Cloning, Overexpression, and Characterization of a Thermostable, Organic Solvent-Tolerant Laccase from *Bacillus pumilus* ARA and Its Application to Dye Decolorization

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**ABSTRACT:** A thermostable and organic solvent-tolerant bacterial laccase from *Bacillus pumilus* ARA has been expressed heterologously and characterized, which shows potential decolorization capacity to various types of industrial synthetic dyes. The optimal temperature and pH were 85 °C and 3.5, respectively, while the purified recombinant laccase B.P.Lacc was stable under 55−75 °C and pH 5.0−8.0 conditions. The apparent kinetic parameters \( K_m \) and \( V_{max} \) of B.P.Lacc for ABTS as the substrate were 0.33 mM and 32.4 U/mg, respectively. Ethanol (1%, v/v) and methanol (2%, v/v) could stimulate the enzyme activity. The recombinant laccase retained over 95% of its initial activity in 10% (v/v) methanol. The optimal expression conditions for the laccase production of B.P.Lacc in LB medium were obtained: induction temperature of 25 °C, 0.4 mM Cu\(^{2+}\), and 1.0 mM IPTG added into the culture. After 5 h, the final laccase production was 1283 U/mL. Moreover, the laccase activity increased to 4822 U/mL after follow-up 2 h stationary cultivation, with about a 3.76-fold increase. The purified B.P.Lacc was able to efficiently decolorize synthetic dyes combined with mediators. Adding 1.0 mM ABTS, more than 90% of BRRB was decolorized by the enzyme, whether at pH 4.0 or pH 7.9. The outstanding enzymatic properties suggested that B.P.Lacc may be suitable for a wide application in future biodegradation fields.

**INTRODUCTION**

Laccase (EC.1.10.3.2), as a member of blue copper-containing polyphenol oxidase families, is well known as a biocatalyst for oxidizing reactions in a range of typical phenolic or aromatic compounds (such as polyphenol, aminophenol, ary diamine, etc.), with concomitant reduction of O\(_2\) to give water.\(^1\sim3\) In addition, some studies have shown that laccases have potential application values in degrading PAH micropollutants, organic pesticides, and lignocellulose raw materials.\(^4\sim6\) Due to the broad substrate spectrum of catalyzed reactions by means of laccase/mediator systems, laccases can also be reacted with lots of nonphenolic aromatic compounds in the presence of some small molecular mediators.\(^7\)

Laccase was first discovered by Yoshida from the secretory protein of Japanese lac tree.\(^8\) From then on, laccase can be divided into two categories: the *Rhus verniciflua* laccase and the fungal laccase. To date, a number of fungal laccases from *Pleurotus ostreatus*, *Trametes versicolor*, etc. have been screened, purified, explored, and applied in various fields, ranging from biocatalysis and biotransformation,\(^9,10\) pulp bleaching, dye decolorization,\(^11,12\) and organic pollutant degradation.\(^5,13,14\) Li et al. screened three laccase isozymes from *T. versicolor* and applied them in dye decolorization industry with good decolorization efficiency.\(^15\) Xie et al. reported a recombinant laccase and degraded chlorpyrifos in several laccase/mediator systems, which led to 98% chlorpyrifos degradation.\(^16\) However, the fungal laccase has an obvious defect, that is, poor heat resistance and easy inactivation at high temperature.\(^17\) At present, laccases are widely found in bacteria through homologous explosion in protein and bacterial genome databases.\(^18\) Compared with the laccases secreting from white rot fungi, bacterial laccases may have some special advantageous properties, such as higher thermostability, salt resistance, organic solvent resistance, and alkali resistance.\(^19\sim22\)

