Fungal laccase – a versatile enzyme for biotechnological applications

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Laccase belongs to the small group of enzymes called the blue multi copper oxidases. Laccase is widely distributed in higher plants and fungi. In fungi, laccase is present in Ascomycetes, Deuteromycetes, Basidiomycetes and is particularly abundant in many white-rot fungi that degrade lignin. Laccases have been subject of intensive research in the last decades due to their broad substrate specificity. In the recent years, their uses span from the textile to the pulp and paper industries, and food applications to bioremediation processes. Laccases also have uses in organic synthesis, where typical substrates are phenols and amines, and the reaction products are dimers and oligomers derived from the coupling of reactive radical intermediates. More recently, they have found applications in other field such as in the design of biosensors and biofuel cells. In this review, the occurrence, mode of action, general properties, production and immobilization of laccases will be discussed. Here, we will also provide discussion of potential applications of these blue enzymes.

Keywords Laccase, White rot fungi, Production, Properties, Potential applications, Pulp and paper industry, Textile industry, Food industry, Bioremediation, Organic syntheses, Biosensors, Biofuel cells

1. Introduction

Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) belongs to the small group of enzymes called the blue copper proteins or the blue copper oxidases along with the plant ascorbate oxidase and the mammalian plasma protein ceruloplasmin [1,2] among others. These proteins are characterized by containing 4 catalytic copper atoms. One copper is placed at the T1 site, where reducing substrate binds, and it is responsible in the characteristic blue-greenish colour in the oxidizing resting state Cu^{2+} [1,3]. The other three coppers are clustered in the called T2/T3 site in which molecular oxygen binds.

Laccase is widely distributed in higher plants and fungi [4] and has been found also in insects and bacteria. Recently a novel polyphenol oxidase with laccase like activity was mined from a metagenome expression library from bovine rumen microflora [5].

Yoshida first described laccase in 1883 when he extracted it from the exudates of the Japanese lacquer tree, *Rhus vernicifera* [1,6]. In 1896 laccase was demonstrated to be present in fungi for the first time by both Bertrand and Laborde [1,6]. Since then, laccases have been found in Ascomycetes, Deuteromycetes and Basidiomycetes; being particularly abundant in many white-rot fungi that are involved in lignin metabolism [7,8]. Fungal laccases have higher redox potential than bacterial or plant laccases (up to +800 mV), and their action seems to be relevant in nature finding also important applications in biotechonology. Thus, fungal laccases are involved in the degradation of lignin or in the removal of potentially toxic phenols arising during lignin degradation [1]. In addition, fungal laccases are hypothesized to take part in the synthesis of dihydroxynaphthalene melanins, darkly pigmented polymers that organisms produce against environmental stress [9] or in fungal morphogenesis by catalysing the formation of extracellular pigments [10].

Concerning their use in the biotechnology area, fungal laccases have widespread applications, ranging from effluent decolouration and detoxification to pulp bleaching, removal of phenolics from wines, organic synthesis, biosensors, synthesis of complex medical compounds and dye transfer blocking functions in detergents and washing powders, many of which have been patented [11]. The biotechnological use of laccase has been expanded by the introduction of laccase-mediator systems,

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which are able to oxidise non-phenolic compounds that are otherwise hardly or not oxidised by the enzyme alone.

2. Occurrence and location of laccases

Laccase is the most widely distributed of all the large blue copper-containing proteins, as it is found in a wide range of higher plants and fungi [8,12] and recently some bacterial laccases have also been characterized from *Azospirillum lipoferum* [13], *Bacillus subtilis* [14], *Streptomyces lavendulae* [15], *S. cyaneus* [16] and *Marinomonas mediterranea* [17]. The occurrence of laccases in higher plants appears to be far more limited than in fungi. Laccases in plants have been identified in trees, cabbages, turnips, beets, apples, asparagus, potatoes, pears, and various other vegetables [6]. The classical demonstration of laccase in *R. vernicifera* is well documented [18]. In addition, the lacquer tree is a member of the Anacardiaceae family, appear to contain laccase in the resin ducts and in the secreted resin [18]. Cell cultures of *Acer pseudoplatanus* have been shown to contain eight laccases, all expressed predominantly in xylem tissue [19]. Other reports are those of Wosilait *et al.* [20] on the presence of a laccase in leaves of *Aesculus parviflora* and in green shoots of tea [21]. Other higher plant species also appear to contain laccases, although their characterization is less convincing [22]. Laccases have been isolated from Ascomyceteous, Deuteromyceteous and Basidiomyceteous fungi [23]. In the fungi, Ascomycetes and Deuteromycetes have not been a clear focus for lignin degradation studies as much as the white-rot Basidiomycetes. Laccase from *Monocillium indicum* was the first laccase to be characterised from an Ascomycete showing peroxidative activity [24]. This chapter will focus on laccases isolated from the white-rot fungi.

