White Rot Fungi Laccases for Biotechnological Applications

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Abstract: White rot fungi have an enzymatic system producing oxidative and hydrolytic enzymes that act on the degradation of certain components of the cell wall. They can be applied in several technological processes, such as paper industry, bio-fuels and environmental pollution.

Laccases are multi-copper enzymes of wide substrate specificity and high non-specific oxidation capacity that use molecular oxygen to oxidize various aromatic compounds, and are highly relevant biotechnological applications.

In this review, we present some significant patents on laccase production and recombinant DNA technology for diverse biotechnology applications.

Keywords: Ligninolytic enzymes, laccases, white rot fungi, biotechnology applications.

INTRODUCTION

White-rot fungi are characterized by the unique ability to degrade recalcitrant wood polymer-lignin. The major enzymes associated with lignin-degrading ability of white rot fungi are lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac) although the above fungi do not have the same set of enzymes. Recently, extensive research on these fungi has been conducted with the aim to isolate new organisms with increased secretion of ligninolytic enzymes as well as enzymes with important properties for industrial applications, such as bioremediation of industrial waste streams polluted with hazardous xenobiotics, pulps biobleaching, wood biopulping, textile dyeing, biotransformation of pharmaceutical and other intermediates, food properties enhancing, biosensors construction, cosmetics, medicine and analytic biochemistry. All these biotechnological applications require large amounts of enzymes at low cost. However, current commercial enzymes are still expensive due to the low yield and high production and isolation costs. Therefore, the tasks should point toward searching for new hipersecretory strains, enhancing cultural conditions and applying heterologous approach.

Numerous researchers and inventors describe and patent new laccases from the diversity of organisms, including fungi. These enzymes are adapted for industrial production from native microorganisms or from recombinant organisms with fungal laccase genes incorporated. New technology is adapted for fungal laccase utilization and focused on cellulose and paper industry, textile industry, biofuels production and research Fig. (**1**). In this review, we comment recently developed patents related to areas showed in the flow diagram.

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LACCASES PRODUCTION

Laccases (EC 1.10.3.2), are multi-copper enzymes, generally extracellular and catalyzes the oxidation of several phenolic compounds, aromatic amines, thiols and some inorganic compounds using molecular oxygen as electron acceptor [1]. Different studies have shown that laccase production is regulated by metal ions such as Cu^{2+} and Fe^{3+} by gene expression induction or through translational or post-translational regulation [2-5]. They have been found widespread in eukaryotes, namely in fungi and less frequently in plants [6-8] as well as in prokaryotes [9-12]. In white rot fungi, they are responsible of delignification during wood attack and they act as fungal virulence factors.

Few years back, *Phlebia*, *Trametes* and *Pleurotus* species, were the most extensively studied white rot fungi. Now it is known that the ligninolytic machinery in white rot fungi is highly regulated by nutrients. Carbon and nitrogen sources, as well as several aromatic compounds and microelements, have been shown to have strong regulating effects. This information provides data for several approaches which were used to promote enzyme production. Inducers like xylidine, ferulic acid, veratryl alcohol, manganese salts, copper, polypropylene glycol, phospholipids, unsaturated fatty acids or detergent, such as Tween 80 or Tween 20, were utilized to increase the production of ligninolityc enzymes [13]. Large-scale commercial and profitable production are hindered by insufficient yields or levels of the ligninolytic enzymes production. There is a need for inexpensive equipment, medium and inducers. Accordingly, to develop commercially viable technologies of ligninolytic enzyme production, the enzyme over-expression is required with a reliable procedure ensuring highest enzyme activity using simple and non toxic medium and fast and inexpensive manufacturing processes. The most relevant approaches are summarized in Table **I**.

