153]
niger USM AI	sugarcane bagasse and palm kernel cake	3.2	Lee et al., [84]
P. citrinum	rice bran	2	Dutta et al., [59]
P. decumbens	wheat straw	52.8	Mo et al., [154]
P. echinulatum 9A02S1	sugarcane bagasse and wheat bran	282.4	Camassola and Dillon, [58]
T. reesei	sugarcane bagasse and palm kernel cake	2.2	Lee et al., [84]
T. reesei LW1	corn straw and wheat bran	452.5	Wang et al., [155]
T. reesei NRRL11460	sugarcane bagasse	154.6	Singhania et al., [38]
T. reesei LM-UC4	bagasse	38.6	Duenas et al., [156]
A. phoenicis QM 329			
T. viride	wheat straw	555	AboState et al., [152]
Thermoascus aurantiacus	wheat straw	1572	Kalogeris et al., [157]

Table 2: Production of cellulase by fungi on various lignocellulosic substrates employing solid-state fermentation.

Statistical optimization

The major challenge in the development of economically feasible bioprocess for the production of industrially significant enzymes is to identify the potential microorganisms, composition of media and the optimization of various process parameters that influence the microbial growth and production of enzyme [63]. Statistical tool such as response surface methodology (RSM) coupled with artificial neural networks (ANN) is generally employed to optimize the parameters influencing a particular biological response, that critically evaluates the interactive effects normally neglected in conventional one-at-a-time cultivation strategy. Moreover, it significantly reduces the number of experiments required for the standardization of a biotechnological process with remarkable reproducibility [64]. Since microbial cellulases are inducible in nature and their production is influenced by several parameters such as pH, temperature, agitation, substrate concentration, type of nitrogen and carbon source etc. RSM is effectively employed to standardize these process parameters. The statistical tools such as Plackett-Burman, Box-Behnken, central composite designs, ANN alone or in their combination are the most commonly used methods for the screening of independent variables that significantly affect the enzyme production. For instance, Singhania et al. [65] employed a combination of Plackett-Burman and central composite designs to maximize the production of cellulase produced by *T. reesei* RUT C30, which resulted in 6.2 folds increase in cellulase production in the presence of 37.5% moisture content and 30°C. Mixed cultures of *T. reesei* and *A. phoenicis* showed β-glucosidase activity and filter paper activity of 0.64 IU/mL and 1.54 FPU/mL, respectively on dairy manure at 27°C and pH 5, which was optimized using Box-Behnken design [66]. Similarly, Singh et al. [67] utilized Box-Behnken design for effectively optimizing the temperature, pH and substrate concentration as 60°C, pH 4.8 and 148.9 mg/mL, respectively [68]. Thus, the statistical methods improve the effectiveness of fermentation processes prior to industrialization, thereby promoting the utilities of various microorganisms as potential sources of bioproducts.

Purification and characterization of cellulase

Purification and characterization are the vital steps required for developing the improved performance/functioning of an enzyme. Enzymes in the culture supernatant could be purified by the classical methods including precipitation, dialysis and column purification [69]. Different types of columns are used for the purification of cellulase, among which sephadex with different sieve sizes is the most popular

matrix used for gel exclusion chromatography [70]. The efficiency of purification is generally analyzed in terms of purification folds and yield. Table 3 represents the columns used, purification fold and yield of cellulase produced by different species of fungi.

Fungi	Column	Yeild (%)	Purification fold	Reference
A. aculeatus	DEAE-Sephadex	25	4	Naika et al., [90]
A. glaucus XC9	Sephadex G-100	22.3	21.5	Tao et al., [70]
A. kawachii	G3000-SW	7.7	494	Iwashita et al., [158]
A. niger	Sephacryl S-300	22	23	Yan et al., [159]
A. niger 322	Sephadex G-75	33	6	Peshin and Mathur, [160]
A. niger IF031125	TSK-gel G2000SW	21.8	46.1	Akiba et al., [71]
A. oryzae	TSK DEAE-5PW	4.5	176.9	Riou et al., [74]
A. terreus AN_1	DEAE-sepharose	1.3	40	Nazir et al., [78]
A. terreus DSM	Sepharose-4B column F11	16.6	15.4	Elshafei et al., [161]
A. terreus M11	Sephadex G-100	14	18	Gao et al., [72]
Alternaria alternata	Sephadex	13	4.2	Macris, [162]
Fusarium oxysporum	Column PBE94	65.4	17.6	Christakopoulos et al., [163]
Mucor circinelloides	Bio-Gel A-0.5 m	3	408	Saha, [164]
P. purpurogenum KJS506	Hydroxyapatite column	22.3	34	Lee et al., [84]
T. harzianum	Sephadex G-50	10.3	21.9	Ahmed et al., [165]
T. viride	Sephadex-G100	2.1	2.3	Nasir [166]

Table 3: Purification fold and yield of fungal cellulase based on various column packing materials.

