Production of spore laccase from *Bacillus pumilus* W3 and its application in dye decolorization after immobilization


**ABSTRACT**

Given that spore laccase from the *Bacillus* genus is heat- and alkali-resistant, it is more suitable for industrial applications than fungal laccase. To determine the optimal culture conditions for spore laccase production, the effects of Cu$^{2+}$ concentration, oxygen content, and culture time on spore laccase production from *Bacillus pumilus* W3 were investigated. The optimal production parameters were 0.2 mM of Cu$^{2+}$, 200 rpm shaking speed, 100 mL liquid loading, and 5 days of cultivation. Spore laccase was efficiently immobilized on amino-functionalized celite. When used in dye decolorization, 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. These insights into the culture conditions and immobilization of spore laccases should be useful in the development of spore laccase as a biocatalyst in the treatment of textile wastewater.

**Key words** | amino-functionalized celite, CotA-laccase, culture conditions, dye decolorization, immobilization, spore laccase

**INTRODUCTION**

Water is essential for the survival of all living creatures. Moreover, water is a valuable natural resource for industrial and agricultural production, economic development, and environmental improvement. Water shortage and water pollution, however, have become global problems in the 21st century. Synthetic dyes are used extensively in textile dyeing and finishing industries. More than $7.0 \times 10^5$ tons and $1.0 \times 10^5$ kinds of commercially available dyes are produced annually worldwide (Strong & Claus 2014). Given that many synthetic dyes are toxic, carcinogenic, and difficult to degrade (Ozmen et al. 2008), textile wastewater has become one of the main sources of severe pollution problems worldwide (Sarayu & Sandhya 2012).

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) are metalloenzymes that contain four copper ions. Laccases catalyze the oxidation of various phenolic compounds and dye molecules with the concomitant reduction of bound dioxygen to water (Hilden et al. 2009; Wang et al. 2014). Combined with their environmental friendliness, laccases have a wide substrate spectrum given their non-specificity towards substrates, thus making them promising biocatalysts for dye decolorization (Dwivedi et al. 2011). Numerous reports have shown the high degradation ability of fungal laccases in dye decolorization (Strong & Claus 2011). However, the decolorization activity of fungal laccases is highly dependent on acidity and is absent at pH values that exceed 7 (Held et al. 2005; Yesilada et al. 2014). This characteristic limits the potential application of fungal laccases in dye decolorization because the pH value of dye wastewater ranges from 5.5 to 11 (Sarayu & Sandhya 2012). By contrast, bacterial laccases are both heat- and alkali-resistant, thus contributing to their potential as dye degradation biocatalysts (Loncar et al. 2014). For example, spore laccase from *Bacillus licheniformis* LS04 efficiently catalyzes the decolorization of various dyes at pH 9 while retaining approximately 16% of its initial activity after 10 h at 80°C (Lu et al. 2012b). Spore laccase from *Bacillus vallismortis* fmb-103 retains more than 90% of its initial activity after 10 h at 70°C and retains its relative activity at 82% and 38% at pH 7 and 9 after 10 days, respectively (Zhang et al. 2012). In addition to the intrinsic properties of spore laccases that meet the requirements of high
temperature and alkaline pH conditions for the treatment of textile dye effluents, the culture time of bacteria is shorter than that of fungi.

Spore laccases are typical bacterial laccases that bind to the surfaces of spores from Bacillus genus strains. They were first discovered in 1996 (Van Waasbergen et al. 1996). CotA, a spore coat protein, is a heat- and alkali-resistant laccase that provides the laccase activity of spore laccases (Brander et al. 2014). CotA-laccase, however, has to undergo an expensive protein purification process prior to its immobilization for use in industrial applications.

Spore laccases have potential advantages over CotA-laccase in industrial applications. To transform spore laccases into industrial biocatalysts, laccase production should be enhanced by optimizing culture conditions and reducing use-cost by immobilizing laccase on supports. However, limited research has been conducted on the effects of culture conditions on spore laccase activity and the use of spore immobilization to improve the reusability and stability of spore laccase.

