



Bacterial laccases: promising biological green tools for industrial applications

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Abstract

Multicopper oxidases (MCOs) are a pervasive family of enzymes that oxidize a wide range of phenolic and nonphenolic aromatic substrates, concomitantly with the reduction of dioxygen to water. MCOs are usually divided into two functional classes: metalloxidases and laccases. Given their broad substrate specificity and eco-friendliness (molecular oxygen from air as is used as the final electron acceptor and they only release water as byproduct), laccases are regarded as promising biological green tools for an array of applications. Among these laccases, those of bacterial origin have attracted research attention because of their notable advantages, including broad substrate spectrum, wide pH range, high thermostability, and tolerance to alkaline environments. This review aims to summarize the significant research efforts on the properties, mechanisms and structures, laccase-mediator systems, genetic engineering, immobilization, and biotechnological applications of the bacteria-source laccases and laccase-like enzymes, which principally include *Bacillus* laccases, actinomycetic laccases and some other species of bacterial laccases. In addition, these enzymes may offer tremendous potential for environmental and industrial applications.

Keywords Laccase · Green catalyst · Mediator · Engineering · Immobilization · Decolorization · Degradation · Delignification

Introduction

Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) belong to a family of blue multicopper enzymes that oxidize a range of substrates, such as phenols (–OH), anilines (–NH₂), arylamines, ascorbic acid, and certain inorganic compounds, coupled to the four-electron reduction of dioxygen to water [1–3]. Laccases are widely distributed in nature. The first laccase was extracted from the latex of the Japanese lacquer tree *Rhus vernicifera* in the late 19th century [4]. To date, laccases are widely distributed in almost all wood rotting fungi [5]. Laccases have also been identified in several higher plant species [4, 6–8], lichens [9], and sponges [10]. Moreover, polyphenol oxidases with

laccase-like activity have also been found in oysters [11], insects [12–14], metagenome libraries of bovine rumen [15], and acidic bog soil metagenome [16]. Among these laccases, fungal laccases have been widely studied. Several studies on the application of *Trametes versicolor* laccase have been conducted in recent years [17–27], but fungal laccases generally fail to work in extreme environments during industrial operations. Laccases are usually suitable under mesophilic and acidic reaction conditions [28]. Therefore, bacterial laccases have attracted research attention [29–42]. Here, we summarized bacterial laccases, mainly including *Bacillus* and actinomycetic laccases.

Laccases are monomeric, dimeric, or tetrameric glycoproteins. First and foremost, bacteria are the sources of prokaryotic laccase. To our knowledge, the molecular structure of typical bacterial laccase usually contains three types of copper ions according to their magnetic and spectroscopic properties, namely, type 1 (T1), type 2 (T2), and double type 3 (T3) copper ions [43, 44]. The center of T1 copper, which is in charge of electron transfer, is responsible for substrate oxidation. T1 copper exhibits strong electronic absorbance around 610 nm, and electro-paramagnetic resonance (EPR)

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can be detected. The T2 site is a mono-nuclear center formed by T2 copper; the site is colorless, and EPR is also detectable. The T3 site is composed of two strongly coupled T3 coppers, which provide a weak absorbance near the UV spectrum (330 nm); here, EPR is not detectable [43]. The trinuclear T2/T3 cluster is composed of one T2 copper ion and two T3 copper ions, which bind and reduce molecular oxygen to water [45, 46]. The copper centers are shown using *Bacillus subtilis* CotA-laccase, a fully characterized and studied laccase (Fig. 1).

The other significant bacterial laccases are from actinomycetes, which belong to broad bacteriology. *Streptomyces* laccases of actinomycetes are the most identified and characterized and are known as extracellular enzymes. A variety of laccases have been identified in species including *S. cyaneus* [30, 47, 48], *S. coelicolor* [49, 50], *S. bikiniensis* [51], and *S. ipomoea* [52]. Among these laccases, *S. coelicolor* laccase is the most extensively characterized. Different from the typical three-domain bacterial laccases, *Streptomyces* laccases are usually two-domain laccases, such as the so-called small laccase (SLAC) from *S. coelicolor* (Fig. 2). Compared with common three-domain laccases, SLAC is composed of only two domains without domain 2. Domain 2 is responsible for the connection and positioning of domains 1 and 3 in three-domain laccase, which is essential for the formation of trinuclear cluster at the interface of domains 1 and 3 [28]. However, to form the trinuclear cluster and intact catalytic site, the homotrimer structure is formed to overcome the lacking domain 2 in two-domain laccases [50, 53]. SLAC contains 12 copper ions that form three pseudo-symmetrically related active units. Each SLAC monomer consists of

two domains (Fig. 2a, domain 1, which includes A1, B1, and C1, and domain 2, which is composed of A2, B2, and C2). Each copper center is formed by two neighboring chains organized in a head-to-tail manner (A1–B2, B1–C2, and C1–A2) and contains four copper ions (Fig. 2c). The three copper ions of type 1 are localized near the surface of the central part of the trimer. Three trinuclear copper clusters are placed between domains 1 and 2 of each of the two neighbor chains of the trimer, contributing strongly to the stability of the trimer [50]. However, the spectroscopic and kinetic properties remained similar to those of the common three-domain laccases [54].

In recent years, bacterial laccases from different microorganisms have been isolated and characterized. The strategies of upgrading the production of laccases have also been summarized [55]. Metagenomic analysis [16] has become the most useful and powerful technological tool for determining potential laccase from natural microorganisms, especially from the genes of uncultured and non-cultivable microbes. Immobilization and genetic engineering technologies remain in demand [39, 56–60]. Nanomaterials and ultrafiltration membranes have been extensively researched as vectors for laccase [18, 25, 61–65]. The applications of bacterial laccases, such as degradation of textile dyes, pollutant degradation, bio-sensor, and paper industry, are increasing due to their notable features in extreme industrial environment. Compared with fungal laccases, bacterial laccases exhibit the most significant advantages of high thermostability, wide pH range, and tolerance to alkaline conditions. Bacterial laccases are regarded as promising biological green tools for industrial applications.

Fig. 1 Overall structure and copper centers of *B. subtilis* CotA-laccase (PDB code 1GSK). **a** Domains, T1, and T2/T3 copper (domains 1, 2, and 3 represented in cyan, magenta, and red, respectively). D2 acts as a bridge between D1 and D3. A short α -helical fragment shown in yellow connects D1 and D2. A large loop segment shows D2 and D3 in green links). **b** Conservative amino acids around the copper center. Molecular representations were generated using PyMOL [190]. **c** Schematic of T1 and T2/T3 centers, including interatomic distances among all relevant atoms [89]

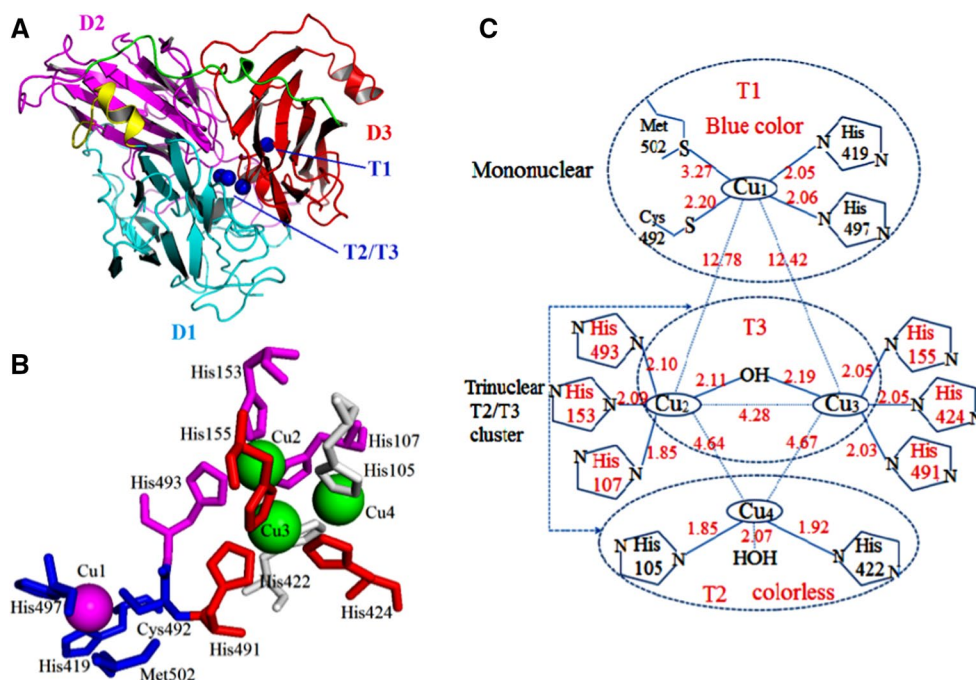
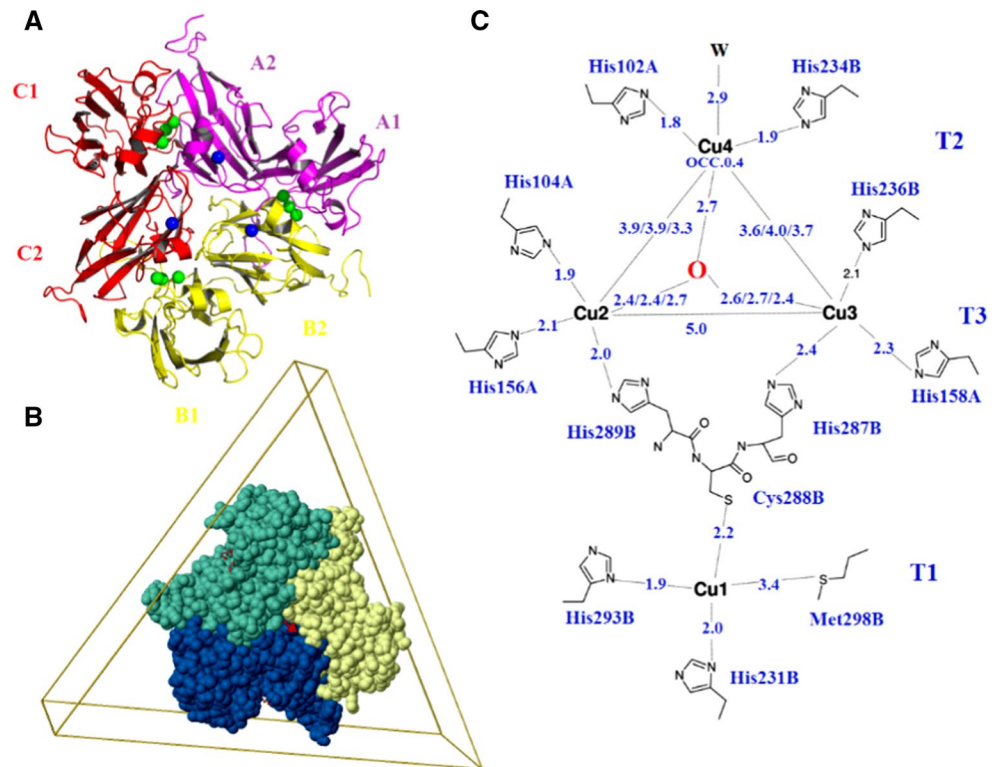


