Brazilian Journal
of Chemical
Engineering

ISSN 0104-6632
Printed in Brazil
www.abeq.org.br/bjche

Vol. 34, No. 01, pp. 41 - 52, January - March, 2017
dx.doi.org/10.1590/0104-6632.20170341s20150492

PREPARATION AND CHARACTERIZATION OF IMMOBILIZED SPORES WITH LACCASE ACTIVITY FROM *Bacillus pumilus* W3 ON DEAE-CELLULOSE AND THEIR APPLICATION IN DYE DECOLORIZATION

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(Submitted: August 5, 2015 ; Revised: November 10, 2015 ; Accepted: November 24, 2015)

Abstract - In order to obtain a more stable and reusable immobilized spore laccase for dye decolorization, the spores from *Bacillus pumilus* W3 were immobilized on diethylaminoethyl cellulose (DEAE-cellulose). Free and immobilized spore laccase retained 34.38% and 46.11% of their initial activity, respectively, after 10 days incubation at pH 9.0. Their residual activity remained at 27.36% and 31.84% after 10 h incubation at 70 °C. Immobilized spore laccase was more stable than free spore laccase in the presence of most organic solvents, metal ions and inhibitors. The tested dyes, including methyl green, methyl red and acid red 1, were removed 86.82%, 78.14% and 88.60%, respectively, by immobilized spore laccase after 24 h at 37 °C, and 74.34% of initial decolorization activity after 7 cycles was retained when it decolorized acid red 1. These properties indicated that immobilized spore laccase may be useful in textile effluent treatment.
Keywords: Spore laccase; *Bacillus pumilus*; DEAE-cellulose; Decolorization; Immobilization.

INTRODUCTION

Synthetic dyes are used extensively in textile dyeing and finishing industries. More than 7.0×10^5 tons and 1.0×10^5 kinds of commercially available dyes are produced worldwide annually (Strong and Claus 2011). Many of the synthetic dyes are toxic, carcinogenic, mutagenic, or teratogenic to various aquatic organisms and are difficult to degrade (Ozmen *et al*., 2008). Growing demand of dyes in the textile industry makes it one of the main sources of water pollution problems (Sarayu and Sandhya 2012).

In early years, some physical and chemical methods, such as adsorption, oxidation, coagulation-flocculation, filtration, and electrochemical methods were the main treatment methods. However, these methods are quite expensive and have some operational problems (Madhavi and Lele 2009). Bacterial aerobic or anaerobic dye degradation is one of the treatment methods for dye-containing industrial effluent. However, bacterial aerobic dye degradation has been confined to chemostat-enriched cultures adapting to a single dye, while under anaerobic conditions the azo dyes are cleaved by azo-reductases to yield potentially carcinogenic aromatic amines (Strong and Claus 2011).

Laccase (EC 1.10.3.2), one of the earliest discovered multicopper oxidases, is able to decolorize

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synthetic dyes efficiently, which attracted a large number of scholars to apply it in dye decolorization. To date, white-rot fungi are the most efficient single class of microorganisms in breaking down synthetic dyes (Wesenberg *et al*., 2003). However, fungal laccase is usually unstable under high temperatures and alkaline conditions, and no decolorization activities are observed at pH values higher than 7 (Held *et al*., 2005). In contrast, bacterial laccase can withstand high temperature and extreme pH values (Zhang *et al*., 2012).

Spore laccase is a typical bacterial laccase. It was mentioned by Held *et al* (2005) for the first time and may be defined as spore-bound laccase (laccase is one of the spore proteins) with the individual spore as the research object. CotA, an outer coat protein of spores from the *Bacillus* genus, is the best-studied bacterial laccase by far and usually considered as the laccase activity supplier of spore laccase. However, there may be some other spore proteins with laccase activity such as MnxG from the ridged outermost layer of the SG-1 spores (van Waasbergen *et al*., 1996). The properties of spore laccase from previous reports (Lončar *et al*., 2014; Lu *et al*., 2012b) have shown their advantages for the biodegradation of industrial textile dyes.

The immobilization of laccase offers several improvements for dye decolorization because the stability of laccase towards pH, temperature and storage is frequently enhanced. Moreover, the reusability of immobilized laccase represents a great advantage compared with free laccase (Fernández-Fernández *et al*., 2013). The immobilization of fungal laccase on different carriers by various means for dye decolorization has been well investigated, such as green coconut fiber via covalent attachment (Cristóvão *et al*., 2012), Fe_3O_4/SiO_2 nanoparticles with particle size below 30 nm by immobilization through glutaraldehyde coupling (Wang *et al*., 2013), alginate-gelatin mixed gel via entrapment (Mogharabi *et al*., 2012), beer spent grain via adsorption and covalent binding (da Silva *et al*., 2012), etc. However, immobilized fungal laccase still prefers acidic conditions (Bayramoglu *et al*., 2012). To date, there are only a few reports (Held *et al*., 2005; Lu *et al*., 2012b) about spore laccase immobilization. These reports showed the advantages of immobilized spore laccase in dye decolorization. However, the optimization of immobilization conditions of spore laccase, the characterization of immobilized spore laccase and its application in dye decolorization still need to be studied further in detail.

In the present work, we describe, for the first time, the immobilization of spores from *Bacillus pumilus* W3 on diethylaminoethyl cellulose (DEAE-cellulose). The decolorization ability of free and immobilized spore laccases was determined by using various synthetic dyes. Furthermore, the characterizations of free and immobilized spore laccases were also assessed in comparison.