In this study, the effects of culture conditions for Bacillus pumilus W3, including Cu²⁺ concentration, oxygen content, and culture time, on the specific activity of spore laccase were investigated. Spore laccase with high activity was then immobilized on amino-functionalized celite. Then, to estimate its decolorization activity, the immobilized spore laccase was used to decolorize the synthetic dyes methyl green and acid red 1.

### MATERIALS AND METHODS

#### Materials

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonate)(ABTS) (purity ≥98.0%), Syringaldehyde (SGZ) (purity ≥99.0%), and 2,6-pyridinedicarboxylic acid (Dipicolinic acid, DPA) (purity ≥99.5%) were purchased from Sigma-Aldrich (St Louis, MO, USA). 3-Aminopropyltriethoxysilane (APTES) (purity ≥99.0%) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (purity ≥98.0%) were purchased from Aladdin Reagents Co. Ltd (Shanghai, China). Celite 545, methyl green (zinc chloride salt, for microscopy), acid red 1 (purity ≥98.0%), and other chemicals were purchased from Sinopharm Chemical Reagent Company (Shanghai, China). All the chemicals were of analytical grade except for Celite 545.

#### Preparation of spore suspension

B. pumilus W3 that was isolated from raw gallnut honey samples (Guan et al. 2014) was cultivated at 30 °C for 48 h on nutrient broth sporulation medium that contained Cu²⁺. The spores were prepared following a published procedure by Jenkinson et al. (1981), harvested via lysozyme treatment and salt washing, and then finally suspended in deionized water at a concentration of 100 mg wet spores/mL.

#### Laccase activity assay

Laccase activity was determined based on the oxidation of 0.05 mM SGZ. Before the reaction, the spores or immobilized spores were incubated in 0.1 M citrate-phosphate buffer (pH 6.8) at 37 °C for 15 min. SGZ oxidation was initiated by adding SGZ into the reaction mixture and was terminated by placing the sample in an ice water bath for 10 min. SGZ oxidation was monitored using an UV-Visible spectrophotometer at 525 nm. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of substrate per minute. The activity yield of immobilized spore laccase was calculated in accordance with the method described by Mouelhi et al. (2014). All assays were conducted in triplicates.

#### Effects of culture conditions on the production of spore laccase

The effects of Cu²⁺ concentration on spore laccase production was investigated by adding 0–0.6 mM Cu²⁺ into the culture medium. The strains were then cultivated at 30 °C at 200 rpm for 2 days. To estimate the effect of culture time on laccase production, the strains were cultivated at 30 °C at 200 rpm for 9 days with 50 mL of liquid loading. The standard DPA curve was constructed using six different concentrations of DPA standard reagent. DPA content was assayed in accordance with the method of Janssen et al. (1958). To estimate the effect of oxygen content on spore laccase production, the strains were cultivated at 30 °C for 5 days. To cultivate sufficient numbers of vegetative cells, samples that were cultivated with a shaking speed of 50 rpm were cultivated with a shaking speed of 200 rpm from 0 h to 8 h. Oxygen content was determined with a portable dissolved oxygen meter (Ohaus, Starter 300D) under normal culture conditions. The oxygen meter was fastened in the neck of a conical flask while the parameter was set; meanwhile, the ventilation property and shaking speed...
remained constant. A glass ball was then submerged below the liquid surface without contacting the bottom of the bottle during the test. All the experiments were performed in triplicates.

**Preparation of amino-functionalized celite and immobilization of spore laccase**

Amino-functionalized celite was prepared in accordance with a published procedure by Kumar et al. (2014) with some modifications. Briefly, celite was soaked in 48 mL of anhydrous ethanol. Then, 2 mL of APTES was added to the celite. The mixture was ultrasonicated for 30 min. Then, the mixture was shaken at 70°C and 200 rpm for 12 h. Finally, the amino-functionalized celite was separated and washed thrice with deionized water.

