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6.45 Application of White-Rot Fungi in Transformation, Detoxification, or Revalorization of Agriculture Wastes: Role of Laccase in the Processes

M Jurado, ÀT Martinèz, and MJ Martinez, Centro de Investigaciones Biológicas, Madrid, Spain
MCN Saparrat, Universidad Nacional de La Plata (UNLP)–CCT-La Plata–Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), La Plata, Argentina

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Glossary

bioaugmentation Strategy leading to the increase in the activity of degrading microorganisms to enhance the performance of bioremediation.
bioremediation Process for removing environmental pollutants through the use of microorganisms (bacteria and/or fungi and their enzymes) or plants, and thus, restoring the original natural surroundings.
biostimulation Strategy for degrading environmental pollutants through the addition of nutrients or substrates to stimulate organisms that can perform bioremediation.
mineralization Process of complete degradation of organic compounds into CO_2 and H_2O, as a result of decomposition.
refiner Equipment used in paper production to improve the physical properties of pulp through a mechanical treatment of fibers.
Solid-state fermentation The growth of microorganisms on solid materials (usually lignocellulosic wastes), which have the property to absorb or contain water, in the absence of free-flowing water.

6.45.1 Introduction

Lignocellulose is a major constituent of the cell wall of vascular plants and contains most of the carbon in the planet, lignin being the barrier that protects cellulose and hemicellulose from microbial attack. In addition, lignin waterproofs the cell wall and is the principal component responsible for strengthening tissue pattern and cross-linking as well as providing the structural support necessary to transport water, minerals, and photosynthetic products through the plant vascular system.

Lignin structure is made up of a complex three-dimensional bulky network, the result of reactions between three *p*-hydroxycinnamyl alcohols, *p*-coumaryl, coniferyl, and sinapyl alcohols, and their acylated forms [16]. A dehydrogenative process, initiated by oxidative enzymes, such as peroxidases and/or laccases, leads to the formation of phenoxy radicals resulting in a heterogeneous and recalcitrant polymer, which may be different depending not only on the type of vascular plant but also on the type of tissue and cell-wall layer within the same plant. Lignin has gained increasing attention during recent years, in parallel with global population growth and industrial development in the world, as its degradation is the key factor in the industrial application of plant biomass [16].

Although, in nature, an enormous quantity of lignocellulose material exists, only a small fraction has been given any economic value, that is, in agriculture or in the paper and textile industries, but most is still managed as waste. Promoting the principle of

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sustainable and environmentally friendly development, lignocellulose could be transformed through tailored biotechnological processes into value-added natural products for animal feed, or for use in the pharmaceutical, chemical, and biofuel industries.

Different microorganisms, including bacteria and fungi, are involved in lignin degradation but only white-rot basidiomycetes have developed strategies to depolymerize and mineralize efficiently this recalcitrant heteropolymer. These organisms have a nonspecific system, including oxidoreductases, low-molecular-mass metabolites, and dehydrogenases, plus activated oxygen species whose concomitant action contributes toward effectively removing the lignin barrier, thus increasing the accessibility of plant carbohydrates. The extracellular oxidoreductases from white-rot fungi, frequently referred to as lignin-modifying enzymes, play an essential role in the process. For more information regarding their occurrence, classification, and targeted use in site bioremediation as well as in the treatment of man-made pollutants, the reader may also consult Chapter 6.16. These oxidoreductases include laccases and peroxidases that catalyze the one-electron oxidation of lignin units subsequently progressing through nonenzymatic reactions and leading to the formation of bond cleavage products. Ligninolytic peroxidases consist of three different kinds of enzymes: lignin peroxidase (LiP, E.C.1.11.1.14), which are able to oxidize directly nonphenolic lignin units; manganese peroxidases (MnP, E.C.1.11.1.13) that act preferentially on phenolic units through the oxidation of Mn^{2+} to Mn^{3+}; and versatile peroxidases (VP, E.C.1.11.1.16), which share catalytic properties with LiP and MnP. The H_2O_2 required for the catalytic activity of peroxidases is provided by extracellular oxidases, such as glyoxal (GLOX, E.C.1.2.3.5) and aryl-alcohol oxidases (AAO, E.C.1.1.3.7), but H_2O_2 also participates on its own in lignocellulose degradation, as it is the precursor of the hydroxyl radical, the strongest oxidizing agent produced by fungi through the iron-catalyzed Haber–Weiss reaction.

On the other hand, laccases (EC 1.10.3.2) catalyze the oxidation of a broad range of phenolic compounds, such as polyphenols and methoxy-substituted phenols, as well as aromatic amines. These enzymes belong to the family of blue multicopper oxidases, which contain four catalytic copper atoms in their molecular structure (**Figure 1**) [18].

