Laccase–Production and It’s Application

Shruti S. Patel, Sanjay N. Parekh

Shree Ram Krishna Institute of Computer Education & Applied Science, Surat, India

A B S T R A C T

Laccase belongs to the small group of enzymes called the blue multi copper oxidases. Laccase is widely distributed in higher plants, fungi, bacteria. By fermentation both submerged and solid state fermentation laccase can be produced by using various carbon and nitrogen sources. 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. More recently, they have found applications in other field such as in the design of biosensors. In this review, the occurrence, structure, production, and application of laccases will be discussed.

Keywords: Laccase, sources, Production, structure, applications, Pulp and paper industry, Food industry, Bioremediation, Biosensors

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1. Introduction

Laccases (p-diphenol: dioxygen oxidoreductases; benzenediol dioxygen oxidoreductases; EC 1.10.3.2) are member of large blue copper proteins or the blue copper oxidases; other enzymes in this group are the plant ascorbate oxidase and the mammalian plasma protein ceruloplasmin [1,2]. Laccases are defined in the Enzyme Commission (EC) nomenclature as oxidoreductases which oxidize aromatic and nonaromatic compounds by a radical-
catalyzed reaction mechanism and use molecular oxygen as an electron acceptor. Laccases catalyze the oxidation of a variety of phenolic compounds, as well as diamines and aromatic amines, with concomitant reduction of molecular oxygen to water [1]. The syringaldazine [4-hydroxy-3,5-dimethoxy benzaldehyde azine] is considered the substrate oxidized only by laccase enzyme [1]. Thus, several organic compounds which contain hydroxyl, acid, or amino groups can act like substrates [3]. Recently a novel polyphenol oxidase with laccase like activity was mined from a metagenome expression library from bovine rumen microflora [3].

2. Structure of Laccase
Laccases often occur as isoenzymes or monomers that oligomerize to form multimeric complexes. Each isoenzyme has four copper atoms and is able to individually carry on the catalytic mechanism of laccases. The molecular mass of the laccase monomers ranges from 40 to 130 kDa with a covalently linked carbohydrate content of 10–25% in fungi and 20-45% in plants. The carbohydrate moiety typically consists of mannose, N-acetylglucosamine and galactose, which may contribute to the high stability of the enzymes [23,24]. Laccases contain 4 copper atoms termed Cu T1 (where the reducing substrate binds) and trinuclear copper cluster T2/T3 (electron transfer from type I Cu to the type II Cu and type III Cu trinuclear cluster reduction of oxygen to water at the trinuclear cluster). These four copper ions are classified into three categories: Type 1 (T1), Type 2 (T2) and Type 3 (T3). These three types can be distinguished by using UV/visible absorption and electronic paramagnetic resonance (EPR) spectroscopy.

Figure 1: Scheme of T1 (Cu1) and T2/T3 (Cu4/Cu2-Cu3) copper sites of laccase

Laccase Production
Laccases occur as extracellular glyco-proteins, which allows for rapid removal from fungal or bacterial biomass [25,26]. 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. [27].

Induction of laccase production
Laccase production has been found to be highly dependent on the conditions for the cultivation [26] and media supporting high biomass did not necessarily support high laccase yields [27]. Laccase production often triggered by nitrogen concentration [28] or when carbon or sulfur became limiting [26]. The addition of aromatic compounds such as 2,5-xylidine, lignin, and veratryl alcohol is known to increase and induce laccase activity [27]. The promoter regions of the genes encoding for laccase contain various recognition sites that are specific for xenobiotics and heavy metals [29]. These can bind to the recognition sites when present in the substrate and induce laccase production. The addition of certain inducers can increase the concentration of a specific laccase or induce the production of new isoforms of the enzyme [30]. The addition of ethanol as an indirect inducer of laccase activity offers a very economical way to enhance laccase production[31].

Influence of carbon sources on laccase production
The carbon sources in the medium play an important role in ligninolytic enzyme production[30]. Hatvani et al. 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 [32]. Glucose and cellobiose were efficiently and rapidly utilized by Trametes pubescens with high laccase activity [33]. Similarly, the replacement of crystalline cellulose or xylan by cellobiose increased laccase activity of C. unicolor by 21- and 70-fold, respectively [34].

Influence of nitrogen sources on laccase production
Monteiro and De Carvalho [34] 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. [28] 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.

Influence of pH on laccase production
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 cultivation [32]. It is reported that an initial pH of 7.0 was the best for optimal growth and laccase production.

