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FUNGAL LACCASES: INDUCTION AND PRODUCTION

LACASAS FUNGALES: INDUCCIÓN Y PRODUCCIÓN

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Abstract

Fungal laccases are phenol oxidases that have been extensively studied due to their relevance in diverse industrial applications including paper whitening, color reduction, elimination of phenolic compounds in wine, detoxification of polluted environments, revaluation of industrial wastes and water treatment. The principal difficulties in the use of these enzymes on an industrial scale are the cost of production and limitations on operation conditions (low stability and low catalytic activity). Over the last few decades, a variety of strategies have been evaluated to increase the productivity and improve the biochemical properties of these enzymes. The identification of inducers and the mechanisms by which gene expression is regulated is crucial for efforts to increase laccase production in fungi. Laccase gene transcription is regulated by various carbon and nitrogen sources, the presence of metal ions, the addition of diverse aromatic compounds related to lignin or its derivatives (phenolic and/or non-phenolic), and even the presence of other microorganisms. Although abundant information is available about the biochemical properties and kinetic parameters of laccases, it is difficult to compare different laccases due to the diversity of laccase producing strains, isoforms, laccase substrates, inducers and operating conditions. This review discusses the literature on the induction and production of fungal laccases.

Keywords: laccase isoforms, induction, laccase regulation, laccase production.

Resumen

Las lacasas fúngicas son oxidasas fenólicas ampliamente estudiadas por su relevancia en diversas aplicaciones industriales, incluyendo el blanqueo de papel, reducción de color, eliminación de compuestos fenólicos en el vino, la biorremediación de ambientes contaminados, en la revalorización de residuos industriales y el tratamiento de aguas residuales. Las dificultades en el uso de lacasas a escala industrial son el costo de la producción y las limitaciones en las condiciones de operación (baja estabilidad y actividad catalítica). En la última década, se han evaluado diferentes estrategias que permiten un aumento de la productividad y la mejora de sus propiedades bioquímicas. Por ello, la identificación de los inductores y de los mecanismos implicados en la regulación de la expresión génica es crucial para el aumento de la producción de lacasa en los hongos. La transcripción de genes de lacasa está regulada por diferentes fuentes de carbono y nitrógeno, la adición de compuestos fenólicos y no fenólicos, la presencia de iones metálicos, y la interacción con otros organismos. Aunque existe información acerca de las propiedades bioquímicas y parámetros cinéticos de las lacasas, es difícil compararlos debido a la diversidad de cepas, número de isoformas, sustratos e inductores y condiciones de operación utilizadas. En esta revisión, se discuten los aspectos generales de la inducción y producción de lacasas fúngicas.

Palabras clave: isoformas de lacasa, inducción, regulación, producción.

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1 Introduction

The widespread laccase enzyme (EC 1.10.3.2 benzenediol: oxygen oxidoreductase) belongs to the family of multi-copper enzymes and is produced by many ligninolytic fungi (Messerschmidt *et al*. 1989). A diversity of industrial applications have been proposed for laccases including in the pulp and paper industry, textile applications, organic synthesis, and environmental, food, pharmaceutical and nanobiotechnological applications (Kunamneni *et al*. 2008). However, its utilization in this wide variety of fields has been ignored because of lack of commercial availability (Imran *et al*. 2012).

The principal use of laccases is in the delignification of lignocellulosic compounds in the pulp and paper industry (Hattaka, 1994). In the industrial preparation of paper, the separation and degradation of lignin in wood pulp and paper was conventionally achieved using chlorine or chemically oxygenated oxidants. In 1994, the whitening of wood pulp with laccase and without the use of chlorine was patented for the first time (Hattaka, 1994).

Recently, laccases have been studied for their ability to solve challenges in the development of sustainable forms of energy such as bioethanol, where laccases are used to detoxify culture media with high furanosyl and other phenolic content derived from raw materials of lignocellulosic origin (Larsson *et al*. 2001; Imran *et al*. 2012). These enzymes are also applied in the production of animal feed with high lignin content to improve digestibility (Kunamneni *et al*. 2008; Sharma *et al*. 2013).

In the food industry, laccases are added to enhance product quality and reduce cost. They can be used to reduce oxygen concentration and increase product life because molecular oxygen can negatively affect the product quality due to unwanted oxidation (Kunamneni *et al*. 2008; Osma *et al*. 2010). The use of laccase to stabilize wine is one of the most important applications in the food industry. In wine, the mixture of different chemical compounds such as ethanol and fatty acids (fragrance) and salts and phenols (color and taste) is important (Osma *et al*. 2010; Imran *et al*. 2012). The use of laccases to improve the sensory parameters of food products is not limited to treatment processes but also extends to diagnostic systems. Various amperometric biosensors have been developed based on laccases to measure polyphenol contents in food products including wine, beer, and tea (Osma *et al*. 2010).

Laccase is also used in the production of antimicrobial and detoxifying agents and personal care products. Laccases are also used in the synthesis of complex medical compounds such as anesthetics, anti-inflammatory agents, antibiotics and sedatives (Kunamneni *et al*. 2008; Imran *et al*. 2012).

