Scientific Bulletin. Series F. Biotechnologies, Vol. XXIII, 2019
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

LACCASE: MACRO AND MICROBIAL SOURCES, PRODUCTION, PURIFICATION AND BIOTECHNOLOGICAL APPLICATIONS

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Abstract

Laccase belongs to the blue multicopper oxidases and participates in degradation of polymers, ring cleavage of aromatic compounds and cross-linking of monomers. It is distributed in higher plants and fungi. It is present in Ascomycetes, Deuteromycete, and Basidiomycetes and abundant in lignin-degrading white-rot fungi. Lacasse has been reported to be produced by different mushrooms (Trametes, Ganoderma, Pleurotus) and by filamentous bacteria (Streptomyces) or fungi (Aspergillus). The article proposes a comparative analysis of the optimal conditions for laccase production in the case of macromycetes and some micromycetes, like fungi or filamentous bacteria, meanwhile describing the isolation, purification and characterization of the laccase produced by such organisms. All these issues will be approached through the biotechnological application of these enzymes (dye decoloration, bioremediation etc).

Key words*: laccase, macromycetes, micromycetes, bacteria, fungi.*

INTRODUCTION

Laccase was first detected in the Japanese lac tree *Toxicodendron verniciflua*. Later, it was found in other plants, in many insects and in fungi, including yeasts (e.g., *Cryptococcus*), molds (e.g., *Penicillium*), mushrooms (e.g., *Agaricus*), and white-rot fungi (e.g., *Pleurotus*) (Mikolasch et al., 2009).

Laccases play an important role in the food proccessing industry, dye decolorization, bioremediation and biodegradation, pulp and paper industry (Couto et al., 2006) and some medical applications. Laccase is an enzyme used for degradation of pesticide and insecticide, organic synthesis and pulp delignification (Couto et al., 2008), waste detoxification, biosensor and analytical applications, textile dye transformation and food technology. Recently the activity of laccases have been efficiently applied to nanobiotechnology due to their ability to catalyze electron transfer reactions without additional cofactor. The technique for the immobilization of biomolecule such as layer-by-layer, self-assembled monolayer technique and micropatterning can be used for preserving the enzymatic activity of laccases (Agematu et al., 1993).

LACCASE – BIOCHEMISTRY

In recent years, enzymes, like laccase, have gained great importance in industries. Laccases are the oldest and most studied enzymatic systems present in nature. These enzymes contain 15-30 % carbohydrate and have a molecular mass of 60-90 kDa.

Laccase is a copper-containing molecule, 1,4-benzenediol: oxygen oxidoreductases (EC 1.10.3.2) found in higher plants and microorganisms. These oxidoreductases are glycosylated polyphenol oxidases that contain 4 copper ions per molecule that carry out 1 electron oxidation of phenolic and its related compound, and reduce oxygen to water (Couto et al., 2006; Gianfreda et al., 1999). When the substrate is oxidized by laccase, it loses a single electron and usually forms a free radical which may undergo further oxidation or non-enzymatic reactions including hydratation, disproportionation, and polymerization (Faccelo et al.,1993). These enzymes are polymeric and generally contain one of each type 1, type 2 and type 3 copper

center/subunit where the type 2 and type 3 are closer together forming a trinuclear copper cluster.

Laccases are divided into "low-redox potential" and "high-redox potential", depending on the structure and properties of the copper center.

The high-redox potential laccases occur mainly in basidiomycetes, especially white-rot fungi, the low-redox potential laccases seem to be widely distributed in molds, bacteria, insects, and plants.

Unlike most enzymes, laccases have the ability to display their activity on a wide range of substrates like monophenols, diphenols, methoxyphenols, polyphenols, aromatic amines, benzenethiols and even some inorganic compounds such as iodine (Burlacu et al., 2018).

Given their versatility and broad substrate specificity, laccases, as a family of copper-containing oxidases catalyzing a variety of oxidations, could become one of the most important biocatalysts in fungal biotechnology. Because of this, their biochemical properties and molecular evolution are of considerable interest and have been summarized in several reviews (Mikolasch et al., 2009).