Wastewater drained from printing and textile industries contains various kinds of industrial synthetic dyes (such as azo, anthraquinone, indigo dyes, etc.), which are harmful to our ecosystem environment and human health.\(^23,24\) For instance, azo dyes, which have teratogenic, carcinogenic, and mutagenic
effects, are harmful to the health of human and animals. However, over 0.7 million tons of different kinds of dyes are produced annually, of which 10% to 20% of dyes is directly discharged into industrial wastewater. In addition, the structures of these synthetic dyes are extremely stable to resist degradation by light, high temperature, and chemicals. In recent years, such coagulation–floculation, adsorption, oxidation, electron chemistry, and other methods were used to decolorize dyes. However, these methods are often costly, inefficient, and even unsafe. By comparison, the extracellular oxidative enzyme laccase remediation of dye wastewater represents a highly free-radical-mediated and non-specific process with a number of desirable characteristics. The laccase LccA from T. versicolor was an environmentally friendly and cost-competitive biocatalyst, which was used in decolorization of Reactive blue KN-R and Acid red 35 with the decolorization rates of 91.33% and 78.96%, respectively. Bacterial laccases have excellent properties of high temperature resistance, alkali resistance, and organic solvent tolerance, so they have great application prospects in the field of environmental pollutant treatment. Hence, using bacterial laccases for decolorizing synthetic dye effluent is supposed to be a promising solution.

In this study, we detected laccase activity in the culture supernatant of a thermophilic and α-arabinosidase high-producing strain, which was isolated, subsequently identified, and designated as Bacillus pumilus ARA by our lab. In order to obtain enough amount of bacterial laccase protein for characterization, a laccase gene B.P-lacc was cloned and overexpressed in Escherichia coli. Finally, we decolorized some synthetic dyes (the different structures are shown in Figure 1) by B.P-Lacc. This research lays a foundation for the further application of bacterial laccase in the biodegradation of industrial dyes.

![Chemical structures of dyes. (a) Acid black ATT; (b) Acid red 35; (c) Reactive blue KN-R; and (d) Direct black 19.](image)

**RESULTS AND DISCUSSION**

Cloning and Heterologous Expression of Bacterial Laccase Gene. The cloned B.P-lacc gene contains 1536 bp, encoding laccase 511 amino acids (Figure 2). At the gene level, the B.P-lacc gene in this study shows 88% sequence identity with the previously reported B. pumilus strain MK001 cotA gene and B. pumilus strain DSKK1 lacc1 gene. The recombinant laccase peptides contain conserved cysteine residues C229 and C322, forming a disulfide bond in Bacillus subtilis laccase CotA, which possibly made bacterial laccases’ higher-order structures more stable. The theoretically predicted protein molecular weight is 58.7 kDa in accord with SDS-PAGE (Figure 3). The total number of atoms in the whole protein B.P-Lacc was 8207, and the extinction coefficient at 280 nm was 77,030 M⁻¹ cm⁻¹. The instability coefficient of B.P-Lacc was 37.19, which indicated that the protein was stable. In addition, the lipase’s aliphatic coefficient was 79.92, and the GRAVY value was −0.491, which indicated that the laccase B.P-Lacc was a hydrophilic protein.

![Figure 2. Agarose gel of the PCR product of B.P-lacc. (lanes 1–4: DNA of B.P-lacc; lane M: marker DL5000).](image)

![Figure 3. SDS-PAGE of the purified recombinant laccase B.P-Lacc from B. pumilus ARA. (lane M: prestained protein markers (10–170 kDa); lane 1: recombinant laccase purified by Ni²⁺-affinity chromatography; lane 2: crude laccase supernatant incubated at 70 °C for 20 min; lane 3: crude laccase supernatant incubated at 65 °C for 20 min; lane 4: crude laccase supernatant).](image)
The amino acid composition of the laccase B.P-Lacc showed that the contents of Pro (9.0%), Glu (7.0%), Leu (8.2%), and Arg (5.9%) were relatively high. Through literature comparison, the amino acid composition of the enzyme system of each thermophilic bacteria family was calculated and analyzed. With the improvement of thermostability, the contents of amino acids such as Glu, Gly, Ile, Pro, and Arg were increased gradually, which indicated that laccase B.P-Lacc would have a certain heat resistance.9

