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**Review Article** 

# Lignin Degradation by Fungal Pretreatment: A Review

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#### Abstract

Lignin is regarded as the most plentiful aromatic polymer contains both non-phenolic and phenolic structures. It makes the integral part of secondary wall and plays a significant role in water conduction in vascular plants. Many fungi, bacteria and insects have ability to decrease this lignin by producing enzymes. Among these fungi are the major player in degradation of lignin. These fungi produce enzymes such as *lignin peroxidases* and *laccases*. The well-known fungi which degrades lignin are white, brown, soft-rot fungi, and deuteromycetes. Currently these fungi are utilized to reduce lignin to produce ecofriendly bioenergy. The primary aim of this paper is to describe the lignin degradation by biological pre-treatment using fungi (white-, brown- soft-rot fungi and molds), as well as biochemical application. Besides, Molecular methods and enzymes regulation engaged in fungal pre-treatment and some of the factors affecting pretreatment will also be briefly discussed.

Keywords: Lignin; Fungi; Degradation; Biological pre-treatment

#### Introduction

Global warming and increasing prices of fossil fuels influencing the improvement of renewable energy and biofuel production technologies [1]. Bioethanol production by biomass digestion promises to be one of the most effective methods for producing renewable and sustainable energy [2]. Lignocellulosic raw materials include agricultural wastes, forest products or energy crops and constitute abundant, widely distributed and inexpensive feedstocks for biofuels production [3]. The major compositions of plant cell wall are cellulose, hemicellulose and lignin. High amount of sugars can be produced from cellulose and hemicellulose, using acid as the catalyst, or enzymatic hydrolysis and reformed into bioethanol by a fermentation procedure [4]. Most physical and chemical pre-treatment using acid, alkali, processes require special instrument and consume a lot of energy and generate inhibitors which will affect enzymatic hydrolysis and fermentation [5]. Many researches displayed that biological pretreatment such as bacteria, fungus (white-, brown-, and soft-rot fungi), deuteromycetes and ascomycetes can enhance the hydrolysis productivity because of generating low inhibitors and limited energy utilization [6-8]. Furthermore, biological pretreatment compared to other pretreatment process such as organosolvant and ammonium fiber explosion (AFEX) is considered as cheap process and have been less investigated [9]. The highest efficiency among the pre-treatment methods has been achieved by lignin degrading white-rot fungi for the soft and brown fungi only attack cellulose. Among the known species of white-rot fungi used until now, Phanerochaete chrysosporium because of considerable growth ratio and remarkable reduction of lignin potentials has the highest productivity [10-12]. Moreover, using white rot fungi consume less environmental damage and less energy conception [13,14]. Through the biological process effective lignin degradation relies on the lignolytic enzymes presented by basidiomycete such as lignin peroxidase, manganese peroxidase and laccase [15-18]. Biological pretreatment using fungi in nature for ethanol production from agricultural residues is a favorable method because of immense benefits such as environmentally friendly and thriftily feasible method for enhancing lignocellulosic digestion rate [19].

#### Lignocellulose

On the world lignocelluloses are the main part of biomass, because it is a renewable resource and the prominent structural component of plant cell wall as well. Lignocellulosic wastes are released in large amounts by many industries. Plant cell wall is generally composed of cellulose (35% to 50%), hemicellulose (20% to 35%), and lignin (15% to 20%) (Figure 1) [20]. Cellulose is the dominant part of lignocellulose and consist of a linear chain of D-glucose linked by  $\beta$  (1-4)-glycosidic bonds to each other. The cellulose strains are connected to each other deliver cellulose fibril. A number of intra- and intermolecular hydrogen bonds are linked cellulose fibers together. Hemicellulose is the second plentiful constituent of lignocellulose, is comprised of diverse pentoses (arabinose, xylose) and hexoses (mannose, galactose, glucose). So, that large amount of hemicelluloses must be eliminated to improve the cellulose digestibility for the enzymatic hydrolysis. Lignin primarily is consisted of *p*-coumaryl phenol (H), guaiacyl (G) and sinapyl alcohol (S). Polymerization of these constituents mainly synthesize lignin and their proportion is different between crops, woody plants and also in the primary and secondary cell wall. Microfibrils formed by cellulose,