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 [6]. In general the fungi were cultivated at temperatures between 25°C and 30°C for optimal laccase production [39].

Inhibition of laccase production
It seems as if the use of excessive concentrations of glucose as carbon source in cultivation of laccase producing strains has an inhibitory effect on laccase production [37]. 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 [40]. A simple but effective way to overcome this problem is the use of cellulose as carbon source during cultivation [37].

3. Purification of Laccase

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In general, plant laccases are purified from sap or tissue extract, whereas extracellular laccases are purified from culture medium (fermentation broth) of the selected organism. Various protein purification techniques are frequently used in purifying laccase. Typical purification protocol involves ultrafiltration, ion exchange, gel filtration, hydrophobic interaction, or other electrophoretic and chromatographic techniques. Affinity chromatography using a phenolic group as ligand can increase purification efficiency. The purity of laccase preparation is often measured by SDS-PAGE and by the ratio of the absorbance at 280 nm to that at 600 nm. Ammonium sulphate is being commonly used for the enzyme purification for many years. But researchers have found much more efficient methodologies such as protein precipitation by ammonium sulphate, anion exchange chromatography, desalt/buffer exchange of protein, and gel filtration chromatography. Single-step laccase purification from Neurospora crassa takes place by using celite chromatography and 54 fold purification was obtained with specific activity of 333 U mg⁻¹ [17]. Laccase from LLP13 was first purified with column chromatography and then purified with gel filtration. Laccase from T. versicolour 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 U mg⁻¹ [41]. Laccase from T. versicolour is purified with Ion Exchange chromatography followed by gel filtration with specific activity of 101 U mL⁻¹ and 34.8-fold purification. Laccase from Stereum ostrea is purified with ammonium sulphate followed by Sephadex G-100 column chromatography with 70-fold purification. Laccase from fruiting bodies is purified with ammonium sulphate precipitation with 40-70% saturation and DEAE cellulose chromatography then 1.34 and 3.07 fold purification is obtained respectively [42].

4. Laccase Application
A few laccases are at present in the market for textile, food and other industries (Table 3), and more candidates are being actively developed for future commercialization [16]. Laccase is important because it oxidizes both the toxic and nontoxic substrates. Laccases have been used in a variety of industrial and environmental applications since they can oxidize a number of natural and synthetic substrates using oxygen, producing water as the only by-product. Laccases are versatile enzymes able to oxidize recalcitrant compounds like lignin, which makes them attractive for use in various biotechnological processes. A vast amount of industrial applications for laccases have been proposed. This enzyme is very specific, ecologically sustainable and a proficient catalyst. Applications of laccases are as follows.

Nanobiotechnology
Since laccases are able to catalyse electron transfer reactions without additional cofactors, their use has also been studied in biosensors or bioreporters to detect various phenolic compounds, oxygen or azides. Moreover, biosensors for detection of morphine and codeine, catecholamines, plant flavonoids, for determination of glucose, aromatic amines and phenolic compounds and also for electroimmunoassay have been developed. The Biotechnological application of such micropatterned surfaces: the production of islands of micrometer size of extracellular matrix, where the pattern of these islands could determine the position and distribution of bovine and endothelial cells [42]. Laccase covalently conjugated to a bio-binding molecule can be used as a reporter for immunochemical (ELISA, Western blotting), histochemical, cytochemical, or nucleic acid-detection assays.

Bioremediation and Biodegradation
Laccases have many possible applications in bioremediation. Laccase may be applied to degrade plastic waste having olefin units. Likely, an oxidation of the olefin units by the LMS, could initiate a radical chain reaction, leading to the disintegration of the plastic. Also this LMS can be used to degrade polyurethanes. LMS facilitated the degradation of phenolic compounds (environmental hormones) from biphenyl and alkylphenol derivatives and also the decomposition of fluorescent brighteners [17]. Laccase may also be used to eliminate odor emitted from places such as garbage disposal sites, livestock farms, or pulp mills. Also, they could be used for decolorizing dye house effluents that are hardly decolorized by conventional sewage treatment plants. In addition to dye house effluents, laccases can decolorize waste waters from olive oil mills and pulp mills by removing colored phenolic compounds. Another potential environmental application for laccases is the bioremediation of contaminated soils, as laccases and LMS are able to oxidize toxic organic pollutants, such as various xenobiotics, polycyclic aromatic hydrocarbons (PAHs), chlorophenols, and other contaminants.