**Enzymatic Characterization of the Recombinant Laccase.** The crude laccase B.P-Lacc was heat-treated at 65 °C, purified by Ni²⁺-affinity column, and eluted by 150 mM imidazole with a final purification of 2.59-fold and a rate of 63.3% (Table 1).

| Purification Step         | Total Activity (U) | Total Protein (mg) | Specific Activity (U/mg) | Yield (%) | Fold Purification |
|---------------------------|--------------------|--------------------|--------------------------|-----------|------------------|
| Culture Extract           | 12,830             | 3899.7             | 3.29                     | 100       | 1                |
| Heat Treatment            | 9725               | 1462.4             | 6.65                     | 75.8      | 2.02             |
| Ni²⁺-affinity Chromatography | 8121               | 953.2              | 8.52                     | 63.3      | 2.59             |

“Substrate for B.P-Lacc was 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS).”6 The cell extracts after sonication were heat-treated at 65 °C for 20 min, then cooled in an ice bath, and centrifuged at 12,000 rpm for 20 min at 4 °C, and the supernatant was kept.

As shown in Figure 3, after heat treatment, a considerable number of hetero-proteins had been removed, which indicated that the purification method of this bacterial laccase enzyme was simple. Also, the expressed protein was shown as a single band on a 10% SDS-PAGE gel with a molecular mass between 58 kDa. Furthermore, the expressed protein was shown as a single band on a 10% SDS-PAGE gel with a molecular mass between 58 kDa. Also, the expressed protein was shown as a single band on a 10% SDS-PAGE gel with a molecular mass between 58 kDa.

Generally, bacterial laccases have high optimal temperatures, which could maintain high activity at 55–75 °C. The optimum temperatures for the oxidation of ABTS and guaiacol of B.P-Lacc were around 85 and 75 °C (Figure 4a), respectively, similar to the other CotA-laccase from *Bacillus* species.28,29 However, they were much higher than that observed for the laccase (temperature of 45 °C) from a marine microbial metagenomic library of the South China Sea based on a sequence screening strategy.20 The optimal pH for ABTS oxidation was 3.5 (Figure 4b), which was lower than the bacterial laccase CotA from *B. subtilis* DB 10428 and slightly higher than laccase from *Aeromonas hydrophila* WL-11.31 When the substrate was guaiacol, the optimum pH was 7.0 (Figure 4b). The extensive pH range may be a potential advantage for applying to biodgradation and biocatalysis fields compared with fungal laccases. In industrial applications, enzyme heat resistance has been a bottleneck for economically viable enzyme use. Therefore, laccase with high thermal stability has become an appreciable property in the actual industrial processes. The recombinant bacterial laccase B.P-Lacc showed high thermostability between 50 and 70 °C, which was higher than laccase from *A. hydrophila* WL-11.32 and similar with the laccase from *Bacillus licheniformis*.52 After incubation for 2 h at 70 °C, more than 80% of the initial laccase activity still remained. However, it lost 70% of the original laccase activity when the temperature was raised to 85 °C (Figure 4c). It was worth mentioning that the enzyme was found to be stable under the faintly acid and neutral conditions (in a pH range of 5.0–8.0) (Figure 4d), which was different from the other *Bacillus* species laccases (pH 7.0–10.0).52 These properties will contribute to exploring the potential applications of the enzyme. The kinetic parameter *Km* and *Vmax* values of B.P-Lacc were 0.33 mM and 32.4 U/mg, respectively, for ABTS as the substrate, which suggested that B.P-Lacc had good substrate specificity for ABTS.

We investigated the effects of different metal ions on the recombinant laccase activity (Figure 4e). The results revealed that Cu²⁺, Zn²⁺, and Al³⁺ (at 0.1 mM concentration) enhanced the enzyme activity, while the laccase activity was inhibited significantly by 0.1 mM Ag⁺, Fe²⁺, Mn²⁺, and Ca²⁺. Especially, the addition of 0.1 mM Ag⁺ reduced the enzyme activity by 50%. Majority of organic pollutants and biocatalytic substrates are insoluble in water. In the actual degradation process, we have to add organic solvents to dissolve them in enzymatic reaction systems. Therefore, the effects of organic solvents on the recombinant laccase activity were investigated (Figure 4f).

