## CELLULOLYTIC MICROORGANISMS: DIVERSITY AND ROLE IN CONVERSION OF RICE STRAW TO BIOETHANOL

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Lignocellulosic biomass is considered to be an inexhaustible biopolymer known to mankind. For the successful exploitation of lignocellulosic biomass, such as rice straw, as a substrate for the production of industrially significant value-added products, such as bioethanol, its conversion into fermentable sugars, sugar acids and phenolics is the most imperative step. Enzymatic hydrolysis of lignocelluloses is performed by cellulases, a complex enzyme system comprising endo-1,4- $\beta$ -D-glucanase, exo-1,4- $\beta$ -D-glucanase and  $\beta$ -D-glucosidase. Cellulases are produced by a diverse range of microorganisms, including bacteria, fungi, actinomycetes, algae, insects and mollusks. However, the cost of cellulase is a major economic barrier for ethanol production. To economize the ethanol production process for its successful utilization at the industrial scale, genetic improvement of microorganisms, and other industrially relevant strategies of enzyme production and recycling need to be adopted.

Keywords: lignocellulose, rice straw, cellulases, hydrolysis, bioethanol

#### **INTRODUCTION**

A spurt in industrialization, booming population growth, injudicious utilization of natural resources over the past few decades have compelled us to exploit alternative renewable energy resources to meet the upsurging energy demands.<sup>1</sup> Lignocellulosic biomass like rice straw is a widely available, cheap, polymeric, renewable resource and has attracted wide interest in the search of alternative ways to meet future energy demands. The chemical composition of rice straw. with 32-47% cellulose, 19-27% hemicelluloses and 5-24% lignin, makes it an ideal substrate for ethanolic fermentation.<sup>2</sup> Complete and efficient hydrolysis of cellulose requires an enzyme complex called cellulase enzyme complex, involving the synergistic action of different enzymes, viz. exo-glucanases, endoglucanases and  $\beta$ -glucosidase. Cellulases belong to the glycoside hydrolase family of enzymes and act by cleaving  $\beta$ ,1-4 glycosidic bonds of polysaccharides and oligosaccharides.<sup>3</sup> Cellulases are produced by diverse types of microorganisms, including

bacteria, fungi, actinomycetes, algae, insects and mollusks. However, the major impediment in the successful commercialization of cellulase is its exorbitant cost.<sup>4</sup> A wide array of strategies, such as the isolation of potential cellulase producing microorganisms, use of low-cost substrates, such as rice bran, rice straw, wheat bran, coconut coir pith etc., and solid substrate fermentation as a mode of enzyme production, improved cellulase synthesis by genetic engineering,<sup>5</sup> and recycling of cellulase by immobilization,<sup>6,7</sup> could appreciably bring down the cost of the saccharification process. The present review synthesizes available information on cellulose, its structure and the historical background of its research, as well as on cellulase complexes, their structure and catalytic mechanisms, the microbial sources of cellulases and their role in the conversion of lignocellulosic biomass (rice straw) to ethanol. Also, the review discusses some challenges in the way of ethanol production and proposes future research directions.

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#### CELLULOSE – FUNDAMENTAL ASPECTS AND HISTORICAL BACKGROUND

Cellulose is known to be the most abundant and almost inexhaustible biopolymer. As per estimations,  $10^{11}$ - $10^{12}$  tons of cellulose is synthesized annually through photosynthesis, either in pure form or in combination with lignin and other polysaccharides. such hemicelluloses. The trend towards these kinds of resources and their customization to obtain novel products to be used for the welfare of mankind has led to a resurgence of integrative cellulose research and an immense interest in using this biopolymer.<sup>8</sup> Cellulose as a polymeric raw material has been used for a wide variety of purposes for centuries, e.g. for the production of paper products, such as cotton, rayon and linen for clothes. Cellulose also acts as a precursor for many chemical conversions involved in the production of stable cellulose derivatives, e.g. nitrocellulose for explosives and cellulose acetate for films. The name <sup>TM</sup>cellulose was coined by Payen, as early as in 1838, who was the first to recognize it as a definitive substance.<sup>9</sup> Important landmarks in the field of cellulose research were the discovery of cellulose nitrate by Schönbein,<sup>10</sup> Schweizer's reagent (cuprammonium hydroxide solution) as the first cellulose solvent in 1857 (Schweizer, 1856, 1857, 1859),<sup>11-13</sup> and cellulose acetate synthesis.<sup>14-15</sup> The fundamental experiments of Staudinger<sup>16-17</sup> on the acetylation and deacetvlation of cellulose led to the evolution of cellulose chemistry as a branch of polymer research.18

## Diversity of cellulose

Cellulose is derived from lignocellulosic materials, such as agricultural residues, plant fibers, grasses and wood.<sup>19</sup> Lignocellulosic biomass is primarily composed of cellulose, hemicellulose, and lignin, the composition of which may vary depending on the type of biomass, e.g. corn stover, straw, wheat straw, algae and others (Table 1). Cellulose is synthesized by a multitude of microorganisms, bacterial species, e.g., Acetobacter, Rhizobium and Agrobacterium.<sup>20</sup> Besides, some pathogenic bacteria have also been found to synthesize cellulose. Cyanobacteria, which represent the most primitive forms of life on the earth, also synthesize cellulose.<sup>21</sup> Eukaryotic organisms like fungi, amoebae, cellular slime molds, and green algae, are also known to produce cellulose. In fact, green alga Valonia ventricosa is known to

produce one of nature's most perfect crystalline forms of cellulose.<sup>22</sup> Cellulose is also synthesized by marine and freshwater algae, including land plants, such as mosses, ferns, angiosperms, and gymnosperms,<sup>22</sup> and even by some animals, such as the tunicates.<sup>23</sup>

### Structure of cellulose

Cellulose exists in nature in two states: crystalline and non-crystalline. The crystalline state has further six different polymorphs: cellulose I, II, III<sub>I</sub>, III<sub>I</sub>, IV<sub>I</sub>, and IV<sub>I</sub>. Cellulose I and II are prevalent and the other polymorphs can be obtained artificially employing chemical or heat treatments. Cellulose I is more abundantly present than cellulose II, and has two allomorphs denominated as I $\alpha$  and I $\beta$ . Based on its structure, cellulose can be defined as a linear homopolymer made up of  $\beta$ -D-glucopyranose units, linked by  $\beta$ -1,4-glycosidic bonds with anhydroglucose (C<sub>6</sub>H<sub>10</sub>O<sub>5</sub>)n, as its monomeric unit (Fig. 1).<sup>38</sup>

The dimeric form of cellulose, cellobiose is a structural repetitive unit of the cellulose chain, which is made up of two glucose residues linked by  $\beta$ -1,4 linkage. The degree of polymerization of cellulose, n, is dictated by the number of monomers present in the cellulose chain and varies between 10,000 to 15,000.<sup>22</sup> Cellulose is an amphiphilic structure with a clear demarcation of polar (OH) and nonpolar (CH) patches. The  $\beta$ -(1,4) linkage between glucose residues confers unique structural characteristics to cellulose. Cellulose exists in the form of microfibrils, with approximately 36 glucose chains stabilized by intra- and inter-hydrogen bonding, which impart microfibrils.39 greater stability to These are microfibrils further organized into superassemblies, such as cell walls, fibers, and pellicles.

