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Chapter 5

Enzymatic Degradation of Lignocellulosic Biomass: A Brief Update

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Abstract

Lignocellulose comprises mainly cellulose, hemicellulose and lignin is the Earth's most abundant renewable source. It is a promising feedstock to produce biofuels, chemicals, sugars, and materials. Lignocellulose is a complex biopolymer therefore a cost effective consolidating bioprocessing microbes that directly convert lignocellulose into valuable end products are exploited. Microbes degrade lignocellulose by producing a battery of enzymes that work synergically. In the near future, processes that uses lignocellulolytic enzymes

could lead to new, environmentally friendly technologies. Diverse mechanisms are used by organisms particularly glycoside hydrolases to deconstruct lignocellulose. Lignin depolymerisation is achieved by white-rot fungi and certain bacteria, using peroxidases and laccases. This study reviews an overview of enzymatic degradation of cellulose, hemicellulose and lignin. In addition, production of lignocellulolytic enzymes by different microorganisms are also outlined.

Keywords: Biomass; Lignocellulose; Biodegradation; Enzymatic degradation

1. Introduction

Production of ethanol from cellulosic biomass utilizes enzyme was first carried out in1980s by US Department of Energy. Although enzymatic hydrolysis of cellulose displayed several advantages such as high yield, low energy cost and operating conditions, it was thought that the technology was too risky for industry to pursue at that time. Later, advancement in biotechnology reduced processing cost made cellulosic ethanol competitive [1]. Lignocellulose is about half of the matter produced by photosynthesis and considered as an alternative source of energy. It is composed of cellulose, hemicellulose and lignin, strongly intermeshed and chemically bonded by covalent and non-covalent forces [2-4]. Besides these, some other materials such as proteins, pectin and ash are present in very less quantity. The proportion of cellulose, hemicellulose and lignin varies depends on the source of origin (**Table 1**). Forestry, agriculture, pulp/paper industry and municipal solids are the main source of lignocellulose biomass. Although a huge amount of lignocellulose produced, only small portion is used in value-added products like ethanol, food additives, organic acids and pharmaceutical building blocks, rest being considered waste [5-8]. These valuable materials are treated as waste since a long time in some developing counties, which raises numerous environmental concerns [9-11].

Hydrolysis of lignocellulose into simple sugars can be achieved either by enzymes or chemically with sulfuric or other acids. While enzymatic hydrolysis displayed several advantages over acid hydrolysis as it entails lesser energy and mild environmental conditions with a generation of fewer fermentation inhibitors but it seems to be a bottleneck due to the high costs of enzyme production. Therefore, continuous efforts have been made for cost-effective production and search for new sources of enzymes. The complex structure of lignocellulose makes it recalcitrant for enzymatic degradation. Additionally, some enzyme absorbed with condensed lignin by non-specific linkages which decreases hydrolysis yield [12-15].

2. Lignocellulytic Enzymes Produced by Microorganisms

Different range of microorganisms, mainly fungi and bacteria have been identified over several years which are producing lignocellulolytic enzymes. These microorganisms depolymerize lignocellulose via a series of hydrolytic and or oxidative enzymes comprising lignin peroxidases, magnese peroxidases, versatile peroxidases, laccases, endoglucanases, cellobiohydrolases and β-glucosidases. These enzymes broadly studied in a laboratory as submerged

and solid culture processes ranging from flask shake to large scale [29-31]. Since bacterial systems lacking lignin peroxidases, therefore, biodegradation of lignocellulose in bacteria is essentially a slow process. Grasses are more susceptible than wood for actinomycete and play a substantial role in humification processes together with bacteria in soils and composts [32-34]. Bacterial enzymes can cleave alkyl-aryl ether bonds in oligomeric and monomeric aromatic compounds, released by fungi during lignin decomposition [35-37]. Therefore, degradation of lignocellulose by prokaryotes is of ecological important while in fungi it is of commercial significance.

Table 1: Percent dry weight composition of lignocellulose materials in some common feedstocks.