One of the greatest environmental problems in the world today is water, soil and air pollution by toxic compounds. Together with industrialization and excessive use of pesticides in agriculture, environmental pollution has become a serious problem. Certain dangerous compounds such as polycyclic aromatic hydrocarbons (PAH), pentachlorophenol (PCF), polychlorinated biphenyl, DDT, toluene, benzene, and TNT are persistent in the environment, and have been demonstrated to be potential mutagenic agents. The capacity of fungi to transform a wide range of toxic substances attracted attention decades ago (Nityanand and Desai, 2006).

Laccases also have many applications in the area of bioremediation. They can be applied to degrade non-desirable toxic compounds, secondary products or waste materials. They have also been used to degrade plastic materials that contain olefin units, where they promote chain reactions that lead to plastic disintegration. Laccase activity facilitates the degradation of phenolic compounds, biphenyl derivatives ("environmental hormones"), and alkyl phenols, and also fluorescent dyes (Kunamneni *et al*. 2008). These oxido-reductases have been used to detoxify (by oxidation) polycyclic aromatic hydrocarbons, promoting the removal of the aromatic rings for their subsequent mineralization/ biodegradation (Bezalel and Cerniglia, 1996). These enzymes are used in the decolorization of textile dye effluents to reduce processing time as well as energy and water consumption (Pedersen and Schmidt, 1992; Imran *et al*. 2012; Osma *et al*. 2010). Enzymatic oxidation of dibenzothiophene by laccase has also been reported (Villaseñor *et al*. 2004).

The presence of phenols in agro-industrial effluents has attracted interest for the application of laccase-based processes to wastewater treatment and bioremediation. The presence of phenolic compounds in drinking and irrigation water or in cultivated land represents a significant health and/or environmental hazard. With government policies on pollution control becoming increasingly stringent, various industries have been forced to look for more effective wastewater treatment technologies (Osma *et al*. 2010).

2 Laccases: General biochemical characteristics and behavior

The best studied laccases to date are produced by fungi belonging to the group of basidiomycetes that cause white rot in wood (Baldrian, 2006). Generally, the catalytic activity and stability can vary between enzymes depending on the origin, temperature, pH, and culture medium used for their production. Laccases are stable at acidic pH (3-6) (Nyanhongo *et al*. 2002), but pH 3 is normally optimal for laccase activity. Laccases can be active over a wide range of temperature (20-55 °C), with an optimum temperature at 55 °C. Nonetheless, thermostable laccases (60-70 °C) have also been purified and characterized (Saraiva *et al*. 2012). The presence of isoforms has been reported depending on fungal growth phase, the presence of inducers and the conditions of the production process. Additionally, it has been demonstrated that the isoforms produced in a particular strain can vary in their pI values and molecular weights. The proportion of different laccase isoforms produced depends on the culture age and the substrate used (Moldes *et al*. 2004). The genes that codify these isoforms are differentially regulated and can be constitutively expressed or induced during the life of the cell (Castanera *et al*. 2012; Piscitelli *et al*. 2011).

Laccase structure. In general, the mature protein is a holoenzyme, and in its active form it can be monomeric, dimeric or tetrameric, with four copper atoms for each monomer (Kunammeni *et al*. 2008; Imran *et al*. 2012). Currently, more than 40 three-dimensional laccase structures are accessible via GenBank, NCBI; the majority of these are white-rot fungal laccases (Benson *et al*. 2012). The molecular weights of these laccases range between 60 and 100 kDa. Only three species of fungi have been described as producers of laccases with molecular weights of 100 kDa and above (Baldrian, 2006). The majority of these enzymes are glycoproteins. These biomolecules can exhibit different levels of glycosylation, generally between 10 and 30 % (Baldrian, 2006). Glycosylation plays an important role in secretion, proteolytic stability (Bertrand, 2010), copper retention capacity and thermal stability (Thurston, 1994). Glycosylation is also believed to play an important role in the pI values of laccase isoforms. Their pI values range from 3-7, and can be as high as 9 in plants (Baldrian, 2006). These enzymes usually exhibit different kinetic parameters, for example different Km, optimum pH, optimum temperature, and Kcat values (Schlosser *et al*. 1997; Tinoco *et al*. 2001).

Laccase catalytic mechanism. The sites T1, T2, T3 and T4, HWH, HSH, HGH and HCH are highly conserved copper binding sites that form part of the laccase active site (Figure 1). In these regions, all the histidine and cysteine residues are critical for the coordination of the copper atoms (Yaver *et al*. 1996). Laccases are characterized by the presence of four copper atoms per molecule, distributed among three different sites, although one isoform was described as having only one copper atom (Schückel *et al*. 2011). The type 1 copper site is responsible for the intense blue color of the enzyme, with an absorbance of 605 nm. Catalysis by laccase starts with the reduction of the site 1 copper atom by the reducing substrate. The electron is then transferred to the copper sites 2 and 3, followed by the concomitant reduction of molecular oxygen (O_2) in the tri-nuclear site complex (Lyashenko *et al*. 2006) (Figure 1). Electron transfer from the substrate to the copper 1 site is controlled by the difference in redox potential. The redox potential of laccase is between 450-800 mV (Nityanand and Desai, 2006).

