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Full Length Research Paper

Isolation and characterization of a novel *Bacillus subtilis* WD23 exhibiting laccase activity from forest soil

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The strain *Bacillus* sp. WD23 exhibiting laccase activity was screened from forest soil. The M9 medium containing Cu^{2+} was used for enriching and isolating bacterial strains capable of oxidizing syringaldazine. One isolated strain was identified as *Bacillus subtilis* WD23 based on the results of physiological and biochemical tests and 16S rDNA sequence analysis. The strain WD23 could grow at temperatures ranging from 20 to 55°C and showed optimum growth temperature and pH at 25°C and 7.0, respectively. The sporulation rate of the strain clearly correlated well with the laccase activity. The temperature half-life of the spore laccase was 2.5 h at 80°C and the pH half life time was 15 d at pH 9.0. Its spore laccase could decolorize 50 - 90% of Remazol brilliant blue R (RBBR), alizarin red, congo red, methyl orange and methyl violet, which suggests the potential application of spore laccase in dyestuff treatment.

Key words: Bacterial laccase, *Bacillus subtilis*, spore, decolorization.

INTRODUCTION

Laccases (benzenediol: oxygen oxidoreductases, EC1. 10.3.2) are multi-copper proteins that can oxidize a wide range of inorganic and aromatic compounds, particularly phenols, while reducing molecular oxygen to water (Alexandre and Zhulin, 2000). Laccases catalyze the removal of a hydrogen atom from phenolic substrates and aromatic amines by one-electron abstraction. The free radicals formed during the reaction are also capable of undergoing further depolymerization, repolymerization, demethylation or quinone formation (D'Annibale et al., 2000; Ullah et al., 2000; Held et al., 2005). The low substrate specificity of laccases and their ability to oxidize many pollutants suggest their industrial and biotechnological applications (Witayakran and Ragauskas, 2009; Steevensz et al., 2009).

Laccases are widely distributed among fungi and plants (Younes et al., 2007). However, it has been found that laccases are also widespread among bacteria (Alexandre and Zhulin, 2000). To date, laccases have mostly been isolated and characterized from plants and fungi but only fungal laccases are used currently in biotechnological applications. In contrast, only a few bacterial laccases have been characterized. Bacterial laccases could oxidize syringaldazine and 2,6-dimethoxyphenol, which are typical substrates for laccases, and also possess the canonical four copper-binding domains. Nervertheless, the overall sequences of bacterial laccases show little similarity with fungal laccases. Therefore, they are often known as "multicopper oxidase" or "(poly) phenol oxidase", and their activity is usually defined as "laccase-like" (Solano et al., 2001). The first report on bacterial laccase was from the non-motile strain of *Azospirillum lipoferum* isolated from rice rhizosphere (Givaudan et al., 1993). This enzyme was identified as a laccase by using a combination of substrates and inhibitors (Givaudan et al., 1993; Diamantidis et al., 2000). Laccases activities were also found in *Bacillus sphaericus* (Claus and Filip,

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Abbreviations: RBBR, Remazol brilliant blue R; **LB,** Luria-Bertani; **PCR,** polymerase chain reaction; **BLAST,** basic local alignment search tool.

1997), *Escherichia coli* (Grass and Rensing, 2001), *Serratia marcescens* (Verma and Madamwar, 2003), *Bacillus halodurans* (Ruijssenaars and Hartmans, 2004) and *Streptomyces. psammoticus* (Niladevi and Prema, 2008).

CotA, which is the endospore coat component of *Bacillus subtilis*, is the most-studied bacterial laccase (Hullo et al., 2001). Since spores allow microorganisms to survive under drastic conditions, spore coat enzymes might also withstand high temperatures or extreme pH values. Since most fungal laccases are unstable at pH values higher than 7.0, their detoxification efficiencies for pollutants often decrease under alkaline conditions. This limits the industrial potential of fungal laccase as many processes are performed in alkaline conditions. Alternatively, spore laccases which are active in the alkaline pH range could be used for bioremediation or application in membrane reactors (Held et al., 2005).

Compared with fungal laccases, bacterial laccases have the advantages of being less sensitive towards halides and alkaline conditions as well as a fast growth rate (Verma and Madamwar, 2003; Jimenez-Juarez et al., 2005). Despite the importance of bacterial laccase in pollutant degradation, only a few novel bacterial strains displaying "laccase-like" activity have been discovered. The lack of a commercially available robust and inexpensive laccase is a major barrier to the widespread application of laccases in various industrial sectors (Dube et al., 2008). Since bacterial genetic tools and biotechnological processes are well established, developing bacterial laccases would be significantly important (Sharma et al., 2007). The present study was carried out to isolate and characterize the bacteria strain *Bacillus* sp. WD23 exhibiting laccase activity from forest soil. The spore laccase of this strain was characterized and used to decolorize several synthetic dyes.