Fig. 2 Structure of the two-domain laccase SLAC from *S. coelicolor* (PDB: 3CG8). **a** SLAC forms homotrimers and monomers are colored in magenta, yellow, and red. T1Cu is shown in blue, and T2/T3Cu is shown in green. **b** 3D view of SLAC. **c** Copper binding scheme in SLAC. Three active sites in the SLAC trimer at domain interfaces A1–B2, B1–C2, and C1–A2. The chain notation corresponds to the interface A1–B2 [50]



Species and properties

Bacterial laccase was first isolated from *Azospirillum* sp. in 1993 from rice rhizosphere [66]. Then, laccases were gradually discovered from numerous other bacteria of different genera, such as Gram-positive bacteria, including *Bacillus*, *Streptomyces*, *Geobacter*, *Staphylococcus*, *Lysinibacillus*, and *Aquisalibacillus*, and Gram-negative bacteria, including *Pseudomonas*, *Delfia*, *Enterobacter*, *Proteobacterium*, and *Alteromonas* [28]. In addition, an increasing number of laccase genes from the metagenome libraries of soil sludge and water are recombinantly expressed [16, 67, 68]. The most characterized CotA-laccases are from *Bacillus*, such as *B. subtilis* [57, 69, 70], *B. pumilus* [42, 44], *B. licheniformis* [71], *B. halodurans* [72], *Bacillus* sp. HR03 [73], *B. valismortis* [74, 75], *B. tequilensis* [76], *B. amyloliquefaciens* [77], *Bacillus* sp. ADR [78], *B. sphaericus* [79], *B. clausii* [80], *B. altitudinis* [42], *B. safensis* [81], and *B. cereus* [82]. *Streptomyces* laccases also exist in a variety of species, such as *S. coelicolor* [49, 83], *S. cyaneus* [48], *S. griseus* [84], *S. lavendulae* [85], *S. psammoticus* [86], *S. ipomoea* [52], *S. svinceus* [53], *S. bikiniensis* [51], *S. violaceusniger*, *S. lividans*, and *S. viridosporus* [87, 88]. Several novel, special sources of laccases are depicted in Table 1.

The molecular weight of the majority of bacterial laccases is predicted to be in the range of 50–70 kDa according to various experimental reports. For example, the molecular weights of *B. subtilis*, *B. pumilus*, and *Bacillus* sp. HR03

CotAs are ~65 kDa [44, 73, 89], whereas that of CotAs from *B. subtilis* are ~67.5 and ~66 kDa [41, 90]. SDS-PAGE analysis revealed that the molecular mass of *S. cyaneus* CECT 3335 is ~69.5 kDa, and Mrlac from *Meiothermus ruber* DSM 1279 possesses a molecular weight of ~50 kDa [30, 33]. However, a special extracellular thermo-alkali-stable laccase, in which laccase is a monomeric protein with a molecular weight of ~32 kDa, has been identified from *B. tequilensis* SN4 [76]. The molecular weights of several other bacterial laccases are shown in Table 1. In CotA from *B. subtilis*, the ~65 kDa form represents the fully denatured protein, and the fast migrating ~30 kDa represents a partially unfolded form of the enzyme [69]. In *B. pumilus* MK001, CotA was boiled for 10 min, showing a band at ~65 kDa; without boiling, a band at ~35 kDa was observed [35].

The most significant biochemical properties of bacterial laccases are their stability under various pH, high temperature, organic solvents, and metal ion conditions. Thermo-stable laccases have been isolated from *S. lavendulae* with a half-life of 100 min at 70 °C and from *B. subtilis* for 112 min at 80 °C [43]. The CotA laccases from *B. pumilus* MK001 and *B. pumilus* W3 exhibit a half-life of 1 and 1.14 h, respectively, at 80 °C [35, 45]. The spore-bound laccase of *B. subtilis* WD23 exhibited a high thermal and pH stability with a temperature half-life of 2.5 h at 80 °C, and its pH half-life is more than 6 months at pH 6.8 and 15 days at pH 9.0 [91]. The laccase also exhibits high tolerance to acetone, petroleum ether, ethyl acetate, and chloroform. The pure CotA

Table 1 Bacterial laccases and laccase-like from different environmental sources and their properties reported in recent years

No.	Laccase source	Expression host	Molecular weight (kDa)	Substrates used in enzyme assay	Optimum temperature (°C)	Thermal stability (°C)	Optimum pH	pH stability	k_{cat}/K_m ($s^{-1} mM^{-1}$)	Activity and function	References
1	<i>Klebsiella pneumoniae</i>	<i>E. coli</i>	~55.6	ABTS, 2,6-DMP	35 (ABTS) 70 (2,6-DMP)	30–70	4.0 (ABTS) 8.0 (2,6-DMP)	5.0–9.0	0.19 (ABTS) 2.36 (2,6-DMP)	Dyes decolorization	[183]
2	Acidic bog soil metagenome	<i>E. coli</i>	~50.3	ABTS, 2,6-DMP L-DOPA Vanillic acid Syringaldehyde Pyrogallol Pyrocatechol	50 (ABTS)	40–60	4.0 (ABTS) 5.0 (2,6-DMP)	–	8.45 (ABTS) 6.42 (2,6-DMP) 173.6 (Pyrogallol)	Decolored azo and triphenylmethane dyes	[16]
3	Metagenome of chemical plant sludge (CueO-G276R)	<i>E. coli</i>	~60	ABTS Benzo[α]pyrene	60 (ABTS)	–	3.5 (ABTS)	–	123 (ABTS)	Oxidize carcinogen benzo[α]pyrene and biological remediation	[68]
4	<i>Proteus hauseri</i> ZMd44 (MCO)	<i>E. coli</i>	–	ABTS	55 (ABTS)	–	2.2 (ABTS)	–	73.03 (ABTS)	Au adsorption, as a biosensor and bioremediation of electronic waste	[182]
5	<i>Bacillus lichniformis</i> LS04	<i>P. pastoris</i>	–	ABTS	70 [WT] 70 [D500G]	70 °C/1.8 h [D500G]	4.2 [WT] 4.6 [D500G]	–	127.27 [WT] 115.12 [D500G]	Dyes decolorization	[103]
6	<i>Bacillus subtilis</i> cjp3	<i>E. coli</i>	~58.5	ABTS SGZ	80 (ABTS)	20–80	5.0 (ABTS)	9.0/10 h	–	Treating waste water containing synthetic dyes	[70]
7	<i>Streptomyces cyaneus</i> CECT 3335	<i>E. coli</i>	~69.5	DMP ABTS Guaiacol	30–90 (DMP)	60 °C/1 h/50% activity	5.5 (DMP)	5.5/24 h/40%	4.737 (DMP) 1.72 (ABTS) 5.17 (Guaiacol)	Biomass degradation	[30]

Table 1 (continued)

No.	Laccase source	Expression host	Molecular weight (kDa)	Substrates used in enzyme assay	Optimum temperature (°C)	Thermal stability (°C)	Optimum pH	pH stability	k_{cat}/K_m ($s^{-1} mM^{-1}$)	Activity and function	References
8	<i>Chromohalobacter salexigens</i>	-	-	2,6-DMP, ABTS, L-Tyrosine, SGZ, catechol, L-DOPA, guaiacol, Gallic acid, tannic acid, pyrogallol, Resorcinol	-	25–55 °C/80% activity	-	6.0–9.0/80%	-	Cellulose fibres extraction, delignification of lignin and lignin-derived industrial wastes	[171]
9	<i>Bacillus valisornis</i> fmb-103	<i>E. coli</i>	~70	ABTS, SGZ, Acetosyringone	84 [H/AS], 85 [Ni-NTA] (ABTS)	70 °C/10 h/> 50% activity	4.8 (ABTS), 7.4 (SGZ)	8.0/10 d/90% [H/AS], 8.0/10 d/80% [Ni-NTA]	$K_m = 35.79 \mu M$ [1], $K_m = 24.4 \mu M$ [2] (ABTS), $K_m = 379.12 \mu M$ [1], $K_m = 302.4 \mu M$ [2] (SGZ)	Malachite green degradation	[74]
10	<i>Setosphaeria turcica</i>	<i>E. coli</i>	~71.5	ABTS	60 (ABTS)	-	4.0 (ABTS)	-	-	Industrial effluents application	[184]
11	<i>Aquisalibacillus elongatus</i>	-	~75	2,6-DMP	40	25–55 °C/6 h/> 80% activity	8.0 (2,6-DMP), 6.0 (ABTS), 7.0 (SGZ)	5.0–10.0/6 h/> 40%	1.23×10^5	Delignification of sugar beet pulp	[170]
12	<i>Thermus thermophilus</i>	<i>E. coli</i>	~53	ABTS, SGZ, 4-fluoro-2-methylphenol	90 (ABTS), 80 (SGZ) [short duration], 65 (ABTS) [longer duration]	-	6.0 (ABTS), 7.0 (SGZ), 8.0 (4-fluoro-2-methylphenol)	-	1.99 (ABTS) [60 °C], 673.20 (SGZ) [40 °C]	Decolorization of industrial dyes	[154]
13	<i>Pediococcus Acidilactici</i> CECT 5930	<i>E. coli</i>	~60	ABTS	28 (ABTS)	28–60 °C/10 min/> 80% activity	4.0 (ABTS), 9.5 (Tyramine)	-	$K_m = 1.7 mM$ (ABTS)	Degrade tyramine in food	[175]
14	<i>Anoxybacillus sp. UARK-01</i>	<i>E. coli</i>	-	Congo Red	90	-	9	-	-	Dyes decolorization	[152]
15	<i>Geobacillus thermopaksistanensis</i>	<i>E. coli</i>	~60	Syringaldazine	60 (SGZ)	-	7–7.5 (SGZ)	-	1179 (SGZ)	Bioremediation of colored wastewater	[185]

Table 1 (continued)