Patent CN101235354 is focused on specific culture media to improve white rot fungi growth and laccase secretion. The invention targets the low laccase yield problem using a defined

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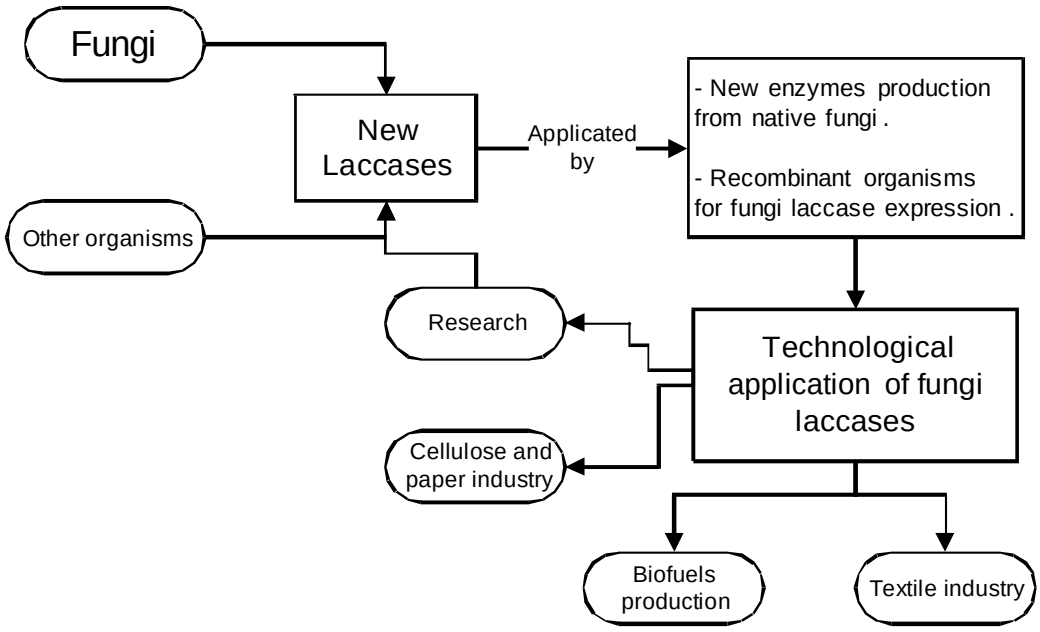

Fig. (1). Flow diagram of new laccase development and application.

Table I. Laccase Production

Nº Patent	Title	Authors
US2009311751A1	Wood-rotting basidiomycetes for production of ligninolytic enzymes	Elisashvili and Rebhun, 2009 [14]
CN101407794A	Laccase inducer and use thereof for improving microbial laccase production ability	Wei *et al*., 2009 [15]
CN101235354	Culture medium for cultivating white rot fungus secretion laccase	Gao *et al*., 2008 [16]
KR20000043999A	Mass production method of laccase by the addition of alcohols during fermentation of white rot fungi	Lee *et al*., 2000 [17]

culture media. The invention comprises corn meal leaching solution supplemented with ammonium tartrate, KH_2PO_4, $MgSO_4.7H_2O$, $CaCl_2$, vitamin solution and buffered with acetic acid/ acetate solution pH 7. At these conditions, the enzyme activity is 5 times higher than the typical culture media [16].

Another approach to increase the yield and decrease the cost of enzymes is to develop new hypersecretory fungi strains and recombinant organisms. In this regard, many patents have been registered for WRF enzyme production with different biotechnological approaches. For example, the aim of US2009311751A1 patent is to create a novel, non-expensive and time-effective overall procedure comprising the use of specific mushroom strains of *Cerrena unicolor* (CBS 117347) and *Trametes versicolor* (CBS 117346) isolated from Georgia and deposited in the Culture Collection of the Netherlands (Centraalbureau voor Schimmelcultures, International Depository Authority). Other aspect of the invention comprises a bioprocess for laccase and manganese peroxidase production of the mentioned strains. A submerged fermentation of the specific strains on a variety of lignocellulosic substrates, such as wastes from ethanol production, wheat grain, mandarin peels and bran is developed. Culturing conditions can be selected to modify the laccase/manganese peroxidase ratio promoting the production of either laccase or manganese peroxidase [14].

