

## Enzymatic Pretreatment of Lignocellulosic Biomass: An Overview

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**ABSTRACT** – Lignocellulosic biomass is nature's most abundant alternative source of biofuels replacing traditional fossil fuels. Globally, more than 70% of renewable energy depends on biomass and contributes 14% of the total energy supply. The pretreatment of lignocellulosic biomass is to remove lignin, modify the lignin structure, reduce the cellulose crystallinity and increase the porosity and surface area of lignocellulosic material. The pretreatment of lignocellulosic biomass is one of the most expensive steps for biomass conversion and consumes about 40% of total costs. The conventional physical and chemical methods that have been used for the pretreatment of lignocellulosic biomass are somehow unsustainable and render negative impact to the environment. Pretreatment by the lignocellulosic laccase enzyme may offer solutions to these problems. In this paper, the pretreatment of lignocellulosic biomass has been studied, focussing on the enzymatic pretreatment of lignocellulosic biomass.

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## INTRODUCTION

Lignocellulosic biomass composed of three fundamental compounds which are cellulose (30-60% of dry matter), hemicellulose (14-40% of dry matter) and lignin (7-25% of dry matter)[1]. The cellulose in lignocellulosic biomass is protected by lignin and hemicellulose. Lignin is highly recalcitrant towards chemical and biological degradation. Lignocellulosic pretreatment aims in disintegration of compact structure of lignocellulosic biomass for saccharification and hydrolysis [1]. The biomass can be converted into value added products like biofuel, chemicals, biomaterials and composites through a mutli-step process involving pretreatment; the breakdown and removal of lignin, hydrolysis; breakdown of polysaccharide into monosaccharide and fermentation to convert monomer sugar into biofuel [2].

However, this promising renewable and alternative energy source often underutilized, compared to cellulose and hemicellulose. As a result, each year 40 to 50 billion tons of lignin waste are abundantly produced globally [3]. These excessive amount of biomasses urge researchers to develop novel, eco-friendly and biodegradable approaches that mitigate alarming environmental pollution by fossil fuels. One of the techniques commonly employed for the degradation of lignin is ligninolytic enzymes laccases. The major reason for the pretreatment of biomass in bioprocess is to reduce the resistance mounted by the components of the cell walls of biomass, for an easy accessibility to the cellulose and hemicellulose content. After pretreatment, the biomass cell wall is disrupted either partially or completely to affect the accessibility of hydrolytic agents into the core of the biomass for better sugar recovery [5].

Lignocellulosic enzyme laccase is a natural biological catalyst with high specificity, selectivity, and catalytic activity [6]. Generally, it demonstrated high activity in mild pH, low temperature, and pressure. Laccase also indicated better catalytic activity than microorganisms in biodegradation [7]. It can easily degrade the complex aromatic polymer lignin. The polymer lignin has been observed to exhibit a recalcitrant property towards biological and chemical degradation [8]. Laccase is essential for the oxidation of lignin in lignocellulosic biomass. The focus of this work is to discuss the literature data on laccase enzyme for the pretreatment of lignocellulosic biomass.

## BIOMASS PRETREATMENT

Pretreatment is one way of causing disruption and hydration on lignocellulosic materials which ensures accessibility of the material to subsequent treatments. Lignocellulosic materials contain polysaccharide molecules in the forms of cellulose and hemicellulose. Efforts have been made to recover these compounds for more efficient use. One of the major challenges faced recently in this regard was how to overcome the resistance of the biomass cell wall to hydrolytic enzymes due to the structural lignin component of the cell wall. The cell wall of plants contains abundant of lignin, a mesh of polysaccharides, which provides it strength and rigidity. This lignin must be overcome to access the sugar components of lignocellulosic materials. A good pretreatment technique will alter the integrity of these plant cell walls through lignin disruption and render the lignocellulosic biomass porous to various treatments for the recovery of simple sugars from its polysaccharide base. So that future treatment can be easily achieved in the overall biomass conversion process [9-10].

Lignocellulosic biomass possesses features that make it resistant to biodegradation. The best pretreatment method and conditions are determined by the types of lignocellulose present, due to the complexity and variety of biomass chemical structures. Several structural and compositional properties, such as cellulose crystallinity, accessible surface area, degree of cellulose polymerization, presence of lignin and hemicellulose, and degree of hemicellulose acetylation, were discovered to have an impact on the biodegradability of lignocellulosic biomass [11]. In general, different pretreatment procedures indicate varying degrees of impact on these parameters.

