Chapter 11
Enzymatic Saccharification Technologies for Biofuel Production: Challenges and Prospects

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Abstract Significant advancement has been made in biomass valorization, especially in the twenty-first century. Reasons for these advancements include population growth, depletion in petroleum and fossil fuels, and growing demand for fuels, lignin derivatives, and petrochemicals. The energy demand is increasing tremendously, and today's energy needs can be met by producing fuels and chemicals from renewable feedstocks. Agricultural by-products and other lignocellulosic biomass (LCB) are abundant feedstocks for this purpose. A plethora of biocatalysts are available for biomass conversion, and the discovery of new and efficient enzymes is ever increasing. The significant challenges faced in this area are bridging the efficient utilization of biomass and developing enzyme cocktails with improved saccharification efficiency in a cost-effective manner. Overcoming the inhibitors generation during pretreatment, understanding biomass complexity, enhancing biocatalyst efficiency, optimizing saccharification, and reducing operating costs are challenging needs. This chapter provides a comprehensive review of biomass feedstocks, the enzymes available for the conversion and saccharification of these renewable substrates, the challenges for optimized conversion, and the production of

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platform chemicals that can serve as substrates for generating other high-value products.

Keywords Biomass conversion · Saccharification · Glycosyl hydrolases · Thermozymes · Platform chemicals

11.1 Introduction

Most biorefineries rely upon the production of biogas, bioethanol, and/or biodiesel from lignocellulosic biomass (LCB). Biogas fuel is generated by breaking down biomass in anaerobic environments using methanogens and acidogenic microbes that produce a biogas mixture of 40% carbon dioxide and 60% methane. Bioethanol is a renewable and ecofriendly liquid fuel that is recovered from the fermentation of sugars released from LCB pretreated with physical, chemical, or biological hydrolysis techniques. Biodiesel is produced by trans-esterification processes that employ feedstocks such as oilseeds and can be used to replace fossil diesel (Nikkhah et al. 2020). Platform chemicals produced from LCB are also gaining attraction owing to the dwindling supply of fossil reserves, fluctuating crude oil prices, and environmental concerns. Whether the finished products are fuels or chemicals, LCB-based bioconversion is of global interest to strengthen economies, minimize climate change, conserve energy, and maximize food security (Limayem and Ricke 2012). In the process of bioethanol production, the cost for LCB saccharification is still extraordinarily high, owing to primarily the cost of cellulase to saccharify the cellulose. The presently available industrial cellulases are not optimal for harsh conditions and lack sufficient enzymes for complete hydrolysis. LCB-based biorefineries opt for cellulases that possess temperature tolerance and wider range of stability to pH, metal ions, and solvents. Additionally, the saccharification process to achieve higher sugar recovery needs to be optimized. Hence, the present chapter focuses on the biocatalysts, their suitability for enhancing the saccharification process, and the issues and challenges about biomass conversion.

11.1.1 History of Feedstock

Historical transitions have occurred in the type of feedstock supplies used for bioenergy production. Accordingly, the first-generation biofuel production plants rely on edible food crops like grains, starchy, and sugar-rich feedstocks, and they are competing with the food supply. The second-generation (2G) biofuel production utilizes non-edible biomass such as energy crops and waste residues from forestry and agricultural processes. Also, the LCBs abundant in nature are highly feasible for use as a substrate in bioenergy. Second-generation biofuels might not affect food security and the environment than first-generation biofuels. Thus, 2G biofuels crops

could be grown on marginal lands without competing with the land used for food industries. The third generation utilize algal sources such as microalgal (*Cholera vulgaris*) and macroalgal (*Ulva sp.*) biomass as a major substitute for biofuel production. The third-generation biofuels present the best possibility for alternative fuels as they show a rich nutritional profile with high lipids and carbohydrates and are easily cultivated in an aquatic environment. However, there are still some limitations in making them economically feasible. The fourth-generation biofuels are derived from genetically modified algae to enhance biofuel production (Raud et al. 2019). However, the potential environmental and health-related risks such as modified algal systems are yet to be studied.

11.1.2 Composition of Lignocellulosic Biomass

LCB remains a sustainable material for use as feedstock in biofuels and bioproducts. LCB contains a carbohydrate fraction of cellulose and hemicelluloses and a non-carbohydrate fraction of lignin, proteins, and extractives (Yoo et al. 2020). Lignin is found at 15–40% of the LCB material. Lignin is a complex macromolecule composed of monomeric units of para-coumaryl, coniferyl, and sinapyl alcohols that are cross-linked via stable covalent bonds between the polysaccharides and lignin polymer. This lignin-based cross-linked matrix complicates the degradation of biomass using microorganisms (Dragone et al. 2020).

The lignocellulosic residues comprise an abundance of complex carbon components derived from plant sources after harvesting or processing. Lignin forms the protective layer for the hemicellulose and cellulose matrix. The cellulose polymer consists of glucose units linked via β-1-4 glycosidic bonds and forms a linear crystalline structure. Cellulose requires three types of enzymes for efficient degradation, including (1) cellobiohydrolases (CBHs) to cleave the cellobiose from the reducing and non-reducing ends of cellulose chains, (2) endo-β-glucanases to cleave the glycosidic bonds, and iii) β-glucosidases for hydrolysis of the free cellobiose and cellodextrin fractions; hemicellulose is located between the cellulose and lignin. It is a complex polysaccharide mainly composed of arabinoxylan with branched heteropolymers of D-glucose, D-galactose, D-mannose, and D-xylan. Xylan and lignin are covalently bound together by cinnamic acids. Hydrolysis of the xylan component of hemicellulose requires endoxylanases and accessory enzymes like β-xylosidases, α-L-arabinofuranosidases, 4-O-methyl-D-glucuronidases, and acetyl xylan esterases (Cintra et al. 2020). Lignin is the most recalcitrant molecule to degrade among the LCB constituents, as it is a complex polymer of phenolic, amorphous, and hydrophobic nature due to its varied precursor components (Pereira et al. 2016).

