Chapter 3
Application of Hydrolytic Enzymes in Biorefinery and Its Future Prospects

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Abstract Biomass is an alternative to the depleting fossil fuels which are primary source of the biofuel and biochemicals. It majorly consists of lignin, cellulose, hemicelluloses along with small fractions of proteins and extractives. Some of the biomass is also rich in pectin and lipids. The two major steps involved in biomass to biofuel conversion is pretreatment and enzymatic hydrolysis. The groups of hydrolytic enzymes that act on these constituents are cellulase, laccases, peroxidases, lytic polysaccharide monooxygenases (LPMOs), hemicellulases, pectinases, amylases, lipases, proteases, etc. The enzymes are obtained from a wide variety of living organisms such as microbes, plants, and animals. The specificity and activity of enzyme differ for different microorganisms. Therefore, search of novel isolates or design of microbes with desired property is essential to attain high yield in biomass-based biorefinery. The chapter gives an insight into source and function application of hydrolytic enzymes along with its techno-economic evaluation. This chapter also discusses the recent advances in the area of enzyme technology to further strengthen the concept of integrated biorefinery.

Keywords Biomass · Biorefinery · Hydrolytic enzymes · Enzymatic hydrolysis · Lytic polysaccharide monooxygenases

3.1 Introduction

The energy and most of chemical requirements are met by non-renewable fossil fuels. The depletion of fossil fuels has led to investigate for alternative energy sources (Kumari and Singh 2018). Biomass is available abundantly in nature which can be exploited for the generation of biofuel and value-added biochemicals.

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In addition, biomass is also an excellent feedstock for enzyme production (Ben-Iwo et al. 2016). The enzymes play a vital role in enzymatic hydrolysis of the biomass and generation of different precursor chemicals (Singh et al. 2019a). The biofuel can be divided into generations based on the type of biomass used, i.e., in the production of first-generation biofuel, food crops rich in sugar and starch are used, whereas in the second- and third-generation biofuels production, lignocellulosic biomass, and algal biomass are used (Kumar et al. 2020) respectively. The biomass consists of several constituents such as lignocellulosic biomass that are rich in celluloses, hemicelluloses, lignin, pectin, amylase, some oils, and proteins, whereas algal biomass mostly constitutes lipids and proteins with cell wall consisting of polysaccharides such as hemicelluloses and celluloses. The enzymes are highly selective that acts on specific constituents of biomass; therefore, different groups of hydrolytic enzymes are required for the breakdown of complex biomass into their monomer units. The group of enzymes that can facilitate the hydrolysis of these components are cellulases, hemicellulases, laccases, peroxidases, lytic polysaccharide monooxygeneases (LPMOs), pectinases, lipases, and proteases (Escamilla-Alvarado et al. 2017). The enzymes are required by several living organisms for their specific requirements; thus, these enzymes can be obtained from several sources such as microbes, plants, and animals. However, micro-organisms such as bacteria and fungus are preferred source due to its ability to grow fast, easy regulation, and manipulation for the desired yield of enzymes (Gurung et al. 2013).

The enzyme can act individually or in combination for efficient removal of lignin (ligninolytic enzymes), hydrolysis of cellulose, starch, hemicelluloses, pectin for the generation of fermentable sugars (Manisha and Yadav 2017). The lipases and proteases are used during transformation of the oils, lipids, and proteins into biofuels (Escamilla-Alvarado et al. 2017). Based on the application of steps at which enzymes during conversion of biomass to biofuel, reaction types are classified as separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), and recently developed consolidated bioprocessing (CP). During SHF, biomass is separately hydrolyzed followed by fermentation with ethanologenic microbes (Gupta et al. 2009). The SSF involves hydrolysis and fermentation together using hydrolyzing enzymes producing microbes or directly enzymes with the ethanologenic microbes (Escamilla-Alvarado et al. 2017; Bhardwaj et al. 2019b). In consolidated bioprocessing, pretreatment (ligninolytic enzymes), enzyme production (cellulases, xylanases, and pectinases), hydrolysis, and fermentation are conducted in a single pot/vessel (Tanimura et al. 2015).

The challenge associated with enzymatic hydrolysis is the development of robust and highly efficient enzymes for cheap conversion of variety of biomass (Bhardwaj et al. 2019c). The understanding of enzyme structure, kinetics, and metabolic pathways allows designing microbes or enzymes with robust applicability (Pröschel et al. 2015). Several private stakeholders such as Novozymes and DuPont have been in the field for long and are not developing and setting benchmark in the area of enzymatic hydrolysis (Brooks and Tchelet 2014). National renewable energy laboratory, USA, is also working exhaustively in the area of developing efficient pretreatment process, robust enzymes, and designing process to obtain bioethanol

and platform chemicals using biomass as starting/precursor material (Gao et al. 2014). Several initiatives by the Indian government is also leading to the development of biobased biorefineries plant in India (TOI 2018). This chapter gives an insight into structure, function, source, application of enzymes in breaking down different constituents of biomass, along with its techno-economic evaluation. This chapter also focuses on the approaches used for improving the yield and efficiency of the enzymes and its roles in the development of consolidated and integrated biorefineries.

3.2 Biomass Structure

The organic material obtained from different living organisms can be collectively defined as biomass, e.g., grass, vegetable, agricultural and forest residues, microbial biomass, and organic wastes generated from meat, fish, food processing industries, and municipal wastes. These biomasses are abundantly available and can be readily converted to bioethanol and bio-oils using microbial enzymatic systems. The biomass is composed of lignin, cellulose, hemicelluloses, pectin, starch, chitin, fats, and oils, with minor quantities of proteins, inorganic materials, and extractives (Mussatto and Dragone 2016).