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Received February 05, 2017; Accepted February 22, 2017; Published February 28, 2017

Citation: Madadi M, Abbas A (2017) Lignin Degradation by Fungal Pretreatment: A Review. J Plant Pathol Microbiol 8: 398. doi: 10.4172/2157-7471.1000398

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Citation: Madadi M, Abbas A (2017) Lignin Degradation by Fungal Pretreatment: A Review. J Plant Pathol Microbiol 8: 398. doi: 10.4172/2157-7471.1000398

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Biomass	Microorganisms	Effect	Reference
Rice straw	Trichoderma viride	56% of lignin reduction	[29]
Poplar wood	White rot fungus	85% of lignin removal	[6]
Wheat straw	white rot fungi (Pleurotus ostreatus)	35% of lignin reduction	[30]
Rice, wheat, sugarcane, and pea straw	Fungal consortium	6.6-folds increase in saccharification	[7]
Eucalyptus grandis saw dust	P. ostreatus P. pulmonarius	Twenty fold increase in hydrolysis	[31]
Plant biomass	Fungal consortium	Complete removal of use of dangerous chemicals	[32]
Corn stover	Fungal consortium	43.8% of lignin removal	[33]
Pine wood	Chrysonilia sitophila	Carbohydrate (18%) and lignin (20%) reduction	[8]
Bamboo culms	Punctualaria sp. TUFC20056	50% of lignin removal	[12]
Milled wheat straw	Penicillium chrysogenum	27.4% lignin mineralization	[54]
Milled wheat straw	Fusarium oxysporum	23.5% lignin mineralization	[54]
Milled wheat straw	Fusarium solani	22.6% lignin mineralization	[54]

Table 1: Effect of different microorganisms in different biomass involved for pre-treatment of lignocellulosic biomass.

hemicellulose and lignin, which make intensity in the plant cell wall (Figure 2) [21-23].

### Fungal Pre-treatment of Lignocelluloses Biomass

The first step of bioethanol production is pre-treatment of biomass which is the most challenging step. It is considered as the critical step and has a large impact on digestibility of cellulose and it strongly affect downstream costs involving detoxification, enzyme loading, waste treatment demands [24]. In plant cell wall, hemicelluloses, and lignin secure cellulose. Hence, it reduces surface area available for enzymatic hydrolysis. Pre-treatment is required to alter the biomass particle size and structure as well as its sub-microscopic chemical composition and structure so that hydrolysis of the carbohydrate fraction to monomeric sugars can be achieved more rapidly and with greater yields. An ideal pre-treatment method should be contains many advantages like biomass size reduction, quick enzymatic hydrolysis with improved monosaccharide yields and limitation in inhibitor enzymes formation compounds and reduce energy requirements and low-cost demand [25,26]. Saccharification of lignocellulosic biomass without pretreatment can yield less than 20% of total sugars, while after pretreatment it can rise to 90% with different pre-treatment methods. The efficiency of pre-treatment depends on chemical composition, physical structure of the biomass and the treatment requirement [27]. Pre-treatment is probably the most energy intensive operation in biomass conversion to fuels or chemicals. From 1950s cellulose and hemicellulose hydrolysis have been investigated, with the fungus *Trichoderma reesei* providing as the ideal microorganism [28]. Table 1 depicts the influence of different biological pre-treatment methods effectively engaged in lignocellulosic biomass pre-treatment.