The amino-functionalized celite (2 g) was redispersed in 34 mL of phosphate buffer (50 mM, pH 7). Then, 4 mL of EDC solution (4 mg/mL) was added and the mixture was sonicated for 10 min. Next, 2 mL of spore suspension was added to the mixture. The mixture was then sonicated for 30 min. After that, the mixture was incubated at 37°C and 200 rpm for 12 h. The immobilized spores were separated using a Buchner funnel and washed thrice with assay buffer. The immobilized spores and supernatant were collected for the evaluation of immobilization efficiency and for further study.

**Dye decolorization**

The decolorization ability of the immobilized spore laccase was evaluated by assaying the decolorization percentage of methyl green ($\lambda_{\text{max}} = 630$ nm) and acid red 1 ($\lambda_{\text{max}} = 531$ nm). The classifications and concentration of dyes vary in accordance with industrial specifications. To make the experiment more convenient and uniform, a reaction system that is suitable for observation and detection was selected through a series of preliminary experiments. The experimental system was composed of 4 mL of reaction mixture that contained 0.1 g of immobilized spores, 4 mM of ABTS (as the mediator), 0.1 M citrate-phosphate buffer (pH 7), and 4 g/L methyl green or 4 g/L acid red 1. The reaction mixture was incubated at 37°C at 220 rpm for 48 h. Decolorization percentage was measured at various times. Amino-functionalized celite without immobilized spores and untreated celite were used as the control samples and were run in parallel. Decolorization percentage was calculated using the following formula: decolorization (%) = $\left[ \frac{(C_0 - C_t)}{C_0} \right] \times 100\%$, where $C_t$ is the absorbance of the controls and $C_i$ is the absorbance of the experimental group. Acid red 1 was used as the substrate to determine the reusability of immobilized spore laccase. The immobilized spores were separated from the reaction mixture via filtration and washed thrice with citrate-phosphate buffer before reuse. All the experiments were performed in triplicate.

**RESULTS AND DISCUSSION**

**Effect of Cu$^{2+}$ on spore laccase production**

Laccases are multicopper oxidases. Their active centers are composed of four copper ions and corresponding proteins. The copper ions transfer electrons to O$_2$. Therefore, the Cu$^{2+}$ content of the culture medium greatly affects laccase activity (Durão et al. 2012). A previous report (Martins et al. 2015) has demonstrated that inactive CotA is still assembled in the spore coat even when copper is deficient. Excess copper, however, may inhibit spore laccase activity. In this study, the effect of Cu$^{2+}$ concentration in the culture medium on the specific activity of laccase and spore yield is shown in Figure 1. The highest spore yield was observed when no CuSO$_4$ was added to the culture medium. Spore yield decreased as Cu$^{2+}$ concentration increased because the high Cu$^{2+}$ content in the culture medium inhibited the growth of *B. pumilus* W3. The specific activity of spore laccase was uncorrelated with spore yield and reached the maximum value of 6.86 U/g wet spores when *B. pumilus* W3 was cultivated in medium that contained 0.2 mM Cu$^{2+}$. *B. licheniformis* LS04 exhibits a similar specific activity when cultivated in a medium that contains 0.2 mM Cu$^{2+}$, where its specific activity is 11.07 U/g dry
spores (Lu et al. 2012b). However, there are no previous reports on the optimum Cu$^{2+}$ concentration in the culture medium for the production of highly active spore laccase. According to Durão’s report (Durão et al. 2008), the spore yield and specific activity of spore laccase are uncorrelated because of the cytotoxicity of Cu. As Cu$^{2+}$ concentration increases, strain proliferation is restrained and spore yield decreases. Increasing Cu$^{2+}$ concentration provides sufficient Cu for the assembly of CotA, which increases the specific activity of spore laccase. However, when Cu concentration increases to a certain value, the specific activity of spore laccase decreases because the low concentration of Cu in the bacterial cytoplasm may impair CotA laccase. Compared with Bacillus laccases, fungal laccases are induced under higher Cu$^{2+}$ concentrations. For example, the activity of laccase from Coprinus comatus is 2.7- to 3.4-fold higher in cultures that are supplemented with 0.5-3.0 mM Cu$^{2+}$ than that in culture medium supplemented in 1.6 μM Cu$^{2+}$ (Lu & Ding 2010).