Lignocellulosic material	Cellulose (wt%)	Hemicellulose (wt%)	Lignin (wt%)	References
Empty palm fruit bunch	59.7	22.1	18.1	[16]
Sugarcane	43.8	27.0	22.6	[17]
Paper	85-99	0	0-15	[18]
Hardwood	40-55	24-40	18-25	[18, 19]
Softwood stems	45-50	25-35	25-35	[18,19]
Wheat straw	41.3	30.8	7.7	[20]
Rice straw	32.1	24	18	[21]
Barley straw	31-34	24-29	14-15	[22]
Sunflower stalks	33.8	20.2	17.3	[23]
Leaves	15-20	80-85	0	[18]
Office paper	68.6	12.4	11.3	[24]
Corn cobs	42.7	34.3	18.4	[25]
Bamboo	26-43	15-26	21-31	[26]
Coconut fiber	17.7	22	34	[27]
Popular	49.9	17.4	18.1	[24]
Primary wastewater solids	8-15	NA	24-29	[18]
Sorghum	35.1	24.0	25.4	[28]

White and brown rot fungi are two main groups, which decompose lignocellulose effectively. White rot fungi degrade more quickly than any other microorganisms [38,39]. Because of insolubility, fungal degradation occurs either exocellular in association with outer cell envelope layer or extracellular. Two enzyme systems are operated for lignocellulose degradation; a hydrolytic system in which hydrolases degrade polysaccharide and a distinctive oxidative and extracellular ligninolytic system, which degrades lignin and unlocks phenyl rings [40,41]. Despite a large number of microorganisms producing lignocellulolytic enzyme, only a few studied broadly. *Trichoderma reesei* and its mutants are extensively employed in the commercial production of cellulases and hemicellulases [42]. Most of the microorganism used in enzymes production is acting mainly on either cellulose or hemicellulose. Only a few group of microorganisms has evolved with the ability to degrade lignin. It has been reported that *T. reesei* produces hemi and cellulolytic enzymes significantly but unable to degrade lignin. The

most efficient lignin degrading microbes are basidiomycetes, white rot fungi *Phanerochaete chrysosporium,* producing plentiful amounts of a unique set of lignocellulytic enzymes which efficiently degrade lignin into CO_2 [43,44]. Other white-rot fungi such as *Daedalea flavida, Phlebia fascicularia, P. floridensis* and *P. radiate* have been established to degrade lignin selectively in wheat straw. So these fungi are used for selective removal of lignin leaving other components intact [45]. Some lignocellulose degrading brown-rot fungi rapidly depolymerize cellulose while only modifying lignin. The strong oxidative capacity and low substrate specificity make some white-rot fungi distinctive as they can degrade several environmental pollutants such as such as industrial dyes, chlorinated/heterocyclic aromatic compounds and synthetic polymers [46].

3. Enzymatic Degradation of Lignocellulosic Biomass

The conversion of lignocellulose into fermentable sugars is divided into two categories. First and primitive one used acids as a catalyst, while the second used an enzyme. Effective pretreatment is a key step in the success of hydrolysis where polymer sugars from cellulose and hemicellulose hydrolyzed into free monomer further undergo fermentation to produce bioethanol. Enzyme hydrolysis is more effective than inorganic catalysts because of high specificity and mild operating conditions. Although enzymatic hydrolysis offers several advantages but mechanism and relationship between substrate structure and function of various glycosyl hydrolases are still not well known. Enzymatic hydrolysis of lignocellulose biomass is a complex process since various enzymes with different specificities are required to degrade all components [47,48].

When enzymatic hydrolysis occurs sequentially, the first hydrolysis followed by fermentation, named separate hydrolysis and fermentation (SHF) but when hydrolysis carried out in presence of fermenting microorganisms, then it is called simultaneous saccharification and fermentation (SSF) [49]. In SHF, lignocellulose first hydrolyzes to produce glucose and then fermented to ethanol in separate reactors. Thus, both the hydrolysis and fermentation take place at optimum temperatures, 50°C for hydrolysis and 37°C for yeast fermentation. Accumulation of hydrolysis products is a foremost drawback because it acts as feedback inhibitor to enzymes. It has been reported that cellulase activity is inhibited mainly by cellobiose and glucose and the effect of cellobiose, a dimer of glucose is higher than the glucose. Cellobiose reduced cellulase activity by 60% at a concentration of 6 g/l [50-52]. In SSF both hydrolysis and fermentation operated in a single reactor so the optimum temperature maintained around 38°C which is between the optimum temperature for hydrolysis (45-50°C) and fermentation (30°C). Glucose released in hydrolysis is directly consumed by fermenting microorganism present in the culture, thus minimized end product inhibition. SSF is preferred over SHF because of low processing costs. *Trichoderma reesei* and *Saccharomyces cerevisiae* are the most preferred microorganisms in SSF [53].