Lignin acts as a physical barrier and hinders the conversion of biomass to biofuel. It affects both the pretreatment and enzymatic hydrolysis process due to its resistant nature and cross-linked networks. Thus, lignin has been targeted by various pretreatment methods like alkaline, alcohol-based organosolv, ionic liquid pretreatment, and biological methods such as enzymes and microbes (Yoo et al.

2020). Ligninolytic enzymes are produced by certain fungi and bacterial strains in large amounts. The efficient and beneficial characteristics of these strains are highly preferable for biocatalyst development for biofuel production, biopulping, textile industries, and platform chemicals (Gaur et al. 2018). The on-site enzyme production and tailor-made enzyme cocktail formulation can be used to pretreat LCB effectively. However, these added enzymes are of high-cost commodity due to the production cost, including nutrient costs, operational and capital cost, formulation, transport cost, and enzyme activity. Moreover, the performance of the enzyme is another limitation that usually differs based on the lignocellulosic substrates (Dragone et al. 2020).

11.2 Renewable Lignocellulosic Feedstocks

Agricultural residues (e.g., rice straw, sugarcane bagasse, corn stover, stalks, and other secondary products), energy crops, forestry residues, and industrially processed residues can be used as feedstocks for the production of biofuels and chemicals (Raud et al. 2019). These plant-based feedstocks are sustainable and have the potential to be generated under harsh conditions like saline, drought, and hot climates. Sweet sorghum is a highly feasible lignocellulosic crop containing both soluble and insoluble sugars to improve the sugar yield for further conversion to biofuel. The sorghum biomass produces (1.26–1.80 t $acre^{-1}$) bioethanol comparatively higher than any other feedstocks (Dar et al. 2018). On the other hand, the biomass from sugarcane, cassava, and plant seeds can also be used as renewable feedstocks based on the availability of bioenergy production resources (Adewuyi 2020). Several crop wastes are also considered for biofuel production. The availability of banana peduncle is 1% compared to that of sugarcane. One in five parts of sorghum is considered waste and has been employed for bioethanol using commercial fermentation using yeast and biogas production (Pazmiño-Hernandez et al. 2019). Alternative biomass for biodiesel production includes plant-based oils (e.g., olive oil, rapeseed oil, and palm oil), waste cooking oil, and crude tall oil derived as a by-product of pulping woody residues.

11.2.1 Industrial and Municipal Solid Waste

Industrial and municipal waste can be used for renewable fuels and chemical production. Municipal wastes, including animal waste, rotten vegetables and fruits, and tubers, have been used for bioethanol production (Adewuyi 2020). Paper mill sludge (PMS) materials from paper and milling industries can also be used as renewable feedstocks for biofuel production using feasible biological conversion approaches. As the PMS materials are obtained from the woody biomass, an increased amount of cellulose and other components like hemicelluloses and lignin

with minimal quantity can be effectively utilized as a feedstock (Tawalbeh et al. 2021).

11.2.2 Macroalgal and Microalgal Sources

Macroalgae and microalgae are useful feedstocks with numerous beneficiary bioproducts. Microalgal oil, seaweeds, and natural algae are large-scale and renewable feedstocks used for biofuel production. Algal varieties with high lipid content, fast growth rate, reduced nutritional requirement, and biological traits amenable to pretreatment methods that reduce production cost include *Chlorella vulgaris* and mixed cultures like *Chlorophyceae* sp., *Cyanophyceae* sp., *Euglenophyceae* sp., *Bacillariophyceae* sp., and *Nannochloropsis* sp. (Japar et al. 2017; Thirugnanasambantham et al. 2020). Industrial effluents, often a menace to the environment, can also be useful resources for bioenergy production. The meat processing industry is one such manufacturing unit where the effluent is often organic rich and amenable for use as a bio-based feedstock for algal cultivation. Techniques like thermal, physicochemical, and biochemical methods are preferred for algal biomass conversion (Okoro et al. 2017). Macroalgae, such as *Ulva* sp., predominantly known as seaweeds, that has high sugars (at least 50%) can be used in biofuel production (Margareta et al. 2020; Nagarajan et al. 2020). Microalgae produces several different kinds of renewable biofuel, such as (a) anaerobic digestion of the algal biomass produces methane, (b) biodiesel from microalgal oil, and (c) biohydrogen through photobiological mechanism (Rajkumar et al. 2014).

11.3 Challenges in Biomass Processing

Biorefinery process designs must consider multiple factors to ensure the system is economically viable. Biomass processing requires optimized conditions like pH, temperature, inoculum, agitation rate, biocatalyst, and the concentration of the final product for efficient conversion. The nature and complexity of the biomass used as feedstock can dictate the combination of pretreatment techniques needed to ensure efficient bioconversion. These factors can lead to technical complications that could render the system economically unprofitable. The utilization of waste resources, while renewable and of limited impact on food security, can harbor undesired variables as the biomass can be diverse and include drastic fluctuations in pathogenic, organic, and moisture content (Okoro et al. 2017).

11.3.1 Consideration of Pretreatment Versus Inhibitors Generated

Pretreatment of biomass is an essential step for overcoming the recalcitrant nature of lignocelluloses and enabling access to the sugars for fermentation. The degradation products produced from pretreatment of lignocellulose depends on both the biomass and the pretreatment conditions, including temperature, duration, pressure, pH, redox conditions, and presence of catalysts (Klinke et al. 2004). Fermentation inhibitors are generated as by-products during pretreatment that interferes with the metabolism of microorganisms during bioconversion and further fermentation. Short-chain aliphatic acids (formic acid, acetic acid, and levulinic acid) are reported as inhibitors (Zhang et al. 2011a, 2016). The concentration and composition of inhibitors generated depend on the raw materials and the pretreatment method (Bellido et al. 2011). The choice of pretreatment often affects inhibitor formation. Acid-based pretreatments often generate aliphatic carboxylic acids, phenolic compounds, furans, and other related by-products. Likewise, hydrothermal processing produces acetic acid and furan aldehydes. Mild alkaline pretreatments methods are considered to be slow processes and may produce several acids and phenolic compounds that can inhibit biocatalysis. Similarly, oxidative methods produce aldonic and aldaric acids, furoic acid, phenolic acids, and acetic acid. Contrary to these methods, ammonia fiber explosion produces inhibitors such as ferulic acid that attack the biofuel process (Chundawat et al. 2010; Jönsson and Martín 2016; Piotrowski et al. 2014).