The lignocellulosic biomass mostly consists of celluloses (30–50% of dry weight) and is located in the secondary cell wall of plant cells. Cellulose is a homopolysaccharide structure made up of β-D-glucopyranose moieties with ~10,000–15,000° of polymerization and linked via β-(1,4)-glucosidic bonds (Crocker and Andrews 2010). Followed by cellulose, the second abundant component in nature is hemicellulose, which is a highly branched hetero-polymeric structure, made up of a wide variety of moieties. Pentoses (xylose and arabinose), hexoses (mannose, glucose, galactose, and rhamnose), and sugar acids (acetic acid, D-glucuronic acid, 4-*O*-methyl glucuronic acid, and D-galacturonic acid) are the major moieties present in the hemicelluloses (Xu 2010). The amount of hemicelluloses can range from 20 to 40% of the dry biomass weight in lignocellulosic biomass (Saha 2003).

Lignin is a heteropolymeric constituent which imparts recalcitrance to lignocellulosic biomass. It is an amorphous structure built up by phenyl propanoid units. The major phenyl propanoid units are coniferyl alcohol, p-coumaryl alcohol, and sinapyl alcohol that are interlocked by highly resistant C-C and *β-O-4*-aryl ether bonds. The aromatic constituents of the different alcohols in lignin are identified as syringyl (S), p-hydroxyphenyl (H) and guaiacyl (G) (Isikgor and Remzi Becer 2015). These components are arranged in a reticulate manner to give lignin a complex structure and is insoluble in most solvents (Isikgor and Remzi Becer 2015). Therefore, for depolymerization of polyphenolic lignin, pretreatment (physical, chemical, biological, physicochemical) steps are required prior to hydrolysis of polysaccharide constituents (cellulose and hemicelluloses) into respective monomers or fermentable sugars (Balan et al. 2009; Adsul et al. 2011). In general, normal hardwood contains

20–28% lignin, whereas normal softwood contains 26–32% lignin herbaceous biomass which usually has a lower lignin content (10–25%) compared to woody biomass.

Several extractives such as resins, waxes, gums, fatty acids, chlorophyll, a variety of phenolic substances, and terpenoids are also present in the biomass (Milagres et al. 2011). Lignocellulosic biomass also consists of extractable minerals, i.e., ash. Inorganic elements such as K, Ca, Si, and Mg are also present in minute quantity as part of ash in LCB (Xu 2010). The ash content of hardwood and softwood is 0.5% and 0.4%, respectively (Demirbas 2009; Mussatto and Dragone 2016).

Pectin is usually part of middle lamella in the cell wall of young plant. Pectin is an acidic heteropolysaccharide made up of galacturonic acid, i.e., galactose-based sugar acids. It constitutes approximately 35% and 5–10% in dicots/monocots and grasses in primary cell wall (Vogel 2008). In lignocellulosic biomass, pectin constitutes only 5–10% of total dry weight; however, agricultural and food waste residues such as fruits and vegetable peels are rich in pectin. Lipids and protein are the constituents of living organisms in varying percentage. Several proteinaceous food crops (jatropha seed, olive seed, etc.), waste stream from oil industries, and oil industry wastes along with microalgae can be used as a rich source of lipid and proteins.

3.3 Application of Hydrolytic Enzymes in Generation of Bioethanol from Biomass

The biomass to biorefinery conversion involves numerous steps such as (1) collection of biomass, (2) biomass preparation, (3) pretreatment (physical, chemical, biological, and physicochemical), (4) hydrolysis/saccharification, (5) fermentation, and (6) downstream processing (distillation). The process of pretreatment, i.e., delignification, usually involves the role of ligninolytic and LPMOs; the hydrolytic enzymes such as cellulases, hemicellulases, LPMO, amylase, and pectinase play important role in the conversion of polysaccharide carbohydrate component to respective monomeric fermentable sugars during saccharification or hydrolysis (Fig. 3.1). The lipases and proteases are important enzymes for the utilization of lipid and protein-rich substrates such as algal biomass and food/oil wastes. Therefore, each hydrolyzing enzymatic families along with their structure, function, source, and application with respect to biorefinery has been discussed below.

3.3.1 Cellulase

The cellulase family constitutes a group of enzymes which act synergistically in order to depolymerizing celluloses (Gupta et al. 2009). As discussed above, cellulose is a homopolymer of glucose with high degree of polymerization. This polymer is

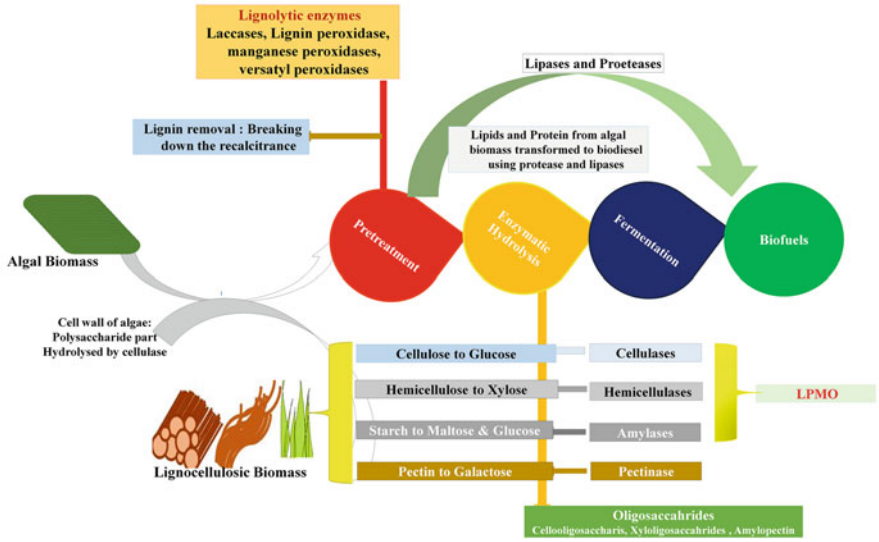

Fig. 3.1 Schematic representation of the role of enzymes: biomass to bioethanol generation

broken down by the systematic action of the constituting enzymes, i.e., endoglucanase and exoglucanase (cellobiohydrolases and β-glucosidase) (Knowles et al. 1987; Lynd et al. 2002; Zhang et al. 2006). First, endoglucanases attack the amorphous regions and randomly cleave at β-1,4 linkages of glucan chain resulting in the generation of cellooligosaccharides. The cellobiohydrolases (1,4-β-D-glucan-cellobiohydrolases) act on reducing and non-reducing ends of cellooligosaccharide chain and result in the formation of cellobiose (Fig. 3.2). Lastly, β-glucosidases (β-D-glucoside glucanohydrolase, EC 3.2.1.21) mediates the conversion of cellobioses to the glucose monomers (Behera et al. 2017). The β-glucosidases also play a vital role in the release of glucose from non-reducing end of cellooligosaccharides (Jørgensen et al. 2007).