Cellulose fibers present in the natural environment lack pure crystallinity. This deviation is believed to be variable and has resulted in the genesis of a notion called "lateral order distribution" of crystallinity, comprising purely crystalline to purely amorphous cellulose fibers with all intermediary degrees of order.<sup>40</sup> The cellulose fibers contain various types of indentations, such as kinks or twists, or voids such as surface micropores and capillaries.<sup>41</sup> In addition to imparting a greater surface area to the cellulose fiber, the structural heterogeneity within the fiber results in partial hydration by water on immersion into aqueous media and also permits the penetration of relatively large molecules like cellulolytic enzymes via the micropores and

capillaries.42

Lignocellulosic biomass		Cellulose	Hemicellulose	Lignin	Deferences
Lignocenulosic	bioinass	(%) (%) (%) Refere		References	
Hardwood	Oak	40.4	35.9	24.1	[24]
	Eucalyptus	54.1	18.4	21.5	[24]
	Hardwood bark	22-40	20-38	30-35	[24]
	Hardwood stems	40-45	18-40	18-28	[25]
Softwood	Pine	42-50	24-27	20	[24]
	Douglas fir	44	11	27	[24]
	Spruce	45.5	22.9	27.9	[24]
	Softwood stem	34-50	21-35	28-35	[25]
	Wheat straw	35.0-39.0	23.0-30.0	12.0-16.0	[26]
	Barley hull	34.0	36.0	13.8-19.0	[24]
	Rice straw	29.2-34.7	23.0-25.9	17.0-19.0	[27,28]
	Barley straw	36.0-43.0	24.0-33.0	6.3-9.8	[29]
	Sugarcane bagasse	25.0-45.0	28.0-32.0	15.0-25.0	[30]
Agricultural waste	Sorghum straw	32.0-35.0	24.0-27.0	15.0-21.0	[24]
	Corn cobs	33.7-41.2	31.9-36.0	6.1-15.9	[31]
	Corn shank	33.8	20.1	31.1	[32]
	Corn stover	37-42	20-28	18-22	[33]
	Areca nut shell	38.4	17.2	25.1	[32]
	Areca nut sheath	17.8	10.4	20.2	[32]
	Bamboo	45.7	26.5	21.1	[31]
	Grasses	25.0-40.0	25.0-50.0	10.0-30.0	[24]
Grasses	Switchgrass	35.0-40.0	25.0-30.0	15.0-20.0	[34]
	Bermuda grass	25.0	35.7	9-18	[32]
	Miscanthus	40	18	23	[35]
Others	Waste papers	12.20	50-70	6-10	[26]
	from chemical pulps	12-20			[30]
	Newspaper	40-55	25-40	18-30	[36]
	Manure solid fibres	8-27	12-22	2-13	[37]

 Table 1

 Chemical composition of different lignocellulosic biomass



Figure 1: Structure of cellulose

#### **CELLULASE COMPLEXES**

The cellulase is the chief enzyme involved in the breakdown of cellulose into simple sugars. Cellulases act by hydrolyzing the  $\beta$ -1,4 linkages in the cellulose chains. The families of catalytic modules of cellulases have been classified based on amino acid sequences and crystal structures.<sup>43</sup> Cellulases also consist of non-catalytic

carbohydrate-binding modules (CBMs) and modules of known or unknown functions, located at the N- or C-terminus of a catalytic module.

Cellulolytic microorganisms have evolved different strategies for cellulose degradation, these microorganisms have either discrete uncomplexed cellulases or complexed cellulases.<sup>44</sup> In general, most aerobic cellulolytic microorganisms degrade cellulose by secreting a set of individual cellulases, each of which contains a CBM adhered to the catalytic module by a flexible linker peptide.<sup>4</sup> In the case of anaerobic microorganisms, large multi-enzyme complexes (approx. 1 million molecular mass), called cellulosomes, are present.<sup>46</sup>

#### Three-dimensional cellulase structure

The function of cellulase enzymes has been unravelled with the help of crystal structures of glycoside hydrolase (GH). It has been elucidated that CBM and a catalytic domain (CD), connected by a non-structured linker peptide are the two domains present in the endo-cellulases and exocellulases.<sup>47</sup> CBMs are non-catalytic, functionally independent domains connected to the catalytic domain of many GHs. Based on fold and carbohydrate recognition, 68 families of CBMs have been classified.<sup>48</sup> The binding of CBM to the cellulose fibers possibly helps to localize the catalytic modules on crystalline cellulosic substrates and target specific carbohydrate structures, as well as aids in the disintegration of lignocelluloses.49 As the catalytic domain of cellulase contains the active site, it must be able to bind, orient, and hydrolyze the  $\beta$ -(1, 4)glycosidic bond. Interestingly, the presence of a tunnel-like structure around the active site seems to be important in the determination of the enzyme's processivity.<sup>50</sup> One such highly processive enzyme is Cel7A, with an enclosed "tunnel" that accommodates a single cellulose chain threaded through the active site. On the other hand, less processive enzymes, which lack these tunnels around their active sites, allow cellulases to bind and arbitrarily release cellulose chains.

#### **Catalytic mechanisms**

There are two major catalytic mechanisms for glycosidic bond hydrolysis: retaining and inverting. During the retaining mechanism, glycosidic oxygen is initially protonated via the acid/base catalyst, along with the formation of a

glycosyl-enzyme intermediate through the nucleophile. The further step involves the subsequent hydrolysis of the intermediate via attack by a water molecule, generating a product with the same stereochemistry as that of the substrate (Fig. 2a). During the inverting mechanism, single-step protonation of the glycosidic oxygen via the acid/base catalyst takes place, along with the nucleophile activated attack of a water molecule, generating a product with the opposite stereochemistry as that of the substrate (Fig. 2b). The type of mechanism, retaining or inverting, is conserved for a given glycoside hydrolase family and is dictated by various factors, such as the architecture of active-site and atomic distance between the acid/base and nucleophilic residues, such as aspartic and/or glutamic acids.<sup>51</sup>