The catalytic properties of laccases can be used to degrade such compounds. 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. Immobilized laccase was found to be useful to remove phenolic and chlorinated phenolic pollutants. 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. LMSs have been also used to oxidize alkenes, carbazole, N-ethylcarbazole, fluorene, and dibenzothiophene. Isoxalflutole is an herbicide activated in soils and plants to its diketonic derivative, the active form of the herbicide: laccases are able to convert the diketonicitrile into the acid. Laccase can be also used to reduce the concentration of synthetic heterocyclic compound such as halogenated organic pesticides in the soil. LMS has been extensively study in the oxidation of recalcitrant PAHs, main components of several ship spills. In this sense, LMS is being included in several enzymatic bioremediation programs. Cerrena unicolor produces laccase in the low nitrogen medium which has the capability of reducing lignin content from sugarcane bagasse up to 36% within 24 h at 30 C.

Future Trends and Perspectives

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This review summarizes the available recent important about the properties, production of laccases and possible industrial and biotechnological use. Laccases are promising enzymes to replace the conventional chemical processes of several industries such as the pulp and paper, textile, pharmaceutical, and nanobiotechnology. However, one of the limitations to the large-scale application of laccases is the lack of capacity to produce large volumes of highly active enzyme at an affordable cost. The use of inexpensive sources for laccase production is being explored in recent times. Thus, efforts have to be made in order to achieve cheap over production of laccase from hosts, and also their modification by chemical means or protein engineering, to obtain more robust, active and less expensive enzymes. Another additional problem is the cost and toxicity of redox mediators. Further investigations should consider different and less polluting mediators such as the natural mediators produced by laccase in a bio-environment during lignin degradation. The current development in laccase catalysis research and the design of mediators along with the research on its heterologous expression opens a wide spectrum of possible applications in the near future. Moreover, laccase can also offer a simple and convenient alternative to using peroxidases with H$_2$O$_2$, because laccases are available on an economically feasible scale. On the other hand, the development of an effective system for laccase immobilisation also deserves great attention. Immobilization could be achieved by chemical modification of the substrates. Hence, micropatterning, SAMs and LbL techniques can be used to functionalise flat and curved surfaces in order to have specific adsorption. Laccase encapsulation with polyelectrolytes will be used as a microreactor for catalytic reactions by changing the permeability properties of the capsule wall. Since the general goal is to obtain stable catalysts with long life times and low cost, the combination of these techniques will enhance:

- The adsorption of laccase on a suitable substrate,
- The lifetime of the laccase activity and
- Reutilisation of the substrate/laccase product.

### Table 1: Sources of Laccase Enzyme And Its Physiological role

<table>
<thead>
<tr>
<th>Sources</th>
<th>Physiological role</th>
<th>References</th>
</tr>
</thead>
</table>
| **Plant** | Rhus vernicifera, cabbages, turnips, beets, apples, asparagus, potatoes, pears, sycamore maple poplar, tobacco, peach, loblolly pine | • oxidize monolignols in the early stages of lignifications  
• involved in the first steps of healing in wounded leaves  
• in defense mechanism against external conditions | [6,7,8,9,10,11,12] |
| **Fungi** | Monocillium indicum, Phanerochaete chrysosporium, Theiophora terrestris, Lenzites, betulina, Phlebia radiate, Pleurotus ostreatus, Trametes versicolor (formerly known as Coriolus versicolor or Polyporus versicolor), Trametes versicolor, Tramete, pubescens, T. hirsuta, T. gallica, Trichoderma atroviride, T. harzianum, T. longibrachiatum, Pleurotus ostreatus, Pycnoporos sanguineus | • delignification of lignocellulosic material,  
• sporulation,  
• pigment production,  
• fungal morphogenesis,  
• protection against toxic compounds,  
• fruiting body formation  
• plant pathogenesis | [1,13,14,15,16,17,18,7,19,20] |
| **Bacteria** | Azospirillum lipoferum, Azospirillum lipoferum, Bacillus subtilis, Streptomyces lavendulae, S. cyaneus | • melanin production for cell pigmentation  
• spore coat resistance against hydrogen peroxide and UV | [21,18,14,22] |
Marinomonas mediterranea, Bacillus subtilis, Streptomyces cyaneus, • involvement in morphogenesis • involved in the solubilization and mineralization of lignin