Cellulases are produced on industrial scale mostly by fungi and bacteria. The fungus isolated from soil, compost, and wood rot are rich sources of cellulases. Several mesophilic fungal strains such as *A. nidulans*, *A. niger*, *A. oryzae.*, and mesophilic aerobic bacteria such as *Cellulomonas fimi, Cellvibrio japonicus*, and *Pseudomonas fluorescens* isolated from soil and compost, *Bacillus brevis*, isolated from animal gut are source of cellulases (Obeng et al. 2017).

3.3.1.1 Cellulases: Application in Biorefinery

During the process of lignocellulosic biomass to biofuel conversion, the biomass is first pretreated with the lignin-degrading enzymes. The delignified biomass is then hydrolyzed to the fermentable sugars through synergistic action of cellulase hemicellulases, pectinases, LMPO, etc. The fermentable sugars are then converted

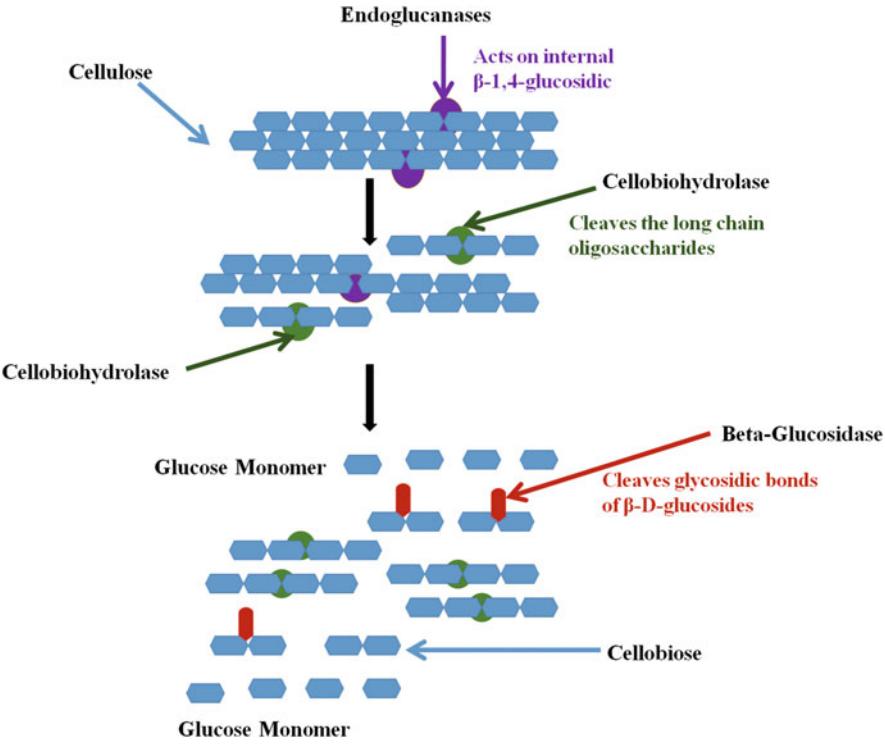

Fig. 3.2 Mode of action of cellulolytic enzyme system

to ethanol by using ethanologenic microbes. Kumar et al. (2019) reported the generation of 7% ethanol by simultaneous saccharification and fermentation of microwave-pretreated rice straw using cellulase enzyme *from S. commune* NAIMC C-F-03379 along with *S. cerevisiae* MTCC 173. Similarly, NaOH-pretreated rice straw was hydrolyzed using *T. harzianum* cellulase followed by its fermentation by *Clostridium acetobutylicum* ATCC 824 resulted in biobutanol yield of 0.16 g/g of glucose generated (Rahnama et al. 2014). Cellooligosaccharides are generated by the action of endoglucanases and CBH (Basholli-Salihu et al. 2013). Thus, simultaneous production of low-cost product like ethanol and high-value chemical such as cellooligosaccharides can help in regulating the overall cost of lignocellulosic biomass-based biorefinery.

3.3.2 Hemicellulases

Hemicellulose is heteropolymer having xylan as its major constituents. The xylan can be completely hydrolyzed into its constituent components through synergistic action of different xylanolytic enzymes. The *endo*-1,4-β-d-xylanases randomly

cleaves the backbone of xylan to generate longer xylooligosaccharides chains which are further broken down to xylose in the presence of β-d-xylosidases. The action of acetylxylan esterase, α-glucuronidase, and α-l-arabinofuranosidases helps in the removal of acetyl and phenolic side chains. The *p*-coumaric esterase and ferulic acid esterase assist in cleaving the ester bond. Thus, these enzyme complex collectively play a vital role in the generation of pentose sugar and high value compounds such as xylooligosaccharides from xylan (Walia et al. 2017; Bhardwaj et al. 2019c).

Several strains of bacteria (*Arthrobacter*, *Bacillus,* and *Clostridium*), fungus (*Aspergillus, Chaetomium,* and *Trichoderma*), and algae (Jensen et al. 2018) are potent source of hemicellulases. Being ubiquitous in nature, xylanase is also found in several protozoa, crustaceans, insects, plants, and seeds (Bhardwaj et al. 2019c). On industrial scale, bacteria such as *Bacillus*, *Actinomycetes*, and *Clostridiales* (Kulkarni et al. 1999; Rabemanolontsoa and Saka 2016) have been widely used. Several filamentous fungi such as *A. niger*, *Humicola insolens*, *A. oryzae*, *Trichoderma reesei, Trichoderma longibrachiatum, Thermomonospora fusca*, and *Trichoderma koningii* (Howard et al. 2003) have been used for the commercial production of xylanase.