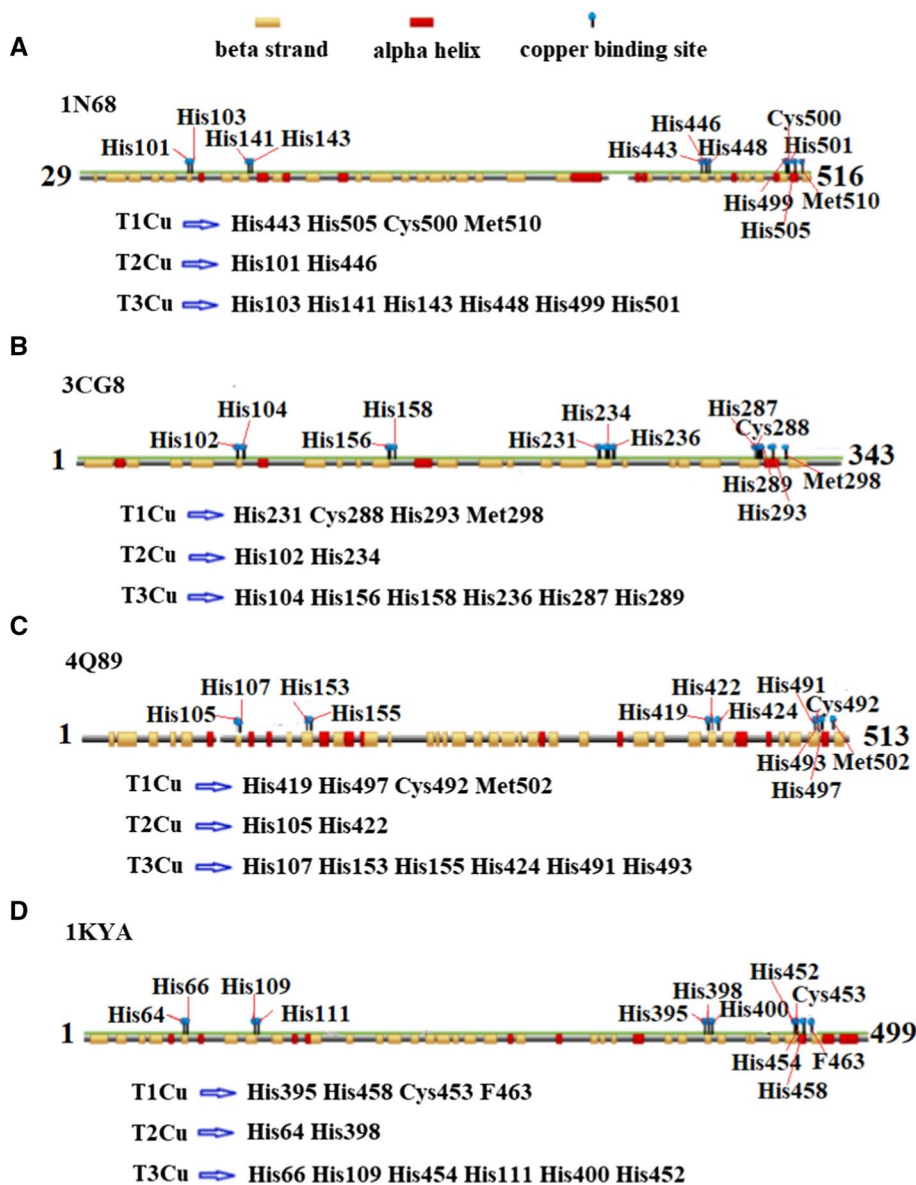
No.	Laccase source	Expression host	Molecular weight (kDa)	Substrates used in enzyme assay	Optimum temperature (°C)	Thermal stability (°C)	Optimum pH	pH stability	k_{cat}/K_m ($s^{-1} mM^{-1}$)	Activity and function	References
16	<i>Spirulina platensis</i> CFTRI	<i>E. coli</i>	~66	ABTS	30 (ABTS)	30 °C/1 h/100%, 50 °C/1.5 h/80%	3.0 (ABTS)	8.0/1 h/100%	–	Decolorize synthetic dyes and treat waste water	[153]
17	A marine microbial metagenome library Lac15	<i>E. coli</i>	~43 to 52 [NaCl concentration]	ABTS SGZ 2,6-DMP Guaiacol Dopamine hydrochloride, Potassium ferrocyanide trihydrate	–	–	–	–	260 [0 mM NaCl] 240 [200 mM NaCl] 270 [500 mM NaCl] 290 [2000 mM NaCl] 310 [2500 mM NaCl] (SGZ)	Biodegradation of organic pollutants and synthesis of novel drugs	[67]
18	<i>Bacillus pumilus</i> W3	<i>E. coli</i>	~65	ABTS	80 (ABTS) [WT]	50 °C/6 h >50%	3.6 (ABTS)	Stable at alkaline pH	157.86 [WT]	Dye decolorization	[102]

laccase is 157% activated by Cu^{2+} and remains stable toward Fe^{2+} [90]. Moreover, special thermophilic and alkaliphilic bacterial strains that contain laccase-like multicopper oxidases genes exist. TtMCO from the thermophilic bacterium *Thermobaculum terrenum* is extremely thermophilic with an inactivation half-life of 2.24 days at 70 °C and 350 min at 80 °C at pH 7.0 [92]. ALRh from an alkaliphilic bacterial strain *Thioalkalivibrio* sp. is a pH-tolerant laccase that is stable in the pH from 2.1 to 9.9 at 20 °C [93]. CotA also exhibited a considerably higher H_2O_2 tolerance than fungal laccases from *T. versicolor* and *T. troglia* [42]. Several laccases and laccase-like bacterial species from different environment sources and their properties were studied in recent years (Table 1). Several factors, including hydrogen bonds and salt bridges, distribution of charged residues on the surface, protein packing, and acid composition [94], contribute to the stability of enzymes. The proline content is apparently associated with increasing protein thermostability [95, 96]. The introduction or increase of proline number is believed to be conducive for improving protein thermostability among many mesophilic bacteria and hyperthermophiles [95]. The percentage of proline residues from *B. pumilus* W3 CotA is 9.0% (46 pro residues and 513 total residues), and those of other laccases of known structure are 8% for TvLa, 8.2% for MaLa, 7.5% for CcLa, and 6.2% for CueO [89]. Copper ions also play key roles in the stability of MCOs [54]. Laccases possess a secondary structure that displays high β -strand (Fig. 3), which is a special characteristic that may explain their high stability.

Substrates and mediators

Laccase is a notably substrate-specific enzyme that oxidizes a wide range of substrates. It can catalyze the synthesis and the breakdown reaction of various organic and aromatic compounds. The breakdown of environmentally harmful pollutants contributes to an eco-friendly environment, and the synthesis of complex compounds by producing non-toxic substances leads to bioremediation [97]. Substrates such as 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,6-dimethylphenol (2,6-DMP), syringaldazine (SGZ), acetosyringone (ACS), guaiacol, and L-DOPA are extensively used, and substrated ABTS, SGZ, and 2,6-DMP are the most used substrates for enzyme assays (Table 1). The oxidation reactions of ABTS, SGZ, and 2,6-DMP usually occur at 420 ($\epsilon = 36,000 M^{-1} cm^{-1}$), 525 ($\epsilon = 65,000 M^{-1} cm^{-1}$), and 468 nm ($\epsilon = 37,500 M^{-1} cm^{-1}$), respectively [93, 98]. Substrate binding with protein by using SGZ is exhibited in Fig. 4. A large number of substrates with large size or high redox potential, such as several azo and anthraquinonic dyes, cannot be oxidized directly by laccase. These substrates require an “electron shuttle,” called

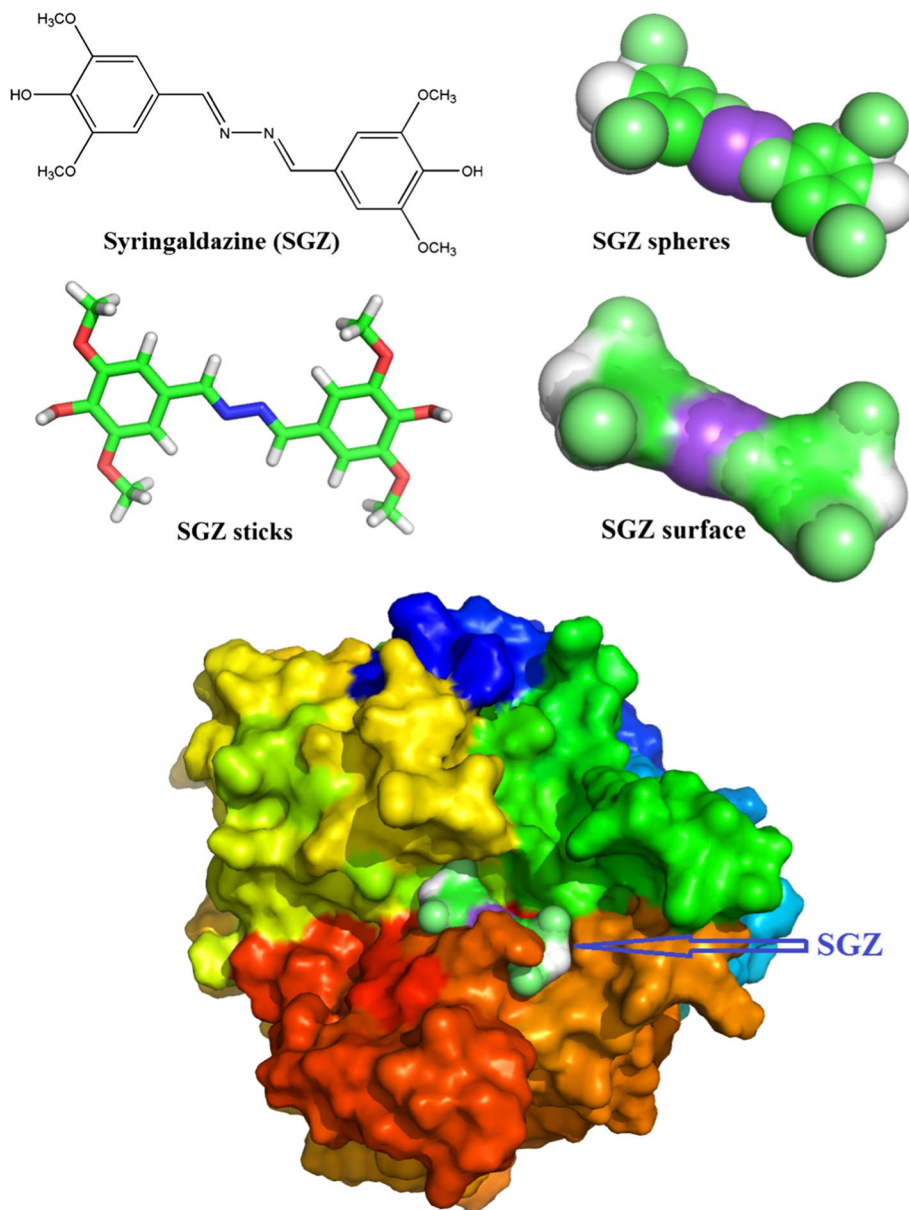
Fig. 3 Secondary structure of four laccases and conservative copper binding site of four typical laccases. **a** *E. coli* laccase (CueO, PDB: 1N68, 488 residues). **b** *S. coelicolor* laccase (SLAC, PDB: 3CG8, 343 residues, SLAC forms homotrimers). **c** *B. subtilis* laccase (CotA, PDB: 4Q89, 513 residues). **d** *T. versicolor* laccase (fungal laccase, PDB: 1KYA, 499 residues; the 1KYA structure contains a total of 4 chains and is represented by one chain)



mediator, between them and laccase [99]. Mediators are low-molecular weight laccase substrates whose enzymatic oxidation causes stable high-potential intermediates; these substrates chemically react with other compounds that cannot be oxidized by laccase alone [100]. ABTS is the first synthetic mediator that was identified to serve as a laccase substrate mediator that enhanced enzyme action [101]. ABTS binds to the enzyme's "pocket" mainly through H-bonds, vander Waals forces, and electrostatic force. The substrate binding pocket and interaction force between ABTS with the amines of laccase are shown in Fig. 5. In recent years, ABTS and especially ACS have been widely used as mediators in dye decolorization because of their high efficiency [70, 74, 102–104]. Several *N*-heterocycles bearing *N*-OH, such as violuric acid, *N*-hydroxyl-*N*-phenyl acetamide, and *N*-hydroxybenzotriazole, are effective mediators [43].