MATERIALS AND METHODS

Materials and Bacterial Strain

2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), syringaldazine (SGZ), 2,6-dimethoxyphenol (2,6-DMP), syringaldehyde (SYR), 1-hydroxybenzotriazole (HBT), vanillin, and acid red 1 were all of reagent grade and obtained from Sigma-Aldrich (St. Louis, MO, USA). DEAE-cellulose (cotton fiber), 4-hydroxybenzoic acid (4-HBA), crystal violet, methyl green, methyl red, lysozyme (twice crystallized), phenylmethylsulfonyl fluoride (PMSF) and other chemicals were all of analytical grade and purchased from Sinopharm Chemical Reagent Company (Shanghai, China). *B. pumilus* W3 was isolated from raw gallnut honey samples (Guan *et al*., 2014a), and deposited at −20 °C.

Preparation of Spore Suspension

B. pumilus W3 was cultivated on nutrient broth sporulation medium containing 0.2 mM Cu^{2+} at 30 °C, 200 rpm for 48 h (Schaeffer *et al*., 1965). Spore suspension was prepared following a procedure published by Jenkinson *et al.* (1981) with some modifications. The culture fluid was harvested by centrifugation (8000 rpm, 10 min, 4 °C) and washing twice with deionized water, then suspended in deionized water containing lysozyme (1 mg/ml) at 37 °C for 2 h to lyse remaining vegetative cells. The pellets were washed with NaCl (1 M) and KCl (1 M), and finally suspended in deionized water to a concentration of 10 mg wet spores/ml. The purified spores were stored at 4 °C.

Enzyme Assay

Laccase activity, for both free and immobilized spore laccases, was assayed at 37 °C using ABTS (0.5 mM), SGZ (0.05 mM), and 2,6-DMP (1.5 mM) as substrates. The oxidation of ABTS was measured at 420 nm, of SGZ at 525 nm and of 2,6-DMP at 469 nm in 0.1 M citrate–phosphate buffer (pH 3.0–7.0) or 0.1 M Tris–HCl buffer (pH 7.0–9.0). Reactions started with the addition of substrates. The oxidation

of substrates was monitored using an UV–Visible spectrophotometer (METASH, UV-6000). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of substrate per minute. All assays were carried out in triplicate.

Immobilization of Spore Laccase

DEAE-cellulose was pretreated according to the previous report (Liu *et al*., 2014). Briefly, dry DEAE-cellulose was suspended in 5 volumes of distilled water and incubated overnight after stirring for swelling and mixing of the slurry. The swelled DEAE-cellulose was filtered on a Buchner funnel and then incubated with 0.5 M HCl for 1 h. It was then washed with distilled water until neutral pH. It was further incubated with 0.5 M NaOH for 1 h, and then washed with distilled water until neutral pH. 0.5 ml of spore suspension (100 mg wet weight per ml) was added to 19.5 ml of 0.05 M citrate–phosphate buffer and thoroughly mixed with the activated DEAE-cellulose at the ratio of 1 ml: 2 g (enzyme: DEAE-cellulose dry weight). The mixture was constantly rotated for full adsorption at 37 °C, 200 rpm overnight. The mixture in full adsorption was washed three times with 0.1 M citrate–phosphate buffer (pH 6.8) and filtered on a Buchner funnel. The filtrate was collected and used for evaluating the immobilization efficiency. The laccase activity of immobilized spores was measured. The immobilization yield was defined as previously described by Valerio *et al.* (2013).

The effects of several factors, including solution pH, spore amount and adsorption time, on immobilization efficiency were investigated, respectively. Scanning electron microscopy (SEM) of DEAE-cellulose before and after spore immobilization was performed with a scanning electron microscope ((FEI Quanta-200, The Netherlands)) at 5.0 kV. All the experiments were performed in triplicate.

Characterization of Free and Immobilized Spore Laccase Activity

The effects of temperature and pH on free and immobilized spore laccase activity and stability were investigated as previously described (Guan *et al*., 2014b) with some modifications. The optimum pH was measured using ABTS, SGZ and 2,6-DMP as substrates at 37 °C in 0.1 M citrate–phosphate buffer (pH 3.0–7.0) or 0.1 M Tris–HCl buffer (pH 7.0–9.0). The pH stability of free and immobilized spore laccases was assayed by pre-incubating the enzyme at pH 3.0, 7.0, and 9.0 at 37 °C for 10 days. The optimum temperature for laccase activity was measured using SGZ as the substrate at different temperatures (25–90 °C) at the optimum pH condition. For thermal stability analysis, the enzyme was incubated at different temperatures (60, 70 and 80 °C) for 0 h to 10 h, and then incubated in ice water for 10 min, and the residual activity was measured.

The effects of organic solvents on laccase activity were investigated in the presence of 10% (v/v) and 50% (v/v) organic solvents. Free and immobilized spores were pre-incubated in 0.1 M citrate–phosphate buffer (pH 6.8) at 37 °C for 2 h, and then the residual activity was determined. The effects of metal ions on the enzyme activity were investigated in the presence of 10 mM of K^+, Ca^{2+}, Na^+, Mg^{2+}, Al^{3+}, Mn^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}. To study the effects of inhibitors on enzyme activity, free and immobilized spores were pre-incubated with various inhibitors at different concentrations at 37 °C for 15 min, and then the residual activities were determined. All the experiments were performed in triplicate at the optimum pH condition. The samples in the absence of organic solvents, inhibitors and metal ions were used as the control, and were run in parallel.