3.3.2.1 Hemicellulases: Application in Biorefinery

The action of xylanase enzyme causes fiber swelling that improves the biomass porosity. This results in the enhanced accessibility of cellulase to biomass causing efficient hydrolysis. Sanjivkumar et al. (2017) performed the production and purification of xylanase from *Streptomyces olivaceus* (MSU3) using Sephadex-G-75 column chromatography resulted in enhanced xylanase-specific activity of 153.11 ± 2.11 IU/mg. The purified xylanase enzyme resulted in reducing the sugar yield of 52.19% with 4.19 g/L bioethanol yield. The commercial substrates for xylanase production are such as beech wood xylan, birch wood xylan, and oat spelt xylan. These substrates are very costly, thus there is an increase in the overall production cost of the xylanase enzymes. Bhardwaj et al. (2017) demonstrated xylanase production by *Aspergillus oryzae* LC1 using different agro-residues such as rice straw, rice husk, wheat straw, wheat bran, and sugarcane baggase. They observed maximum xylanase yield with rice straw as substrate. They also demonstrated the saccharification of different lignocellulosic biomasses, i.e., rice husk, rice straw, wheat bran, wheat straw, sugarcane bagasse, groundnut shell, barley husk, and pearl millet husk with the saccharification yield of 7.8%, 34.5%, 16.7%, 8.9%, 15.6%, 28.1%, 19.3%, and 26.2%, respectively. Later, the same group has purified *Aspergillus oryzae* LC1 xylanase using single-step aqueous two-phase system which helped in enhanced substrate specificity during hydrolysis. The application of partially purified xylanase resulted in the generation of wide range of xylooligosaccharides from different lignocellulosic biomass mentioned above (Bhardwaj et al. 2019a).

3.3.3 *Ligninolytic Enzymes*

The ligninolytic enzyme family comprises a group of enzymes, i.e., laccases, lignin peroxidases (LiP), manganese peroxidases (MnP), and versatile peroxidases (VP) which play a key role in the biodegradation of lignocellulosic biomass and several organic wastes. These enzymes act on phenylpropanoid aryl-C3 unit linkage of several complex organic compounds such as lignin, dyes, and recalcitrance compounds via oxidation with H_2O_2 as the first electron acceptor. The peroxidases contain iron containing hemo group as co-factor (Robles-González et al. 2012).

Laccases (E.C. 1.10.3.2) belong to multicopper consisting oxido reductase class and are often called as p-diphenol oxidase or benzenediol. Laccase is an inducible enzyme whose production is induced by the existence of copper, dyes, or recalcitrance molecules (Minussi et al. 2007). Laccase has low substrate specificity and uses oxygen as co-factor and oxidizing agent. The lignin peroxidase (LiP) (EC 1.11.1.14) and manganese peroxidase (MnP) (EC 1.11.1.13) are collectively known as heme-peroxidases due to the presence of iron in the prosthetic group (protoporphyrin IX). LiP mediates the decomposition of numerous aromatic compounds such as methoxy benzenes and 3,4-dimethoxybenzyl (veratryl alcohol). The catalytic action of MnP results in the formation of Mn^{3+} that helps in oxidization of the aromatic compounds (Hofrichter 2002). The Mn^{+3} can easily pass through the lignified cell wall due to its small size and high redox potential. Therefore, it is key to biomass delignification and penetration of other enzymes to biomass (Martínez 2002; Hammel and Cullen 2008). Versatile peroxidase (EC 1.11.1.16) is also a hemoprotein with a broad range of specificity for phenolic as well as non-phenolic substrates and can oxidize them even in the absence of manganese (Polak and Wilkolazka 2012).

Ligninolytic enzymes are found in microorganisms such as bacteria and fungi. The ligninolytic enzyme can be obtained from wood decaying bacterial strains belonging to α-proteobacteria and γ-proteobacteria and actinomycetes groups. Lacasses have been found to be produced by several plants such as tobacco and Zea mays and insects as well (Plácido and Capareda 2015) (Table 3.1).

3.3.3.1 Ligninolytic Enzymes: Application in the Biorefinery

The ability of laccase to degrade the polyphenolic aromatic recalcitrance compound is used in the removal of lignin from lignocellulosic biomass also referred as biological delignification/pretreatment prior to biofuel production (Moreno et al. 2012). The ligninolytic enzyme also plays a key role in simultaneous waste decomposition and electricity generation in microbial fuel cells with wastewater or industrial effluents as substrate collectively known as biofuel cell. The concept of biological delignification has been described below.

Table 3.1 Hydrolytic enzymes used in biorefinery: its EC number, CAZy family classification, used substrate, and resulting products

Enzyme systems	Enzymes	Substrates/sites for acting of enzyme	Products	References
Cellulases	Endo-1,4-β-glucanase	Amorphous cellulose	Cellooligosaccharides	Lynd et al. (2002), Kumar et al. (2018), Benedetti et al. (2019)
	Cellobiohydrolase	Reducing and non-reducing ends of cellulose	Cellobiose	
	β-Glucosidase	Cellobiose	Glucose	
Hemicellulase	*Endo*-1,4-β-d-xylanases	Xylans	Xylooligosaccharides	Alalouf et al. (2011), Bhardwaj et al. (2019b)
	β-d-xylosidases	Xylooligosaccharides	Xylose	
	Acetyl xylan esterase	Acetyl xylan esters residues	Removal of acetyl groups from xylans	
	p-Coumaric esterase or ferulic acid esterase	Side group of ferulic acid and p-coumaric acid	Removal of side chains such as ferulic acid and p-coumaric acid	
Ligninolytic	Laccases, lignin peroxidase, manganese peroxidase, versatile peroxidase	Lignin, aromatics, veratryl alcohol, methoxybenzenes	Oxidized products, guaiacol, protocatechuic acid and vanillic acid	Rabemanolontsoa and Saka (2016), Agrawal et al. (2018)
Lytic polysaccharide monooxygenase (LPMOs)	Lytic polysaccharides monooxygenase	Cellulose and chitin	Oligosaccharides	Payne et al. (2015), Chylenski et al. (2019)
Amylases	α-amylase	Starch at random site	Short oligosaccharides such as maltotriose and glucose	El-Enshasy et al. (2013), Escamilla-Alvarado et al. (2017)
	β-Amylase	Non-reducing end of the starch molecule	β-Maltose	
	Glucoamylase or γ-amylase	Amylose, amylopectin	Glucose	
	Pullulanase	Amylose, amylopectin	Glucose	