Laccase-mediator systems (LMSs) involve three main categories of mediators, namely synthetic mediators, natural mediators, and polyoxometalates (POM). An increasing number of synthetic mediators, such as ABTS, 1-hydroxybenzotriazole (HBT), violuric acid, and 2,2',6,6'-tetramethylpiperidyl-1-oxy (TEMPO), have been identified. The chemical structures of several synthetic mediators are shown in Fig. 6b. The synthetic mediators can be used in lignin degradation, dye decolorization, and polycyclic aromatic hydrocarbon (PAH) oxidation. However, three major drawbacks of synthetic mediators are as follows: (1) high cost for application at industrial scale; (2) possible formation of toxic derivatives that inactivate laccases [105]; and (3) poor regeneration capacity and high ratio of mediator/substrate is high [106].

Fig. 4 Structure of syringaldazine (SGZ) and docking model of laccase from *B. pumilus* W3 (homology modeling using CotA from *B. subtilis* PDB code: 1GSK) with substrate using Autodock Vina [191]

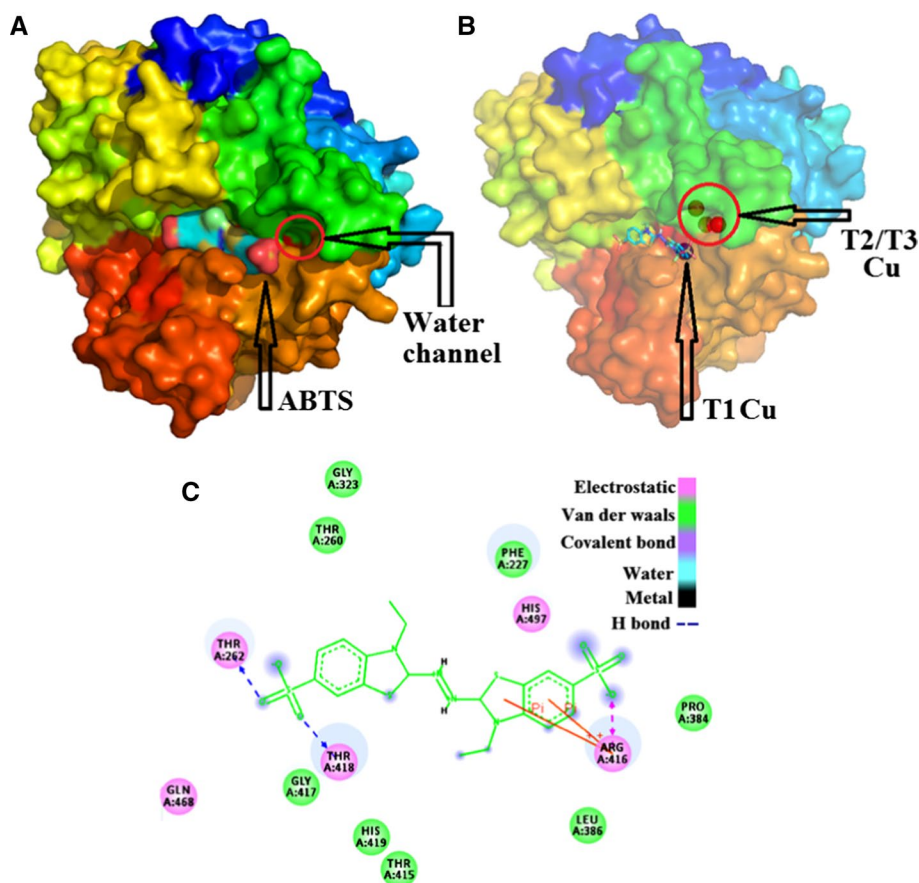


Inexpensive and eco-friendly natural mediators from plants and industrial by-products [107–109] are available. Most natural mediators are a series of phenol compounds with low chemical potential that can be rapidly oxidized by laccases. By analyzing the interactions between CotA and sinapic acid (SA), the presence of methoxy groups in the *ortho*-position of the phenolic structure is crucial for substrate recognition by CotA-laccase [110]. Acetylacetone (AA) can be used to form a laccase-AA system and enhance the stability of laccase [111]. Moreover, SA, AS, and *p*-coumaric acid (*p*-PCA) can improve the stability of laccase during the degradation of flax pulp [112]. The chemical structures of certain natural mediators are shown in Fig. 6a.

Polyoxometalates, a class of polymetal oxygen cluster compounds formed by the transition metal ions through

oxygen linkages, is a kind of bifunctional catalyst characterized by redox and catalysis [113]. POM can be used as mediator of laccase because of its highly stable structure and catalytic activity. It has also been used for lignin degradation and dye bleaching [114–116]. In recent years, LMSs mixed with other enzymes have been widely used in reaction systems. For example, the alkalophilic bacterial xylanase, mannanase, and LMS were combined for biobleaching of mixed wood kraft pulp, and the results demonstrated a reduction of 30% chlorine and 44.4% H_2O_2 consumption after the triple enzyme treatment, thereby revealing an eco-friendly alternative for total chemical bleaching [117].

Fig. 5 Binding pocket of substrate (ABTS as an example) near T1Cu and the interaction force between ABTS with amines of laccase. Docking using AutoDock Vina and Pymol software. Interaction force is shown using Discovery Studio 3.5 Visualizer [192]. **a** ABTS binds to the enzyme's "pocket", **b** Shown by 40% transparency, **c** The substrate binding pocket and interaction force between ABTS with the amines of laccase



Mechanisms of laccase and LMS

Polyphenols can be oxidized by certain enzymes, such as laccase (EC 1.10.3.2), catechol oxidase (EC 1.10.3.1), and cresolase (EC 1.18.14.1) [118], that exhibit oxidase activities. As shown in Fig. 1, laccase contains four copper atoms that form the active center. The binding pocket of the substrate is near the T1Cu center (Fig. 5b). T2/T3 trinuclear cluster is responsible for the reduction of oxygen to water. Laccase catalysis is believed to involve three steps. First, T1Cu is reduced by a reducing substrate. Then, internal electron transferring from T1Cu to T3Cu and T2Cu occurs through a Cys–His pathway that is highly conserved among multicopper oxidases. Finally, oxygen is reduced to water at the T2/T3 trinuclear cluster center [28, 43]. The reaction mechanism of O_2 to H_2O is shown in Fig. 7. The reaction of fully reduced enzyme with O_2 occurs in two two-electron steps. Thus, the reaction is a four-electron process. According to Solomon et al. [119], the first step is slower than the second step; thus, the first step is the rate determining step, which is driven by the presence of an anionic Asp residue near the T2Cu. The second step indicates the large driving force for the two-electron reduction of peroxide combining with the trinuclear center, which presents a triangular topology. The reaction of the fully reduced enzyme with O_2 can

generate the native intermediate (NI). Without substrates, the NI undergoes a gradual decay into a resting enzyme form. NI and resting form are both fully oxidized forms, but with a difference: type 2 and coupled-binuclear type 3 Cu are isolated in the resting enzyme whereas these are all bridged by μ_3 -oxo (O–Cu–O) ligand in NI. Moreover, the T2 OH[−] ligand and T3 OH[−] bridge are maintained in the resting enzyme [120].

The LMS can enhance reaction efficiency and enlarge the range of reaction. Three mechanisms govern the function of mediators in LMS: (1) electron transfer (ET); (2) hydrogen atom transfer (HAT); and (3) ionic mechanism type (IM). The cation radical is significant for LMS. For example, ABTS is a commonly used substrate for bioassay and mediator for oxidizing other substrates, and $ABTS^{2+}$ is the most useful radical. ABTS is oxidized in two stages (Fig. 8). The first stage is the formation of $ABTS^{+\bullet}$ cation radical, followed by slow oxidation of $ABTS^{+\bullet}$ to $ABTS^{2+}$ [100]. According to Morozova et al. [100], $ABTS^{+\bullet}$ can interact only with lignin phenolic groups, and $ABTS^{2+}$ is required for the degradation of nonphenolic lignin structures. Anisyl alcohol and benzylalcohol can be better oxidized by $ABTS^{+\bullet}$ than by $ABTS^{2+}$ [43]. The redox states of ABTS are stable and reversible with formal redox potentials of 0.472 V for $ABTS/ABTS^{+\bullet}$ couple and 0.885 V for $ABTS^{+\bullet}/ABTS^{2+}$

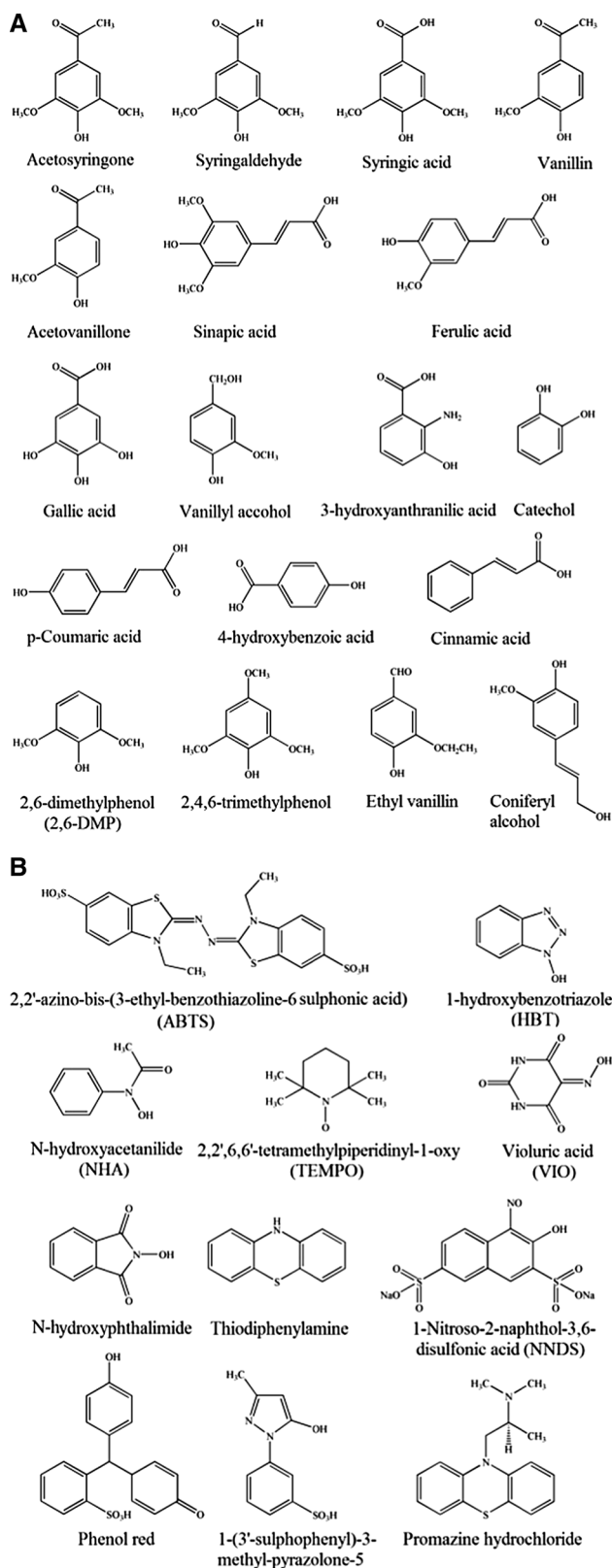


Fig. 6 Chemical structures of several natural and synthetic mediators. **a** Natural mediators; **b** Synthetic mediators