Dye Decolorization

Dye decolorization experiments were carried out using methyl red, acid red 1 and methyl green. The reaction mixture (4 mL) contained reaction buffer, 0.1 ml mediator (4 mM), 0.1 mL dyes (4 g/L) and appropriate quantities of spores or immobilized spores with the same weight as free spores. The free spores acted as the catalyst to find the most suitable mediator, a variety of compounds, including HBT, ABTS, SYR, vanillin, and 4-HBA, were added to the reaction mixture, respectively, and the best promotion effect on dye decolorization was selected. The optimum pH, spore amount, dye concentration and treatment time for dye decolorization were investigated using the free spores as the catalyst. The maximal absorbance wavelength of several dyes and the decolorization ability of enzyme was determined by spectral scanning and absorbance detection using an UV–Visible spectrophotometer (METASH, UV-6000), respectively. The samples without the addition of enzyme, as the control, were run in parallel, and all samples were incubated and rotated at 37 °C, 200 rpm for 24 h. Decolorization percentage was recorded as $(A_c-A_e)/A_c*100\%$, where A_c and A_e are the control and experimental group, respectively, which is more reasonable than calculating the decrease of absorbance relative to the initial absorbance because lots of dyes decolorize automatically.

The reusability of immobilized spore laccase was evaluated by measuring the percentage decolorization of dyes by immobilized spore laccase after seven cycles. The immobilized spores were separated from the reaction mixture through filtration and washed three times for reuse. The measuring method was the same as the method described above. All the experiments were performed in triplicate.

RESULTS

Immobilization of Spore Laccase

Effect of pH on Immobilization Efficiency

The effect of pH on immobilization efficiency of spore laccase was investigated in the range of pH 5.0–10.5. As shown in Figure 1, the immobilization yield of spores always remained above 80%, which increased with increasing pH and reached a maximum (99.19%) at about pH 10.0. The laccase activity of immobilized spores also increased with increasing pH and reached the highest value, 3.01 U/g wet spores, at pH 10.0.

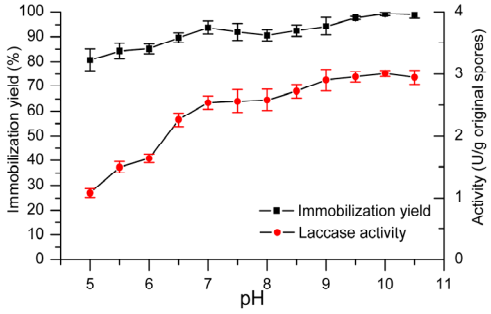

Figure 1: Effect of pH on immobilization efficiency of spore laccase. Adsorption buffer: 0.05 M citrate–phosphate buffer for pH 5.0–7.0, 0.05 M Tris–HCl buffer for pH 7.5–9.0 and 0.05 M sodium carbonate–sodium bicarbonate buffer for pH 9.5–10.5.

Effect of Spore Amount and Adsorption Time on Immobilized Efficiency

The effect of spore amount on immobilization efficiency was investigated ranging from 10 to 150 mg wet spores per g dry weight DEAE-cellulose (Figure A1a). The relative activity of immobilized spore laccase increased rapidly with the increase of spore amount when the spore concentration was 10 mg/g to 50 mg/g, and increased slowly when the spore concentration exceeded 50 mg/g. Nonetheless, only a trace amount of enzyme activity was found in the supernatant (about 0.2%-5% of the total activity). The relative activity increased with prolonged adsorption time from 0 to 24 h (Figure A1b). The relative activity of immobilized spore laccase arrived at 98% at 5 h, and there was no laccase activity increase after 10 h.

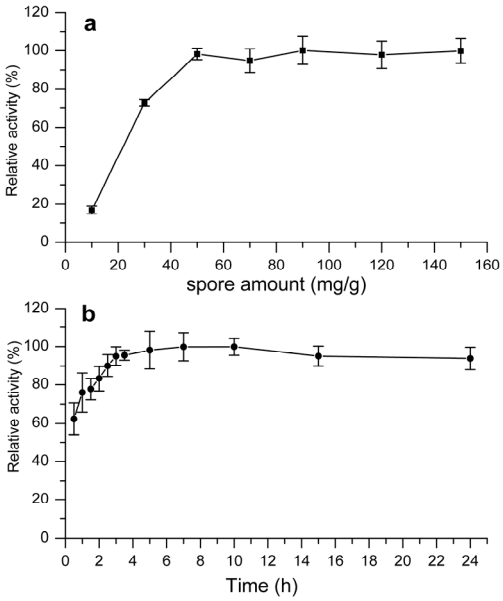

Figure A1: Effects of spore amount (a) and absorption time (b) on immobilization efficiency. The maximum activity was set as 100%. The unit "mg/g" means mg wet spores per g dry DEAE-cellulose in (b).

Structure Characterization of Immobilized Spores

SEM images of the DEAE-cellulose before and after spore immobilization are shown in Figure 2. The images show the diameter of the fiber to be, on average, about 14–21 μm, and the spores to be, on average, about 1.0–1.2 μm long and 0.4–0.6 μm wide. Spore distribution on the fibers surface is not uniform (Figure 2c to 2e). After a period of preservation and drying, some spores seem to blend into the fibers, and form a relief-like morphology (Figure 3f).

Characterizations of Free and Immobilized Spore Laccases

Effect of pH on the Activity and Stability of Free and Immobilized Spore Laccase

The effect of pH on free and immobilized spore laccases is shown in Figure 4. Both free and immobilized spore laccases demonstrated that there is a broad pH range for catalyzing substrates (Figure 3a). The

optimum pH of free and immobilized spore laccases for oxidizing ABTS was pH 3.2 and pH 3.4, respectively, for oxidizing SGZ was 6.8 and 7.0, respectively, and for oxidizing 2,6-DMP was 7.0 (both of them). The pH stability of free and immobilized spore laccases is shown in Figure 3b. The residual activity of immobilized spore laccase respectively retained 52.46% and 46.11% of its initial activity at pH 7.0 and 9.0 after 10-day incubation, while free spore laccase retained 37.51% and 34.38% of its initial activity under the same conditions. However, both free and immobilized spore laccases are unstable at pH 3.0, and retained 17.25% and 29.73% of their initial activity, respectively, after 10 days.