Laccase activity was determined using guaiacol as substrate. The reaction mixture contained 1 ml enzyme sample, 3 ml sodium acetate buffer (10 mM, pH 5.0) and 1 ml guaiacol (2 mM). The mixture was incubated at 30^{o}C for 15 min. The changes in absorbance due the oxidation of guaiacol in the reaction mixture were recorded by spectrophotometer at 450 nm, with a molar extinction coefficient for guaiacol (Ɛ450 = 6740 M-1 cm^{-1}). One unit of enzyme activity (U/ml) is defined as the amount of enzyme that oxidized 1 µmol of guaiacol per minute (Nicolcioiu et al., 2018).

MACROBIAL SOURCES OF LACCASE

Research studies in recent decades have been focused on the different mushroom species, which belong particularly to Macrofungi (Macromycetes). Ascomycetes and Basidiomycetes are two of the most important group of Macrofungi that have been intensively investigated regarding various aspects. Enzymatic activity research of these mushrooms was, is and will be one of the important topics for understanding their physiological and biochemical features in order to emphasize the considerable potential for biotechnological and industrial applications.

Laccase is one of the most studied enzymes, because, according with Krupodorova et al. (2014), its positive reaction was detected in 21 species, frequently in 2-4 days after inoculation. It formed a reddish-brown zone around the mycelium. The most active producers of laccase were *Lentinus edodes*, *L. luscina* and *Coprinus comatus* (the latter two mentioned species are referred to soil saprotrophic). Krupodorova et al. (2014) mentioned laccase presence in two out of four brown-rot species (*Agrocybe aegerita* and *Fomitopsis pinicola*). Souza et al. (2008) found silencing genes of laccase in the genome of brown-rot microorganisms. Investigations showed laccase activity for the species *L. luscina, Crinipellus schevczenkovi, A. aurea, Hypsizygus marmoreus, L. schimeji, Oxyporus obducens* and *S. litschaueri* for the first time.

The existence of laccase has been detected in earlier studies in similar species: *A. agerita*, *C. comatus*, *Fomes fomentarius*, *Fomitopsis pinicola, Flamulina velutipes, Ganoderma lucidum*, *G. applanatum, Grifola frondosa, L. edodes*, *P. eryngii* and *P. ostreatus*. This enzyme has been found in *P. djamor, H. myxotricha, L. sulphureus* and *T. versicolor* (Krupodorova et al., 2014).

Nicolcioiu et al. (2018) research regarding laccase activity has lead to the conclusion that among all tested isolates *Pleurotus ostreatus* var. 'Florida', *Trametes versicolor* and *Ganodrema applanatum,* the fungal strains were more efficient in laccase production than *Laetiporus sulphureus* and *Flammulina velutipes*.

MICROBIAL SOURCES OF LACCASE

Considering microbial sources, laccase is produced by a large variety of bacteria (Table 1) and filamentous fungi (Table 2).

In lower fungi, as *Zygomycetes* or *Chytridiomycetes*, the production of laccase was not demonstrated (Baldrian et al., 2006).

There are also laccase-producing yeasts, like *Kluyveromyces dobzhanskii, Pichia manshurica* (Wakil et al., 2017) and *Cryptococcus neoformans*, but they have far fewer known representants than other classes of microorganisms.

Table 1. Bacterial laccase sources (after Desai and Nityanand, 2011)

Sources	References
Bacteria	
Azospirillium lipoferum	Givaudan et al., 1993; Faure et al., 1994
Bacillus subtilis	Martins et al., 2002
S. maltophilia AAP56	Galai et al., 2009
Streptomyces coelicolor	Dube et al., 2008

Table 2. Fungal laccase sources (after Baldrian, 2006)

Sources	References
Fungi	
Aspergillus nidulans	Scherer and Fischer, 1998
Botrytis cinerea	Slomczynski et al., 1995
Chalara paradoxa	Robles et al., 2002
Chetomium thermophilum	Chefetz et al., 1998
Magnaporthe grisea	Iyer and Chattoo, 2003
Mauginiella sp.	Palonen et al., 2003
Melanocarpus albomyces	Kiiskinen et al., 2002
Monocillium indicum	Thakker et al., 1992
Myrothecium verrucaria	Sulistyaningodyah et al., 2004
Neurospora crassa	Froehner and Eriksson, 1974
Ophiostoma novo-ulmi	Binz and Canerascini, 1997
Podospora anserina	Durrens, 1981
Rhizoctonia solani	Wasaki et al., 1967
Trichoderma giganteum	Wang and Ng, 2004

