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Cellulases: Classification, Methods of Determination and Industrial Applications

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Abstract Microbial cellulases have been receiving worldwide attention, as they have enormous potential to process the most abundant cellulosic biomass on this planet and transform it into sustainable biofuels and other value added products. The synergistic action of endoglucanases, exoglucanases, and β-glucosidases is required for the depolymerization of cellulose to fermentable sugars for transformation in to useful products using suitable microorganisms. The lack of a better understanding of the mechanisms of individual cellulases and their synergistic actions is the major hurdles yet to be overcome for large-scale commercial applications of cellulases. We have reviewed various microbial cellulases with a focus on their classification with mechanistic aspects of cellulase hydrolytic action, insights into novel approaches for determining cellulase activity, and potential industrial applications of cellulases.

Keywords Biofuel · Cellulose · Cellulases · Classification · Cellulase assays · Industrial applications

Introduction

Fossil fuel reserves are depleting at an alarming rate which has developed a grim situation globally drawing the attention of scientific world. The fluctuating costs of these fuels and their harmful environmental impacts have shifted the concerns to exploit renewable biomass sources for the production of biofuels as alternative to petroleum fuels. Worldwide research is focussed to utilize the inexhaustible natural cellulosic biomass efficiently as a raw material

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to develop novel processes for the production of various biofuels. Biotechnological conversion of cellulose biopolymer using microbial cellulases is considered as a foreseeable and sustainable approach for judicial utilization of abundant agricultural lignocellulosic residues to obtain value added chemicals/products. Cellulases account for 8 % share of the industrial enzymes demands globally [1, 2] but the cellulase market is expected to expand up to US$ 400 million per year [3]. Despite enormous research underway, the main obstacle in the path of success is the high cost of cellulases necessitating the need to develop economically profitable processes involving the re-use of the enzymes or to lower the production cost of the same.

Cellulose, an eminent representative of naturally available nanostructure renewable matter, is a component of all plants, algae, and tunics of marine creatures. It has been evaluated that in terrestrial ecosystems, half amount of the fixed carbon is stored in the form of cellulose per annum. Cellulose is synthesized primarily by plants; nonetheless, some microorganisms including algal species and animals are also able to produce it [4, 5]. Cellulose has a complex multilevel architecture made up of bundles of microfibrils. Each microfibril may contain 36–1200 cellulose chains which are held together by hydrogen bonds and van der Waal forces ultimately forming a highly ordered crystalline structure. Each cellulose chain is a non-branched chain of D-glucose monomers that may range from 100 to 20,000 linked together by β-1, 4 glycosidic bonds [6]. The basic repetitious unit of cellulose is cellobiose, a disaccharide not glucose in contrast to other glucan polymers. These well-organized crystalline structures are interspersed with disordered or disorganized domains (amorphous cellulose) constituting 5–20 % of the microfibril [1]. Hydrogen bonding interactions in these amorphous regions are suboptimal, thus accessible for water molecules and enzymatic attack [7]. Complete enzymatic degradation of cellulose to glucose is achieved by synergistic action of three enzymes including endoglucanases, exoglucanases, and β-glucosidases.

Cellulases and Taxonomic Diversity

Cellulases are produced by various microorganisms including actinomycetes, bacteria, and fungi. A lot of reviews have listed the different types of microorganisms involved in the degradation of cellulose [8–11]. It is now reported that cellulases are also produced by some animals like termites and crayfish, but their function in animal system is still unclear [12]. Microorganisms with cellulolytic potential are listed in Table 1. Cellulolytic members are distributed among the entire kingdom of fungi, including primitive ones (Chytridiomycetes) to advanced groups (Agaricomycetes). A large number of cellulolytic species have been reported in subdivisions Ascomycetes, Agaricomycetes, and Deuteromycetes, whereas in Zygomycetes, only a few members have the cellulose degrading ability [13]. Within the Eubacteria, cellulolytic species mainly belong to phyla Actinobacteria, Bacteroids, Fibrobacteres, Firmicutes, Proteobacteria, Spirochaetes, and Thermotogae. Out of these, anaerobic order Clostridiales (phylum Firmicutes) and predominately aerobic order Actinomycetes (phylum Actinobacteria) comprise maximum number (80 %) of cellulolytic members [14]. Cellulose degrading bacteria can be classified into different physiological groups: (i) aerobic gram-positive bacteria (*Cellulomonas* and *Thermobifida*); (ii) aerobic gliding bacteria (*Cytophaga* and *Sporocytophaga*), and (iii) fermentative anaerobes: mostly gram-positive bacteria (*Caldicellulosiruptor*, *Clostridium*, and *Ruminococcus*) and a few gram-negative bacteria (*Acetivibrio*, *Butyrivibrio*, and *Fibrobacter*) [14]. Cellulose hydrolyzing strategy differs in anaerobic and aerobic groups of bacteria. Anaerobic bacteria degrade cellulose

mostly via complexed cellulase system (cellulosome) [15] with some exceptions [16, 17], whereas aerobes via non-complexed cellulose system, i.e., extracellular free cellulases that can be recovered from the supernatant [18]. Aerobic fungi and bacteria are gaining biotechnological attention regarding cellulose degradation because of their characteristic fast growth and high yields of enzymes. Screening and isolation of cellulose degrading microorganisms from different habitats are of crucial importance to get novel cellulases with unique characteristics.

Classification of Cellulases

Cellulases produced from microorganisms are either cell bound or extracellular and also differ on the basis of their modes of action. Following types of cellulases have been described with their mechanisms of action [10, 19]:

Table 1 Micro-organisms with cellulose degrading ability

Fungi	Aerobic bacteria	Anaerobic bacteria	Actinomycetes
Aspergillus aculeatus, *A. candidus*, *A. flavus*, *A. heteromorphus*; *A. niger*; *Bulgaria* sp.; *Chaetomium* spp.; *Cladosporium* sp.; *Coriolus* sp.; *Fusarium* sp.; *Geotrichum* sp.; *Helotium* sp.; *Myrothecium* sp.; *Paecilomyces* sp.; *Penicillium waksmanii*; *Phanerochaete* sp.; *Poria* sp.; *Schizophyllum* sp.; *Serpula* sp.; *Trichoderma aureoviride*, *T. reesei*; *Tricothecium roseum*	*Acetobacter oboediens*, *A. pasteurianus*; *Acidothermus cellulolyticus*; *Acinetobacter amitratus A. junii*; *Aeromonas sp.*; *Anoxybacillus sp.*; *Bacillus amyloliquefaciens*, *B. cereus*, *B. circulans*, *B. flexus*, *B. licheniformis*, *B. pumilus*, *B. subtilis*, *B. thuringiensis*; *Bacteroides sp.*; *Botrytis sp.*; *Cellulomonas bioazotea*, *C. cellulans*, *C. fimi*, *C. flavigena*, *C. uda*; *Cellvibrio gilvus*; *Citrobacter freundii*; *Cytophaga hutchinsonii*; *Enterobacter sp.*; *Erwinia sp.*; *Escherichia coli*; *Eubacterium cellulosolvens*; *Geobacillus pallidus*, *G. stearothermophilus*, *G. thermodenitrificans*; *Gluconacetobacter entani*, *G. europaeus*, *G. hansenii*, *G. intermedius*, *G. xylinus*; *Halomonas caseinilytica*, *H. muralis*; *Klebsiella pneumonia*; *Microbispora bispora*; *Paenibacillus curdlanolyticus*; *Proteus vulgaris*; *Pseudomonas aeruginosa*, *P. cellulose*, *P. fluorescens*; *Rhodothermus marinus*, *Salinivibrio sp.*; *Serratia liquefaciens*	*Acetivibrio cellulolyticus*; *Butyrivibrio fibrisolvens*; *Clostridium acetobutylium*, *C. cellulolyticum*, *C. papyrosolvens*, *C. thermocellum*, *Fibrobacter succinogenes*; *Ruminococcus albus*	*Streptomyces drozdowiczii*, *S. lividans*; *Thermomonospora curvata*, *T. fusca*

Endoglucanase or Glucanohydrolase (EC 3.2.1.4)

This enzyme attacks randomly at the internal sites of the amorphous region of the cellulose chain thereby generating new chain ends and oligosaccharides with varied lengths. It is active against soluble forms of cellulose like carboxymethyl cellulose (CMC) and amorphous cellulose.

Exoglucanase or Cellobiohydrolase (EC 3.2.1.91)

This enzyme acts on reducing or non-reducing ends of cellulose chain. On the basis of major product obtained after its action, it is named as glucanohydrolase (liberating glucose) and cellobiohydrolase (liberating cellobiose). It is active on crystalline substrates like avicel, cellooligosaccharides, etc.

Exoglucanase or Cellodextrinase (EC 3.2.1.74)

It acts to remove cellobiose from cellooligosaccharides. It is generally inactive against amorphous cellulose or soluble forms like CMC.

β-glucosidase (EC 3.2.1.21)

It acts on non-reducing ends and hydrolyzes cellooligosaccharides and cellobiose to glucose. This is inactive against both crystalline and amorphous cellulose.

Cellobiose Phosphorylase or Cellobiase (EC 2.4.1.20)

Also known as orthophosphate α-D-glucosyl transferase, it catalyzes the reversible phosphorolytic cleavage of cellobiose to glucose.