The white-rot basidiomycetes are the most efficient degraders of lignin and also the most widely studied. The enzymes implicated in lignin degradation are: (1) lignin peroxidase, which catalyses the oxidation of both phenolic and non-phenolic units, (2) manganese-dependent peroxidase, (3) laccase, which oxidises phenolic compounds to give phenoxy radicals and quinones; (4) glucose oxidase and glyoxal oxidase for H_2O_2 production, and (5) cellobiose-quinone oxidoreductase for quinone reduction [24]. The veratryl alcohol oxidase and some esterases may also play roles in the complex process of natural wood decay. The different degrees of lignin degradation with respect to other wood components depend on the environmental conditions and the fungal species involved. It has been demonstrated that there is no unique mechanism to achieve the process of lignin degradation and that the enzymatic machinery of the various microorganisms differ. *Pleurotus ostreatus,* for instance, belongs to a subclass of lignin–degrading microorganisms that produce laccase, manganese peroxidase and veratryl alcohol oxidase but no lignin peroxidase [25]. *Pycnoporus cinnabarinus* has been shown to produce laccase as the only ligninolytic enzyme [26] and *Pycnoporus sanguineus* produces laccase as the sole phenol oxidase [27]. In plants, laccase plays a role in lignification, whereas in fungi laccases have been implicated in many cellular processes, including delignification, sporulation, pigment production, fruiting body formation and plant pathogenesis [1,11]. Only a few of these functions have been experimentally demonstrated [28].

Ligninolytic enzymes have mostly been reported to be extracellular but there is evidence in literature of the occurrence of intracellular laccases in white–rot fungi [29]. Intracellular as well as extracellular laccases were identified for *Neurospora crassa* by Froehner and Eriksson [30], who suggested that the intracellular laccase functioned as a precursor for extracellular laccase as there were no differences between the two laccases other than their occurrence.

3. Mode of action of the laccase enzyme

Laccases contain 4 copper atoms termed Cu T1 (where the reducing substrate place) and trinuclear copper cluster T2/T3 (where oxygen binds and is reduced to water). As a one-electron substrate oxidation is coupled to the four-electron reduction of oxygen the reaction mechanism cannot be entirely straightforward. Laccase can be thought to operate as a battery, storing electrons from individual

oxidation reactions in order to reduce molecular oxygen. Hence the oxidation of four reducing substrate molecules is necessary for the complete reduction of molecular oxygen to water.

In general terms, substrate oxidation by laccase is a one-electron reaction generating a free radical. The initial product is typically unstable and may undergo a second enzyme-catalysed oxidation or otherwise a non-enzymatic reaction such as hydration, disproportionation or polymerisation. The bonds of the natural substrate, lignin, that are cleaved by laccase include, Cα- oxidation, Cα-Cβ cleavage and aryl-alkyl cleavage (Figure 1a).

Laccases are similar to other phenol-oxidising enzymes, which preferably polymerise lignin by coupling of the phenoxy radicals produced from oxidation of lignin phenolic groups [7]. Due to this specificity for phenolic subunits in lignin and its restricted access to lignin in the fibre wall, laccase has a limited effect on pulp bleaching unless redox mediators (eg. 2,2´-azino-bis(3-ethybenzthizoline-6-sulfonic acid (ABTS)) will be introduced in the reaction (Figure 1b) [31].

