

## REVIEW ARTICLE

## Fungal Laccases – Properties and Applications: A Review

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Received 09 Dec 2013; Revised 05 Apr 2014; Accepted 18 Apr 2014

**ABSTRACT**

Laccases are metal containing glycosylated blue oxidases that are widely distributed in fungi, higher plants and bacteria. It exists as a monomer, dimer or tetramer and uses copper as co-factor and molecular oxygen as co-substrate. It's found that while most of the multicopper oxidases are highly stereospecific in nature where the fungal laccases are not. It has been the subject of study since the end of the last century. Laccases are diverse group of enzymes having great biotechnological potential and high market expectative due to their broad substrate specificity in the diverse fields of industrial applications such as dye decolorization, bioremediation and biodegradation of xenobiotics such as chlorophenols, herbicides, PAH and benzopyrene. However, one of the limitations for the large-scale application of laccase is the lack of capacity to produce large volumes of highly active enzyme at an affordable cost. Enzymes of terrestrial microorganisms have been widely studied whereas that of marine microorganisms which has a vast resource of useful enzymes is yet to be exploited to its maximum potential. This review deals about the distribution, mechanism of action, production and characterization of laccases with special reference to that of marine fungal lacasses. It will also enlighten the immobilization of laccases and its applications especially in the field of dye degradation and treatment of xenobiotics. Thought it has been studied from the last century it remains a topic of research till today due to its enormous hidden potential.

**Key words:** laccase , lignin degrading enzyme, marine-fungi, biodegradation.

**INTRODUCTION**

Laccases (benzenediol:oxygen oxidoreductases; EC 1.10.3.2) are copper containing lignin degrading enzymes. These multi-copper enzymes belong to the group of blue oxidases. Laccases are extracellular glycoproteins, it uses copper as a co-factor and molecular oxygen as co-substrate. Under different cultivation conditions, laccases are expressed as their isoenzymes<sup>[1]</sup>. In the wild, they become resistant to the attack of protease by exhibiting a high degree of glycosylation. Depending on the redox potential (RP), the laccases are classified in to two groups i.e. Low RP (0.4-0.6 V) and High RP (0.6-0.8V)<sup>[2]</sup>. It catalyzes the oxidation of a variety of phenolic compounds, as well as diamines and aromaticamines, which is accompanied by reduction of molecular oxygen to water<sup>[3]</sup>. Laccase contains four copper atoms which are reduced while the substrate is oxidized. When the laccase receives 4 electrons, it reduces the molecular oxygen to water and returns to the native state. Lignin is an amorphous

polysaccharide with no specific molecular structure, laccase are capable of degrading lignin i.e. they undergo a non specific type of cleavage. This non specificity of laccase makes it to acts on a wide variety of substrates. It has been found that the syringaldazine [4-hydroxy-3,5-dimethoxy benzaldehyde azine] could be oxidized only by laccase enzyme<sup>[3]</sup>. Laccase can be made to act on non phenolic substrates with the help laccase mediator systems. Due to their increased stability and a wide range of substrate specificity, it gains its significance in industrial applications which includes denim bleaching, decolorization and detoxification of dye-containing textile effluents, lignin related compounds and biobleaching of pulp for paper industries<sup>[4]</sup>. Laccases have been applied in the removal of large number of environmental pollutants, such as alkenes, chlorophenols, dyes, herbicides, polycyclic aromatic hydrocarbons and benzopyrene<sup>[5,6]</sup>. When compared with peroxidases, laccases are more stable, versatile and dependent only on

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atmospheric oxygen. These enzymes can be used effectively in bioremediation, detoxification, pulp and paper industry and also for removal of Phenolic pollutants and polycyclic aromatic hydrocarbons from waste water and contaminated soil.

### OCCURRENCE OF LACCASE

Laccases are ubiquitous in nature. The presence of laccases has been found in higher plants, fungi, insects and recently in some bacteria such as *S.lavendulae*, *S.cyaneus*, and *Marinomonas mediterranea* [7,8]. Depending on the source of laccase they can be broadly classified into two major groups viz., those from higher plants and those from fungi [9]. Laccase from plants have been identified from trees and vegetables such as cabbages, potatoes, apples etc. [10]. Fungi seems to hold more laccase producers when compared to that of plants. The fungal laccases are of secretory type, they are produced in the cells and then secreted and accumulated outside the hyphal filaments. All the classes of fungi produce laccases. More than sixty fungal strains belonging to Ascomyceteous, Deuteromyceteous and Basidiomyceteous fungi have been identified [11]. Basidiomycetes such as *Phanerochaete chrysosporium*, *Theiophora terrestris* and *Lenzites, betulina* [12] and white-rot fungi such as *Phlebia radiate* [13], *Pleurotus ostreatus* [14], and *Trametes versicolour* [15] also produce laccase. Many *Trichoderma* species such as *T. atroviride*, *T. harzianum* [16], and *T. longibrachiatum* acts as a source of laccase.