## ENZYME SYSTEM FRAMEWORKS IN MICROORGANISMS

#### Free enzyme system

In nature, complete cellulase hydrolysis is mediated by the collaborative action of three principal enzymes, viz., endoglucanase (EC 3.2.1.4). exoglucanase including cellobiohydrolases (CBHs) (EC 3.2.1.91) and βglucosidase (EC 3.2.1.21).<sup>52</sup> The amorphous (internal) regions of the fibrils are preferentially hydrolyzed by endoglucanases, by randomly cleaving β-glycosidic bonds; cellobiohydrolases are exoglucanases, which act to release the cellobiose from the termini of cellulose chains, while  $\beta$ -glucosidases hydrolyze cellobiose and other cellodextrins into glucose units, thus completing the degradation process. The free cellulases are produced by fungi, bacteria, protozoans, plants and animals, and belong to the glycoside hydrolase (GH) families<sup>53</sup> (Table 2). More than 48,000 CBM sequences have been classified into 71 CBM families based on sequence similarity, and the structures of 271 representative CBMs have been reported until now.<sup>54</sup> Based on the structure and potential to affect the function of associated catalytic domain, three types of CBMs (types A, B and C) have been identified.<sup>55</sup> Type A CBMs interact with the planar surfaces of cellulose by aromatic amino acid side chains of Trp, Tyr and Phe.<sup>56</sup> Type B CBMs interact with amorphous regions of cellulose and hemicelluloses by an open cleft associated with it.<sup>57,58</sup> Type C CBMs are believed to bind short soluble oligosaccharides.<sup>57</sup> Due to the variable binding strategies of these CBMs,

they have great effects on the catalytic rates of the attached enzyme.<sup>59</sup>

#### Endoglucanase

Endoglucanase or carboxymethyl cellulases arbitrarily hydrolyze carboxymethyl cellulose (CMC), resulting in a rapid shortening of cellulose and a gradual increase in reducing sugar concentration. Both the reducing and nonreducing ends of the cellulose chains are exposed by the activity of endoglucanases.<sup>60</sup> Different catalytic modules belonging to families 5-9, 12, 44, 45, 48, 51 and 74 are produced by archaea, bacteria, fungi, plants and animals. Generally, fungal endoglucanases contain a catalytic module, with or without a CBM, while bacterial endoglucanases may contain multiple catalytic modules. CBMs, and other modules with unknown function. A cleft/grove-shaped active site is the characteristic feature present in the catalytic modules of most endoglucanases. The cleft-like structure aids in binding and cleaving the cellulose chain and consequently generates an array of small and insoluble cellulose fragment. However, some endoglucanases act "processively", based on their ability to hydrolyze crystalline cellulose and generate cellobiose or longer cellodextrins as the major products.<sup>61</sup>

#### Exoglucanase

Exoglucanase acts at the non-reducing end of the cellulose chain and degrades it by splitting the cellobiose units. Exoglucanases are known to act on swollen, partially degraded amorphous substrates and cellodextrins, but are incapable of hydrolyzing the soluble derivatives of cellulose like carboxymethyl cellulose and hydroxyethyl cellulose. Cellobiohydrolase (CBH) is the moststudied exoglucanase. Different CBHs are produced by many bacteria and fungi, with catalytic modules belonging to families 5, 6, 7, 9, 48 and 74 glycoside hydrolases. Aerobic fungal CBHs are in families 6 and 7 only; aerobic bacterial CBHs in families 6 and 48; anaerobic fungal CBHs are in family 48; and anaerobic bacterial CBHs are in family 9, as well as 48.62

#### $\beta$ -D-glucosidase

 $\beta$ -Glucosidases (BG's) hydrolyze soluble cellodextrins and cellobiose to glucose.  $\beta$ -Glucosidases do not contain a carbohydratebinding module (CBM). Their activity of BG on insoluble cellulose is negligible. Different BGs are produced by various archaea, bacteria, fungi, plants, and animals, with different catalytic modules belonging to families 1, 3 and 9.





GH family	Enzymes	Catalytic mechanism	Commercial enzyme provider
GH1	β-Glucosidase, β-galactosidase, β-mannosidase, and β-glucuronidase; but not β-xylosidase activity	Retaining	Megazyme; Nzytech; Prozomix
GH3	β-Glucosidase and β-xylosidase activities; glucan 1,3- β-glucosidase, glucan 1,4-β-glucosidase and exo- 1,3(4)-glucanase activities	Retaining	Megazyme; Prozomix
GH5	Cellulase and hemicellulase activities, including endoglucanase, xylanase, 1,3-mannanase; $\beta$ - mannosidase, glucan 1,3- $\beta$ -glucosidase, glucan endo-1,6- $\beta$ -glucosidase, endo-1,6- $\beta$ - galactanase, and xyloglucan-specific endo-1,4- $\beta$ - glucanase activities	Retaining	Megazyme; Nzytech; Prozomix
GH6	Cellulase activities in both aerobic bacteria and fungi: both endo- and exo-glucanase activities	Inverting	-
GH 7	Cellulase activities (fungi): both endo- and exo- glucanase activities	Retaining	
GH8	Major cellulase family, with members exhibiting lichenanase and xylanase activities	Inverting	Nzytech; Prozomix;
GH9	Endo-, processive endo-, and exoglucanase activities in bacteria, plants and fungi (rare in archaea)	Inverting	-
GH12	Endoglucanase, xyloglucanase, and $1,3(4)$ - $\beta$ -glucanase in the three domains of life (archaea, bacteria and eukarya)	Retaining	Megazyme
GH44	Endoglucanase and xyloglucanase activities, mainly in bacteria	Retaining	Nzytech; Prozomix
GH45	Endoglucanase activity, mainly in fungi (some bacteria)	Inverting	-
GH48	Cellobiohydrolases acting from the reducing ends of cellulose and endoprocessive cellulases; mainly in bacteria	Inverting	Nzytech
GH74	Xyloglucanase and endoglucanase activities	Inverting	Nzytech
GH124	Endoglucanase	Inverting	-

 Table 2

 Glycosidic families exhibiting cellulase activity48

Based on experimental data or structural homology analysis, it has been established that the stereochemistry of families 1 and 3 BGs is of the retaining type, while that of family 9 BG is of inverting type. Most of the time, aerobic fungi produce extracellular BGs, and anaerobic bacteria restrict the BGs to the cytoplasm. The BGs consist of a pocket-shaped active site, which allows to bind a non-reducing glucose unit and clip off the glucose from cellobiose or cellodextrin.<sup>63</sup>

#### Self-assembling, aggregated enzyme complex

Some bacteria, such as *Clostridium thermocellum*, and a few rumen bacteria, such as *Fibrobacter succinogenes* and *Ruminococcus albus*, as well as fungi, have evolved an alternative plant cell wall-degrading enzyme paradigm that involves large, multienzyme complexes commonly bound to the cell surface by long linker peptides and large glycoproteins, called scaffoldins. In contrast to monofunctional enzyme systems, a gigantic, multifunctional mega-Dalton-sized complex, called cellulosome is present in many anaerobic bacteria, produced as a result of non-covalent linkage of a multitude of enzymes to scaffoldin. Cellulosome producing bacterial species are known to be present in diverse ecosystems, including forest and pasture soils, hot spring pools, sewage sludge, compost piles, and the microbiota of both vertebrates and invertebrates.<sup>64,65</sup> The first cellulosome complex was discovered in the early 1980s, in the highly cellulolytic thermophilic anaerobe Clostridium thermocellum.<sup>66</sup> Cellulosomes are mainly made up of two building blocks: dockerin containing enzymes or ancillary protein, and cohesin containing structural proteins, called as

scaffoldins (Fig. 3). The complementary modules - cohesins and dockerins - are tightly bound to each other and their high-affinity, type- and species-specific, non-covalent interaction dictates the integration of enzymes into the complex, as well as its final architecture.<sup>67</sup> Scaffoldins also contain a dockerin module, which may bind to other scaffoldins and a CBM, which is joined by a short polylinker region to the catalytic domain at the N-terminal. The function of the CBM is to direct the entire cellulosome complex to the target site, *i.e.* cellulosic biomass. The CBM is composed of approximately 35 amino acids, while the polylinker region is predominantly made up of serine and threonine.<sup>68</sup> Cellulosomes are either attached to the bacterial cell surface or are released as cell free cellulosomes.<sup>69</sup> Amongst the bacterial species, mesophiles such as Clostridium cellulolyticum,<sup>70</sup> Clostridium cellulovorans,<sup>71</sup> Clostridium josui1, Clostridium papyrosolvens,<sup>72</sup> Ruminococcus albus and Ruminococcus