**Effect of culture time on the production of spore laccase**

DPA, which comprises 5–15% of dry spores, is a unique characteristic component of spores. Thus, the amount of detected DPA provides a good estimate of bacterial spore content (White & Harmon 2004; Wang & Lin 2007). As shown in Figure 2, the specific activity of spore laccase is positively correlated with the DPA content of spores. Laccase specific activity and spore DPA content increased from days 1 to 5 of culture. Both parameters, however, decreased from days 5 to day 9 of culture. The maximum specific activity and DPA content were observed after day 5 of cultivation. This result is different from that previously reported by Claus & Filip (1997), where the maximum specific activity and DPA content were observed on day 8 of cultivation. Moreover, in this study, a large number of spores formed on day 1 and did not obviously increase until day 5 of cultivation. These results suggested that the specific activity of spore laccase is the same as that of the DPA content of spores, and that both are closely related to the stage of spore formation and purity of spores. Confusingly, laccase specific activity and spore DPA content decreased, whereas spore yield increased, from days 5 to 9 of culture. A possible speculation for this phenomenon is that a large number of germinated spores produced more strains, which, in turn, produced more spores. However, during spore germination, the spores exist for a period of time when the large depot of DPA and the associated Ca$^{2+}$ in spore cores are released (Setlow 2003; Cheng et al. 2010). During this process, the assembly of spore coat proteins may also be affected, thus further affecting laccase activity that relies on CotA protein. Another possible explanation for this observation is that the growth environment of the strains worsens over time. In extreme environments, some genetic mutations or other spore abnormalities decrease DPA output per g spores (Magge et al. 2008) and decrease the specific activity of laccase. However, the total laccase activity and DPA amount did not significantly decrease because of the increase in spore amount.

**Effect of oxygen content on spore laccase production**

The oxygen content of the culture medium can affect the growth of strains. Therefore, selecting the suitable oxygen level is essential. Moreover, the oxygen content of the culture medium may affect the incorporation of copper in CotA (Durão et al. 2008). The effect of oxygen content in the culture medium on spore laccase production is shown in Table 1. In this study, the effect of oxygen concentration on the specific activity of laccase is very different from that

![Figure 2](https://iwaponline.com/wst/article-pdf/76/1/147/451842/wst076010147.pdf)
in Durão’s report (Durão et al. 2008), which showed that cells accumulate higher amounts of Cu when grown under microaerobic conditions in Cu-supplemented medium than when grown under aerobic conditions. The role of molecular oxygen in the regulation of cotA expression in the spore coat may explain the dependence of spore laccase activity on the oxygen content of the medium. Li et al. (1995) have demonstrated that molecular oxygen induces mnp expression in the presence of manganese. Another possible reason is that spores produced under anaerobic conditions may have some abnormalities; this hypothesis, however, requires further study. Furthermore, the heterologous expression of a fully Cu-loaded enzyme under aerobic conditions is impaired by the low cellular concentration of copper ions (Durão et al. 2008). The highest specific activity, which was obtained from strains that were cultured at a shaking speed of 200 rpm and 100 mL of liquid loading, may be a result of balance between oxygen induction and copper incorporation.

**Immobilization of spore laccase**

Diatomite is a low-cost porous material that is abundant in many areas of the world. Given its unique properties, diatomite can be used in dye absorption, metal ion removal, and beverage filtration and as a catalytic carrier (Bello et al. 2014; Cesarini et al. 2014). In this study, using unmodified celite to absorb spores resulted in low immobilization efficiency and laccase activity. Both EDC and glutaraldehyde have been used as crosslinkers to conjugate spores and amino-functionalized celite. The use of EDC as a crosslinker produced higher activity yield (83.16%) than the use of glutaraldehyde (data not shown). EDC activates the carboxyl group of enzymes for attachment to the amino group of another compound/matrix or enzyme (Kumar et al. 2014). In the crosslinking reaction, EDC has high specificity for reactive groups and does not participate in the composition of the connecting arm (Figure 3), which may increase the activity yield of immobilized enzymes. In this study, immobilization was terminated at pH 7, which may reduce the hydrolysis of EDC. The maximum hydrolysis of EDC occurs at acidic pH and disappears at neutral and higher pH regions (Nakajima & Ikada 1995). Moreover, a previous experiment indicated that EDC-mediated amide bond formation occurs between pH 4.5 and 7.5, and that a mildly alkaline pH condition limits the polymerization of proteins while facilitating the coupling of a carboxylate-containing molecule at a low substitution level per protein (Hermanson 2008).