During ethanol fermentation, acetic acid affects the growth of *Saccharomyces cerevisiae* by a prolonged lag phase (Pampulha and Loureiro-Dias 2000; Zhang et al. 2011b). Similarly, several compounds of phenols, furans, ionic liquids, and other types of inhibitors are generated during pretreatment when harsh processes are employed. The presence of furan aldehydes in the fermentation media during ethanol production can decrease the specific growth rate and ethanol yield. Inhibition problems are increased due to the by-products accumulation during water recirculation and the high solid loads that are used to obtain more amount of sugar (Jönsson and Martín 2016).

The inhibitors generated after pretreatment include dehydrated sugar monomers (furans), degraded lignin polymers (phenols), and small organic acids). The major degradation products of glucose and xylose are 5-(hydroxymethyl)-2-furaldehyde (5-HMF) and furan-2-carbaldehyde (furfural), respectively (Damião Xavier et al. 2018; Rasmussen et al. 2014). 5-HMF may result from the dehydration of hexoses and furfural, resulting from the dehydration of pentoses during pretreatment. Pretreatments involving high temperatures and high acid concentrations for lignin removal result in undesirable compounds such as furans (Kabel et al. 2007). It was observed that there was a significant decrease in ethanol yield and productivity due to the synergistic combination of acetic acid, furfural, and lignin derivatives than due to the combined inhibition of individual compounds (Nigam 2001). In ethanol fermentations, furfural is more toxic than HMF, promoting the inhibition of enzymes

acting on carbon catalysis, including acetaldehyde dehydrogenase, alcohol dehydrogenase, aldehyde dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, and pyruvate dehydrogenase (Guo et al. 2008).

The phenolic compounds generation depends on the molecular weight, polarity, and side-chain characteristics of the lignin structure and pretreatment method applied. Phenolic compounds inhibit cellulases and increase the pretreatment severity with liquid hot water, resulting in the solubilization of phenolic compounds (Michelin et al. 2016; Ximenes et al. 2011). Phenolic compounds affect the integrity in biological membranes, cell growth, ability of cell membrane to serve as barriers and enzymatic matrices, decrease the cellular assimilation of sugars, and inhibit protein synthesis. Low-molecular-weight phenolics or salts are more toxic by penetrating the cell membranes, whereas fermentation inhibitors with high molecular weight affect the transporters of sugar and ion (Kang et al. 2012; Klinke et al. 2004).

11.3.2 Lignin Complexity

Lignin in LCB acts as solid adhesive to cellulose and hemicellulose and contributes for the compactness and integrity of the structure. Lignin contains diverse phenolic acids such as p-coumaryl, coniferyl, guaiacyl, syringyl, and sinapyl, which is one of the dominant compounds that can release various inhibitory by-products during the pretreatment (Kim 2018). Pretreatment is the primary step in producing biofuel production from LCB, followed by saccharification or hydrolysis of the biomass. The removal of lignin enables efficient access to the cellulosic biomass for enzymatic hydrolysis. The saccharification process is the rate-limiting process since the utilization of all sugar in the biomass is vital to achieve the maximum end product. The high bulk lignin content in softwood might be responsible for strong inhibitory effect. Removing bulk lignin can improve enzymatic hydrolysis (Yoo et al. 2020). The inhibitory role of lignin on enzymatic hydrolysis revealed that the type of lignin and molecular weight influenced the inhibition. Similarly, kraft pine lignin precipitated on the cellulose surface, preventing it from contacting with the enzyme. The low-molecular-weight lignin could bind enzyme non-productively, and when the molecular weight increased, the steric repulsion was caused by lignin deposition on cellulose. The lignin structural features like functional groups and syringyl/guaiacyl ratio affected the behaviors of lignin in enzymatic hydrolysis. The high aliphatic hydroxyl groups and low carboxylic groups lead to high surface hydrophobicity, increasing the adsorption between lignin and enzyme. In addition, substrate reactivity is also an essential factor that affects enzymatic hydrolysis (Li and Zheng 2017). The extent of lignin inhibition on enzymatic hydrolysis is closely related to how lignin undergoes non-productive binding and physical blocking of the enzyme biocatalyst (Kumar et al. 2012; Li and Zheng 2017). It has been shown that the bulk lignin can be more inhibitory than the extractable lignin owing to differences in the physicochemical properties and condensed subunit content of these lignin fractions. Milled wood lignin possess a higher enzyme adsorption capacity, leading

to the stronger inhibitory effects of residual lignin during enzymatic hydrolysis, as compared to extractable lignin. Milled wood lignin from softwood exhibits a stronger inhibitory effect on enzymatic hydrolysis of Avicel than pretreated sweetgum (Lai et al. 2015, 2017).

Of the several pretreatment strategies, biological-based methods are promising, as they minimize inhibitor formation, consume less energy, and are eco-friendly. A combination of more than one pretreatment method is also found to enhance delignification efficiency (Wang et al. 2012). Recently, coupling hydrodynamic cavitation with laccase was successful in LCB pretreatment (Thangavelu et al. 2018). The cavitation effect of degrading lignin moieties generates highly reactive radicals (-H and -OH) (Davis et al. 2016). In this hydrodynamic cavitation reactor (HCR)—laccase process, phenoxy radicals are released, eliminating recalcitrant portions of LCB and improving delignification. Coupling a multi-copper oxidase (LccH) from the hyper laccase-producing fungus *Hexagonia hirta* MSF2 in a HCR was also found to be successful for delignification of corn cob and wood biomass (Kandasamy et al. 2016).

11.3.3 Economics of Enzyme Production

Enzyme production is the most costly process in converting LCB to bioethanol, which covers about 40% of the total cost of the conversion process (Du et al. 2010) (Kabel et al. 2007). Finding cheaper methods of producing cellulase and hemicellulase fractions to use as substrates is another challenge for meeting the economics of biofuel production. Improved means of enzyme production and commercially economic enzyme on a large scale are some of the most pressing needs of the industry. Discovering new thermostable enzymes and optimizing methods to produce enzymes from natural polymers through solid-state fermentation (SSF) is envisioned as cost cutting and efficient bioconversion approaches. While doing so, the simultaneous saccharification and enzymes of cellulase and hemicellulase productions are gaining momentum. In this regard, a thermotolerant enzyme cocktail that includes a novel GH family 13 enzymes from the thermophilic fungi *Chaetomium thermophilum* EDWF1 has registered endoglucanase (EGL) activity of 484.10 $IU.mL^{-1}$ under SSF along with xylanase activity (Saranya 2017).