### STRUCTURE OF LACCASE

Laccase is a multi copper containing enzymes that catalyzes the oxidation of phenolic compounds and electron rich substrates. Laccases are found mostly as monomers, dimers or even as a tetramer and are generally extracellular glycoproteins. The glycoproteins have an average molecular mass of 60-70 KDa and their carbohydrate content varies from 10-20 %. The covalently linked carbohydrate moiety of the enzyme was formed by mannose, N-acetyl glucosamine and galactose. Each monomer contains four copper atoms. The four copper atoms differ in their electro paramagnetic resonance (EPR) signals. These copper atoms form three redox sites viz., T1, T2 and T3. T1 and T2 sites contains one copper atom each and T3 contains two copper atoms which forms the trinuclear cluster [17]. The apoprotein is made up of three domains. In the holoenzyme T1 copper is seen in domain 3, where the tri-nuclear cluster is present in between domain 1 and 3. T1 exists in

the oxidized state and laccase can be observed as blue color at 610nm which is due to the formation of copper-cysteine bonds [18,19]. At T1 site the oxidization takes place. The T1 copper contains two histidines and one cysteine. T2 is detectible by EPR and colorless in nature, which is coordinated by two histidines. Both T1 and T2 are paramagnetic in nature where T3 is diamagnetic in nature. At the oxidized state T3 forms a binuclear center and could be absorbed at 330nm. T3 is coordinated by six histidines [20,21]. It exhibits a weak absorption at 600 nm and has no EPR signal [22]. The tertiary structure of laccase was determined X-ray crystallography [23]. Under different cultivable conditions several fungal laccases are expressed as multiple isoenzymes [24].

### PRODUCTION OF LACCASE

Laccase are produced during the secondary metabolism of fungi on natural substrates or submerged culture [25]. Generally laccase is produced extra cellularly by fungi [26] but with the exceptions of Zygomycetes and Chytridiomycetes [27]. Saprophytes and Basidiomycetes hold the large number of fungi which are well known to produce laccase in variable quantity [28]. Since the laccase has a wide range of applications it needs to be produced in higher quantity [29]. The major factors that governs the laccase productions are discussed below.

#### Carbon and Nitrogen Sources:

Based on the fungal strain, laccase production varies when glucose is provided as carbon source. Since it could be easily metabolized by fungus it plays a positive role in *Galerina* sp but not with that of *Trametes pubescens* [30], *Trametes versicolor* [31] and *Phlebia* sp. [32]. Fructose, lactose, glycerol and cellobiose also serve as a potential carbon source. Like that of carbon, nitrogen is also important for the production. In *Pleurotus ostreatus* usage of nitrogen at low levels gives the better results [33] whereas high concentrations are needed for *Trametes gallica* [34]. These differences in the requirements of carbon and nitrogen sources differ from strain to strain widely. They can also be fulfilled by the use of agricultural wastes, as they are cheaper and seems to reproduce better results.

#### pH

Depending on the substrate the optimum pH of laccase varies greatly and it ranges from 3-7 for fungal laccases but it may extend up to 9 if it is from plant sources [35]. In general the laccase activity forms a bell shape profile, this variation

depends on the substrate. At higher pH the substrate oxidation is higher due to the potential difference between the phenolic substrate and T1 copper site, while the hydroxide ion binds to T2 and T3. It has also been suggested that the hypersecretion of laccase occurs when they are cultured in the pH range of 5, though the optimum pH is between 4.5-6 for enzyme production. Syringaldazine had been used as a substrate to determine the effect of pH and enzyme activity was observed in the range of 3.0–8.0 [36]. The optimum pH becomes more acidic between 3-5 when ABTS is used as a substrate [37].

### Temperature

The optimum temperature of laccase varies greatly depending on the strain from which it is produced. The optimal temperature for laccase production falls in the range of 25-30°C [35]. When the temperature is raised beyond 30°C, the activity of the enzymes reduces accordingly. However preincubation of the enzyme at 40-50°C results in the increased efficiency of laccase activity. D'Souza-Ticlo in 2009 [38] recorded the optimum temperature for laccase in the crude culture filtrate was 60°C and 70°C for purified Lac II. *C.unicolor* a terrestrial isolated strain showed activity at 40°C [39].