*bromii*,<sup>73,74</sup> are known to produce simple cellulosome systems, with a single scaffoldin incorporating up to nine enzymatic subunits depending on the number of cohesin modules on the scaffoldin. The cellulosome complex has also been found in anaerobic fungi belonging to the Orpinomyces genera Piromyces, and Neocallimastix, but are markedly different from bacterial cellulosomes as they lack authentic cohesins and bonafide dockerins.<sup>75</sup> In grampositive bacteria, some enzymes, e.g. a family 5 cellulase from *Caldicellulosiruptor*,<sup>76</sup> a family 5 endoglucanase from *Clostridium josui*,<sup>77</sup> are connected directly to the cell wall by a specialized type of module, the SLH (S-layer homology) module.<sup>78</sup> Though the modular architecture of such enzymes is complicated due to the presence of different modules in a single polypeptide chain, it helps in judicious degradation of insoluble substrates.79



Figure 3: Schematic representation of cellulosome

#### **Designer cellulosomes**

Artificial enzymatic complexes or designer cellulosomes, which are known to efficiently degrade crystalline cellulose, have caught the attention of researchers over the past few decades. There has been a spurt in designer cellulosome related research, as it tends to enhance biomass waste solutions and biofuel production at a chimeric worldwide scale. А scaffoldin containing 6 cohesins is the largest designer cellulosome reported to date. The scaffoldin is regarded with certain technical considerations like limits to the cohesin additions, as a large number of it can result in unstable scaffoldin polypeptide and low expression levels, besides limits to the numbers of available cohesin-dockerin

specificities.<sup>80</sup> Each enzyme in designer cellulosomes interacts specifically with one of the cohesin modules of the chimeric scaffoldin via the dockerin module. А cellulose-specific carbohydrate-binding module of artificial scaffolding helps to translocate the enzyme complex to the desired cellulosic biomass. As an outcome of differential specificity, cohesin modules from different natural cellulosomal species interact with desired matching dockerinbearing enzymes, e.g. type I cohesins will bind type I dockerins of some species, but not others.<sup>81</sup> Designing cellulosomes can be achieved by assembling the components of native and nonnative cellulosomes, as well as chimeric monofunctional enzymes possessing relevant

dockerin modules. To achieve the target of generating highly specific cellulolytic complexes, only the desired number of enzymes with specific activity is added in an ordered fashion.<sup>82</sup> This ordered incorporation of selected enzymes over the random incorporation helps to enhance the scaffoldin flexibility, improves the proximity of the cellulases in the complex and facilitates the specific targeting of CBM on the substrate, eventually improving the synergism between cellulases.<sup>83</sup> The ability of the adaptor scaffoldin to incorporate a large number of enzymes and attach to an additional scaffoldin has helped in the construction of hexavalent adaptor scaffoldins with three type II cohesins for enzyme integration, a single type II dockerin for interaction with an additional scaffoldin, and a carbohydrate-binding module for specific targeting to cellulosic biomass.<sup>84</sup> In a recent study by Kahn *et al.*,<sup>85</sup> a hyper-thermostable cellulosome complex, comprising three pairs of cohesins and dockerins, was reported to exhibit maximum enzymatic activity on microcrystalline cellulose at 75 °C.

## Multifunctional enzyme systems

Multifunctional enzymes are considered to be a prototype, intermediate between monofunctional enzymes and cellulosomes. The multifunctional enzymes have several characteristic features: i) they are products of a single gene, ii) have high molecular weight, iii) have two or more catalytic activities, iv) have one or several CBMs.<sup>86</sup> In nature, they exist in both monofunctional enzyme systems and cellulosomal systems. Based on substrate specificities, monofunctional enzymes are grouped into four different classes: cellulasecellulase, cellulase-hemicellulase, hemicellulasehemicellulase, and hemicellulase-carbohydrate esterase systems.<sup>87</sup> A study by Yarbrough et al.<sup>88</sup> has reported that the multifunctional enzyme complex from *Caldicellulosiruptor* bescii outperforms the free enzyme system of Trichoderma reeseii in terms of total cellulose conversion, sugar production, and nano-cellulose production.

#### DIVERSITY OF CELLULOLYTIC MICROORGANISMS

Cellulolytic microorganisms, which include bacteria, fungi and protozoans, are known to be present in diverse habitats and perform their characteristic activities, *e.g.* free-living microbes convert the cellulosic biomass to the assimilable form of sugars, are present in the digestive tract of ruminants, in the guts of wood-degrading termites and worms, in soils, swamps, marshes, water bodies and seawater sediments, in rotting grasses, leaves, wood, in cotton bales, sewage sludge, silage and compost heaps, in muds and decaying vegetable matter, in extreme environments, like hot and volcanic acid, and alkaline springs.<sup>89</sup> They are also known to be present in symbiotic association with secondary microorganisms.<sup>90</sup>

## **Cellulolytic actinomycetes**

The past few decades have witnessed an increase in the interest for the isolation of cellulolytic actinomycetes. Actinomycetes are known to efficiently degrade lignocellulosic biomass, as they produce a host of extracellular hvdrolases. like cellulases, hemicellulases, xylanases, proteases, pectinases and amylases. The cellulase producing actionomycetes have been reported to belong to the following genera: Cellulomonas, Streptomyces, Micromonospora, Actinoplanes, Nocardia, Microbispora, Saccharopolysopra, Thermobifida, Streptoverticillium, Pseudonocardia, Streptosporangium, Actinopolyspora, Intrasporangium and Thermoactinomycetes.<sup>44,91-93</sup> In most of the actinomycetes, cellulases are secreted extracellularly, using specific secretion pathways, *i.e.*, Sec general secretion system and sec independent twin-arginine translocation (TAT) systems, discovered in *Thermobifida*.<sup>94</sup>