11.3.4 Biomass Size, Complexity, and Utilization Factors

Enzymatic saccharification of LCB is affected by various inhibitors that limit enzyme activity. In order to attain effective conversion of cellulosic substrates, the factors negatively affecting saccharification productivity must be overcome (Su et al. 2017). The main factors influencing enzymatic hydrolysis are the type of substrate and enzyme-related factors. In general, the two main chemical and physical

parameters that affect substrate saccharification using cellulases are (1) the cellulose crystallinity and its degree of polymerization and (2) the complexity of the lignin-cellulose structure that acts as a physical barrier that blocks the enzymes from reaching the cellulose (Cateto et al. 2011; Fockink et al. 2016; Zhang and Lynd 2004). The lignin and hemicellulose content, the particle size, and the accessible surface area of the substrate also affect the saccharification efficiency.

Furthermore, cellulase-mediated hydrolysis includes three major steps: (1) cellulase adsorption to substrate surface, (2) fermentable sugar production, and (3) desorption of the cellulase. However, the substrate content, enzyme level, and reaction condition influence the above steps. The biomass particle sizes influencing the sugar recovery were studied using biomass with different sizes from 0.5 to 2.5 cm. The particle size of 1.0 and 0.5 cm gave 99.6% glucan and 67% xylan recovery, while the particle size of 2.5 cm yielded the maximum sugar conversion (100% for glucan and 83% for xylan). With the particle size increase, the surface area of pretreated biomass significantly increased with a decreased crystallinity index of pretreated biomass resulting in maximum hydrolysis and sugar conversion. The large particle size of corn stover biomass also helped in better mixing during steam explosion pretreatment (Liu et al. 2013). Therefore, conditions including the size of the biomass must be optimized to achieve maximal sugar recovery.

11.3.5 Product Inhibition During Saccharification

Metal ions are reported to act as potentiators or inhibitors of the enzymatic saccharification of LCB. Metal ions that potentiate or inhibit cellulases and hemicellulase activity include Co^{2+}, Mg^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+}, Ni^{2+}, Cu^{2+}, Hg^{2+}, Zn^{2+}, Ca^{2+}, Na^{+}, and K^{+} (Mandels and Reese 1965). Metal ions association with the enzyme catalyst alters enzyme activity and the formation of various complexes. The ions interact with the carboxyl and amino groups and affect the enzyme structure (Pereira et al. 2016). Metal ions formed during the acidic processing of biomass may corrode equipment and release metal ions, such as copper, nickel, chromium, and iron, and can be inhibitory to fermenting microorganisms(Watson et al. 1984). Other cations, viz., Na, Ca, and Mg, may result from the chemicals used in pretreatment or pH adjustment (Jönsson and Martín 2016). Biofuel end products themselves are inhibitory. Ethanol and isobutanol produced during saccharification can act as end-product inhibitors that reduce enzyme activity. Implementing ethanol-tolerant microbes for fermentation can address this latter issue.

11.4 Biomass Hydrolyzing Enzymes

Biomass hydrolyzing enzymes require synergistic action of many enzymes, and there are different classes of enzyme with unique functionality. The complexity of biomass varies enormously, and the enzymes for its hydrolysis also vary considerably. Plants have unique cell walls composed of (1) middle lamella, (2) primary cell wall, and (3) secondary cell wall structures. In general, the plant cell wall composition, including lignin content, varies among monocots, dicots, softwood, and hardwood (Rytioja et al. 2014; Vogel 2008). The major polysaccharides of the plant cell walls are cellulose, hemicellulose, and pectin, and its complexion with lignin makes the plant cell wall recalcitrant. The depolymerization of LCB requires the synergistic action of numerous oxidative, hydrolytic, and non-hydrolytic enzymes (Sistakameshwar and Qin 2018). According to the CAZy database, plant biomass polysaccharide-degrading enzymes and their subunits can be divided into six major families: (1) glycoside hydrolases (GHs), (2) glycosyl transferases (GTs), (3) polysaccharide lyases (PLs), (4) carbohydrate esterases (CEs), (5) carbohydrate-binding modules (CBMs), and (6) auxiliary activities (AAs) based on structural or sequence similarities (Lombard et al. 2014).

The group of enzymes involved in cellulose hydrolysis are classified into cellulases, hemicellulases, lignin-modifying enzymes, and non-hydrolytic proteins. In general, redox enzymes catalyze the auxiliary activities (AAs) that can assist and work simultaneously with other GHs to saccharify LCB. Cellulose decomposition was thought to be mediated primarily through the hydrolytic action of cellulases. Later, polysaccharide degradation was discovered to be mediated by oxidative reactions catalyzed by CBM33s (chitin-binding proteins in bacteria) and GH61s (EGs in fungi) (Vaaje-Kolstad et al. 2010). These are called lytic polysaccharide monooxygenases (LPMOs) and are reclassified as AA families 10 and 9, respectively, in the CAZy database (Levasseur et al. 2013).

