Chapter

Overview of the Process of Enzymatic Transformation of Biomass

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Abstract

Cellulase is an enzyme which depolymerizes the cellulose into glucose. Cellulases are produced by a diverse array of microbes including fungi, bacteria, yeast and actinomycetes. Considerable research for understanding the mechanism of cellulases began in early 1950s because of the significant use of these enzymes in various industries. This review provides a general account structure and availability of lignocellulosic biomass, pretreatment strategies for effective digestion, cellulase producing organisms, cellulase activity assay, and enzymology of cellulose degradation. Cellulase production, optimization, purification and characterization studies in addition to the industrial application of cellulase have also been discussed. At last a brief account of present market scenario of cellulases and future prospects of the study are also taken into account.

Keywords: cellulases, lignocellulosic biomass, fungi, pretreatment

1. Introduction

Cellulases are inducible enzymes which breakdown cellulose (the most widely available source of fermentable sugars on earth) into glucose and synthesized during the growth of microorganisms on cellulosic substrates [1, 2]. Cellulase is biotechnological important enzyme due to various industrial applications including biofuel production [3]. Variety of microorganism having cellulose degrading capability, few of them produce considerable quantity of extracellular enzymes. Fungi are the main cellulase producing microorganisms. *Trichoderma* and *Aspergillus* are found to be most potent cellulase producers, to be used for agricultural and industrial purpose [4, 5].

A large number of industries are based upon the agricultural raw materials and it alone accounts for about 10% of the total wages from export. At present, in terms of agricultural production, country holds 2nd position in world (http://www.agrifest.in/aboutagrifest.php). Availability of lignocellulosic

biomass varies from one region to another region in our country because of specific patterns of cultivation of crops in different regions. As estimated by the Ministry of New and Renewable Energy (MNRE), Report 2009, Government of India (GOI) every year about 500 Mt/yr residues are generated in India. Out of total residue generated, highest contributor is Utter Pradesh (60 Mt/yr), followed by Punjab (55 Mt/yr) and Maharashtra (46 Mt/yr). Among different crops, cereals crops contribute for the generation of 352 Mt residue followed by fiber crops (66 Mt/yr), oilseed (29 Mt/yr), pulses (13 Mt/yr) and sugarcane (12 Mt/yr). Among the cereal crops up to 70% is contributed by rice, wheat, maize and millets. Rice crop alone accounts for 34% followed by wheat contributing 22% of total residue generated by cereal crops. As depicted above, out of total residues generated from all crops, 13% is contributed by fiber crops. Among fibers, cotton holds 1st position by generating 53 Mt/yr (11% of crop residues) and coconut ranks 2nd with 12 Mt/yr of residue generation. The sugarcane residue (foliage and tops) generates 12Mt/yr, i.e., 2% of crop residues (**Figure 1**) (www.nicra.iari.res.in/Data/FinalCRM.doc).

The amount of crop residues, which have not any valuable uses is either left in the fields to rot or burnt away as such, is termed as surplus biomass. A brief idea about the amount of residue generated in different states of India, surplus residues left behind after conventional use, residue burned as reported by IPCC and [6] is shown in **Table 1**. Two reports dictated the burnt surplus agricultural biomass approximately 83.66 Mt/yr and 92.81 Mt/yr respectively. The data from two reports vary by 11% and this difference can be due to the climatic conditions, geographic separation, sample size and time of sampling used in above mentioned studies. However, in comparison to the total surplus residues, observed difference can be considered as insignificant. Besides biomass a massive quantity of industrial residues is disposed off as such in environment generating pollution and other related problems [7]. This huge amount of lignocellulosic biomass can likely be converted into different valuable products including biofuels, cheap energy sources for microbial fermentation, enzyme production and useful fine chemicals [8].

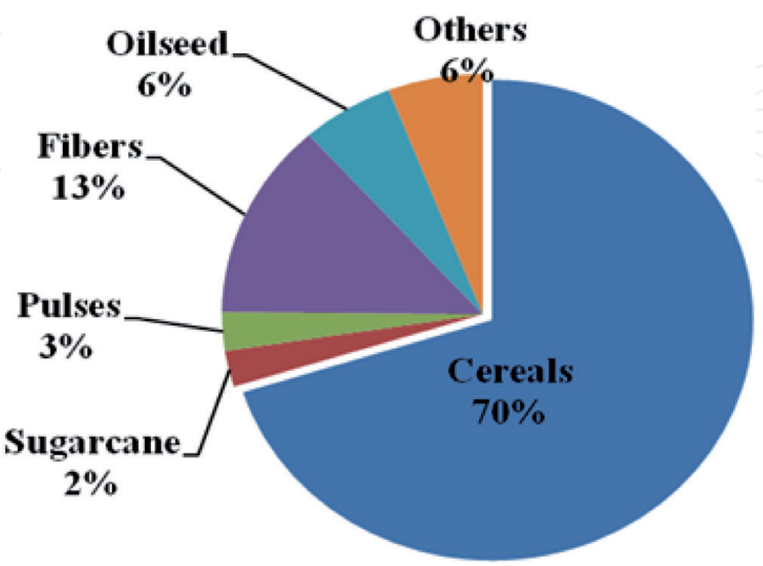

Figure 1.
Contribution of various crops in residue generation (www.nicra.iari.res.in/Data/FinalCRM.doc).

States	Residue generation (MNRE, 2009)	Residue surplus (MNRE, 2009)	Residue burned (IPCC coeff.)	Residue burned [6]
		Mt/yr		
Andhra Pradesh	43.89	6.96	5.73	2.73
Arunachal Pradesh	0.4	0.07	0.06	0.04
Assam	11.43	2.34	1.42	0.73
Bihar	25.29	5.08	3.77	3.19
Chhattisgarh	11.25	2.12	1.84	0.83
Goa	0.57	0.14	0.08	0.04
Gujarat	28.73	8.9	6.69	3.81
Haryana	27.83	11.22	5.45	9.06
Himachal Pradesh	2.85	1.03	0.20	0.41
Jammu and Kashmir	1.59	0.28	0.35	0.89
Jharkhand	3.61	0.89	1.11	1.10
Karnataka	33.94	8.98	2.85	5.66
Kerala	9.74	5.07	0.40	0.22
Madhya Pradesh	33.18	10.22	3.46	1.91
Maharashtra	46.45	14.67	6.27	7.41
Manipur	0.9	0.11	0.14	0.07
Meghalaya	0.51	0.09	0.10	0.05
Mizoram	0.06	0.01	0.01	0.01
Nagaland	0.49	0.09	0.11	0.08
Orissa	20.07	3.68	2.57	1.34
Punjab	50.75	24.83	8.94	19.62
Rajasthan	29.32	8.52	3.58	1.78
Sikkim	0.15	0.02	0.01	0.01
Tamil Nadu	19.93	7.05	3.55	4.08
Tripura	0.04	0.02	0.22	0.11
Uttarakhand	2.86	0.63	13.34	21.92
Uttar Pradesh	59.97	13.53	0.58	0.78
West Bengal	35.93	4.29	10.82	4.96
India	501.76	140.84	83.66	92.81

Table 1.
Residue generated, surplus and burned (www.nicra.iari.res.in/Data/FinalCRM.doc).

2. Lignocellulosic biomass

Lignocellulosic biomass is consist of cellulose, hemicelluloses, lignin, water, protein and other compounds (**Table 2**). Cellulose and hemicelluloses provide strength to fiber and lignin act as the concrete which hold the fibers [9].