Although laccases have lower redox potentials than LiP and VP, the interest in these enzymes was strongly increased after discovering that they can oxidize nonphenolic aromatic compounds in the presence of low-molecular-mass compounds, which act as redox mediators. Moreover, laccases have an advantage compared to ligninolytic peroxidases, as they use oxygen, a nonlimited electron acceptor, for their catalytic activity, which makes them more suitable for industrial and environmental purposes.

The application of white-rot fungi and their enzymes in biotechnology is not only restricted to the transformation or disruption of the lignin barrier from plant material, but also used for the transformation of other molecules. Several organic compounds not readily biodegradable, which persist and bioaccumulate up to toxic levels through trophic chains, are released into the environment as components of emissions of the incomplete combustion of wood and petroleum or generated in wastes and wastewater from agro-industrial activities. Most of these compounds, environmentally undesirable due to their estrogenic, mutagenic, and/or carcinogenic properties on the biota, are structurally related to lignin; hence, the same nonspecific system secreted by white-rot basidiomycetes involved in lignin degradation can participate in the oxidation of these pollutants (for specific details *see also* Chapter 6.16).

The accumulation of huge amounts of lignocellulosic agro-industrial residues from human activity may cause environmental problems. The use of white-rot fungi and their enzymes is being considered as an attractive process for treating these agro-industrial residues, which can either be converted into value-added byproducts as animal food or bioethanol, or be used to produce active primary or secondary metabolites, such as enzymes, antioxidants, antibiotics, flavors, and alkaloids.

In this chapter, we discuss recent progress that has been made in employing white-rot basidiomycetes in biotechnological applications for transforming agricultural wastes, as well as in the degradation of recalcitrant compounds causing environmental problems, focusing on the role of laccases in the processes of revalorization, bioremediation, and detoxification of wastes and wastewater.

6.45.2 Fungal Transformation of Hazardous Organic Compounds in the Bioremediation of Polluted Soils and Industrial Wastewaters

The use of microorganisms and plants, as well as their enzymatic systems, to detoxify soils and waters constitutes the strategy called bioremediation. Microbial bioremediation is therefore associated with the action of microorganisms, such as bacteria, yeasts, or filamentous fungi, which can be either naturally occurring or deliberately introduced into a polluted site. These organisms can detoxify the environment, consuming and breaking down pollutants until their mineralization (complete transformation into CO_2 and H_2O) or conversion into harmless byproducts such as simpler organic compounds and immobilizing them into soil humic substances. Whereas biostimulation involves the mere addition or supplementation of limiting nutrients and environmental modification to stimulate indigenous micro-degraders in a polluted system/site, bioaugmentation consists of inoculating efficient degrader microorganisms to facilitate transformation, which can be carried out both on site and off site. Therefore, naturally occurring organisms with an ability to degrade specific substances are isolated, cloned, and further manufactured as formulations in large quantities to be introduced into hazardous waste sites to eliminate specific contaminants. In the case of white-rot fungi, it is expected that by encompassing the degradation of available lignocellulose, from which the fungus obtains its source of carbon for mycelial growth, contaminated soil could be detoxified via its ligninolytic enzymatic complex. However, successful bioremediation of ecosystems depends on the availability of appropriate fungi, their degradative performance and ecology, as well as their ability to integrate with compatible environmental conditions that favor the cleanup process with low impact on native biota. We will now discuss recent advances made in the application of white-rot fungi and their laccases on bioremediation processes.

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6.45.2.1 White-Rot Fungi as Potential Tools in Bioaugmentation Strategies

White-rot fungi are promising bioremediation agents because of their ability to degrade or transform a large variety of aromatic pollutants that can reach soil and water, thus reducing their toxicity. These pollutants include: (1) the BTEX group, an acronym that stands for benzene, toluene, ethylbenzene and xylenes, all of them representing a significant percentage of petroleum products; (2) chlorophenols, including any derivatives of phenol where one or more hydrogen atoms have been replaced by chlorine atoms, which are frequently used as pesticides; (3) polycyclic aromatic hydrocarbons (PAHs), which originate from the partial combustion of organic material with aromatic rings; (4) polychlorinated biphenyls (PCBs), consisting of two benzene rings where chlorine takes the place of two or more hydrogen atoms, massively used in electrical installations until these compounds were banned; and (5) dyes with different chromophore groups such as anthraquinonic, azo, heterocyclic, and others (e.g., phthalo-cyanine and triphenylmethane).

White-rot fungi can tolerate a broad range of environmental conditions, involving nutrients, pH, and moisture content, and, more importantly, they can use lignocellulose for growth, making them suitable for inoculation into contaminated soils. In addition, white-rot fungi can exert a positive effect on the growth of other autochthonous microorganisms, improving the porosity and water-holding capacities of soil and making the total degradation of recalcitrant pollutants easier.