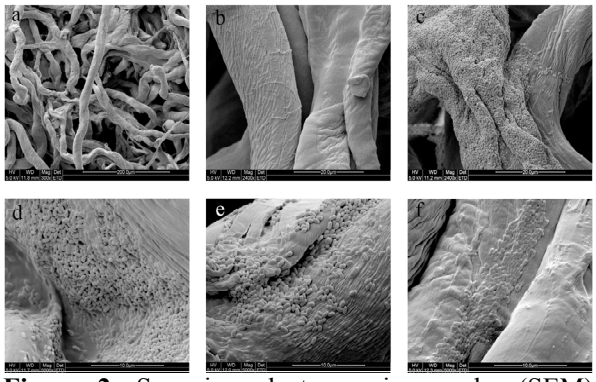

Figure 2: Scanning electron micrography (SEM) showing the surface morphologies of DEAE-cellulose before (a, b) and after (c to f) spore immobilization.

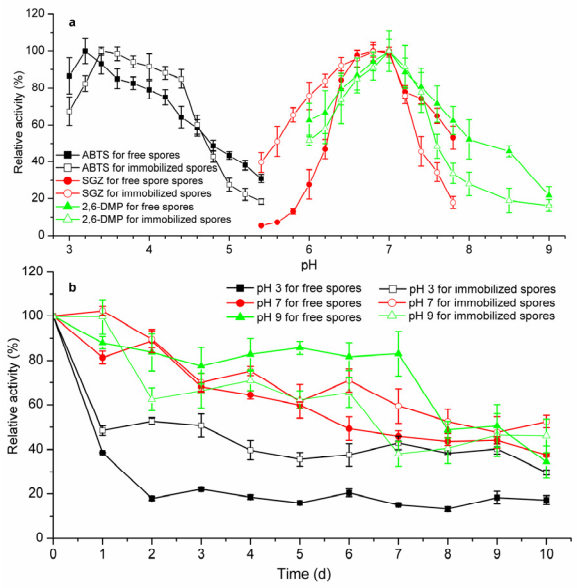

Figure 3: Effect of pH on free and immobilized spore laccase activity (a) and stability (b) at 37 °C. (a) ABTS, pH 3.0–5.4; SGZ, pH 5.4–7.8; 2,6-DMP, pH 6.0–9.0; (b) substrate: pH 3.0, ABTS; pH 7.0, SGZ and 2,6-DMP.

Effect of Temperature on the Activity and Stability of Free and Immobilized Spore Laccase

The effect of temperature on free and immobilized spore laccases towards SGZ is shown in Figure 4. The optimum temperature of immobilized spore laccase is 10 °C lower than that of free spore laccase, and are 55 °C and 65 °C, respectively (Figure 4a). Both free and immobilized spore laccases were very stable at 60 °C after 10 h and retained 85.57% and 73.75% respectively, of their initial activity (Figure 4b). The half-life of free and immobilized spore laccases was about 5.5 h and 4.5 h at 70 °C, 2.2 h and 2.5 h at 80 °C, respectively, and retained 27.36% and 31.84% of their initial activity after 10 h at 70 °C, respectively. Immobilized spore laccase lost all activity after 10 h at 80 °C while free spore laccase lost all activity after 8 h at 80 °C.

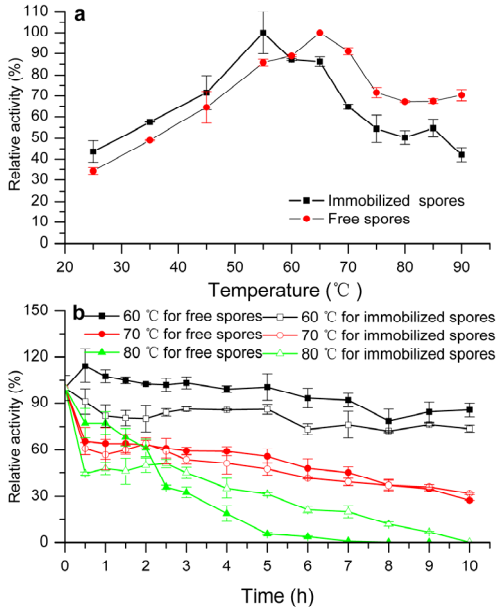

Figure 4: Effect of temperature on free and immobilized spore laccase activity (a) and stability (b) at pH 6.8. Laccase activity was measured using SGZ as the substrate.

Effect of Organic Solvents on Free and Immobilized Spore Laccase Activity

As shown in Table 1, after 2 h of incubation at 37 °C in the presence of 10% (v/v) organic solvents, the residual activity of free spore laccase changed a little, except that it was partly inhibited by dimethylsulfoxide and dimethylformamide, while some organic solvents, including acetone, acetonitrile, ethyl acetate and dimethylformamide, promoted the immobilized spore laccase activity greatly. In the presence of 50%

(v/v) organic solvents, the free spore laccase showed low residual activity, between 0 and 48.62% of its initial activity, while immobilized spore laccase maintained high stability and, specially, it retained more than 80% of its activity in 50% (v/v) methanol or dichloromethane.

Table 1: Effects of organic solvents on the activity of free and immobilized spore laccases.

