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Review Article

Biotechnological and Industrial Applications of Laccase: A Review

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Abstract

Laccase is a polyphenol oxidase, highly glycosylated that mainly presents as monomeric proteins with varying mass of 50-90 kDa. This enzyme oxidizes lignin using molecular oxygen which produces water as the only by-product but it shows specificity to broad range of substrates such as phenols including ortho- and para-diphenols, amino phenols, methoxy phenols, polyphenols, polyamines, aryl diamines and ascorbate. Laccase can be found in fungi, plants, insects and bacteria. Laccases are involved in a wide range of biological functions including pigment formation in fungi, ectomycorrhizal symbiosis, metabolism of proanthocyanidins, virulence of pathogen fungi and sexual development. Regarding its unique function it is getting more attention for novel applications in biosensors, microfuel and bioelectrocatalysis. In addition, it is used in food, pharmaceutical and cosmetic, pulp and paper and textile industries. It has especial potential to be used in bioremediation to remove water and soil pollutions resulted from different industries. This has made researchers to produce transgenic plants containing heterologous laccases to be able phytoremediate polluted soil and water resources with chemicals including different organophosphorus pesticides and nerve agents. Additionally, hydroponic culture of these transgenic plants can be considered as an inexpensive approach for commercial production of laccase exploiting rhizosecretion strategy.

Keywords: Laccase, Fungi, Transgenic Plants, Phytoremediation, Pollution

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Introduction

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) is a polyphenol oxidase which belongs to the multicopper oxidases [1-4] which can be classified as metalloxidases and laccases [5]. Laccases contain 15–30 % carbohydrate and are mostly monomeric proteins of mass of 50–90 kDa [6]. Laccase is the most abundant member of the multicopper protein family that catalyses the oxidation of lignin, using molecular oxygen as the oxidant [7-9] The very interesting catalytic feature of laccase is that water is the only by-product, introducing laccases as excellent catalysts [2]. The enzyme was reported for the first time in Japanese lacquer tree *Rhus vernicifera* in 1883 [10]. These enzymes are widely spread in plants (pears, turnips, cabbages, apples, potatoes, asparagus and other vegetables), bacteria (*Streptomyces lavendulae*, *Streptomyces cyaneus*, and *Marinomonas mediterranea*) and fungi [2, 11, 12] and have also been found in insects [2]. In fungi, laccases exist more than higher plants, which are monomeric globular proteins of approximately 60 – 70 kDa having acidic isoelectric point (pI) around pH 4.0, but some exceptions can be found [13]. They are also important in regular processes like pigment formation in fungi [14], *Phanerochaete chrysosporium*, *Theiophora terrestris*, and *Lenzites betulina* and white-rot fungi such as *Ganoderma* sp, *Phlebia radiate*, *Pleurotus ostreatus* and *Trametes versicolor* are amongst laccase-producer Basidiomycetes. *Monocillium indicum* was the first ascomycetes, its Laccase was characterized with peroxidase activity. White-rot fungi are laccase rich fungi which are the only organisms able to dgrade the whole wood components. [2]. It also has been reported in some archaea, too [15]. Although, very few laccases have been reported from ectomycorrhizal fungi. *Tricholoma matsutake*, a famous wild edible mushroom, is an ectomycorrhizal fungus which belongs to *Tricholomataceae* [12].

Activity (Temperature, pH, Mode of action and mediators)

-Thermal stability

The *Tricholoma matsutake* laccase has high thermal stability and maintains most of its activity in varying temperature between 20°C to 80°C [12]. The optimal temperature for laccase activity differs greatly between different strains. This temperature for fruiting body formation and laccase production is 25°C in the presence of light, but when the cultures are incubated in the dark it shifts to 30°C for laccase production [2].

In *Pleurotus ostreatus* laccase maintains its activity in the temperature range of 40 – 60°C, showing the maximum activity at 50°C. There are little information available on effect of pH on laccase production, but mainly it has been reported that initial pH between 4.5 and 6.0 that is suitable for enzyme production [17]. Phenols including ortho- and para-diphenols, amino phenols, methoxy phenols, polyphenols, polyamines, aryl diamines and ascorbate are natural substrates of laccase. This substrate oxidation by laccase occurs as a reaction producing a free radical with reduction of oxygen to water [2, 10].