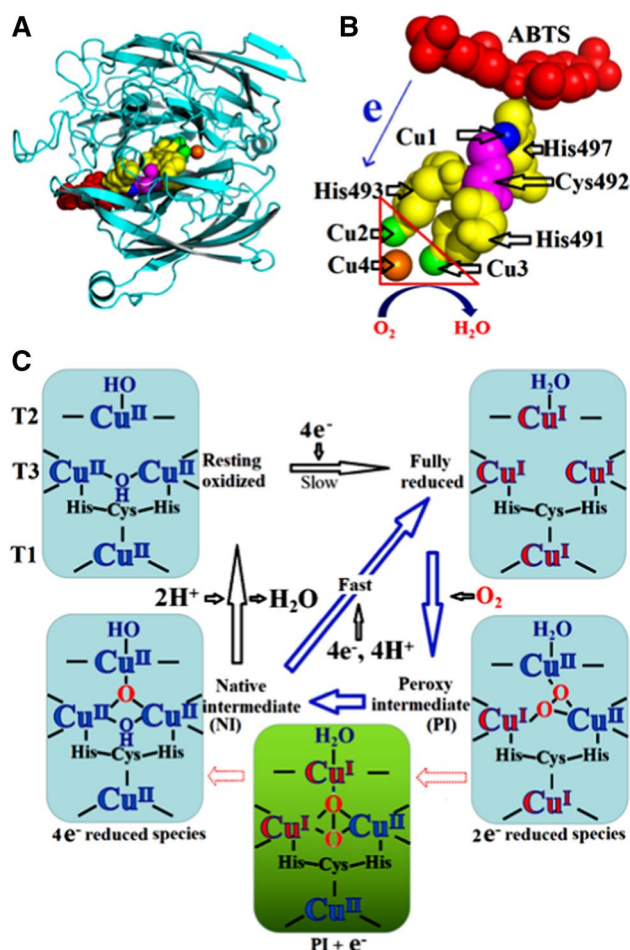


Fig. 7 Reaction mechanism of O₂ to H₂O of bacterial laccases. **a** The model of ABTS with Cu center in laccase. **b** The abstracted electron moves from the T1Cu center to the trinuclear cluster through a Cu–Cys–His pathway. **c** Mechanism of O₂ reduction to water. Blue arrows indicate the steps in the catalytic cycle of the laccases. Black arrows indicate the steps that are not part of the catalytic cycle but can be experimentally observed. The green box indicates the transfer of an electron from T1Cu to the T2Cu to yield PI + e⁻, which occurs in the transfer from PI to NI but is not experimentally observed [119]

according to cyclic voltammetry studies [100]. In the ET mechanism, the C_α–C_β bonds of nonphenolic aromatic substrates are disconnected by a cation radical and generate H–C_α=O as the final product (Fig. 9a). The oxidation of nonphenolic lignin structures by this mechanism includes ABTS and coordinated transition metals. The HAT mechanism is usually generated with a mediator that contains N–OH or phenoxy-group, such as HBT, PCA, VIO, and NHA. In these mediators containing N–OH, the formation of N–O[•] is significant. The mechanism of HAT is preferable for the formation of O=C_α–C_β group, such as the oxidation of nonphenolic lignin model compounds (4-methoxybenzylalcohol) by LMS (Fig. 9) [100]. The IM mechanism that is independent of the redox features of the substrate is

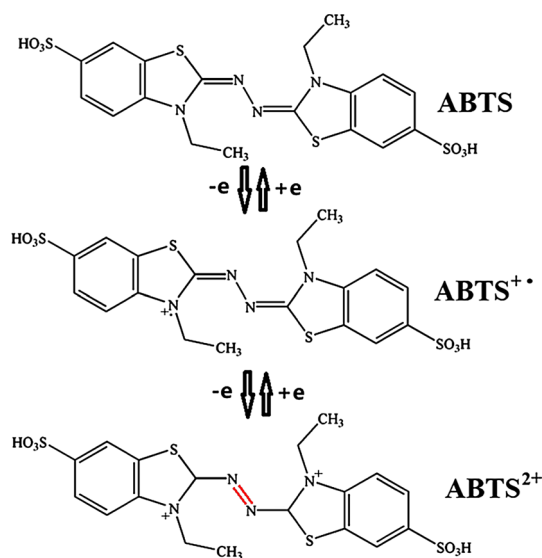


Fig. 8 Oxidation of ABTS to $ABTS^{2+}$ catalyzed by laccase

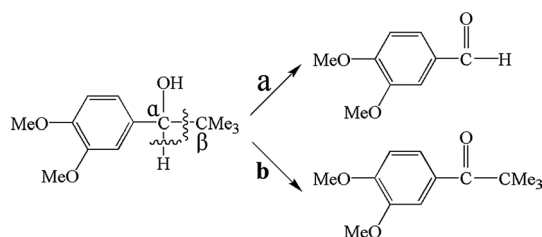


Fig. 9 Oxidation of nonphenolic lignin model compounds (4-methoxybenzylalcohol) by laccase mediators. a Oxidation mechanism of ET; b Oxidation mechanism of HAT

suggested with the laccase/TEMPO system. TEMPO can be oxidized by laccase to form oxoammonium ion, and then the oxoammonium ion oxidizes the alcoholic substances to form carbonyl product or hydroxylamine. The oxidation of alcohols by laccase/TEMPO is shown in Fig. 10 [121].

Polycyclic aromatic hydrocarbons can be oxidized by LMS, but different mediators show distinctions. Taking the oxidation of benzo[α]pyrene for example, laccase/ABTS, laccase/PCA, and laccase/HBT all oxidize benzo[α]pyrene-generated quinone. However, only the accumulation of 6-benzo[α]pyrene acetate intermediate in the laccase/ABTS system is observed (Fig. 11). ABTS-mediated reaction follows an ET route, whereas HBT (nitroxyl) radicals oxidized the aromatic substrate by HAT route, and PCA phenoxyl radicals act similarly to nitroxyl radicals [122, 123]. Laccases not only catalyze the synthetic reaction but also degradation. The formation of C–O, C–N, C–S, and C–C bonds and the reaction of O–O can be attained by oxidative reaction of laccase. Five examples are shown in Fig. 12 [99, 124, 125]. Using a bacterial laccase, the lignin model syringylglycerol β -guaiacyl ether is successfully coupled using the phenolic compound tyramine

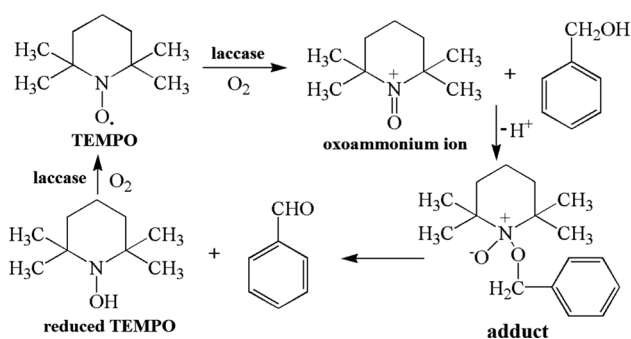


Fig. 10 Oxidation of alcohols with laccase/TEMPO

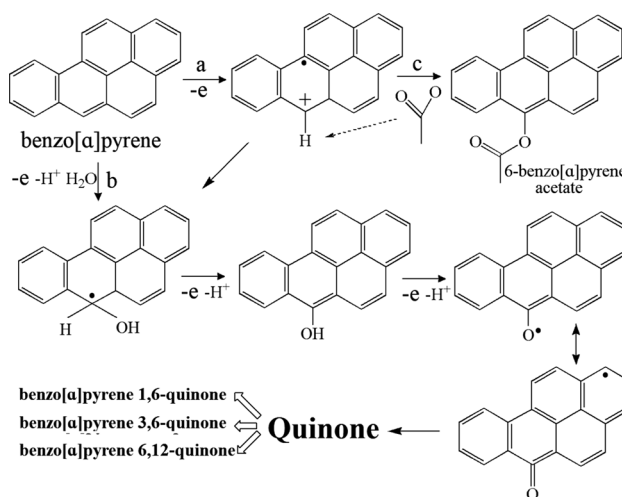


Fig. 11 Three oxidation routes of benzo[α]pyrene by laccase-mediator system. a ET route by laccase/ABTS; b HAT route by laccase/PCA or laccase/HBT; c Nucleophilic attack of the acetate ions of the reaction mixture toward the benzo[α]pyrene radicals generated by laccase/ABTS system. Three different quinones are the final products

as substrate [126], and indole is trimerized to form 2,2-bis(3'-indolyl)indoxyl [127]. The mechanism of degradation, that is, the degradation of perfluorooctanoic acid, is complicated and can form approximately 10 byproducts from ECOHRs in the mineral buffer and Cu^{2+} solution [128]. The catalytic process of laccases is exhibited in Fig. 13.

Engineering of bacterial laccases

At present, bacterial laccases are not widely used in industries because of their low expression levels and catalytic activity compared with fungal laccases. To meet the industrial demands for high expression level, high catalytic activity, stability, and reduced production cost, many researchers paid attention to bacterial laccase engineering. In recent years, heterologous functional expression and

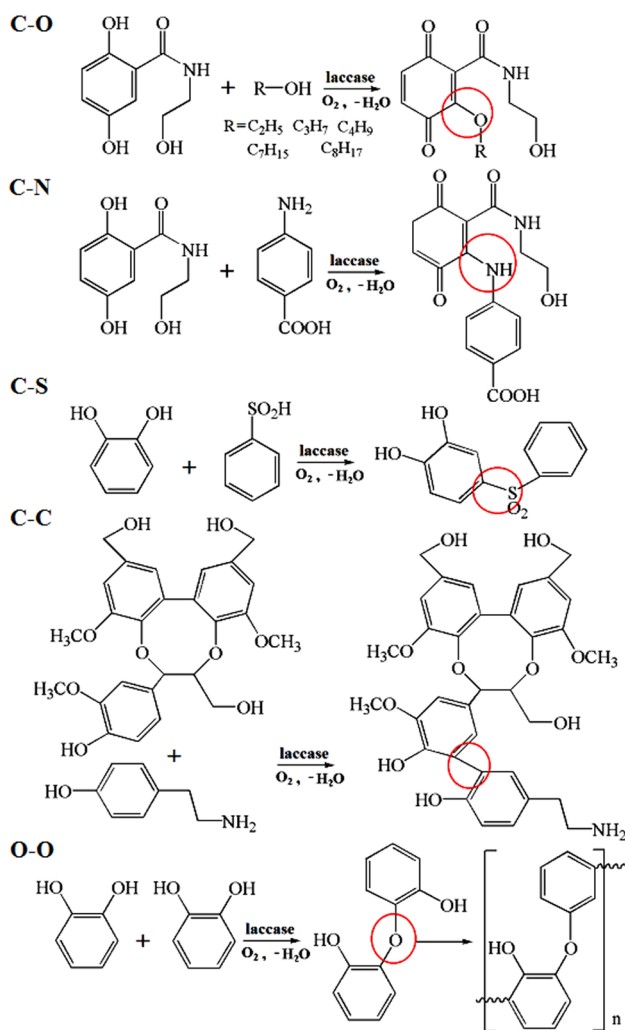


Fig. 12 Formation of C–O, C–N, C–S, and C–C bonds, as well as reaction of O–O, can be attained by oxidation of laccase

rational, semi-rational, or directed evolution approaches have been used to convert bacterial laccases into high value-added biocatalysts (Table 2).