Lignocellulosic materials	Cellulose (%)	Hemicelluloses (%)	Lignin (%)	Reference
Sugar cane bagasse	42	25	20	[11]
Sweet sorghum	45	27	21	[11]
Hard wood	40–55	24–40	18–25	[12]
Soft wood	45–50	25–35	25–35	[12]
Corn cobs	45	35	15	[13]
Corn stover	38	26	19	[14]
Rice straw	32.1	24	18	[13]
Nut shells	25–30	25–30	30–40	[15]
Newspaper	40–55	25–40	18–30	[16]
Grasses	25–40	25–50	10–30	[12]
Wheat straw	29–35	26–32	16–21	[17]
Bagasse	54.87	16.52	23–33	[18]

Table 2.
Composition of lignocellulosic materials [10].

About 50% of the CO_2 fixed by plants through photosynthesis get stored in cell wall in the form of cellulose [19]. It is a homo-polysaccharide of glucose residues connected by β-1,4 linkages in linear un-branched fashion (**Figure 2**). Basic repeating unit of the cellulose polymer is a cellobiose unit, made up of two glucose anhydride [20]. The long-chain cellulose polymers are attached to each other by van der Waals and hydrogen bonds which results in packing cellulose chains into microfibrils [21, 22]. Overall structure is found to be consisted of two different types of regions: region where the chains are highly ordered is crystalline and the region with less ordered chain is amorphous [23]. The crystalline regions of cellulose are highly stiff thus these are not easily reachable to endo-cellulases [24]. Amorphous region is more readily hydrated and more accessible to enzyme.

Other significant component of lignocellulose is hemicellulose (**Figure 3**). Hemicellulose usually contributes for about 25–35% of the mass in dry wood, about 28% of softwoods, and 35% of hardwoods [26]. As compared to cellulose these possesses low molecular weight. These are found to consist of comparatively shorter chains of about 500–3000 monosaccharide units as compared to 7000–15,000 glucose residues cellulose [27]. The monosaccharides of hemicelluloses

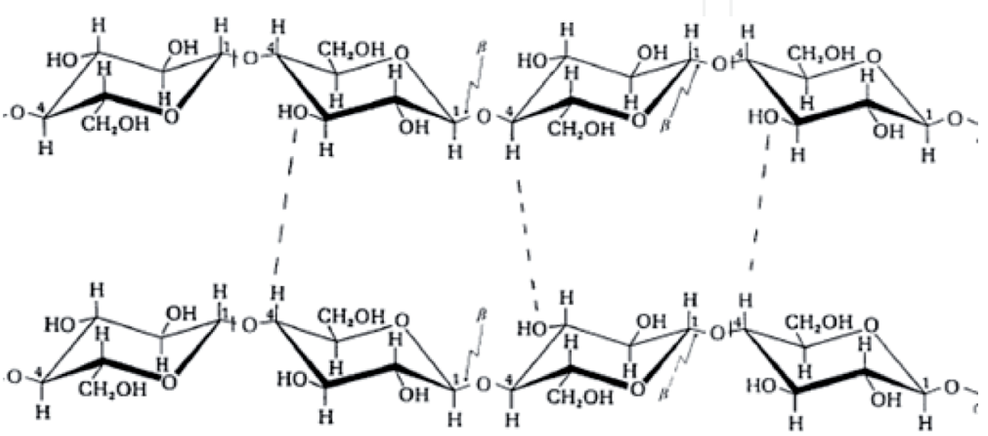

Figure 2.
Structure of cellulose [25].

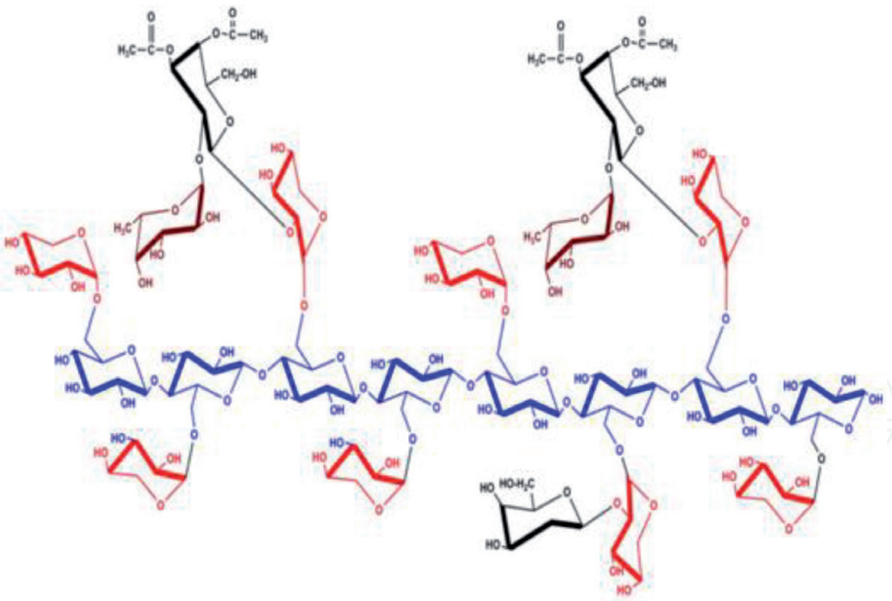

Figure 3.
Xyloglucan: a component of hemicelluloses [29].

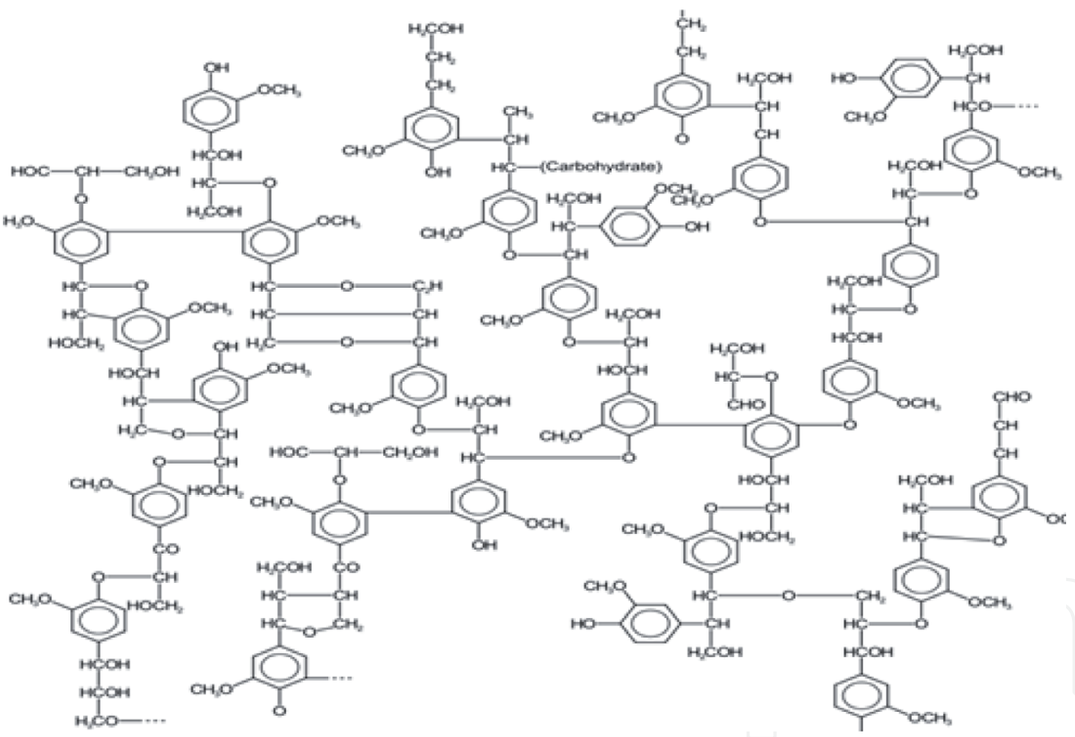

Figure 4.
Chemical structure of lignin (https://en.wikipedia.org/wiki/Lignin).

include pentoses (arabinose, rhamnose and xylose,), hexoses (glucose, galactose and mannose), and uronic acids (D-glucuronic, D-galacturonic acids and 4-o-methylglucuronic). The backbone of hemicelluloses can be a homopolymer or a heteropolymer having β-1,4 or sometimes β-1,3 glycosidic linkages. In hardwood, xylose is the principal pentose sugar but in various agricultural residues and other herbaceous, arabinose is the chief pentose sugar of hemicelluloses [28].