## Cellulolytic bacteria

There has been tremendous interest in the exploration of bacterial species for their cellulolytic activities, as compared to other microorganisms due to several reasons, like high growth rate and ability to inhabit distinctly wide types of habitats that produce cellulolytic enzymes resistant to environmental stresses.<sup>95</sup> Most of the bacterial species can catalyze the conversion of soluble derivatives or amorphous regions of crystalline cellulose. However, a few cellulolytic bacteria called "true cellulolytic" can bring out the extensive hydrolysis of the crystalline cellulose, whereas those bacterial species that do not produce a complete cellulase system are called "pseudocellulolytic".<sup>96</sup> The best characterized aerobic cellulolytic bacteria species are Cellulomonas biazotea, Cellvibrio gilvus, Microbispora bispora and Bacillus sp., while anaerobic cellulolytic bacteria are Acetivibrio cellulolyticus, Clostridium thermocellum, *Ruminococcus albus* and *Fibrobacter succinogenes*.<sup>68</sup> Several cellulolytic bacterial genera dwelling in the marine environment have also been identified.<sup>97</sup> *Saccharophagus degradans* 2-40<sup>T</sup> is reported to be the first marine bacterium capable of degrading cellulosic algae and higher plant material.<sup>98</sup> The genomic analysis of *S. degradans* 2-40<sup>T</sup> revealed that its genome codes 15 have exceptionally long polypeptides in the range of 274 to 1600 kD.<sup>99</sup>

## Cellulolytic fungi

Among all the microorganisms, fungi are considered to be the most versatile cellulolytic microorganisms, contributing to cellulose breakdown to the level of 80 percent on the earth.<sup>100</sup> The cellulolytic fungi are predominantly the members of ascomvcota, basidiomvcota, and deuteromycota, as well as some chytrids present in the rumen of some animals. The fungal species are preferred for the production of cellulase, as they secrete highly adaptive, extracellular cellulase in large quantities.<sup>101</sup> Fungal cellulases can hydrolyze lignocellulosic biomass by secreting a diverse set of hydrolytic and oxidative enzymes.<sup>102</sup> The white-rot fungus. e.g. Phanerochaete chrysosporium, and soft-rot fungi, e.g. Fusarium solani, Penicillum funiculosum, Talaromyces emersonii, Trichoderma koningii and Trichoderma reesei, are the best characterized cellulolytic fungus. The most accepted commercial microbes with highly cellulolytic potential are Aspergillus fumigatus, Aspergillus nidulans, Aspergillus acculeatus, Aspergillus niger, Aspergillus oryzae, Melanocorpus albomyces, Trichoderma koningii, Trichoderma viride, Penicillium fumiculosum, Talaromyces emersonii, Humicola grisea, H. insolens, Fusarium solani, Irpex lactius, Sclerotium rolfsii etc.<sup>101,103</sup> The crude enzymes produced by Trichoderma and Aspergillus are widely available commercially, as they are known to be copious producers of cellulase.<sup>104-106</sup>

#### Cellulolytic gastropod mollusks

One of the most diverse groups of living organisms, comprising up to 50,000 species, is constituted by *Phylum mollusca*. The phylum is divided into seven classes, *i.e.* aplacophora, polyplacophora, monoplacophora, gastropoda, cephalopoda, pelecypoda and scaphopoda, of which gastropods constitute the lagest group (80 percent).<sup>107</sup> The most dominant species of gastropoda are slugs and snails, *e.g. Achatina* 

fulica, Achatina achatina and Archachatina marginata. Their evolutionary successful invasion is attributable to their ability to feed on a great variety of organic matter, including lignocellulosic biomass, not only by the action of their enzymatic reservoir, but also by the activity of characteristic and highly adaptive microbiota that is present in their gut as symbiont.<sup>108</sup> The gut microbiota consists of bacteria, such as Fibrobacter succinogenes, Ruminococcus flavefaciens and Ruminococcus albus and some fungi, which are responsible for the degradation of plant cell walls constituents like cellulose and hemicelluloses.<sup>109,110</sup> The metagenomic analysis of the microbiota of giant land snail Achatina *fulica* revealed the presence of coding sequences for oligosaccharide-degrading enzymes, as well as for novel cellulase and hemicellulase.<sup>111</sup> Pinheiro et al.<sup>112</sup> demonstrated the presence of cellulolytic flora belonging to Aeromonas species in the gastrointestinal tract of snail Achatina fulica. Pawar et al.<sup>113</sup> reported the presence of members of Enterobacteriaceae in the gastrointestinal (GI) tract of Achatina fulica exhibiting potential CMCase and/or avicelase activity.

## Cellulolytic insects

Insects represent a highly variable group of organisms, with about 9 million different kinds of living insects, an estimated biomass of 10<sup>19</sup> approximately.<sup>114</sup> However, their niche and food source varies widely. Over time, insects have been able to evolve strategies for effective degradation of lignocellulosic biomass as source of energy.<sup>115</sup> Termites (order: Isoptera), includes seven families: Serritermitidae, Termitidae, Kalotermitidae, Mastotermitidae, Termopsidae, Rhinotermitidae. Hodotermitidae. Serritermitidae, Termitidae, with about 2,300 species belonging to 200 genera.<sup>116</sup> The gut of termites is a rich source of diverse microorganisms, like bacteria, fungi, and protozoans, which have the potential to efficiently degrade cellulose and hemicelluloses.<sup>117</sup> The first insect cellulase was discovered in 1998 in termite *Reticulitermes speratus*, encoding an endo- $\beta(1,4)$ glucanase by the activity of RsEG.<sup>118</sup> The cellulase producing genes are also known to be found in Hymenoptera (wasps, honeybees), Hemiptera (aphids) and Phthiraptera (lice).<sup>119</sup> There have been numerous reports on the cellulolytic activity of various insects, such as Reticulitermes flavipes,<sup>120</sup> Anoplophora glabripennis,<sup>121</sup> Tenebrio molitor,<sup>122</sup> Pachnoda

marginata,<sup>123</sup> Zootermopsis angusticollis,<sup>124</sup> silver cricket Lepisma sp.,<sup>125</sup> Tipula abdominalis,<sup>126</sup> Saperda vestita, Dendroctonus frontalis,<sup>127</sup> and Pachnoda marginata.<sup>128</sup>

## Cellulolytic microalgae

In 2012, it was experimentally established that not only heterotrophic organisms, but also photosynthetic microalga Chlamydomonas reinhardtii can utilize cellulose for growth in Csources limiting conditions by secreting endocellulases.<sup>129</sup> It has been found that the sequences of catalytic domains (CDs) of algae belong to the inverting GH9 family of CAZymes (carbohydrate-active enzymes). Glucanases (GH family 9) are the most conserved cellulases and are prevalently found in bacteria, fungi, amoebozoa, invertebrate metazoans, mosses, angiosperms.<sup>130</sup> gymnosperms and ferns. Guerriero et al.<sup>131</sup> reported that CDs of all algal enzymes have endoglucanase and mixed exo/endo types of activities, along with the presence of novel CBMs and PS-rich linkers, which make them an ideal candidate for cellulosic biomass degradation.