Improvement of catalytic activity and substrate specificity

With the resolution of the crystal structure of several laccases, the laccase structure–function relationships have been elucidated. The “substrate binding pocket” or in the vicinity of the catalytic coppers affected the activity. A useful “platform” from *B. subtilis* spores is available for directed evolution studies to broaden substrate specificities. The ABTS-bound structure shows the substrate surrounded by 23 amino acids (Pro226, Ala227, Phe228, Cys229, Thr260, Arg261, Thr262, Gly321, Cys322, Gly323, Gly324, Gln378, Gly382, Pro384, Leu386, Thr415, Arg416, Gly417, Thr418, His419, Ile494, His497, and Met502). Among these amino acids, His419, His497, and Cys492 are coordinated with T1Cu, and a disulfide bond is formed between Cys229 and Cys322. The five residues are not altered [129]. The residues of the substrate binding pocket of CotA are randomly modified by saturated mutagenesis to increase the specificity of the enzyme, and the mutant (L386W/G417L) is 132 times more specific for ABTS over SGZ [129]. This finding is also observed in *B. pumilus* CotA, and the catalytic efficiency of its mutant L386W/G417L for ABTS is 4.3-fold higher than that of WT CotA-laccase [130]. A CotA mutant T260L from *B. subtilis* spore is 120-fold more specific for ABTS compared with the baseline [131]. According to Xie et al. [110], Arg416 in CotA-laccase plays an important role in substrate oxidation, and the flexibility of Arg416 facilitates the binding of various substrates. Several other site mutations in *Bacillus* laccases enhance catalytic activity (Table 2). In addition to positive mutations, unalterable conservative sites, such as Met502 of CotA-laccase, occur and act as the axial ligands of T1Cu according to several studies. Catalytic activities decrease strongly after substitution of this residue [132]. The substrate binding pocket of SLAC from *S. coelicolor* has also been redesigned through site-directed mutagenesis to improve its activity toward compounds of interest as redox mediators. The substitution of the two Met of the pocket by small residues (Ala and Gly) substantially increased the catalytic efficiency with

Fig. 13 Catalytic process of laccases

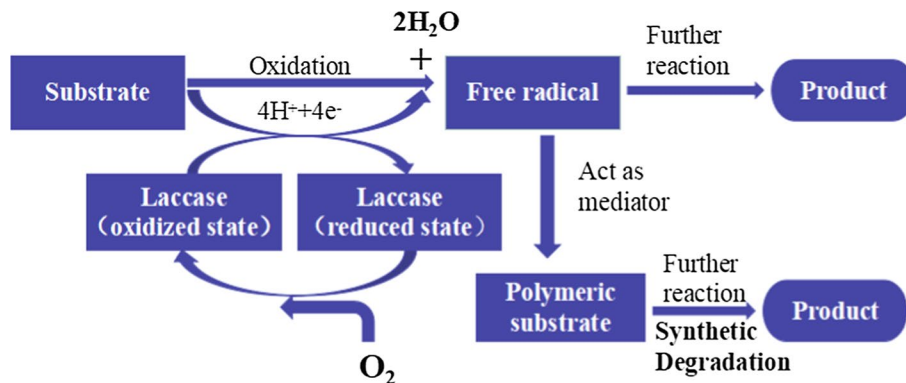


Table 2 Several typical bacterial laccases engineering

Source	Expression host	Modification	Main results	References
<i>Bacillus subtilis</i>	<i>E. coli</i>	M502L M502F	Redox potential increased 100 mv; but catalytic activity is compromised; T1 copper depletion determine thermodynamic stability	[132]
<i>Bacillus subtilis</i>	<i>E. coli</i>	L386A I494A	Both redox potentials downward; and catalytic efficiency decreased	[134]
<i>Bacillus licheniformis</i>	<i>E. coli</i>	K316N D500G K316N/D500G	11.4-fold higher expression level; high dimerization of phenolic and decolorization of industrial dyes	[46]
<i>Shigella dysenteriae</i>	<i>E. coli</i>	E106F Deletion α -helix	Promoting both enzymatic activity and thermostability	[138]
<i>Bacillus subtilis</i>	<i>E. coli</i>	E498D E498T E498L	Catalytic impairment; decreased affinity; Glu498 plays a key role in the protonation events	[186]
<i>Bacillus subtilis</i>	<i>B. subtilis</i>	T260L	120-fold more specific for ABTS	[129]
<i>Bacillus</i> sp. HR03	<i>E. coli</i>	E188K E188R E188A	Thermal stability increased; k_{cat}/K_m of E188R enhancement	[137]
<i>Streptomyces coelicolor</i>	<i>E. coli</i>	Y108A Y108F	Tyr108 does form an integral part of the active site and affects enzyme kinetics	[187]
<i>Bacillus subtilis</i>	<i>E. coli</i>	D116A D116N D116E	Catalytic properties severely compromised; the position 116 in CotA is important for catalysis	[188]
<i>Streptomyces coelicolor</i>	<i>E. coli</i>	Y229A Y230A	Over 10-fold increase in activity	[49]
<i>Streptomyces coelicolor</i>	–	M168G M168A Y199W M266A M266W M168G/M266A M168G/M266W	Enhanced kinetic parameters with a phenolic substrate; enhancement of the ability to decolorize indigo carmine in the presence of commercial mediators (methylsyringate and TEMPO)	[133]
<i>Bacillus</i> sp. HR03	<i>E. coli</i>	D500E D500G D500S G-insertion	Increase in the expression level up to threefold (D500G)	[135]
<i>Bacillus subtilis</i>	<i>P. pastoris</i>	–	76-fold increase in laccase activity through sorbitol addition and pH adjustment in <i>P. pastoris</i>	[104]
<i>Bacillus subtilis</i>	<i>E. coli</i>	T415A R416A	Arg416 is crucial in the oxidation of ABTS and SGZ	[110]
<i>Aquifex aeolicus</i>	<i>E. coli</i>	M449T, I441L K245R, R471G P58S, I199T Y172C, V19A F55S	M449T, K245R, I441L, Y172C, V19A, F55S, and in particular I199T have likely contributed individually or synergistically to improve oxidation for aromatic substrates; R471G and V19A is significant in the stabilization	[189]
<i>Bacillus subtilis</i>	<i>E. coli</i>	R146K, R429K R476K, L431F A478F, T480A T480F	The residues Arg146, Arg429, and Arg476 are essential for the oxidation of ABTS and syringaldazine. T480F was identified to be more specific for ABTS than syringaldazine	[155]
<i>Bacillus subtilis</i>	<i>B. subtilis</i> 1S101	E29V, L343S E498G, T480A T480A/E498G E29V/L343S/E498G	An organic-tolerant and acid-stable variant T480A/E498G had a $t_{1/2}$ 62.1 times increased than wt-CotA	[57]
<i>Bacillus amyloliquefaciens</i>	<i>E. coli</i>	D501G	Better stability and catalytic efficiency	[39]
<i>Bacillus subtilis</i>	<i>E. coli</i>	α -Hemolysin secretion system and YebF secretion system	A simpler approach for extracellular production of recombinant CotA laccase in <i>E. coli</i>	[40]

Table 2 (continued)

Source	Expression host	Modification	Main results	References
<i>Bacillus licheniformis</i>	<i>P. pastoris</i>	D500G	2.1-fold higher activity expressed in <i>P. pastoris</i> and 9.3-folds higher expression than wild-type enzyme	[103]
<i>Bacillus pumilus</i>	<i>E. coli</i>	L386W, G417L L386W/G417L L386W/G417L/G57F	Higher catalytic efficiency for ABTS and decolorization of four industrial dyes. Higher thermostability and resistance to alkaline environment	[130]
<i>Bacillus pumilus</i>	<i>E. coli</i>	K317N/WLF D501G/WLF K317N/D501G/WLF	Enhanced functional expression and remained high thermostability, high decolorization of azo, anthraquinonic and triphenylmethane dyes	[102]

Fig. 14 C-terminal sequence alignment of bacterial and fungal laccase genes. The conserved regions are represented by the red background. The box shows the different residues in the purpose position