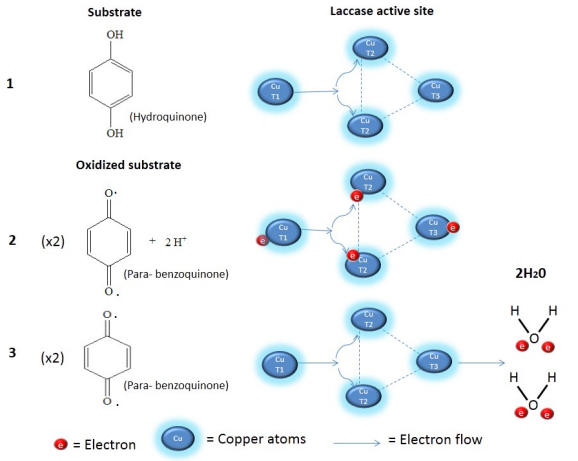

Fig. 1. The catalytic mechanism of laccase. 1-The substrate (in this case, hydroquinone) comes into contact with the active site of laccase. 2-The substrate is oxidized when it loses its electrons to the Type 1 Cu, and Cu is therefore reduced. The electron (smaller circles) is then transferred internally from Type 1 Cu to a tri-nuclear cluster made up of the Type 2 and Type 3 Cu atoms. 3-Two O_2 molecules are reduced to water at the tri-nuclear cluster. A total of four electrons (extracted from two hydroquinone molecules) are needed to complete the laccase catalytic cycle.

A low oxidation potential or a high redox potential at the copper site 1 normally results in a greater oxidation velocity of the substrate (Lyashenko *et al*. 2006).

Laccases are copper-containing proteins that are capable of catalyzing the oxidation of polyphenols, substituted phenols and diamines by the reduction of oxygen to water (Osma *et al*. 2010; Nityanand and Desai, 2006; Imran *et al*. 2012). The typical reaction mechanism of laccases consists in the oxidation of the phenolic substrate that generates a free radical (phenoxyl) (Osma *et al*. 2010; Kunammeni *et al*. 2008) (Figure 1). These active species can be converted to quinones during a second oxidation stage. Both the quinone and the free radical undergo non-enzymatic coupling, leading to their polymerization, generating insoluble compounds that can be easily retrieved (Osma *et al*. 2010; Nityanand and Desai, 2006; Imran *et al*. 2012). Laccases are inhibited by various agents such as small anions such as halides, azides, cyanide and hydroxyls, that act by forming bonds with the copper sites type 2 and 3, interrupting electron transfer. They are also inhibited by fatty acids, di-sulfur agents, hydroglycine and cationic detergents with quaternary ammonium structures (Riva, 2006).

Laccase mediator system (LMS). In contrast to other ligninolytic enzymes, laccases can only oxidize phenolic fragments of lignin due to their random polymeric nature and to the lower redox potential of laccase. Small natural low molecular weight compounds with higher potentials than laccase, called mediators, can be used to oxidize the non-phenolic part of lignin (Nityanand and Desai, 2006; Kunammeni *et al*. 2008). In recent years, the discovery of new and efficient synthetic mediators has extended the laccase activity towards xenobiotic substrates (Nityanand and Desai, 2006). A mediator is a small molecule that acts as an "electron shuttle" between the enzyme and the lignin and causes polymer de-branching and degradation (Kunammeni *et al*. 2008). The activity of an LMS towards lignin depends on two main factors: first, the redox potential of the enzyme, and second, the stability and reactivity of the radical, resulting from the oxidation of the mediator (Kunammeni *et al*. 2008).

3 Laccase genes: gene organization, expression and induction features

At first, differences among laccases were believed to be due to post-translational variations in one gene. However, various fungal species possess more than one gene encoding for laccases enzymes (Fujihiro *et al*. 2009; Kilaru *et al*. 2006; Palmieri *et al*. 2000). Phylogenetic reconstructions indicate that the sequence diversity among fungal laccases is moderate and that the isoforms described to date originate from the same common ancestor (Valderrama *et al*. 2003; Necochea *et al*. 2005). Genetic analysis confirms the fact that isoforms sometimes originate from different genes in the genome (Castanera *et al*. 2012). Two laccase genes were detected in *Agaricus bisporus* (Palmieri *et al*. 2000), three genomic sequences for the basidiomycete I-62 and *Pleurotus ostreatus* (Tlecuitl-Beristain *et al*. 2008). Yaver *et al*. (1996) reported three laccase isoforms in *T. versicolor*. Four different mRNA sequences were detected by cDNA synthesis in *Rhizoctonia solani*, and five in *Trametes villosa*. Seventeen non-allelic laccase genes were found in the genome of *Coprinopsis cinerea* (Kilaru *et al*. 2006); in the case of this specific fungus, two subfamilies were defined based on the positions of the introns and the similarity of the determined genome, one with 15 members (lcc1-lcc15) and the other with two members (lcc16, lcc17). The first subfamily of deduced proteins forms a branch of the phylogenetic tree with smaller groups that most likely reflect recent gene duplication events. The diversity of laccase genes came about by frequent codon changes, (synonymous and non-synonymous). Synonymous codon changes are reflected in alleles, with a total difference of up to 12 % in the codons in a given pair of alleles. Valderrama *et al* (2003) presented the reconstruction of the fungal laccase loci evolution inferred from the comparative analysis of 48 different sequences. The topology of the phylogenetic trees indicated that a single monophyletic branch exists for fungal laccases and that laccase isozyme genes may have evolved independently, possibly through duplication-divergence events. Additionally, the genome of *P. ostreatus* includes 12 laccase genes. Six of the genes appear to be clustered at the sub-telomere region of chromosome IV, and the others map to chromosomes IV, VI, VII, VIII and XI. However, only six *P. ostreatus* laccase isoenzymes have been characterized to date (Castanera *et al*. 2012).