CN101407794A invention discloses a compound inducer to improve the capacity of white rot fungi strain XG8 for producing a laccase, which comprises bagasse, ethanol and a bluestone liquid. Laccase activity in the fermentation liquid is improved by 10 times than that of the strain not added with the compound inducer. The inducer of the invention is quite suitable to the large-scale production of industrialized laccases [15].

White rot fungi such as Trametes versicolor, Coriolus hirsutus, Phanerochaete flavido-alba, Grifila frondosa, Phlebia radiata, Pycnoporos cinnabarinus and Botrytis cineria can oversecrete laccase by the addition of nontoxic and cheap inducers instead of toxic and expensive aromatic ones. KR20000043999A patent describes a mass production method conducted by fermentation of fungi with inducers at pH 5.5-6.5. Laccases are obtained from the broth after one week cultivation, aerated and agitated properly. Aliphatic alcohols such as methanol, ethanol or isopropylalcohol are used to increase the production of laccase. 2% addition of isopropyl alcohol, methanol and ethanol in the medium improved 4, 9.5 and 15.6 times laccase activity, respectively [17].

NEW LACCASES PATENT

Laccases genes studies including DNA and mRNA sequencing analysis and proteins three-dimensional structure revealed important findings. Laccases has shown conserved regions in which histidine residues are abundant and important to bind four copper atoms distributed in two domains that are essential for the enzymatic activity [13]. The presence of conserved domains was the starting point for the design of molecular techniques by which the structure of new laccase genes and their cloning were obtained [18]. Table **II** resume new patent laccases.

Some patents claim genes expressed in recombinants organisms for laccase overexpression. Patent MX200900-5733A refers to novel laccases, nucleic acid sequences encoding such laccases, and vectors and host cells for expressing the laccases. The novel laccase enzymes can be employed in conjunction with mediators to provide an improved method for bleaching denim fabrics [19].

The invention CN1560257A is a new laccase expression carrier, expression microbial strain, expressed laccase protein and its application, where the expression carrier contains a sequence from site 85 to 1551 (including the site 1551) in DNA sequence ID incorporate in description, or a sequence having above 90% consanguinity with the former sequence. The strain is a microbe strain containing one of the expression carriers or the above sequence. The expressed laccase protein is a protein which contains a protein sequence ID incorporate in description and can be used for all known laccases [20].

Invention of US2002192792A1 provides enhanced laccase properties through variants secreted by *Coprinus*, *Polyporus pinsitus*, *Phlebia radiata* and *Myceliophthora thermophila* with improved oxidative stability as compared to the parent Coprinus-like laccase. Some specific structural parts or specific amino acid residues related to the oxidative stability of a laccase can be identified. Further aspects of invention relates to DNA encoding such variants and to use the variants for various industrial purposes. Because of the homology found between the above-mentioned laccases, they are considered as the same class of laccases, namely the class of "Coprinus-like laccases" [22].

Patent NZ501312 describe DNA sequences which code proteins with thermophilic laccase activity as well as the expression of these DNA sequences Their wide use include delignifying cellulose, depolymerizing high-molecular aggregates, de-inking waste paper, polymerizing aromatic compounds in waste liquids, especially waste liquids from cellulose bleaching, oxidizing colorants and activating colorants to produce pigments [23].

Laccases have also been produced by applying heterologous approach such as *Aspergillus oryzae* fungus [28, 29], and *Pichia pastoris* yeast [30]. The expression of a laccase from the *Coprinus* genus in the *Aspergillus* genus is described in the patent publication WO199827198A1 [24]. Similarly, the expression of the laccase from *Polyporus pinsitus* species and the laccase from the *Scytalidium* genus in the *Aspergillus* genus are described in the patent publication EP1294859 [21].

Patent US5750388A also reports an isolated nucleic acid which constructs a sequence encoding a *Scytalidium* laccase, and the laccase proteins encoded thereby. *Scytalidium thermophilum* is a thermophilic deuteromycete, and a member of the *Torula-Humicola* complex which are recognized as dominant species in mushroom compost [25].