3.1. Degradation of Cellulose

Cellulose is the main component of plant cell wall, constitutes approximately 40-50% dry weight of wood. In terms of production cost and availability, it is one of the most promising raw materials for the preparation of biofuels and several value-added products [54,55]. Cellulose can be hydrolyzed by a series of enzymes with different specificities, working together called cellulosome. It is associated with the cell wall of bacteria and some fungi. Hydrolysis can be operated by the synergistic action of three distinct class of enzymes namely cellobiohydrolases (EC 3.2.1.91), endo-β-1,4-glucanases (EC 3.2.1.4) and β-glucosidases (EC 3.2.1.21) [56,57]. According to CAZy (Carbohydrate-active enzymes) classification system, all these enzymes are grouped into glycosyl hydrolase family. These enzymes display structural resemblance in sequence homology and hydrophobic cluster analysis [58]. Cellobiohydrolases acting at the end of cellulose chains while endoglucanases hydrolyze internal β-1,4-glucosidic linkages randomly. The third enzyme, β-glucosidases acts on the hydrolyzed products called cellobiose and cello-oligosaccharides [59]. Structurally, cellobiohydrolases and endoglucanases have two domain: a carbohydrate binding module (CBM) and a catalytic domain (CD). These two domains connected together by a linker region [60]. Molecular weight ranges between 25 to 50 kDa and optimal activity is acidic pH. Endoglucanases have an open active site which enables its action in the middle of glucan chain while exoglucanases have tunnel shape active site, hydrolyze only ends and side chains [61]. CBM works to bring enzyme catalytic module close contact to a substrate in a proper orientation. It has been reported that in the absence of CBM, an activity of cellobiohydrolases on crystalline cellulose decreased remarkably but no changes occurred for soluble and amorphous substrates. So CBM increases a concentration of enzyme on the surface of solid substrate [62,63]. The synergistic degradation of lignocellulose does not follow Michaelis-Menten kinetics. Additionally, heterogeneous nature of lignocellulose makes hydrolysis mechanisms complex [64].

To date number of fungi are discovered, producing a remarkable amount of cellulolytic enzymes and these number increasing continuously. Fungal species like brown-rot fungi (*Fomitopsis palustris, Fomitopsis palustris*), ascomycetes (*Trichoderma reesei*) and few anaerobic species (*Orpinomyces sp.*) show great potential in lignocellulose degradation at industrial scale [65-67]. Apart from fungi, many bacterial strains such as *Cellulomonas fimi* and *Thermomonospora fusca* produce cellulolytic enzymes and grouped into aerobic and anaerobic bacteria as well as actinomycetes [68-70]. Recently, *Clostridium thermocellum* and other related microorganisms are largely exploited for single-step conversion of biomass into desired products [71,72].

3.2. Degradation of Hemicellulose

Hemicelluloses in wood are made up of xylan and glucomannans. Xylan is a major

carbohydrate and its composition varies. Degradation of glucomannans and xylans require several synergistic enzymes, endoxylanases and endomannanases hydrolyse main backbone of xylans and glucomannan, respectively. Xylanases are placed in glycosyl hydrolase families 10 and 11 and differ from each other with respect to their catalytic properties. The catalytic domains of these two families are different in their molecular masses, net charges and isoelectric point. These properties might play some role in specificity and activity [73]. Complete hydrolysis of xylans into free monomers requires numerous enzymes like endo-1,4-β-xylanase, acetylesterase, α-glucuronidase and β-xylosidase. The major difference between endo-1,4-β-xylanase and 1,4-β-xylosidase are; former generate xylan oligosaccharides while later works on oligosaccharides generated by endo-1,4-β-xylanase to produce xylose [74]. Tenkanen and co-workers stated that enzymes from *Trichoderma reesei* synergistically hydrolyze beechwood xylan. Later it was perceived that endoxylanases produced by single fungi show different specificities towards xylans, showing complex nature of the substrate. It has been demonstrated that the α-glucuronidases, α-arabinosidases, and acetyl esterases are varying in specificities with respect to neighboring substituents and xylan chain length [75]. In addition, *Clostridium stercorarium* produced eight different enzymes to degrade arabinoxylan, however, only three of them required for hydrolysis. Therefore, efficient hydrolysis of native xylan appears to comprise not only four different enzymes but also multiple isoenzyme systems [76].