Laccase gene regulation. Cells often respond to changing circumstances and to signals from other cells by altering the amount or type of proteins they express. The fundamental units of gene regulation are the three types of specific DNA sequences that determine the level of expression under particular physiological conditions. Promoters, originally defined as elements that determine the maximal potential level of gene expression, are recognized by RNA polymerase and contain all the information necessary for accurate transcriptional initiation. Operator sequences are recognized by repressor proteins, which inhibit transcription that would otherwise occur beginning at the promoters. Lastly, positive control elements are recognized by activator proteins that stimulate transcription at the promoter. The functions of activators and repressors can be modulated by specific physiological conditions, thus permitting regulated expression of the cognate genes (Struhl, 1999).

Laccases can be expressed constitutively or can be induced; they can also be differentially expressed. The positions of laccase genes and the control elements under which they are located can directly affect the regulation of these genes (Missall *et al*. 2005; Castanera *et al*. 2012). The promoters of laccase genes have been well studied, and various differentially distributed response elements have been discovered. The promoter region of the *T. pubescens* laccase isoenzyme LAP 2 extends up to 1420 bp upstream of the start codon ATG. The promoter region was found to have metal response elements (MRE), CreA consensus sequences (related with carbon metabolism), and also heat shock elements (HSE) (Piscitelli *et al*. 2011). In fungal species such as *G. graminis C. subvermispora, P. sajor-caju* and *Trametes sp*., ACE elements (cup1 protein activation), NIT2, and xenobiotic response elements (XRE) responsible for the regulation of laccases by copper, nitrogen and aromatic compounds related with lignin or its derivatives were observed, respectively (Piscitelli *et al*. 2011; Collins and Dobson, 1997).

Castanera *et al*. (2012) examined the expression profiles in different fungal strains under different conditions (submerged and solid cultures) and in the presence of wheat straw extract. Their results suggest that certain isoforms (in this case *Lacc2* and *Lacc10*) are up-regulated in submerged cultures and down-regulated in solid fermentation. Isoform Unk1 did not appear to be induced by the straw wheat extract, and could be associated with physical culture conditions rather than with the presence of phenolic inducers. Furthermore, the high percentage of genes with altered transcriptional responses in the straw wheat induced cultures revealed a complex regulation mechanism that could be related to the sensitivity of the laccase gene family to phenolic compounds and sugars present in the inducer extract. Apart from natural and synthetic inducers, culture conditions and oxidative stress and the presence of virulent strains affects the regulation of fungal laccases. *Cryptococcus neoformans* encodes two laccases that are both regulated by oxidative and nitrosative stresses (Missall *et al*. 2005). Although an abundance of information is available about the response elements in the promoter regions of laccase genes, only a few reports have been published on the molecular mechanisms of their regulation (Collins and Dobson, 1997; Kilaru *et al*. 2006; Piscitelli *et al*. 2011).

Investigating the regulation of the expression of laccase genes can be very useful in understanding the physiological roles of different isoforms produced by the same organism (Piscitelli *et al*. 2011). The physiological mechanisms that occur during mycelial development can modulate the relative expression levels of laccase isoforms. Some isoforms have been detected in the lag and exponential phases of fungal fermentation, and therefore must be involved in substrate degradation, while other isoforms have been detected in the stationary phase, and may be related to morphogenesis and pigmentation processes (Bourbonnais *et al*. 1995; Piscitelli *et al*. 2011). Laccase synthesis and secretion are also influenced by nutrition levels, culture conditions and the addition of a wide range of inducers to the culture medium, with variations in these effects observed among fungal species and between different isoforms of the same species. For the majority of the reported examples, laccase expression is regulated by several factors acting synergistically and antagonistically (Piscitelli *et al*. 2011).

Compounds that have the capacity to function as inducers of laccase synthesis have been explored in a variety of fungal species. These compounds have structures that are very similar to or are analogues of lignin, and serve as cellular signals to produce specific laccases (Bertrand *et al*. 2013). It has been postulated that genes that codify various laccase isoforms are differentially regulated, while others are constitutive (Collins and Dobson, 1997).

There have been various reports on the differential expression of laccase isoforms in fungal basidiomycetes following the addition of phenolic compounds and copper to the culture media (Bollag and Leonowicz, 1984; Collins and Dobson, 1997;

Goudopoulou *et al*. 2010). In fact, low concentrations of various laccases are produced in solid and submerged media, while higher concentrations are induced with the addition of phenolic compounds such as xylidine and ferulic acid (Bollag and Leonowicz, 1984; Collins and Dobson, 1997).

4 The laccase secretion pathway

Fungal secretomes have been described as nature's tool boxes. By adapting their metabolism to different carbon and nitrogen sources, fungi secrete an arsenal of extracellular enzymes, the secretome, which allows for the degradation of lignocelluloses and other biopolymers (Bouws *et al*. 2008). Filamentous-like fungi species such as *Aspergillus* and *Trichoderma* have an extraordinary capacity to secrete large quantities of proteins, metabolites and organic acids into the growth media (Conesa *et al*. 2001). White rot of wood is possible due to the secretion of organic acids, secondary metabolites, oxidative metalloenzymes, heme peroxidases and laccases codified in divergent gene families in the genomes of these fungi (Lundell *et al*. 2010).