In this work, we selected four commonly used organic solvents with different concentrations of 1%, 2%, 5%, and 10% (v/v). In the presence of the 5% (v/v) tested organic solvents (except ethanol), the recombinant laccase retained over 85% of its initial activity. In addition, the bacterial laccase B.P-Lacc has excellent methanol resistance, which showed that more than 95% of laccase activity remained in 10% of methanol.

**Optimization Expression Conditions of the Recombinant Laccase.** For investigation of induction conditions such as isopropyl-beta-D-thiogalactopyranoside (IPTG) concentration, Cu²⁺ concentration, induction temperature, and induction time, the best recombinant strain was chosen and induced by different conditions to express the laccase.

IPTG, as a substrate analogue of β-galactosidase, is a very effective inducer of lactose operon. In addition, IPTG is not metabolized by Exogenous, so it is very stable and is often used to express exogenous proteins in *E. coli*. As shown in Figure 5a, laccase production increased with the IPTG concentration from 0.2 to 1.0 mM, but the activity slightly decreased when the IPTG concentration was up to 2.0 mM. In general, the optimal concentration of IPTG was 1 mM. The reason was that the inducer IPTG can induce laccase production and affect the growth and reproduction of *E. coli* together, while the low IPTG concentration had no significant effect on the growth of *E. coli*.

Laccase is a copper-dependent polyphenol oxidase. Copper ions play an important role in protein folding. Different Cu²⁺ concentrations were added to the LB medium to the final Cu²⁺ concentrations of 0, 0.10, 0.25, 0.40, 0.50, and 0.75 mM. Laccase activity increased as the Cu²⁺ concentration altered from 0 to 0.50 mM. When Cu²⁺ concentration reached 0.50 mM, laccase activity decreased. According to the laccase activity assay, the results showed that the optimal Cu²⁺ concentration was 0.40 mM (Figure 5b). Under different induction conditions, laccase production was different, which is shown in Figure 5c. Although the optimal growth temperature of *E. coli* is between 37 and 39 °C, the highest temperature of laccase activity was not the optimal growth temperature but was about 25 °C. The reason was that the specific growth rate of *E. coli* was higher at high temperature,
so it was easy to accumulate metabolic byproducts and reverse the fermentation process, which can inhibit the growth of *E. coli* and the expression of laccase. Moreover, too high temperature would affect the stability of the expression plasmid. When the induction temperature was too low, the growth of expression host was limited, and the ability to express the target protein was also affected. The effect of induction time on laccase production is shown in Figure 5d, and with the extension of the induction time, the laccase activity increased. When the induction time was 5 h, the laccase activity was the highest, which was up to 1283 U/mL. In the process of laccase expression, there was a very interesting phenomenon. After IPTG induction, the laccase activity was significantly increased for stationary cultivation subsequently. Therefore, we investigated the effect of different stationary cultivation times on laccase activity after IPTG induction. The result is shown in Figure 5e. After follow-up stationary cultivation for 2 h, the laccase expression was the highest (4822 U/mL), which increased by 3.76-fold. The reason may be that, during the induction of laccase expression, laccase as a kind of blue ketone polyphenol oxidase, which protein could not be folded correctly due to the rapid translation speed of *E. coli* expression system. This result could also be seen in Figure 5b, when the concentration of Cu²⁺ was increased to 0.6 mM, the activity of laccase B.P-Lacc did not increase, which suggested that the binding sites between Cu²⁺ and laccase B.P-Lacc were limited. Some Cu²⁺ could not fully be binded to laccase protein. Therefore, in the later stationary cultivation, due to the timely translation of more laccase by the *E. coli* expression system, more laccase B.P-Lacc and Cu²⁺ binding sites were released, which contributed to more production of all copper laccase. However, with the further increase in stationary cultivation time, *E. coli* autolysis occurred, which resulted in cell damage and laccase enzyme activity decrease.