The biotransformation of pollutants by white-rot fungi involves several types of processes initiated either by ligninolytic enzymes or mycelial-bound redox systems, that generate products such as free radicals, which can then either undergo another enzyme-catalyzed oxidation, or other nonenzymatic transformations via the process of enzymatic combustion. However, until complete pollutant mineralization, the presence of some compounds with different structure and toxicity makes the use of different tests necessary in order to ensure low ecotoxicity and long-term stability of the products.

The first studies on pollutant degradation by white-rot fungi were carried out with *Phanerochaete chrysosporium*, an organism capable of degrading a broad spectrum of pollutants. As this well-studied fungus often demands specific conditions for growth and expression of its ligninolytic system, more white-rot fungi species have been screened for their ability to transform and detoxify soil pollutants under different environmental conditions. Several *Pleurotus* species, including *P. eryngii*, *P. ostreatus*, *P. pulmonarius*, and *P. sajor-caju*, showed themselves to be highly effective at degrading aromatic pollutants such as 2,4-dichorophenol, benzo (*a*) pyrene and chlorinated biphenyls in either submerged cultures or under solid-state fermentation (SSF) conditions [19]. Most of the white-rot basidiomycetes, including different species from *Bjerkandera*, *Coriolopsis*, *Phlebia*, and *Trametes* are able to transform the pollutants, although their degradation capability greatly depends on environmental conditions. Thus, the particular conditions leading to detoxification should be analyzed in each case, paying special attention to nitrogen and carbon contents, as well as to supplementation with the cofactor copper and other inducers, which can greatly enhance laccase production. Therefore, although the use of these microorganisms seems very promising, further research is still needed to achieve maximum efficiency in the fungal removal of pollutants and move our experimental work from artificially contaminated soils to contaminated soils present in the environment due to industrial activities.

6.45.2.2 Laccases as Green Agents in the Transformation of Pollutants

Toxic, carcinogenic, and mutagenic compounds as well as endocrine-disrupting chemicals released by industries in wastewater, or which have been released from conventional water-treatment processes, might be transformed and detoxified by laccases. The use of laccases overcomes some of the deficiencies present in chemical treatment processes which might have a negative ecological impact, and may also be preferred on some occasions over biological treatment with whole fungal cells (fungal cultures), where long time periods are involved. Several fungal laccases, mainly from white-rot fungi, are currently being studied for their ability to transform and degrade different pollutants causing environmental damage. These enzymes might be used to develop treatments for hazardous wastes in bioreactors or in polluted areas, as they catalyze the direct oxidation of chlorophenols, anthraquinone and phenolic azo dyes, hydroxylated biarylic compounds such as 2-hydroxydibenzofuran, and, to a certain extent, some PAHs. Moreover, in the presence of a low-molecular-mass mediator, the so-called laccase-mediator system (LMS), could be a useful tool for detoxifying recalcitrant compounds in wastes. These mediators should be nontoxic, inexpensive, and highly efficient. Despite its importance, the mechanism of pollutant transformation by laccases using synthetic redox mediators, such as ABTS (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)) and HBT (1-hydroxybenzotriazole) among others, is still being studied due to the high cost and potential toxicity of these compounds. Many recent papers have been dedicated to the search for new laccase enhancer mediators. Phenolic aldehydes, ketones, acids, and esters derived from natural degradation of the three monomer units in lignin were among the best natural mediators to decolorize different types of dyes attaining similar decolorization efficiency than that obtained with synthetic mediators [5]. In this respect, the future looks promising since different environmentally friendly compounds, such as naturally occurring substituted phenols present in soil, have been recently described as efficient mediators for oxidative transformation of azo and indigo dyes, halogenated pesticides, and other PAHs, which cannot be oxidized by laccases on their own [5, 6].

The action of laccases upon pollutants occurs through two main degradation pathways or coupling reactions. The main reactions involved in laccase-induced degradation include depolymerization, demethoxylation, decarboxylation, and ring opening [10,16,19]. Many of these reactions generate active oxygen species, and these free radicals continue the oxidation to more simple compounds, which could show lower toxicity than their parent. However, other pollutants are detoxified by coupling reactions of free radicals generated in the reactions that render compounds unavailable to the organisms either by precipitation or by their immobilization in the humic matrix of the soil or river sediments, which slowly can be mineralized to carbon dioxide by these fungi and/or other soil microorganisms. Therefore, it is essential to make a precise analysis of toxicity and stability of coupling reaction products for each recalcitrant compounds and compare them with the original compounds.