### Agitation

Agitation plays a key role in the production of enzymes in general. When they are cultivated in conical flasks, it needs to be baffled so as to ensure the higher oxygen transfer. When the fungus was grown in stirred tank reactor, the agitation causes damages to the fungal mycelia and results in decreased production of laccase [40]. But in the case of laccase from *T.versicolour* it has been found that they are not capable of affecting the production of laccase [41].

### Role of Inducers and Inhibitors of Laccase

Higher concentrations of laccase are produced while using older non-induced cultures. In some cases where the laccase is required in large quantities, the production can be increased by the use of inducers which comprises of phenolic or aromatic compounds related to lignin or its derivatives. The various response elements in the promoter regions of laccase genes can be induced by certain xenobiotics and heavy metals [42]. Many inducers that were able to increase the expression of laccase have been described [11,43,44]. It includes veratryl alcohol [45], 2,5-Xylidine [46], guaiacol [47], gallic acid and ferulic acid [48] ethanol [49] and copper. In case of lignin degrading basidiomycete

CECT 20197, laccase production was increased up to 10 folds with the usage of veratryl alcohol. Like that of basidiomycetes, in case of ascomycetes *Botryosphaeria* sp, which produces two extracellular laccases, the laccase production was increased up to 100 and 25 folds respectively in the presence of veratryl alcohol [50]. The syringaldazine and guaiacol which were generally used for the screening of laccase producing fungi are also capable of playing the role of inducers. Syringaldazine when used at the concentration of 0.11µm acts as the most efficient inducers. Laccase production increases up to 20 folds when a non lignin related compound, ethanol was introduced into the medium. The ethanol triggers the laccase gene expression. Though the copper is toxic to most of the micro organisms it is needed in lesser quantity to promote the laccase production. Copper which is the part of laccase itself acts as an inducer in *Pleurotus ostreatus* [14]. Like that of inducers several compounds are able to inhibit the growth of fungi or production of laccase which includes azide, cyanide and fluorides. These inhibitors binds to the T2/T3 and prevents the electron transfer from T1 thus reduces the enzyme production. Other than those metal ions and fatty acids denatures the protein. Sulphanic acid, malonic acid and EDTA acts as effective inhibitors.

### IMMOBILIZATION OF LACCASES:

Immobilization of an enzyme is defined as its attachment to an insoluble support [51]. Their high enzyme activity, selectivity and wide range of substrate specificity combines together and makes them to perform complex chemical processes under natural conditions as well as experimental conditions [52]. Their stability and high production costs are the major limitations for using it in industrial scale purposes [53,54]. These limitations can be overcome by immobilization [55]; this results in the increased stability and durability of the enzyme and also makes the enzyme to be reused. Though several methods are available for enzyme immobilization on a solid support, selection of the methods involves a great deal which determines the process specification. This includes chemical and physical methods. Chemical immobilization methods mainly include, adsorption, enzyme attachment to the matrix by covalent bonds, cross-linking between enzyme and matrix, and enzyme cross-linking by multifunctional reagents. Physical methods involve the entrapment of enzyme molecules within a fiber or an insoluble gel matrix and/or

within a reverse micelle and encapsulation in an organic or inorganic polymer support<sup>[56]</sup>.

### Entrapment and Encapsulation:

Physical entrapment seems to be having more advantages when it is immobilized on a polyacrylamide gel. In case of entrapment the enzyme is immobilized on a porous solid matrix, such as polyacrylamide, collagen, alginate or gelatin. It is the easiest and causes no structural changes of the enzyme. Encapsulation is similar to that of entrapment wherein both the cases the enzymes are trapped and contact with the environment is prevented and the major limitation is mass transfer<sup>[57]</sup>. In microencapsulation, the enzyme was surrounded by a semipermeable membrane for example polymers or inorganic materials<sup>[58]</sup>. The layer-by-layer (LbL) technique is another emerging microencapsulation technique which seemed to be a promising one in the recent years<sup>[59,60]</sup>.

### Adsorption:

Based on ionic and/or other weak forces of attraction laccase was immobilized on a support by adsorption. It is an easier and inexpensive method and attains a great significance in commercial applications<sup>[57,61]</sup>. The pH, ionic strength of the medium and the hydrophobicity of the support determines the fate of the immobilized enzyme<sup>[62-66]</sup>.

### Covalent binding:

It is the most attention-grabbing as well as extensively utilized method for industrial applications of laccase. Here the reaction takes place between the activated chemical groups on the support and nucleophilic groups on the enzyme. Covalent binding depends on the inert or commercially available active carriers<sup>[67]</sup>. Different supports used here, includes silica-based supports such as kaolinite or mesoporous silica nanoparticles<sup>[68-70]</sup>.