Cellodextrin Phosphorylase (EC 2.4.1.49)

Also known as 1,4-β-D-oligoglucan orthophosphate α-D-glucosyl transferase, it catalyzes the reversible phosphorolytic cleavage of cellodextrins (cellotriose to cellohexose) to glucose. It does not act on cellobiose.

Cellobiose Epimerase (EC 5.1.3.11)

This catalyzes the epimerization of disaccharides like cellobiose into 4-*O*-β-D-glucosylmannose.

All the cellulases target β-1, 4 glycosidic bonds of the cellulose polymer either by exo- or endo-cleavage. Exoglucanases are found to be active on crystalline regions but endoglucanases are typically active on more soluble amorphous regions of cellulose [20, 21]. The cellulase system hydrolyzes cellulose by collective action of all the enzymes which are described as synergism. In literature, different forms of synergism have been suggested: (i) intramolecular synergy between catalytic domains (CDs) and cellulose binding modules (CBMs); (ii) exo-exo synergy between exoglucanases cleaving the reducing and non-reducing ends of cellulose chains; (iii) endo-exo synergy between endoglucanases and exoglucanases; and (iv) synergy between exoglucanases and β-glucosidases [22, 23].

Methods for Quantification of Cellulases

All the existing methods used for the determination of cellulase activity are classified on the basis of the (a) amount of products accumulated after substrate hydrolysis, (b) decrease in the quantity of substrate during assay, and (c) change observed in the physical properties of the substrate after assay [3]. Most common assay methods measure the accumulated hydrolysis products like total sugars, reducing sugars, and chromophores. Two commonly used methods for cellulase activity measurement include Dinitro salicylic acid (DNS) method and Nelson-Somogyi method; as they have broad detection range of reducing sugars as well as low interference from protein. Two strategies can be applied in order to improve the detection range of sugar assays: (1) dilution after the color development and (2) variation in the sample volume added per sample before carrying out the reaction. However, there is a poor stoichiometric correlation between the glucose standard and cellodextrins in these assay methods [24, 25]. On the other hand, ferricyanide, 2, 2 Bicinchoninic acid (BCA), and *p*-hydroxy benzoic acid hydrazide (PAHBAH) assay methods are highly sensitive in detection of reducing sugars but major disadvantage is the non-specific interference of protein. Assay methods involving the detection of total sugars like phenol-H_2SO_4 and anthrone-H_2SO_4 are much sensitive and less affected by proteins but they are limited to be used for pure cellulases [3]. A lot of other assay methods have also been reported to determine cellulase activity [3, 26–28]. Reduction in substrate quantity also drew the attention to determine cellulase activity by chemical methods and gravimetric methods. But these methods did not gain much popularity as they were very tedious to perform. Chemical methods to calculate substrate loss include phenol-H_2SO_4 [25, 29] and anthrone-H_2SO_4 methods [30, 31]. Activity assay including change in physical properties of substrate have also been utilized to quantify cellulase activity. As viscosity is related to the concentration of polymer, so cellulase activity can be determined with quite accuracy by measuring the rheological behavior of carboxymethyl cellulose (CMC) solutions [32]. A lot of commercial kits based on these assay methods have been available for cellulase activity. Assay kits based on calorimetric method include Megazyme D-glucose oxidase/peroxidase (GOPOD) assay kit, NZYTech D-glucose GOPOD assay kit, and Sigma glucose assay whereas assay kits based on fluorometric estimation are also available like Abcam cellulase assay kit, MarkerGeneTM Fluorescent cellulase assay kit, etc. All these assay kits are highly sensitive and work at microplate level.

Endoglucanase Assay

Endoglucanases cleave in a random fashion on the internal β-1, 4 glycosidic linkages of cellulose chains. Soluble cellulose derivative such as CMC with varying degree of polymerization can be used for measuring endoglucanase activity. Endoglucanase activity can be measured by determining the change in substrate viscosity and/or by reducing sugar assay. Reducing sugar assay measures the increase in reducing ends due to intra-molecular cleavages in the substrate chains. Carboxymethyl cellulase (CMCase) activity is mainly measured by the method described by Mandels et al. [33]. According to this method, one unit (IU) of CMCase activity is described as the amount of CMCase needed to release 1 μmol of reducing sugars per milliliter per minute under the given assay conditions. Other methods to measure the reducing sugars include Nelson-Somogyi method [34, 35], glucose oxidase (GOD)/peroxidase reagent [36], and high-performance liquid chromatography (HPLC) [37, 38].

Although CMC is commonly used substrate [39–41] but there are several issues associated with it. This method is non-reproducible, with linearity observed within a limited degree of hydrolysis of CMC due to interference by substituents. This is because substituted glucose units available in different types of CMC are also hydrolyzed by cellulase and thus result in non-reproducibility [3, 42]. Furthermore, the amount of reducing sugars produced in the assay is highly affected by type of CMC used ultimately affecting the enzyme units calculated [42]. To avoid these misinterpretations, non-ionic substituted cellulose such as hydroxyethyl cellulose (HEC) is gaining preference [43, 44]. Endoglucanase activity can also be determined using various soluble oligosaccharides and substrates substituted with chromophores, e.g., methylumbelliferyl-β-D-glucosides, p-nitrophenyl glucosides, etc. The basis of these methods is to detect the release of chromophore or the oligosaccharide fragments. The formation of oligosaccharide fragments can be detected by HPLC or thin layer chromatography (TLC) [38, 45, 46].

Some other methods of qualitative determination of endoglucanase activity are gaining attention in which a dye is added to the soluble cellulose derivatives and hydrolysis zone after incubation with enzyme is analyzed as these dyes are retained by long chain polysaccharides only. But these methods are semi quantitative as there is no linear relationship between halo zones and enzyme activity. Some dyes like Brilliant Red 3B-A, Cibacron Blue 3GA, and Reactive Orange 14 have been reported to be added to the cellulose derivatives or chemically substituted to produce chromogenic cellulose substrate and used further for plate assays [47]. Other dyes used in CMCase assays were Remazol Brilliant Blue R and Ruthenium Red, but Gram's iodine has also been reported as a fast and easy detection dye for determining enzyme activity [48].

Exoglucanase Assay

Also known as cellobiohydrolases as they are exo-acting enzymes that cleave β-1, 4 glycosidic bonds from the chain ends of the cellulose thereby generating cellobiose and other cellodextrins. Commercially, available avicel (microcrystalline cellulose or hydrocellulose) is usually used for measuring exoglucanase activity or avicelase activity as it is crystalline in nature and has a low degree of polymerization. Enzymes showing relatively high activity on avicel but little or no activity on carboxymethyl cellulose are classified as exoglucanases. But as some soluble cellodextrins and amorphous cellulose are also present in avicel, which can be hydrolyzed by both endo- and exo-glucanases thus supporting the fact that there is no highly specific substrate for determining exocellulase activity [49, 50]. Assays reported till date have some sort of limitations in assessing exoglucanase activity. 4-methylumbelliferyl-β-D-lactoside was reported as an efficient substrate to determine cellobiohydrolase I (CBH I) of *Trichoderma reesei*, where 4-methylumbelliferone (fluorescent signal molecule) is produced along with lactose and phenol [51]. But this substrate was not hydrolyzed by CBH II of *T. reesei* thus cannot be used for determining the true exoglucanase activity of this strain [52, 53].

Another method to detect exoglucanase activity with endoglucanase and β-glucosidases being present was reported by Deshpande et al. [54] which was based on the specific hydrolysis of agluconic bond, yielding cellobiose, and *p*-nitrophenol after the hydrolysis of p-nitrophenyl-β-D-cellobioside. In this assay, addition of D-glucono-1, 5-δ-lactone repressed the interfering β-glucosidase activity [55] whereas the interference of endoglucanases was also compensated for by carrying out the prior standardization of the procedure of the assay using a purified endoglucanase from the studied mixture. But CBH II activity of *T. resei* cannot be

measured using this substrate owing to the fact that specific activity of the purified endoglucanases used may not be a true measure of the endoglucanases activity determined in the mixture. In addition, the products obtained after the endoglucanases action might be influenced by the action of exoglucanases [3]. Numerous synthetic conjugates (cellobiosides) of the disaccharide subunit of cellulose have been reported so far for the determination of cellulase activity. But most of them are time consuming and difficult to quantitate. Different microplate assays have been developed commonly employing fluorogenic substrates (cellobiosides) like 4-methylumbelliferyl β-cellobioside (4-MUCB) [56], MU-β-D-cellobioside (MU-C) [57], resorufin-β-D-cellobioside (Res-CB) [58], chromogenic substrates like 5-bromo-4-chloro-3-indolyl-β-D-cellobioside (X-CB) [59]; as well as non-chromogenic substrates like bacterial microcrystalline cellulose (BMCC) [60], phosphoric acid-swollen cellulose (PASC) and regenerated amorphous cellulose (RAC) [44].