Lignocellulosic microfibrils are found to be surrounded by a complex aromatic heteropolymer known as lignin which provides a tough protective shield to highly energetic cellulose fibers [30]. Lignin comprises of β-aryl ether, biaryl ether, phenylcoumaran, pinoresinol, or diaryl propane linked p-coumaryl, coniferyl

and sinapyl alcohol units (**Figure 4**). It is categorized as softwood lignin when the coniferyl alcohol derivatives predominant, hardwood lignin where both coniferyl and synapyl alcohol derivatives exist together and grass lignin where it chiefly consisted of p-coumaryl alcohol derivatives [31].

3. Pretreatment

Lignin is a recalcitrant component of the lignocellulosic biomass. Resistance to chemical and enzymatic attack increases with increase in lignin content [32]. Lignin the natural cement, acts as a ceiling for microbial/enzymatic attack. Hence, it is one of the major hurdles in using lingo-cellulosic materials in fermentation. Pretreatment is one of the most important steps in the process of converting renewable lignocellulosic biomass into useful products. The main target of any pretreatment is to alter or remove structural and compositional resistant to hydrolysis which further enhance digestibility of biomass [33]. It exposes cellulose and hemicellulose chains by breaking the crystalline matrix (**Figure 5**). To remove the obstacles for enzymatic scarification of lignocellulosic material following pretreatment used.

3.1 Mechanical treatment

Major mechanical treatment includes chiping, grinding and milling to reduce the particle size which is responsible to increase surface area and increased surface area responsible for better interaction between substrate and enzyme [21, 35]. Physical treatment includes un-catalyzed steam explosion, hot water pretreatment and high energy radiations. By the process size reduces to 10–30 mm after chipping the biomass and finally after milling or grinding 0.2–2 mm size is attained.

3.2 Steam explosion

Mason [36] first time introduced steam explosion in which biomass is pretreated at 180–240°C under 1–3.5 MPa pressure for 1–10 min with hot steam, followed by

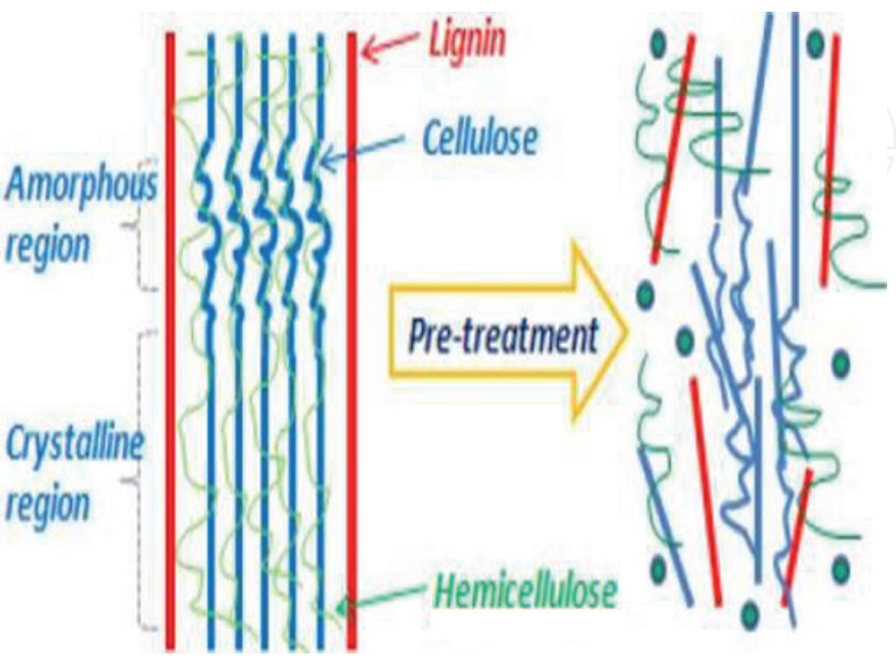

Figure 5.
Effect of pretreatment on lignocellulosic biomass [34].

an explosive decompression which bursts the rigid biomass fibers [37]. Nature of material to be processed and particle size are the determining factor for relationship between temperature and time [38]. Quick expansion in steam explosion vaporizes the saturated water present in fibril structure linkages between molecules, and produces a better lignocellulosic matrix [39]. Recoveries ranged from 46 to 90% indicated that significant autohydrolysis and degradation of sugars can occur during this pretreatment process [40]. Steam provides an effective mean to rapidly attain the required temperature without diluting the resulting sugar syrup. At the end, a rapid release of pressure brings temperature down and arrests the reaction [41].

3.3 Ultrasonic pretreatment

Scanning electron microscopy images reveal that ultrasonic treatment have the capacity to modify structure of lignocellulosic biomass [42]. Ultrasonic waves work by creating pressure difference within a solution [43]. The pressure wave travels through the liquid medium creating alternate regions of high (compression) and low (rarefaction) pressure (**Figure 6**).

3.4 Acid pretreatment

In this method lignocellulosic material is dipped in an acidic solution (typically H_2SO_4), and subjected to optimum temperature. Dilute sulfuric acid had been used at commercial scale for pretreatment of various biomasses such as Switch grass [44] Corn Stover [45] and Poplar [46]. By acid catalyzed hydrolysis (**Figure 7**) most of the hemicelluloses are almost removed from the micro fibrils of the biomass but delignification is achieved to a lesser extent. Dilute acids are highly effective in removing hemicelluloses as dissolved sugars as a result of which glucose yield from cellulose increase to almost 100%. The optimal conditions to attain maximum sugar yield depends on the target to be achieved [47].

3.5 Alkaline pretreatment

It is responsible for the saponification of inter molecule delignification of the hemicelluloses. The biomass is exposed for the enzymatic hydrolysis of cellulose and hemicelluloses. As compared to other methods of pretreatment, alkali pretreatment is carried out for longer duration at low temperature and pressure [39]. It is supposed to act by saponification of inter-molecular ester bonds which are found to present between hemicelluloses and other components [48] (**Figure 8**). It is mainly responsible for

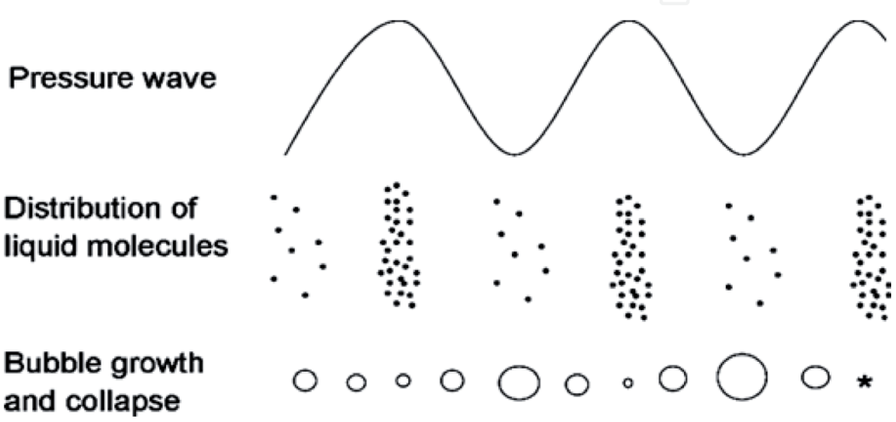

Figure 6.
A pressure wave traveling through a solution [36].