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The use of laccases could be enhanced by enzyme immobilization as this process usually increases pH and temperature stability and allows the reuse of the biocatalyst. Several processes have been developed to immobilize the enzyme on solid supports for the treatment of effluents with phenolic compounds, polycyclic aromatics and endocrine-disrupting chemicals with aromatic structure. In these treatments, both water consumption and effluent toxicity could be reduced by means of immobilized laccases, in the presence or absence of redox mediators, showing promising results for the removal of some pollutants from different industrial effluents at laboratory scale. However, further studies will be necessary to know the transformation pathways involved in the degradation of the different pollutants, especially those from endocrine-disrupting chemicals where the nature of the chemicals produced has to be elucidated [3] .

6.45.3 Application of White-Rot Fungi and Laccases in the Pulp and Paper Industry

The principal nonfood industrial utilization of plant biomass is in the production of pulp and paper. During its production, it is necessary to separate the cellulose fibers from lignin and this is done by employing mechanical or chemical methods. In mechanical pulping, the fibers are separated by stone grinding and refiner and the pulps are usually bleached by using H_2O_2.

In chemical pulping, lignin is solubilized by chemicals resulting in an oxidized brown residual material after cooking, which must be eliminated to manufacture white paper. In these pulps, elemental chlorine has been used for a long time in the bleaching stage, but currently totally chlorine-free (TCF) sequences are being developed, although they are less efficient in achieving a high degree of brightness due to the lower delignification power of oxygen and hydrogen peroxide compared to chlorine reagents.

White-rot basidiomycetes have been considered as potentially useful agents for biological pulping, because they not only reduce energy consumption in the process, especially in mechanical pulps, but can also contribute to reducing chemicals, thus minimizing the environmental impact of traditional pulping processes. The efficiency of fungal pretreatment using different lignocellulosic material has been reported and several patents have been issued, the one proposing the use of *Ceriporiopsis subvermispora* being the most optimized [11]. However, engineering the process, together with the long period required by fungi to be effective, makes its exploitation difficult.

On the other hand, the use of TCF bleaching sequences in kraft pulps in the substitution of sequences using chlorine has significantly increased lipophilic deposits (named pitch), because these compounds, present in wood extractives, survive the kraft cooking and bleaching [11]. Wood pretreatment with white-rot basidiomycetes, on the other hand, is effective in preventing pitch problems as these fungi degrade efficiently triglycerides as well as free and esterified sterols involved in pitch deposition. Some basidiomycetes could therefore be considered because of both their biopulping and biodepitching effects. However, the same problem regarding the exploitation of this technology exists and, for this reason, most interest has been focused on the use of ligninolytic enzymes, such as laccases and peroxidases, as substitutes for chlorinated reagents used in paper pulp bleaching or biodepitching processes, as more environmentally friendly alternatives.

6.45.3.1 Use of Laccase in Bleaching Processes

Since discovering how laccase acts on nonphenolic lignin in the presence of ABTS, as a low-molecular-mass redox mediator, the interest of this enzyme for biotechnological applications has been increasing. In fact, laccase is being studied to delignify paper pulp by using synthetic redox mediators such as ABTS, HBT, or violuric acid, among others. Many studies have confirmed the efficiency of the so-called LMS for bleaching different kinds of pulps, even in integrative industrial-type sequences (**Figures 1 and 2**) [13]. However, there is a need to resolve the high cost of the different synthetic mediators studied, together with their possible negative environmental impact in some cases, before their possible industrial implementation.

The first metabolite reported as a laccase natural mediator was the 3-hydroxyanthranilic acid secreted by *Pycnoporus cinnabarinus*, a fungus producing laccase only as a ligninolytic enzyme [8]. Since then, interest in phenolic natural mediators derived from lignin for delignification has increased, especially for applications in the paper pulp industry. In this sense, it has been recently reported that both acetosyringone and syringaldehyde are efficient natural laccase mediators for delignifying and bleaching eucalypt paper pulp without altering significantly pulp properties [4]. Although the pulp treatment conditions must be optimized, these phenolic compounds are abundant in black liquors of eucalypt kraft pulping and could be potentially cost-effective as natural mediators for environmentally friendly bleaching processes.

An alternative solution for delignifying pulp using laccase might be through a laccase/xylanase system (LXS). This enzyme-assisted system is highly effective at delignifying pine kraft pulp with good selectivity as an LMS [23]. Therefore, LXS should be analyzed in more detail as an effective option for reducing the use of expensive chemicals in the paper and pulp industry, in such a way that its application could contribute to an economically sustainable technology that is environmentally friendly.

6.45.3.2 Other Potential Uses of Laccases

Laccases have also been described as potential eco-friendly biocatalysts for functionalizing cellulose fiber. It has been reported that laccase treatment of coupled phenolic amines in cellulose leads to the successive radical formation of these compounds which polymerize, thus improving adhesion of the fibers and their resistance. LMS could also be used to improve the intrinsic properties of pulp or introduce new ones such as antimicrobial, antioxidant, or optical properties [1].