Laccase genes (part of the secretome) in many filamentous fungi have sequences that exhibit a common pattern, and code for polypeptides of approximately 520-550 amino acid residues, including a signal peptide found on the N-terminus. Laccases require at least three processing steps (Yaver *et al*. 1996). The signal peptide is involved in enzyme maturation, guiding the laccase into the extracellular space, a secretion route where several events occur: A) co-translation folding in the endoplasmic reticulum where di-sulfide bridges are formed (Freeman *et al*. 1993), B) the incorporation of a precursor sequence (glucose 3 mannose 9-glucose-N-acetylglucosamine-2) that bonds with the asparagine of the majority of the NXT/S sequences of the protein (Gavel and Von, 1990) and C) binding of calcium ions that stabilize the resulting apolaccase. Subsequently, in the Golgi apparatus, the copper ions are added (Taylor *et al*. 2005) along with additional carbohydrates before secretion (Rodríguez-Rincón *et al*. 2010). Laccases also contain an N terminal secretion sequence abundant in arginine and lysine, which suggests processing during biosynthesis. Not all laccases are extracellular. Missall *et al*. (2005) showed that laccases are differentially localized in *C. neoformans*. Lac1 is localized to the cell wall, while Lac2 is cytoplasmic. This difference may in part account for the greater ability of Lac1 to produce melanin, as a substrate in the medium is more accessible to the cell wall-localized enzyme and the deposition of melanin in the cell wall would not require additional transport. Differences in location may contribute to differences in substrate specificity.

5 Laccase production

Laccase production by basidiomycetes of genera *Trametes, Pleurotus, Lentinula, Pycnoporus, Phanerochaete* and *Agaricus* have been widely studied due to the ease with which these microorganisms are cultured in vitro, and because these laccases are excreted into the culture medium. Studies of laccase production have evaluated the effect of production systems (solid or liquid media) (López-Pérez *et al*. 2010; Díaz *et al*. 2011a; Díaz *et al*. 2011b; Neifar *et al*. 2011; Poojary and Mugeraya, 2012), carbon source (sugars or lignocellulosic residues), and the use of inducers (phenolic or non-phenolic compounds, natural or synthetic compounds) using different ligninolytic fungal strains (Table 1). These factors affect productivity and the relative amounts of the various secreted laccase isoforms. The observed laccase profiles and concentrations are very important because the different isoforms possess particular catalytic properties.

In recent years, there has been a surge in the tendency towards the effective use and valuation of organic wastes such as wastes from the agriculture, forest, and food industries as raw materials and substrates for solid-state fermentation (Moldes *et al*. 2003; Risdianto *et al*. 2010). Moreover, the majority of these wastes contain lignin and/or cellulose or hemi-cellulose, which in turn act as inducers of ligninolytic activity. Most of these wastes have high sugar contents, making the processes more economical (Risdianto *et al*. 2010). The use of these types of wastes not only provides an alternative substrate source but also aids in solving environmental pollution problems (Moldes *et al*. 2003; Neifar *et al*. 2011). Solid state fermentation (SSF) is considered one of best methods for the culture of filamentous fungi and for the production of ligninolytic enzymes because they are grown under conditions that emulate their natural habitat, and as a result are able to produce certain enzymes at high levels, as high as those obtained in submerged fermentation conditions (Kumar and Mishra, 2011; Neifar *et al*. 2011).

Nutshells were used as lignocelullosic wastes by

Gómez *et al*. (2005), resulting in a 25-fold increase in the production of laccase using the fungus *Coriolopsis rigida* by means of SSF. Moldes *et al*. (2003) used grape seeds as a lignocellulosic source to increase the activity of *T. hirsute* 10-fold.

The main focus of our investigations is the production of enzymes with high impacts on environmental biotechnology. We continue to evaluate the advantages of the use of agro-industrial lignocellulosic wastes such as sawdust of different wood types and compost. Sawdust from different wood sources is a very cheap and effective source of natural inducers used for fungal laccase production. We have conducted various studies on the induction and production of laccase isoforms under conditions of solid state and submerged fermentation using the *T. versicolor* HEMIM-9 strain. High laccase production levels were achieved under these conditions. Additionally, the lignocellulosic source was shown to have a direct effect on the laccase isoforms secreted, and these isoforms exhibited significant biochemical differences in their capacities to oxidize synthetic Azo-dyes (Bertrand *et al*. 2013). We are currently evaluating the potential of these isoforms (presented as a cocktail) in fungal supernatants for their potential use in the oxidation of diverse compounds with high environmental impacts in the area of water and soil pollution. In the same way, we assessed the production of laccases for the oxidation of phenol and polyphenolic compounds using aqueous extracts of the residual culture medium of the mushroom *Agaricus bisporus* (compost). Our results demonstrated that the residual compost after culturing *A. bisporus* is a potential source of laccase that could become a cost-effective waste management alternative for some phenolic compounds (Trejo-Hernández *et al*. 2001) and polyaromatic hydrocarbons (Mayolo-Deloisa *et al*. 2011).

6 Improvement of laccase production by different types of inducers

Ligninolytic enzyme production by wood rotting fungi is a phenomenon involving the interaction between fungal physiology and the composition of the essential media used for cultivation (Table 2) (Bakkiyaraj *et al*. 2013).