## Cellulolytic rumen microorganisms

The ruminant stomach contains crude fiber, such as cellulose, starch and xylan, not completely converted to animal products.<sup>132</sup> These materials are fermented by the microbial community, including bacteria, protozoa.<sup>133</sup> Rumen microbial fungi and ecosystem comprised  $10^{10}$ - $10^{11}$  bacterial cells/mL,  $10^4$ - $10^6$ protozoan cells/mL, and 10<sup>3</sup>-10<sup>5</sup> anaerobic fungi zoospores/mL.<sup>134</sup> The predominant cellulolytic bacteria present in the rumen are Bacillus, Paenibacillus. Pseudomonas. Clostridium, Cellulomonas, Thermomonospora, Ruminococcus, Bacteroides, Erwinia, Acetivibrio, Methanobrevibacter, Gluconacetobacter, Rhodobacter, Fibrobacter, Ruminococcus.<sup>135-141</sup>

## **Cellulolytic thermophiles**

There has been a manifold increase in the demand for thermostable enzymes lately, which has proved to be a major thrust for the intensive research on such enzymes. There are several advantages of using thermostable enzymes, particularly cellulase, at the industrial scale – for instance, at higher temperatures cellulose swells up, facilitating its easy breakdown and thus significantly improving the overall economy of the process.<sup>142,143</sup> A large number of bacterial and

fungal species have been reported to produce thermostable cellulase.

## Thermophilic cellulolytic bacteria

A large number of bacterial species, both aerobic and anaerobic like Bacillus and Clostridium, have been reported to produce enzymes.<sup>144</sup> thermostable cellulase Α thermostable cellulase with optimal activity at 60 °C has been reported to be produced by Bacillus strains isolated from hot springs, India.<sup>145</sup> Archaebacteria, like Pyrococcus furiosus and Pyrococcus horikoshii, have been reported to produce thermostable cellulases with optimal activity at 102-105 °C.<sup>146</sup> Sulfolobus solfataricus MT4. Sulfolobus acidocaldarius and Sulfolobus shibatae have been shown to produce significant amounts of thermostable  $\beta$ -glucosidases.<sup>147</sup> Highly thermostable cellulases acting at 95 °C have been reported from Thermotoga maritima MSB8.<sup>148</sup> *Thermotoga* sp. FjSS3-B1 also produces highly thermostable cellobiase active at 115 °C.<sup>149</sup> Endocellulase with maximal activity at 85-95 °C was isolated from the thermophile Anaerocellum thermophilum.<sup>150</sup> A thermophilic strain Stenotrophomonas maltophilia isolated from Nambor reserve forest reported maximum cellulase activity of 0.43 IU/mL.<sup>151</sup> А thermophilic cellulase-producing bacterium isolated from a hot spring showed maximum activity at 60 °C.152

## Thermophilic cellulolytic fungi

Thermophilic fungi grow at a maximum temperature of 50 °C or above, and a minimum of 20 °C or above.<sup>63</sup> The best source of cellulases is Trichoderma sp., however, the major bottleneck with Trichoderma cellulases is that it produces very low  $\beta$ -glucosidase activity and the enzyme is subject to product inhibition. Kalogeris et al.<sup>153</sup> reported that thermophilic fungus Thermoascus aurantiacus under optimal growth conditions resulted in 1572 U of endoglucanase and 101.6 U of  $\beta$ -glucosidase per g of carbon source. Myceliophthora thermophila JCP 1-4 on solidstate fermentation using lignocellulosic materials substrates produced endoglucanase as β-glucosidase (357.51 Ug/L), (45·42 Ug/L), (931·11 Ug/L) and xylanase avicelase (3.58 Ug/L), found to be active at 55-70 °C and stable at 30-60 °C.<sup>154</sup> Reddy et al.<sup>155</sup> isolated thermophilic robust cellulolytic fungal strains identified as A. niger, A. flavus, Nigrospora sphaerica, Chaetomium globosum, Cladosporium cladosporides, Trichoderma sp. and Penicillium from different soil samples. Chaetomium thermophilum, Sporotrichum thermophile, T. emersonii and Thermoascus aurantiacus grew well and decomposed cellulose very rapidly by producing thermostable cellulases.<sup>156</sup> Table 3 presents an overview of microbial cellulases and their cellulase activity.

Microorganism	Enzyme activity	Fermentation	Reference
Wheroorganism	Enzyme activity	type	Kelefenee
Fungi			
	Exoglucanase, endoglucanase and		
T. harzianum MTCC 8230	cellobiase activities of 0.127 IU/mL, 0.15	SmF	[157]
	IU/mL and 1.65 IU/mL, respectively		
T. reesei ZU-02	5.48 IU/mL (FPase)	SmF	[158]
A. fumigatus Z5	4.94 IU/mL (β-glucosidase)	SmF	[159]
A. flavus BS1	5408.5 IU/g (FPase)	SSF	[156]
Bacteria			
Paenibacillus terrae	2.08 IU/mL (CMCase)	SmF	[160]
Pseudomonas fulorescens E.	0.48 HJ/ml 0.56 HJ/ml 0.54 HJ/ml on d		
coli, Bacillus subtilis, and	0.48  IU/mL, 0.36  IU/mL, 0.34  IU/mL and 0.58  IU/mL (CMC are)	SmF	[161]
Serratia marscens	0.58 IU/mL (CMCase)		
Cellulomonas sp.YJ5	60 IU/mL (CMCase)	SmF	[162]
Bacillus sp.	68 IU/mL (CMCase)	SmF	[163]
Bacillus sp.	246 IU/mg protein (CMCase)	SmF	[164]
Paenibacillus sp.	2655 IU/mg protein (CMCase)	SmF	[165]
Thermophilic Bacteria			
Caldibacillus cellulovorans	32 IU/mL (CMCase)	SmF	[166]
Bacillus licheniformis C55	0.13 IU/mL (CMCase)	SmF	[167]
Caldicellulosiruptor owensensis	532.4 mU/mg (β-D-glucosidase)	SmF	[168]
Bacillus vallismortis RG-07	4105 IU/mL (CMCase)	SmF	[169]
Thermophilic fungi			
	Endoglucanase (357.51 IU/g), B-		
Myceliophthora thermophila	glucosidase (45.42 IU/g), xylanase (931.11	SSF	[170]
JCP 1-4	IU/g) and avicelase (3.58 $IU/g$ )		[]
	X vlanase (72.38 $IU/g$ ) and $\beta$ -glucosidase		
Aspergillus fumigatus M.7.1 and	(40.4 IU/g) by A. fumigatus M.7.1: 1044.6	0.075	54 <b>5</b> 4 3
Myceliophthora thermophila	IU/g of xylanase and 54 IU/g of CMCase	SSF	[171]
M.7.7	by <i>M. thermophila</i> M.7.7		
	CMCase (26.2 $IU/mL$ ), FPase (18.2		
Aspergillus fumigatus JCM	IU/mL), B-glucosidase (0.87 IU/mL), and	SSF	[172]
10253	xylanase (2.6 IU/mL)		L · J
Thielavia sp.	20 IU/ml (CMCase)	SSF	[173]
Actinomycetes			
Streptomyces argenteolus		<b>6 5</b>	54 5 43
AE58P	0.42 IU/mL (CMCase)	SmF	[174]
Streptomyces ruber	25.6 U/mL (CMCase)	SmF	[175]
Streptomyces sp	0.26 IU/mL (CMCase);18.54 IU/mL	SmF	[176]
Sirepioniyees sp.	(Xylanase)	SIII	[1/0]
Streptomyces G12 strain	0.1 IU/mL (CMCase)	SmF	[177]
Streptomyces longispororuber	25.08 IU/mL (CMCase)	SmF	[178]