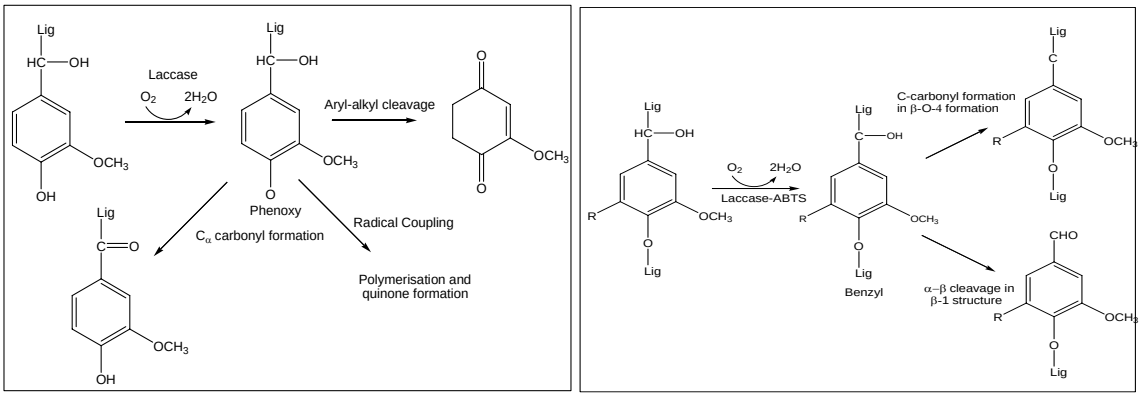

Figure 1.Oxidation of (a) phenolic subunits of lignin by laccase and (b) non-phenolic lignin model compounds by a laccase mediator system.

4. Laccase mediator system

With respect to other ligninolytic enzymes, laccase can oxidize only phenolic fragments of lignin due to the random polymer nature of lignin and to the laccase lower redox potential [32,33]. Small natural low-molecular weight compounds with high redox potential than laccase itself (> 900 mV) called mediators may be used to oxidize the non-phenolic part of lignin [26] (Figure 1b). In the last years the discovery of new and efficient synthetic mediators extended the laccase catalysis towards xenobiotic substrates [26,34,35].

A mediator is a small molecule that acts as a sort of 'electron shuttle': once it is oxidized by the enzyme generating a strongly oxidizing intermediate, the co-mediator (oxidized mediator), it diffuses away from the enzymatic pocket and in turn oxidizes any substrate that, due to its size could not directly enter into the active site. Futhermore, the use mediators allows the oxidation of polymers by side-stepping the inherent steric hindrance problems (enzyme and polymer do not have to interact in a direct manner) (Figure 2) [36].

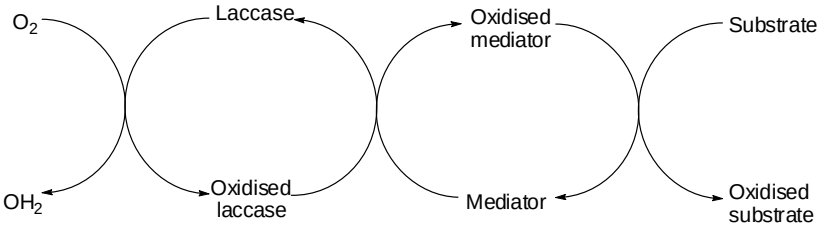

Figure 2. Catalytic cycle of a laccase-mediator oxidation system (reproduced from Banci *et al.* [36]).

Alternatively, the oxidized mediator could rely on an oxidation mechanism not available to the enzyme, thereby extending the range of substrates accessible to it [37]. It is therefore of primary importance to understand the nature of the reaction mechanism operating in the oxidation of a substrate by the oxidized mediator species derived from the corresponding mediator investigated. In the laccase-dependent oxidation of non-phenolic substrates, previous evidence suggests an electron-transfer (ET) mechanism with mediator ABTS, towards substrates having a low oxidation potential. Alternatively, a radical hydrogen atom transfer (HAT) route may operate with N-OH type mediators, if weak C-H bonds are present in the substrate [38].

More than 100 mediator compounds have been described but the most commonly used are the ABTS and the triazole 1-hydroxybenzotriazole (HBT) [7,34,35]. Various laccases readily oxidize ABTS, by free radicals, to the cation radical $ABTS^{+\cdot}$ and the concentration of the intensely colored, green-blue cation radical can be correlated to the enzyme activity (ε_{418}= 36000$M^{-1}cm^{-1}$). It is well known that cation radicals represent an intermediate oxidation step in the redox cycle of azines and, upon extended oxidation and abstraction of the second electron, the corresponding dications can be obtained. The redox potentials of $ABTS^{+\cdot}$ and $ABTS^{2+}$ were evaluated as 0.680 V and 1.09 V respectively [39].