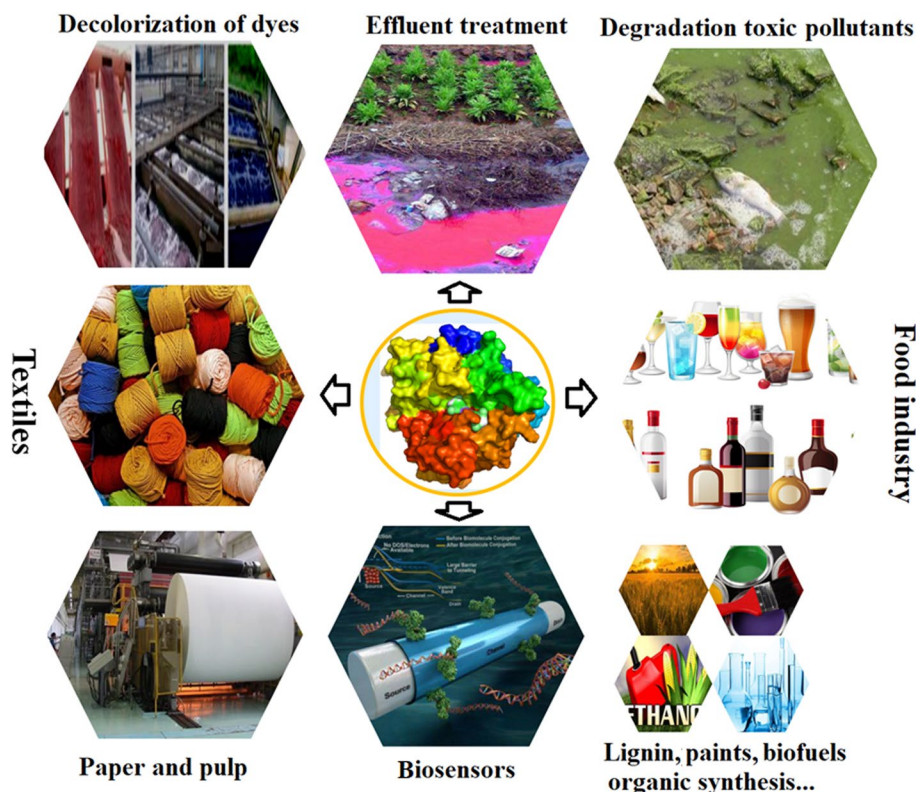
<i>B. pumilus</i> W3	FVPYSG. . . RYVWHCHILEHEDYDMMRPMDIIQ.	510
<i>B. pumilus</i> MK001	FVPYTG. . . RYVWHCHILEHEDYDMMRPMDIIQ.	510
<i>B. subtilis</i>	FGPYSG. . . RYVWHCHILEHEDYDMMRPMDITDPHK.	513
<i>B. licheniformis</i>	FAPYSG. . . RYVWHCHILEHEDYDMMRPLEVTDVRHQ.	513
<i>E. coli</i>	FNHDAPKEHAYMAHCHLLEHEDTGMLLGFTV.	488
<i>T. thermophilus</i>	LREKG. . . RTVFHCHIVEHEDRGMGVLEVG.	462
<i>B. halodurans</i>	AKNPGN. . . WMFHCHIEFHASGGMVAEIHVEGFELPF. . . TPDNPNMPE. . .	500
<i>Ascorbate oxidase</i>	IRFVADNPGVWAFHCHIEPHLHMGVVFVAEGVEKVG. . . IPTKALACGCTAKS	544
<i>T. versicolor</i>	IRFRDNPWPFLHCHIDFHLEAGFAVVFAEDIPDVAS. . . ANPVPQAWSDLCP	490
<i>M. albomyces</i>	LAFRTDNPAGAWLFHCHIAWHVSGGLSVDFLERPADLRQRISQEDDDFNRCDE	542

DMP and the decolorization of indigo carmine mediated by methylsyringate and TEMPO [133]. According to site-directed mutagenesis by Sherif et al. [49], 17 amino acid residues, including 10 His involved in copper coordination, are crucial for SLAC activity. In particular, the Y229A and Y230A mutants showed over 10-fold increase in inactivity compared with WT SLAC. In *S. sviveus*, several Met residues (Met195, Met220, Met293, and Met295) located in the putative substrate binding site of the enzyme are substituted with Leu. Moreover, a truncated mutant without 17 residues corresponding to the C-terminus of the laccase was evaluated. All variants exhibited increased redox potentials ranging from 16 and 81 mV over the WT enzyme [53]. According to Durão et al. [134], the changes in amino acid residues in direct contact with metal center (including ligands) significantly affect the properties of the T1 copper sites of laccase and suggested that the redox potential may be modulated without compromising the overall reactivity through changes in residues away from the immediate contact shell. The mechanisms influencing the catalytic efficiency through mutation is the change in interaction between the enzyme and substrate, as well as the variety of redox potential in the T1Cu site. Moreover, the quantity and distance of H-bonds may also be changed between laccase pocket residues with substrate.

Enhancement of expression level

E. coli, *B. subtilis*, and *P. pastoris* are typically used as expression hosts. CotA-laccase mutants are more easily expressed in *E. coli* compared with other hosts, but isolation of high enzyme quantities from its cell extracts in practical industrial applications is expensive. The advantage of *B. subtilis* and *P. pastoris* is their exocrine expression, but mutation is more easily conducted successfully in *E. coli*. In recent years, site-directed mutagenesis technology has been used to improve functional expression and achieve success in *B. licheniformis*, *Bacillus* sp. HR03, and *B. pumilus*. The soluble expression in *E. coli* of endospore laccase from *B. licheniformis*, which is similar to *B. subtilis* CotA, is enhanced 11.4-fold by a combination of random and site-directed mutagenesis [46]. One of the selected mutations (D500G) is found to be adjacent to the axial Met of T1Cu, which is responsible for an eightfold increase in soluble expression. After sequence alignment, an Asp residue in this position has only been observed in laccases from *Bacillus* genus, whereas other bacterial and fungal laccases present Gly. This mutation is subsequently introduced in laccase from *Bacillus* sp. HR03, which also caused a threefold increase in the expression in *E. coli* [135] and in *B. pumilus* (D501G/WLF), thereby inducing a 4.48-fold increase in the expression level compared with WLF [102]. The position Asp⁵⁰¹ (equivalent to Asp⁵⁰⁰ in *B. licheniformis*) is located in the C-terminal segment and close to the T1Cu center that lies in a conserved region

Fig. 15 Application areas of laccases



(Fig. 14). One to three amino residues (X) in the conserved region (His–Cys–His–XXX–His–XXXX–Met) connected with copper ligands are acidic in numerous multicopper oxidases. Asp⁵⁰¹ in *B. subtilis* CotA-laccase is found at the surface of a water channel [136].

In addition to site-directed mutagenesis, different expression hosts also enhance expression level. According to Wang et al. [104], protease-deficient *P. pastoris* strain SMD1168H is selected for the heterologous expression of the CotA-laccase from *B. subtilis*. The enzyme production phase is prolonged, and the expression level of rCotA is effectively improved (sorbitol together with pH adjustment enhanced the rCotA production by 76-fold). A D500G mutant of *B. licheniformis* LS04 laccase, which is constructed by site-directed mutagenesis, demonstrates 2.1-fold higher activity when expressed in *P. pastoris*, and the protein yield under the optimized conditions is approximately 59 mg L⁻¹, which is 9.3-fold higher than that of WT enzyme [103]. Moreover, an interesting experiment on CotA-laccase extracellular production in *E. coli* was conducted by a simple strategy. Two secretion systems (α -hemolysin and YebF secretion systems) were used to achieve the secretion of recombinant CotA into the culture medium. The uropathogenic *E. coli* α -hemolysin (HlyA) secretion system is the most used secretion system for recombinant protein production. Meanwhile, by optimizing the induction parameters, the extracellular yield of

recombinant CotA-laccase was improved by 15-fold (157.4 to 2401.3 U L⁻¹) [40]. The enhancement of expression level renders laccases an increasingly effective catalyst for industrial applications.

Improvement of laccase stability

For industrial application of laccases, highly stable robust enzymes that are active under harsh operational conditions are required. According to a study by Mollania et al. [137], the introduction of positive charge in a connecting loop between domains 1 and 2 promotes the thermal stability of laccase from *Bacillus* sp. HR03. They further demonstrate that not only the reduction of negative charges but also the size of newly created positive residues can affect laccase stability. Another mutant T480A obtained from *B. subtilis* is screened for organic solvent resistance. Then, a T480A/E498G variant is constructed, and the $t_{1/2}$ is 62.1 times larger than that of WT-CotA [57]. In addition to substitution, fragment deletion can enhance thermostability. For example, deletion of helix-5 creates a WlacD that is more thermostable than wild-type Wlac. Other factors that improve protein thermostability include deletion of surface loop, increased hydrophobic residues with branched side chains, and increased proportions of charged residues [138]. According to Enguita et al. [89], additional proline content

and increasingly tightly packed residues in the interface region between domains I and II are apparently associated with enhanced protein thermostability in a bacterial multicopper CotA. These methods can be referenced by other unstable enzymes.

Applications of bacterial laccases

Biocatalysis is regarded as a key component for the development of a sustainable bio-economy. The application of enzymes as biocatalysts is becoming increasingly popular in numerous industries. Laccases are promising biological green tools that work in air and generate water as the only by-product. Hence, laccases, especially fungal laccases, are widely applied in different areas (Fig. 15), such as decolorization of dyes, degradation of toxic pollutants, biosensors, effluent treatment, textiles, food industry, paper and pulp production, organic synthesis (phenolic compounds, alkaloids, antibiotics, and bioactive polymers), cosmetics, paints, furniture, and nanobiotechnology [99, 125, 139, 140]. As environmentally friendly enzymes of prokaryotic laccases, bacterial laccases are being increasing researches investigated in terms of their applications. In the present study, we summarize the main application studies on bacterial laccases conducted in recent years.

Laccase immobilization

Immobilized enzyme is a new technology that was developed in the 1960s. Immobilized enzymes are water-soluble enzymes that are physically or chemically treated to render them water-insoluble but still enzymatically active. Enzyme immobilization methods can be roughly classified into physical and chemical methods. Physical methods include adsorption and embedding, whereas chemical methods include coupling and cross-linking. Each method presents specific advantages and disadvantages depending on the application purpose. In general, immobilization methods are developed to facilitate enzyme recovery and reusability and to increase enzymatic stability. Materials for laccase immobilization include alginate gel, gelatin, polyacrylamide, hybrid nafion/sol-set silicate film, chitosan film, silica spheres, and other magnetically separable particles [43, 141].

The utilization of laccases for practical application is usually limited due to their high production costs. The use of enzyme immobilization technology in industrial processes, in comparison with the use of soluble enzymes, could reduce process costs by reducing the quantity of enzyme required, since the immobilized biocatalyst can be recovered at the end of a reaction cycle and reused, as long as the enzyme remains active. In addition, immobilization technology can be applied in order to improve enzyme properties such as

activity and selectivity as well as stability, because the major advantages of laccase immobilization are the enhancement in the thermostability of the enzyme and its resistance to extreme conditions and chemical reagents. There are many useful methods of laccase immobilization described in the research review paper [142].

Recently, a spore laccase from *B. pumilus* W3 was efficiently immobilized on amino-functionalized celite. The immobilized spore laccase removed 84.15% of methyl green and 69.70% of acid red 1 after 48 h of treatment. Moreover, the immobilized spore laccase retained 87.04% of its initial decolorization activity after six cycles in the decolorization of acid red 1. This laccase can be a useful biocatalyst in the treatment of textile wastewater [60]. Fan et al. [143] loaded hollow microspheres obtained from *Ganoderma lucidum* spores with Fe_3O_4 nanoparticles to prepare novel magnetic spore microspheres. The magnetic microspheres loaded with CotA-laccase, which can be easily and quickly recovered by an external magnetic field, were used for dye decolorization. As a result, 99% of indigo carmine was removed using 10 mg of microspheres after 1 h, and the immobilized CotA retained 75% of activity after 10 consecutive cycles. The magnetic spore microspheres are regarded as good support materials for enzyme immobilization. Laccase- $\text{Cu}_3(\text{PO}_4)_2$ hybrid microspheres with hierarchical structure are successfully prepared and loaded on a treated copper foil surface. Furthermore, the microspheres exhibit higher decoloration efficiency and decoloration rate (nearly 3.6 times) on Congo red dye solution after 3 h compared with free laccase [144]. In addition, numerous new materials, including different biopolymers, such as agar-agar, polyacrylamide, and gelatin, were utilized as bolster materials for immobilization of fungal laccase (*T. versicolor*) and commercialization [145]. Laccase immobilized on nanocopper-incorporated electrospun fibrous membrane successfully removed 2,4,6-trichlorophenol [146]. Poly (glycidyl methacrylate) (PGMA) microspheres can act as ideal supports for enzyme immobilization [147]. These materials and methods can be referenced to affix bacterial laccases.