The non-hydrolytic proteins that take part in the amorphogenesis of cellulose include swollenin (SWO1), which resembles plant expansins can degrade crystalline cellulose. *Trichoderma reesei,* SWO1s possess close amino acid sequence similarity to the plant expansins (Arantes and Saddler 2010; Gourlay et al. 2012). Similar to the expansins, SWO1s with no catalytic activity appear to disrupt the structure of cellulose microfibers, possibly by breaking hydrogen bonds (Saloheimo et al. 2002). SWO1 synergistically enhances endoxylanase and then endoglucanase or cellobiohydrolase activities during enzymatic hydrolysis of pretreated corn stover (Gourlay et al. 2013). The proposed mode of action of SWO1 is that the protein renders the xylan portion of LCB more accessible for degradation by xylanases and thereby indirectly promotes the action of cellulases. The two proteins CIP1 and CIP2 (cellulose-induced protein), which are induced along with most of the cellulases (Brown et al. 2003), are shown to be essential to degrade lignocellulose efficiently (Banerjee et al. 2010). CIP1 has synergistic activity with swollenins, while CIP2 cleaves hemicellulose-lignin cross links. CIP1 consists of a GH family 1 CBM connected via a linker region to a domain with yet unknown function. Though

CIP1 lacks lyase activity, it shows structural similarities with lyases (Jacobson et al. 2013). CIP2 is a glucuronoyl esterase of the carbohydrate esterase family 15. The glucuronoyl esterase could separate the lignin from hemicelluloses by hydrolysis of the ester bond between 4-O-methyl-D-glucuronic acid moieties of glucuronoxylans and aromatic alcohols of lignin (Pokkuluri et al. 2011). Expansins cell wall loosening action weakens the lignocellulose structure and enhances cellulose hydrolysis by cellulases (Baker et al. 2000).

11.5 Glycosyl Hydrolases (GHs)

11.5.1 Cellulases

The discovery of *T. reesei* (then *T. viride*) for its astonishing extracellular cellulases producing potential is exploited by many industries. Predominant biorefineries use *T. reesei* enzymes to saccharify lignocellulose from renewable plant biomass in order to produce bio-based fuels and chemicals (Bischof et al. 2016). Among 14,000 molds screened for cellulase, *Trichoderma* sp. QM6a was found to display the ability to degrade native crystalline cellulose. This strain was regarded as the *T. reesei* reference strain, and most of the mutants used in industry today have been derived from this strain. Subsequently, a 20-fold increase in the extracellular protein produced by the original strain QM6a was achieved through mutagenesis, which opened its industrial applicability (Bischof et al. 2016). By the end of the 1990s, *Hypocrea jecorina*, the sexual form of *T. reesei*, was discovered. Since then, numerous cellulolytic microorganisms have been discovered, and their cellulases have been characterized.

Cellulolytic microorganisms have developed two major cellulase strategies: discrete non-complexed cellulases and complexed cellulases (Lynd et al. 2002; Zhang and Lynd 2004) (Fig. 11.1). Most aerobic cellulolytic microorganisms degrade cellulose by secreting a set of individual cellulases, which possess a CBM linked N-terminus or C-terminus to the catalytic module. In contrast, most anaerobic microorganisms produce large (> one million Da molecular mass) multienzyme complexes, called cellulosomes, which are attached to the cell surface of the microorganisms (Bayer et al. 2004). Only a few of the enzymes in cellulosomes contain a CBM, but most of them are attached to the scaffolding protein that contains a CBM. Certain anaerobic bacteria produce both cellulosomes and free cellulases.

The whole process of cellulose bioconversion to glucose occurs in two steps. The first step is catalyzed by exoglucanases and endoglucanases that reduce the degree of polymerization in the liquefaction stage, releasing cellobiose; the second step is performed by β-glucosidase that cleaves cellobiose to glucose. Synergism has been observed between endo- and exo-β-glucanases as well as among exo-β-glucanases that act from the reducing and non-reducing ends. Four different types of synergism exist among these enzymes as proposed by Teeri (1997): (1) endo–exo synergy between endoglucanases and exoglucanases, (2) exo–exo synergy between

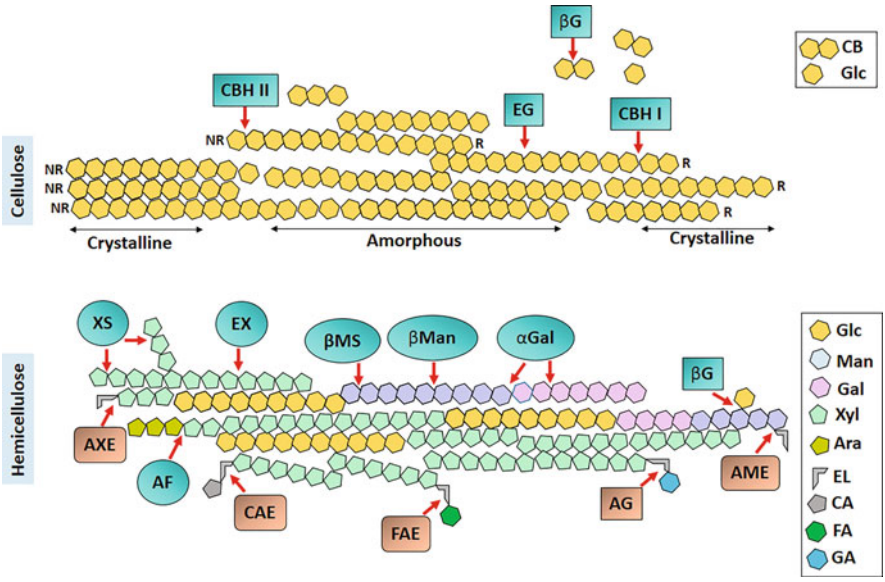

Fig. 11.1 Schematic portrayal showing enzymatic depolymerization of cellulose and hemicellulose. CBH I & II hydrolyze the cellulase chain from the non-reducing end (NR) and the reducing ends (R) of the cellulose chain, respectively, liberating glucose or cellobiose. EG hydrolyze the cellulose chain randomly in the amorphous region of the cellulose. βG acts on the cellobiose to produce glucose units. Hemicellulose is a branched polymer consisting of many different sugars. The complete hydrolysis of hemicellulose requires the concerted action of many enzymes. The enzymes that participate in xylan biomass hydrolysis include endo-1,4-β-xylanase (EX), exo-1,4-β-xylosidase or β-xylosidase (XS), β-mannanases (βMan), β-mannosidase (βMS), α-D-galactosidase (αGal), α-L-arabinofuranosidase (AF), α-D-glucuronidase (AG), acetyl xylan esterase (AXE), ferulic acid esterase (FAE), para-coumaroyl esterase (CAE), and acetyl mannan esterase (AME); βG also act on glucose and mannose linked units to liberate free sugars. *CBH* cellobiohydrolase, *EG* endoglucanase, *βG* β-glucosidase, *Glc* glucose, *CB* cellobiose, *Man* mannan, *Gal* galactose, *Xyl* xylose, *Ara* arabinose, *EL* ester linkage, *CA* para-coumaric acid, *FA* ferulic acid, *GA* glucuronic acid. Red arrows represent the enzyme action on glycosidic bonds or ester linkages present in the biomass component

reducing-end exoglucanases and non–reducing-end exoglucanases, (3) synergy between exoglucanases and β-glucosidase, and (4) intramolecular synergy between CBMs and catalytic modules. The CBMs aid in disrupting the cellulose fibers as well as helping the cellulases bind to the cellulose (Zhang and Zhang 2013).