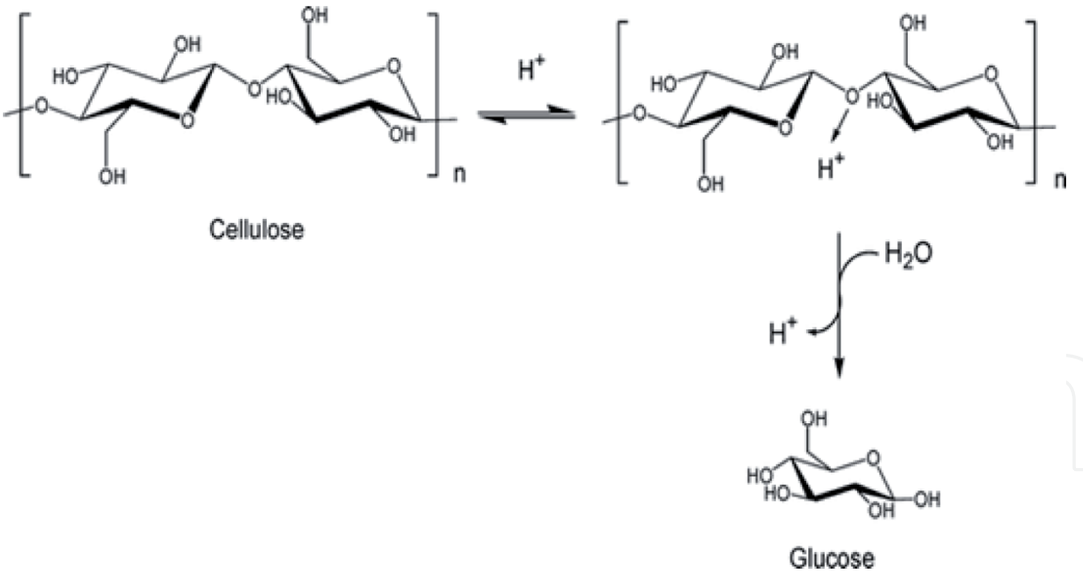

Figure 7.
Cellulose hydrolysis in acidic media [47].

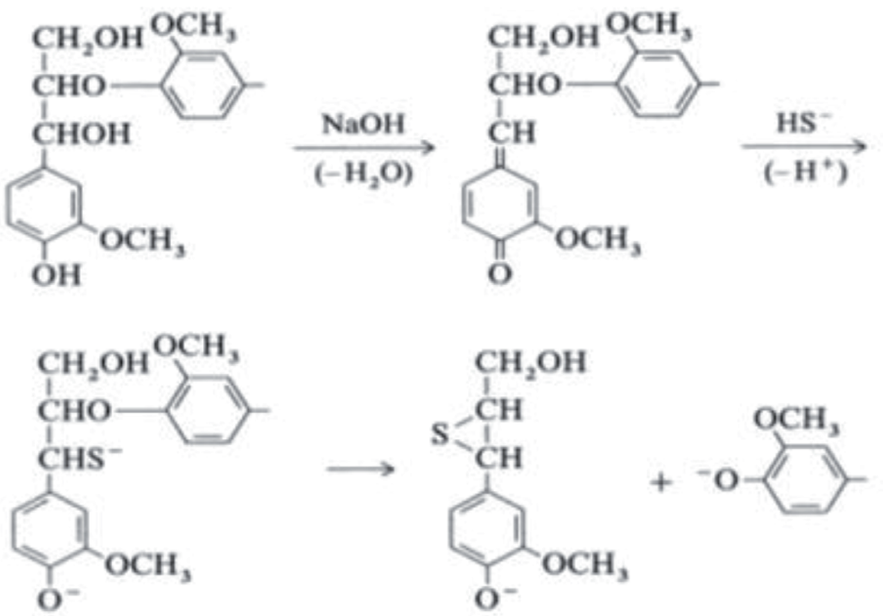

Figure 8.
Ether bond cleavage in alkaline solution [48].

delignification of lignocellulosic biomass. But it also removes some acetyl and uronic acid substitutions on hemicelluloses, which expose the biomass for enzymatic hydrolysis of cellulose and hemicelluloses [49]. A major limitation of alkaline pretreatments is formation of some salts which are either irrecoverable or incorporated as salts into the biomass [50]. Reactor costs for alkali pretreatment are lower than those for acid pretreatments [51]. For a given quantity of biomass, lowest operating cost is for lime pretreatment [39]. However the use of more pricey salts at higher concentrations is the major drawback that poses environmental threats and may also hinder the recycling process [52].

4. Enzymology of cellulose degradation

Cellulases are classified as hydrolases, i.e., they add water molecules to cleave glycosidic bonds. Cellulases purified from different microorganisms found to poses

different molecular characteristics including molecular weight, amino acid composition, isoelectric point) absorbability for cellulose, catalytic activity and substrate specificity [53]. Three chief classes of cellulases recognized to date are:

1. Endo-β-1,4-glucanases (Cx) attacks soluble cellulose derivative in a random fashion forming nonreducing ends, producing new chain ends to be attacked by exoglucanases. These enzymes may be processive or nonprocessive. In processive enzymes, enzyme-substrate complex formation is followed by several successive breaks in a polysaccharide chain [23].

2. Exo-β-1,4-glucanases (C1) (avicelase) attack the reducing or nonreducing end of the cellulose polymer. Processive exo-β-1,4-glucanases are named as cellobiohydrolases. The end product of exo-glucanase hydrolysis are cellobiose and glucose units,

3. β-Glucosidases finally breaks cellobiose to glucose.

These enzymes act synergistically (**Figure 9**) [54]. An endo-acting enzyme generates new reducing and nonreducing ends. Exo-acting enzyme releases cellobiose from ends produced by endo-enzymes acting which is finally hydrolyzed by β-glucosidases to glucose [55]. Mainly four types of synergism have been identified [56]:

i. Endo-exo: among exo-glucanases and endo-glucanases.

ii. Exo-exo: among exo-glucanases those processing from different ends (reducing and nonreducing ends).

iii. Synergy between exo-glucanases and β-glucosidases that removes cellobiose.

iv. Intramolecular synergy between catalytic domains and CBHs.

In general cellulases comprise of two distinct domains, i.e., Small cellulose-binding module (CBM) which is noncatalytic, Large domain having catalytic characteristics

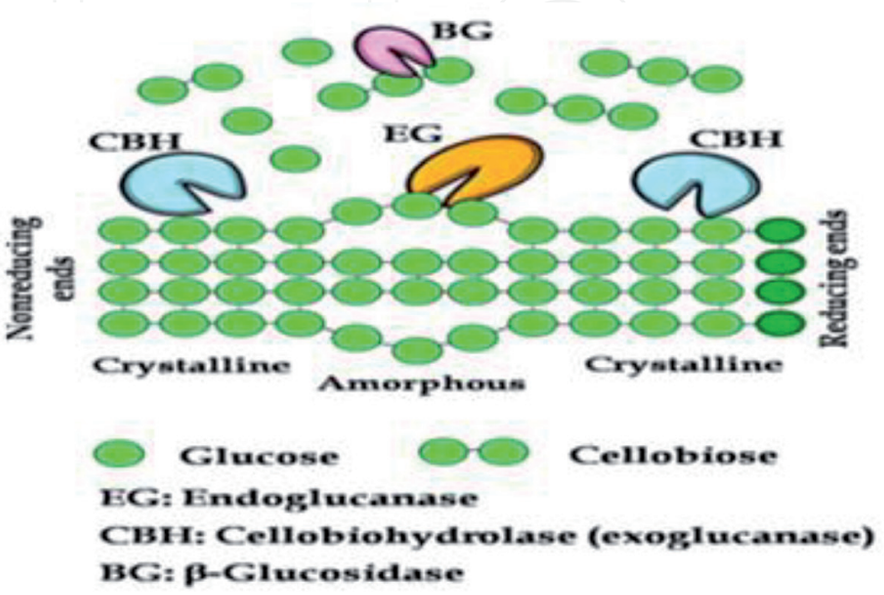

Figure 9.
Mechanism of action of cellulases [54].