β-glucosidase Assay

The procedure for quantification of β-glucosidase activity was given by Kubicek [61]. Different kinds of chromogenic or fluorescent compounds releasing substrates like 6-bromo-2-naphthyl-β-D-glucopyranoside, 4-methylumbelliferyl-β-D-glucopyranoside, β-naphthyl-β-D-glucopyranoside, and p-nitrophenyl β-D-1, 4-glucopyranoside [3, 57] as well as non-chromogenic substrates like cello-oligosaccharides or cellobiose [62, 63] can be utilized to determine activity. They hydrolyze cellobiose or other cello-oligosaccharides with degree of polymerization up to six ultimately producing glucose in the liquid phase. Therefore, these enzymes should not be named as 'cellobiases' as this term is misleading because they are able to hydrolyze substrate beyond the degree of polymerization (DP) of 2. DNS method can be used to measure the β-glucosidase activity by using non-chromogenic polysaccharides as substrate and reducing sugars released will be measured [64]. In DNS method, enzyme activity is expressed as the amount of enzyme that liberates 1 μmol of glucose or reducing sugars per milliliter per minute [33]. Cellobiose can also be used as a substrate to determine the β-glucosidase activity with available commercial kits based on GOD method. Usually, chromogenic substrate *p*-nitrophenol-β-glucoside (*p*NPG) is used to quantify β-glucosidase activity which is defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol per minute under the assay conditions [3, 39, 65–67]. There are some other substrates gaining attention to detect β-glucosidase activity like gentiobiose, sophorose, amygdalin, salicin, daidzin, genistin, barley, laminarin, etc. [67–72].

Total Cellulase Activity

Filter Paper Assay (FPA) is a true method for analysis of total cellulase activity, utilizing Whatman No. 1 Filter paper as the substrate [73]. Total cellulase activity includes the synergic action of endoglucanases, exoglucanases, and β-D-glucosidases on cellulose. But there are many concerns associated with the use of FPA to measure the total cellulase activity due to non-reproducible nature of this method. Moreover, FPA is less sensitive, laborious, and time-consuming and also requires large amounts of reagents. Most natural cellulase complexes lack β-glucosidase activity [74] thus affecting its reproducibility and sensitivity. Coward-Kelly et al. [74] however worked on a different aspect to improve FPA by supplementing the enzyme mixture with external β-glucosidase. Various insoluble substrates can be utilized for the evaluation of FPA like algal cellulose, bacterial cellulose, microcrystalline cellulose,

Sigmacell-20, Whatman No. 1 filter paper, and cellulose-containing substrates like dyed cellulose, α-cellulose, and pretreated lignocellulose [3]. Filter paper assay measurement is not only perfect but is also simple, reproducible, quantitative, and predicts enzyme action under practical saccharification conditions [42]. Nordmark et al. [75] modified the FPA assay for enzyme preparation with activities too low to be detected by traditional International Union of Pure and Applied Chemistry (IUPAC) approved filter paper assay. The modified assay utilized potassium chloride and Bovine serum albumin (BSA) as protein stabilizers to reduce the enzyme inactivation with time during the traditional enzyme assay. This modified assay measured relative filter paper activities taking into account the time needed for hydrolysis of 3.6 % of the traditional substrate, which can be compared to results observed in traditional IUPAC assay. Camassola and Dillon [76] made certain adaptations to the FPA to create a low-cost, easier, faster, less labor intensive, and less polluting alternative while maintaining similar results to the standard method. The modified method involved the use of 50 μl of enzyme into a deep well plate and incubating with 1 × 6 cm Whatman no. 1 filter paper strip after adding 150 μl of buffer. Although the collected data indicated the possibility of realizing measurement of total cellulases in small volumes than those proposed in the standard method as in a less laborious way.

Novel Approaches for Determining Cellulase Activity

A few novel approaches of cellulase assays came into light with enhanced features like ease of operation, better sensitivity, greater reproducibility, and high-throughput screening (Table 2). Decker et al. [77] has exemplified the complete automation of traditional FPA using Cyberlabs C400 robotics deck. The automated version created by this group has customized reagent storage, incubation, and plate reading capabilities in order to reduce operator error during assay. This high-throughput technique significantly reduced the usage of reagents in addition to lower reagent disposable costs. Similarly, a more sensitive assay was introduced by Helbert et al. [78] where fluorescent microfibrils have been prepared from bacterial cellulose by using 5-(4, 6-dichlorotriazinyl) aminofluorescein (DTAF) as a grafting agent. The DTAF bear dichlorotriazinyl groups which can interact with the hydroxyl group of polysaccharides thus justifying its use in enzyme assays following the digestion of cellulose resulting in liberation of fluorescent compounds like cellodextrins and reducing sugars. Thus, this method is efficient enough to differentiate between exo- and endoglucanase activities as the amount of released fluorescence can be compared with the amount of released reducing sugars. They also provided possible automated version of the assay by casting films of fluorescent microfibrils to the bottom of microtiter plates. The main advantage of the assay was its efficiency and sensitivity to quantify even minute amounts of enzyme activity. Natural cellulose was used for assay instead of other substrates like CMC which are quite different from native cellulosic substrate. Excess β-glucosidase addition resulted in the enhancement of the assay sensitivity.

Miniatured assays are coming up and standardization of these assays has greatly influenced the screening process. Mini assay is advantageous as it reduces the quantity of reagents ultimately resulting in reduction of time, cost, and error during quantification. Downsizing the FPA to 96-well microtiter plates has improved the use of assay in evaluating a large number of samples simultaneously [79]. The IUPAC filter paper assay is labor-intensive, consumes a lot of time and requires large amount of reagents. 96-well microplate assay is adequately sensitive and reproducible unlike IUPAC FPA. Similarly, miniatured CMCase

Table 2 Determination of cellulase activity using novel techniques and their advantages

S. No.	Cellulase assay	Enzyme activity	Substrate	Advantages	References
1.	Automated FPA	FPA	FP	Reproducible, high-throughput, reduced reagent usage	[77]
2.	Fluorescent microfibrils	Endoglucanase, Exoglucanase activity	Bacterial cellulose	Possible automation, native cellulose, sensitive cellulose detection	[78]
3.	Miniaturized colorimetric assay	FPA	FP	Reproducible, sensitive, High-throughput, comparable, reduced reagent usage	[79]
		CMCase	CMC	High-throughput, reproducible, comparable, reduced reagent usage	[80]
		Cellulase activity	FP, Avicel, corn stalk, switchgrass, CMC, arabinoxylan	High-throughput, comparable results, various substrates, reduced reagent usage	[81]
		Cellulase activity	Wheat straw, Spruce	Fully automated, high throughput, reproducible, lignocellulose substrates	[82]
4.	Gravimetric analysis	Cellulase activity	Cellulose acetate films	Improved sensitivity, highly efficient	[83]
5.	Filter paper collapsing method	FPA	FP	Improved sensitivity, rapid screening	[84]
6.	Fluorescence Resonance Energy Transfer	CMCase	Labeled CMC with 5-(aminomethyl) fluorescein	Rapid real time investigation, highly sensitive, comparable results	[85]
7.	Quartz crystal microbalance dissipiation	Cellulase activity	FP, Avicel, CMC, lignocellulosic nanofibrils (LCNFs) films	Various substrates, Easy to implement. Results comparable with traditional assays	[88]
8.	Isothermal Titration calorimetry	Cellulase activity	Cello-oligosaccharides (cellobiose, cellotriose, cellotetraose, cellopentaose, cellohexaose)	Precise determination, High sensitivity and reproducibility	[89, 90]
		Cellobiohydrolase activity	Bacterial cellulose, Cellobiose, RAC	Complex substrates, Precise, Highly sensitivity and reproducibility	[91]
		Endoglucanase activity	Cellulose	Quantitative precision, Highly sensitive and reproducible, complex substrates	[92]
9.	Surface Plasmon Resonance	Cellulase activity	Cellulose microfibrils, β-glucan, CMC, PASC, Avicel	Complex substrates, high sensitivity, real time detection	[93–96]
10.	Electrochemical Biosensor	Cellobiose dehydrogenase	Avicel		[104]

Table 2 (continued)

S. No.	Cellulase assay	Enzyme activity	Substrate	Advantages	References
				Rapid, readily implemented, nondestructive, comparable precision	
		cellobiohydrolase	Avicel, RAC	High sensitivity, low detection limit, fast response time	[98]
		Cellobiohydrolase	Avicel, BMCC	Anomer unspecific, High operational stability, sensitivity and specificity	[99]
		Cellobiohydrolase	Avicel	Fast response, comparable results, High sensitivity and specificity, resistant to electrode fouling	[100]
11.	Fluorescence activated cell sorting	Endoglucanase	CMC	Ultra-high-throughput, automated, rapid, 12-fold enrichment of cellulase-expressing cells	[107, 108]
12.	Droplet microfluidics	Endoglucanase, β-glucosidase	CMC, Fluorescein Di-β-D-Galactopyranoside (FDG)	Ultra-high-throughput, sensitive, flexible, automated, precise, 300-fold enrichment of cellulase-expressing cells	[109, 110]
13.	Calorimetric method	Cellulase activity	Wheat straw, Filter paper	Rapid, sensitive, easy method, low detection limit, no boiling or heating required, complex substrates	[111]
14.	CMC gel layers	Endoglucanase	CMC	Reliable, rapid, inexpensive, spatial mapping of cellulolytic activity	[112]
15.	Metagenomic screening	Cellulase activity	Dinitrophenol (DNP)-cellobioside substrate	High-throughput screening, automated, rapid, sensitive	[113]
		Cellulase Cellobiohydrolase β-glucosidase	AZCL-HE-Cellulose, 4-MUB-β-D-Cellobiose, PNP-β-D-Glucoside	Fully automated, rapid, high sensitivity, and reproducibility Ultra throughput, various substrates	[114]