Incompatible pretreatment procedures for specific biomass samples can be costly, labor-intensive, and time-consuming, putting the economic feasibility of the process and the creation of inhibitory by-products in doubt. Pretreatment should produce reactive cellulosic fibres for enzymatic attack, decrease energy demand and by-products, avoid degradation of lignocellulosic components, and avoid potential inhibitors for hydrolytic enzymes and fermentative microorganisms in order to be effective and cost-efficient [12].

The pretreatment procedure removes lignin and hemicellulose, reduces cellulose crystallinity, and increases the porosity of lignocellulosic materials. Pretreatment must meet the following criteria: (1) increase sugar formation or the ability to create sugars by hydrolysis, (2) avoid carbohydrate degradation or loss, (3) avoid the development of by-products that limit subsequent hydrolysis and fermentation processes, and (4) cost-effective [13].

## TYPES OF BIOMASS PRETREATMENT PROCESS

### Physical Pretreatment

Mechanical crushing, ultrasonic, and microwave pretreatments are examples of physical pretreatment methods for lignocellulosic biomass. Mechanical crushing is the breaking of cellulosic biomass into powders by mechanical milling which reduces the crystallinity of cellulose, increases the surface area between the catalyst and the raw materials in the reaction process, and improves hydrolysis efficiency. Ball-milling, which is one of milling methods where powder particle grinded into fine particle, has been investigated in several studies [14-15].

This mechanical technique cleaving hydrogen bonds in cellulose thus reduces particle size and crystallinity, increasing glucose production [16-17]. The particle size of cellulose was reduced through jet-milling treatment without reducing crystallinity index, while the crystallinity of cellulose was converted into amorphous within 1 h by ball-milling and rob-milling [18-19].

Steam explosion, discovered by Mason in 1925, has been considered as a suitable pretreatment method of lignocellulosic biomass [23], which was originally applied to the fiberboard industry. In the "Mansonite Wet-Form Process," abrupt decompression disrupted the biomass structure. This method is relatively inexpensive because there is no need of external catalyst addition [20].

The disadvantage of mechanical crushing pretreatment is that, it consumes more energy as the particle size of the raw material decreases. The molecular architectures of hemicellulose, cellulose, and lignin in cellulosic biomass are modified by ultrasonic and microwave pretreatments. After the ultrasonic and microwave pretreatments, the crystalline structure of cellulose is broken, the crystallinity is decreased, and the raw material hydrolysis efficiency is enhanced [21]. Chen et al. [41] discovered that the ultrasonic treatment at a higher output power (> 1000 w) reduced the diameter of cellulose to 5–20 nm. This pretreatment process is expensive and insufficient for industrialised large-scale applications [24].

### Chemical pretreatment

Chemical pretreatment reduces lignocellulosic biomass by using chemicals (acid or base). There are two types of chemical pretreatment methods; alkaline pretreatment and acid pretreatment. Chemical pretreatments use a variety of chemicals to break down the complex structure of lignocellulosic biomass, including acids, bases, ionic liquids, and organic solutions [25]. The chemicals such as acids, bases, and ionic liquids is used to alter the chemical and physical properties of lignocelluloses [26].

In alkaline pretreatment methods, weak or strong alkaline solutions are used to treat the lignocellulosic biomass. This brings about a reduction in lignin recalcitrance based on the lignin content of the biomass. With alkaline pretreatment methods, the recovery of more cellulose is established at room temperature within a short period of time [28]. The hydroxide of calcium, potassium, sodium and ammonium are suitable alkaline pretreatment chemicals. Generation of products that inhibits sugar production is a concern and there is a possible loss of fermentable sugars during the pretreatment process [29]. Utilization of alkaline pretreatment causes major effect on biomass such as swelling which decreases degree of polymerization and cellulose crystallinity while increasing the internal surface area of biomass [30].

Pretreatment with an organic solvent effectively degrades lignin, which is mostly utilized using osmosis to break and degrade the internal chemical bonds of cellulose and hemicellulose. This pretreatment method can also produce pure lignin, cellulose, and hemicellulose with hardly any structural modification, which can be used as high-value-added products. Organic solutions pretreatment methods can effectively eliminate the barrier action of wheat straw components. However, the main issues were solutions recovery, the huge costs (in some cases) as well as ignitability and volatility which can be regarded as its disadvantages. Organisms might also pose adverse effects on the subsequent strain growth, fermentation, and environment, hence proper extraction and separation procedures, such as evaporation are recommended to be carried out along the main process [31].