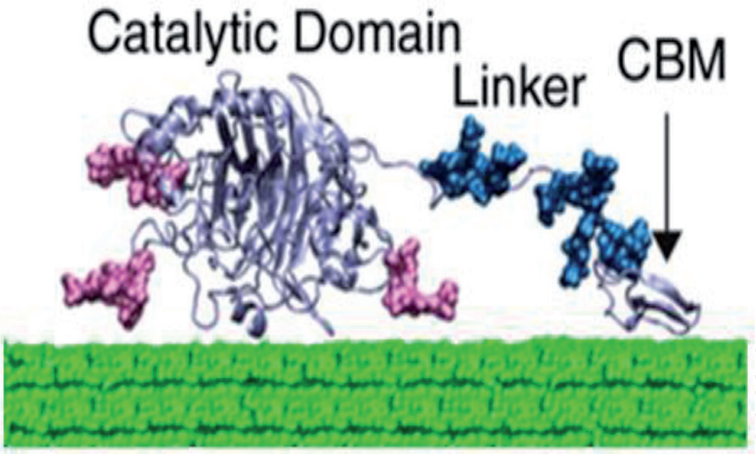

Figure 10.
Cellulases [59].

(CD). Both the domains are found to be connected by a linker region (**Figure 10**) [57]. Till date, about 300 different CBMs have already been identified. CBMs are categorized into 45 families on the basis of their amino acid similarity [58]. This variation in affinity may be due to variation in spatial structure created by the presence of CBMs [60].

4.1 Source of cellulase

Cellulases are the hydrolytic enzymes which are produced by a diversity of microbes like actinomycetes, bacteria and fungi when grown on cellulosic substrates [61]. Among these organisms fungi are studied most extensively [62]. Filamentous fungi are the chief sources known for producing cellulases and hemicellulases [63]. Crude cellulases from *Trichoderma* and *Aspergillus* genera production are commercially available for agricultural and industrial use [64]. Representatives of *Trichoderma* genus secretes comparatively large quantities of endo-β-glucanase and exo-β-glucanase but low level of β-glucosidase, while those of Aspergillus genus secretes moderately high level of endo-β-glucanase and β-glucosidase with low level of exo-β-glucanase [65]. Cellulases isolated from thermophilic fungi are of great interest because of their industrial application on account of thermo stability. Thermophilic fungi producing cellulases include *Chaetomium thermopile*, *Humicola insolens*, *Humicola agrisea*, *Myceliopthora thermophila*, *Talaromyces emersonii* and *Thermoascus aurantiacus* [66]. Unlike thermophiles, cellulase producing alkaliphilic fungi are very rare [67]. The alkaline tolerated cellulases producing marine fungi Chaetomium sp. (NIOCC36) from mangrove leaves. Surprisingly, no any thermophilic archaea showing cellulolytic behavior have been described [68]. Bacterial cellulase generally forms complex systems (cellulosomes). Historically fungal cellulases have been easier to study than bacterial system, as the bacterial enzyme tend to form aggregates. *Cellulomonas*, *Bacillus* and *Micrococcus* spp. isolated from coir retting effluents of estuarine environment were also employed to study endoglucanase activity [69]. Gaor and Tiwari reported organic solvent thermostable cellulases from *Bacillus vallismortis* RG-07 [70]. *Bacillus thuringiensis* strains [71], *Bacillus pumilus* EB3 [72] are also reported as good cellulase producers. Wild-type and mutants stains of *Pseudomonas fluorescens* were used by Bakare and co-workers to produce cellulases [73]. Interestingly, research findings are reported even for the production of cellulases from several species of insects in the orders of dictyoptera, orthoptera, and coleoptera by their own in the mid gut or salivary glands. These findings challenged the traditional view of cellulose digestion that it is mediated by microbial cellulases in the gut of insect [74]. The first endogenous cellulase

from insect was discovered in 1998 in the termite (*Reticulitermes speratus*), which was found to be capable for feeding wood even after the removal of its gut fauna [75]. Acquisition of digestive enzymes has also been explored in other xylophagous arthropods, molluscs, including snails, a sea slug, a periwinkle and some bivalves. Various possible sources are reported for these endogenous enzymes such as the hepatopancreas, gastric teeth, and crystalline styles (needlelike structures made of crystalline proteins forming a motor organ in the stomach of bivalves [76].

4.2 Cellulase activity assay

Two fundamental approaches used for measuring cellulase activity are:

1. Measuring individual cellulase (endoglucanases, exoglucanases and β-glucosidases) activities.

2. Measuring the total cellulase (FPase) activity [77].

Quantitatively cellulase activity can be assayed in three ways:

1. Accumulation of products after hydrolysis.

2. The reduction in substrate quantity.

3. Change in the physical properties of substrates.

The first one is ideal for measuring individual cellulase activity within a short time however the third one is a chosen for measuring total enzyme activity within a given time [77].

Total cellulase activity assay is always performed using insoluble substrates having pure cellulosic substrates such as Whatman No. 1 filter paper. The filter paper activity (FPase activity) is the key method for analysis of total cellulase activity which was developed by Mandels, cotton linter, microcrystalline cellulose, bacterial cellulose, algal cellulose and cellulose-containing substrates such as pretreated lignocellulose [78]. This standard filter paper method has been revised by Ghose which was established and published by the International Union of Pure and Applied Chemistry (IUPAC) [79]. He used Whatman No. 1 filter paper (1 × 6 cm strip) as the substrate. It is used as the standard substrate because of its readily availability and inexpensiveness [80].

Commercial avicel is also used for measuring exoglucanase activity because it has a low degree of polymerization (DP) and it is moderately hard to be attacked by endoglucanases [81]. Endoglucanase activity can be measured using a soluble cellulose derivative with a high degree of polymerization (DP) such as carboxymethyl cellulose (CMC). It can be measured by both methods, i.e., reduction in substrate viscosity/increase in reducing sugar. CMCase activity using CMC is measured by determining reducing sugars released after 5 min of enzyme reaction with 0.5% CMC at pH 4.8 and 50°C [78]. Exoglucanases are known to cleave the easily accessible ends of cellulose molecules liberating glucose and cellobiose. β-glucosidases cleaves soluble cellobiose and other cellodextrins having DP up to 6 and liberates glucose as end product [82]. Various chromogenic and nonchromogenic substrates could be evaluated. In chromogenic method, p-nitrophenol-β-glucoside (P-NPG) can be used as the substrate. However, in the case of nonchromogenic substrates different methods used are based on nature of substrates. For example, when oligo or disaccharides (such as cellobiose) are used, released glucose can be evaluated by the GOD (glucose oxidase)

method with a commercial kit but when polysaccharide are used a substrate, reducing sugars released is measured by the DNS (dinitrosalicylic acid) method [81].

4.3 Production of cellulases

The technique which are mainly used for the enzyme production are Submerged fermentation (SmF) and solid state fermentation (SSF) [83].