The properties of laccase includes: extracellular localization, molecular weight of approximatively 60 to 70 kDa (typical in fungi), isoelectric pH point between 3.0 and 7.0, optimal pH in fungi between 3.6 and 5.2 (highly dependent on the substrate), usually monomeric structure (homodimeric laccases were found too in *Gaeumannomyces graminis, Monocillium indicum, Podospora anserina*), optimal temperature around 55-60°C (for *B.cinerea*). In spectrophotometry, purified laccase has a blue appearance around 600 nm.

Bacterial laccases have a higher thermostatbility and halotolerance than fungal laccases and are thought to be more valuable in dye decolorization, biofuel production and biobleaching.

There are some actinomycetes with lignolytic-activity, like *Trichoderma* sp. or *Botryosphaeria* sp., that displayed laccase production.

The laccase produced by *Monocillium indicum* was the first *Ascomycetae* laccase characterized, that had similar immunological features with *Coriolus versicolor* and *Agaricus bisporus* laccases and with lignin peroxidase activity similar to *Phanerochaete chrysosporium* laccase (Thakker et al., 1992; Shraddha et al., 2011).

Pseudomonas putida was revealed to be a laccase-producing bacteria, with the attribute of decolorizing synthetic dyes and industrial effluents.

Streptomyces cyaneus and *Streptomyces ipomoea* exhibit laccase activity, the former having an activity tens of times higher (Margot et al., 2013). Although it is known that *Streptomyces griseus* is also a producer of laccase, in the cited assessment it did not show this kind of enzymatic activity - the localization of the laccase could be responsible for the absence of activity detected.

A fungal laccase, produced by and isolated from the Ascomycete *Chaetomium* sp., was characterized with the ability to decolorize different dyes, even in the presence of high concentrations of sodium chloride (Mtibaa et al., 2017). The purified laccase from this fungus is able to degrade or transform various synthetic dyes, such as Acid Orange, Direct Red, Direct Blue and RBBR (Remazol Brilliant Blue R), with and without mediators.

Another laccase, able to decolorize synthetic dyes, is the one produced by *Spirulina platensis*, a cyanobacterium. Afreen et al. (2017) purified and characterized the laccase from this *Spirulina* genus and evaluated its decolorization property on Reactive Blue 4, the results indicating an almost complete decolorization, without any mediators.

A psychrotolerant bacterial strain of *Serratia marescens* was studied for its laccase production (Kaira et al., 2015). It was proved to synthesize laccase even under extreme

conditions, which is likely to be beneficial for biotechnological applications.

The laccase obtained from Ascomycetae *Myceliophtora thermophila* is suitable for industrial pulp bleaching and delignification (Babot et al., 2011).

ISOLATION, PURIFICATION AND CHARACTERIZATION OF LACCASE

Isolation of laccase

When the enzyme is immobilized, it becomes more resistant to alteration in the environment, allowing easy recovery and reuse of enzyme for multiple purposes. That is why researchers are moving towards the efficient methods of immobilization that influence the properties of the biocatalyst. Laccase has been studied with a wide range of different immobilization methods and substrates (Shraddha et al., 2011).

A variety of methods include immobilization on polyamide matrices, on glass supports, on epoxy-activated carriers, on magnetically separable silica spheres, on magnetic chitosan microspheres, on nanoparticles and on kaolinite. Laccase may be immobilized by entrapment in alginate - chitosan microcapsules or in Cu-Al and Cu-alginate beads. In most cases, immobilization lead to increased enzyme stability and improved resistance to changes in temperature and pH (Mikolasch et al., 2009).