*Trichoderma viride* is a white rot fungi which can be used for digestion of lignin (56%) resulting in biomass enzymatic digestibility enhancement [29]. In a study, pre-treatment of wheat straw for five weeks by *Pleurotus ostreatus* (white rot fungi) has reduced lignin in the original wheat straw about 34%, but in the un-treated samples only 12% lignin reduction occurred [30]. It has shown by Taha et al. [7] that straw digestibility with fungal pre-treatment is more effective than bacteria pre-treatment. The outcome of this study exhibit that gene actions of fungal were two-fold more than those from bacteria.

Biological pretreatment of eucalyptus grandis saw dust degradation patterns and saccharification kinetics with white rot fungi was reported by [31]. In the saw dust fibers structural exchanges presented by treatment and after pre-treatment sugars reduction improve approximately twenty fold. The treatment with P. ostreatus and Pleurotus pulmonarius resulted in selective degradation of lignin which is evidenced by FTIR and microscopic analysis [31]. Many researchers have reported simultaneous pre-treatment and saccharification (SPS) using a cocktail of hydrolytic and oxidizing enzymes from fungal consortium. Lactase efficiently worked as a removing toxins agent. This is the first report on improvement of an eco-friendly SPS method. This process completely eliminates the use of hazardous chemicals [32]. Song et al. [33] has shown fungal pre-treatment effectively removed lignin and altered biomass structure for enhanced enzymatic hydrolysis in corn stover. There was 43.8% lignin removal after pre-treatment for 42 days with fungi. The saccharification efficiency was seven fold higher when compared to raw corn stover. Suhara et al. [12] reported selective lignin degrading basidiomycetes and biological pre-treatment of bamboo culms for bioethanol production. Fifty-one fungal identifies were acquired and they belong to white rot basidiomycete Punctularia sp. TUFC20056 and an unknown basidiomycete TUFC20057. They showed preferential lignin removal (50%) than Ceriporiopsis subvermispora FP90031 and Phanerochaete sordida YK624. Pretreatment with Punctularia sp. TUFC20056 improved hydrolysis efficiency.

#### Lignin Reduction by Microorganisms

Aerobic and anaerobic organisms produce carbon dioxide and glucose release, relatively. The most important group of microorganisms in cellulose reduction are *Basidiomycetes* (white- and brown-rot fungi), *Ascomycetes*, *Deuteromycetes* (soft-rot fungi), and anaerobic (rumen) fungi [34]. Lignin biodegradation because of its complex structure and macromolecular features is complicated. A few microorganisms are identified to reduce lignin partly, which decrease just the polysaccharide component [35]. Nevertheless, lignin virtually includes sugars, it is probably that these procedures rely on energy obtained from the sugars. It takes maximum time to achieve roughly 10% lignin degradation under 1,000 Daltons in molecular weight. For the hydrolysis of lignin oxygen is an essential principle [36].

Many different environmental factors influence lignin degradation. The environmental parameters motivate lignin degradation by increasing growth and metabolism of the fungi. Temperature, acidity, carbon and nitrogen sources are the major prominent parameters that affect fungal growth. In the fungal growth, combined nitrogen at the low and high contents perform an efficient function. Lignin degradation is optimum at low nitrogen level [37,38]. The hydrolyzation of cellulose by fungi is more harder than hemicelluloses hydrolyzation. White-rot fungi are one of the extremely skilled fungi in lignin reduction in nature [39].