**Dye Decolorization.** Anthraquinonic dyes (such as Reactive brilliant blue KN-R (RBBR)) and azo dyes (such as Acid black ATT, Acid red A35, and Direct black 19) are the commonly used dyes worldwide. However, the dyeing and physicochemical treatment processes produce potentially
harmful byproducts, resulting in serious environmental pollution. Among dye treatment technologies, the use of a green catalyst of laccase has been the focus of attention.\textsuperscript{33,34} Laccase is so far known to have the ability to decolorize various classes of dyes, such as the laccase from \textit{T. versicolor}, \textit{Vitreoscilla} sp., and \textit{Chaetomium globosum}.\textsuperscript{35−37} Redox mediators, such as ABTS, could increase a decolorization rate of 45%. Thus, laccase combined with a mediator has potential to degrade a wide range of compounds such as dyes. In this paper, the decolorization rates of dyes were tested in several laccase/mediator systems. The five synthetic or natural mediators (ABTS, 1-hydroxybenzotriazole (HBT), 5-hydroxyiminobisbarbituric acid (VA), acetosyringone, and guaiacol) were the common mediators when laccase was used to degrade phenolic compounds. As shown in Figure 6, the purified recombinant laccase B.P-Lacc was able to efficiently decolorize synthetic dyes with different structures at pH 4.0 (Figure 6a) and pH 7.9 (Figure 6b) in the presence of various mediators. Yan et al.\textsuperscript{38} reported that a laccase derived from \textit{P. ostreatus} could decolorize 70% of malachite green in 24 h incubation. Li et al.\textsuperscript{15} cloned a laccase from \textit{T. versicolor} and used it to decolorize the Reactive blue KN-R and Acid red 35 with the decolorization rates of 91.33% and 78.96%, respectively. However, in this study, the decolorization rate by B.P-Lacc was very low in the absence of mediators. Acid black ATT and Acid red A35 were difficult to be decolorized by the recombinant laccase, and less than 60% decolorization rate was observed at any laccase/mediator systems during 4 h incubation, which was lower than the immobilized laccases applied to the indigo decolorization reported by Wu et al.\textsuperscript{39} By adding 1.0 mM ABTS, more than 90% of BRRB was decolorized by the enzyme, whether at pH 4.0 or pH 7.9. In the presence of any tested mediators, Direct black 19 was completely decolorized at pH 4.0. As shown in Figure 6c, the decolorization effects of Direct black 19 were very obvious at pH 4.0 when different mediators were added, and the decolorization rates could all reach over 80%, while it was only 32.6% when no mediator was added. This was also consistent with the report of Fang et al. that adding some chlorides could significantly promote the decolorization rate of bacterial laccase for Dark blue M-2GE.\textsuperscript{30} In contrast, the decolorization rate for Direct black 19 was all less than 40% at pH 7.9 when adding 1.0 mM HBT, VA, and acetosyringone. Thus, it is of great significance to explore and determine the optimal conditions for the use of mediators for laccase application. The structure−effect relationships of mediators in the dye decolorization process require to be further studied, which is what we are aspiring.
MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Chemicals. B. pumilus ARA was isolated in the Microbial Technology Research Laboratory (Nanjing Forestry University, China). E. coli JM109 (Invitrogen, USA) as an expression host was grown at 37°C in Lysogeny Broth medium (LB) and supplemented with ampicillin (0.1 mg/mL) when required. The cloning and expression pET-20b was from Novagen, USA, and restriction endonucleases NdeI, XhoI, and Ex-Taq DNA polymerase were purchased from TaKaRa (Dalian, China). ABTS was purchased from Sigma (St. Louis, MO, USA).