Purification of laccase

Ammonium sulfate is being used for the enzyme purification. Researchers have found much more methods such as protein precipitation by ammonium sulfate, anion exchange chromatography, gel filtration chromategraphy and desalt/buffer exchange of protein. Single-step laccase purification from *Neurospora crassa* takes place by using celite chromatography and 54 fold purifycation was obtained with a specific activity of 333 Umg^{-1} (Grotewold et al., 1998). Laccase from LLP13 was first purified with column chromategraphy and then purified with gel filtration (Kiiskinen et al., 2004a; Kiiskinen et al., 2004b). Laccase from *T. versicolor* is purified by using ethanol precipitation, DEAE-Sepharose, Phenyl-Sepharose and Sephadex G-100 chromatography which is a single monomeric laccase with a specific activity of 91.443 Umg^{-1} (Hess et al., 2002). Laccase from *T. versicolor* is purified with ion exchange chromatography followed by gel filtration with a specific activity of 101 UmL^{-1} and 34.8-fold purification (Cordi et al., 2007). Laccase from *Stereum ostrea* is purified with ammonium sulfate followed by Sephadex G-100 column chromatography with 70-fold purification (Viswanath et al., 2008). Laccase from fruiting bodies is purified with ammonium sulfate precipitation with 40-70% saturation and DEAE cellulose chromategraphy then 1.34 and 3.07 fold purification is obtained, respectively (Shraddha et al., 2011).

Laccase can be immobilized on different pyrolytic graphite (best support), ceramics supports and on a carbon fiber electrode where it acts as a biosensor. At the 12th day, maximum laccase activity of 40.774 UL^{-1} was achieved (Minussi et al., 2007). An optical biosensor is fabricated by using stacked films for the detection of phenolic compounds; 3-methyl-2-benzothiazolinone hydrazone (MBTH) was immobilized on a silicate film and laccase on a chitosan film (Alimin Abdul et al., 2009).

Characterization of laccase

Laccase was first characterized when it was extracted from the Japanese lacquer tree *Rhus vernicifera* in 1883. Later, in 1896, it was demonstrated that laccases were also present in fungi (Desai and Nityanand, 2011).

In higher plants, laccases can be found in *Rhus vernicifera, Rhus succedanea, Lactarius piperatus, Prunus persica*, *Acer pseudoplatanus, Chaetomiaceae* sp.

According to Takao Saito et al. (2003), the purified laccase produced one band on an SDS-PAGE gel at the apparent molecular mass of approximately 73 kDa (Figure 1A).

This laccase was used in gel filtration chromatography on a HiLoad 16/60 Superdex 200 pg column, the molecular mass of the native enzyme being estimated at 80 kDa. The isoelectric point (pI), determined by analytic isoelectric focusing, was 3.5 (Figure 1B). This pI is similar to that of the laccase from the basidiomycete PM1 (Coll PM et al., 1993), which has an acidic isoelectric point.

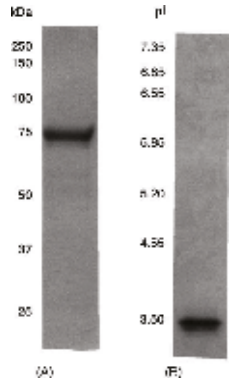

Figure 1. SDS-PAGE (A) and IEF (B) of the purified strain I-4 laccase (Takao Saito et al., 2003)

To determine the state of its catalytic center, the laccase was characterized spectroscopically. The purified laccase had a blue color, typical of copper-containing proteins. The UV-VIS spectrum of laccase showed a peak absorption at about 611 nm, typical for the type I Cu(II), that is responsible for the deep blue color of the enzyme. A shoulder at about 333 nm suggests the presence of type III binuclear Cu(II) pair (Eggert et al., 1996). The EPR spectrum of the laccase showed the superimposed signals from type I and type II Cu(II), each in a different coordination environment. The parameters of the type I Cu(II) signal were gII = 2.21 and AII = 8.3×10^{-3} cm^{-1}, and those of the type II Cu(II) signal were gII = 2.25 and AII = 1.93×10^{-2} cm^{-1}. These spectral characteristics are similar to those of other blue copper proteins that have four copper atoms (Dedeyan et al., 2000; Shin KS et al., 2000).