HBT belongs to the *N*-heterocyclic coumpounds bearing *N*–OH groups mediators [40]. Consuming oxygen HBT is converted by the enzyme into the active intermediate, which is oxidized to a reactive radical ($R–NO^{\cdot}$) [34] and HBT redox potential has been estimated as 1.1-1.2 V [41]. Mediated laccase catalysis has been used in a wide range of applications, such as pulp delignification [31,42,43], textile dye bleaching [35], polycyclic aromatic hydrocarbon degradation [44], pesticide or insecticide degradation [45], and organic synthesis [46]. In pulp and paper industry, novel enzymatic bleaching technologies are attracting increasing attention because of concerns regarding the environmental impact of the chlorine-based oxidants currently being used in delignification or bleaching [31,42,47]. However, synthetic mediators are toxic, expensive and generally at concentrations above 1 mM inactivate the laccase. Novel approaches to overcome this hurdles are coming up (from searching for natural mediators such as *p*-coumaric acid, 4-hidroxybenzoic acid, syringaldehyde etc) [35] to the directed evolution of laccases [48].

5. General properties of laccase enzymes

Most fungal laccases are monomeric, dimeric or tetrameric glycoproteins. Glycosylation of fungal laccase is believed to play a role in secretion, susceptibility to proteolytic degradation, copper retention and thermal stability [43]. Upon purification, laccase enzymes demonstrate considerable heterogeneity. Glycosylation content and composition of fungal glycoproteins can vary with growth medium composition [49]. For this reason data can be heterogeneous. The molecular mass of the monomer ranges from about 50 to 100 kDa. An important feature is a covalently-linked carbohydrate moiety (10–45% of total molecular mass), which may contribute to the high stability of the enzyme [50]. The sugar composition has been analyzed in several examples, such as *Podospora ansenna,*and *Botrytis cinerea* [42], *Trametes hirsuta*, *Trametes ochracea, Cerrena maxima* and *Coriolopsis fulvocinerea* [51] and *Melanocarpus albomyces* [52].

5.1 Isozymes

Many laccase producing fungi secrete isoforms of the same enzyme [8]. These isozymes have been found to originate from the same or different genes encoding for the laccase enzyme [53]. The number of isozymes present differs between species and also within species depending on whether they are induced or non-induced [23]. They can differ markedly in their stability, optimal pH and temperature and affinity for different substrates [23,54]. Furthermore, these different isozymes can modulate different roles in the physiology of different species or in the same species under different conditions [23]. *Cerrena unicolor* secreted two laccase isoforms with different characteristics during the growth in a synthetic low-nutrient nitrogen/glucose medium [55]. Various laccase encoding gene sequences have been reported from a

range of ligninolytic fungi; these sequences encode for proteins between 515 and 619 amino acid residues and close phylogenetic proximity between them is indicated by sequence comparisons [7].

5.2 Substrate specificity of laccase

Laccases are remarkably non-specific as to their reducing substrates, and the range of substrates oxidised varies from one laccase to another. These enzymes catalyse the one-electron oxidation of a wide variety of organic and inorganic substrates, including polyphenols, methoxy-substituted phenols, aromatic amines and ascorbate with the concomitant four-electron reduction of oxygen to water [1]. Laccase has broad substrate specificity towards aromatic compounds containing hydroxyl and amine groups, and as such, the ability to react with the phenolic hydroxyl groups found in lignin [56].
Kinetic data of laccases from different sources were reported [57]. K*m* values are similar for the co-substrate dissolved oxygen (about 5-10 M), but V*max* varies with the source of laccase (50–300 M/s). The turnover is heterogeneous over a broad range depending on the source of enzyme and substrate/type of reaction. The kinetic constants differ in their dependence on pH. K*m* is pH-independent for both substrate and co-substrate, while K*cat* is pH-dependent.

5.3 Influence of pH on laccase activity and stability

The pH optima of laccases are highly dependable on the substrate. When using ABTS as substrate the pH optima are more acidic and are found in the range 3.0-5.0 [54]. In general, laccase activity has a bell-shaped profile with an optimal pH that varies considerably. This variation may be due to changes in the reaction caused by the substrate, oxygen or the enzyme itself [58]. The difference in redox potential between the phenolic substrate and the T1 copper could increase oxidation of the substrate at high pH values, but the hydroxide anion (OH^-) binding to the T2/T3 coppers results in an inhibition of the laccase activity due to a disruption of the internal electron transfer between the T1 and T2/T3 centres. These two opposing effects can play an important role in determining the optimal pH of the bi-phasic laccase enzymes [58]. Laccase produced by *Trametes modesta* was fully active at pH 4.0 and very stable at pH 4.5 but its half-life decreased to 125 min at pH 3.0 [59].