FP filter paper, *FPA* filter paper activity; *CMC* carboxymethyl cellulose, *PASC* phosphoric acid-swollen cellulose, RAC regenerated amorphous cellulose, *BMCC* bacterial microcrystalline cellulose

assay has been found to be comparable in accuracy to that of standard IUPAC procedure and reduces the amount of enzyme and reagents relatively by 15-folds [80]. This microplate assay is simple, reproducible, and cost effective with adequate accuracy that can be used rapidly to determine the CMCase activity. Thus, this modified CMCase assay was significantly appropriate for high-throughput large-scale screening of samples for assessing endoglucanase activity. Furthermore, King et al. [81] determined the total cellulase activity based on 96-well microtiter plate assay in which enzyme extracts with different type of lignocellulosic substrates like arabinoxylan, avicel, CMC, corn stalk, filter paper, and switchgrass were incubated. The use of complex biomass substrates can provide insight into the mechanism of enzyme hydrolysis thus providing a useful platform for high-throughput screening of novel enzymes with unique biomass degrading properties. The use of DNS to measure reducing sugars produced in a miniatured platform represented a technological breakthrough truly. But most of the reported methods were not fully automated thus influenced by operator errors at one or more steps. Keeping this in mind, Navarro et al. [82] described a completely automated system, i.e., without any manual step to measure the reducing sugars released from lignocellulosic biomass substrates during the assay. This method involved two separate automated steps, wherein the first step was the formation of 96-well substrate microplates which were prepared with the slurry of micronized substrate (wheat-straw or spruce) and frozen until use. The second step was the enzyme assay after the addition of enzymes to the substrate plates. The whole process until the quantification of reducing sugars released was autonomously performed by robots. This method represented a high-throughput, fully automated screening method for determining cellulase activity with great efficiency. The advantages of this method involved (1) automation of the entire assay; (2) availability of automatic plate sealer avoiding evaporation issues reliably; and (3) micronization of the substrate which did not change the structure of the biomass substrate but allowed uniform distribution into microplates.

In addition, Jang et al. [83] reported the determination of cellulase action by gravimetric analysis of the substrate loss. The uniform cellulose acetate (CA) films in the presence of buffer were incubated with cellulase on a constant temperature rotator shaker and biodegradation of ultravoilet (UV) irradiated CA films was estimated. The SEM analysis of the surface of the CA films was also done to confirm the degradation. The cellulase action was determined gravimetrically by measuring the % weight loss of the cellulase-treated CA films in comparison to the untreated CA films. The cellulase activity can be determined with increased efficiency and sensitivity thus improving the screening process. In an another approach, Toyama et al. [84] reported filter paper collapsing method involving Whatman no. 2 filter paper as substrate and assay was carried out with a constant temperature oscillator and cellulase activity was calculated in relation to collapse of filter paper. The term ‘collapse’ referred to the condition where only fibers were present in the reaction mixture but no filter paper fragments. The reaction mixture was filtered after collapse and reducing sugars were measured to quantify cellulase activity.

Some novel real-time approaches like fluorescence resonance energy transfer (FRET), quartz crystal microbalance (QCM), isothermal titration calorimetry (ITC), and electrochemical sensors have also added new dimensions to the existing knowledge regarding mechanistic and molecular aspects of cellulase kinetics. The FRET has found a wider application in both biological and biophysical fields to evaluate the binding properties of an enzyme and its substrate. It was employed successfully in molecular studies related to the temperature dependence of cellulase binding to cellulose. The real-time cellulase–cellulose interaction was investigated on the basis of FRET signal obtained from donor-conjugate (CMC) to the

acceptor–conjugate (cellulase) in a homogenous liquid environment detected using steady-state fluorescence spectrometer [85]. Another choice is QCM piezoelectric-sensing technique which is a very easy method to implement for measuring cellulase activity in terms of changes in the crystallinity of the substrate [86–88]. Quartz crystal has piezoelectric property, and when enzyme mixture is applied to the cellulose films, molecular binding interactions reduce the sensor's frequency. In situ and *real-time* quantification of rate of degradation of the cellulose film can be performed using piezoelectric resonators [86]. Changes in frequency of quartz crystal are also used to measure the change in density and viscosity of a solution after enzymatic hydrolysis of cellulosic substrate. Thus, cellulase activity can be determined by piezoelectric sensing device and are closer to those obtained by reducing sugar measurement method. This method eliminates the need for color development as well as flexible in dealing with the properties of the substrate used. The piezoelectric sensing presented a simple and efficient method to monitor the kinetics and mechanism of enzymatic degradation [87]. QCM is a promising application in determination of cellulase activity as it uses a simple operation principle. It is also a reliable method in situations where substrates other than pure cellulose are to be used. Kumagai et al. [88] studied in situ quartz crystal microbalance with dissipiation (QCM-D) monitoring to investigate the impact of steam treatment on the hydrolysis of lignocellulosic nanofibrils by cellulases. The QCM-D results suggested that rapid degradation of hemicellulose occurs first resulting in the exposure of cellulose microfibrils for main enzymatic hydrolysis. Thus, QCM-D monitoring is a powerful method to provide valuable information about the impacts of individual components of lignocellulose on enzymatic hydrolysis. With the technological advances, ITC is becoming a powerful and mainstream tool in the study of enzyme kinetics [89, 90]. The ITC is a feasible data analysis method with a real-time approach which provides a way to evaluate enzyme kinetics by detecting the heat generated in an enzyme catalyzed reaction. Karim and Kidokoro [89, 90] studied the kinetics of a cellobiohydrolase with high sensitivity and reproducibility. They conducted normal-phase HPLC along with the ITC to probe the cleavage patterns of cello-oligosaccharides continuously thus elucidating the time course of enzymatic reaction with more efficacy and precision. Murphy et al. [91] developed an enzymatic signal amplification system for studying the reaction kinetics of cellobiohydrolases using ITC. The ITC amplification system evaluated that the heat flow can be considered as true measure of the cellobiose production. This method is advantageous as it is a real-time approach that measures reaction rate directly using complex substrates thus it may become valuable to probe mechanism of cellulolytic action. Moreover, initial kinetics studies of endoglucanase activity on cellulose substrate were studied using ITC [92]. They studied the molecular origins of the endoglucanase activity of *T. resei* on cellulose based on the calorimetric and chromatography data and then the amperometric detection of glucose and cellobiose.

Surface plasmon resonance (SPR) has also been used as another real-time approach to analyze the kinetic behavior of the cellulose hydrolysis by studying the binding interactions [93]. The kinetic data on the recombinant protein binding with CMC was collected using thermostatted IAsys™ resonant mirror biosensor (An affinity sensor). This method provided insights into the binding properties of endoglucanase with immobilized CMCase thus determining kinetic parameters of the protein-ligand interaction [94]. Allen et al. [95] studied the cellulase mixture interaction with cellulose microfibrils present on thin-layered thioglucose invested onto a gold film using SPR imaging. The decrease in thickness of the microfibrils is measured with time using SPR imaging providing real-time insights into effectiveness of enzyme on cellulose. This technique is an effective measure of the enzyme activity as a

function of time with great sensitivity. Jeon et al. [96] measured the real-time interactions of the cohesions and the cellulosomal enzymes of *Clostridium cellulovorans* by SPR. The study employing SPR along with Immunoflourescence and Enzyme linked Immunosorbent Assay (ELISA) elucidated the functional role of recombinant endoglucanase in the cellulosomal system as well in cellulose hydrolysis.