APPLICATIONS OF LACCASE IN BIOTECHNOLOGY

Food Processing Industry

In the food industry, laccase is used for the elimination of undesirable phenolic compounds in wine and beer stabilization, in baking, in juice processing and in the bioremediation of wastewater (Shraddha et. al., 2011).

Wine browning, due, primarily, to enzymatic and chemical oxidation of phenolic compounds, represents one of the most unwanted processes that can occur in wine-making. During the crushing of the grapes, the release of laccase from *Botrytis cinerea* affected the beans in the must, thus resulting in a significant reduction of phenolic compounds. The important polyphenols in wine, including their major classes (phenolic acids, catechins, anthocyanins, tannins and stilbenes), are converted to the corresponding quinones, that will pass into dark-colored polymers. These polymers are insoluble in water and aqueous solutions and precipitates from the must and wine. Moreover, the oxidation of the phenolic compounds can adversely affect the sensorial and nutritional properties of the wine.

The storage of beer depends on various factors, such as the haziness, the oxygen content, the temperature. The haziness is caused by small amounts of proanthocyanidins, which are naturally occurring polyphenols and proteins that could cause precipitation. This type of complex is known as “cold haze” and occurs during chilling – can be redissolved at room temperature or at higher temperatures. Even the products that do not have this disorder when packaged, could form it during long storage. The usage of laccase for the oxidation of polyphenols as an alternative to traditional therapy has been tested many times. Also, laccase is used to eliminate the oxygen at the end of the production process of beer (Osma et al., 2010).

Sugar beet pectin is a functional aliment, which can form thermo-irreversible gelatins. These types of gelatin are very interesting and can be used in the food industry because they can be warming while retaining the gel structure. Compared with peroxidase, which is used as a food additive, laccase proved to be more effective and safer for consumption.

One of the biggest problems in the fruit juice processing is the enzymatic and the chemical browning. The color and the taste of the fruit depends on the phenolic compounds, which should be selectively removed from the composition, in order to prevent any alteration of taste, flavor, and color – attributed to oxidation of polyphenols. To prevent the discoloration of the fruit beverages, by replacing the chemical adsorbents, enzymes are being used, such as laccase. This enzyme has the potential to eliminate unwanted phenols responsible for browning and disorder in many beverages, such as fruit juice, beer, and wine.

In the bread-making process, it is known that additives are added to improve bread and bread dough, from which results improved texture and flavor, a larger volume and longer freshness. In recent years, enzymes have been increasingly used as enhancing agents, including laccase. Even if the laccase used in the preparation of doughs could be of any origin, inclusively vegetal, it is preferable to be of microbial origin, because it is easier to handle and produce on a larger scale (Si, 2001).

Dye Decolorization

In the textile industry, there are used numerous chemicals (which vary from organic to inorganic compounds) and a large volume of water. These chemical compounds make the dyes resistant to fading when exposed to water, to light, or to other chemicals. Laccase is able to degrade this kind of dyes, which is why there were created industrial processes based on laccase treatment of the syntethic dyes (Dominguez et al., 2005; Hou et al., 2004).

Blanquez et al. (2004) manipulated *Trametes versicolor* into pellets, with which they treated black liquor, to decompose its aromatic compounds, to reduce its color and its chemical oxygen consumption. Their results showed that the color and the content of aromatic compound was decreased up to almost 80% and the COD (chemical oxygen demand) was reduced up to 60%. Their conclusions were the following: *Trametes versicolor* produces laccase, which is able to completely decolorize five dyes (Acid Red 27, Reactive Blue 15, Congo Red, Reactive Black 5 and Acid Orange 6) without absorbing them and to partially decolorize other three dyes (Remazol Brilliant Blue R, Brilliant Yellow and Brilliant Red) while absorbing them. Also, they discovered that the toxicity of a few of the dyes remained the same, while the toxicity of others decreased all the way to becoming non-toxic.

The laccase-based hair dyes are less irritant and easier to handle that standard hair dyes, because the enzyme replaces the oxygen peroxide in the formula.