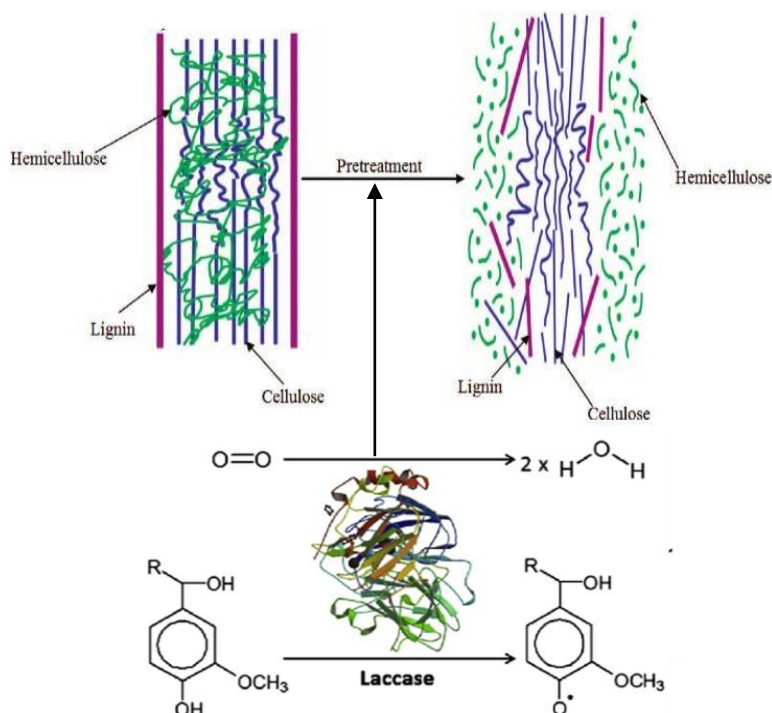
Mineral acids such as sulfuric acid, phosphoric acid or nitric acid are used to pretreat biomass either in concentrated or diluted form [17]. This is targeted to cause conformational changes in the structure of the biomass either by random

cleavage or a continuous cleavage to reduce it into smaller forms for easy accessibility of hydrolytic enzymes. Some of the acids could be sprayed on the surface of the material to be pretreated or the material wholly immersed in the acid and allowed for about 30 - 90 minutes in contact with the acid. The excess acid retained in the biomass after soaking could be expressed by physical methods [21]. Using acid in concentrated or diluted form has some disadvantages which have raised concerns regarding its use and environmental safety. Acids in concentrated form cause corrosion on the body of the instruments used in constructing pretreatment chambers, and this corrosion effect will lead to a search for more anti-corrosive materials when designing the instrument for concentrated acid pretreatment. Dilute acid on its part has a high tendency of generating inhibition products which affects hydrolysis and thereby reducing the digestibility of the yielded sugar [23].

### Biological pretreatment

Natural microorganisms are used in the biological pretreatments. They secrete hydrolytic and ligninolytic enzymes, which result in lignin depolymerization and lignocellulose degradation [32]. The cell wall structure of biomass is damaged during biological pretreatment, which aids in the hydrolysis of cellulose and hemicellulose [5]. Lignocellulosic derivatives such as furfural, 5-Hydroxymethylfurfural (HMF), and levulinic acid (LA) are derived from monomeric sugars produced from biopolymers during biological processing [33]. Generally, biological pretreatments mainly involve the use of bacteria, fungi, and their metabolites, particularly the enzymes (lignin-degrading enzymes, cellulose-degrading enzymes, hemicellulose-degrading enzymes, etc.) [34]. All of these enzymes are important in the degradation/hydrolysis of lignocellulosic biomass constituents (lignin, cellulose, hemicellulose, and polyphenols) [17].

Biological pretreatment requires low energy and mild operation conditions. Biological pretreatment has various advantages over physical/chemical pretreatments, including minimal energy consumption, high yield of desired products, and no toxicants production [35]. However, biological pretreatment requires strict control of growth conditions and a large availability of space, and it takes longer time to complete [1].



**Figure 1.** Schematic of enzymatic pretreatment of lignocellulosic materials (adapted from [9]).

### LACCASE PRETREATMENT OF LIGNOCELLULOSIC BIOMASS

Laccases (benzenediol: oxygen oxidoreductases), are a blue multicopper oxidase (MCOs). In general, multicopper oxidases oxidize their substrate by accepting electrons at a mononuclear copper center and transferring them to a trinuclear copper center; dioxygen binds to the trinuclear center and, following the transfer of four electrons, is reduced to two molecules of water [15].