Orgnanic compounds	Concentration (%, v/v)	Residual activity (%)	
		Free spores	Immobilized spores
Control		100.00±0.73	100.00±1.14
methanol	10	110.74±4.29	99.53±3.38
	50	16.68±1.98	82.54±2.11
ethanol	10	104.2±3.22	86.2±5.88
	50	40.79±2.56	51.24±2.23
acetone	10	106.12±4.23	193.87±2.18
	50	1.8±0.23	21.34±1.08
acetonitrile	10	126.09±4.48	180.78±6.56
	50	3.42±0.33	55.23±1.27
Dimethylsulfoxide	10	84.64±1.19	108.02±4.59
	50	6.78±0.56	33.55±1.76
ethyl acetate[a]	10	100.84±5.23	149.41±6.78
	50	–	+
Dichloromethane[b]	10	36.47±3.23	22.05±1.22
	50	48.62±3.12	103.24±5.59
Dimethylformamide	10	79.42±4.56	128.89±3.45
	50	3.54±0.75	2.33±0.54

Note:
[a] The products generated from laccase oxidizing substrate were in the water phase. It could not be measured in the presence of 50% (v/v) ethyl acetate because the reaction mixture was an emulsion. "–" represents no reaction and "+" represents weak reaction.
[b] The products generated from laccase oxidizing substrate were in the organic phase. The sample containing dichloromethane was measured with the water phase. The products extracted into the organic phase were not measured (only the sample containning 10% of dicloromethane has the situation that a small amount of products extracted into the organic phase). In addition, half of its absorbance value was taken as the final value when the samples containing 50% dichloromethane were measured.

Effect of Metal Ions and Inhibitors on the Free and Immobilized Spore Laccase Activity

The effects of various metal ions (10 mM) on the free and immobilized spore laccases are listed in Table 2. The results showed that free spore laccase was severely inhibited by Mn^{2+}, Fe^{3+} and Co^{2+}, while it was not inhibited by Na^{+}, K^{+}. Other metal ions had partly inhibitory effects on free spore laccase activity. In contrast, the activity of immobilized spore laccase was improved remarkablely in the solution containing metal ions except it was still severely inhibited by Mn^{2+} and Fe^{3+}. Some metal ions, such as Al^{3+}, K^{+}, Ca^{2+} and Zn^{2+} turn from inhibitors into activators after spore immobilization.

Table 2: Effects of metal ions on the activity of free and immobilized spore laccases.

Metal ions (10 mM)	Residual activity (%)	
	Free spores	Immobilized spores
Control	100.00±1.42	100.00±1.10
Na^{+}	97.22±2.67	102.67±3.03
Mg^{2+}	93.09±4.18	98.78±6.44
Al^{3+}	91.55±3.50	111.22±2.16
K^{+}	96.52±2.51	122.78±6.02
Ca^{2+}	91.32±1.34	113.56±5.14
Mn^{2+}	17.48±1.50	28.44±2.26
Fe^{3+}	2.07±0.78	5.00±1.62
Co^{2+}	30.36±4.17	92.78±2.31
Ni^{2+}	75.25±2.84	96.11±4.67
Cu^{2+}	79.21±2.59	95.11±4.30
Zn^{2+}	78.32±1.50	110±6.35

The effect of several typical laccase inhibitors on the activity of free and immobilized spore laccases is shown in Table 3. The activity of free spore laccase was completely inhibited by 1 mM of cysteine, while it was partially inhibited by NaN_3 and PMSF in various concentrations. In contrast, the immobilized spore laccase was found to manifest significant tolerance against inactivation by cysteine, NaN_3 and PMSF. Specifically, higher residual activity of immobilized spore laccase was observed in high concentrations of NaN_3, while free spore laccase retained its maximum residual activity when the concentration of NaN_3 was 0.005 mM. Both free and immobilized spore laccases manifest low tolerance towards 1 M of NaCl.

Table 3: Effects of inhibitors on the activity of free and immobilized spore laccases.

Inhibitors	Concentration (mM)	Residual activity (%)	
		Free spores	Immobilized spores
Control		100.00±1.22	100.00±2.87
EDTA	10	78.18±4.23	64.69±4.68
	25	75.63±6.14	66.67±3.74
	50	59.25±1.75	51.38±1.39
Cysteine	0.01	73.93±2.35	131.49±2.87
	0.1	5.77±1.22	89.33±2.01
	1	0.36±0.15	71.01±1.13
NaN3	0.0005	68.89±1.33	37.68±0.66
	0.005	79.01±2.26	85.38±1.28
	0.05	51.35±1.39	98.81±3.35
NaCl	100	97.49±2.22	82.87±5.59
	500	99.37±1.38	89.33±1.19
	1000	12.35±0.51	1.58±0.33
SDS	0.1	89.61±6.88	92.23±5.29
	1	50.2±4.75	81.29±3.01
	10	30.25±3.35	10.14±1.15
PMSF	0.1	96.91±3.33	91.44±5.43
	1	101.25±2.34	91.88±4.56
	10	26.44±0.69	65.22±1.76

Dye Decolorization

Measuring of the Maximum Absorbance Wavelength and Mediator Screening

The maximum absorbance wavelength of dyes was investigated because it may be influenced by the solvent and pH. The results showed that the maximum adsorption wavelengths of methyl green and acid red 1 were observed at λ=630 nm and λ=531 nm, respectively, while the maximum adsorption wavelength of methyl red is λ=520 nm in acidic conditions and λ=435 nm in alkaline conditions. In this work, ABTS was demonstrated to be the best mediator compared with SYR, HBT, vanillin and 4-HBA for dye decolorization, and was selected for further study (data not shown).

Effects of Different Decolorization Conditions on Decolorization Efficiency

In order to find out the characteristics of spore laccase in dye decolorization and to select the suitable condition for further study, the effects of pH, spore amount, dye concentration and treatment time on decolorization efficiency were investigated with free spore laccase (Figure 5). The results indicated that the decolorizaion efficiency of methyl green, methyl red and acid red 1 is the highest at pH 7.0, pH 5.0 and pH 6.0, respectively (Figure 5a). Meanwhile, the basic dye (methyl green) decolorized itself severely with the increase in pH value. The decolorization percentage of all the tested dyes (methyl green, methyl red and acid red 1) increased with the increase of spore amount and reached 86.24%, 66.31% and 65.60%, respectively, for 2.5 mg/ml of spores (Figure 5b). The highest decolorization efficiency was observed when the concentration of methyl green, methyl red and acid red 1 was 100 mg/L, 50 mg/L and 50 mg/L, respectively (Figure 5c). The effects of treatment time on the decolorization efficiency varied due to dye differences (Figure 5d). The decolorization percentage of the dyes rose dramatically as the treatment time increased from 0 to 24 h. The decolorization percentages of methyl green and acid red 1 increase slowly when the treatment time exceeded 24 h.