Cellulases of bacteria are ideal compared to the fungal enzymes owing to the fast multiplication, various genetic diversity, and ease of genetic manipulation (Chandel et al. 2010). Many bacteria produce endoglucanases that can hydrolyze amorphous celluloses viz. carboxymethyl cellulose (CMC) but can be limited in the efficient hydrolysis of crystalline cellulose (Wilson 2011). Only few *Bacillus* spp. produce microcrystalline cellulose (Avicel)-degrading endoglucanases (Han et al. 1995). Furthermore, thermotolerant bacteria identified to synthesize cellulases with β-glucosidase activity can overcome the rate-limiting steps of the saccharification

process leading to increased glucose yield (Bhalla et al. 2013). However, successful biomass hydrolysis and synergistic action of cellulase rely mainly on an optimum pretreatment process.

11.5.2 Endoglucanase, Exoglucanase, and β-Glucosidase

Endoglucanase, 1,4-β-D-glucan-4-glucanohydrolase, or carboxymethylcellulases (CMCases) (EC 3.2.1.4) are found to cut randomly at the β-1,4-bonds of cellulose chains, generating new ends. EGLs hydrolyze cellulose at the amorphous regions and produce accessible free chain ends for the further action of CBH (Fig. 11.1). In general, fungal EGLs possess a catalytic module with or without a CBM, while bacterial EGLs may have multiple catalytic modules, CBMs, and other modules with unknown functions. The catalytic modules of most EGLs possess a cleft/grove-shaped active site, which allows the endoglucanases to bind and cleave the cellulose chain that generates glucose, soluble cellodextrins, or insoluble cellulose fragments. Certain EGLs act "processively," to hydrolyze crystalline cellulose and produce the major products as cellobiose or longer cellodextrins (Cohen et al. 2005; Medve et al. 1998).

Exoglucanases, 1,4-β-D-glucan glucohydrolases (EC. 3.2.1.74), or CBHs acts on the reducing or non-reducing ends of cellulose chains, releasing either cellobiose or glucose as major products. CBHs join with the ends of cellulose microfibrils and then processively slide down the strands and cleave off cellobiose. The processive nature of CBHs is mediated by tunnel-like active sites, which can only accept a substrate chain via its terminal regions. These exo-acting CBH enzymes function by threading the cellulose chain through the tunnel, removing cellobiose units in a sequential manner (Kurašin and Väljamäe 2011; Yeoman et al. 2010). The CBHs also act on swollen, partly degraded amorphous substrates and cellodextrins but do not hydrolyze soluble derivatives of cellulose like carboxymethyl cellulose and hydroxyethyl cellulose (Sajith et al. 2016).

β-Glucosidase is also called as cellobiase (EC 3.2.1.21) that completes the process of cellulose hydrolysis by cleaving cellobiose and removing glucose from the non-reducing end of oligosaccharides. The β-glucosidases hydrolyze β-glucosidic linkages present in disaccharides, oligosaccharides, or conjugated glucosides. Based on substrate specificity, β-glucosidases are divided into three groups: aryl-β-glucosidases, cellobiases, and broad-specificity β-glucosidases. Aryl-β-glucosidases prefer hydrolysis of aryl-β-glucosides, whereas cellobiases only hydrolyze cello-oligosaccharides and cellobiose. Broad-specificity β-glucosidases show significant activity on both substrate types and represent the most commonly observed group in cellulolytic microbes (Bhatia et al. 2002). β-Glucosidase is the rate-limiting enzyme because it hydrolyzes the final step of lignocellulose breakdown in which cellobiose and short cellodextrins are converted into glucose.

11.5.3 Hemicellulases

Hemicellulose is complex and heterogeneous, and the complete hydrolysis of hemicellulose requires the interactive action of several hydrolytic enzymes (Beg et al. 2001). In hemicelluloses, xylanase is involved in the enzymatic hydrolysis of xylan. Based on the mode of action on the substrate, endo-1,4-β-xylanase or endoxylanases (EC 3.2.1.8) and exo-1,4-β-xylosidase or β-xylosidase or xylobiase (EC 3.2.1.37) hydrolyze the hemicellulose. The xylan hydrolysis demands the use of endo-β-1,4-xylanases, acting randomly on the internal bond of xylan to release a diverse range of products, such as xylobiose, xylotriose, xylotetraose, and longer and/or branched xylooligomers (Collins et al. 2005). Reducing-end xylose-releasing exooligoxylanases are called Rexs (EC 3.2.1.156). Rexs hydrolyze the xylan backbone or xylo-oligosaccharide (XOS) from the reducing end producing short XOSs and xylose (Malgas et al. 2019). β-xylosidase hydrolyzes the non-reducing ends of xylose chains, xylobiose, and xylo-oligomers to release xylose but do not hydrolyze xylan (Huy et al. 2015; Knob et al. 2010; Yan et al. 2008). Several supplementary enzymes, such as α-L-arabinofuranosidase (EC 3.2.1.55), α-D-glucuronidase (EC 3.2.1.139), α-D-galactosidase (EC 3.2.1.22), acetyl xylan esterase (EC 3.1.1.72), and feruloyl esterase (EC 3.1.1.73), participate in xylan biomass hydrolysis (Fig. 11.1).