Characterization of cellulase for analyzing its optimum temperature and pH is required to reveal its possible industrial applications. Most of the fungal cellulases are active at a temperature range from 40 to 70°C and are acidophilic in nature. For instance, the cellulase produced by *A. niger* IFO31125 showed an optimum temperature of around 70°C at pH 6 with stability for 2 h [71]. The pH and the temperature optima of the cellulase produced by *A. terreus* M11 was found as 2 and 60°C, respectively; and 60% of the initial activity was maintained for 1 h at 70°C [72]. Two endoglucanases, RCE1 and RCE2 produced by *Rhizopus oryzae* showed 55°C as the optimum temperature for both the enzymes with different pH optima, i.e., 5 and 6, respectively [73]. *A. oryzae* secretes a highly glucose tolerant cellulase with the optimum activity at 50°C and pH 5 [74], while the cellulase produced by *A. oryzae* S/92Gbr showed the optimum activity at pH 5 and 45°C [75].

Influence of metal ions, detergents and surfactants

Various metal ions and chemical compounds may influence the cellulase activity. It was reported that usually metal ions such as Hg^{2+}, Cu^{2+}, Zn^{2+}, Mg^{2+}, Fe^{3+}, Mn^{2+}, Ag^{+}, Mn^{2+}, K^{+} are slightly or completely inhibitory to cellulase, whereas metal ions such as Ca^{2+}, Na^{+} and Co^{2+} stimulate or unaffect the activity of cellulase [59,76-78]. Various chemicals or reagents such ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulphate (SDS), dicyclohexyl carbodiimide, sodium azide, β-mercaptoethanol, dithiothreitol, triton X-100, urea, etc. may increase, decrease or abolish the activity, based on the nature of enzymes [79]. Riou et al. [74] investigated the effect of different metal ions and detergents on cellulase produced by *A. oryzae*, and found that Ag^{+}, Hg^{2+} Fe^{3+}, SDS, diethylpyrocarbonate, castanospermine, dithiothreitol were slightly or completely inhibited the cellulase activity; whereas Mn^{2+} enhanced the activity. Similarly, the activity of cellulase produced by *A. terreus* M11 was increased in the presence of Mn^{2+}; but the presence of Hg^{2+}, Cu^{2+}, Pb^{2+} and detergents slightly decreased or completely abolished the activity of cellulase [72]. Yang et al. [80] demonstrated that the cellulase produced by *Paecilomyces thermophila* was strongly inhibited by Hg^{2+}; while the activity was highly enhanced in the presence Zn^{2+}.

Enzyme kinetics

The maximum velocity (V_{max}) and Michaelis-Menton constant (K_m) are the two constants describing the kinetic characteristics of the enzyme action. The V_{max} describes the specific point in an enzymatic reaction at which the rate of the reaction is catalyzed to the maximum, if other factors are optimum; the V_{max} is attained only when the substrate concentration is sufficiently available to fill the enzyme's active site. K_m is the substrate concentration required to fill half of the enzyme's active site. High K_m describes that the enzyme has less affinity towards substrate; whereas low K_m indicates high affinity towards substrate, thereby higher activity. Liu et al. [81] demonstrated the K_m and V_{max} of two cellulases produced by *A. fumigatus* Z5 as 37.8 mg/mL and 437.3 μmol/min/mg; 51.8 mg/mL and 652.7 μmol/min/mg, respectively. The K_m and V_{max} of cellulase produced by *A. niger* BCRC31494 were found to be 134 mg/mL and 4.6 U/min/mg [82]; while K_m and V_{max} of *P. pinophilum* were: 4.8 mg/mL and 72.5

U/mg, respectively [83]. Lee et al. [84] confirmed the K_m and V_{max} of *P. purpurogenum* KJS506 as 1.15 mg/mL and 220 U/mg, respectively.

