# Research Progress on Microbial Sources of Laccase and its Applications in Food Industry

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**Abstract:** Laccases are increasingly used in food industry and their sources are mainly plants, bacteria and fungi. A research review on the production methods and the characteristics, as well as the applications in food industry of laccases derived from bacteria and fungi is given in the article. It also looks forward to the development of laccases in the future food industry.

## 1. Introduction

Laccases are mainly found in cell walls in higher plants. In 1883, the Japanese scholar Yoshida discovered a protein in the sap of Rhus *verniciflua* that catalyzes phenolic substances. In 1884, Bertrand named the protein laccase [1]. Laccases are found in higher plants, fungi, insects, and bacteria. Fungi are an important source of laccases, particularly large basidiomycetes and ascomycetes, and more than 120 fungi have been reported. In essential, Laccases were a p-binary polyphenol oxidase (binary phenol oxidase, EC 1.10.3.2) that can catalyze a double-substrate enzymatic reaction. By exploiting the oxidizing properties of copper ions, the substrate lost electrons to form free radicals and, the oxygen molecules got the electrons to form water under the involvement of oxygen molecules. [2] The catalysis ways of laccases could be specifically divided into three types: laccases oxidize substrate molecules directly to compounds with free radicals; or catalyze the high oxidation-reduction potential substrates with the participation of mediators; or laccases first oxidize mediators, and then the Luciferase. Afterward the oxidized Luciferase was used to catalyze the substrate and achieved regeneration through the dehydrogenase. [2] As shown in Fig. 1, the catalysis of laccases was a single-electron oxidation process. The mononuclear center $T1Cu^{2+}$ was the initial electron capture site, and its reduction was the rate-limiting step in laccase catalytic process. The four electrons of the reducing substrate were captured by $T1Cu^{2+}$ to form a semiquinone product. These electrons were captured by the tri-nuclear center $T2Cu^{2+}$ and $T3Cu^{2+}$ via the His-Cys-His pathway coordinated with $T1Cu^{2+}$, and finally obtained by the oxygen molecules which were reduced to water at the end. The semiquinone product could continue to undergo other non-enzymatic decomposition, conversion or polymerization reactions after leaving the enzyme molecules [6].

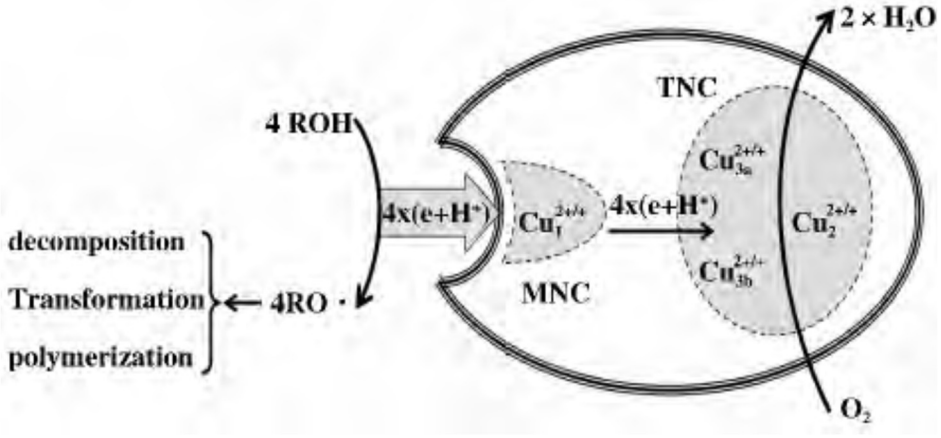


Fig. 1 Schematic function of the two redox centers in laccase [6]

The laccases currently used in industrial production are mainly microbial sources laccases, namely

bacterial laccases and fungal laccases. In recent years, there have been many studies on the enzyme characteristics, catalytic mechanism, and production methods of laccases derived from microorganisms. This article summarizes the recent research progress (bacterial laccases and fungal laccases) of microbial laccases and their applications in food industry.