Considering the cost of spore production, decolorization efficiency and the requirements of textile wastewater treatment, a decolorization condition was selected for further study, which was pH 7.0, 2.5 mg/mL of spore, 100 mg/L of methyl green or 50 mg/L of methyl red or acid red 1, and 24 h of treatment time.

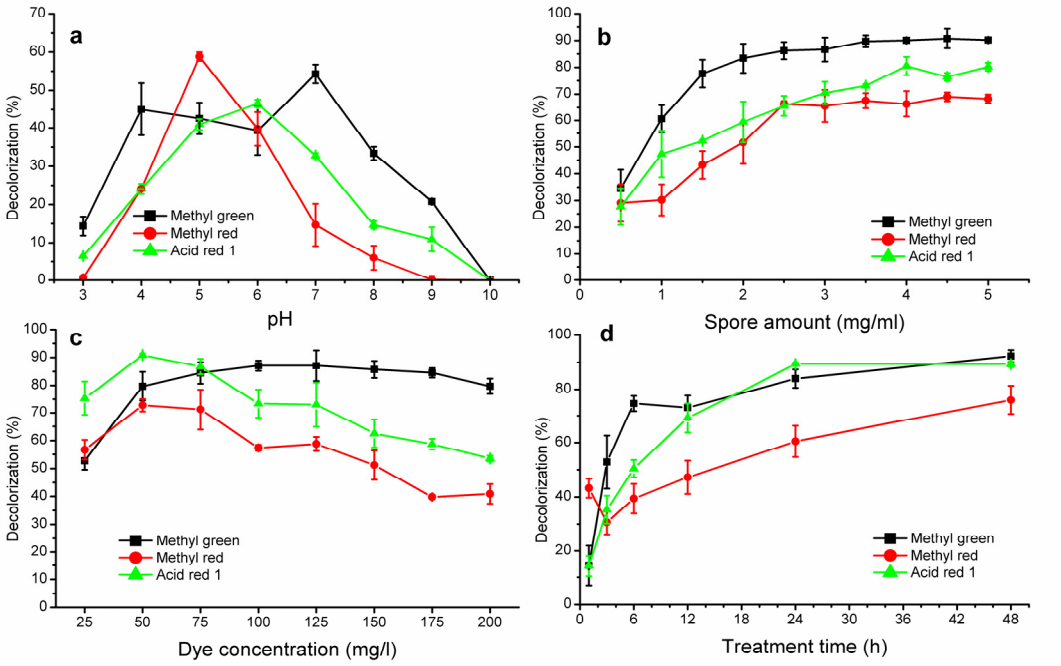

Figure 5: Effect of pH (a), spore amount (b), dye concentration (c) and treatment time (d) on decolorization efficiency. (a) The reaction mixture (4 ml) contained 1 mM ABTS, 100 mg/L dyes and 1.0 mg/mL free spores. The samples were incubated at 37 °C, pH 3.0–10.0 and rotated at 200 rpm for 24 h. Subsequent experimental conditions were set according to the optimum conditions from the previous experiment: (b) pH 7.0; (c) 2.5 mg/mL spores; (d) methyl green (100 mg/L), methyl red (50 mg/L) and acid red 1 (50 mg/L).

Dye Decolorization by Free and Immobilized Spore Laccases

The decolorization ability of free and immobilized spore laccases was evaluated with two acid dyes (methyl red and acid red 1) and a basic dye (methyl green) at 37 °C and 60 °C at pH 7.0. As shown in Figure 6, methyl green, methyl red and acid red 1 were removed 86.82%, 78.14% and 88.60%, respectively, by immobilized spore laccase, while they were removed 76.61%, 59.03% and 81.03%, respectively, by free spore laccase at 37 °C. In addition, the DEAE-cellulose without spores had high adsorption ability towards acid dyes (methyl red and acid red 1). It absorbed 42.49% of methyl red and 77.19% of acid red 1, while methyl green was decolorized 6.85% at 37 °C. There was no significant difference of the decolorization efficiency whether the reaction mixture was incubated at 37 °C or 60 °C.

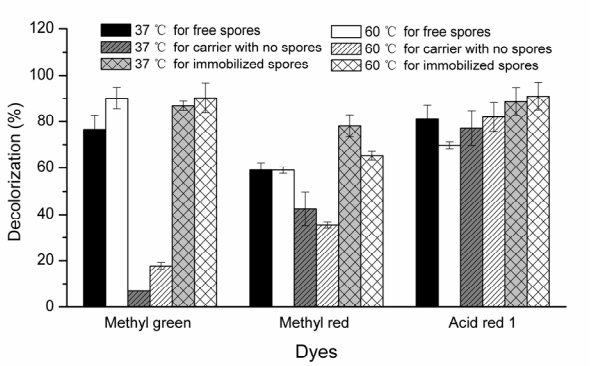

Figure 6: Decolorization of synthetic dyes by free and immobilized spore laccases. Each reaction sample contained 10 mg spores or immobilized spores (does not include the DEAE-cellulose weight). Their decolorization ability was evaluated by measuring the residual absorbance of methyl green (100 mg/L), methyl red (50 mg/L) and acid red 1 (50 mg/L) after 24 h incubation at 37 °C, pH 7.0 and 200 rpm.