4.3.1 Submerged fermentation

When fermentation is performed with some free flowing nutrient media; it is termed as SmF [84]. In industry, enzymes are produced mostly by SmF, primarily due to the much simplified processes associated with scale-up compared to those involved for scale-up in SSF [85]. In fact, some other important factors like indulgence in controlling process parameters, monitoring and downstream processing makes SmF more significant [86]. Only a few designs are available in literature for SSF based bioreactors. This is principally due to several problems encountered in case of SSF for controlling various parameters like pH, temperature, aeration and moisture content. Fungal cellulase production is largely dependent on media composition and culture conditions. Thus development of a suitable fermentation strategy is necessary for full exploitation of potential of microorganism used for fermentation [87]. Several reports are available for cellulase production using SmF. Karthikeyan et al. [88] reported cellulase production from *Penicillium* strain K-P in liquid medium supplemented with different carbon and nitrogen sources at varying pH and temperature, maximum cellulase activity was observed on fifth day (pH 3.0 and 30°C) in the presence of fructose and ammonium nitrate as carbon and nitrogen source respectively. Narasimha et al. [89] reported maximum cellulase production using *A. niger* on medium (pH 5) supplemented with 1% CMC or sawdust.

4.3.2 Solid state fermentation

When fermentation is performed on nonsoluble materials in the absence of free flowing nutrient media, so that the material used can serve as a platform for support as well as nutrients; it is termed as solid state fermentation. While compared for their potential it was found SSF offers various opportunities over SmF because they are eco-friendly on account of lower energy requirements, produce lesser wastewater and they are based on employment of waste solid biomass [90]. Further advantages of SSF over SmF include prevalence of nonaseptic conditions, a wide variety of substrate are available, low capital cost, inexpensive downstream processing [91], higher product concentration, high reproducibility, lesser space requirements (compact fermenters), easy contamination management [92]. It is observed that production cost was decreased about 10 fold in SSF over SmF.

4.3.3 Fermentation conditions

Fermentation condition play the main role for the standardization of process parameters such as incubation period, inoculum size, pH, carbon and Nitrogen source, metal ions, etc. Maximum cellulase production may vary from 1 day to weeks. It is usually observed that fungal cultures require longer incubation period for cellulase production than bacterial cultures. The highest cellulase level was achieved 96 hrs of the fermentation while using *T. harzianam* and *P. chrysosporium* [93]. Maximum cellulase production was observed after 96 h by *A. niger* [94].

Optimal cellulase secretion from *Aspergillus niger* was achieved at a time of 72 h in maize straw while 96 and 120 h were the growth period in millet and guinea corn straws respectively [95].

The age and concentration of inoculum also plays an important role in the production of cellulases. An increase in inoculum size up to an optimum limits results in rapid proliferation and biomass synthesis which leads to produced higher amount of cellulase [96]. On the other hand higher inoculum volume beyond optimum size leads to increases in the water content of medium in case of SSF creating aeration problems in SSF and it will responsible for reduction in overall yield [97].

Bacterial and fungal cellulase production found to be significantly affected by pH. Milala et al. [95] reported maximum cellulase activity at pH 4.0 by *A. niger*. Devi and Kumar [98] optimized condition of cellulase production in fungal strain *A. niger* against the lignocellulosic bio wastes like saw dust, paper cellulose at varying environmental parameters of pH (4.0–7.0) and maximum activity was observed at pH 5. Gao et al. [99] studied the production of extracellular cellulases by a newly isolated thermoacidophilic fungus *Aspergillus terreus* M11 on the lignocellulosic materials in solid-state fermentation (SSF) and the high-level cellulase activity was observed at pH 3.0. However, the results appeared to contradict previous results reported by Solingen et al. [100] of an alkaline novel *Streptomyces* sp. isolated from east African soda lakes that have an optimal pH of 8.0, highlighting the effect of alkaline environment on the adaptation of these *Streptomyces*.

The fermentation temperature plays a very significant role on the growth and metabolic activity of microbial cells. Optimum temperature for cellulase production under solid-state fermentation by *Trichoderma reesei* RUT C30 was 33°C [101]. Fatma et al. [102] studied ethanol production from rice straw using cellulase produced by *T. reesei* F-418 cultivated in alkali treated rice straw under SSF and reported 162 U/g substrate cellulase activity when fungus was cultivated incubation at 28°C. Maximum enzyme production (3.9 U/ml) was achieved at 45°C temperature by *Aspergillus niger* using paper cellulose [98]. Gao et al. [99] studied production of extracellular cellulases by a newly isolated thermoacidophilic fungus *Aspergillus terreus M11*, on the lignocellulosic materials in solid-state fermentation (SSF) at 45°C. Jang and Chen [103] described a CMCase produced by a *Streptomyces* T3-1 with optimum temperature 50°C. Schrempf and Walter, [104] described a CMCase production by *S. reticuli* at an optimum temperature 55°C.

Various carbon sources such as metabolizable sugars, commercial cellulose and agricultural residues/by-products have been used for cellulase production. Some carbon sources resulted good growth with low enzyme production while some supported good growth along with high yield of enzyme secretion. Commercially available carbon sources used for cellulase production were Powdered cellulose by *A. niger* [105], and CM Lactose by *Mucor circinelloides* [81]. Several studies focused on cellulase use in the bioconversion of agro-industrial waste [106]. Chandra et al. [107] studied effect of several carbon sources including groundnut fodder, wheat bran, rice bran and sawdust on cellulase production by *A. niger.* They found that highest titers of cellulolytic enzymes in solid state fermentation on wheat bran. Azzaz, [108] studied effect of several carbon sources including banana wastes, rice straw, wheat straw, corn stalks and pure cellulose powder on cellulase production by *A. niger* and *A. flavus* NRRL 5521. He observed that wheat straw gave the highest cellulase production when fermented with *A. niger* (0.177 U/mL) while rice straw gave the highest (0.046 U/mL) cellulase production when fermented with *A. flavus* NRRL 5521. The lignocellulosic residues offer cheaper substituent of pure cellulose available commercially for the production of cellulase. Mixed substrates like wheat bran and corn cob are used as best carbon source in case of *A. niger* NRRL3 for cellulase production under SSF [109]. Milala et al. [95] used different agricultural

Supplement	SmF (U/mL)		SSF (U/gDMB)	
	CMCase	FPase	CMCase	FPase
Carbon sources (5% w/v in SmF and 4% w/w in SSF)				
Control	0.7	0.4	3.7	2
Glucose	1.52	0.54	11.1	6.5
Xylose	1.2	1.42	15.7	6.6
Lactose	3	1.71	18	10.9
Maltose	1.51	1.5	17.5	6.3
Sucrose	1.54	1.51	13.7	6.2

Table 3.
Effect of supplementation of various carbon sources [106].

wastes millet, guinea corn straw, rice husks and maize straw as carbon sources for cellulase production by *Aspergillus niger*. According to Mrudula and Murugammal [85] lactose was found to be the best inducer in SmF and SSF (**Table 3**). Prasanna et al. [110] also reported lactose as the most excellent carbon source for cellulase production by *Penicillium* sp. followed by carboxymethyl cellulose and galactose.

Different researchers studied the effect of various nitrogen sources for cellulase production by employing different microbes. Peptone was reported as most effective nitrogen source for Penicillium sp. [110], *Penicillium waksmanii* F10-2 [111], urea for *A. niger* [89] and NH_4NO_3 for *Trichoderma reesei* NRRL 11460 [112]. Although the addition of beef extract and peptone (as organic nitrogen source) leads to enhanced growth and enzyme production but they were not economically fit because of their higher cost.