Molecular weight of fungal cellulases

The MW of cellulase produced by different fungal species may vary from 12 kDa to 126 kDa [85,86]. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is the most commonly used method for judging the apparent MW of enzymes [87-89]. Fungal cellulase may be of monomeric [90] or dimeric [91] in nature. Cellulase produced by *T. viride* was purified to homogeneity using DEAE-sepharose column and the MW was estimated as 87 kDa by SDS-PAGE [92]. *P. pinophilum* MS 20 produced a monomeric cellulase with MW of 42 kDa, which appeared as a single band on SDS-PAGE gel [83]. It was reported that *A. awamori* VTCC-F099 and *Fomitopsis pinicola* produced monomeric thermo active cellulase with a MW of 32 kDa [93,94]. The cellulase produced by *A. niger* revealed a MW of 60 kDa on SDS-PAGE gel [95]. In all the aforesaid studies, the purified celluase appeared as single band upon SDS-PAGE, indicating that the cellulase produced by these fungi are active in solution as monomers or homodimers, consequently migrates through the SDS-PAGE gel according to their MW so as to segregate as single band. In contrast, some studies reported the identification of hetero-dimeric cellulases or isoforms which appeared as separate bands upon SDS-PAGE. For instance, *A. niger* Z10 produced two cellulase bands on SDS-PAGE gel with MWs 50 and 83 kDa [5,6]. Similarly, another strain of *A. niger* also reported as producing dimeric cellulase with MWs of 23 and 36 kDa; whereas *A. fumigatus* produced dimeric cellulase with MWs of 21 and 32 kDa [96]. Kaur et al. [97] reported 40 and 50 kDa isoforms of cellulase produced by *Melanocarpus* sp. MTCC 3922 with MW as judged by SDS-PAGE.

Applications of cellulases

Cellulases occupy the third most significant industrial enzymes on the global market (i.e, ≈15%) after amylase (≈25%) and protease (≈18%). Enzymatic hydrolysis of cellulosic biomass offers an attractive alternative for the generation of sugars, which can serve as the raw materials for the production of various value added products of commercial interest such as bioethanol [98]; organic acids [99,100]; free sugars, antibiotics and animal feeds [101]. Enzymatic hydrolysis of cellulose is favorably superior to acid and alkali hydrolyses - because enzymes are recoverable, specific, low in energy requirements, and nonpolluting. The production cost and the low yield of cellulase are the major constraints in the economics of the process, which hinder its application in industries. Therefore, the discovery of novel microbial species or mutant strains secreting higher levels of cellulases is still an emerging area of research to develop economically competitive bioprocess strategies applicable on large scale [102]. Cellulase has wide applications in various industries including detergents, textiles, in the production of paper and pulp, bioethanol and organics; some of them are discussed below.

Paper and pulp industries

Cellulases are mainly used for the pulping and deinking of waste papers. Bio-mechanical methods are widely used now-a-days to obtain the suspension of fibres from wood, i.e., the pulp. Application of cellulase for pulping enhances the energy efficiency of the process, and also improves the physical properties such as inter-fibre bonding and mechanical strength of the final paper product [103]. Moreover, it provides environment-friendly processes, limiting the use of harmful chemicals. For instance, cellulase was effectively used for refining the bleached Eucalyptus globules kraft pulp, which enhanced the drainability of the pulp by about 80% without any change in the energy consumption [104]. Similarly, cellulase was used for the modification of cellulose fibres of kraft/sulphate pulp, resulting in improved physical properties of the sheets [105]. Cellulase was also used for deinking of waste papers. During deinking, the ink attached to the surface of recycled cellulose fibres was released by the enzymatic hydrolysis of carbohydrates, leading to the peeling of individual fibres or bundles [106]. The waste papers from various fields offer as important raw material for the pulp and paper industries, as the recycling of used papers reduces the solid wastes and also lessens the burden of deforestation for wood fibres [107,108]. Enzymatic deinking of waste papers, especially employing the mixtures of cellulase and hemicellulase, enhance the quality and brightness of the recycled paper [109-111]. Cellulases are also used to improve the drainage of several paper mills by dissolving clogged fibre residues [112]. Moreover, the cellulase preparations are also used to make easily biodegradable cardboards, tissue papers and sanitary papers [113-115].