(continued)

Table 3.1 (continued)

Enzyme systems	Enzymes	Substrates/sites for acting of enzyme	Products	References
Pectinases	Pectin acetyl esterase Pectin methyl esterase Polymethylgalacturonase	Pectin	Galacturonic acids and galactose	Kiran et al. (2014), Garg et al. (2016)
	Rhamnogalacturonan endolyase (RGL	Rhamnogalacturonan		
Lipases	Lipases	Triglycerides	Monoacylglycerols, diacylglycerols, glycerol	Bajaj et al. (2010)

Biological Delignification

The existence of complex and highly recalcitrance lignin makes it difficult for the application of lignocellulosic biomass in biomass-based biorefinery. Therefore, the ligninolytic enzyme-producing microbes basically fungus or obtained ligninolytic enzymes can been used for biomass delignification. Based on the type of applications, Plácido and Capareda (2015) classified the fungal delignification into four broad categories. Considering the role of all the types of microorganisms and its ligninolytic enzymes, we can further modify those classification as (a) microbial delignification (MD), (b) enzymatic delignification (ED), (c) laccase-mediator system (LMS), and (d) integrated microbial fermentation (IMF).

(a) **Microbial Delignification**: This process involves direct application of the ligninolytic microorganisms subjecting the biomass in submerged (Lu et al. 2010; Martin-Sampedro et al. 2011) or solid state fermentation (Wan and Li 2010; Salvachúa et al. 2011). The microorganisms selectively degrade lignin and sometime hemicelluloses as well, while it is expected that cellulose remains intact (Moreno et al. 2015).
(b) **Enzyme Delignification**: The long duration of 13–50 days required for microbial delignification can be reduced to 1–4 days by the direct application of enzymes (Asgher et al. 2013). During enzymatic delignification, in-house crude extracts, partially purified, purified. or commercially available ligninolytic enzymes are used (Mattinen et al. 2011). Agrawal et al. (2019) used partially purified laccase from *Myrothecium verrucaria* ITCC-8447 for lignin removal from wheat straw, and a delignification of 64.7% was achieved.
(c) **Laccase Mediator System**: Laccase has often reported to have low redox potential thus often not able to break recalcitrance molecules on its own, thus delignification efficiency is limited. The other factors which also affect laccase ability to degrade the biomass are its size, complexity, and composition of the substrate (Agrawal et al. 2018). Therefore, the researchers have come up with the addition of compounds having high redox potential which act as a bridge between enzyme and substrate for electron transfer. These mediator compounds are either chemicals such as 1-hydroxybenzotriazole (HBT), and 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) or exist naturally such as vanillin and syringaldehyde (Fillat and Roncero 2009).
(d) **Integrated Microbial Fermentation (IMF)/Consolidated Processing**: The multistep process of biomass to biofuel conversion is not only tedious but also costly. Therefore, there is a requirement of a single-step process to convert biomass to biofuel directly. The application of a microorganism or a group of microorganisms for direct bioethanol generation from different biomass in a single vessel, the process is also known as consolidated bioprocessing (Lynd et al. 2005). Several reports suggest that thermophillic Gram-positive (+ve) Firmicutes belonging to *Thermoanaerobiales and Clostridiales* order can be used potentially in consolidated bioprocessings.

3.3.4 Lytic Polysaccharide Monooxygenases (LPMOs)

Lytic polysaccharide monooxygenases (LPMOs) are also referred as PMO, and structurally LPMO are multi-copper enzyme with immunoglobulin- or fibronectin-type III-like structure. LPMOs consist of β-sandwich made up of 8–10 β-strand (Quinlan et al. 2011). LPMOs have a characteristic property, i.e., presence of "histidine brace" that binds to the copper (Quinlan et al. 2011; Ciano et al. 2018). LPMOs have high affinity toward copper and have ubiquitous presence of copper (Quinlan et al. 2011; Aachmann et al. 2012). Earlier studies showed that catalytic action of LPMO requires one reductant, two electrons, and one molecular oxygen and reaction (Beeson et al. 2015; Walton and Davies 2016; Meier et al. 2017) and is represented in Scheme 3.1.

Scheme 3.1 $RH + O_2 + 2e^- + 2H^+ \rightarrow R\text{-}OH + H_2O$ (Vaaje-Kolstad et al. 2010)

Bissaro et al. (2017) used mass spectrometry, enzyme assays, and experiments with labeled oxygen atom to establish the fact that H_2O_2 is a more preferred co-substrate rather than O_2 for the LPMOs. The reaction is represented in Scheme 3.2.

Scheme 3.2 $R\text{-}H + H_2O_2 \rightarrow R\text{-}OH + H_2O$ (Bissaro et al. 2017)

LMPOs are naturally produced by several bacteria (*Bacillus licheniformis, Serratia marcescens*), fungi (*Myceliophthora thermophila, Phanerochaete chrysosporium, T. reesei, and Aspergillus oryzae*), and insect (*Thermobia domestica*) (Hemsworth et al. 2015; Sabbadin et al. 2018). However, most of the characterized LPMOs are recombinant in origin, such as *E. coli* for bacterial and *Pichia pastori*s and fungal hosts for fungal LPMOs (Eijsink et al. 2019).