Cellulase production by some microorganisms has been found to be influenced by metal ions, chelators, detergents and surfactants. It was reported that usually metal ions such as Ag^{+}, Cu^{2+}, Hg^{2+}, Fe^{3+}, K^{+}, Mn^{2+}, Mg^{2+}, and Zn^{2+} are slightly or completely inhibitory of cellulase, whereas metal ions such as Ca^{2+}, Co^{2+} and Na^{+} either stimulate or does not affect the cellulase activity [113]. Addition of Tween20 leads to a significant increase in endoglucanase and xylanase production by *Melanocarpus* sp. MTCC 3922 [114]. Cellulase activity increased with Tween80 and reduced with SDS [115]. Enhancement in enzyme production by Tween80 may be due to increase in permeability of cell membrane allowing rapid secretion and synthesis of the enzymes [116].

5. Purification of cellulase

It is an important step to remove any contaminants that are found to be present in the mixture. Hence, it is a vital step required for improving performance/functioning of an enzyme. Enzymes in the culture supernatant could be purified by the conventional methods which include ammonium sulfate precipitation and dialysis followed by column chromatography [117]. The most common matrix for gel exclusion chromatography is the Sephadex with different pore sizes which is employed in the purification of cellulase [118]. The purification folds and % yield are the two most important factors which are used to evaluate the efficiency of purification. First step (ammonium salt precipitation) is based upon difference in protein solubility. The solubility of protein firstly increase and then starts decreasing with increase in salt concentration and finally protein gets precipitate. This

process is called Salting out [119]. Ammonium sulfate ($(NH_4)_2SO_4$) is often used for this purpose because of its high solubility in water. Devi et al. [120] reported protein precipitation by addition of solid ammonium sulfate up to 80% saturation. Chen et al. [121] reported precipitation with $(NH_4)_2SO_4$ at 40–60% saturation. Precipitation is followed by a concentrating step that separates proteins from salts called dialysis. For next step chromatographic technique is most widely used for the direct recovery of protein and other charged molecules. Various types of chromatography methods (gel filtration: Sephadex G-100 [73], ion exchange: DEAE-Cellulose [122] and affinity: swollen avicel [123] have been used for purification of cellulase from various fungal strains.

6. Characterization of cellulase

Different researchers reported different temperatures for maximum cellulase production. It is reported that the optimal temperature for cellulase production varies from strain to strain of microorganisms [69]. The optimum temperature of fungal cellulases ranges from 40 to 60°C and pH found to be 4.8. A battery of thermophilic fungal strains are known to produce thermostable enzymes which are stable and active at such high temperature which are not optimum for the growth of the microorganism. Filamentous fungi, e.g., *Talaromyces emersonii*, *Thermoascus aurantiacus* and *Chaetomium thermophilum* are reported to produce cellulases having high-cellulase activity at elevated temperature [124]. The Km value is used for the measurement of enzyme affinity towards the substrate. An increase in substrate concentration made more binding sites available for the enzymes to adhere and the rate at which product formation would be achieved therefore would be faster [125]. In literature, different ranges of Km and Vmax for different fungal species have been reported. Genetic variability may be a factor for the above reported variation [126]. Taha et al. [127] reported cellulase showing optimum activity at pH 6 and 50°C with (Vmax) of 75 g l^{-1} min^{-1} mg^{-1} with its corresponding Km value of 2.5 × 10^{-5} g/l.

7. Applications of cellulases

According to Sajith et al. [87] on the global enzyme market cellulases occupy the third place (i.e., ≈15%) after amylase (≈25%) and protease (≈18%). Cellulases are currently being produced on commercial scale by several industries all over the world and widely used in various industrial applications [128].

7.1 Paper and pulp industries

Today, 90% of paper pulp is made of wood. Recycling one ton of newsprint and printing or copier paper saves about 1 ton and more than 2 tons of wood respectively [129]. Usually, the industrial process for eradicating wastepaper pollutants involves re-pulping, screening, cleaning, washing and flotation [130]. According to Shrinath et al. [131] the conventional recycling of waste papers is costly and hazardous to the environment due to the use of chemicals (hydrogen peroxide, sodium hydroxide and sodium silicate). Cellulases are mainly used for the pulping and deinking of waste papers. Enzymatic deinking as whole is an environmental friendly process [132]. Cellulase based pulping process is not only energy efficient, environment-friendly but also improve mechanical strength of the final paper product by improving the inter-fiber bonding [133]. When used with hemicellulases, cellulases improve the brightness and quality of the recycled paper [134].

Besides deinking and pulping, cellulases are also used in paper mills for drainage of clogged pipes by dissolving fiber residues [61] and for manufacturing easily biodegradable cardboards, sanitary papers [135].

7.2 Textile industry

Among the application textile industry dominated in the market in 2017. Cellulase application in textile play main role in the growth of textile industry. In textile industry worn-out look is given to the denim using stone washing. But stone washing have some disadvantages. It causes wear and tear of the fabric, huge loss of water due to extensive washing step and high labor cost, etc. Cellulases used for bio-polishing of cotton cloths and enzyme based stoning of jeans to impart stone-washed look for denims. Cellulase treatment gives a smooth and glossy appearance to fabric by removing short fibers, surface fuzziness and improves color brightness, hydrophilicity and moisture absorbance [136]. Most of the cotton and cotton mixed garments tend to become fluffy and dull during repeated washing due to detachment of microfibrils on the surface of garments. Cellulase treatment can restore a smooth surface and original color to the garments by removing these microfibrils [137]. According to a statistics of India Brand Equity Foundation (IBEF), Indian textile market has increased from US$ 99 Billion in 2014 to US$137 Billion in 2016 and exhibited a CAGR of 17.6% during the period 2014–2016.

7.3 Food and feed processing

Cellulases are found to be highly valuable for feed and food Recently BIO-CAT introduced a cellulase (Cellulase C500) at IPPE 2016. The enzyme have been derived from a non-GMO, AAFCO approved microbial strain. Addition of Cellulase to animal feed increases its digestibility (http://www.bio-cat.com/introducing-cellulase-c500-animal-feed-enzyme/).

Use of cellulases in feed processing leads to improvement in feed digestibility and animal performance. As a component of macerating enzyme complex (cellulase, xylanase and pectinase) these are used for extraction and clarification of fruits and vegetable juices, nectars and oils [138]. Along with others, cell wall degrading enzymes cellulases can be used to reduce bitterness and increase the taste and aroma of citrus fruits [61].

7.4 Detergents

Nowadays liquid laundry detergent containing anionic or nonionic surfactant, citric acid or a water-soluble salt, protease, cellulose and a mixture of propanediol and boric acid or its derivatives are employed to improve the stability of cellulases [61]. Cellulases are added to detergents for the breakdown of hydrogen bonding under harsh environmental conditions such as alkaline or thermophilic conditions [139]. Cellulases are mixed with detergents to enhance brightness and hand feel, dirt removal from cotton and cotton blended garments because they are capable of modifying the structure of cellulose fibrils [62].

7.5 Biofuel production

With the fast exhaustion of fossil fuels the need to find a substitute source for renewable energy and fuels is intensifying day by day. Thus interest in the saccharification of lignocellulosic biomass using cellulases and other related enzymes is also increasing [14, 16]. In other words, the cellulase market could be expanded

considerably by using cellulases for saccharification of pretreated cellulosic material to sugars which can be fermented further to bioethanol and other bio-based products on large scale [77]. By 2020 biofuels, especially bioethanol from renewable resources is expected to replace 20% of the fossil fuel consumption [140]. Cellulases produced by various filamentous fungi mainly *Aspergillus*, *Trichoderma* and *Penicillium* have a potentially to be used successfully for bioethanol production using sugarcane bagasse, corn straw, rice straw, wheat straw and wheat bran as raw materials [141–143].