## APPLICATIONS OF LACCASE

Laccases degrades a wide range of compounds due to its less substrate specificity which includes phenolic and non-phenolic compounds. They are also capable to detoxify environmental pollutants. Laccase has many applications in agricultural, medicinal and industrial areas. Industrial effluents such as effluents from the paper industry, pulp, textile & petrochemical industries are acted up on by laccase in order to degrade the hazardous compounds present in them, which may cause a severe effect when released in to the environment as such. They are also used in the medical

diagnostics and for the removal of some herbicides and pesticides from soil.

### Decolourisation of textile dyes:

More than 10,000 different dyes and pigments are used in dyeing and printing industries worldwide. About 10% of the dyes gains its entry into the environment through various water channels and affects or alters the physical as well as chemical properties of the water. Biological decolorization of dye using microorganisms is an Eco friendly and cost effective as well as alternative to chemical methods. The ability of laccases to act on chromophore compounds such as azo, triarylmethane, anthraquinonic and indigoid dyes leads to the suggestion that they can be applied in industrial decolorization processes. Laccase from *pleurotus florida* was screened and characterized and its ability to decolourize dye in in vitro condition was studied<sup>[71]</sup>. A several fold enhancement in laccase production was found during treatment of colored effluents from textile waste. The predominant laccase producers, *Peniophora* sp.hpF-04 and *Phellinus* sp. hpF-17 were employed for the decolorization of textile dyes in liquid cultures. Strain *Peniophora* sp. hpF-04 showed more than 80% of decolorization of six out of 14 textile dyes, where as *Phellinus* sp.hpF-17 showed nine out 14 dyes<sup>[72]</sup>. Donna D'Souza-Ticlo<sup>[73]</sup> studied the effects of various synthetic medium components and their interactions with each other ultimately impact laccase production in *Cerrena unicolor* MTCC 5159. Concentration of carbon and nitrogen acted antagonistic with respect to laccase production. A combination of low nitrogen and high carbon concentration favored both biomass and laccase production. Addition of a surfactant such as Tween 80 positively impacted biomass and increased the laccase activity. Synthetic dyes were decolorized by the *T. modesta* laccase most efficiently under acid conditions (pH 3–6) and increased with the rise in temperature to 50–60°C<sup>[74]</sup>. A laccase from *Panus rudis* was produced without induction in a shaken liquid culture which degraded anthraquinone dye (Acid Green 27) without any redox mediators, as well as azo and indigo dyes (Acid Violet 7 and Indigo Carmine)<sup>[75]</sup>. Laccase obtained from *Pleurotus ostreatus*, a common fungus had shown a better decolorization and degradation of blue HFRL dye, which is present in most of the textile mill effluents. The Decolorization and Detoxification of Textile Dyes with a Laccase from *Trametes hirsute* and also against indigoid, azo, and anthraquinonic dyes

were tested. Laccase immobilized on alumina enhanced the thermal stabilities of the enzyme and its tolerance against some enzyme inhibitors, such as halides, copper chelators, and dyeing additives. Treatment of dyes with the immobilized laccase reduced their toxicities up to 80% (anthraquinonic dyes). Laccase from lignolytic basidiomycete fungus, *Funalia trogii* showed complete rhemazol brilliant blue - R, decolorization 2,2'-azino-bis (3-ethylbenzthiazoline)-6-sulfonate.

#### Degradation of PAH :

Laccases have also shown their ability for the removal of toxic compounds through oxidative enzymatic coupling of the contaminants, leading to insoluble complex structures [76,77]. Immobilized laccase was found to be useful to remove phenolic and chlorinated phenolic pollutants [78,79]. Laccase transformed 2,4,6-trichlorophenol to 2,6-dichloro-1,4-hydroquinol and 2,6-dichloro-1,4-benzoquinone [80]. Laccases from white rot fungi oxidized alkenes, carbazole, N-ethylcarbazole, fluorene, and dibenzothiophene in the presence of HBT and ABTS as mediators [81,82]. A herbicide Isoxaflutole which exists as its diketone nitrile derivative (active form) is converted into the acid [83].

#### CONCLUSION

The catalytic ability of laccases has led to diverse biotechnological applications of this enzyme. Though the Laccases have immense application, the major limitation is the cost of redox mediators and the availability of laccase for industrial purpose that can be overcome by the use of genetically engineered micro organisms or selecting naturally hypersecretory strains. The hyper secretion relies on several environmental factors, so selection of a strain capable of producing high concentrations of enzyme under optimised conditions for laccase production by the selected organism is necessary so that it can be taken to several industrial applications.

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