The implementation of biosensor for determining the cellulase activity gained a lot attention as they were very simple and convenient. A biosensor is a device that contains a biological component acting as the sensor and an electronic component that detects and transmits the signal. Electrochemical biosensors are emerging as a novel efficient approach for real-time measurements of mechanistic aspects of cellulolytic action [97–100]. The electrochemical approach is a widely applicable as a highly sensitive, real-time detector for analysis of multiplicity of hydrolytic enzymes. Electrochemical amperometric enzyme biosensors based on immobilized enzymes include glucose oxidase (GO_x) [101] and pyrrolo-quinoline quinine-dependent glucose dehydrogenase (GDH) [102] to detect enzyme activity has been well reported. In glucose oxidase biosensor, enzyme GOD is used in the membrane of the electrode to detect and finally relay glucose concentrations. But this biosensor is unable to detect small oligosaccharides and results may be affected due to some interfering reactions [103]. Another amperometric biosensor which has found potential application in cellulase determination is the amperometric redox polymer-based biosensor containing cellobiose dehydrogenase (CDH) [104]. The CDH biosensor is found to be faster, reliable, and more convenient method in rapid analysis of as much as 30 samples in an hour. This method allows precise and sensitive detection of reducing sugars except glucose. An amperometric biosensor based on pyrroloquinoline quinone-dependent glucose dehydrogenase which was immobilized on benzoquinone-mixed carbon paste electrode was introduced for the detection of cellobiose. This biosensor was quite efficient in continuous and direct observation of cellulose hydrolysis by CBH [105]. But all these biosensors could detect the β-anomeric form of the analytes formed and unable to detect α-anomer product. Cruys-Bagger et al. [98] implemented an amperometric enzyme biosensor which utilized *Phanerochaete chrysosporium* cellobiose dehydrogenase (PcCDH) for the continuous detection of cellobiose. They presented a novel CDH-based biosensor in which PcCDH was immobilized and cross-linked on the carbon paste electrode surface. It can be used for monitoring cellobiohydrolases activity on cellulosic substrates with real-time approach. The response obtained after enzymatic reaction was amended for the β-anomer specificity of PcCDH, and this approach was validated using HPLC. This biosensor was a rapid, highly sensitive with low detection limit which can be utilized to gain insight into the molecular mechanism of enzymatic degradation. Recently, Cruys-Bagger et al. [99] described a novel mediator amperometric biosensor based on immobilized pyranose dehydrogenase (PDH) to measure the kinetic analysis of hydrolyzing activity of cellulase on insoluble cellulose. This biosensor was anomeric unspecific and can be exploited to measure real-time activity over different time scales of both retaining and inverting cellulases. The PDH biosensor has showed high operational stability of several weeks with daily use. Recently, an electrochemical approach based on graphene screen-printed carbon electrode (SPCE) tested for the enzymatic action of cellobiohydrolase Cel7A from *Hypocrea jecorina* on microcrystalline cellulose [100]. A graphene-modified SPCE is advantageous as it can overcome the limitation of electrode fouling due to the oxidation products of *p*-nitrophenol. The current method provided a useful information for the analysis of different molecular states of cellulases thus helpful in development of a better description of molecular mechanism of enzymatic action. The information derived from this current approach

can be used to develop kinetic models to understand and identify the rate limiting processes for different types of enzymes including cellulases.

Some progress has been made in the determination methods for cellulase screening with the involvement of microfluidics [106] or fluorescence-activated cell sorting (FACS) [107, 108]. These techniques tremendously increase the speed of the samples screening and also reduce effectively the amount of reagents required. A high throughput screening method based on FACS in coordination with double emulsion technology has been developed for detecting cellulase expressing cells [108]. Using this approach, a 12-fold enrichment can be obtained in one sorting round proving itself as a promising approach for directed protein evolution studies. But in FACS-based cellulase screening system, some false signals can also be obtained due to effects using polydisperse double emulsions and it is impossible to differentiate fluorescence changes due to enzyme activity from these effects. Droplet-based microfluidics hold an enormous potential to be used for high-throughput screening applications [109, 110]. Ostafe et al. [110] proposed a modified method for ultra high-throughput screening for cellulase activity in samples based on microfluidic sorting. They reported a method based on droplet microfluidics for sorting libraries containing different percentage of cells with cellulase activity and further enrichment of this population. The design of the assay used a chain of coupled enzymatic reactions, leading to the formation a fluorescent compound that could be detected on a chip. Thus, fluorescence corresponding to cellulase activity can be measured with 300-fold enrichment of the cellulase expressing cells. The microfluidic method was found to be more sensitive, flexible, and quantitative than the FACS-based system related to enzyme activity detection.

Recently, a novel, quick, sensitive, and a facile Chit-Oligosaccharide (ChitO)-based calorimetric method was proposed by Ferrari et al. [111] which can be applied for high-throughput screening of cellulases. This assay was based on the use of two oxidases including ChitO and its mutant oxidase ChitO-Q268R for determining chitinase and cellulase activity, respectively. Hydrogen peroxide was released by this oxidase upon oxidation of cellulase produced hydrolytic products. A second enzyme horseradish peroxidase (HRP) along with a chromogenic substrate was used to monitor hydrogen peroxide produced during the reaction. The use of oxidase in combination with HRP constituted a fast and sensitive method to detect cellulase activity without involving a boiling step, commonly required in other assays. ChitO-Q268R-based calorimetric method represented a fast sensitive method capable of detecting cellulase activity in the range of 6 to 375 mU.

Moreover, Johnsen and Krause [112] reported a new approach for cellulase detection in plant tissue extracts by making use of the CMC ability to form gel-like layers on its own. High sugar content, chromophores-like anthocyanin, carotenoids, and chlorophyll as well as phenolics of the plant extracts interfere with the spectrophotometric detection of cellulase activity, affecting its sensitivity. Till date, no appropriate method is available for the determination of the cellulolytic activity of plants qualitatively or quantitatively. The property of CMC to form gel-like layers on polystyrene plates or nylon membrane formed the basis of a novel cellulase detection method being sensitive and quantitative in nature. These CMC gel layers are stainable with Gram's iodine to detect cellulase activity spatially. This method is rapid, inexpensive, and potentially applicable for screening of plant tissue extracts for mapping cellulase activity spatially in a zymogram-like fashion. These zymograms in the form of tissue print can aid in detection of cellulase activity without the obstruction caused by intrinsic sugars.

A vast majority of microbes in nature are non-cultivable and resist laboratory cultivation. Metagenomic screening process is a promising strategy for bridging the gap in research

dealing with identification of novel enzymes. These processes have been found to be quite efficient in recovering a lot of enzymes from non-cultivable microorganisms present in environment. Mewis et al. [113] reported a novel approach using chromogenic dinitrophenol (DNP)-cellobioside substrate in 384-well microplate for the detection of cellulase activity based on absorbance measurement using a liquid handler and a plate reader. This method was quantitative, automated high throughput screening method with a throughput of 100X 384-well microplates with high sensitivity for biomining cellulase activity. Nyyssonen et al. [114] described a high-throughput functional screening method coupled with next generation sequencing technique to identify bio-polymer degrading enzymes in metagenomic libraries. Multiple complex substrates like cellulose, hemicellulose, starch, and chitin have been used thus enabling direct, rapid, and simultaneous screening of targeted activities. This solution-based screening method has high throughput with 12,160 clones per day with increased sensitivity and reproducibility.

Applications of Cellulases

Microbial cellulases have gained worldwide attention as biocatalysts due to their enormous industrial applications and commercial significance. Cellulases have become indispensable in numerous fields of commercial sector as discussed below:

Textile Industry

A lot of constraints have been imposed on the textile industry due to rising environmental concerns, and in order to combat this situation, enzymatic treatment has emerged as an environmentally friendly solution.

Biostoning Denim is a type of cotton with heavy grade which is dyed with indigo dye that mainly adheres to the surface of the fiber or yarn. Blue jeans or denim garments have become appreciably popular in the past few years. Washings of these garments repeatedly showed the aged/faded effect laying the foundation of whole denim business. Traditionally, this wash down effect was achieved by chemical washing with sodium hypochlorite or potassium permanganate also known as pumice stone (stone washing). The abrasive washing of the garments with pumice stones had several disadvantages associated with it. Pumice stones were needed in large quantity causing wear and tear of machines resulting in low productivity. These stones were removed manually from the denim folds thus adding more to the problem. Environmental pollution and large amount of backstaining (redeposition tendency of the indigo dye on the denim garment) called for the urgent need of an alternative to chemical stone washing. In 1980s, perfect solution to this problem was provided by biotechnology in the form of microbial cellulases and the process was named as ‘Biostonewashing’ or ‘Biostoning’. Cellulases caused the removal of the dye from the surface of the fibrils without affecting the strength of the fiber creating the shaded look of the fabric. The use of cellulases has several advantages over stone washing with pumice stones including high productivity, less work-intensive, safer environment, short treatment times, less wear and tear of machines, etc. [115, 116]. Numerous cellulases are available which can be used either alone or in combination of other enzymes like proteses to achieve a particular finish [116–118]. Major problem associated with the use of microbial cellulases is the backstaining as it hide the shaded appearance of the

garment. *T. reesei* endoglucanase II has been found to be very efficient candidate for biostoning [119]. Genetically, engineered strains of *T. reesei* are able to produce four times more endoglucanase than parent strain thus has established themselves as strong applicant for biostoning [120]. It has been observed that acidic endoglucanase of *T. reesei* causes better abrasion and less backstaining as compared to the neutral endoglucanase of *Humicola insolens* [121]. To overcome the problem of backstaining, cellulases have been immobilized on pumice stones thus reducing the cost of the process [122]. The combination of cellulases and laccases was used to improve the lightness and decrease the staining on both back of denim garment and on white pocket [123]. An alkali stable cellulase in combination with xylanase from *Thermomonospora* sp. has been well reported for biostoning and biofinishing of denim garments with reduced backstaining [124].

Biopolishing Cellulose is elemental to almost all types of fabrics like cotton, linen, etc. and very much prone to fuzz formation, i.e., short fibers protruding out from the surface of the fabric. Fuzz attached loosely to the fabric forming a fluffy ball-like appearance is called pill and give an untidy/unattractive look to the fabric. To escalate the commercial value of cotton fabric, prevention of fuzz formation, and pilling must be achieved at economical rates [115]. Cellulases have emerged as an ideal solution to smoothen the surface of fabric in a process known as biopolishing. Fuzz is most susceptible to cellulase attack and is easily released from the surface of the fabric material giving a cleaner and smoother look enhancing the color brightness of the fabric. Due to fuzz removal, the pilling tendency reduces greatly upgrading the finishing and quality of garment [122]. The role of acidic cellulase of *T. reesei* [125] and *A. niger* [126] in biopolishing has been well reported. Improvements observed in the fabric due to cellulase treatment are permanent, environmentally safe, and economical as compared to the chemical treatment [118].