Hemicellulose in softwood has mannan as the major component. Mannan is primarily composed of mannose residues. This polysaccharide is known as glucomannan when combined with glucose residues, galactomannan when combined with galactose, and galactoglucomannan with all three sugar units are present. β-mannanases or endo-β-1,4-mannanase (EC 3.2.1.78) hydrolyze mannan linkages via cleaving β-1,4 bonds and producing new reducing and non-reducing ends. Most of the β-mannanases are active on oligosaccharides containing three or four monomers. β-mannanases hydrolyze mannan with the help of β-mannosidase or exo-β-1,4-mannosidases (EC 3.2.1.25) and produce the terminal, non-reducing β-D-mannose residues. β-glucosidases can cleave the bond between one mannose and one glucose residue during glucomannan degradation. In softwood, endomannanases also catalyze internal linkages in mannan chains, constituting galactoglucomannans and glucomannans (Andlar et al. 2018). Acetyl mannan esterase (AME) (EC 3.1.1.6) plays a key role in removing side-chain acetyl substituents attached at various points on the mannan structure.

Generally, debranching enzymes can remove side groups linked to the main chain of the polysaccharides or oligomers. α-L-arabinofuranosidases cleaves arabinose residues from arabinan, arabinoxylan, or pectin. This activity facilitates the debranching and degradation of xylan and disrupts the lignin-carbohydrate complex. Similalry, α-glucuronidases catalyze the release of glucuronic acid or 4-O-methylglucuronic acid from xylan, showing a synergistic effect with endoxylanases. α-D-Galactosidases are involved in the cleavage of terminal α-1,6-linked galactose residues of galactomannans, galactoglucomannans, and oligosaccharides (Ademark et al. 2001; Lei et al. 2016).

Carbohydrate esterases act synergistically for efficient hemicellulose degradation. These accessorial enzymes are acetyl xylan esterase (AXE) (EC 3.1.1.72), feruloyl esterase (FAE) (EC 3.1.1.73), para-coumaroyl esterase (CAE) (EC 3.1.1.B10), exo-acting α-L-arabinofuranosidase (EC 3.2.1.55), endo-acting arabinofuranosidase (EC 3.2.1.99), xylan α-1,2-glucuronosidase (EC 3.2.1.131), and α-glucuronidase (EC 3.2.1.139). The esterases are considered as hemicellulases since they hydrolyze the ester bonds between hemicellulose and other components (Andlar et al. 2018; Malgas and Pletschke 2019; Zhang et al. 2011b). AXEs are involved in the liberation of acetic acid from acetylated polysaccharides by hydrolysis of ester bonds, thereby the main chain is accessible to GHs. FAEs cleave ester bonds between a hydroxyl-cinnamate and acetyl xylan, liberating phenolic acids including ferulic acid or *p*-coumaric acid (Wong et al. 2013). Glucuronoyl esterases (EC3.1.1.B11) cleave ester bonds between lignin-aliphatic alcohols and the 4-O-methyl-D-glucuronic acid substituents of glucuronoxylans (Arnling Bååth et al. 2016). Ferulic and para-coumaric acid esterases hydrolyze ester bonds between hydroxycinnamic acids and sugars and release ferulic acid and para-coumaric acid from these polymers. α-Glucuronidase catalyzes the hydrolysis of xylan into glucuronic acid or 4-O-methyl-glucuronic acid. The action of esterases can enhance the accessibility of the cellulose fibers and be used to produce bioactive chemicals and biofuels (Polizeli et al. 2005). Pectinases (EC 3.2.1.15) depolymerize (hydrolases and lyases) and deesterify (esterases) pectic substances present in the plant cell wall.

11.6 Thermophilic Biocatalysts Hydrolyzing Plant Biomass

The industrial conversion of LCB necessitates a pretreatment step that facilitates the subsequent enzymatic saccharification. This step is often characterized by a combination of extremely harsh conditions (high temperatures, pressures, and pH). Thermozymes are enzymes that works under high temperatures. These highly stable enzymes offer advantages during pretreatment steps to minimize the cost and complication of varying process conditions, including enzymatic hydrolysis steps. Of the extremozymes, polyextremophilic enzymes simultaneously withstand a combination of more than one harsh condition such as high temperature and pressure (thermopiezophilic), low temperature and high pressure (psychropiezophilic), or high temperature and low pH (thermoacidophilic). These enzymes allow saccharification at higher temperatures, shortens the reaction time, and avoids contamination (Guerriero et al. 2015).

Physical, chemical, and biological pretreatment processes can be customized based on the nature of the LCB. Laccases, also called green catalysts, hold a critical role in biological pretreatment processes and provide flexibility to the pretreatment process when these enzymes are expressed at high levels in a stable form. One such example is the laccase of the halophilic archaeon *Haloferax volcanii* (LccA). LccA is secreted at high levels into the culture supernatant of *H. volcanii* US02 with peak laccase activity detected at the stationary phase, thus, finding application in

biorefineries. LccA is tolerant to high salt, mixed organosolvents, and high temperatures, with a half-life of inactivation at 50 °C of 1.3 days (Uthandi et al. 2010, 2012; Hepowit et al. 2012). A hyper laccase-producing white-rot fungus, *Hexagonia hirta* MSF2 (1944.44 $U.mL^{-1}$), is also found to hold promise in pretreatment strategies as it delignifies wood and corncob biomass to a level of 28.6 and 16.5%, respectively (Kandasamy et al. 2016). HCR coupled with *H. hirta* laccase pretreatment shows 47% delignification efficiency in corn cob in 1 h (Thangavelu et al. 2018). As inhibitors are typically not generated using biological pretreatments, robust enzymes are needed to develop economic and efficient LCB bioconversion processes. Xylitol was produced from the pretreated corncob biomass (Ariyan and Uthandi 2019; Yamunasri et al. 2021).