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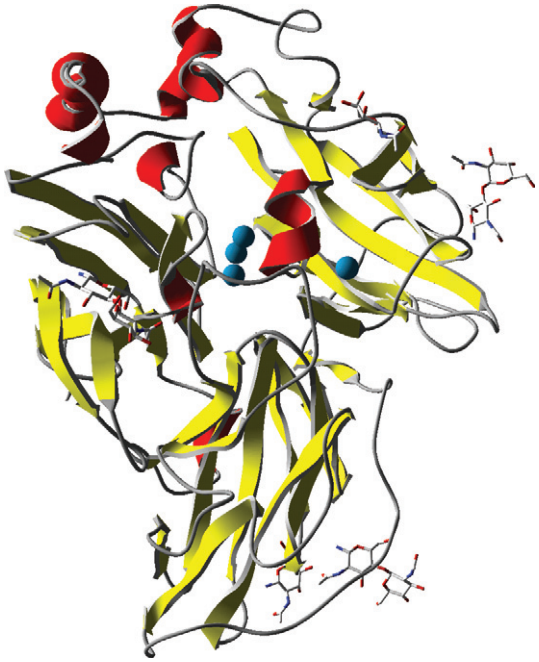

Figure 1 Ribbon diagram of *Trametes versicolor* laccase showing the catalytic coppers (as blue spheres), some carbohydrate moieties (coloured bars) and three catalytic domains described in the crystallized protein formed by several β-strands (yellow) and some short helices (red) (PDB model, entry code 1GYC).

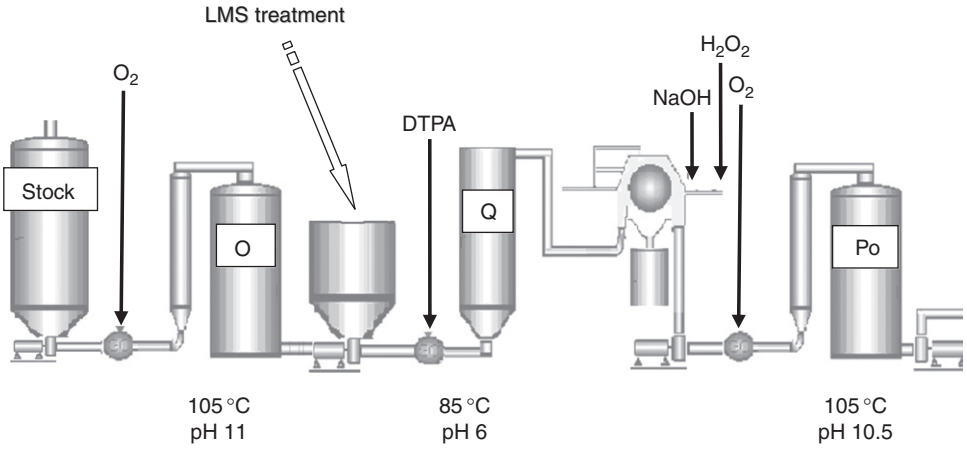

Figure 2 Scheme of industrial manufacturing of *Eucalyptus glubulus* pulp showing the standard TCF sequence: O–O–Q–PoP (O, oxygen stage; Q, quelation stage using DTPA (diethylenetriaminopentaacetic acid); PoP, alkaline peroxide stage. Adapted from Ibarra D, Camarero S, Romero J, *et al.* (2006) Integrating laccase-mediator treatment into an industrial-type sequence for totally chlorine free bleaching eucalypt kraft pulp. *Journal of Chemical Technology and Biotechnology* 81: 1159–1165.

On the other hand, although there are different patents on the use of lipases and sterol esterases to prevent pitch deposition during softwood or hardwood pulping [11], it has been reported that LMSs are also effective at removing problematic lipophilic compounds involved in pitch deposition [16]. The efficiency and selectivity of the LMS makes its use attractive in both biobleaching and biodepitching processes, and this has led to an increasing interest in the study of new laccases and mediators as a serious proposal in the pulp and paper industry.

6.45.4 Revalorization of byproducts from Agriculture

Agriculture has expanded and intensified worldwide over the past century in order to feed a growing global population. This has contributed not only to an increase in waste production but also to the appearance of new types of pollutants. Some of the waste generated by agro-industrial activities includes lignocellulosic residues from both herbaceous and woody biomass that can be

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recycled for microbial production of enzymes such as laccases for future use in other industrial processes. Although fungal cultivation on lignocellulose can be performed on submerged cultures, SSF cultures are now being considered as an attractive alternative for treating solid agro-industrial residues. This is because the conversion of these residues into value-added byproducts such as animal food or their use in the production of enzymes and active secondary metabolites, such as antioxidants, antibiotics, or flavors, is now considered economically feasible.