Xylanases are produced by many species of bacteria, fungi and plants. The optimum temperature from the bacterial and fungal origin are ranging between 40 to 60°C but thermostability of bacterial xylanases are higher than fungal enzymes. A tadpole-shaped endoglucanases from *T. reesei* of almost 5 nm in diameter and 20 nm long showing acidic pH optima [64]. Two glycoproteins of 38 and 62 kDa with acidic pH optima were purified from *Irpex lacteus* which depolymerizes larch xylan [77]. The pH optima of fungal xylanases ranges between pH 4.5-5.5 while bacterial enzymes displayed maximum activity at pH 6.0-7.0. Xylanases from *Bacillus sp*. and *Streptomyces viridosporus* are active at alkaline pH [78,79].

Mannanases are the heterogeneous group of enzymes similar to xylanases. The complete hydrolysis of O-acetylgalactoglucomanann require many enzymes such as endomannases, α-galactosidases, acetylglucomannan esterases and β-mannisidases. Degradation opens with rupturing of a polymer by endomannases; acetylglucomannan esterase removes acetyl groups, similar to xylan esterase in xylans. After that α-Galactosidases remove substituted galactose residues and finally β-mannosidase and β-glycosidase breakdown β-1,4 bonds and release oligomers. Mannanases are larger proteins than xylanases with acidic isoelectric points. The molecular weight ranges between 30-90 kDa. Similar to a cellulolytic enzyme, multidomain structure is reported in mannanase of *Trichoderma reesei*; a catalytic core domain and a cellulose binding domain, separated by a linker. In addition to these groups of enzymes, hemicellulose degradation required some supplementary enzymes like xylanesterases, ferulic

and p-coumaricesterases, α-l-arabinofuranosidases and α-4-O-methyl glucuronosidases for the efficient hydrolysis of xylans and mannans [80].

Endomannases usually found in white-rot fungi like *Irpex lacteus, Haemato stereum-sanguinolentum and Coriolusversicolor* as well as gram-positive and gram-negative bacteria. They are extensively studies in several nonwood decaying ascomycetes such as *Sporotrichum cellulophilum, Trichoderm areesei,* and *Sclerotium rolfsii*. Additionally, α-galactosidases, acetylglucomananeesterases and β-mannonidases are explored in *Aspergillus niger* and *Polyporus sulfureus* [81,82].

3.3. Degradation of Lignin

Degradation of lignin is challenging due to structural complexity. High molecular weight, insolubility and heterogeneous nature make less accessibility for enzymes. Lignin has inter-unit carbon-carbon and ether bonds, therefore, degradation mechanism is oxidative rather than hydrolytic. Degradation of lignin required nonspecific oxidative enzymes since the polymer is stereo-irregular [83]. Enzymes employed are lignin peroxidase (LiP, ligninase, EC 1.11.1.14), manganese peroxidase (MnP, Mn-dependent peroxidases, EC 1.11.1.13) and laccase (benzenediol, oxygen oxidoreductase, EC 1.10.3.2). It has been evidenced that these enzymes act on lower molecular weight intermediaries. Besides these, some additional enzymes like glyoxal oxidase and aryl alcohol oxidase (EC 1.1.3.7) are also taking part in hydrogen peroxide production [84]. Some white-rot fungi produce all three enzymes while others produce either two or even only one [85]. Several isoenzymes of LiP and MnP but not for laccase were produced by *Phanerochaete chrysosporium* while their genome contains ten LiP and five MnP genes [86,87]. Among several lignin degrading microorganism, white-rot basidiomycetes such as *Coriolus versicolor, Phanerochaete chrysosporium and Trametes versicolor* are widely studied [88,89].