Patent EP0850306A1 describes mutants of a blue multicopper oxidase of a *Rhizoctonia* and *Myceliophthora* laccase created by substituting one or more amino acid residues with other amino acid residues, inserting one or more amino acid residues and/or deleting one or more amino acid residues, wherein the substitution, insertion or deletion is carried out at a position which is not greater than 15Å from a Type I copper site [26].

MX9606726A patent relates to isolated nucleic acid fragments encoding a fungal oxidoreductase enzyme and the purified enzymes produced thereby. It also relates to nucleic acid fragments encoding a *Polyporus pinsitus* laccase [27].

Table II. Patents on New Laccase Description

Nº Patent	Title	Authors
MX2009005733A	Novel laccases, compositions and methods of use	Wang *et al*., 2009 [19]
CN1560257A	Expression carrier of laccase, microorganism strain of expression, laccase protein of expression and application thereof	Weimin, 2005 [20]
EP1294859	Novel laccase enzyme and the gene encoding the enzyme	Kruus, 2003 [21]
US2002192792A1	Laccase mutants	Schneider *et al*., 2002 [22]
NZ501312A	Thermophilic laccases, dna sequences encoding them and their use in paper manufacture	Pfaller and Wich, 2000 [23]
WO199827198A1	Laccases mutants from the *Coprinus* genus in the *Aspergillus* genus	Pedersen *et al.*, 1998 [24]
US5750388A	Purified scytalidium laccases and nucleic acids encoding same	Berka *et al*., 1998 [25]
EP0850306A1	Laccases with an altered pH activity profile	Xu *et al*., 1998 [26]
MX9606726A	Purified polyporus laccases and nucleic acids encoding same.	Yaver *et al*., 1997 [27]

TECHNOLOGICAL APPLICATIONS OF LACCASES OF FUNGI

Laccases are also capable of polymerization, depolymerization, methylation and demethylation reactions in relation to highly relevant biotechnological applications [9].

There are many laccase applications on different fields, such as wood or pulp treatment to reduce pitch, as reporter genes, as part of a fermentation mixture, for oxidation and bleaching processes (Table **III**).

Materials, such as lignocelluloses or pulp and paper mill wastes can be treated with fungal laccases. In this regard, there are many applications using fungal laccases in the cellulose and paper industry. In the past decade, Call in 1992 (CA2103260A1), patented a process for the delignification of material containing lignocellulose, bleaching and treatment of white water by laccases from white rot fungi *Coriolus versicolor* and other laccase formers with extended efficiency capable of lignin degradation and enzymatic deinking waste paper [31].

Wang *et al*., WO2007035481A1, developed a method of treating wood chips or sawdust, prior to refining, with a formulation comprising various enzymes (including laccases) to reduce the total extractives content of the wood chips or sawdust and to modify wood structure. The treatment leads to a decrease in the apparent pitch content during pulping and reduced energy requirements, increased paper strength, improved paper machine runability, and lower costs associated with paper manufacturing. Wood extractives in wood chips, commonly known as pitch, have a significant impact on pulping and papermaking processes. Minimizing or preventing pitch deposits are critical to minimize the equipment fouling and down time, maximizing production efficiency, and improving paper product quality [32].

Pitch deposition can also be reduced by treating pulp stocks with similar enzyme formulation applied at any of sev-