7.6 Wine and brewery industry

Microbial glucanases and related polysaccharides are usually used to produce alcoholic beverages including beers and wines by fermentation [144]. In wine production various enzymes such as pectinases, glucanases and hemicellulases plays an important role in improving wine quality and stability by improving color extraction, skin maceration, must clarification and filtration [145]. According to the precedent literature about 10–35% increase in the wine must extraction, a 70–80% increase in the rate of must filtration, 50–120 min decreased pressing time, and 30–70% decreased must viscosity, 20–40% energy saving while cooling thus a considerably improved wine stability. Thus supplementation of enzymes like cellulase and pectinase to the process are expected to enhance the productivity of brewing production [143]. β-Glucosidases can enhance the aroma of wines by modifying glycosylated precursors. Macerating enzymes also improve the juice, press ability and settling of grapes used for wine fermentation. A number of commercial enzyme preparations are now available to the wine industry.

7.7 Medical industry

Cellulolytic bacteria like Bacteroides cellulosilyticus and Ruminococcus champanellensis can be employed for the treatment of phytobezoars disease, which causes concretion of indigestible vegetable and fruit fibers in the gastrointestinal tract that may leads to surgical intrusion [128]. Moreover, cellulases have been utilized as excellent antibiofilm agents against pathogenic biofilms [146]. Further research is required to unravel yet unknown applications of cellulases in medical field.

8. Cellulase market demand

Demand for industrial enzymes in developed countries such as the US, Western Europe, Japan and Canada was relatively stable during the recent times while in developing economies of Asia-Pacific, Eastern Europe, Africa and Middle East regions, demand is increasing day by day [147]. Currently, by dollar volume cellulases are the third largest industrial enzyme globally, because of their extensive applications in animal feed additives, as detergent enzymes, cotton processing, juice extraction and paper recycling. However, cellulases may become the largest quantity industrial enzyme, if ethanol produced from lignocellulosic biomass through these enzymes becomes the major transportation fuel [112, 148]. They contribute to 8% of the worldwide industrial enzyme demand [149]. The international market for biofuel enzymes is expected to reach $9.0 billion by 2017 [150]. Global demand for industrial enzyme's projected to grow 4.0% per year to $5.0 billion in 2021. Key players in the global cellulose market are Amano enzyme U.S.A, Worthington Biochemical Corporation, MP Biomedical LLC, Sigma-Aldrich Co. LLC, Prozmix LLC, Creative Enzymes, bio-WORLD, Amano

Enzyme samples	Supplier	Source
Cellubrix	Novozymes, Denmark	*Trichoderma longibrachiatum* and *Aspergillus niger*
Novozymes 188	Novozymes	*Aspergillus niger*
Viscostar 150L	Dyadic (Jupiter, USA)	*Trichoderma longibrachiatum/Trichoderma reesei*
Multifect CL Genencor	Intl. (S.San Francisco, CA)	*Trichoderma reesei*
Energex L	Novozymes	*Trichoderma longibrachiatum/Trichoderma reesei*
Ultraflo L	Novozymes	*Trichoderma longibrachiatum/ Trichoderma reesei*
Viscozyme L	Novozymes	*Trichoderma longibrachiatum/Trichoderma reesei*
GC 440	Genencor-Danisco (Rochester, USA)	*Trichoderma longibrachiatum/Trichoderma reesei*
GC 880	Genencor	*Trichoderma longibrachiatum/Trichoderma reesei*
Spezyme CP	Genencor	*Trichoderma longibrachiatum/Trichoderma reesei*
Accelerase® 1500	Genencor	*Trichoderma reesei*
Cellulase AP30K	Amano Enzyme	*Aspeergillus niger*
Cellulase TRL	Solvay Enzymes (Elkhart, IN)	*Trichoderma longibrachiatum/Trichoderma reesei*
Econase CE	Alko-EDC (New York, NY)	*Trichoderma longibrachiatum/Trichoderma reesei*
Cellulase TAP106	Amano Enzyme (Troy, VA)	*Trichoderma viride*

Table 4.
Suppliers and sources of enzyme samples [122].

Enzyme Inc., Zhongbei Bio-Chem Industry Co., Ltd., Hunan Hong Ying Biotech Co., Ltd., Genencor and Novozyme are major producers they are known worldwide for cellulase production. All above companies played a noteworthy role for reducing production cost of cellulase several folds by their active research and are still continuing to bring down the cost by assuming novel technologies [112]. A few suppliers and source of enzyme samples are list below (**Table 4**). North America accounted for largest market share in global cellulose production in 2017. Production is depended on the increasing production of biofuel. According to a report by United States Energy information Administration in July 2018, the production of biofuel has increased in the U.S. from 1891 trillion butane to 2332 trillion, increasing at a CAGR of 5.4 during 2013 to 2017.

9. Future prospects

The demand for cellulases is increasing day by day due to its volatile and the rise in oil prices which induced a shift in interest towards the application of cellulases in producing biofuel using lignocellulosic biomass [151]. Enhancing the cellulase activity and reducing the cost of production of enzyme are two key issues regarding the enzymatic hydrolysis of cellulosic biomass. Genetic techniques can be used to clone the cellulase coding sequences into bacteria, yeasts, fungi, plants and animals to create new cellulase producing systems with improved production and activity of enzyme [152]. One of the major drawbacks of SSF is the low thermal conductivity of the solid medium used in SSF which restricts the removal of excess heat generated by microbial metabolism. The elevated temperature in bioreactors may lead

to denaturation of thermo labile proteins [153]. Thus the thermo stable, modified fungal and bacterial strains are also good future prospects for cellulase production [62]. Interchangeably more advanced strategy is to engineer microbes for producing all major enzymes involved in cellulose hydrolysis in optimum ratio which may decrease the expenditure greatly [154]. Although the cellulase enzyme cost has dropped due to improvements in expression vectors and on-site production still there is a necessity of engineering a new generation cellulase cocktails that would further reduce cellulase cost. Efforts have to be made via hunting both diversity rich environments and extremophilic niches for identification of novel cellulase producers [150]. It can be made possible through following four approaches:

i. Mining novel cellulase genes via culturable/nonculturable strategies.

ii. Improving production technologies by using novel bioreactors.

iii. Designing novel cellulases through protein and metabolic engineering by understanding molecular mechanism and mode of interaction of cellulases with substrates.

iv. Using mathematical, biophysical and enzymological approaches for cellulase production through consolidated bioprocessing in a cost-effective manner.

10. Conclusion

Lignocellulosic biomass is the most abundant biomass on the earth. They are the potential source of biofuels, and other useful chemicals. But one of the most severe hindrances in this process is the structure of biomass itself. This problem can be resolved up to a greater extent by various types of pretreatments and enzymatic hydrolysis, engineered cellulases and by consolidated bioprocessing.

Consolidated bioprocessing includes cellulose production, hydrolysis of cellulose and fermentation of Pentose and Hexose sugars in a single step which will reduce production cost and increase production/conversion efficiency as compared to the processes performing dedicated cellulase production. A good pretreatment should result in increased cellulose content and decreased hemicelluloses/lignin content of biomass. Another problem is the yield and efficiency of enzyme. Yield of enzyme can be increased by optimization of different parameters involved in enzyme production using one variable or statistical approach (RSM). Alternatively novel proteins with enhanced production can be synthesized by protein and metabolic engineering. Enzyme engineering must be focused on (1) to increase cellulase specific activity on pretreated biomass through enzyme cocktail (2) to increase cellulase stability for cellulase recycling, and (3) to reduce enzyme production costs. Consolidated bioprocessing microorganisms or consortium would simplify the whole process and increase productivity. The above three approaches would be integrated together for maximizing the process for lignocellulosic biomass management/conversion in to value added products.

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