#### White-rot fungi

White-rot fungi is the only organism that can decrease lignin faster than other organisms. Moreover, in nature the responsibility of white-rot fungi in lignin saccharification is high. *Basidiomycetes* are the important identified white-rot fungi. Under aerobic environment *P. chrysosporium* can reduce one gram of different separated lignins in two days. It influence in creation of about 70% CO<sub>2</sub> and 30% lowmolecular-weight water-soluble compounds [10]. The fungus uses lignin, hemicelluloses, and cellulose as substrate [40]. The lignin reduction happens at the end of primary growth by cooperation of other metabolism like nitrogen [8,40]. The oxidation reactions are involved in the fungal attack which decreases methoxy, phenolic, and aliphatic content of lignin. These reactions also cleave aromatic rings, and forms new carbonyl groups [40,41]. Selective type of degradations involves degradation of lignin and hemicellulose compared to cellulose. For example, *C. subvermispora, Dichomitus squalens, P. chrysosporium* follow selective decay mechanism. Similarly, simultaneous, or non-selective type of degradation is the type of digestion in which all the components of lignocellulose are decayed irrespectively (e.g. *Trametes versicolour* and *Fomes fomentarius*) [8,11]. White-rot fungi are identified to grow on woody and herbaceous plants. The most examined white-rot fungi for lignin reduction in a selective way are *P. chrysosporium* and *Phlebia* while in a non-selective way *Trametes versicolor* decrease lignin [10,11,41].

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At the high concentration, white-rot fungi can decrease pollution. In addition, since the lignin reduction system is non-particular and free white-rot fungi have an ability to decrease various contaminations [40]. For the cultivation of fungi cost-effective substrates and various liquid medias are being used. Fungi have special oxygenic radicle which has ability to oxidize to biomolecules of other organisms that cause the death of the particular microorganism. By altering medium pH other microbe cannot easily growth into the medium, because of preventing of fungus. Moreover, many different genes create by fungi which can convert lignin into water-soluble compounds [40,42].

#### Soft-rot fungi

Most of the soft-rot fungi have identified from Deuteromycotina or Ascomycotina. These fungi are very skilled to decrease lignin in woody plants more than herbaceous crops [11,43]. Soft rot fungi are degrading wood components very slowly as compared to white-rot and brown-rot fungi [10]. The best place for the growth of soft-rot fungi are compost, soil, piles of woodchips, straw [44]. Soft-rot fungi can reduce cellulose and hemicellulose of woody plants in a slowly way, whereas lignin reduction is somewhat slight [41,45]. The adaptation of soft-rot fungi in various temperature, different pH and limited oxygen is higher than other fungi. Figure 3 shows *in vitro* growth of lignin degrading fungi [46]. Soft rot fungi are no doubt the most efficient fungi to degrade lignin in mixed microbial populations [25,47].

#### **Brown-rot fungi**

Brown-rot fungi degrades cellulose and hemicellulose more faster than lignin. Moreover, Compare with other fungus and bacteria the way of digestibility of plant cell wall by brown-rot fungi is entirely different, because the reduction mechanism is non-enzymatic and lacks of *exoglucanases* [48]. Phenolic and non-phenolic de-methylation result in chemical alteration in lignin [49] which outcome of aromatic hydroxylation and ring splitting [50]. In the wood presence lignin de-methylation is operated by brown-rot [51]. Brown-rot fungi more effectively grown on herbaceous crops than woody plants [52]. Among the brown-rot fungi generally *Serpula lacrymans* and *Gloephyllum trabeum* can destruct the structure of woody plants without difficulty. The residues of brown-rot fungi is brown in colour which composed of changed lignin and also remains in the nature lacking any more hydrolysis [48,53].

#### Molds

Most of deuteromycetes and certain ascomycetes which are actually called Microfungi or molds, i.e. are usually thought to degrade mainly carbohydrates in soil, forest litter, and compost, can also degrade lignin in these environments. These molds are able to mineralize grass lignin up to 27%. Among the molds the *Penicillium chrysogenum*, *Fusarium* 



Figure 3: In vitro growth of lignin degrading fungi.