Lignin peroxidases (LiPs) are heme-containing glycoproteins, catalytic properties are similar to other peroxidases [90]. Molecular mass ranges between 38 to 43 kDa with acidic pH optima and pI. LiPs and a series of their isoenzymes are found in fungi, encoded by different genes. LiPs are most effective peroxidases so far studied. Besides natural substrates like phenols and aromatic amines it oxidizes variety of other aromatic ethers, amines and polycyclic aromatics [91]. Catalysis of LiPs is H_2O_2 dependent oxidative de-polymerization, where oxidation begins with an abstraction of one electron from the aromatic ring of donor substrate resulted in aryl cation formation which acts as both cations and free radical thus generates variety of degradation fragments. LiPs catalyze Cα-Cβ bond cleavage, ring opening and many other reactions [82]. Piontek and coworkers reported that, heme group in LiPs is buried inside protein and acted on substrates through a channel, therefore, it catalyze only small substrates because the size of channel is not appropriate for larger polymer [92].

Similar to LiPs, MnPs are extracellular glycoproteins with slightly higher molecular masses (45-60 kDa). These enzymes are secreted in multiple isoforms having a heme molecule as iron protoporphyrin IX. Catalytic mechanism of MnPs is very similar to conventional peroxidase with a slight difference by means of Mn (II) acting as a substrate. During the catalytic reaction, Mn(II) is converted into Mn(III) and oxidizes phenolic rings to phenoxyl radicals leading to decomposition of substrate. It has been reported that Mn(II) must be chelated via bidentate organic acid chelators so that, product Mn(III) stabilized and released easily. Chelated Mn(III) complex is a diffusible low molecular weight redox-mediator that can act at some distance from the enzyme. LiPs can only act on phenolic substrates such as simple phenols, amines, dyes and phenolic dimers because of weak oxidation nature of these substrates [93-95]. White-rot fungi produce MnPs but lacking LiPs, can also degrade nonphenolic lignin substructures, directing towards other ligninolytic mechanisms [96]. Wesenberg et al reported that oxidation of non-phenolic lignin occurs in presence of Mn(II) through peroxidation of unsaturated lipids. These MnP/lipid peroxidation systems strongly depolymerize phenolic and non-phenolic lignins more efficiently [97]. Camarero et al described a novel versatile peroxidase having activities of both manganese peroxidase and lignin peroxidase and degrades natural lignin more effectively. Versatile peroxidase can oxidize hydroquinone even in the absence of exogenous H_2O_2 but require Mn(II) thus, it promoting chemical oxidation of hydroquinones [98].

Laccases are a blue-copper oxidoreductase, utilizes molecular oxygen as oxidant. They oxidize a number of phenolics, aromatic amines and other electron-rich substrates [99]. The reaction starts with a reduction of molecular oxygen into the water with one-electron oxidation mechanism. These enzymes oxidize phenolic unit into phenoxy radicals which cause aryl-Ca cleavage. In this reaction, free radicals acting as an intermediate substrate for enzyme. The catalytic center of a molecule has four copper atoms which can be differentiated by UV-vis spectroscopy. Usually, laccases oxidize phenolics but in presence of redox mediators like ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid). They effectively oxidize non-phenolic compounds also [100].

Wood-rotting fungi are the main producers of laccases but most of the enzymes isolated and characterized belong to white-rot fungi. The common laccase producing white-rot fungi are *Lentinus tigrinus, Pleurotus ostreatus* D1*, Trametes versicolor, Trametes sp.* strain AH28-2*, Trametes pubescens* and *Cyathus bulleri.* Laccases from different organisms display extensive diversity in substrate specificity, pH optima, molecular weight and other properties. The molecular mass of laccases in white-rot fungi ranges between 60 to 80 kDa with acidic pIs and pH optima. Laccases have significant biotechnological applications, used in biosensors, soil bioremediation, food and textile industries and synthetic chemistry [101-103].

4. Conclusions

The important socioeconomic issues today are energy and environmental crises, food security and agro-processing. Some of these issues in developing countries can be addressed by lignocellulose biotechnology where most of the radially available biomass waste can utilize and converted into numerous value-added products. Additionally, lignocellulose biomass can be used to produce bioenergy to replace exhausting fossil fuels. The major hurdles in enzymatic bioconversion of lignocelluloses are the crystalline nature of cellulose, protection of accessible surface area by lignin and sheathing by hemicellulose. This study presented an overview of current knowledge on lignocellulose degradation by a variety of microbial enzyme systems. Cellulosic degradation is multi-step process require complex enzyme system for conversion of biomass into fermentable sugars. Although synergy and interaction between cellulases have been well-established, lignin and hemicellulose are more diverse, therefore, further research is required towards enzymatic degradation of hemicelluloses and lignin.

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6. References

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