Textile industry

Cellulases are widely used to improve the softness and appearance of cellulose-based textiles. In textile industry, cellulases are mainly used for bio-polishing of cotton cloths and biostoning of denim jeans to impart stonewashed look for denims. During the biostoning process, cellulases hydrolyse the small fibre protrusions from the surface and release the indigo dye attached to it, resulting in the dull look of the jeans. It replaces the conventional pumice stones used for the purpose, thereby reducing the fibre damage and human labour [116]. Cellulase produced by *Humicola insolens* and *Trichoderma* is generally used for biostoning of jeans [40,117]. During the process of biopolishing, cellulase hydrolyzes the small protrusions of the fibres from the surface of cotton clothes, thus removes the fussiness of the surface so as to create a smooth and glossy appearance [107,118]. Cellulases are also used to improve the dye absorbance of the fibres and to remove excess dye, giving a colour gradient to the fabrics [112]. Cellulases found potential applications as additive in household laundry detergents for improving fabric softness and brightness. Mild alkaline and thermotolerent cellulases produced by fungi such as *T. viride, T. hurzianum, T. reesei, A. niger* and *Humicola insolens* are generally used for the purpose [119,120].

Food and feeds

Cellulases found potential applications in food and feed processing industries as well. They are the integral part of the macerating enzyme complex (cellulase, xylanase and pectinase), that are used for the extraction and clarification of fruits and vegetable juices, nectars, oils and purees [121,123]. Cellulase-assisted extraction of flavanoids from flowers and seeds enhanced the yield and reduced the extraction time and heat damage, as compared to the conventional acid/alkali/organic solvent/heat extraction methods [101]. Cellulase in combination with other cell wall degrading enzymes can be used to increase the taste and aroma of citrus fruits by reducing the bitterness [112]. As cellulases favour the enhanced release of simple sugars, they found potential applications in alcoholic beverages including beer and wine. It was found that application of cell wall degrading enzymes during malting and fermentation improved liquor yield, aroma and stability [124]. Besides, cellulases are also used in fermented foods and feeds for improving the nutritional quality and digestibility.

Bioethanol

The massive exploitation and utilization of fossil fuels have insistently reduced its natural reserves and caused severe environmental pollution via the release of green house and toxic gases. Hence, the world economy is now focused on biofuels, especially bioethanol from renewable resources, which is expected to replace 20% of the fossil fuel consumption by 2020 [125,126]. The most actively investigated application of cellulase is the production of biofuels, especially bioethanol. Cellulases actively convert the cellulosic renewable resources in to glucose and other simple fermentable sugars that can be used as substrates for the production of bioethanol. Production of bioethanol from lignocelluloses is a multistep process. Initially, the lignocellulosic biomass is subjected to pre-treatment - either mechanically, chemically or enzymatically to remove lignin and hemicelluloses fractions, followed by the treatment with cellulase to release fermentable sugars (pentoses and hexoses). Then the hydrolyzed cellulosic residue is used for the microbial fermentation to produce bioethanol [127]. Agriculture residues such as sugarcane bagasse, straw of wheat, rice and corn; wheat bran, corn stover, etc. were successfully used as raw materials for the production of bioethanol, employing cellulases produced by various filamentous fungi including *Aspergillus, Trichoderma*, and *Penicillium* [41,128-130].

Other applications

Cellulases are also used to generate plant protoplast for genetic manipulation [131], to control industrial slime [132] and to produce cellulase-based chitosan with antibacterial, immunomodulatory and antitumour activities [133,134]. Han and He [135] reported that the commercially available cellulase from *T. reesei* successfully decomposed the straw thereby increasing the soil fertility and plant growth.

Conclusion

Overwhelming demand for natural products has elevated the significance of industrial enzymes; among which, cellulases occupy a pivotal position. The major bottleneck in the commercialization of cellulase is the lacuna in the economically feasible process and to improve the functioning/catalysis of cellulase in tune with the demand. However, the utilization of lignocellulosic wastes has proven as a main contender to overcome the problem to a great extent. Still, the exploration of sustainable substrates, microorganisms and fermentation strategies are to be evolved so as to achieve higher productivity, quality and economic feasibility. Moreover, further studies should be accelerated for the expansion of cellulase research by manipulating the ability of microorganism via gene/protein engineering for the effective utilization of biomass, facilitating better methods for bioconversion as well as solid waste management. In fact, the upper hand of SSF in alleviating the environmental burden due to the heaping up of lignocellulosic biomass has to be exploited with an economic and industrial perspective.

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