5.4 Influence of temperature on laccase activity and stability

The optimal temperature of laccase can differ greatly from one strain to another. The laccases isolated from a strain of *Marasmius quercophilus* [60] were found to be stable for 1 h at 60°C. Farnet *et al*. [60] further found that pre-incubation of enzymes at 40°C and 50°C greatly increased laccase activity. Another technique that can be used to increase the stability of laccase is to immobilise the enzyme on glass powder by means of air-drying [61]. This technique also has potential for the enzyme to be used on the glass powder matrix in specific biotechnology applications where stability is required [61]. The laccase from *P. ostreatus* is almost fully active in the temperature range of 40°C-60°C, with maximum activity at 50°C. The activity remains unaltered after prolonged incubation at 40°C for more than 4 h [62]. Nyanhongo *et al*. [59] showed that laccase produced by *T. modesta* was fully active at 50°C and was very stable at 40°C but half-life decreased to 120 min at higher temperature (60°C).

5.5 Influence of Inhibitors on enzyme activity

In general, laccases responds similarly to several inhibitors of enzyme activity [63]. Many ions such as azide, halides, cyanide, thiocyanide, fluoride and hydroxide bind to the type 2 and type 3 Cu, resulting in the interruption of internal electron transfer and accordingly therefore inhibition of activity. Other inhibitors include metal ions (e.g. Hg^{+2}), fatty acids, sulfhydryl reagents, hydroxyglycine, kojic acid, desferal and cationic quaternary ammonium detergents, the reactions with which may involve amino acid residue modifications, confirmational changes or Cu chelation [64,42]. Confirmational changes are

highly depended on the state of oxidation of the copper atoms. This is one of the reasons for the sensitivity towards chelating agents. The selective removal of Cu by chelaing agents (EDTA, dimethyl glyoxime, N,N'-dimethyldithiocarbamate, NTA) leads to a loss of catalytic activity.

6. Production of fungal laccases

Laccase activity was detected in the cultures of a wide range of fungi, from Ascomycetes to Basidiomycetes, and from wood- and litter-decomposing fungi to ectomycorrhizal fungi [63]. White-rot fungi have been studied extensively for application in biological pulping and bleaching [65] because they are of the only organisms that are able to degrade lignin efficiently [54]. White-rot fungi, such as *Coriolus versicolor* and *P. sanguineus,* Murr. are known producers of lignolytic enzymes that are involved in the natural delignification of wood [42]. This group of fungi is the only known (micro)organisms that have evolved complex enzymatic systems that enable them to degrade lignin [66]. In general, laccases occur as extracellular glyco-proteins, which allows for rapid removal from fungal biomass [53,54]. One of the major limitations for the large-scale applications of fungal laccases is the low production rates by both wild type and recombinant fungal strains according to Galhaup *et al.* [67].

White-rot fungi constitutively produce low concentrations of various laccases [68] when they are cultivated in submerged culture or on wood. Higher concentrations can be induced by the addition of various aromatic compounds such as 2,5-xylidine and ferulic acid. High concentrations of laccase have also been observed in old non-induced cultures [7]. The mechanisms of metabolism in microorganisms are used and controlled by its environmental conditions and medium composition [69]. There are various response element sites in the promoter regions of laccase genes that can be induced by certain xenobiotic compounds, heavy metals or heatshock treatment [70].

6.1 Induction of laccase production

Laccase production has been found to be highly dependent on the conditions for the fungus cultivation [54] and media supporting high biomass did not necessarily support high laccase yields [71]. Ligninolytic systems of white-rot fungi were mainly activated during the secondary metabolic phase and were often triggered by nitrogen concentration [72] or when carbon or sulfur became limiting [54]. Laccases were generally produced in low concentrations by laccase producing fungi [73], but higher concentrations were obtainable with the addition of various supplements to media [74]. The addition of aromatic compounds such as 2,5-xylidine, lignin, and veratryl alcohol is known to increase and induce laccase activity [71]. Many of these compounds resemble lignin molecules or other phenolic chemicals [75,76]. Veratryl alcohol is an aromatic compound known to play an important role in the synthesis and degradation of lignin. The addition of veratryl alcohol to cultivation media of many white-rot fungi has resulted in an increase in laccase production [77]. Some of these compounds affect the metabolism or growth rate [30] while others, such as ethanol, indirectly trigger laccase production [74].