Table III. Patents on Technological Laccase Applications

Nº Patent	Title	Authors
CA2103260A1	A process for the delignification of material containing lignocellulose, bleaching and treatment of white water by means of laccases with extended efficiency	Call, 1992 [31]
WO2007035481A1	Treatment of wood chips using enzymes	Wang *et al*., 2007 [32]
US2007261806A1	Treatment of pulp stocks using oxidative enzymes to reduce pitch deposition	Wang *et al*., 2007 [33]
US2010018658A1	Laccases for Bio-Bleaching	Kerovou *et al*., 2010 [34]
US2006054290A1	Novel catalytic activities of oxidoreductases for oxidation and or bleaching	Call, 2006 [35]
US2008070284A1	Oxidative, reductive, hydrolytic and other enzymatic systems for oxidizing, reducing, coating, coupling or cross-linking natural and artificial fiber materials, plastic materials or other natural or artificial monomer to polymer materials	Call, 2008 [36]
PT102779A	Textile materials pretreatment based whiteness enhancement consists of oxidative bleaching with peroxide after treatment with laccases	Artur Cavaco *et al*., 2003 [37]
US2001047852A1	Methods for deinking and decolorizing printed paper	Franks, 2001 [38]
WO0015899A1	Methods for deinking and decolorizing printed paper	Franks, 2000 [39]
WO2004029193A1	Fermentation methods and compositions	Grichko, 2004 [40]
US2003205247A1	Use of solutions containing enzymes for cleaning fermentation for storage tanks	Lengling and Kluschanzoff, 2003 [41]
WO9915137A1	Enzymatic foam compositions for dyeing keratinous fibres	Soerensen, 1999 [42]
US5948121A	Laccases with improved dyeing properties	Aaslyng *et al*., 1999 [43]
WO2006034811A2	Use of laccases as reporter genes	Karos *et al*., 2006 [44]
WO9745549A1	DNA sequences coding for laccases, and their applications in the field of plant lignin content control.	Faye *et al*., 1997 [45]
WO0002464A1	Use of a phenol oxidising enzyme in the treatment of tobacco	Kierulff *et al*., 2000 [46]
WO9923887A1	Antimicrobial activity of laccases	Johansen *et al*., 1999 [47]
US6030933A	Detergent compositions comprising immobilized enzymes	Herbots *et al*., 2000 [48]
US6893470B1	Keratinous fibre oxidation dyeing composition containing a laccase and dyeing method using same	Lang and Cotteret, 2009 [49]

eral locations during the pulping and/or papermaking process as a solution to the pulp stock [33].

Recently, isolated laccase enzymes, nucleic acids encoding them and mediators for laccase reactions were applied to oxidize lignins and other phenolic and aromatic compounds. The applications US2010018658A1 include biological bleaching and decolorization of wood pulp under high temperature and pH conditions. There are important environmental benefits due to a substantial reduction using bleaching chemicals. The conventional bleaching process requires application of harsh chemicals and energy-intensive conditions. The wood pulping process can involve alkaline conditions. However, most known laccases are acidic enzymes [34].

An enzyme-based process for oxidation and bleaching comprising laccases or peroxidases separately or in combination was developed by some authors.

US2006054290A1 uses the enzyme combination, co-substrate, enhancer compound and carbonyl compound generate active oxygen species that act as oxidizing and/or bleaching agents [35]. In 2008, the same inventors patented an oxidizing method (pulp delignification/bleaching), for carrying out coupling reactions (grafting polymer materials) or for carrying out cross-linking reactions on natural or artificial monomers to polymers or of mixtures of natural and artificial polymers or of fibre materials, lignocellulose-containing natural polymers or fibre materials such as pulp, and textiles like cotton and wool. These enzymes may be able to couple enzymatically special enzyme substrates *via* generating of ester or ether bounds or *via* radical reactions [36].

Artur Cavaco *et al*., 2003, patented a method for treating textile materials with laccases to whiteness enhancement employing an economical technique. The laccase treated step renders superior material quality after oxidative bleaching of the cellulose fibres when compare with two successive peroxide treatments [37].

US2001047852A1 and WO0015899A1 patents describe laccase application as deinking and decolorizing aid on printed paper. The enzyme is added to a pulp slurry dislodging ink, decolorizing dyes and separating the released ink from the pulp slurry. The process can be used as fungal laccase isolated from some white rot fungi, such as *Chrysosporium*, *Phlebia*, *Pleurotus*, *Pycnoporus*, *Schizophyllum*, *Sclerotium*, *Sporotrichum*, *Stagonospora* and *Trametes* species [38, 39].

WO2004029193 provides an enhanced fermentation method, for using in an ethanol production process. The improved fermentation process includes applying laccases (among other enzymes) with the addition of various growth stimulators for the fermenting microorganisms [40].