Bioscouring It is the process of removing non-cellulosic material from the surface of the cotton fabric with the help of enzymes alone or in combination. Pectinases play a key role in enzymatic scouring by digesting the pectin thereby removing the connection between cuticle and the main body of the cotton fiber. Pectinases when used in combination with cellulases result in increased bioscouring efficiency as cellulases penetrate the cuticle and hydrolyze primary cellulosic wall ultimately destructing the cuticle [117]. Cellulases open up the space for the action of other hydrolytic enzymes like pectinases by hydrolyzing cellulosic wall. Thus, cellulases break the wall from the cellulose side whereas pectinases break the linkage from the cuticle side [118]. Enzymatic scouring has several advantages over traditional treatment as former helped in retaining fiber strength with increased fabric softness as well as environmentally safe for the workers [116].

Bio-carbonization and Wool Scouring It is a biological method of cleaning fabric from the cellulosic and vegetable matter impurities with the help of enzymes [118, 127]. In polyester/cotton blends or pure cotton blends, some traces of cellulosic material remain on the surface thus deteriorating its finishing and quality. Traditional methods for carbonization involved the use of sulfuric acid which was hazardous, corrosive, and unsafe. Enzymatic carbonization came up as a perfect alternative for the removal of cellulosic component as this process was non-hazardous, non-corrosive, eco-friendly, and least affected the print color as well as feel of the fabric. Wool scouring involves the removal of the vegetative impurities from the surface of raw wool with the aid of cellulases [118]. Cellulases are very efficient in removal of impurities alone or in combination of pectinases [127]. They result in the maintenance of fiber weight and strength which was otherwise lost after chemical treatment.

De-fibrillation of Lyocell Lyocell represents pure form of cellulose fiber obtained from wood pulp after solvent-spun with amino oxide as solvent system. It is a very quality fabric but major drawback with lyocell fiber is fibrillation (small primary fibrils present at the surface of the fiber in tangled form). Cellulases are very efficient in the treatment of fibrillation of lyocell [115, 128]. Acidic cellulases are more effective in removal of fibrils from pure lyocell whereas mixed lyocell fabrics have been successfully treated with cellulases active at neutral pH. The major advantages of cellulase treatment over other methods include (a) increased softness and enhanced appearance, (b) fuzz and pill prevention, and (c) improved appearance even after repeated washings [115].

Pulp and Paper Industry

Since 1980s, the use of enzymes in recycling of pulp has come into account to overcome the limitations of other methods [129]. Cellulases have been successfully used in deinking and removal of pollutant particles without affecting the brightness and strength of the paper [129]. They are used in different aspects like bioremediation of industry wastes, deinking, pulping, bleaching and fiber enhancement, etc. [115, 130–132].

Pulping Mechanical pulping including grinding and refining woody material is usually associated with high-energy consumption. But bio-pulping with the aid of cellulases and other enzymes is an eco-friendly, less energy consuming approach. Refining can generate small particles of the pulps that ultimately cause reduction in pulp's drainage rate in the course of papermaking [129]. Cellulases are very effective in hydrolyzing these particles thereby improving the drainage ability of the pulp [130]. Cellulases along with hemicellulases play a crucial role in modification of coarse pulp material and strengthening of handsheets [133, 134]. Pretreatment of the bleached kraft pulp with CBDs of *Trichoderma* sp. results in improvement in the beating degree of the pulp, reduction in energy consumption, and improvement in the tensile index of the handsheet [135].

De-inking Main benefit of the bio-deinking is the avoidance of alkali use during the process which ultimately prevents yellowing of the fiber. The release of ink from the fiber surface is efficiently achieved with the help of cellulases alone or in combination with hemicellulases by hydrolyzing the carbohydrate part partially [136]. Acidic enzymes have been found to be better in treatment than basic and neutral ones as they prevent alkaline yellowing besides improving fiber brightness and cleanliness [115]. Enzymatic de-inking has several advantages like pulp freeness, clean look, enhanced brightness, and improved strength of the fiber as well as reduction in environmental pollution [137, 138].

Bio-modification and Bio-characterization of Fibers Mixture of cellulases and hemicellulases has been used successfully in the treatment of fibers to modify properties ultimately improving beatability, runnability, and drainage during papermaking. Treatment of pulp with enzymes before refining is done usually to improve the beatability of pulp or modification of fiber properties. Crude cellulases from *A. niger* improved the beatability of simao pine kraft pulp and decreased the energy consumption of papermaking process as analysed by Fourier transform infrared spectroscopy (FT-IR) [139]. On the other hand, enzymatic treatment of the pulp after beating results in improvement of drainage of pulp fibers

[130]. Enzymatic hydrolysis presents an important tool for characterization of fibers. Partial or complete hydrolysis of the pulp fiber by cellulases alone or in combination with other hydrolytic enzymes can be achieved followed by subsequent characterization of the fibers by various techniques like scanning electron microscopy (SEM) and HPLC. Cel9B cellulase had a refining effect on the flax fibers as it increased collapsibility, inter-fiber bonding in fibers ultimately facilitating the tensile index [140].

Laundry and Detergent Industry

Cellulase preparations capable of modification of cellulose fiber are commonly used in detergents for cleaning textiles. They improve the color brightness and feel of the fabric by removing dirt particles entangled in the garments. Mostly, cotton and cotton-based fabric after repeated washings become fluffy and dull due to partial detachment of microfibrils present on the fabric surface. Cellulases present in the detergent can result in the removal of these microfibrils thereby increasing the smoothness of the surface and restoring the original color of the garment. Softness of the fabric is also enhanced after treatment with detergents containing cellulases. The CBH I and endoglucanase from *T. longibrachiatum* were added to detergent composition in different ratios resulted in superior cleaning ability and imparted softness to the fabric [141]. Cellulases from *Humicola* (*H. insolens* and *H. griseathermoidea*) were found to active in mild alkaline conditions at high temperatures and used commonly in washing powders and detergents [142]. Cellulases like EG III and CBH I are particularly used in most commonly used detergents for textile washing [141, 143]. Granular bleach-containing laundry detergent comprising a specific cellulase from *Trichoderma* sp. and having a 1 % solution pH between 7.5 and 10 was reported to provide superior cleaning and whiteness performance as compared to detergent compositions without cellulases [144]. Cellulases from *Bacillus* sp. were found to possess excellent properties like color restoration, softening, anti-graying, wrinkle inhibition, and least damage to the fabric [145]. Cellulases from *Trichoderma* (*T. reseei*, *T. viride*, and *T. harzianum*) and *A. niger* has been well exploited in detergent preparations at industrial scale [146]. The use of cellulases in combination with proteases and lipases has been recently explored [147]. The alkaline cellulases are considered as potential industrial candidates to be used as additives to conventional detergent ingredients due to their ability to remove soil and dirt particles present in the interfibril spaces [8, 45]. Recently, liquid laundry detergents have been used to increase the stability of cellulases. The liquid detergent comprises various components including an anionic or non-anionic surfactant, protease, cellulase, citric acid or water soluble salt, a mixture of propanediol, and boric acid or its derivatives. The composition of the detergent was achieved by first adding diol and boric acid followed by addition of citric acid/salt, as the order of addition is important to enhance the stability of cellulases. The cellulases remove the rough protuberances or cellulose aggregates still attached to the fabric and result in a glossier smoother look of the fabric [144].

Animal Feed Industry

The use of fibrolytic enzymes like cellulases in processing of animal feed (poultry, pigs ruminants, pets, and fish) has received considerable attention during recent years as it resulted in improved animal performance. Enzyme preparations containing cellulases and hemicellulases are utilized for numerous activities like milk yield, body weight gain, and feed

utilization by the ruminants [134]. The major role of the enzymes in treatment of animal diet is to remove anti-nutritional factors (ANF) present in vegetables and grains [115, 134].

Effect on Feed Digestibility and Nutritional Availability The use of *Trichoderma* cellulase as feed additive significantly improved the feed conversion ratio as well as digestibility of the cereal based food [148]. Forage feed of ruminants is quite complex in composition containing cellulose, hemicellulose, pectin, and lignin. A number of other studies have suggested that it is possible to use cellulase preparations to enhance the forage digestibility [149–152]. Cellulases play a crucial role in increasing the rate and extent of fiber digestion thus affecting the natural gastrointestinal processes of the ruminants which ultimately lead to increased nutrient digestibility [152–157]. Cellulases cause partial hydrolysis of lignocellulose materials, β-glucans, de-hulling of cereal grains leading to better emulsification, and forage digestion ultimately improving the nutrition availability to the animals [158, 159]. Quality of pork can be enhanced significantly with the use of cellulases alone or in combination with proteases. Enzyme preparations containing mixture of xylanases and glucanases greatly reduce the viscosity of high fiber diet used to feed poultry and pigs, thereby increasing digestibility. Improved digestion and nutrient absorption has a positive effect on the health of animals causing weight gain in animals [115, 134, 160].