Laccase is also used in the dechlorination processes. In the presence of xylidine, which is a laccase inducing agent, that modifies the enzymatic activity by increasing it, the concentration of dissolved oxygen is reduced (Unal et al., 2001).

Romero et al. studied *Stenotrophomonas maltophilia* bacteria and found that it is able to decolorize Congo Red, Toluidine Blue, Methylene Blue, Methyl Green and Methyl Orange (Shraddha et al., 2011).

Bioremediation and Biodegradation

Laccase is used in bioremediation, biosolubilization, and desulfurization, in the production of biosensors and biofuels and also in the production of fiberboards. Laccase is used for degradation of industrial wastes, like paper, oil, leather, pharmaceutical, pesticides because of her oxidizing ability towards phenolic and non-phenolic compound (Senthivelan et al., 2016).

The presence of phenols in the industrial wastewater attracted interest in the application of bioremediation processes and their treatment with laccase. The presence of phenolic compounds in drinking water is a real danger. The distillery wastewater is generated during the production of ethanol by the fermentation of sugar cane molasses. This produces a major environmental impact, due to the high content of soluble organic matter and due to its dark brown color. Fungal laccase showed better properties in the reduction of total phenolic compounds in color than the laccase from other sources.

The intensive use of pesticides and the fast industrialization cause the pollution of the soil, the water and the air. Chemical substances persists in the environment and can be removed with high difficulties. *Trametes versicolor* and *Pleurotus ostreatus* are used in the bioremediation of the soil and in the degradation of polychlorinated biphenyls, pyrocatechines, protocatechuic acid and benzoic acid (Udayasoorian et al., 2005).

Ahn et al. (2002) conducted an experiment with two types of soil and *Thermopsis villosa* laccase, each sample with different percentage of water in it. They used free laccase and immobilized laccase (on montmorillonite) for this research. *Thermopsis villosa* laccase has proved to be able to degrade dichlorophenol

from the soil, most likely from the immobilized form.
The laccase from another source, *Cerrena unicolor*, had the capacity of decreasing the lignin amount from sugarcane bagasse, up to almost 40%, within 24 hours (D'Souza-Ticlo et al., 2009).

Pulp and Paper Industry
The industrial manufacture of paper involves the separation and degradation of lignin, by treating the wood pulp with ozone or chlorine dioxide. To reduce the pollution of chlorine-based processes, an alternative involved laccase-based processes, that provided a milder and cleaner delignification (Kunamneni et al., 2007).
TEMPO oxidation by laccase is used in the production of hydrophobic cotton fibers and hydrophobic jute fibers (with dodecyl gallate in this case).

Medical applications
The biosensors used in biomedical engineering are also produced with laccase. In the determinations of chemical compounds, in nanobiotechnology and biomedicine and cosmetics is used laccase too.
The effect of poison ivy dermatitis, which is caused by urushiol, can be reduced with laccase treatment. It has been shown that laccase can oxidize the urushiol to a quinone derivative, that is innocuous (Madhavi et al., 2009).
Some important drugs, like anti-cancer drugs, antioxidants, hormones and hormone derivatives are prepared with the help of laccase (by oxidation) and are added to some cosmetics (Senthivelan et al., 2016).
Laccase can also oxidize iodide to iodine, which is used as a disinfectant.

CONCLUSIONS

Laccases are the versatile enzymes which catalyze oxidation reactions coupled to four-electron reduction of molecular oxygen to water. They are multicopper enzymes which are widely distributed in higher plants and fungi. They are capable of degrading lignin and are present abundantly in many white-rot fungi. They decolorize and detoxify the industrial effluents and help in wastewater treatment. They act on both phenolic and nonphenolic lignin-related compounds as well as highly recalcitrant environmental pollutants which help researchers to use them in various biotechnological applications.
They can be used and act as a biosensor in textile industries, paper and pulp industries, xenobiotic degradation, and bioremediation. Laccase has been applied to nanobio-technology which is an increasing research field and catalyzes electron transfer reactions without additional cofactors.
Recently, several techniques have been developed for the immobilization of biomolecules such as micropatterning, self-assembled monolayer, and layer-by-layer technique which immobilize laccase and preserve their enzymatic activity.

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