Table 1. Laccase production using different types of substrates.

Species	Inducer	Laccase production	Improvement	Reference
Schyzophyllum commune	Corn stover and Banana stalk	1270000 U L^{-1}	1.3-fold	Yasmeen *et al*. (2013)
Pleurotus ostreatus	Glucose Yeast extract Malt extract	906000 U L^{-1}	0.8 fold	Periasamy and Palvannan (2010)
Coriolous versicolor MTCC138	Nut shell and Cyanobacteria biomass	163000 U L^{-1}	2.6-fold	Mishra *et al*. (2008)
Phellinus noxius hpF17	Glucose Ammonium tartate Tween 80	780 U L^{-1}	1.4-fold	Poojary and Mugeray (2012)
T. hirusta	Barley	25889 U L^{-1}	1.8-fold	Bakkiyaraj *et al*. (2013)
Rigidoporous sp.	Rice bran, Wheat bran and Corn husk	4.25 U g^{-1}	3.5-fold	Sridah *et al*. (2012)
Fomes fomentarius	Wheat bran	150 U g^{-1}	2.3-fold	Niefar *et al*. (2011)
WRF-1	Nut shell and Cyanobacteria biomass	352 U g^{-1}	1.3-fold	Kumar and Mishra (2011)
Coriolus sp.	Barley	2661 U g^{-1}	6.5-fold	Mathur *et al*. (2013)

Table 2. Improvement of laccase production using synthetic inducers.

Species	Inducer	Laccase production	Improvement	Reference
Cerrena unicolor	Pyrogallol	151600 U L^{-1}	2.5-fold	Elisashvili *et al*. (2010)
T. versicolor	TNT	8400 U L^{-1}	2.8-fold	
Coprinus comatus	Copper	150 U L^{-1}	3.4-fold	Lu and Ding (2010)
	Managnese	225 U L^{-1}	4.4-fold	
	Caffeic acid	188 U L^{-1}	3.3-fold	
Pleurotus ostreatus	copper	8000 U L^{-1}	4.0-fold	Tinoco *et al*. (2011)
Pleurotus ostreatus	Copper + lignin	12000 U L^{-1}	10-fold	Tinoco *et al*. (2011)
P. plumonarius	Ferulic acid and vanillin	250000 U g^{-1}	10 fold	D'souza (2004)
T. versicolor	Grey Lanaset (GLG) and Alizarin Red (AR)	1200 U g^{-1}	7.0-fold	Casas *et al*. (2013)
G. applanatum	Copper	18830 U g^{-1}	49.2-fold	Fonseca *et al*. 2010
Peniophora sp	Copper	27132 U g^{-1}	19.7-fold	

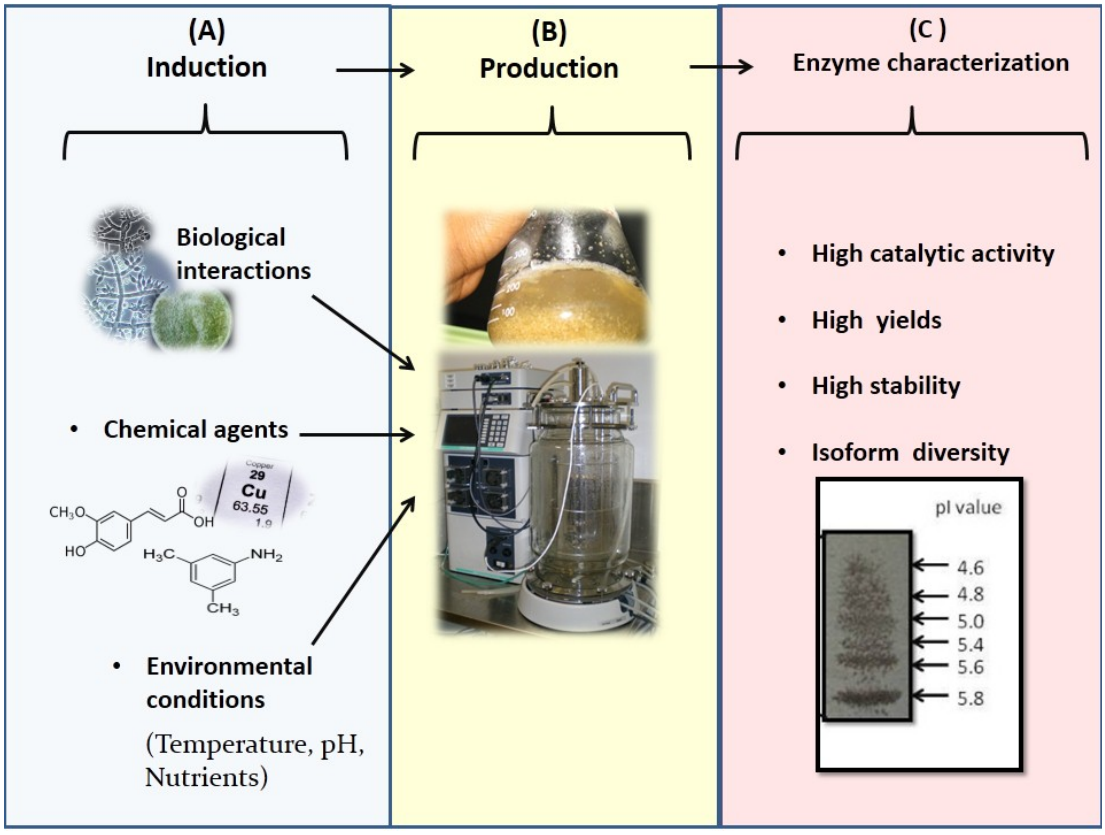

Fig. 2. Strategies for increasing laccase production. The first step is to determine an adequate way to induce laccase production. The second step to produce laccase isoforms in shaking flasks or in fermentation reactors. The third step is the biochemical characterization of laccase isoforms to look for features such as high enzyme activity and stability.