**Dye decolorization**

Diatomite can be directly used for dye removal. However, the decolorization ability of diatomite for textile effluents relies on surface charges. The surface charge of diatomite changes as the pH changes, which affects the adsorption capacity of diatomite for dyes. Under alkaline conditions, diatomite adsorbs anionic dyes because the surface hydroxides of diatomite have been deprotonated; thus, its surface becomes anionic (Al-Ghouti et al. 2003). In this study, methyl green (cationic dye) and acid red 1 (anionic dye) were decolorized with the immobilized spores, unmodified celite, and amino-functionalized celite (Figure 4). The decolorization percentages of methyl green and acid red 1 increased constantly with treatment time and reached 84.15% and 69.70%, respectively, at 48 h of treatment. However, the dye decolorization ability of the celite before and after surface modification exhibited little change. Moreover, decolorization ability almost disappeared as the treatment period was extended. This likely resulted from the low amount of available celite for dye decolorization. Notably, all the decolorization percentages of dyes via celite absorption decreased constantly and reached the minimum value at 6 h of treatment. In addition, the absorbance value of methyl green after celite absorption was 20% higher than that of the control after 6 h of treatment. This situation may be explained by the autodecolorization of methyl green in the control samples. By contrast, methyl green that was absorbed by celite did not undergo autodecolorization for unknown reasons. Given that acid red 1 does not autodecolorize, its final decolorization percentage was approximately 0 instead of a negative value. This also demonstrates that acid red 1 that absorbed on celite was fully desorbed starting from 6 h of treatment. A similar...
situation was observed in previous reports (Sun et al. 2011; Gong 2014). The decolorization percentage reached the maximum when the dye was treated with modified diatomite for 10–60 min. The dye was then desorbed constantly with prolonged treatment time. Therefore, laccase oxidation can remedy the inadequate adsorption capacity of diatomite. In fact, both the decolorization of methyl green and acid red 1 were terminated by the oxidation of spore laccase in this study.

Reusability of immobilized spore laccase

Reusability is a key advantage of immobilized biocatalysts. To determine the reusability of immobilized spore laccase, acid red 1 was used as the experimental object because it will not autodecolorize. The result showed that immobilized spore laccase retained 87.04% of its original decolorization percentage after six cycles of usage (Figure 5). Held et al. (2005) reported that immobilized spore laccase on alumina pellets exhibited excellent decolorization ability for various dyes; their study, however, did not describe the reusability of the immobilized spore laccase. Spore laccase that is immobilized on calcium alginate beads manifests higher reusability (at least 15 times) than that immobilized on celite (Lu et al. 2012a). However, calcium alginate beads will swell and dissolve in phosphate buffer for ion exchange between Ca$^{2+}$ and other metal ions under some conditions (Bajpai & Sharma 2004). This study confirmed that immobilized spore laccase on amino-functionalized celite is a stable, reusable, and effective biocatalyst.

CONCLUSION

Several key factors influence the activity and yield of spore laccase. High yields of spore laccase with high activity can be harvested in a short duration by adjusting oxygen content, Cu$^{2+}$ concentration, and culture time. By
immobilizing spores onto low-cost diatomite, laccase can decolorize various dyes via oxidation. Moreover, diatomite may be used to adsorb and filter wastewater. Spore laccase was efficiently immobilized on amino-functionalized celite and can be reused several times in dye decolorization. Moreover, the immobilized spore laccase on celite do not require a regeneration process and can be used continually and directly until the immobilized enzyme is deactivated. 

These insights into culture conditions and immobilization are useful for developing spore laccases as biocatalysts in the treatment of textile effluents.

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REFERENCES