Laccases can also be applied as a component of aqueous cleaning solutions for fermentation or storage tanks in the beverage industry. They can especially remove left over residues from fermentation processes [41].

Laccase secreted by a strain of *Polyporus*, *Myceliophthora*, *Fomes*, *Lentinus*, *Pleurotus*, *Phlebia* and *Coriolus* can work as enzymatic foam for dyeing fibres as described in patent WO9915137A1. Enzymatic foam compositions can be applied for dyeing of keratinous fibres, e.g. hair, fur, hide or wool. The method includes one oxidation enzyme, typically laccase, one foaming agent, one dye precursor and one modifier. The foamed compositions of the invention provide an improved uniformity of the dyeing effect [42].

In the same way, patent US5948121A also refers to an enhanced permanent dyeing composition comprising a small amount of enzyme protein of microbial laccase. It can be applied for dyeing keratinous fibers, such as hair, fur, hide, and wool. The production strain for the laccase enzyme is the *A. oryzae* strain Mt. The strain contains the gene encoding laccase from *Myceliophthora thermophila*. *M. thermophila* is a thermophilic fungus that occurs in decaying manure, silage, wood chips, etc., in Europe and North America [43].

Research applications of fungi laccase include laboratory methods for determining the regulatory properties of a genetic element, comprising the introduction of a promoter sequence, whose regulatory properties are to be determined into a cell, being linked to a sequence encoding a laccase (WO2006-034811A2), and applications in plants lignin content control (WO9745549A1) [44, 45].

Other industrial applications of fungal laccases include unrelated areas. For example WO0002464A1 describes an improved tobacco product having a reduced amount of phenolic compounds that can be obtained by treating the tobacco material with a phenol oxidising enzyme, such as a laccase derived from *Trametes villosa* and *Pycnoporus cinnabarious*, among other microorganisms. This is an alternative or a complement to a process in which the phenolic compounds are adsorbed into the insoluble carrier polyvinylpolypyrrolidone (PVPP) [46]. Other examples are used as antimicrobial treatment of microorganisms and/or viruses (WO9923887A1) and the immobilized enzymes, such as laccases as part of detergent compositions (US6030933A) [47, 48]. The immobilization of one or more enzymes on activated polymers offers the following advantages: improved thermal stability of the immobilized enzymes, reduced deposition of the immobilized enzyme(s) on fabrics due to increased rinse-away, better whiteness maintenance, etc.

Biotechnological applications of laccases from fungi are diverse. In 2009, Lang and Cotteret presented the invention of US6893470B1, which relates to a composition for the oxidation dyeing of keratin fibres, particularly human hair fibres, comprising a suitable medium for dyeing, the dyeing process and at least one laccase-type enzyme among other compounds. The authors intend to use at least one type of laccase from many organisms, such as white rot fungi *Trametes versicolor*, *Pleurotus ostreatus*, *Ganoderma lucidum*, *Cerrena unicolor*, *Coriolus hirsutus*, *Ceriporiopsis subvermispora*, *Coprinus cinereus*, *Panaeolus papilionaceus*, *Schizophyllum commune*, and from variants thereof [49].

CURRENT & FUTURE DEVELOPMENTS

Recent publications of the sequence of many laccases genes and fungi genome will accelerate the discovery of new enzymes with outstanding properties and the development of new biotechnological approaches. The exhaustive analysis of the enzymatic degradation procedures and the elucidation of

molecular pathways for enzyme production will broaden the existing biotechnological boundaries.

In particular, current patents in laccase production are the standpoint to the future developments. New techniques are able to detect mRNA expression and to test microarray technologies, others demonstrate the introduction of specific antibodies to detect proteins families, leading Biotechnology into Transcriptomic and Proteomic fields. Simultaneously, many recent inventions developed alternative bioprocess with new ligninolytic enzymes.

All these findings demonstrate the need to get deeper into the understanding of those molecular mechanisms involved in the fungal enzymes induction and secretion.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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