Laccase production by ligninolytic fungi has been comprehensively investigated due to the ability of these microorganisms to grow on economic substrates, excrete enzymes and oxidize xenobiotic compounds with a useful capacity (Shah and Nerud, 2002; Ikehata *et al*. 2004). Although laccase production is principally related to lignin degradation, these enzymes have a wide range of physiological functions including biosynthesis of the pigments of conidia, and participation in the detoxification of phenols through polymerization (generated as a defense mechanism by plants against attack by phytopathogens) (Mayer, 1986; Baldrian, 2005). Additionally, laccase production is stimulated under conditions of limited nutrition, in particular under limited carbon and nitrogen conditions (Kirk and Farell, 1987; Valderrama *et al*. 2003).

For efficient laccase expression, it is essential to optimize all conditions and compositions of the media used for production. Figure 2 shows various strategies to increase the production of the extracellular laccase and its activity and to achieve various industrially desirable features such as high yield, high catalytic activity, isoform diversity and high stability. Various authors have implemented experimental designs such as RSM, Plackett-Burman, and Box-Behnken to optimize laccase production using their systems (Kumar and Mishra, 2011; Poojary and Mugeraya, 2012; Nandal *et al*., 2013; Srihdar *et al*., 2012; Neifar *et al*., 2011). Media supplemented with barley enabled 200 times greater production of laccase than the control culture (Moldes *et al*., 2004). This study demonstrated that the type of substrate also has a significant influence on the relationship between the two laccases produced (LacII/LacI), varying from 0.9 U L^{-1} (barley bran) to 4.4 U L^{-1} (grape pulp). The profiles obtained are very important because in the case of effluent decolorization from the textile industries, Lac1 exhibits more attractive catalytic properties. Selective induction of laccases by *Trametes sp*. was reported by Xiao *et al*. (2004). These authors revealed that cultures supplemented with cellobiose increased the production of several isoforms, while cultures where 3, 5- dihydroxytoluene was added produced only one isoform.

Note that even though the great majority of studies on laccase induction and production have been performed in submerged cultures, SSF cultures of ligninolytic fungi are also a potential source of laccase (Diaz *et al*. 2011a and b). The most important reports in this area are related to the use of compost waste, generated after the harvest of edible fungi, as a rich source of laccases with interesting catalytic characteristics. One of the first reports of laccase production in the fungus *Agaricus bisporus* was presented by Bonnen *et al*. (1994). Their main objective was to study the role that these enzymes play in lignin degradation during commercial production. Rodríguez-Couto *et al*. (2002) described the production of laccase by *T. versicolor* in the semi-solid state using different supports (polyurethane foam, wheat straw, barley straw, wood shaving and barley bran). This group claimed that the best conditions were achieved with barley bran, reaching an activity level of approximately 1200 U L^{-1}.

7 Laccase induction by phenolic compounds

Laccase production by ligninolytic fungi can be considerably stimulated by a wide variety of aromatic compounds related to lignin and its derivatives (Marques de Souza *et al*. 2004). Aromatic compounds or phenols are generally considered laccase inducers not only because they increase laccase production but also because they modify the isoform profile. The type and composition of the medium culture and the use of inducers play important roles in the productivity and profile of the laccases obtained. For example, in *Trametes sp*., the genes *lcc1* and *lcc2* were induced in cultures where veratrilic acid was added, while the gene *lcc3* was not induced by this compound and was repressed by glucose (Mansur *et al*. 1998). The use of ferulic acid and vanillin increased laccase production 10 times in submerged cultures of *Pleurotus pulmonaris* (Marques de Souza *et al*. 2004). These authors demonstrated that while LacI and LacII were produced in non-induced cultures, the cultures supplied with vanillin and ferulic acid only produced lacII and lacIII. Rodríguez-Couto *et al*. (2002) reported the production of laccase in *Trametes versicolor* in semi-solid state fermentation and proved that the addition of xilidine caused laccase activity to reach approximately 1700 U L^{-1}. These authors argue that the change in the laccase isoform profile most likely represents a detoxification mechanism of the microorganism in response to the inducers in question. These findings are supported by the fact that high concentrations of the inducers can be detrimental to cellular health (Bertrand *et al*. 2013; Bourbonnais *et al*. 1995).