In other cases, lignocellulosic residues are byproducts derived from crop manipulation, whose elimination can be problematic because of the presence of toxic compounds formed during industrial processing, which may have a negative impact on ecosystems. In the next section, we will consider the case of residues generated by the olive-oil industry whose proper handling and disposal can reduce environmental pollution, while their transformation may generate an added-value product that could be used for agro-industrial purposes.

6.45.4.1 Bioconversion of Olive-Oil Solid Wastes

Olive-oil production generates substantial quantities of effluents and solid byproducts with very high organic load, recalcitrant in nature. Several physicochemical, biological, and combined processes, including advanced oxidation processing, are being considered for the treatment of olive-mill wastewater. Both liquid and solid wastes are deemed to be toxic for plants and microorganisms, causing serious pollution problems in both soil and water [10]. The proportion and composition of solid/liquid fractions depends on the process used to extract the oil, which includes pressing, and decanting in either two or three phases. The two-phase extraction of oil generates a semisolid byproduct called alpeorujo, which is then subjected to *n*-hexane extraction to recover residual oil, generating a dry solid residue. Many approaches have been explored to detoxify this kind of residue, including thermal processes, electrolysis, ozonation, and evaporation among others, because of its potential as fertilizer or amendment due to its high organic content and mineral nutrients. Bioremediation using white-rot fungi has gained importance in recent years, because ligninolytic enzymes may transform the phenolic fraction, which is considered to be the principal toxic constituent. Laccases have been shown to be useful for decolorizing and degrading aromatic compounds from olive-oil mill wastewater, and they can be easily applied [10]. However, in the case of solid byproducts, it is necessary to inoculate the fungus, as an SSF process, which complicates the process. Although the role played by ligninolytic enzymes during the detoxification process has been questioned, it has recently been demonstrated that laccase action is an important mechanism involved in the reduction of toxicity in this kind of industrial waste as well as benefiting *Azospirillum brasilense*, a soil bacterium which stimulates plant potential by nitrogen fixation [20]. These results suggest that laccase secreted by basidiomycetous fungi could assist plant-promoting bacteria, contribute to the revalorization of olive-mill wastes as fertilizer and have considerable impact on integrative agricultural systems.

6.45.4.2 Production of Laccases by Fungi on Agriculture Residues

In order to feasibly apply fungal laccases in various technological areas, high levels of the enzyme must be available. Many reports have focused on convenient, reliable production systems that include inexpensive, optimized media for large-scale production. A promising alternative is the growth of laccase-producing fungi using agricultural residues, as they are abundant and readily available, provide the needed nutrients for fungal growth, and as the presence of lignin acts as an inducer of ligninolytic enzymes [7]. Although fungi can be grown on lignocellulose in submerged cultures, SSF conditions are usually preferred as it has been well established that this method provides higher enzyme stability and enzyme yields [7].

However, despite the numerous advantages of using lignocellulose for laccase production under SSF conditions, some limitations exist, as in the case of alpeorujo to scale laccase production. Although it has shown potential as a growth supporter and laccase inducer, its direct use is limited by certain structural features, such as porosity and particle size, which determine compaction and aeration processes, as well as its inhibitory effect on fungal growth and activity in the presence of whole nondiluted wastes. This illustrates that, in some cases, a chemical or mechanical pretreatment of the substrate is required in order to modify particle size, increase accessibility to polysaccharides, or reduce the presence of inhibitors. Although the use of lignocellulose for enzyme production seems to be very suitable, new designs for bioreactors operating under SSF conditions should be developed in the future, as optimal regulation and control of parameters, such as aeration, moisture, and agitation, could lead to more efficient processes.

6.45.5 The Role of White-Rot Fungi and Their Enzymes on Second-Generation Bioethanol

There is a growing need to find sustainable alternatives to avoid further increases in global warming due to the greenhouse effect. The utilization of renewable resources for ethanol production has been given much attention in a drive to find a substitute for petrochemical fuels. Bioethanol is produced by the alcoholic fermentation of sugar or starch-based crops such as maize, wheat, sugarcane, or sugarbeet, among others. In recent years, increasing interest has been shown in second-generation bioethanol produced from lignocellulosic materials in agricultural and forestry waste. The main advantages in favor of using second-generation bioethanol is that the raw materials are sufficiently abundant and do not compete with food chains.

The process to obtain ethanol from lignocellulose involves the enzymatic hydrolysis of cellulose (**Figure 3**), and, in some cases, hemicellulose, into fermentable monomers. However, the hydrolysis of these polysaccharides is hampered by the presence of lignin and the compact architecture of the cell wall, which makes their degradation to fermentable sugars with hydrolytic enzymes much more difficult than that for starch.