Thermophilic bacteria are bioprospected for LCB-modifying enzymes. *Bacillus* spp. including *Bacillus tequilensis, Bacillus subtilis,* and *Bacillus licheniformis* were isolated for this purpose by in situ enrichment methods from the hot springs of Manikaran (~95 °C), Kalath (~50 °C), and Vasist (~65 °C), The Himalayas, India (Thangappan et al. 2017). Cellulases and xylanases identified by this approach are found tolerant of temperatures up to 80 °C and pH 7. The identified endoglucanases also exhibit high-level activity in the presence of calcium and potassium ions (Thankappan et al. 2018). Under submerged conditions, the thermophilic bacterium *B. aerius* CMCPS1, isolated from paddy straw compost, showed maximum activity of FPAse of 4.36 IU mL^{-1} and endoglucanase of 2.98 IU mL^{-1} at 44 h (Ganesan et al. 2020). The GHs encoding genes from thermophilic fungi engineered in a suitable yeast-based vector system are also a feasible technology for the optimal and sustainable production of GHs from thermophilic fungi. While bioprospecting endophytes for biomass conversion, perennial grasses are also unique sources of GHs. Endophytes from a C4 perennial grass *Neyraudia reynaudiana* L viz., *Bacillus tequilensis* BT5 and *Alcaligenes faecalis* B12, show FPAase, β-glucosidase, and xylanase activities (Vegnesh et al. 2019).

11.7 Accelerated Saccharification

Multifunctional cellulases are showing high-temperature tolerance, work at harsh conditions, and accelerate saccharification (Bhalla et al. 2013). The cellulase with high catalytic efficiency would reduce the viscosity of the medium and simultaneously increasing the diffusion of simple sugars from complex polysaccharides. Thus, screening diverse cellulases suitable for industrial requirements is an important goal (Krahe et al. 1996; Mozhaev 1993).

Multi-functional cellulases of the *Bacillus subtilis* CMCPS1 recorded a saccharification efficiency of 55% at 50 °C and pH 5.0 (Ganesan et al. 2020). Similarly, thermophilic fungi are more efficient than bacteria, as they produce good yields of GHs. However, the maintenance of thermophilic fungi under laboratory conditions is challenging (Saranya and Uthandi 2017). The thermophilic fungus *Chaetomium thermophilum* EDWF1 was isolated from elephant dung and produces

thermotolerant and alkali-tolerant cellulases, endoglucanases, and beta-glucosidase (Saranya and Uthandi 2017).

A novel one-pot enzyme technology that comprises laccase, cellulase, and β-glucosidase have been co-immobilized to facilitate bioethanol production from *Typha angustifolia, Arundo donax, Saccharum arundinaceum,* and *Ipomoea carnea*. The co-immobilized enzyme system is more stable at different temperatures compared to free enzymes. Enzymatic saccharification of *S. arundinaceum* recorded the highest reducing sugar of 205 mg/g and the highest bioethanol yield of 63% with *I. carnea* among the LCB (Sankar et al. 2018).

The enzymes involved in cellulose degradation are not produced at an optimal level in a single microbe, and cellulases from a single organism may not be hydrolyzing different feedstocks. The enzyme-producing firms make cocktails of cellulase by enzyme assembly (multienzyme mixtures) or use of engineered microorganisms to express the desired combination of enzymes. Enzyme cocktails are also often produced from the co-fermentation of several microorganisms. The most productive major source of cellulases comes from the filamentous fungi and mutant strains of *Trichoderma* (*T. viride, T. reesei,* and *T. longibrachiatum*). The two leading companies that supply commercial cellulases are Novozymes and Genencor, supported by the US Department of Energy. Genencor has launched four new blends: Accelerase®1500, Accelerase®XP, Accelerase®XC, and Accelerase®BG. Each of these enzyme blends includes two or more enzymes. Accelerase®1500 includes exoglucanase, endoglucanase, hemicellulase, and β-glucosidase. Accelerase®XP improves both xylan and glucan conversion. Accelerase®XC comprises of hemicellulase and cellulase activities. Accellerase®Duet has exoglucanase, endoglucanase, β-glucosidase, and xylanase enzymes and can hydrolyze LCB into fermentable monosaccharides such as glucose and xylose (Genencos 2010). In contrast, Accelerase®BG includes only β-glucosidase enzyme designed as an accessory product to supplement whole cellulases deficient in beta-glucosidase.

Similarly, Cellic CTec in combination with Cellic HTec produced by Novozymes can be helpful for the conversion of the carbohydrates in biomass materials into simple sugars using a wide variety of pretreated feedstocks, such as sugarcane bagasse, corn cob, corn fiber, and wood pulp. Most of the commercial cellulases are optimally active at 50 °C and pH of 4.0–5.0. Similarly, enzyme mixtures produced Biocellulase A and Cellulase AP 30K produced by Quest Intl. (Sarasota, Fl) and Amano Enzyme Inc., respectively can work at higher temperatures from 50 to 60 °C (Verardi et al. 2012).

11.8 Conclusion and Perspective

Many times, the combination of more than one pretreatment method is helpful in effective delignification and deconstruction, resulting in maximal saccharification efficiency. Regardless of the pretreatment method, it should aim for the recovery of monomers without the generation of inhibitors. Another approach for effective and

economical conversion of biomass is to find suitable multi-functional thermophilic GHs. Applications of GHs in biorefineries to produce sugars and concomitant fermentation products can be accelerated by using enzyme that possess multi-stability of pH, temperature, metal ions, and organic solvents. Therefore, methods to enhance saccharification efficiency are urgently needed and may be solved by staggered enzyme loading, assembly of enzyme cocktails, and optimizing the conditions of monosaccharide generation. Additionally, candidate microbial strains that produce multi-functional GHs should possess cellulase activity in the presence of hydrophobic solvents at thermo-alkali conditions. Such strains are more potent in terms of activity and stability and, thereby, make the strain a cost-efficient resource. High-value commodity chemicals from biomass can be produced using the optimized process of in-house thermophilic GHs production through submerged and solid-state fermentations. While producing GHs through SSF, cheaply available renewable biomass materials, such as corn cob and *Erianthus*, may be used as substrates.

Valorization of lignin has gained importance in recent years for the production of low-molecular-weight value-added products in industries because of the abundance and aromatic polymeric structure of lignin. The integrated biorefinery approach of catalytic depolymerization of lignin using enzymatically pretreated LCB seems to be a viable technology for lignin routed high-value commodities. However, this combined and sequential process needs to be perfected for the recovery of high-value platform chemicals. Hence, catalytic and biocatalytic approaches of deconstructing LCB for lignin-derived platform chemicals holds promise.

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