-Carbon source

Glucose, maltose, sucrose, fructose, glycerol and lactose are the commonly used carbon sources. Fructose was shown to be a good carbon source for laccase production

in Pleurotus sajor-caju, cellobiose in *Toxicodendron pubescens*, and lactose or glycerol in *Pseudotrametes gibbosa*, *Coriolus versicolor* and *Fomes fomentarius*. Higher concentrations of glucose inhibited laccase production in various fungal strains while excessive sucrose concentrations reduce the laccase production to the constitutive level. Addition of polymeric substrates such as cellulose behaves differently and increases enzyme production during cultivation. [18].

-Nitrogen

Fungal laccases are mainly triggered by nitrogen depletion [18], while in some strains laccase activity has no relation with nitrogen concentration [19]. Low carbon to nitrogen ratio has caused high laccase activity in some studies [20], while high carbon to nitrogen ratio has increased laccase production in some studies. Laccase production was promoted in fungi growing in a nitrogen rich media rather than nitrogen-limited media [2].

-Copper sulphate

Different copper sulphate concentrations efficiently affected laccase synthesis by *Nigrospora* sp. and *Arthopyrenia* sp. in a liquid culture medium [3]. It has been shown that increasing copper ion concentration from 1 to 10 mM/L has increased laccase activity from *Setosphaeria turcica* [21], in agreement with marked increase of Agrobacterium laccase activity [22]. Despite those, inhibitory effect of copper ion even at 0.5 mM for *Fusarium solani* has been reported elsewhere [23]

-Chloride

NaCl had an increasing inhibitory but reversible effect on purified *Trametes polyzona* and *Trametes versicolor* laccase activity for 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) by more than 50% in the presence of 20 mM NaCl [24], but it was stimulatory rather than inhibitory for laccase activity from *Bacillus halodurans* for syringaldazin at alkaline pH proposing this enzyme as an ideal biocatalyst for paper production [25].

-Other elements

Different cations and anions have different effects on laccase activity depending on the conditions such as element type and concentration. Fe^{3+}, Mn^{2+} increased *Setosphaeria turcica* laccase activity expressed in *Escherichia coli* by approximately 434.8% at 10 mM/L and at 5 mM/L, respectively, while Na^{+} increased activity at 1 mM/L but inhibited activity at 5 and 10 mM/L. Sodium Dodecyl Sulphate (SDS) had increasing effect on laccase activity at 1 mM/L, but inhibited activity at 5 and 10 mM/L [21]; while Fe^{2+} inhibited *Trametes polyzona* laccase expression by 95% at 5 mM, and complete inhibition by 0.1 mM NaN_3[24]. It also has been reported that sodium thioglycolate is a laccase inhibitor and its function is related to fungi growth [26].

Laccase application and functions

-Food Industry

Laccase application in the food industry is based on its ability to polymerize molecules. Laccases can be applied to certain processes that enhance or modify the colour appearance of food or beverage for the elimination of undesirable phenolics, responsible for the browning, haze formation and turbidity in clear fruit juice, beer and wine. Laccase is also employed to ascorbic acid determination, sugar beet pectin gelation, baking and in the treatment of olive mill wastewater. Researcher showed that a laccase from the white-rot fungus *Trametes hirsuta* increased the maximum resistance of dough and decreased the dough extensibility in both flour and gluten dough [2]. Also laccase in a bi-enzyme system with novel cellobiose dehydrogenase-3-ethylbenzothiazoline-6-sulphonic acid (CDH-ABTS-laccase) has been used for fast oxidation of lactose to lactobionic acid (LBA) which is a valuable organic acid, with numerous applications in pharmaceutical, food, and cosmetics industries [27].

-Pulp and paper industry

Laccases are able to depolymerize lignin and delignify wood pulps, kraft pulp fibers and chlorine-free in the biopolpation process. Laccases are more easily able to delignify pulp when they are used together with mediators. Mediators may be used to oxidize the non-phenolic residues from the oxygen delignification. The mediator is oxidized by laccase and the oxidized mediator molecule further oxidizes subunits of lignin that otherwise would not be laccase substrates. Laccase mediator systems can also be applied to remove pitch and dyes from wood-based materials. Laccases can be used for binding fiber-, particle- and paper-boards [2]. Pulp and paper mills generate large volumes of intensely colored black-liquors that contain toxic chlorinated lignin degradation products like chlorolignins, chlorophenols, and chloroaliphatics. These paper mill effluents are highly alkaline and alter the pH of the soil and water bodies where they are discharged. Laccase significantly affects the color remediation and toxicity of these samples [28]. Laccase catalyzed decolorization of Acid Blue 92, indicated pH, enzyme activity, and dye concentration [4].