Table 3 An overview of microbial cellulases and their cellulase activity

# CONVERSION OF RICE STRAW INTO BIOETHANOL

The depletion of fossil fuel, global warming, and the reduction of natural resources have directed the research attention to bioethanol production from lignocellulosic biomass as an alternative energy source.<sup>179</sup> Although, extensive research has been carried out on ethanol production from rice straw,<sup>180</sup> the manufacturing process of the ethanol has not been realized yet, because of the high costs involved in collection and preservation from rice field ridge, high cost of

cellulase, fermentation system, *etc.* Therefore, the development of effective and cost-efficient ethanol production systems from rice straw is the need of the hour to achieve the aim of novel energy supply.<sup>181</sup>

#### **Bioethanol: an eco-friendly fuel**

Bioethanol is one of the largest sources of renewable and sustainable energy due to its unique economic and environmental benefits. Bioethanol holds high prospects for mitigating the problem of greenhouse gas emission as it represents a closed carbon dioxide cycle (Fig. 4). It also plays an important role at the community level by boosting the income of smallholder farmers, as farmers can cultivate energy crops that yield an income, while meeting their food needs at the same time.<sup>182</sup> The bioethanol contains 35% oxygen, complete combustion of which results in exhaust emissions with less toxicity as compared to conventional fuels.<sup>183</sup> It has been estimated that gasoline blending with ethanol has reduced CO<sub>2</sub>equivalent greenhouse gas emissions from the transportation sector by 55.1 million metric tons in 2018.<sup>184</sup> Although bioethanol is currently produced worldwide, the United States is the world's leader in bioethanol production (Fig. 5).<sup>185</sup>

#### **Generations of bioethanol**

There are various generations of bioethanol categorized as first through fourth (Fig. 6). Firstgeneration biofuels are produced from starch or sugar-based feedstocks.<sup>186</sup> However, firstgeneration biofuels are replete with serious setbacks, as they pose a stiff competition to food production.<sup>187</sup> Thus, the focus has been shifted to second-generation biofuels that are produced from lignocellulosic feedstock. The lignocellulosic biomass consists of forest, agricultural and municipal wastes, including non-food and dedicated energy crops.<sup>188,189</sup> The third generation of biofuels is based on microalgae or unicellular microorganisms derived from eukaryotes and prokaryotes, including cyanobacteria, such as Chlorella, Nanochloropsis or Botryococcus.<sup>190</sup>



Figure 4: Biofuel carbon cycle

Figure 5: Global fuel ethanol production in 2019 by country<sup>185</sup>



Figure 6: Schematic representation of different generations of bioethanol

#### Cellulase



Figure 7: Pretreatment methods of rice straw

Fourth-generation biofuels are also called photobiological solar fuels and electrofuels. Photobiological solar fuels are based on direct conversion of solar energy into fuel, using inexhaustible, cheap, and widely available raw materials. The revolutionary development of photobiological solar fuels depends on the synthetic biology of algae and cyanobacteria, and requires the discovery of new-to-nature solutions, construction of the synthetic living factories, and designer microorganisms. Likewise, electrobiofuels are based on the combination of photovoltaics or inorganic water-splitting catalysts with metabolically engineered microbial fuel production pathways.<sup>191</sup>

## Rice straw: an ideal feedstock for cellulase and bioethanol production

Rice straw has been widely acclaimed as the most attractive feedstock for bioethanol production. According to the Food and Agriculture Organization (FAO), approximately 1140 million tons of rice straw is generated globally.<sup>192</sup> About half of this waste is burnt in the fields, resulting in massive amounts of obnoxious gases, such as carbon monoxide, nitrous oxides, volatile organic compounds, and, suspended particulate matter.<sup>193</sup> Thus, the problem to this

solution lies in the conversion of surplus rice straw into value-added products like bioethanol. Rice straw predominantly comprises cellulose (32-47%), hemicelluloses (19-27%) and lignin (5-24%), with 18.8% ashes.<sup>194</sup> However, to disrupt the recalcitrant structure of rice straw, in which hard and crystalline cellulose is entangled with lignin and hemicelluloses, physical and/or chemical pretreatment or a combination of these processes is required as a prerequisite step (Fig. 7). The physical treatment includes steaming,<sup>197</sup> steam explosion,<sup>196</sup> grinding and milling,<sup>197</sup> and irradiation.<sup>198</sup> The chemical treatment includes alkali,<sup>198</sup> acids,<sup>199</sup> and ammonia treatment.<sup>200</sup> The biological pretreatment has been carried out using white-rot fungi, e.g. Phanerochaete chrysosporium, Ceriporiopsis subvermispora and Pleurotus ostreatus, which can metabolize lignin by secreting a set of ligninolytic enzymes like manganese peroxidase, lignin peroxidase, and laccase.<sup>201</sup> Likewise, several actinomycetes produce enzymes involved in lignocellulose degradation. The combined processes involve two-step pretreatment with dilute sulfuric acid and aqueous ammonia,<sup>202</sup> microwave with acid and alkali,<sup>203</sup> ultrasonic radiation and ionic liquids.204

Apart from utilizing rice straw as a feedstock for bioethanol production, it can also serve as a promising substrate for cellulase production. The rice straw as a substrate not only provides solid support for fungal growth, but also acts as a viable source of carbon and nutrients.<sup>205</sup> As reported by Singla et al.,<sup>206</sup> the addition of soybean pod husk to paddy straw in equal proportions resulted in significantly higher filter paper, CMCase, cellobiase, and xylanase activities, of 89.54 nmol/min/gds, 405.37 nmol/min/gds, 171.26 nmol/min/gds and 3765.76 nmol/min/gds, 72 hours after incubation by Aspergillus fumigatus CST2. Kogo et al.<sup>207</sup> evaluated rice straw as a carbon source for Trichoderma reesei and Humicola insolens, to produce enzymes for rice straw hydrolysis. The authors reported that the enzyme activity of T. reesei cultivated in medium containing NH4OHtreated rice straw was 4-fold higher than enzyme from cultures grown in Avicel (form of refined cellulose) medium. Rice straw pre-treated with sodium hypochlorite (NaClO) resulted in the highest FPase activity of 24.14 U by Phanerochaete chrysosporium on the 5th day of incubation.<sup>208</sup> In contract to the above findings. untreated rice straw showed higher activity of FPase, CMCase,  $\beta$ -glucosidase and xylanase with values of 6.25 g/substrate, 111.31 g/substrate, 173.31 g/substrate and 433.75 g/substrate, respectively, as compared to 1.72 g/substrate, 23.01 g/substrate, 2.18 g/substrate and 45.46 g/substrate for FPase, CMCase, β-glucosidase and xylanase, respectively, for 0.5% (w/v) NaOH pretreated rice straw. The authors opined the lower activity in pre-treated rice straw is due to the decline in absolute crystallinity of cellulose owing to alkali pretreatment, which is otherwise required for the induction of cellulase enzyme.<sup>209</sup>