## 2. Bacterial Sources of Laccases

When laccases were first discovered, scientists believed that it existed only in eukaryotes. In 1993, Givaudan isolated a strain of *Azospirillum lipoferum* that could produce laccases in the soil around rice. At this point, prokaryotes had also been confirmed to produce laccases. [27]

The function and catalytic properties of bacterial laccases are different from others. Unlike fungal laccases, bacterial laccases had more advantages in terms of $Cu^{2+}$ resistance, glycosylation, thermal stability, and the suitable pH value [4]. Most bacterial laccases belong to endospores. Although the catalytic process of bacterial laccases had the similarity with others, its reaction kinetics was significantly different from those of other sources because bacterial laccases usually consisted of one peptide chain [6]. Laccases were mainly the complex polymers with the combination of phenols, aromatic or aliphatic amines and their macromolecular. The substrate specificity of the laccases was not very high; however, the types of substrates that can be catalyzed were relatively extensive. It was also the main reason that laccases have attracted wide attention in biotechnology applications. According to the reaction mode of laccases proposed by Riva and others [7] in 2006, bacterial laccases mainly achieved the catalytic oxidation of substrates in two modes as shown in Fig. 2.

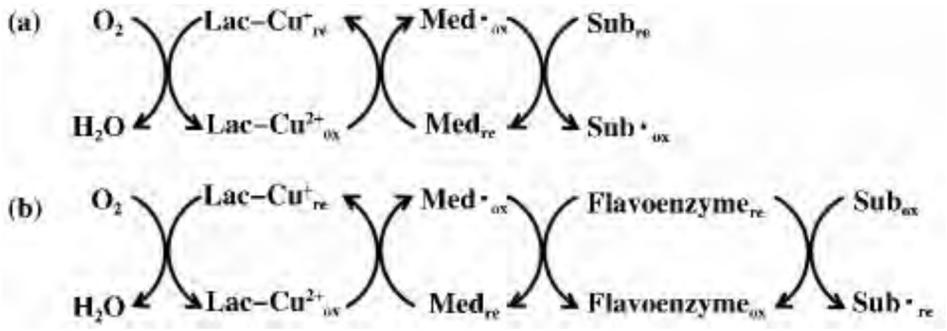


Fig. 2 Oxidation reduction cycle schematic function of bacterial laccases catalyzing the oxidation of substrates with mediator

In the two modes of Fig.2, the mediator is involved in the catalytic oxidation of substrates by laccases. According to the summarization of Gao Jian [6] and other researchers, with the catalysis of laccases, the mediator produced a higher potential and more stable oxidized forms of free radicals, which had strong oxidative ability and could spontaneously reacted with substrates with itself reduced to original status. In the second mode, it was also necessary for Flavin to participate in conducting electron transfer.

Bacterial laccases could be produced by a variety of bacteria, which included *Acillus subtilis, E. coli, Aquifexaeolicus, P. vulgaris, T. thermophilus, Bacillus subtilis,* etc [3, 6].

Claudia and others found that *P. vulgaris* can produce a heat-resistant and alkali-resistant extracellular laccase, and its enzymatic activity could be significantly improved in the presence of metal and ionic surfactants [5]. Unlike other laccases, most of the bacterial laccases are not glycosylated in terms of molecular composition and one peptide chain constitutes the entire laccase molecule. Only individual bacterial laccases are oligomerases, such as laccases from *Streptomyces griseus* and *Azospirillum lipoferum*, which are homogeneous and non-homogeneous trimers respectively. Most bacterial laccase molecules contained four copper ions with a few of them were replaced by other metal ions such as iron and zinc ions. Another structural feature of bacterial laccases was that disulfide bonds can be found in a few bacterial laccases, whereas they were common to find in fungi and plant laccases. [6]

The production methods of bacterial sources laccases have also become diversified, and some

methods to increase bacterial laccases production have been made. Koschorreck and others demonstrated that cell pyrolysis can rapidly separate laccases, and thermal lysis can increase the specific activity of *T. thermophilus* laccase by 460 times [3]. Sharma [8] and others used deep and solid-state fermentation, respectively, to increase the laccase production of *Rheinheimera. sp* and *Lysinibacillus. sp*. Furthermore, they took an entrainment on agricultural and industrial residues containing flavonoids, thus increasing their laccase production.

## 3. Fungi Sources of Laccases

Fungal laccases are mostly acidic proteins, with molecular weights generally between 50-90KD. The redox potential of the fungal laccases is generally lower than that of the lacquer laccases. Fungal laccases also have broad substrate selectivity and can catalyze the oxidation of a range of substrates. The major sources of fungal laccases were: *Basidimycetes*, *Polyporus*, *Asomycetes*, *Neurospora*, *Podospora*, and *Aspergillus*. [9]. Fungal laccases were mainly involved in the process of lignification, de-lignification and detoxification in fungi [11].