Table 2: Production of laccase under solid state fermentation

<table>
<thead>
<tr>
<th>Source</th>
<th>Support</th>
<th>Reactor</th>
<th>Production (U/l)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galerina sp. HCl</td>
<td>Orange peels</td>
<td>Cotton plugged Erlenmeyer flasks</td>
<td>720</td>
<td>Laura Mendoza et al. 2010</td>
</tr>
<tr>
<td>Pycnoporus cinnabarinus</td>
<td>Sugarcane bagasse</td>
<td>Packed reactor</td>
<td>10000</td>
<td>Meza et al. 2006</td>
</tr>
<tr>
<td>Trametes hirsuta</td>
<td>Grape seeds</td>
<td>Tray</td>
<td>18715</td>
<td>Rodríguez Couto et al. 2006</td>
</tr>
<tr>
<td>Trametes hirsuta</td>
<td>Kiwi fruits</td>
<td>Cotton plugged Erlenmeyer flasks</td>
<td>5399</td>
<td>Rosales et al. 2005</td>
</tr>
<tr>
<td>Trametes hirsuta</td>
<td>Nylon sponge</td>
<td>Tray</td>
<td>6898</td>
<td>Rodríguez Couto et al. 2006</td>
</tr>
<tr>
<td>Trametes hirsuta</td>
<td>Orange sponge</td>
<td>Tray</td>
<td>12000</td>
<td>Rosales et al. 2007</td>
</tr>
<tr>
<td>Trametes pubescens</td>
<td>Banana skin</td>
<td>Cotton plugged Erlenmeyer flasks</td>
<td>1570</td>
<td>Osma et al. 2007</td>
</tr>
<tr>
<td>Trametes versicolor</td>
<td>Barley bran</td>
<td>Immersion</td>
<td>600</td>
<td>Rodríguez Couto et al. 2003</td>
</tr>
<tr>
<td>Trametes versicolor</td>
<td>Barley bran</td>
<td>Tray</td>
<td>3500</td>
<td>Rodríguez Couto et al. 2003</td>
</tr>
<tr>
<td>Trametes versicolor</td>
<td>Nylon sponge</td>
<td>Expanded bed</td>
<td>229</td>
<td>Rodríguez Couto et al. 2003</td>
</tr>
<tr>
<td>Trametes versicolor</td>
<td>Nylon sponge</td>
<td>Immersion</td>
<td>229</td>
<td>Rodríguez Couto et al. 2003</td>
</tr>
<tr>
<td>Trametes versicolor</td>
<td>Nylon sponge</td>
<td>Tray</td>
<td>343</td>
<td>Rodríguez Couto et al. 2003</td>
</tr>
</tbody>
</table>

Table 3: Commercial preparations based on laccases for industrial processes.

<table>
<thead>
<tr>
<th>Industry</th>
<th>Main application</th>
<th>Brand name</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food industry</td>
<td>Brewing</td>
<td>Flavourstar</td>
<td>Advanced Enzyme Technologies Ltd. (India)</td>
</tr>
<tr>
<td></td>
<td>Colour enhancement in tea, etc.</td>
<td>LACCASE Y120</td>
<td>Amano Enzyme USA Co. Ltd.</td>
</tr>
<tr>
<td></td>
<td>Cork modification</td>
<td>Suberase</td>
<td>Novozymes (Denmark)</td>
</tr>
<tr>
<td>Paper industry</td>
<td>Pulp bleaching</td>
<td>Lignozym-process</td>
<td>Lignozym GmbH (Germany)</td>
</tr>
<tr>
<td></td>
<td>Paper pulp delignification</td>
<td></td>
<td>Novozym 51003 Novozymes (Denmark)</td>
</tr>
<tr>
<td>Textile Industry</td>
<td>Denim bleaching</td>
<td>Bleach Cut 3-S</td>
<td>Season Chemicals (China)</td>
</tr>
<tr>
<td></td>
<td>DeniLite</td>
<td></td>
<td>Novozymes (Denmark)</td>
</tr>
<tr>
<td></td>
<td>Zylite</td>
<td></td>
<td>Zytex Pvt. Ltd. (India)</td>
</tr>
<tr>
<td></td>
<td>Denim finishing</td>
<td>Cololace BB</td>
<td>Colotex Biotechnology Co. Ltd. (Hong Kong)</td>
</tr>
<tr>
<td></td>
<td>Ecostone LC10</td>
<td></td>
<td>AB Enzymes GmbH (Germany)</td>
</tr>
<tr>
<td></td>
<td>IndiStar</td>
<td></td>
<td>Genencor Inc. (Rochester, USA)</td>
</tr>
</tbody>
</table>
5. Conclusion

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 to organisms. 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 bleaching processes. The laccase enzyme has a wide field of application including food industries, the pulp and paper industries, the treatment of various industrial effluents, enzymatic decolouring of material and bioremediation of soils. 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.

6. Reference


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