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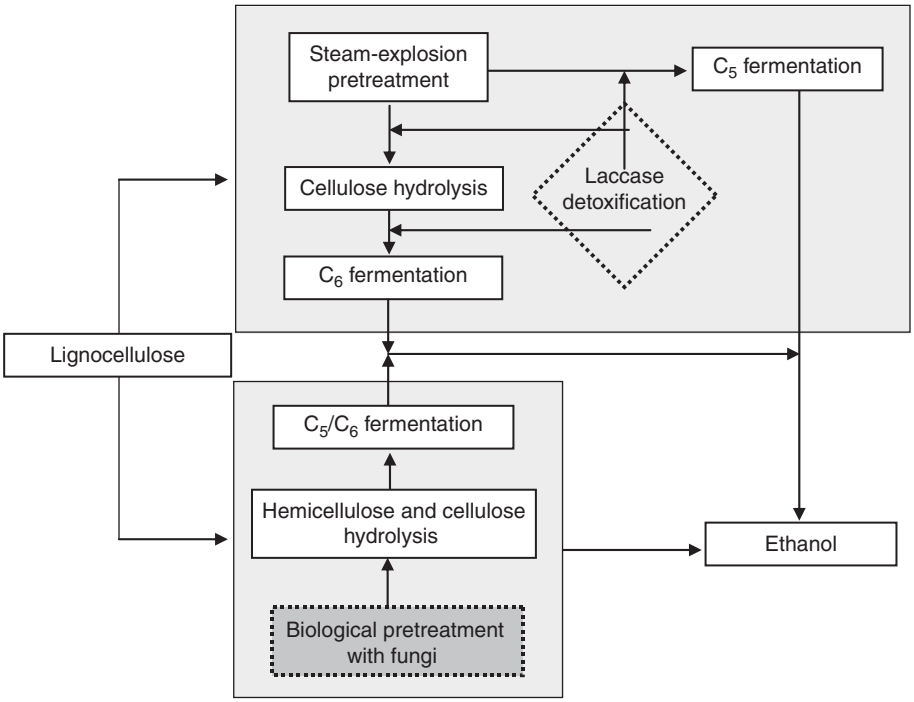

Figure 3 Flowchart of the production of bioethanol from lignocellulose, showing the integration possibilities of biological pretreatment with fungi or laccase treatment for steam-exploded lignocellulose material detoxification.

Therefore, a pretreatment of this material is necessary to remove or alter the lignin in order to improve the rate of enzymatic hydrolysis of the sugar fraction. White-rot fungi have been studied as an alternative biological pretreatment for degrading the lignin barrier. Moreover, ligninolytic enzymes from white-rot basidiomycetes have been employed for reducing the free phenol content released after physicochemical pretreatment, which might have a negative impact on both enzymatic hydrolysis of sugars and fermentation by microorganisms [9, 12].

6.45.5.1 Biological Pretreatment of Lignocellulose for Ethanol Production

The biological pretreatment of lignocellulose is an environmentally friendly alternative to other chemical and physical methods, which are expensive, pollute the environment, and generate a number of inhibitors, including acetic acid, furfural or hydroxymethyl-furfurals, and free phenols, which remain embedded in the biomass and can affect downstream processes. The biological pretreatment basically consists of an SSF process in which microbes grow on the lignocellulosic biomass, preferentially degrading lignin and increasing the accessibility of cellulose and hemicellulose.

White-rot basidiomycetes are the most effective organisms for the biological pretreatment of lignocellulosic materials, as previously commented. The studies with these fungi for biopulping and animal feed applications have created the basis for lignocellulose biomass use in other biotechnological applications, as in the case of second-generation bioethanol production. The drawback in this case, as in the case of biopulping processes, is the long period required by fungi to delignify cell walls and make the cellulose and hemicellulose accessible to the hydrolytic enzymes. It is worth noting that levels of free phenolic compounds after fungal pretreatment of lignocellulose decrease due to either their polymerization or their mineralization [19]. As phenolic compounds present in lignocellulose material, which has been pretreated using physicochemical methods, can inhibit fermentation by yeasts, biological pretreatment would do away with the need for a detoxification step, as is the case with steam explosion treatment. Although further research is needed to shorten incubation time and increase lignocellulose digestibility in order to be competitive with other types of pretreatment, the economic and environmental advantages of biological pretreatment with fungi is currently attracting much attention with a view to improving and optimizing the results.