Eggert *et al.* [26] found that the addition of 2,5-xylidine as inducer had the most pronounced effect on laccase production. The addition of 10 μM 2,5-xylidine after 24 h of cultivation gave the highest induction of laccase activity and increased laccase activity nine-fold [26]. At higher concentrations the 2,5-xylidine had a reduced effect, probably due to toxicity [26].

The promoter regions of the genes encoding for laccase contain various recognition sites that are specific for xenobiotics and heavy metals [70]. These can bind to the recognition sites when present in the substrate and induce laccase production. White-rot fungi were very diverse in their responses to tested inducers for laccase. The addition of certain inducers can increase the concentration of a specific laccase or induce the production of new isoforms of the enzyme [68]. Some inducers interact variably with different fungal strains [26].

Lee *et al*. [74] investigated the inducing effect of alcohols on the laccase production by *Trametes versicolor.* The enhanced laccase activity was comparable to those obtained using 2,5-xylidine or veratryl alcohol [78]. It was postulated that the addition of ethanol to the cultivation medium caused a reduction in melanin formation. The monomers, when not polymerised to melanin, then acted as inducers

for laccase production [74]. The addition of ethanol as an indirect inducer of laccase activity offers a very economical way to enhance laccase production.

Lu *et al*. [79] found that there is a strong correlation between hyphal branching and the expression and secretion of laccase. The addition of cellobiose can induce profuse branching in certain *Pycnoporus* species and consequently increase laccase activity [79]. The addition of cellobiose and lignin can increase the activity of extracellular laccases without an increase in total protein concentration [66,79,80]. Osma *et al*. [80] showed that soya oil was the best inducer of laccase activities, attaining 4-fold higher than those obtained in the reference cultures.

The addition of low concentrations of Cu^{+2} to the cultivation media of laccase producing fungi stimulates laccase production [23]. Palmieri *et al*. [81] found that the addition of 150 µM copper sulphate to the cultivation media can result in a fifty-fold increase in laccase activity compared to a basal medium. Employing copper sulphate as laccase inducer or supplementing the culture medium with veratryl alcohol, led to maximum values of laccase activity [82]. A new basidiomycete, *Trametes* sp. 420, produced laccase in glucose medium and in cellobiose medium with induction by 0.5 mM Cu^{+2} and 6 mM *o*-toluidine [83].

6.2 Influence of carbon sources on laccase production

The carbon sources in the medium play an important role in ligninolytic enzyme production. Mansur *et al.* [78] showed that fructose induced 100-fold increase in laccase production of *Basidiomycete* sp. I-62. *T. versicolor* is an excellent producer of laccase in fermentation of mandarin peels [84]. Glucose and cellobiose were efficiently and rapidly utilized by *Trametes pubescens* with high laccase activity [85]. Similarly, the replacement of crystalline cellulose or xylan by cellobiose increased laccase activity of *C. unicolor* by 21- and 70-fold, respectively [86]. Furthermore, in *T. versicolor* lignocellulosic material (barly bran) increased almost 50-fold laccase activity compared to the control culture with glucose [87]. In the medium with the best carbon sources (mandarine peels and grapevine sawdust), both *Pleurotus eryngii* and *P. ostreatus* strain No. 493, showed the highest laccase activity [88]. Glucose showed the highest potential for the production of laccase [89].

6.3 Influence of nitrogen sources on laccase production

White-rot fungi ligninolytic systems are mainly activated during the secondary metabolic phase of the fungus and are often triggered by nitrogen depletion [90]. Monteiro and De Carvalho [69] reported high laccase activity with semi-continuous production in shake-flasks using a low carbon to nitrogen ratio (7.8 g/g). Buswell *et al*. [72] found that laccases were produced at high nitrogen concentrations, although it is generally accepted that a high carbon to nitrogen ratio is required for laccase production. Laccase was also produced earlier when the fungus was cultivated in a substrate with a high nitrogen concentration and these changes did not reflect differences in biomass. Elisashvili *et al*. [91] observed highest laccase activity in *C. unicolor* IBB 62 in a medium with ammonium sulphate as the nitrogen source. D'Souza-Ticlo *et al*. [92] showed that well defined organic nitrogen sources such as glutamic acid and glycine were better than beef extract and corn steep liquor for laccase production. Heinzkill *et al.* [54] also reported a higher yield of laccase using nitrogen rich media rather than the nitrogen-limited media usually employed for induction of oxidoreductases.