The optimum temperature and pH value of the fungal laccases strongly depend on its substrates. Different substrates have different optimum environmental conditions. The optimum temperature is generally 50-80°C. Most fungal laccases exert their maximum catalytic activity at pH value between 2 and 5, or when the reaction conditions are acidic. A small number of fungal laccases also had high catalytic activity under alkaline conditions. For example, *Panaeolus papilionaceus* laccase had an optimum pH value of 8.0 when DMP was used as its substrate [14].

Laccase mediator system (LMS) had been widely used in pulp delignification, fuel cell, organic pollutant transformation, biosensor and dye degradation [10]. Dalel and others studied the catalytic oxidation of bisphenol A (BPA) by different fungal laccases using 1-hydroxybenzotriazole (HBT) as the catalytic mediator. The result of the study showed that BPA was catalyzed and oxidized faster by *Coriolopsis gallica* in different fungal laccases [12]. Hafiz and others extracted laccases from *Aspergillus niger* and used them to assist in the preparation of green pathways for poly (3-hydroxybutyrate) ethylcellulose (EC)-based green composites with new properties [13].

At present, many fungal laccases have been crystallized to analyze the structure. Generally, fungal laccases were also single-peptidase laccases, which contained four copper ions. Depending on the differences between spectroscopy and magnetic properties, these four copper ions could be classified into three types: one type I copper ion, one type II copper ion and two type III copper ions. These four copper ions correspond to the three redox sites T1, T2, and T3 [15]. T1Cu (Type I) was a mononuclear center and a place where the redox of the reducing substrate proceeds. The strong light absorption could be found at 610 nm, and confer endowed the laccases blue-green color. T2-Cu (Type II) was a single electron acceptor with no characteristic absorption spectra. T3-Cu (Type II), forming a coupled ion pair, was a two-electron acceptor. It had diamagnetism and a broad absorption band near 330 nm. With the combination between T2-Cu and T3-Cu, the trinuclear copper ion center where oxygen molecules received four electrons in the center and were reduced to produce water was formed. [15]

## 4. The Industrial Process of Laccases

Industrial laccases can be obtained through fermentation, including solid and liquid fermentation. Liquid fermentation is the main production method of laccases. The microbial strain is involved in bacteria and fungi. In 2017, Koschorreck and others demonstrated that cell pyrolysis was a simple and rapid method for the isolation of bacterial laccases, which could separate and partially purify the recombinant laccase [3]. The bacterial fermentation has a short cycle and a high expression level. However, the applications of laccases are limited because of its low redox potential. Although Fungal laccases have high redox potential, they grow slowly and have a low expression level.

At present, most researches on the production of laccases are concentrated on the shaking flask level. Only a small number of strains have been tried at the fermenter level with a small scale. The potential fungal strains at the fermenter level are mainly *Trametes* and *Pycnoporus*. Fonseca and

others evaluated the secretion ability of laccase produced by four newly discovered strains *Trametes sp*. and found that the laccase secreted by one of the strains was highly thermostable and showed its potential in the development of biotechnology [16]. Chen Qionghua [17] and others used *Ganoderma weberianum* TZC-1 as a production strain and tried to enlarge the strain in a 50L fermentor under the conditions of optimized shake flask culture and a small test production process on a 5L fermentor. According to the experiment, maintaining DO at 15%-30% for 72 hours before fermentation was conducive to mycelial growth. After 72 hours, if DO maintained at 10-15%, the laccases would be better accumulated. After 144 hours of fermentation, the 18.8 L fermentation broth was obtained, and its laccase activity was 27,667.7 U/L, which was 2.5 times that of shake flask fermentation. Afreen [18] and others studied the laccase production potential of photosynthetic nitrogen-fixing cyanobacteria, *Arthrospira maxima* (SAE-25780). The study indicated that the laccases output rate of *A. maxima* (SAE-25780) reached 80% in the presence of the inducer guaiacol.

## 5. Application of Laccases in Food Industry

Laccases have a wide range of applications in the food industry as the products of its catalysis are simple and environmentally friendly with water as the only by-product, while microbial sources laccase are more widely used.

Fungal laccases are involved in the processing of beverages, the production of medicinal bacteria, and the cross-linking of food molecules, etc.