Decolorization of dyes

Effluents produced in textile industries are always strongly colored; consequently, their disposal into the receiving waters reduces light penetration and subsequently disrupts the photosynthetic activity of aquatic plants [148]. The affected wastewater poses a severe environmental risk and may be mutagenic or carcinogenic because of the presence of metals, chlorides, and dye breakdown products [149, 150]. At present, treatment of textile effluents by expensive physicochemical methods that generally fail to degrade the pollutants but only cause dye accumulation as sludge results

in disposal problems. Enhanced microbial decolorization may provide a cost-effective and environment-friendly method [151, 149].

The first report on dye degradation involved an alkali-tolerant bacterial laccase from γ -proteobacterium JB that degraded indigo carmine at pH 9.0 at 55 °C by using syringaldehyde, *p*-hydroxybenzoic acid, and vanillin as mediators. According to Guan et al. [148], CotA gene of *B. pumilus* W3 achieved efficient secretory expression in *B. subtilis* WB600 by screening a suitable signal peptide. The toxicity of the CotA–ACS-treated effluent is markedly lower than that of the crude effluent. *B. pumilus* W3 CotA-laccase mutant is also used to decolorize five industrial dyes (acid red 1, acid blue 129, methyl green, malachite green, and methyl blue), and these variants maintain high decolorization rates [102]. In the presence of ACS as a mediator, the CotA from *B. subtilis* cjp3 can decolorize all tested dyes (reactive black 5, indigo carmine, and reactive blue 19), but it cannot decolorize the dyes without mediators [70]. Similarly, laccase from *B. vallismortis* fmb-103 with ABTS, ACS, or syringaldehyde can efficiently degrade malachite [74]. In addition, certain new bacterial laccases can decolorize different dyes without mediators. For example, a new thermophilic soil bacterium with hyperthermostable alkaline laccase activity named *Anoxybacillus* sp. UARK-01 can decolorize approximately 1.64 nM of Congo Red per microgram protein in 30 min at 90 °C and pH 9 [152]. Pure *Spirulina platensis* CFTRI laccase alone can efficiently decolorize anthraquinonic dye reactive blue 4 in 4 h without any mediators [153]. Pure TthLAC (*T. thermophilus* laccase) decolorized green dye, orange dye, and acid red dye by itself [154]. Except for the thermostable and pH-stable laccase from *K. pneumoniae*, both bacterial laccase-like enzymes in an acidic bog soil metagenome can efficiently decolor/oxidize sundry dyes in the absence of redox mediators [16, 153]. Studies on the decolorization mechanism of different dyes by bacterial laccases are limited and thus may be a new research topic.

Paper and pulp industry

Bacterial laccases have successfully showed effectiveness for biobleaching and kraft pulps. The capability of laccases to oxidize nonphenolic compound, such as veratryl alcohol, in the presence of ABTS provides new possibilities for the use of bacterial laccases in the pulp and paper industry. The laccase from *S. cyaneus* CECT 3335 with ABTS as mediator in the biobleaching of eucalyptus kraft pulps resulted in a significant decrease in the kappa number (2.3 U) and a significant increase in the brightness of the pulps [47]. Laccase from *S. ipomoea* CECT 3341, with ACS as natural mediator, was also used to enhance the conventional chemical bleaching process of an industrial eucalyptus kraft pulp

[52]. In addition, a recombinant laccase from hyperthermophilic *T. thermophilus* was applied for the biobleaching of wheat straw pulp. With the ABTS (5 mM) as mediator, pulp brightness considerably increased by 1.5% ISO [156]. According to Sondhi et al. [157], an extracellular thermo-alkali stable laccase from *B. tequilensis* SN4 can be used for pulp biobleaching. An increase in brightness by 7.6% and decrease in lignin content by 28% are retained without *N*-hydroxy-benzotriazole as mediators, whereas 12% improvement in brightness and 47% decrease in lignin content were observed in the presence of a mediator. An effective method for deinking and biobleaching involves the co-production of thermo-alkali stable ligninolytic and hemicellulolytic enzymes by growing two different *Bacillus* spp. in the same solid-state fermentation medium. The combination of laccase and xylanase reveals a synergistic effect for enhancing pulp properties. The dual cultivation not only improves the utilization rate of substrates but also enhances enzyme yield and the inhibitory effect on the growth of non-desirable microorganisms [28, 158].

Biomass delignification and degradation

Lignin degradation and delignification by laccases are important both in the environment (lignocellulosic bio-waste) and in commercial biofuel production [159, 160]. Laccase digestion provides a mild, clean, and efficient treatment method for bio-delignification of lignocellulose without damaging the cellulose [161, 162]. However, according to Rocha-Martin et al. [163], laccases are supplemented to the enzymatic hydrolysis resulting in contradictory results depending on the pretreatment and substrate used. The addition of laccase on the hydrolysis of softwood increased the glucose conversion, while an inhibitory effect was observed during the hydrolysis of hardwood or agricultural residues like wheat straw. They used two strategies: simultaneous laccase treat enzymatic hydrolysis of pretreated sugar cane straw and corn stover (strategy 1); and a previous laccase treatment and a subsequent hydrolysis step of both pre-processing substrates (strategy 2). Significant reduction of the glucose concentration after enzymatic hydrolysis was found when any of the two strategies were used. The results do not support the use of laccases to detoxify pretreated lignocellulosic materials for improving the bioethanol production [163]. In addition laccase-derived compounds affect negatively the enzymatic hydrolysis being the level of inhibition dependent on the type of phenol, besides phenoxyl radicals and oligomeric phenols [164]. Laccase enzymes are promising detoxifying agents during lignocellulosic bioethanol production from wheat straw. However, they affect the enzymatic hydrolysis of this material by lowering the glucose recovery yields. This work revealed that a grafting process of phenoxy radicals onto the lignin fiber could be the cause

of diminished accessibility of cellulases to cellulases in pre-treated wheat straw [165].

According to Singh et al. [166], an SLAC from *Amycolatopsis* sp. 75iv3 enhances the delignification of steam-pre-treated poplar. Their study established the lignolytic activity of SLAC on woody biomass and highlighted the biocatalytic potential of bacterial enzymes. In addition, numerous agricultural residues are efficiently degraded by various bacterial laccases, such as wheat straw by *S. ipomoeae* laccase [167, 168], peanut shell bio-waste by *Aquisalibacillus elongatus* laccase [169], sugar beet pulp by *A. elongatus* laccase [170], almond shell bio-waste by *Chromohalobacter salexigens* laccase [171], and paddy straw by *S. griseorubens* srr38 [172]. Moreover, co-expressed mixture methods are used for lignocellulose degradation. Fonseca-Maldonado et al. [173] demonstrated that synergism occurs between an endoxylanase and laccase with milled sugar cane bagasse as a lignocellulose substrate. As examples, the three genes of bacterial laccase (*cotA*), pectate lyase (*pel*), and endoxylanase (*xyl*) are simultaneously cloned in single vector *E. coli*. This enzyme cocktail is important in the pretreatment of lignocellulosic residues for biofuel production [174].

Pollutant degradation

Polycyclic aromatic hydrocarbons and biogenic amines are pollutants that are widely distributed in natural environments, such as soil, air, or aquatic environment. Most of these pollutants and their intermediates are toxic for living beings [28]. To date, increasing reports have shown that bacterial laccase can degrade xenobiotic compounds. Recently, several bacterial laccase CueO mutations from the metagenome of chemical plant sludge displayed that the mutants G276R, G276N, G276Y, and G276K oxidize carcinogen benzo[α]pyrene more efficiently than the WT-enzyme [68]. According to Callejón et al. [175], recombinant laccase from *Pediococcus acidilactici* can degrade the biogenic amine tyramine at pH 9.5 and 4.0 with or without ABTS as mediator. Tyramine degradation by laccases can solve the problems generated in food due to the presence of this toxic compound. In addition, gallic acid, syringaldehyde, vanillin, and catechol can be degraded by bacterial-derived laccase [176]. Several micropollutants, such as BPA (bisphenol A), inflammatory drug DFC (diclofenac), and MFA (mefenamic acid), can also be oxidized by laccases [177, 178]. Thus, bacterial laccases are potential biological green tools for the environment.

Other applications

In addition to the above-mentioned applications, laccases can be used in polymer production [36], synthesis of indo

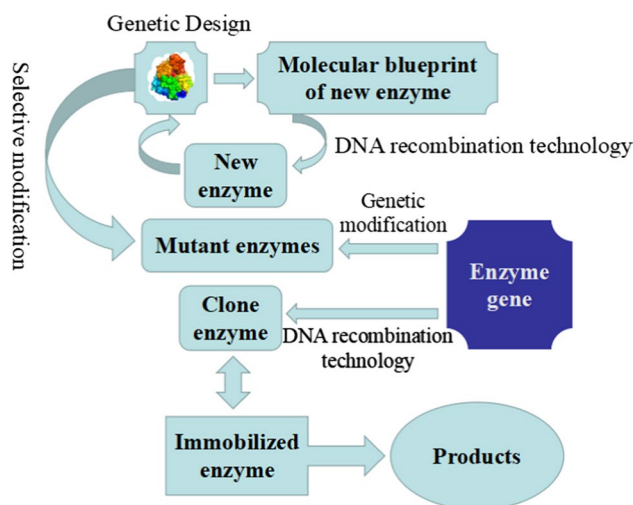


Fig. 16 Biological enzyme engineering

dyes [179], detection [180, 181], biosensor, and bioremediation [182]. In a word, laccases have become important industrially relevant enzymes as promising biological green tools for the environment and in industries.

Concluding remarks

The current review provides a detailed and comprehensive study of bacterial laccases, their species and properties, and substrates and mediators, as well as the mechanisms of laccase and LMS. Moreover, the strategies for further enhancement of the catalytic activity and substrate specificity, expression level, and laccase stability by genetic engineering are summarized. Bacterial laccases exhibit a wide range of applications for decolorization of dyes, effluent treatment, degradation of toxic pollutants, and other fields (textile, food, and paper industries). Biological enzyme engineering (Fig. 16) is a powerful and useful technical area for environment-friendly and industrial production. However, the main obstacles for the large-scale application of bacterial laccases include low expression level, high price of mediator, and inadequate capacity to produce large volumes of highly active enzyme at low cost. Future research should focus on the following areas: (1) expansion of the production scale using exocrine strain as expression vector; (2) separation and screening of novel laccases or transformation of current laccases to new enzymes, which offer high application potential in the absence of mediators, to reduce the cost; (3) development of immobilized laccases using new environmentally friendly nanoscale material that can be easily reused and recycled; (4) reaction engineering to optimize the synthesis of specifically desired products of economic value; and (5) potential of laccases for Au adsorption which may

become a novel application for bioremediation in the future. In addition, investigations on dye degradation–detoxification mechanism by laccases should be conducted. Hopefully, these questions will attract researchers' attention in the future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

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