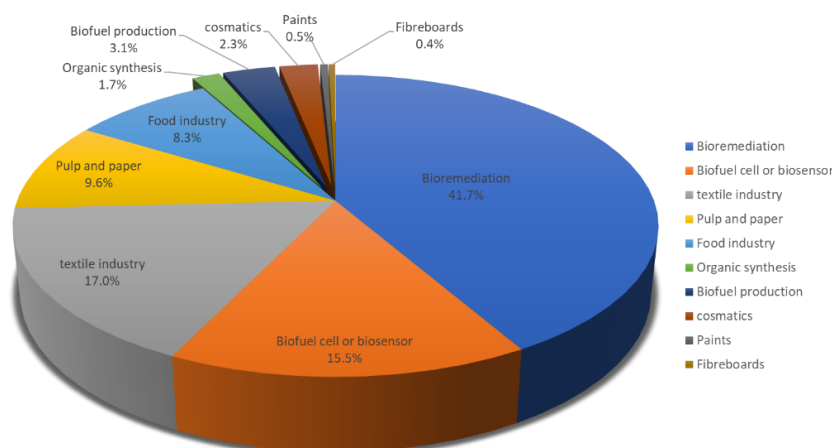
The primary goal of enzymatic pretreatment is to overcome the recalcitrant nature of cellulosic biomass. The pretreatment step aims to increase surface area and pore volume while simultaneously lowering cellulose crystallinity [18]. The main goal of the pretreatment in the pulp and paper industries is to preserve cellulose while removing both hemicellulose and lignin [22]. Laccases plays important roles in textile, pulp, and paper industry (Figure 2). They are widely used in lignocellulosic biomass pretreatment and hydrolysis. Laccase also decolorizes azo dye and degrades xenobiotic components by oxidizing the chemicals. [6][22]. Laccases have significant biotechnological potential because of their ability to oxidize a wide variety of substrates used in different sectors of industry.

Laccases are increasingly popular as they can oxidize both phenolic and non-phenolic aromatic compounds. Laccase is known for its oxidation catalysis of a wide range of heterocyclic compounds, aromatic amines and phenolic groups in ortho-para position [12], via its high and low redox potential. Usually fungi contain high redox potential while bacteria, plants and insects laccases are low redox potential enzymes [13]. Fungal laccases are mostly used in biotechnological fields. Many species of fungi including white [14] and brown rot fungi [15], yeasts [16], filamentous ascomycetes [17] and mycorrhizal species [18] have been documented for the potential of laccase production. Although intracellular activity of laccase have been reported, fungal laccases are mostly monomeric enzymes with extracellular activity [19].

Extracellular laccases have been intensively investigated in fungi for their roles in lignocellulose breakdown, organic material recycling, oxidative stress reduction, and pathogenicity toward plants and mammals [20]. The three-dimensional structure of laccase from fungi and bacteria has been studied in x-ray crystallography for a few times in recent years. But the structure of laccase from plant and animals still needs to be reported. The presence of multiple copper residue is most notably common in all crystallography structure of laccases. Based on the multicopper oxidase evolutionary, phylogenetic tree laccase can be considered as one of the earliest metalloproteins. Laccase consisted of two, three and six cupredoxin-like domain [21].

The major purpose of enzymatic pretreatment is to facilitate the recovery of the sugar components that it contained. A good pretreatment technique provides an accessible avenue to biomass and make it easy to hydrolyze by enzymes for the production of simple sugar. Qualities of a good pretreatment process include;

- (i) Ability to provide an accessible route for the enzymatic mechanism, by reducing the lignin recalcitrance while producing little or no product which will inhibit further steps in the sugar recovery pathway.
- (ii) To make way for the recovery of sugar from biomass during hydrolysis [10].