## Role of cellulase in the conversion of rice straw to bioethanol

Post pretreatment, hydrolysis of biomass results in the conversion of cellulose and hemicelluloses into simple sugars. The cellulose contains glucans, whereas hemicelluloses contain polymers of several sugars, such as xylan, galactan, mannan and arabinan. Consequently, glucose is the predominant product of cellulose hydrolysis, whereas the hemicelluloses yield pentoses and hexoses.<sup>210</sup> The hydrolysis process can be accomplished in two ways; enzymatically by (hemi) cellulolytic enzymes or chemically by sulphuric or other acids.<sup>211</sup> However, enzymatic hydrolysis is a preferred mode as it offers several advantages such as the generation of compounds with lower toxicity, hyper-production of desired product, low input of chemicals and energy, and subtle environmental conditions.<sup>212</sup> Table 4 presents the enzymatic hydrolysis of various feedstock using indigenous cellulases. The overall schematic of bioethanol production from rice straw is presented in Figure 8.

#### CHALLENGES IN CELLULOSIC ETHANOL PRODUCTION AND FUTURE RESEARCH

The cellulosic ethanol production faces several technical and economic hindrances, such as the need for year-round availability of feedstock, efficient and economical pretreatment and saccharification technology, as well as potent ethanologenic strains.<sup>222</sup> The cost of cellulase production and the development of more efficient. specific and stable cellulases are the real objectives of future research on cellulases. Though a large number of culturable, cellulolytic microorganisms have been isolated from diverse sources, such as compost piles, decomposed organic matter, faeces of ruminants, and hot springs, many habitats, such as synthetic extreme environments, e.g. carwash effluent tanks and drains, which could be potential sources of cellulolytic microorganisms, remain unexplored. Besides, non-culturable microbes are also an excellent source of cellulolytic genes. The most widely recognized approach to exploit these microbes is to construct a metagenomic library, followed by its functional screening.<sup>223</sup> The cost of cellulase, which greatly contributes to the overall process cost, can be cut short by recycling or immobilization of cellulases. Previous studies point to immobilization of cellulase on nonporous and porous silica, with a retention of >50% activity for at least four rounds of recycling;<sup>224</sup> and to recycling of endocellulases immobilized on activated carbon, zeolite, ion exchange resin, and polystyrene in two different shapes (drop or sheet) and mixtures thereof. The enhancement of cellulase production is another strategy being attempted consistently for reducing the cost of cellulases.

Pretreatment	Cellulase source	Enzyme dose	Process conditions	Outcome	Referenc e
2% (w/v) NaOH	T. harzianum SNRS3	FPase, 6.25 IU/gs; CMCase 111.31 IU/gs and β- glucosidase 173.71 IU/gs	Substrate conc. 5% (w/v); incubation temp. 50 °C and agitation 150 rpm; incubation period 96 h	29.87 g/L reducing sugars	[213]
2% (w/v) NaOH	Aspergillus CP1	FPase, 0.62 IU/mL, CMCase 5.9 IU/mL and β-glucosidase 1.11 IU/mL	Substrate conc. 12% (w/v); incubation temp. 55 °C and agitation 150 rpm; incubation period 36 h	159.1 g/L sugars	[214]
Untreated	Aspergillus niger	10 FPU/g substrate	Substrate conc. 12% (w/v); agitation 150 rpm, incubation temp. 45 °C, incubation period 96 h	3.62 g/L fermentable sugars	[215]
2% NaOH	Trichoderma reesei ZM4-F3	$10^6$ spores/mL at the rate of $2\%$ (v/v)	Substrate conc. 3% (w/v); temp. 35 °C, agitation 180 rpm	2.23 g/L reducing sugars	[216]
1% Sodium hydroxide	Trichoderma reesei F-418	1.2 FPU/mL	Substrate conc. 5% (w/v), incubation period 16 h, agitation 100 rpm	1 .07% glucose	[102]
Steam treatment by autoclaving	Aspergillus terreus F98	2.0 mL spore suspension (Spore count ≈10 <sup>6</sup> spores/mL)	Saccharification under SSF conditions, Incubation temp. 30 °C, incubation period 7 days	17.0 g/L total reducing sugar, 44.0% saccharification	[217]
Phenarocheate chrysosporium NCIM 1197	Aspergillus niger	$1 x 10^5$ condia/mL	Substrate conc. 5% (w/v), agitation 180 rpm, incubation temp. 28 °C, incubation period 24 h	480 mg/g reducing sugars	[218]
0.5 M NaOH at 121 °C for 1 hour at the ratio of 1:10 (w/v)	Aspergillus fumigatus NITDGPKA3	40 IU/g CMCase	Substrate conc. 2% (w/v), incubation temp. 50 °C, agitation 120 rpm, incubation period 36 h	0.522 g reducing sugar/g dry substrate	[219]
0.5 M KOH for 4 h followed by 0.1 N $H_2SO_4$ for 1 h (bath ratio 1:10), at room temp.	Aspergillus niger BK01	2 % (v/v) (12.0 U/gds CMCase)	Substrate conc. 10% (w/v), incubation temp. 40 °C, incubation period 2.5 h	23.78% sugars; 35.96% saccharification	[220]
Milled and sieved (100 µm)	Bacillus carboniphilus CAS 3	250 U/mL CMCase	Substrate conc. 2% (w/v) incubation temp. 50 °C; agitation 100 rpm, incubation period 96 h	15.56 g/L of reducing sugars	[221]

 Table 4

 Recent studies on hydrolysis of rice straw using indigenous cellulases



Figure 8: Overall schematic of bioethanol production from rice straw

The use of lignocellulosic biomass for enzyme production,<sup>225</sup> co-culture or consortium-mediated cellulase production,<sup>222</sup> the development of recombinant strains by genetic engineering,<sup>226</sup> protein engineering for remodeling the cellulolytic systems in the microbes,<sup>227</sup> random mutagenesis by chemical or physical mutagens,<sup>228</sup> innovative bioreactor geometries and process strategies, *e.g.* biomass conversion at high-solids loads, submerged fermentation (SmF)<sup>229</sup> are popular techniques for achieving enhanced synthesis or secretion of cellulases in microbial strains.

#### CONCLUSION

An overwhelming demand for natural products has elevated the significance of industrial enzymes; among which, cellulases occupy a pivotal position. In the environment, microbes (i.e., fungi and bacteria) are essential for the deconstruction of complex carbohydrates (e.g., cellulose). The most actively investigated application of cellulase is production of biofuels, especially the bioethanol, from lignocellulosic biomass like Cellulases actively rice straw. convert cellulosic renewable resources into glucose and other simple fermentable sugars that can be used as substrates for the production of bioethanol. However, the cost of cellulases is a major bottleneck in the economic production of cellulosic ethanol. Several approaches, such as non-culturable cellulolytic microbes, genetic and protein engineering *etc.*, can be employed to achieve economic and industrial dividends.

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