-Textile industry

Laccase are considered as a potential solution for textile effluent problem because of its ability to degrade dyes are currently being used in this industry [2]. Laccase are also used in stone-washing along with cellulose to confine fiber damage to outer fibers and protects inner ones [29]. It also can be used for decolorizing dyed fabric with these Indigo to create a brighter shade and the decrease blot used after stone washing operation [30]. Laccase has been used to decolorize Malachite green (MG) which is a triphenylmethane dye used in aquaculture to control protozoan and fungal infections of farmed fish. MG is also used in food, medical and textile industries [31].

-Bioremediation

Immobilized laccases are good bioremediating agents which and can be used continuously like many other enzymes. Laccase is immobilized in polyvinyl alcohol (PVA)-based polymers cross-linked either by nitrate or boric acid [32]. Laccases are considered green biodegrading agents due to their ligninolytic activity [33]. This enzyme degrade xenobiotics, polycyclic aromatic hydrocarbons (PAHs), arising from natural oil deposits and fossil fuels, phenolic and chlorinated phenolic pollutants, including diesel which increasing its concentration in the soil increases laccase activity in plant tissues [2, 34].

-Pharmaceuticals and cosmetics

Laccases are biomolecules which act specifically; this make pharma-tech companies to use this enzyme for syn-

thesis of complex medicinal compounds such as anesthetics, anti-inflammatory, antibiotics, sedatives, etc. [35]. More recently cosmetics for skin lightening have been developed which are laccase-based hair dyes could be less irritant and safer than current hair dyes. Protein engineered laccase may be used as deodorants, toothpaste, mouthwash, detergent, soap, and diapers with reduced allergenicity [2].

-Metabolism of proanthocyanidins

Flavonoids, including proanthocyanidins (PAs; also called condensed tannins), play a multitude of roles in plants. Laccase-like polyphenol oxidase is involved in proanthocyanidins oxidation in *Arabidopsis* [36].

-Laccase as a virulence factor and a marker of fitness

The ability to produce melanin pigments was strongly correlated with virulence in early studies of cryptococcal infection. The first genetic evidence that laccase was important for virulence came from studies in which the gene encoding the laccase enzyme, was deleted in a strain of *Cryptococcus neoformans* var. neoformans. Laccase has been associated with the production of immunomodulatory catecholamines from brain, as well as protection from antifungals and oxidative products of macrophages. In addition, laccase activity has been found to be a marker of stress, as induction of laccase correlated with substrate starvation and the presence of potentially toxic metals. It may be partly due to an additional ability of laccase to act as a genetic marker for cellular processes that are important for virulence [37].

-Herbal treatment

Laccase that is extracted from oyster mushroom (*Pleurotus ostreatus*) is being used as herbal medicine to inhibit the HCV (hepatitis C virus) replication rate [38].

-Biosensor

Laccase has been used in the development of biosensors for phenolic substrates, and extensively used for electrocatalytic reduction of oxygen. Laccase biosensor differs from peroxidase biosensors in that it does not require hydrogen peroxide to oxidize phenolic substrates. Biosensors based on tyrosinase suffer from low enzyme stability and significant inhibition of the enzyme by reaction products, which make laccase as an alternative, strong candidate for application in biosensor for the determination of phenolic compounds [39].

-Microfuel

Laccase is being used in microfuel cells to reduce dioxygen by itself as a cathode [39].

-Bioelectrocatalysis

Laccase bioelectrode utilizing conversion of redox active substrates. The laccase catalyzes the 4e- reduction of O_2 to H_2O by using copper centers of three different types. Typically, laccase based electrodes act using mediators. The application of laccase in bioelectrocatalytical systems is associated with development of biosensors and cathodes of biofuel cells which makes laccase as greener alternative for chemical oxidation based fuel cells [40, 41].

-Natural Vanillin

Using a three-enzyme-system to degrade curcumin to natural vanillin, laccase catalyzed formation of a phenol radical, radical migration and oxygen insertion at the benzylic positions, can result in the formation of vanillin. As vanillin itself is a preferred phenolic substrate of laccases, the formation of vanillin oligomers and polymers is inevitable just after vanillin release [42].