**Figure 2.** Applications of laccase (Prepared from Scopus database search results)

## LACCASE FOR BIOMASS PRETREATMENT

Laccase is primarily used for the delignification of lignocellulosic biomass. Pretreatment is a prerequisite step to break down the complex lignocellulosic biomass into cellulose, hemicellulose, and lignin (Figure 1). Cellulose and hemicellulose subjected to enzymatic hydrolysis produce fermentable carbohydrates. While lignin is not fermentable due to their phenolic composition [27]. Thus, delignification is a crucial part of pretreatment of lignocellulosic biomass. Lignolytic enzymes such as laccase are primarily responsible for delignification of lignocellulosic biomass. Palm oil empty fruit branch (OPEFB), aloe vera leaf, sugarcane bagasse, rice Straw, hard wood, sweet sorghum bagasse, pineapple leaf waste, agave biomass, corncob, wheat straw are some of the biomass that have been pretreated by laccase. Giacobbe et al. [29] reported to obtain effective removal of lignin in agrofood waste (AFW) (Apple pomace, potato peels, and coffee silverskin) resulting 80% sugar yield. Besides, 7.83g/L butanol obtained within 190 hours from laccase pretreated brewer's spent grain [28-29]. Microalgal biomass pretreatment with laccase during waste water treatment increases biomethane production to 74% [30].

Bhardwaj et al. [31] used laccase from *Myrothecium verrucaria* for rice straw delignification to produce bioethanol. Rejeswari et al., [32] utilized laccase from the 76.67% delignification of aloe vera leaf rind (AVLR), collected from industrial waste to elevate reducing sugar yield available for bioethanol production. Raj et al. [33] (used cellulase and laccase cocktail to enhance total sugar yield of sugarcane bagasse (SCB). The laccase mediated SCB hydrolysis produced relatively higher 78.4% sugar during low energy biomass conversion process. Singh et al. [34] reported that the delignification of woody biomass has enhanced about 6 fold by using small amount of laccase enzyme (sLac). Rizal et al. [35] used laccase combined with superheated steam (SHS) for the delignification of palm oil empty fruit branch (OPEFB) to enhance enzymatic hydrolysis by 4.6-4.8-fold from oil palm empty fruit branch (OPEFB) and oil palm



mesocarp fiber (OPMF). The combined superheated steam with laccase pretreatment to utilize oil palm biomass (OPEFB) into glucose that increase glucose production by 4.8 fold compared to the untreated OPEFB [35].

## FUTURE PERSPECTIVES AND CHALLENGES

One of the major challenges that the biorefinery concept faces is to find suitable raw materials. The major raw material source in a biorefinery is likely to be second-grade or waste materials. Straw, bagasse, tree roots, branches, forest thinning, and other items fall into this category. However, a large part of the published research which deals with woody materials are often covered on wood chips of high quality. This is indirect opposition to the interests of pulp producers. On the other hand, the residues from agricultural can often be converted into other valuable products. However, not all waste material can be removed from the field in order to maintain soil quality such as nutrient levels but as an alternative, processed residues can be returned to the field. Other types of waste materials include municipal solid waste, waste textile, waste-construction materials, etc. Thus, various production methods are required to be able to process a variety of raw materials [36].

The sensitivity to denaturant chemicals and the difficulties to be reclaimed has limited the usage of enzymes. Enzyme immobilisation could improve enzyme stability and reuse in different catalytic cycles, but it would have a negative influence on economic-technical feasibility. Enzymatic reusability is critical for lowering the cost of converting biomass into biofuels. Elucidating the structural changes of lignin via pretreatment improve the understanding of the mechanism in enzymatic conversion [23]. A variety of pretreatment techniques degrade lignocellulose's recalcitrant structure, causing significant and particular alterations in lignin structure. Lignin modification is achieved by incorporating other chemicals during the pretreatment process, which reduces adverse affects of lignin. In several cases, pretreatment with enzymatic hydrolysis stimulates in-situ lignin alteration in various circumstances, lowering biofuel costs and increasing yields [33]. The effects of different pretreatments on structural changes of lignin influence the enzymatic conversion of biomass into biofuel. The principles involved in the interactions between lignin and enzymatic conversion remain unknown due to the complicated physicochemical characteristics of lignin and enzymes. On the other hand, valorization of lignin is critical and valuable to the comprehensive utilization of biomass which includes both carbohydrates and lignin fractions [39].

The scaling-up opportunity for a broad application of laccase is hindered due to some obstacles, mainly the expense of the commercially available catalyst [9]. Furthermore, various issues linked to its instability under operational conditions, such as rapid activity loss and difficulty reclaiming enzymes and products have restricted its industrial utilization [6]. To reduce the high cost of the enzymatic process, many approaches were undertaken to improve the efficiency of enzyme biocatalytic systems. Enzyme immobilisation improves enzyme stability, activity and reusability which is one of the most important techniques [37-38]. This technique reduces the cost of the process by 50% through an enhanced enzymes recyclability [40].

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