The Reusability of Immobilized Spore Laccase

In order to evaluate the reusability of immobilized spore laccase, the decolorization percentages of all the tested dyes were determined after multiple reusage as shown in Figure 7. The immobilized spore laccase retained more than 95% of its initial activity in the 2nd cycle of dye decolorization. With increasing repeat cycles, the immobilized spore laccase showed a different reusability for the three dyes. The immobilized spore laccase retained 51.03%, 62.56% and 74.34% of its initial decolorization activity for decolorizing methyl green, methyl red and acid red 1 after 7 cycles of reusage.

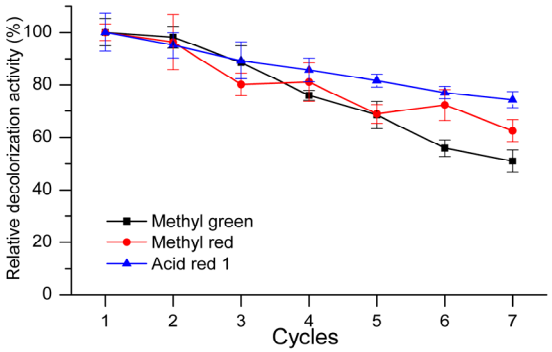

Figure 7: Reusability of immobilized spore laccase for dye decolorization.

DISCUSSION

DEAE-cellulose is a positively charged resin. In ion exchange adsorption, the adsorption pH is the most important parameter to determine which object is adsorbed (Nakatani *et al*., 2012). The optimum pH of spore laccase for immobilization (pH 10.0, Figure 1) is different from the immobilization of puerarin glycosidase on DEAE-52 cellulose, where the adsorption on DEAE-52 cellulose decreased when the pH was higher or lower than 6.5 (Liu *et al*., 2014). The optimum pH of immobilization is related to the isoelectric point of the protein and its resistance towards acid and alkaline conditions. When an anion exchanger is used as the immobilization carrier, the higher the isoelectric point, the higher the adsorption pH condition of immobilization. Besides, the more alkali-resistant the enzyme, the broader the acceptable pH range of immobilization. The properties of alkali-resistance of spore laccase contribute to getting a more stable immobilized spore laccase through ionic bonding, because a pH far away from the isoelectric point of the molecule of interest will give stronger binding and increased capacity (Amersham Pharmacia Biotech, 1999). However, the chemical structure of puerarin glycosidase might be destroyed under alkaline conditions, which made the activity of immobilized puerarin glycosidase decrease fast when the pH was higher than 6.5 (Liu *et al*., 2014).

The current results are similar to the immobilization of nuclease p1 on DEAE-cellulose (Shi *et al*., 2010). DEAE-cellulose can absorb a lot of spores in a short time, but the spore distribution on the fiber surface is not uniform (Figure 2). Those fibers whose surface is crinkly or dehiscent coupled a large number of spores, while most fibers that have a smooth

surface often coupled less spores. This may be related to the contact area between spores and fibers, steric hindrance and the distribution of di-ethyl amino-ethyl tertiary amine functional groups on the surface of the fibers. The inner spores were affected by diffusion constraints when a large number of spores were stacked together on the surface of fibers, so that the laccase activity of immobilized spores did not increase with increasing spore amount (Figure 2c) (Cao 2006). The dry immobilized spore laccase with a relief-like morphology may generate stronger binding between spores and fibers, but it could also affect the enzyme activity adversely (Figure 2f).

The pH preferences of spores after immobilization show no significant changes (Figure 3a). The optimum pH of free and immobilized spore laccases for oxidation of ABTS is different from most *Bacillus* laccases (Lončar *et al*., 2013; Guan *et al*., 2014b; Lu *et al*., 2013) where their optimum pH for laccase activity was between pH 3.8 and pH 4.8. The optimum pH of free and immobilized spore laccases for oxidation of SGZ and 2, 6-DMP corresponds to that of other *Bacillus* laccases (Wang *et al*., 2011; Koschorreck *et al*., 2008) in the pH range of 6.5–7.5 (Zhou *et al*., 2015). However, fungal laccase has lower pH preference than spore laccase. They usually oxidize ABTS in the pH range of 2.0–4.0 and oxidize SGZ and DMP in the pH range of 3.0–6.2 (Baldrian 2006). The pH stability of free and immobilized spore laccase from *B. pumilus* W3 is similar to that of laccase from *Bacillus vallismortis* fmb-103 (Zhang *et al*., 2012; Zhang *et al*., 2013), and stronger than that of spore laccase from *Bacillus* SF (Held *et al.* 2005). In contrast, fungal laccases are extremely unstable under alkaline conditions, for instance, a laccase from *Trametes versicolor* was inactivated completely after 1 h at 60 °C at pH 7.0 or pH 8.0 (Carvalho *et al*., 1999). Moreover, the pH stability of spore laccase after immobilization was improved significantly (Figure 3b). These results showed the advantages of immobilized spore laccase applied under alkaline conditions.

The optimum temperature of free and immobilized spore laccase (Figure 4a) is similar to that of some *Bacillus* laccases such as spore laccase from *B. licheniformis* LS04 (Lu *et al*., 2012a). According to a previous report (Zhou *et al*., 2015), the optimum reaction temperature of laccase from *Bacillus* strains is usually between 60 °C and 80 °C, while the optimum temperature of fungal laccase is usually between 50 °C and 70 °C (Baldrian 2006). *Bacillus* laccase is more thermostable than fungal laccase, even though there is little difference between their optimum temperatures. In the report by Hiden *et al.* (2009), the half-life of fungal laccase was usually not more than 1 h, while in this study the half-lives of free and immobilized spore laccase were more than 4 h at 70 °C. The results (Figure 4b) also indicated that free laccase can retain higher activity at short times and lower temperatures, while immobilized spore laccase has the advantage at long times and high temperature.