6.4. Influence of pH on laccase production

There is not much information available on the influence of pH on laccase production, but when fungi are grown in a medium of which the pH is optimal for growth (pH 5.0), the laccase will be produced in excess [1]. Most reports indicated initial pH levels set between pH 4.5 and pH 6.0 prior to inoculation, but the levels are not controlled during most cultivations [73]. Nyanhongo *et al*. [59] reported that an

initial pH of 7.0 was the best for optimal growth and laccase production by a newly isolated strain of *T. modesta*.

6.5 Influence of temperature on laccase production

It has been found that the optimal temperature for fruiting body formation and laccase production is 25°C in the presence of light, but 30°C for laccase production when the cultures are incubated in the dark [1]. In general the fungi were cultivated at temperatures between 25°C and 30°C for optimal laccase production [73]. When cultivated at temperatures higher than 30°C the activity of ligninolytic enzymes was reduced [59].

6.6 Inhibition of laccase production

It seems as if the use of excessive concentrations of glucose as carbon source in cultivation of laccase-producing fungal strains has an inhibitory effect on laccase production [26]. An increase in the amount of glucose in the media resulted in a delay of the laccase production [69]. An excess of sucrose or glucose in the cultivation media can reduce the production of laccase, as these components allow constitutive production of the enzyme, but repress its induction when applicable [63]. A simple but effective way to overcome this problem is the use of cellulose as carbon source during cultivation [26].

6.7 Heterologous expression

Most commercial laccases are produced in *Aspergillus* hosts. The functional expression of the *Myceliophthora thermophila* laccase in *Saccharomyces cerevisiae* by directed molecular evolution was recently reported, becoming the mutant T2 an idoneous scaffold for further improvements towards biotechnological applications [48]. Another efficient expression system was developed for the basidiomycete *P. cinnabarinus* and this was used to transform a laccase-deficient monokaryotic strain with the homologous laccase gene. The yield was above 1.2 g of laccase per litre and represents the best laccase production reported for recombinant fungal strains [93].

7. Laccase immobilization

Enzymes exhibit a number of features that make their use advantageous as compared to conventional chemical catalysts. However, a number of practical problems exist that reduce their operational life-time, such as their high cost of isolation and purification, their non-reusability, the instability of their structures and their sensitivity to harsh process conditions. Many of these undesirable limitations may be overcome by the use of immobilized enzymes [94,95].

Immobilization is achieved by fixing enzymes to or within solid supports, as a result of which heterogeneous immobilized enzyme systems are obtained. By mimicking the natural mode of occurrence in living cells, where enzymes for the most cases are attached to cellular membranes, the systems stabilize the structure of enzymes, hence their activities. In the immobilized form enzymes are more robust and more resistant to environmental changes allowing easy recovery and multiple reuse [96]. Compared with the free enzyme, the immobilized enzyme has usually its activity lowered and the Michaelis constant increased [50]. These alterations result from structural changes introduced to the enzyme by the applied immobilization procedure and from the creation of a microenvironment in which the enzyme works, different from the bulk solution. Enzymes may be immobilized by a variety of methods (adsorption, entrapment, crosslinking and covalent bonding) mainly based on chemical and/or physical mechanisms.

Since the methods for the immobilization procedures greatly influence the properties of the resulting biocatalyst, immobilization strategy determines the process specifications for the catalyst [97].

Laccase immobilization was extensively studied with a wide range of different methods and substrates [50,96,98]. The adsorption of chromophoric-oxidized products on the surface of the immobilization support often leads to enzyme inactivation phenomena [99].

8. Applications of fungal laccase in biotechnology

A number of industrial applications for fungal laccases have been proposed and they include paper processing, prevention of wine decolouration, detoxification of environmental pollutants, oxidation of dye and their precursors, enzymatic conversion of chemical intermediates, and production of chemicals from lignin. Before laccases can be commercially implemented for potential applications, however, an inexpensive enzyme source needs to be made available [11]. Two of the most intensively studied areas in the potential industrial application of laccase are the delignification and pulp bleaching and the bioremediation of contaminating environmental pollutants [29].