Effect on Milk Production Cellulase preparations used as additive in the ruminant feed had a positive effect on the health of the animals. Lactating dairy cows consume more feed when pre-treated with cellulase preparations and produce more milk [152, 161, 162]. Cellulases when added during feeding or before feeding result in improved nutrient availability which confers a positive health benefit on ruminants. Fat and protein content of the milk has been found to be more when cows are fed with cellulase-treated diet [163]. Studies also demonstrated that there is a change in energy and protein digestion as well as enhancement in microbial protein synthesis in the rumen [164]. However, several factors like mode and time of enzyme addition, stability and activity of the cellulases, addition to specific dietary component, amount of enzyme preparations, and stage of lactating animal affect the use of cellulases as additive in animal feed [157, 163–165].

Food Processing Industry

Cellulases are considered as valuable tools in food biotechnology due to increase in their application to numerous processes. They are utilized worldwide due to their promising potential to be exploited in various processes involved in food biotechnology like juice clarification, reducing the viscosity of nectars, concentrating purees, alteration of fruits sensory properties, olive oil extraction, and improving the quality of bakery products [115, 130–132, 134, 166].

Treatment of Juices, Nectars, and Purees The cloudiness of fruit juices is due to the presence of polysaccharides like cellulose, hemicelluloses, lignin, pectin, starch, metals, proteins and tannins [167]. The utilization of commercial enzyme preparation ‘Rapidase pomaliq’ containing cellulases, hemicellulases, and pectinases from *Trichoderma* and *Aspergillus* at industrial scale, benefitted the food industry to an appreciable level [168]. These enzymes were known as macerating enzymes and utilized in the food industry for clarification and extraction of fruit juices [166, 169–172]. The production of juices from fruits like apple

and pears includes the crushing of fruits to pulp mash which is separated into solid phase (pomace) and clear fruit juice by mechanical processing [170]. The yield as well as the process performance has been found to be increased by the use of macerating enzymes to clarify the fruit juice of apple [173]. They are usually utilized in two steps: (1) after crushing of the fruit, the pulp is macerated which results in increased juice yield and decreases processing time in addition to extraction of beneficial components of fruits, and (2) after the extraction of juice, clarification is carried out with pectinases, thus increasing the product stability [11, 174]. There was 53 % reduction in viscosity of passion fruit juice when combination of amylases, cellulases, and pectinases was used [175]. The enzymatic clarification of juices has several advantages over mechanical processes. These include an increase in juice recovery, enhanced clarification, higher juice yield, enhanced pulp liquefaction, increased total soluble solids in juices, decrease in turbidity, and viscosity [11, 166, 176–179]. The use of exogenous enzymes in black carrot juice processing remarkably enhances the antioxidant properties due to increase in phenolics and flavonoids [180].

Fruit nectars are prepared by blending pulpy juices of fruits with citric acid and sugar syrup in order to prepare ready-to-drink beverages. The most attractive feature of these drinks to be maintained is stability of the cloud. The cloud stability of nectars and concentration of puree has been improved to an appreciable extent by the use of exogenous enzymes. Commercial enzyme preparations like Rohapect TF, Rapidase LIQ^{+}, Pectinex Ultra SP, or containing the combination of different enzymes like cellulases, hemicellulases, and pectinases have been found to make the puree concentration easy and decrease the viscosity of nectars considerably [181]. Viscosity of fruit nectars is an important factor to be addressed seriously to increase the quality and ultimately the market value of the product. Fruit purees obtained from Table mango varieties are generally too viscous to be processed due to the presence of higher amount of suspended insoluble content and this viscosity pose a barrier to the export of these products. Different enzyme preparations have been tested to improve the rheological properties of the puree from Table mango varieties and Rapidase® Carrot Juice and Rapidase® Pomaliq were found to yield best results. These preparations have high content of pectinases, cellulases and xylanases can reduce the viscosity of puree drastically in minutes to modify the rheological parameters suitable for commercial use [182].

Modification of Sensory Parameters of Food The sensory properties of foods include aroma properties, flavor, and texture of fruits which play a crucial role in food biotechnology. The sensory properties of various fruits and vegetables can be altered with the infusion of enzymes like pectinases and cellulases [183]. Enzyme compositions have been found to have an enhancing effect on nutritional value as well as aroma of fruits [184]. An immobilized β-glucosidase on alginate when added to tea beverages resulted in aroma enhancement to a significant level due to increase in the content of essential oils [185]. The immobilization of the β-glucosidase resulted in the storage as well as thermal stability. Immobilized enzyme was able to work at lower temperatures as compared to the free enzyme so browning of the tea infusion could be avoided. The use of immobilized enzymes in the food biotechnology to enhance the texture, aroma, flavor, and nutritional value of foods is accelerating tremendously [186]. Enzyme-assisted treatment of fruit juices has been found to improve the color quality, juice yield, and health benefits. An appreciable increase in acidity, total soluble sugar, and β-carotene content of carrot juice with decrease the viscosity has been observed due to enzymatic processing thus improving the sensory scores of color, flavor, and overall acceptability after storage for 3 months [179].

Olive Oil Extraction Extraction of olive oil involves a series of steps like crushing and grinding of olives, moving the minced olive paste through decanters, and finally, centrifugation at high-speed for the recovery of the oil [115]. The first enzyme preparation, Olivex (consisting of high pectinase amount with low levels of cellulase and hemicellulase) obtained from *A. aculeatus* was used at commercial level to improvise olive oil extraction [187]. Numerous studies have well established the potential role of enzymes in improving the extraction processes [188–190]. The use of cellulases alone or in combination with other hydrolytic enzymes like pectinases has been found to have an enhancing effect on the extraction as well as the quality of olive oil [191]. Exogenous enzyme cocktail is required in the mixing step as the native enzymes present in the olive fruit lose their activity during extraction processes [190, 192–194]. These enzymes efficiently act on the colloidal and lipoprotein structures present in the olive paste and significantly result in release of the oil with enhanced quality and appearance [190, 193] [195, 196]. The use of various enzymes during the extraction of olive oil has attracted the world market due to various health claims [191, 197]. Enzyme formulations when added to the olive paste during the malaxation step result in significant enhancement in phenolic content and antioxidant activity in the extracted olive oil thereby improving its quality [197, 198].

Improvement of Bakery Products Enzymes have gained a lot of attention in bakery industry due to their profound effect on improving bread and dough quality. Cellulases with addition of other hydrolytic enzymes like amylases, proteases, and xylanases result in the increase in the loaf volume, improvement in bread quality, and production of softer crumb [199]. Enzyme combinations containing cellulases and other hydrolytic enzymes have been utilized to improvise the volume, quality, and storage stability of the bread thereby reducing the use of emulsifiers in the bread making processes [200]. Addition of carboxymethyl cellulase to the dough improves the farinographic parameters of the dough like water absorption, development time and stability time, etc. [178]. Enzyme cocktail containing cellulases, hemicellulases, amylases, lipases, and phospholipases results in dough conditioning with improvement of flavor, prolonged shelf life, and increase in volume after baking [201].

Bread stalling is an important phenomenon related to bread storage which is caused by water migration and changes in starch. It includes loss of crispiness due to increase in moisture in the crust, increase in crumb firmness, and increase in the crystallinity of the starch. Enzyme additives are gaining attention in the bread-making industries to reduce staling. Yurdugul et al. [202] reported the isolation of an enzyme complex possessing cellulase activity from *Neocallimastix* spp. and studied the effect on bread quality at various concentrations. An optimal concentration (0.3 ml) of cellulase containing enzyme preparation when added into the bread dough caused decrease in gumminess, chewiness, and hardness resulting in a softer crumb. Enzyme formulation being a natural additive increased the consumer's acceptability and extended shelf life with retardation of bread staling. Similar results were reported by Oliveira et al. [203] where an enzyme cocktail containing xylanases, endoglucanases, amylases, and proteases exerted a remarkable effect on the moisture redistribution in the system resulting in increase in the specific volume of bread and decrease in the firmness of the bread crumb. Improvement in the quality of the wheat bread with a profound decrease in bread staling was observed with the use of enzyme preparation.

Pigment Extraction from Plants and Plant Products The use of enzyme combination in pigment extraction is a relatively new approach carotenoids are widely used as natural food

colorants due to the important biological properties like natural origin, no toxicity, and wide range colors. A combination of cellulases and hemicellulases was used for carotenoid extraction from sepals of *Physalis alkekengi* L. which resulted in enhanced recovery of pigments as compared to solvent extraction [204]. Enzyme-based pigment extraction is gaining importance because they release pigments still attached to proteins unlike solvent-based extraction. The extraction of the pigment in conjugated form prevents oxidation of pigment and helps to maintain color stability [131].

Lycopene is a highly conjugated acyclic hydrocarbon molecule mainly extracted from the tomatoes possessing a high nutraceutical value. A high degree of conjugation confers antioxidant properties to the carotenoid making the molecule a potent antioxidant. The major bottleneck is high cost of commercially available product and less yields with solvent extraction. The enzyme preparations with cellulase and pectinase were found to be the most effective in carotenoid extraction with an up to 10-fold increase [205]. An enzyme preparations with pectinolytic, cellulolytic, and hemicellulolytic activities significantly enhanced (8–18-folds) lycopene recovery from the tomato skins. Enzyme-assisted extraction of lycopene from solid waste tomato pomace or tomato is likely to provide economically beneficial way to exploit waste product for pigment extraction with better yields [205–208].