6.45.5.2 Enzymatic Detoxification of Steam-Exploded Lignocellulose

Steam explosion is one of the most commonly used methods for lignocellulose pretreatment, as it partially degrades and solubilizes lignin and hemicellulose due to the high pressure and temperature conditions used in the process. However, as mentioned previously, during this process, several compounds are generated, which adversely affect ethanol production. Much effort has been devoted analyzing the conditions during the steam-explosion pretreatment with the aim of increasing sugar yield after enzymatic hydrolysis, but, at the same time, minimizing the formation of inhibitory compounds [21, 22]. In general, higher temperatures and the use of diluted sulfuric acid during pretreatment increase cellulose accessibility, as well as the concentration of

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fermentation inhibitors. The use of sulfuric acid is particularly important, as it permits an almost complete solubilization of the hemicellulose fraction, which may constitute as much as 35% of the dry matter of lignocellulosic raw materials. The fermentation of this fraction is essential to obtain a positive balance from the process, but it is currently being investigated because, although, selected yeasts have been reported to ferment pentose in synthetic media, they cannot grow on steam-exploded wheat straw.

Phenolic compounds are one of the main groups of inhibitors formed during the steam explosion of lignocellulose material. The removal of free phenols present in steam-exploded wheat straw by laccase polymerization reduced the toxic effect on *Saccharomyces cerevisiae*, resulting in higher yeast growth and improved ethanol production [14]. The inhibition of yeast growth is based on a decrease in the ability of the membrane to serve as a selective barrier, and is caused by phenols, which reduce both cell growth and sugar assimilation. After laccase treatment, the yield of bioethanol was greatly improved in exploded wheat straw under both soft conditions (water) and harsh conditions (acids), thus confirming the negative effect that free phenolic compounds have on the process yield [2]. The benefits derived from treatment with ligninolytic enzymes have been demonstrated in several steam-exploded lignocellulosic biomass materials such as wood, sugarcane bagasse, or wheat straw. However, the impact on the ethanol yield seems to depend to a large degree not only on the kind of raw materials used, but also on the severity of conditions during pretreatment, which greatly affect the inhibitors generated. This has been studied particularly in the case of wheat straw, where the increase in ethanol yield obtained for samples pretreated under mild conditions after laccase treatment was higher than that obtained after the same treatment on samples pretreated under more severe conditions [14]. In this case, the higher concentration of nonphenolic inhibitors in samples pretreated under harsher conditions reduced the effect of the treatment with oxidoreductases.

Although laccases seem to be very effective at detoxifying phenolic inhibitors, if we take into account the costs, it would be better if a separate detoxification step was unnecessary. A promising alternative would be the use of genetically engineered yeasts for active laccase production as a host for heterologous enzyme expression. A *S. cerevisiae* strain expressing a laccase enzyme from *T. versicolor* has already been shown to reduce coniferyl aldehyde concentrations, resulting in higher growth and increased ethanol production [15]. The development of new, modified yeast strains in the future will provide higher fermentation efficiency.

6.45.6 Concluding Remarks

In a world with uncontrolled industrial development and population growth, further efforts are needed to ensure that the planet is environmentally sustainable. Over recent decades, significant progress has been made in understanding lignocellulose degradation by white-rot basidiomycetous fungi and the enormous potential that these organisms and their enzymes possess, if applied in environmentally sustainable green and white biotechnology processes.

These fungi, especially those able to degrade, preferentially, lignin compared to cellulose, provide the possibility of efficiently reutilizing the carbon fixed through photosynthesis, as they increase accessibility to plant polysaccharides for different industrial applications. In addition, white-rot basidiomycetes are also involved in the degradation and detoxification of aromatic pollutants, which cause environmental problems. However, in industrial processes where conditions must be controlled, as in the case of biopulping, biodepitching, and bioethanol production, the long period of treatment required by fungi to be effective makes their application difficult.

On the other hand, the use of ligninolytic enzymes for controlled processes has advantages, such as the shorter treatment times and their high substrate specificity for environmentally problematic compounds. In this sense, laccases are considered the most promising enzymes, especially in the presence of a low-molecular-mass redox mediator to degrade nonphenolic recalcitrant compounds. These enzymes use oxygen as an electron acceptor and their efficiency has been reported for different industrial applications, including bleaching and depitching in the paper industry and degradation and detoxification of recalcitrant environmental pollutants. However, it is still necessary to solve some problems in order to employ this biocatalyst on an industrial level. Therefore, one solution could be the tailoring of more robust laccases by protein engineering as well as new, effective, cheaper, and environmentally friendly mediators. The analysis of the recently sequenced genome from basidiomycetes such as *P. chrysosporium* [17], or *C. subvermispora*, and *P. ostreatus*, currently in process (Joint Genome Institute, California, USA), as well as the rational design of new biocatalysts through site-directed mutagenesis and directed evolution, which have emerged as powerful enabling technologies, will permit optimal performance in selected applications in industrial processes, and, in the near future, will contribute toward the development of enzymatic tools for sustainable environmental solutions.

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