3.3.4.1 LPMOs: Application in Biorefinery

The monooxygenases or LPMOs show auxiliary activities (AA) that act synergistically along with several other hydrolytic enzymes (GH) such as cellulase, hemicellulase, chitinase, and amylases thus able in better hydrolysis of cellulose, hemicelluloses, chitin, and starch as compared with hydrolyzing enzymes. The efficient cellulose degradation requires collegial action of endo-1,4-β-glucanases (random cleavage), cellobiohydrolases (generation of cellobiose), and β-glucosidases (glucose from cellobiose). Several commercial cocktails have blending of LPMOs which increase the overall hydrolytic efficiency. Hu et al. (2014, 2015) and Müller et al. (2015) suggested that spiking of Celluclast and Novozyme 188 mixture with TaAA9A LPMO can result in improvement in cellulose conversion efficiency by 18–63% from a wide range of pretreated lignocellulosic biomass (i.e., corn stover, poplar, and loblolly pine) and commercial substrate Avicel. Several studies suggest that the LPMO can play a vital role in biomass processing, fermentative valorization of biomass. However, to utilize the potential of LPMO at

industrial scale requires changes in the process design, e.g. the regulation of the addition of co-substrates (oxygen or hydrogen peroxide) need to be done when LPMO is used with the ligno-hemicellulosic cocktail (Müller et al. 2018).

3.3.5 Amylases

The amylase family consists of four enzymes, i.e., α-amylase (EC 3.2.1.1), glucoamylase or γ-amylase (EC 3.2.1.3), β-amylase (EC 3.2.1.2), and pullulanase (EC 3.2.1.41). α-Amylases are metalloenzymes (require metal ions Ca^{+2} to initiate reaction) that cleave randomly on α-1,4-glycosidic bonds of the linear chain of starch molecule breaking it into shorter oligosaccharides. β-Amylase or 1,4-α-d-glucan maltohydrolase is an exoenzyme that acts on non-reducing end of linear starch molecule breaking the α-1,4-glycosidic linkage and generates β-maltose as major and β-limit dextrin as minor end products. Glucoamylase or γ-amylase or glucan α-1,4-glucosidase is an exoamylase that acts on non-reducing end of amylase and amylopectin at last α-1,4-glycosidic linkages and generates glucose. Pullulanases acts on the α-1,6 linkages of the partially hydrolyzed amylopectin. Pullulanases are usually used in combination with the glucoamylases and α-glucosidases to improve the saccharification yield in starch-rich substrates (El-Enshasy et al. 2013).

Microorganisms (bacteria, fungi, and Actinomycetes), plants, and animals are source of amylase enzyme. It plays a vital role in human digestion and is found predominantly in the saliva and pancreatic fluids. Several bacterial strains from *Bacillus* species have a desired character needed for its industrial application (Kathiresan and Manivannan 2006). Several hyper-thermophilic bacteria and Archaea such as *Rhodothermus marinus* and *Clostridium thermosulfurogenes* are also capable of producing amylases specifically pullulanases (Gomes et al. 2003; Gangadharan and Sivaramakrishnan 2009).

Several fungal strains from genus *Aspergillus* (*A. flavus*, *A. oryzae*) and *Penicillium* (*P. chrysogenum*) and several other fungal strains such as *Cryptococcus flavus*, *Pycnoporus sanguineus*, and *Mucor* sp. have shown amylase production potential (Sundarram and Murthy 2014).

3.3.5.1 Amylases: Application in Biorefinery

The first-generation biofuels are obtained from the conversion of mainly edible parts of food crops rich in starch, sugars, or oils (Kumar et al. 2020). The role of amylase is of great importance in the conversion of the starch-rich substrate to bioethanol and bio-butanol. Through a series of steps, the starchy biomass can be transformed to ethanol. The bio-conversion of carbohydrate (starch) to fermentable reducing sugar requires the role of these amylases (Lewis and Van Hulzen 2013). The starchy materials are first grounded to pulp and are then subjected to liquefaction by the action of amylases. The liquefaction process involves the action of α-amylase

conversing pulp to water soluble maltodextrin oligosaccharides which are subsequently hydrolyzed to fermentable sugars such as glucose, maltose, and iso-maltose and dextrins via the action of pullulanase and glucoamylase. The fermentable sugars thus generated are subsequently converted to ethanol via action of the ethanologenic microbes (Lewis and Van Hulzen 2013).

3.3.6 Pectinases

The pectic substrate is degraded by the de-esterification and de-polymerization reaction carried out by esterases and hydrolases/lyases, respectively (Singh et al. 2019b). Basically, the pectinases are grouped based on its mode of action on pectin. Pectin acetyl esterase (E.C. 3.1.1.6) and pectin methyl esterase (E.C. 3.1.1.11) are two esterases that act on pectin. The depolymerization reaction of pectin is catalyzed by depolymerases (Kiran et al. 2014; Garg et al. 2016).

Naturally, pectinases help in natural ripening of the fruits, thus it is produced by plants, but commercially, the pectinases are produced by the microbes usually bacteria, fungi, and yeasts. Strains from genus *Aspergillus* (*A. niger* and *A. fumigates*) and *Penicillium* (*P. notatum* and *Penicillium occitanis*) and other fungal strains such as several bacterial strains such *B. subtilis, Sclerotium rolfsii*, *Chryseobacterium indologenes*, and *Pectobacterium carotovorum* are potent pectinases producing bacterium (Singh et al. 2019c). Till date very few report on the application of pectinases in the biorefinery. However, the bio-waste generated from the fruits, vegetables, and food processing industries can be used for the further biofuel and value-added compound generation. The food, fruit, and vegetable industry wastes can be valorized by the action of pectinase enzyme.

3.3.7 Lipases

Lipases (EC 3.1.1.3) also known as triaglycerol acyl hydrolases can efficiently hydrolyze the fats (lipids) such as triacylglycerols, fatty acids, oils, and glycerol. Unlike conventional lipases, phospholipases and sphingomyelinases exist in nature which catalyzes hydrolysis of glycerophospholipids (attack on ester and phosphodiester bond) (Murakami and Kudo 2002) and sphingomyelin. The lipases catalyze different reaction by the acidolysis, alcoholysis, aminolysis, and transesterification (Saxena et al. 2003).