B. pumilus ARA was grown at 50°C in the medium containing 1.5 g/L xylan, 0.3 g/L K2HPO4, and 0.2 g/L yeast extract.26 The genomic DNA of B. pumilus ARA was extracted as a template.

Cloning, Expression, and Puriﬁcation of the Recombinant Laccase. The laccase gene named B.P-lacc was successfully ampliﬁed by PCR with the forward primer 5′-CCCCCATATG AACCTAGAAAAATTTGTTGAC-3′ (introducing an NdeI site, underlined text in the sequence) and the reverse primer 5′-CCCCTCGAGCTGGATGATATC-3′ (introducing an XhoI site, underlined text in the sequence). The PCR product of B.P-lacc was cloned into vector pET-20b. Then, the pET-20b-B.P-lacc vector was transformed into E. coli TOP10F′ to amplify and overexpress in E. coli JM109. The recombinant E. coli JM109 (an isolated colony from a plate) was cultivated at 37°C and 180 rpm in LB medium supplemented with 0.1 mg/mL ampicillin (150 mL volume in a 500 mL shaking flask). With a cell density of 1.0–2.0 (OD600), the expression of B.P-lacc gene was induced by adding 1.0 mM IPTG and 0.40 mM CuSO4. The induction temperature and time were 25°C and 5 h, respectively. Another 2 h static incubation time can improve the cellular copper content to increase the laccase activity. The cells were harvested by centrifugation at 10,000 rpm for 30 min and resuspended in 20 mM Tris-HCl (pH 7.9). Subsequently, the resuspended cells were broken up by ultrasonication and centrifugation at 12,000 rpm and 4°C for 30 min. The supernatant was incubated at 65°C for 20 min and centrifuged at 12,000 rpm and 4°C for 20 min to remove some thermolabile host cell proteins. The crude laccase supernatant was subsequently puriﬁed by Ni-chelating aﬃnity chromatography, and the enzyme protein was collected by eluting in 150 mM imidazole (pH 7.9). SDS-PAGE was carried out according to Laemmli’s protocol with a 5% stacking gel and 10% resolving gel. The molecular mass of the laccase was determined by calculating the relative mobility of a low molecular mass marker.

Analysis of Laccase Activity and Biochemical Characterization. The recombinant laccase activity was assayed at 50°C, with ABTS as the substrate in sodium citrate buﬀer (0.1 M, pH 4.0). The reaction system contained 200 μL of 15 mM ABTS and 2780 μL of 100 mM sodium citrate buﬀer (pH 4.0), and the reaction was triggered by adding 20 μL of recombinant laccase. The oxidation of ABTS was monitored at 420 nm. The change in absorbance for 5 min was recorded every minute spectrophotometrically using a full wavelength microplate reader (Molecular Devices, USA).10 One unit of enzyme activity is deﬁned as the amount of enzyme required to oxidize 1 μmol of ABTS per minute.

The biochemical characteristics of the puriﬁed B.P-Lacc were investigated using ABTS and guaiacol as the substrates. The optimum temperature for recombinant laccase activity was determined at a temperature range of 40–90°C using ABTS (420 nm) and guaiacol (465 nm) as substrates. Similarly, the effect of pH on laccase activity was determined within pH 3.0–8.0. In order to investigate recombinant laccase temperature stability, B.P-Lacc was kept at a certain temperature for