8.1 Delignification and pulp bleaching

In the industrial preparation of paper the separation and degradation of lignin in wood pulp are conventionally obtained using ClO_2 and O_3. Oxygen delignification process has been industrially introduced in the last years to replace conventional and polluting chlorine-based methods. In spite of this new method, the pre-treatments of wood pulp with laccase can provide milder and cleaner strategies of delignification that also respect the integrity of cellulose [100]. Lignocellulose is a common substrate for laccase and the laccase ability to break down nonphenolic ligno-cellulose is provided by certain phenolic compounds acting as mediators [34]. More recently, the potential of this enzyme for cross-linking and functionalizing lignocellulose compounds was discovered.

Laccases can be used for binding fibre-, particle- and paper-boards [101]. However, different wood-decaying basidiomycetes have shown a highly variable pattern of laccase formation, and this subject requires more detailed experiments [102].

8.2 Bioremediation

Laccases have also shown to be useful for the removal of toxic compounds through oxidative enzymatic coupling of the contaminants, leading to insoluble complex structures [103]. The degradation of a variety of persistent environmental pollutants, in particular phenols, was also observed.

Phenolic compounds are present in wastes from several industrial processes, as coal conversion, petroleum refining, production of organic chemicals and olive oil production among others [104]. Immobilized laccase was found to be useful to remove phenolic and chlorinated phenolic pollutants [98]. Laccase was found to be responsible for the transformation of 2,4,6-trichlorophenol to 2,6-dichloro-1,4-hydroquinol and 2,6-dichloro-1,4-benzoquinone [105]. Laccases from white rot fungi have been also used to oxidize alkenes, carbazole, N-ethylcarbazole, fluorene, and dibenzothiophene in the presence of HBT and ABTS as mediators [106]. Isoxaflutole is an herbicide activated in soils and plants to its diketonitrile derivative, the active form of the herbicide. Laccases are able to convert the diketonitrile into the acid [107]. The study of the laccase-mediator system in the bioremediation of polycyclic aromatic hydrocarbons (PAHs) has been extensively reported [108,109]. In particular, the combination of several mediators looking for synergetic effects along with the use of natural mediators open new alternatives in this field.

8.3 Alternative applications

Laccases do not only show potential for biological delignification of pulp but also for other applications. Laccases can be applied for the treatment of and detoxification of soils containing phenolic pollutants as well as other polluted systems due to the broad substrate range of the enzyme [110]. The application of

laccase for dyeing of materials with sulfur and reduced vat dyes has been patented [41]. The use of laccase for the treatment of textile [35] and bleach-plant effluents [111] has also been investigated with success. Recently, increasing interest has arises on the application of laccase as a new biocatalyst in organic synthesis [102,112]. The use of laccase for the production and treatment of beverages and as biosensor for the estimation of phenol or other enzymes in fruit juice has also been proposed [64,113]. Recently, they have applications in other field such as in the design of biofuel cells [114].

9. Concluding remarks

Laccases are widespread in nature, being produced by a wide variety of plants, fungi and also bacteria. The functions of the enzyme differ from organism to organism and typify the diversity of laccase in nature. Laccases catalyse the oxidation of phenolic compounds whilst simultaneously reducing molecular oxygen to water. The catalytic ability of laccases has, not surprisingly, led to diverse biotechnological applications of this enzyme.
The introduction of the laccase-mediator system provides a biological alternative to traditional chlorine bleaching processes. The laccase enzyme has a wide field of application including the pulp and paper industries, the treatment of various industrial effluents, enzymatic decolouring of material and bioremediation of soils. One of the limitations to the large-scale application of the enzyme is the lack of capacity to produce large volumes of highly active enzyme. These problems can be solved with the use of recombinant organisms or screening for natural hypersecretory strains. Environmental factors influence the ability of fungi to produce high titres of laccase, and different strains react differently to these conditions. One should thus select a strain capable of producing high concentrations of a suitable enzyme and then optimise conditions for laccase production by the selected organism. It is therefore not surprising that this enzyme has been studied intensively since the nineteenth century and yet remains a topic of intense research today.

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