The lipases are produced by microbes as well. Bacterial strains from genus *Bacillus* (*B. subtilis*), *Pseudomonas* (*Pseudomonas aeruginosa*), *Streptomyces* (*Streptomyces aureus*, *Streptomyces hyicus*, *Streptomyces epidermidis*, *Streptomyces* sp.), and other strains such as *Aeromonas hydrophila* and *Xenorhabdus luminescens* reported to be as potent lipase producer. Fungal strains from genus *Penicillium* (*Penicillium aurantiogriseum*), *Rhizopus* (*Rhizopus rhizopodiformis*),

Aspergillus (*Aspergillus carneu*), and *Candida* (*Candida cylindracea*) have shown good lipase production potential using oil-rich plant components as substrates such as olive oil, olive bagasse, olive mill wastewater, wheat bran, soybean oil, and oleic acid (Singh and Mukhopadhyay 2012).

3.3.7.1 Lipases: Application in Biorefinery

The biotechnological application of lipases is food, dairy, laundry, organic synthesis, medicine, health, cosmetics, etc. With respect to the biorefineries, the lipases can be used for the conversion of crude/waste oils, lipids, and fats to the biodiesel (Bajaj et al. 2010; Singh and Mukhopadhyay 2012; Kiran et al. 2014). The first- and third-generation (1G, 3G) fuels are based on transesterification of vegetable oils (crude or waste) and third-generation fuel based on lipids (algal biomass) to biodiesel through trans-esterification. Therefore, the lipases are important for 1G and 3G biofuels.

3.3.8 Proteases

Proteases are the enzymes that catalyze the proteolysis, i.e., breaking down protein into smaller fragments of polypeptides or even to single amino acids. The proteases are ubiquitous in nature existing in all types of the living organisms from prokaryotic to eukaryotic or single cell to complex organisms. Microbial proteases are classified into three different types based on the activity at the respective pH, i.e., alkaline proteases (9–11), acidic proteases (3.8–5.6), and neutral proteases (5–8) (Pushpam et al. 2011; Vadlamani and Parcha 2011; Razzaq et al. 2019). Different strains of bacterial genus *Bacillus* such as *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus* sp., and *B. lentus* have been reported to be used in commercial production of the proteases (Razzaq et al. 2019). Fungal strains from genus *Aspergillus*, *Penicillium*, and *Rhizopus* have been used as microbial proteases (Kiran et al. 2014).

3.3.8.1 Proteases: Application in Biorefinery

The proteases find application in leather, detergent, dairy, food, and pharmacy due to its ability to break down proteins which are intermediate or starting material for the product being generated in these industries. Algae or microalgae are primary substrates for third-generation biofuel, as they are rich in the protein/lipids which need to be hydrolyzed for being converted to biofuel or biochemicals (López-Otín and Bond 2008; Sari et al. 2016; Li et al. 2018; Tavano et al. 2018; Razzaq et al. 2019). Several chemical and enzymatic methods have been used, and proteases and lipases can be used for enzymatic hydrolysis of algal protein for its conversion to biofuels. The global need of petrochemical is decreasing due to the depletion of petroleum

reserve. The proteases can play an important role to accelerate bio-based chemicals in order to meet the global need arising from chemical requirement (Li et al. 2018).

3.4 Strategies Employed for Improving the Hydrolytic Enzyme Yield and Efficiency

3.4.1 Immobilization of Enzyme

The use of enzyme in biorefinery is limited due to the high cost of enzyme and lack of efficient method to reuse the enzymes. The immobilization of enzymes enables the enzymes to be more stable and enables its reusability without losing much of the activity (Manisha and Yadav 2017). Different methods for immobilization of enzyme on a durable and reusable matrix are suggested such as covalent binding (support such as polyaniline via glutaraldehyde, HP-20 (styrene-divynilbenzene adsorbent resin) with glutaraldehyde) (Kapoor and Kuhad 2007; Madakbacs et al. 2013), ionic binding (using Q-sepharose, Dowex-50W) (Kapoor and Kuhad 2007; Hu et al. 2018), physical absorption, crosslinking, entrapment, encapsulation (Reis et al. 2019), magnetic nanoparticles, and carbon nanotubes (Long et al. 2017).

These encapsulation methods help to reuse the immobilized enzyme upto 5–15 cycles of hydrolytic process (Hu et al. 2018). Hu et al. (2018) performed immobilization of xylanase into alginate beads using glutaraldehyde. They suggested that lowering the glutaraldehyde loading can improve enzyme immobilization and xylo-oligomer conversion efficiency (~65%). The immobilization leads to improvement in the β-xylosidase and endoxylanase activities by 40% and 80%, respectively. The efficiency of the immobilized enzyme remains almost similar even after five cycles of application.

3.4.2 Screening of New and Robust Isolates from Extreme Habitats

The industrial application of enzymes requires characteristics property such as high specificity (specific to substrate), activity, and stability. The industrially potent enzymes need to endure a wide range of temperature and pH, metal ions and solvent concentrations, highly alkaline, and halophilic. Usually the enzymatic hydrolysis during conversion of biomass to biofuel requires high temperature; therefore, there is a need of thermophilic enzymes (Bhardwaj et al. 2019c). These thermophilic enzymes are key to the development of simultaneous saccharification and co-fermentation (SSCF) strategy and consolidated biorefinery (CP) system. The thermophillic enzymes may be obtained from the microbes from the thermophillic environments such as hot spring. Zarafeta et al. (2016) isolated a bacterial strain

belonging to *Thermoanaerobacterium* sp. from Icelandic hot springs. The isolate has been characterized to have a GH5 cellulase gene (*CelDZ1*) which is thermotolerance and halotolerance. An extremely halophilic bacterial isolates from genera *Salinicoccus* were able to produce amylase and proteases extracellularly (Jayachandra et al. 2012). Bhardwaj et al. (2017, 2019a) isolated a fungal strain of *A. oryzae* from leaf litter samples collected from the forests of Assam. The xylanase obtained from the isolated strain is found to be alkali and thermo-tolerant and efficient in the saccharification of biomass to sugars and xylooligosaccharides. Similarly, Kumar et al. (2018) demonstrated production and purification of thermo-alkali stable cellulase from *S. commune* NAIMCC-F-03379. The purified cellulase showed significant potential to be in biomass hydrolysis.