-Melanins synthesis

Amongst three classes of melanins synthesized in fungi, the eumelanin DOPA-melanin (3,4 dihydroxyphenylalanine melanin) and the allomelanin DHN-melanin (1,8-dihydroxynaphthalene melanin) are the two most common and best characterized melanins in fungi which require the action of laccases for their production [43].

Expression of laccase gene

The multigene family of laccases is a common feature in fungi. The first example of the multigene family of laccases was described in *Agaricus bisporus* [44, 45]. Since then, two laccases have been characterized from *Pycnoporus cinnabarinus*, 11 genes from *Trametes versicolor*, 17 from *Coprinopsis cinerea*, three from *Pleurotus eryngii*, 11 laccases from *Laccaria bicolor*, 12 from *Pleurotus ostreatus*, and 11 from *Flammulina velutipes* have been characterized [46]. The laccase gene identificate in the Shiitake Mushrooms (*Lentinula edodes*) laccase can regulate sexual development, for example sexual reproduction in fungal pathogens such as Cryptococcus provides natural selection and adaptation of the organisms to environmental conditions by allowing beneficial mutations to spread. However, successful mating in these fungi requires a time critical induction of signaling [47, 48]. Today, microorganisms such as bacteria, fungi and Yeasts are exploited produce the enzyme. Traditionally, *E. coli* lacks glycosylation of expressed proteins, therefore, a glycosylated protein cannot be produced using bacterial system. Fungi themselves grow slowly but yeast which grow fast and has the glycosylation machinery is a useful system for laccase production [49]. Considering the demands for industrial enzymes (Laccase) and their environmental benefits, an efficient system for heterologous protein production is greatly needed. Microbial expression systems have mainly been developed for this purpose, using various promoters and incubation conditions but plants are novel systems are used for production of heterologous proteins including laccases [6].

-Expression of recombinant laccase gene in Tobacco

A fungal laccase from *Pleurotus ostreatus* was expressed in tobacco chloroplasts using an expression system containing enhancing sequences for effective protein translation and stabilization fused to a laccase gene under the control of the *rrn* promoter for production of recombinant laccase in the tobacco plant trnI-trnA sites bordering the construct allowed the insertion of laccase into the trnI-trnA region of the chloroplast basesd on homologous recombination. The laccase expressed efficiently but it did not show any considerable activity in citrate buffer pH 5 [6], while another group could not produce laccase transplastomic lines successfully [50]. On the contrary there are several reports for successful expression of heterologous laccases using plants. For example efficient expression of laccase gene in transgenic maize [51], tobacco plant [52], and tobacco BY-2 cells [53] has been reported.

-Regulation of laccase by copper and nitrogen

Using *Trametes versicolor* cultures containing various nitrogen and copper concentrations was shown that in-

creasing laccases mRNA transcripts and activities are function of increment of nitrogen and copper concentration [54].

The genome of *P. ostreatus* possesses three groups of 12 Lacc2 and Lacc10 genes which are overexpressed in the mycelia of *P. ostreatus* in submerged culture containing wheat straw extract in a chemically defined medium. while expression of the two laccases are negligible in sawdust medium at all developmental stages indicating that expression of different laccases depends on the medium conditions the mushroom grows on. Although Lcc6 was expressed in all developmental stages, Lacc1 and Lacc3 were specific to the mycelial stage in solid medium; whereas Lacc5 and Lacc12 were specific for fruiting bodies and primordia, respectively; concluding different laccase isozymes have different expression pattern depending on the nutrients available and age of organism [55].

Conclusion

Laccases are versatile enzymes in plants, participate in lignin biosynthesis, carrying out the oxidative polymerization of monolignols and additional physiological processes, such as cytokine in homeostasis, resistance to phenolic pollutants, flavonoid polymerization in seed coats, and iron metabolism and anthocyanin degradation [56]. In this Regards, laccases are enzymes with very wide functions among different expressing organisms at different developmental stages. Their very strong oxidation potential has made them considerable proteins with various applications in different industries such as food, textile, paper, fuel, and environment section. Overexpression of laccases in plants and yeasts are part of efforts to produce them commercially; and additionally laccase expressing transgenic plants secreting recombinant laccase especially by rhizosecretion are considered new candidates for phyroremediation.

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