The effects of several organic solvents such as methanol, ethanol, etc. on the laccase activity of free and immobilized spores are very different. Spore laccase after immobilization has obvious advantages in organic solvents (Table 1) may relate to the fact that free and immobilized spore laccases have different preferences towards water activity and aquaphilicity. According to a previous report (Cao, 2008), different carriers and protein amount influence the activity of immobilized enzymes because they may change the water activity of the solution. The effects of metal ions on the activity of free and immobilized spore laccase from *B. pumilus* W3 (Table 2) are similar to that of other *Bacillus* laccases whose activity is usually activated by Li^{+}, Na^{+}, Mg^{2+}, Ca^{2+} and Ba^{2+}, and inhibited by Ag^{+}, Hg^{2+} and Fe^{3+} (Zhou *et al*., 2015). The spore laccase after immobilization improved its stability towards metal ions, which may be related to the change of structure of the laccase active center when the spores are immobilized. A similar situation can also be found in a previous report (Asghe *et al*., 2012). However, the effects of metal ions on the catalytic activity of enzymes are very complex and there are no one-to-one relationships between individual metals and functions (Andreini *et al.* 2008). So, the mechanism of metal ions effects on spore laccase should be studied further. The free spore laccase was stability towards some typical laccase inhibitors similar to other *Bacillus* laccases (Zhou *et al*., 2015) and its activity was inhibited to varying degrees (Table 3). Specially, similar to a previous report (Zhang *et al*., 2012), it was strongly inhibited by cysteine, NaN_3 and PMSF. In contrast, the immobilized spore laccase manifested strong resistance towards high concentrations of cysteine, NaN_3 and PMSF. What caused this difference is unclear and needs to be studied further.

One of the most important factors that influences the decolorization efficiency is pH. The optimum pH (pH 5.0–7.0) for decolorizaiton of methyl green, methyl red and acid red 1 by spore laccase from *B. pumilus* W3 (Figure 5a) is higher than that of the spore laccase from *B. vallismortis,* which has high decolorization efficiency in the pH range of 4.0–6.0, and that of the laccase from *Trametes modesta,* which has no decolorization activity when the pH

value is higher than 7 (Held *et al.* 2005; Zhang *et al*., 2012). However, the pH of combined effluents from textile industries is usually in the pH range of 6.0–11.0 (Yu *et al*., 2013). This indicates the potential of spore laccase from *B. pumilus* W3 in textile dye wastewater treatment. Similar to some other spore laccases (Lu *et al*., 2012a; Lončar *et al*., 2013), the spore laccase from *B. pumilus* W3 has high decolorization ability when the dye concentration is between 25–200 mg/l (Figure 5c). Considering the time and decolorization efficiency, 24 h of treatment time is suitable for spore laccase in dye decolorization (Figure 5d). Some *Bacillus* species cultures can also degrade synthetic dyes with high efficiency, but usually need a longer time than spore laccase (Dawkar *et al*., 2009; Kadam *et al*., 2013).

Both free and immobilized spore laccases had high decolorization ability towards three dyes (Figure 6). The lowest decolorization efficiency calculated for free spore laccase oxidizing methyl red was still 58.96%. Immobilized spore laccase with the same weight of spores as free spore laccase showed a higher decolorizing percentage for all the tested dyes compared to free spore laccase. Similar results were obtained by Mirzadeh *et al*., (2014), where the immobilized laccase had higher decolorization efficiency than free laccase. DEAE-cellulose can adsorb some dyes and its adsorption efficiency may be decided by its charge. Specially, acid red 1 could be efficiently absorbed by DEAE-cellulose and be decolorized 81.95% at 60 °C. This situation showed an advantage of immobilized spore laccase since the adsorption of carrier towards dyes can promote the decolorization efficiency of dyes, and can also decolorize some dyes that can not be decolorized by spore laccase.

The immobilized spore laccase can be reused for at least 7 cycles in dye decolorization (Figure 7). This is much higher than the reusage times of immobilized laccase on a similar carrier, green coconut fiber, where immobilized laccase lost 40%–100% of its decolorization activity towards different dyes after 3 reusages (Cristóvão *et al*., 2012). In the previous report, spore laccase immobilized in calcium alginate beads showed a higher reusability than this study and could be reused at least 15 times (Lu *et al*., 2012b). However, calcium alginate beads usually exhibit a lot of water uptake and subsequently dissolve in phosphate buffer under alkaline conditions (Bajpai and sharma, 2004). The dissolution is caused by the ion-exchange between Ca^{2+} and Na^{+} in phosphate buffered saline, and may also be caused by the ion-exchange between Ca^{2+} and other metal ions in other solutions. This limits the application of immobilized spore laccase in calcium alginate. In contrast, considering that the loss of activity in multi-cycle reusage may be caused by desorption of spores, adding spores to re-immobilize after 7 cycles of reusage may improve the reusage times of immobilized spore laccase greatly and could finish with a simple adsorption.

In conclusion, on the basis of previous reports, this study presents detailed research on the immobilization and characterization of spore laccase and its application in dye decolorization (Table A1). The immobilized spore laccase from *B. pumilus* W3 was prepared by a simple method and from cheap materials, has high stability towards various harsh environments, and can decolorize various dyes efficiently and be reused many times. These properties demonstrate that immobilized spore laccase might be useful for textile effluent treatment.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (31472003 & 31101331), the Open Project Program of the Key Laboratory of Industrial Biotechnology, Ministry of Education, China (KLIB-KF201304), a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions, the 111 Project (No. 111-2-06), and the Jiangsu province "Collaborative Innovation Center for Advanced Industrial Fermentation" industry development program. We thank Julie from Wuxi Joy English School for language assistance.

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