oxysporum, and *Fusarium solani* have been identified for their lignnolytic activities in forests. These molds or microfungi mineralized 27.4%, 23.5%, and 22.6% of a 14 C-labeled lignin prepared from milled wheat straw. However, lignin prepared from pine was much less degraded, and mineralization rate of less than 3% was obtained [54]. The degradation of lignin has been also being studied in the red mold of bread (*Chrysonilia sitophila*). These fungi caused 20% weight loss of pine wood in 3 months, with the losses of carbohydrate and lignin being 18% and 25%, respectively. Analysis of the decayed lignin suggested that oxidative Ca  $\pm$  Cb and b-O-aryl cleavages occurred during lignin degradation [8]. Recently another mold *Neurospora discrete* was found to degrade lignin in sugarcane bagasse and produced nearly 1.5 times the amount of lignin degradation products in submerged culture. Based on this data, *N. discrete* is recorded to have high lignin degrading capability than previously reported lignin degrading fungi [55].

#### Lignin-degrading enzymes from fungi

The structure of lignin mainly composed of phenolic and nonphenolic components. Lignin forms an integral part of secondary walls in plants and it plays an important role in enhancing the efficiency of water conduction in vascular plants. In the lignin, digestibly many various fungi and bacteria are proficient for genes production. These enzymes comprising of lignin peroxidases (e.g. lignin peroxidase (E.C. 1.11.1.7), and manganese peroxidase (E.C. 1.11.1.7)) and laccases as well. These are hemecontaining glycoprotein which requires hydrogen peroxide as oxidant. *Lignin peroxidase* degrades non-phenolic lignin units. *Manganese peroxidase* acts on phenolic and non-phenolic lignin units through lipid peroxidation reactions [16,56]. It oxidizes Mn<sup>2+</sup> to Mn<sup>3+</sup> which oxidizes phenol rings to phenoxy radicals leading to decomposition of compounds.

*P. chrysosporium, Ceriporiala cerata, Cyathus stercolerus, C. subvermispora, Pycnoporus cinnarbarinus,* and *Pleurotus ostreaus* produce enzymes which are involve in lignin degradation [14,57].

*Laccases* (E.C. 1.10.3.2.) are copper containing enzymes which are involved in lignin degradation. *Laccases* acts along with *lignin peroxidase* and *manganese peroxidase* leading to complete degradation of lignin. It catalyzes the oxidation of phenolic units in lignin and phenolic compounds and aromatic amines to radicals. The capability of laccase in lignocelluloses degradation is improved by phenolic components such as 3-hydroxyanthranilic acid, 3-ethylthiazoline-6sulfonate which will act as redox mediators. Without the role of redox mediators *laccases* have a limited effect [15,58].

# Molecular Methods and Enzymes Regulation Engaged in Fungal Pre-treatment

Molecular techniques can be employed to improve lignin degradation potential of fungi. Earlier studies revealed that expression of white rot fungal genes encoding lignolytic enzymes is differentially regulated at the transcriptional level based on the conditions used in biological pre-treatment. Expression of P. chrysosporium genes are strongly influenced by nitrogen and carbon limitation. Regulatory elements present in the promoter regions of genes encoding lignolytic enzymes play an important role in transcriptional activation. Transcription levels are collinear to enzyme activities in culture media [60]. Heterologous expression investigations exhibited that nearly in most case the features and activities of heterologous expressed enzymes are the same as that of native enzymes, yields obtained are too low. introduced It has shown that mnp cDNA of P. ostreatus in Coprinus cinereus combined the high MnP production of P. ostreatus and fast growth of C. cinereus resulting in higher lignin degradation after 16 days [61]. The effect of substrate on isozymes production using C. subvermispora and carbon and also nitrogen has an effective function in enzymes expression which are engaged in the degradation of lignin [62].

#### **Factors Influencing Biological Pre-treatment**

Biological pre-treatment is not only involved in generating any inhibitors and environmentally friendly methods, but also, it's a quietly time consuming method. To enhance it by choosing the most efficient strain and culture conditions can make the method more effective by decreasing the treatment time and carbohydrate loss [63]. Important process factors affecting biological pre-treatment comprise the nature, component of biomass, and other factors such as variety of organisms engaged in incubation time and temperature, acidity (pH), inoculums concentration, moisture content and aeration rate [64].