3.4.3 Genetic Engineering

Several molecular approaches such as recombinant DNA technology and genetic engineering that can be used for the enhanced enzyme yield with high specific activity (Gopinath et al. 2017). Genetic engineering or protein engineering as in case of enzymes can be broadly divided into three approaches: (1) rational approaches, (2) semi-rational, and (3) directed evolution. Rational approaches utilize the available structural information in the enzyme sequence to be applied for site direct mutation leading to specific sequence modification (Plácido and Capareda 2015). Deletion of transcription factor encoding gene amyR of *A. niger* CICC2462 resulted in the enhanced amylase production specifically with no or low background protein secretion (Zhang et al. 2016). During semi-rational approaches, a selected amino acid in the hotspot region of enzyme is replaced with other amino acid (Mate and Alcalde 2015). This approach has even helped to improve the enzyme efficiency by three- to eight-folds (Andberg et al. 2009). In the absence of the structural information, the directed molecular evolution is applied by utilizing random mutation followed by recombination and selection of efficient mutant. This approach has resulted in the improvement of the solvent tolerance to catalytic activity (Mate and Alcalde 2015).

Recombinant DNA technology involves several steps first being selection of suitable gene and second the gene is inserted in a suitable vector system. The third step of RDT involves transformation to an efficient expression system such as *E. coli* and yeast, and the fourth step is the selection of the recombinant followed by downstream processing and characterization of the protein thus produced by the recombinant. Bhardwaj et al. (2020) showed that xylanase gene (*XynF1*) of *A. oryzae* LC1was transferred to *E. coli* BL21(DE3) (a prokaryotic system), for the production of recombinant xylanase having very high titer (1037.3 U/mg) of specific activity that is higher than that of the native strain by 9.3-fold.

3.4.4 *Metagenomics Approach for the Identification of the Potential Hydrolytic Enzyme*

All the microbes are cultivable under laboratory condition; therefore, in order to explore this microbiome, metagenomics approach is used. Metagenomics is a technology of directly isolating DNA from the natural habitat such as soil, water, and composed leaves. The open reading frame of the isolated DNA is screened in order to identify the novel genes for different enzymes. Several lipases from forest topsoil, activated sludge, cold-sea sediment, and fat contaminated soil have been identified using metagenomic approach (Lee et al. 2004; Roh and Villatte 2008; Jeon et al. 2009; Glogauer et al. 2011; Manisha and Yadav 2017).

The beginning of highly advanced next-generation sequencing (NGS) technology has arrived as a boon to the field of metagenomics. The advancements have enabled the discovery of potential source of hydrolytic enzymes. The application of multi-substrate-derived microbial consortium approach has taken a leap due to the combination of NGS-assisted metagenomics. By using this approach, 25 GH families have been identified from anaerobic beer under high-temperature condition (Yang et al. 2016).

3.5 Integrated Biorefineries: Future of Biomass-Based Biorefinery

Previously, the bio-refineries usually used the commercial enzymes for the generation of bioethanol which resulted in the high cost of the overall process. Therefore, it was suggested to produce the enzymes in-house, and also the waste generated in the overall process is further used up for the generation of high-value chemicals, making the overall process more feasible economically. Several studies such as one by Bozell and Petersen (2010) at the US Department of energy suggested that chemicals such as aspartic acid, fumaric acid, glutamic acid, glycerol, itaconic acid, malic acid, levulinic acid, sorbitol, succinic acid, and xylitol can be generated along with the bioethanol/biofuel in the biomass-based biorefinery. Further, the integrated biorefinery can be attributing to the generation of essential precursor molecules such as benzene, ethylene, propylene, toluene, and xylene as well. Thus, the process of integrating the process from collection to complete valorization can be defined as integrated biorefineries.

Robles-González et al. (2012) demonstrated an integrated biorefinery during the conversion of agave to mescal. The overall process involves first extraction of juice, and the bagasse generated can be used for in-house enzyme production along with the production of compost. The juice is fermented to produce mescal, and the microbial biomass generated during the process was suggested to be used for the generation of enzymes such as laccase, peroxidases, biofuel such as methane and other value-added compounds such as antioxidants. The four-stage H-M-Z-S

model-based integrated biorefineries are suggested to use the in-house generated hydrolytic enzymes for the conversion of municipal solid waste to bio-hydrogen, for hydrogen fuel cells. Also, methane produced during the process is used to meet the energy need of the biorefineries (Escamilla-Alvarado et al. 2014). The role of enzyme is important for success of the integrated biorefinery concept and may even help in fulfilling the dream of biomass-based circular bio-economy.

3.6 Summary

Enzyme is the key player in the biomass-based biorefinery with (1) polysaccharide hydrolysis mediated by cellulase, amylase, xylanase, and pectinase, (2) removal of lignin and generation of platform chemical by ligninolytic enzyme, and (3) transformation of oils, lipids, and protein-rich biomass by lipases and proteases. The microbial sources are needed to be further exploited to attain high yield of these enzymes at lower cost by approaches such as genetic engineering and process optimization. The conversion of biomass to bioethanol is a multistep process which eventually leads to higher cost. Thus, there is also need to minimize the number of steps by using approaches such as consolidated bioprocessing which may be developed using advance technologies such as metagenomics and cell surface engineering. There is a need to have better understanding of the biochemical and kinetic properties of different hydrolytic enzymes. This improved understanding coupled with advance technologies such as metagenomics, next-generation sequencing, and cell surface engineering can help to develop microbes that can be used in developing integrated biorefineries.

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