Extraction of Bioactive Compounds from Plants

Bioactive compounds are the natural compounds that either confer a health benefit or are toxic when ingested. Increasing demand for novel bioactives with important nutraceutical applications has intensified the research related to extraction of these compounds from plant cells. Enzymes alone or in combination were found to enhance the release of bioactives from plant cells. Stevioside, a non-nutritive sweetener was extracted from *Stevia rebaudiana* by enzyme-assisted environment friendly extraction with enhanced yields [209]. Enzyme aided extraction of natural compounds is provides an eco-friendly reproducible process saving a lot of time and energy. Cellulases have established themselves as commercially attractive candidates due to their increasing demand in the extraction of numerous bioactive compounds from plants including carotenoids, anthocyanins, glycosides, flavonoids, phenolics, vanillin, etc., thus enzymes emerges as versatile candidates catalyzing process for the release of various natural compounds which can be used as food supplements, nutraceuticals and functional foods, etc. [180, 209–214].

Removal of Bacterial Biofilm

Bacterial cells were found to secrete sticky exopolysaccharides (EPS) when they remain attached to an inert surface for a longer duration. The role of EPS was to develop a matrix allowing the growth and division of the bacterial cells resulting in the formation of a biofilm. Wiatr [215] reported that slime layer formation on cooling towers in waste water treatment plants was prevented significantly for months by using a composition of two parts cellulases, one part α-amylase, and one part protease. It has been suggested that α-amylase and protease provide access sites for action of cellulases by causing nicks in the slime layer. The exopolysaccharide layer was targeted by a combination of enzymes including α-glucosidase, galactosidase, galacturonidase, rhamnosidase, xylosidase, fucosidase, and arabinosidases. The enzyme combination was found to be effective in slowing down the formation of biofilm by

Klebsiella pneumoniae, IPC500, and *Pseudomonas aeruginosa* ATTC10145 when treatment was given for 72 h [216]. Various compositions containing carbohydrate degrading enzymes like amylases, cellulases and proteases have been used to remove the bacterial biofilm formation [217]. Recently, kits containing varied combinations of enzymes have been developed to get the slime mold removal. These kits contain different amount of various enzymes like cellulases, hemicellulases, proteases, amylases, and esterases. Kits were tested against *P. aeruginosa* to prevent the biofilm formation on solid surfaces. The kits were significantly effective to remove 80 % of the slime mold at acidic, neutral, and basic pH [218, 219].

Biorefinery

The most important and commercially attractive application of cellulases is the production of value added chemicals utilizing the most abundant lignocellulosic waste in an economically profitable manner. The utilization of this renewable source of cellulose to form industrially relevant compounds is still limited due to high costs of enzymes. The attention of the scientific community to overcome this obstacle is focused on the screening of cellulases with novel characteristics from newer environments as well as genetic improvement of the existing enzymes to meet up the industrial demands. Grape fruit peel waste was utilized for the production of fermentable sugars by employing an enzyme combination containing cellulases and pectinases [220]. The development of biochemicals, energy, and value added products by using plant or algal biomass has been ascribed as biorefinery concept. Over the years, biorefineries based on lignocellulosic biomass have received substantial attention and this sector is growing with a great pace. Biofuel production using discarded biomass including agro-industrial wastes (brewer's spent grain, citrus fruit waste, sugarcane bagasse, waste paper and paper sludge, etc.), municipal solid wastes, and kitchen wastes by enzymatic hydrolysis has gained paramount importance as extensive studies dealing with this aspect were reported [40, 70, 72, 221–244]. Statistical optimization of the process steps has been achieved by Celiktas et al. [229] utilizing wheat bran as lignocellulosic source and hemicellulases as enzyme complement in the biorefinery concept suggesting the development of a sustainable technology which reduced environmental pollution. The concept of biorefinery based on the use of Lactic acid bacteria has also been receiving substantial attention owing to their characteristic ability in generation of lignocellulosic biomass into biofuels and other industrially important products [230]. A multistage biorefinery concept has been introduced suggesting the use of coffee and other crop wastes for the production of biofuels. In this multi-stage concept, coupled thermochemical and biochemical processes have been involved with the use of mutant yeast strains for the generation of bioethanol from sugars [231]. Kiran et al. [232] nicely demonstrated the utilization of food wastes generated worldwide into variety of value-added chemicals viz. organic acids, biopolymers, etc. using the biorefinery approach thereby reducing the emission of environmental pollutants. Recently, an integrated approach involving anaerobic digestion and aerobic fungal fermentation has been developed utilizing the combination of cellulases and amylases for the conversion of corn stover and animal manure as main feedstocks. This represented an advanced self-sustaining process for biodiesel production with great efficacy [243]. Jin et al. [244] have discussed the development of second generation biorefineries dealing with the use of lignocellulosic biomass to produce commercially valuable lipids. As diverse waste products are available to be used in biorefinery concept, the need of the hour is to focus on the optimization of process steps including the involvement of better catalysts, optimization of the processes, and efficient recovery of the target molecules.

Pharmaceutical Industry

Cellulases have emerged as commercially attractive biocatalysts to be exploited in pharmaceutical industry. These have been found to play a pivotal role in the release of medicinally relevant compounds from plants or plant products. The enhanced yields of ellagic acid from ellagitannins correlated well with the hydrolytic activities of enzymes like cellulase, xylanase, and ellagitannin acyl hydrolase [245]. The enzymes including cellulases, proteases, amylases, etc. were found to have an enhancement effect on the extraction of bioactive polyphenols from different cereals [246, 247]. The compounds like polyphenols possess anti-oxidant properties and considered as free radical scavengers reducing the incidence of numerous diseases. Antioxidant properties of wheat were drastically enhanced when fermented with filamentous fungi *Aspergillus oryzae* and *Aspergillus awamori* due to increase in polyphenol extraction by hydrolytic enzymes. The polyphenol enrichment was in compliance with the action of enzymes like xylanase, amylase and β-glucosidase released by fungi and ultimately enhanced the free radical scavenging property of wheat [246]. As humans are unable to digest cellulose so cellulase supplements are gaining popularity these days. The appliance of cellulases as digestive aids (e.g., Digestin, P-A-L Plus Enzymes, Polyenzyme Plus, etc.) to treat people suffering from metabolic disorders is evolving as a promising strategy [134].

The employment of cellulases in prebiotics is coming up as a novel prospect that is accelerating dramatically. Prebiotics in diet include non-digestible fibers which pass upper gastrointestinal tract and act as a substrate for the growth of useful bacteria in the large intestine. Glucomannans were found to act as effective prebiotics in depolymerized state as compared to intact native form [248]. The depolymerization of glucomannans can be achieved by action of enzymes like cellulases and mannanases efficiently thereby boosting the prebiotic effect [248, 249]. The appliance of cellulases and mannanases hydrolysates as effective prebiotics is evolving rapidly as suggested by numerous reports [248–250]. Similarly, Albrecht et al. [251] produced mixtures of Konjac Glucomannan (KGM) oligosaccharide from a KGM polysaccharide by the action of endo-β-(1, 4)-mannanase and endo-β-(1, 4)-glucanase. These KGM oligosaccharide mixtures were monitored for changes in structure during 72 h in vitro fermentation with human colonic flora by the application of laser-induced fluorescence detection (CE-LIF) and matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS). It was suggested that the Konjac Glucomannan (KGM) oligosaccharide mixture produced by endo-β-(1, 4)-glucanase action exhibited larger degradability during in vitro fermentation in human gut flora in comparison to endo-β-(1, 4)-mannanase. Similarly, glucomannan hydrolysates (GMH) produced by the action of cellulase were found to increase the population of advantageous bacteria *Bifidobacterium* and *Lactobacilli* in fecal mixed cultures [252]. GMH selectively stimulated the increase in population of advantageous microbiota in human fecal gut models and modulated the microbiota composition with propionic acid rich short chain fatty acid (SCFA) profile. It was studied that increased propanoic acid concentration decreases cholesterol synthesis thus decreasing the cholesterol concentration in humans [253]. Recently, an equivalent mixture of cellulase–mannanase hydrolysates produced from konjac glucomannan was found to have role in promotion of the growth of lactic acid bacteria (LAB) *Lactobacillus* and *Bifidobacterium* in milk with great efficacy. It was also investigated that konjac glucomannan treated with cellulase alone showed better improvement on LAB growth in comparison to those containing konjac glucomannan mannanase hydrolysates or glucose [254].

Conclusion

Microbes hold immense potential to be exploited for the production of cellulases and thus intensified the research dealing with the processes involved in their production. Microbial cellulases are gaining worldwide attention due to their broad applicability in textile, detergent, food, pharmaceutical, biofuels, and other industries. Moreover, they are emerging as potential candidates judicially utilizing the lignocellulosic waste thereby reducing environmental pollution. With the advances in the biotechnological fields, newer strategies will open up novel prospects in the field of industrial microbiology.

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