The optimum temperature during biological pre-treatment varies with the type of microorganism employed. Most of the white rot *ascomycetes* fungi grow optimally around 39°C while the white rot basidiomycetes grow optimally around 25°C and 30°C. The metabolism of these fungi generates heat and develops temperature gradients in solid state media. The accumulated heat can destroy or inhibit fungal growth and metabolism. Various optimal temperature for biological pre-treatment of biomass is because of fungal physiology, fungal strain and type of substrate [64]. Incubation time requested for microorganisms pre-treatment differs depending on the strain and component of the biomass utilized for pre-treatment. Long incubation time due to low delignification rate is one of the major barriers for large scale application of biological pre-treatment [65].

Acidity (pH) plays a prominent function in the cultivation of fungi and it is very complicated to control it in a solid culture condition. Production of lignolytic gene is influenced by the initial pH of the medium. In the pH of 4 to 5 most of the white rot fungi can grow

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Other enzymes like *aryl alcohol dehydrogenase*, *cellobiose*, *aromatic acid reductase*, *vanillate hydroxylase*, *dioxygenase* and *catalase* are regarded to contribute an important function in lignin reduction. The number of research investigations on the soft-rot fungi enzyme system and their degradation of lignin is low. *Peroxidases* and *laccase* provide by *F. oxysporum*, *Xylaria* sp., and *Altenaria* sp., *Botrytis cinerea*, *Myceliophthora thermophila*, *Chaetomium thermophilium* and *Paecilomyces farinosus*. Although, laccase provide by soft-rot fungi in a low proportion compared to white-rot fungi [59].

J Plant Pathol Microbiol, an open access journal ISSN: 2157-7471

properly and also the substrate acidity decrease their growth [66]. Inoculum concentration performs a significant function in biological pre-treatment. The time required for the colonization of the substrate is affected by the type and amount of inoculums. Spores are the commonly used inoculum. Larger quantity of inoculum leads to shorter time for colonization of the substrate [63].

High substrate concentrations have to be used for biological pretreatment to make the process economically viable. Generation of inhibitor compounds increase by using high dry material that may unfavourably influence sugar yield reduction. Hence pre-treatment to be carried out with a compromised condition to minimize the generation as well as accumulation of inhibitory compounds. Initial moisture content is essential for the establishment of microbial growth in the biomass. Initial moisture content critically affects the fungal growth and enzyme production and significantly affects lignin degradation [63]. The production and pH of lignolytic enzymes mainly affected by aeration. Aeration has many functions which are including oxygenation, CO<sub>2</sub> removal, heat dispersion, humidity conservation and also dispersal of volatile combinations produced during metabolism. Since lignin degradation is an oxidative process, oxygen availability is important for ligninase activity of white rot fungi. High aeration could improve delignification rate and hence controlled aeration is essential for improvement of biological pre-treatment. Efficiency of manganese peroxidase is not considerably influenced by aeration [67].

#### Conclusion

Bioethanol production from lignocellulosic biomass serves as an alternative source of renewable energy. Fine tuning of pre-treatment technologies for different biomass types and development of an economically viable process are still needed. In biological pretreatment for the degradation of lignin the most used microorganisms are brown-, white- and soft-rot fungi. Using white rot fungi that can decrease lignin seems favourable since they consume less environmental damage and less energy conception. Biological pre-treatment has several advantages over conventional chemical/physical pre-treatment strategies, several challenges need to be addressed before implementing at the commercial scale. To address these drawbacks consequential study and improved schemes are required for decreasing the pre-treatment costs and enzymatic hydrolysis procedures, reactor formation to reduce heat production during biological pre-treatment and determination of effective lignin hydrolyzing microbes by using improved molecular systems.

#### Acknowledgments

The assistance of our friends for their valuable technical and practical assistance is gratefully Acknowledged.

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