### 5.1 Application of Laccases in Fruit Juice Production

Laccases can be used to clarify juice in the process of juice production. Tannins, anthocyanins and other phenolic substances in juice increased the turbidity of the juice. The macromolecular polymers of arylamine compounds and protein-phenol compounds were even more difficult to control for secondary turbidity [20]. These are all the affecting factors of the quality of fruit juice. Laccases can specifically and efficiently remove most of the phenolic substances in the juice by making phenols into free radicals without destroying some of the amino acids in the juice thus saving the nutritional of the juice. Lettera [23] and others experimentally demonstrated that the use of laccases in the production of orange juice improved the organoleptic properties of the product, and the flavanone molecules in the juice were not affected by this process. They optimized the covalent immobilization of *Pleurotus ostreatus* recombinant POXA1b laccase on epoxy-activated poly (methacrylate) beads using response surface methodology. The fixing efficiency was 98%. By characterizing the immobilized biocatalyst, Lettera and others tested such biocatalyst in the clarification of juice. The result of test showed a 45% degradation ratio of phenol with the flavanone in a healthy standard. Piacquadio and others applied the HPLC analysis to measure the phenolic composition of apple juice treated with laccase immobilized on copper-chelate carrier. Significant reductions were obtained for all the phenolic compounds identified by untreated juice.

### 5.2 Application of Laccases in Wine Brewing

Wines are complex mixtures of different phenolic compounds such as tannins, styrene acid and catechol, affecting organoleptic characteristics and stability of wines. For instance, tannin in grape seeds directly affects its organoleptic properties. The traditional way to remove phenols is to add sulfur dioxide, oxidants, proteins, etc. Cantarelli [29] and others found that 70% of catechol, 90% of ferulic acid and 90% of anthocyanidins in the sample can be removed by the laccase from *Polyporus versicolor* and 50% of total polyphenols can be removed by the laccase treated with the black grape juice [21]. Under the mild condition, Laccases can remove phenolic substances in wine and keep its sensory properties unchanged. Combining with the analytical methodology (capillary zone electrophoresis) and regarding phenol removal kinetics as the potential change of total antioxidant, Minussi [25] and others evaluated the potential of laccases from *Trametes versicolor* for the stabilization of essential phenols in wine and found that the use of laccases in white wine was totally feasible. According to this evaluation, wine can be treated in a more gentle and ecological way, which can save the finished cost and avoid the quality deterioration due to long-term storage.

### 5.3 Application of Laccases in Edible Mushroom Cultivation

Laccase, as an indispensable enzyme molecule in the life history of edible fungi, has important significance for the growth, development, differentiation, and defense of fruiting bodies, shortening the production cycle of edible fungi, reducing production costs, and improving antibacterial ability. Adding laccase inhibitors in the cultivation of edible fungi can promote the growth of mycelium, shorten the production cycle and increase the yield. Laccases could oxidize and catalyze the low-molecular-weight phenolic compounds, participate in the formation of cell walls and play a role in the defense of fruit-body entities. For example, laccases could degrade tannins and other compounds in plants, protect the fungi themselves in the presence of tannins and toxic phytoalexins and avoid the toxicity of the plant's own compounds on the fungi [22]. Munk studied the electron acceptor, substrate, reaction environment, and oxidation-reduction potential difference of the laccase oxidation process in fungi. He proved that laccase participated in the degradation of lignin and provided nutrition for the growth of edible fungi by establishing a lignin model for discussion of the correlation between fungal lignification and laccases [26].

## 6. Application of Laccases in the Field of Color Bleaching

Monssef and others [19] compared the decolorization of laccases on aging paper and parchment. FTIR analysis showed that the structure of the extracted fungal pigment has aromatic rings and phenol groups. The crude laccases could decolorize different pigment structures and show good decolorization efficiency for the extracted yellow pigment produced from Asp. Ochareceous could be used to treat 200 microliters purified enzyme. During the 30 days, laccases were used for paper and parchment papers inoculated with spore suspensions on paper and parchment stained with bleaching pigments secreted by extinct pigment fungi. The results showed that the fungal pigment had higher paper removal efficiency (71.21%) than that of the parchment sample (32.39%).

## 7. Outlook

Laccases, as an environmentally friendly enzyme, has been used into many fields. This article describes the application potential of laccases in food industry, papermaking and bleaching. However, the problem of how to produce a large number of highly active laccases remains an important factor limiting the industrial application of laccases. At present, there are few reports on the selection of fine strains and the exploration of suitable culture conditions, which are still hot topics in the future. On the other hand, elucidating the structure, function and catalytic mechanism of laccase at the molecular level contribute to the development of new laccases and promote their applications in industry.

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