Figure 6. Decolorization of synthetic dyes by the puriﬁed recombinant laccase B.P-Lacc from B. pumilus ARA. (a) Decolorization rate under pH 4.0 without or with mediators; (b) decolorization rate under pH 7.9 without or with mediators; and (c) decolorization eﬀect of Direct black 19 with different mediators.
different incubation times (40, 60, 90, and 120 min at a range of temperatures of 50, 60, 70, and 85 °C, respectively). After that, ABTS oxidation activity was determined at 50 °C. For pH stability of recombinant laccase measurements, the laccase was prepared by pre-incubating the purified recombinant laccase enzyme solution at 4 °C for 24 h in various pH buffer systems (pH 3.0–8.0) and then the residual laccase activity was determined at 50 °C using ABTS as the substrate. Kinetic parameters (Km and Vmax) of the purified enzyme were determined at room temperature using different concentrations of ABTS as the substrate. The Michaelis–Menten constant was determined in 50 mM sodium citrate buffer at the optimal pH and temperature. We added 0.1 mM Cu2+, Zn2+, Co2+, Mn2+, Ca2+, Ag+, Mg2+, Ba2+, Li+, K+, Fe2+, Na+, and Al3+ in the reaction system to evaluate the effect of metal ions on laccase activity. The organic solvent tolerance of recombinant laccase was also investigated in this study. The laccase activity was determined in the presence of 1%, 2%, 5%, and 10% (v/v) of several common organic solvents, including ethanol, methanol, dimethylsulfoxide (DMSO), and acetonitrile. A control without organic solvents was also prepared.

Optimization of Laccase Expression Conditions. The recombinant B.P-Lacc was incubated in 30 mL of LB medium for 12 h at 37 °C with constant shaking at 180 rpm. When OD600 reached values between 0.6 and 0.8, the seed solution was inoculated in the fresh 50 mL LB medium. The effects of IPTG concentration (0.2, 0.5, 0.8, 1.0, 1.5, and 2.0 mM), Cu2+ concentration (0, 0.25, 0.5, 0.75 mM), induction temperature (20, 25, 28, 37, and 42 °C), and induction time (3, 4, 5, 6, 8, and 10 h) on the enzyme production were investigated. Only one variable was set in each experiment, and other conditions were the same. The effects of different liquid stationary incubation times on enzyme activity were investigated. Under the optimal IPTG concentration and temperature, the culture mode was changed to static culture after adding IPTG for 5 h, and different static times (0, 2, 5, 12, 18, and 24 h) were set to investigate the effect of IPTG on enzyme production.

Dye Decolorization by Recombinant Laccase. The purified recombinant laccase B.P-Lacc was used to decolorize different dyes as follows. The decolorization rate of dyes was determined by measuring absorbance at dye’s maximum wavelength, and results are expressed in terms of percentage.

Four dyes (structures are shown in Figure 1), one anthraquinonic dye (Reactive brilliant blue KN-R, RBBR) and three azo dyes (Acid black ATT, Acid red A35, and Direct black 19) (Zhejiang Runtu Chemical Group Limited Company, China), were chosen to evaluate the decolorizing efficiency of recombinant laccase in the presence of various mediators at different pH values of 4.0 and 7.9. A control sample was added in some deactivated enzymes. The absorption wavelengths of the four dyes were 610 nm for RBBR, 620 nm for Acid black ATT, 505 nm for Acid red A35, and 646 nm for Direct black 19. The decolorization rate of each dye was calculated by means of the following formula:

\[
\text{decolorization rate (\%) } = \left( \frac{C_i - C_f}{C_i} \right) \times 100
\]  
(1)

where C_i is the initial concentration of the dye and C_f is the dye concentration with time. The concentration of the dye was calculated according to the value of absorbance.

The molecular mediators included ABTS, HBT, VA, acetosyringone, and guaiacol. The concentrations of mediators and dyes were 1.0 mM and 50 mg/L in the different pH buffer systems (5 mL), respectively. Reactions were initiated by adding 1.0 U/mL recombinant laccase and incubated for 4 h at 50 °C.

■ CONCLUSIONS

To sum up, the recombinant bacterial laccase B.P-Lacc from B. pumilus ARA exhibited great thermostability and considerable tolerance to some organic solvents. The enzyme was very stable under the faintly acid and neutral conditions, which differed from most of bacterial laccases found so far. Moreover, some synthetic dyes were able to decolorize effectively by the purified laccase B.P-Lacc in the presence of mediators. These properties will contribute to exploring its potential application in various industries without negative effects on the ecological system.

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Notes
The authors declare no competing financial interest.

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