8 Laccase induction by metal ions

The induction of laccase by toxic metals can be explained in terms of defense from toxic stress, as laccase is involved in the synthesis of pigments to prevent metal uptake (Lorenzo *et al*. 2006). Different isoforms exhibit different characteristics with respect to copper in *P. ostreatus* (Tinoco *et al*. 2001). The addition of copper sulfate (150 μM) to the medium culture resulted in an increase in the production (up to 500-fold) of certain laccase isoforms, as in the case of laccase isoform POXA1b, while isoforms were not affected, such as POXA1w (Palmieri *et al*. 2000). Genetic regulation at the transcriptional level by copper and nitrogen was demonstrated in *T. versicolor* 290. RT-PCR demonstrated an increase in the levels of laccase mRNA with increases in copper and nitrogen concentrations (Collins and Dobson, 1997). The presence of Mn^{2+}, Cd^{2+} or Zn^{2+} in the culture medium increased the LacI/LacII proportion by nearly 100% in comparison to the control cultures. Furthermore, the metal concentration added to the culture medium affected the LacI/LacII ratio. The highest LacI/LacII activity ratio (approximately 0.51) was obtained from cultures with 5 mM copper sulfate, attaining values 360% and 155% higher than those obtained from cultures with 2 to 3.5 mM copper sulfate, respectively (Lorenzo *et al*. 2006). Pleurotus ostreatus produces four different laccases in potato-dextrose medium supplemented with yeast extract and $CuSO_4$ (Palmieri *et al*. 2000). Likewise, the same authors demonstrated that copper increases the transcription of the laccase genes poxc and poxa 1b of Pleurotus ostreatus (Faraco *et al*. 2003). In other studies, Klonowska *et al*. (2001) reported that Marasmius quercophilus produces only one laccase (LacI) in liquid medium with malt extract. This same medium supplemented with $CuSO_4$ permits the induction of three other isoforms, increasing the total activity10 times. Additionally, cultures supplemented with $CuSO_4$ and *p*-hydrobenzoic acid exhibited a 30-fold increase in total activity compared to the basal production. Considering that laccase contains four copper atoms in its active site, it is not surprising that copper has an effect on laccase activity.

9 Laccase induction by biological interaction

Laccases are thought to be important to the virulence of many fungal pathogens or as a defense mechanism in lignolytic fungi (Missall *et al*. 2005). Laccase induction and production have been examined in cultures of *Lentinula edodes, Trametes versicolor* and *Pleurotus ostreatus* infected with *Trichoderma sp*. Some authors suggest that laccase plays an important role in the basidiomycete defense mechanisms against the microparasite. Researchers are primarily interested in these types of responses because some *Trichoderma* strains are aggressive microparasites of edible lignolytic fungi (*Pleurotus, Agaricus* and *Lentinula*), leading to serious economic losses in commercial cultures. In the same way, Savoie *et al*. (1998) reported the induction of laccase from *Lentinula edodes* in liquid cultures challenged with *Trichoderma sp*. These authors made evident that induction occurs to a greater extent in co-cultures. However, the supernatant obtained apart from the *Trichoderma* culture also increased laccase production in liquid media. Subsequently, the same group showed that laccase induction in co-cultures was not from *Trichoderma*, but likely from *Lentinula* as a defense mechanism against the micro-parasite (Savoie and Mata, 1999). Hatvani *et al*. (2002) also studied the effects of changes in extracellular enzymatic activities during fungal co-cultures. Their results established the existence of laccase repression in *Lentinula* during co-culture with different strains of *Trichoderma* and induction with their supernatants.

Baldrian (2006) reported an increase in production in solid-state laccase cultures by 18 strains of ligninolytic fungi in response to a strain of *Trichoderma harzianum*. On analyzing the laccase production by a *Trametes versicolor* strain in liquid medium, only the co-cultures (*Trametes-Trichoderma*) exhibited an increase in laccase production, and no increase was observed when *Trichoderma* supernatants were used.

The production and profile of laccase isoforms from *Agaricus bisporus* and *Pleurotus ostreatus* as a function of the type of infection with *Trichoderma sp*. was explored by Flores *et al*. (2009). In their studies, solid cultures were used to evaluate the effects of the interaction with extracellular metabolites. These results revealed an increase in the production of laccase in *in vitro* cultures (in a solid medium) of *Agaricus bisporus* and *Pleurotus ostreatus* infected with non-laccase producing strains of *Trichoderma* (Flores *et al*. 2009). Additionally, an increase in laccase production was observed by *Pleurotus ostreatus* and *Trichoderma viride* co-cultures in submerged fermentation (Flores *et al*. 2010).

Concluding remarks

Abundant information published over the last decade or so reflects the significant industrial potential of laccases in the environmental (bioremediation of soil and water), food processing (alcoholic beverage stabilization and fruit juice) and pharmacy (morphine determination and ascorbate) fields.

Despite the discovery of new laccase producing fungal strains with high productivities, the most important limitation remains the high cost of enzyme production. This has contributed to the search for lower cost production processes and to the use of cheap substrates including residues and wastewater from the agriculture, food, and paper industries. The reduction of production time through the use of enzyme inducers has been widely demonstrated, and increases in production levels have been reported up to 500%. Additionally, these processes must be optimized to enable industrial scale application, as most studies have been conducted at the laboratory level in shaking flasks using experimental designs.

Laccases are relatively stable in extracellular extracts for long periods of time, but enzyme stability must be improved to enable industrial use. On the other hand, it is important to highlight the analysis of the effects of regulators on laccase gene transcription, and in the presence of specific responses that are translated into the induction of enzyme production. Few studies have elucidated the molecular mechanisms that prevail in laccase regulation as a response to different stimuli. The current analyses suggest the existence of a set of complex phenomena regulating laccase expression. However, much more work must be performed to fully understand the general mechanisms of the regulation of laccase transcription.

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