MATERIALS AND METHODS

Sample collection

The soil samples were obtained from the forest of Liangshui National Nature Reserve in Heilongjiang, China (128° 53′ E and 47° 10′ N). Soil samples were collected in sterilized plastic bags from a depth of 5 cm below the earth's surface.

Enrichment and isolation of microorganisms

For enrichment of laccase-producing bacterial strains, the 250-ml flasks containing 100 ml M9 culture medium supplemented with 0.2 mmol/L Cu^{2+} were inoculated with 10 g of soil particles and incubated at 37°C on a rotary shaker (150 rpm) for 2 days. Then 5 ml cultures were transferred to 100 ml Luria-Bertani (LB) culture medium containing 0.2 mmol/L Cu^{2+} and incubated at 37°C at 150 rpm for 7 days. Stable enrichment cultures were obtained after subculture.

To isolate pure cultures, the enriched cultivated products were appropriately diluted with sterile saline solution (0.9% NaCl) before spreading onto LB/Cu^{2+} plates. The plates were incubated at 37°C for 3 days. Individual bacterial colonies from the plates were dropped with 0.1% (m/v) syringaldazine for checking laccase activity. The colonies displaying pink were streaked on new LB/Cu^{2+} plates for purification. Re-inoculation was performed after identification with syringaldazine as described above. The isolation process was repeated several times until the isolates were found to be pure.

Morphological and metabolic characterization

Gram staining was performed according to standard protocol. Gram's characteristics and cell morphology of the isolated strain were determined by microscopy. For carbon sources utilization, the pure cultures were inoculated respectively into peptone-water culture medium containing 1% substrates, and incubated at 37°C for 24 h. The results were determined by the variation of both the turbidity and the color of the culture medium. Control tubes contained uninoculated medium and were incubated at the same conditions.

Some biochemical metabolic ability was determined by inoculating isolates onto selective media such as casein, urea, balsam and starch agar, to identify protease, urase, lipase and amylase producers, respectively. All assays were carried out in triplicate.

Isolation of genomic DNA and 16S rDNA PCR amplification

Bacterial cells were collected by centrifugation at 12,000 rpm for 2 min and incubated with 100 µg/ml lysozyme at 37°C for 1 h followed by the treatment of lysis solution (1% SDS, 1 mmol/L EDTA, 20 mmol/L CH_3COONa and 40 mmol/L Tris-HCl, pH 8.0). After adding 5 mmol/L NaCl into the lysis solution, the mixture was extracted with phenol/chloroform/isoamyl alcohol (25:24:1). The supernatant was collected and then precipitated by absolute ethanol. The obtained genomic DNA was dissolved in sterile deionized water and stored at -20°C for further use.

For polymerase chain reaction (PCR), primers specific for eubacterial 16S rDNA 27F: 5'-GAGTTTGATCMTGGCTCAG-3' (M=A+C), 1492R: 5'-TACGGYTACCTTGTTACGACTT-3' (Y=C+T) were used (Wang et al., 2005). The PCR was run in a Gene Amp PCR system 9700 (Applied Biosystems, Singapore). The amplification reaction consisted of an initial denaturation at 93°C for 5 min, followed by 30 cycles of 94°C for 18 s, 56°C for 15 s and 72°C for 78 s, and a final extension step at 72°C for 7 min. The PCR products were analysed by electrophoresis in 1.0% (w/v) agarose gel and photographed using Bio Imaging System (Gene Genius, USA).

Amplification products were cloned using a commercially available pMD18-T vector cloning kit and transformed into competent *E. coli* JM109. Positive clones were identified by PCR amplification with above primer pairs.

Nucleotide sequencing, alignment, and phylogeny

16S rDNA sequencing of the isolated strain was done by Shanghai Sangon Biotechnology Company. Related sequences were obtained from the GenBank database using the Basic Local Alignment Search Tool (BLAST) search program. Multiple sequences alignment was performed using Clustal X 1.81. Phylipwx package was used to calculate similarity values and construct a phylogenetic tree.

Determination of optimal growth conditions

The optimal growth conditions with regard to pH and temperature were determined. The strain was inoculated in LB medium with varying pH values (4 - 11) and incubated at 15 - 55°C. The optical

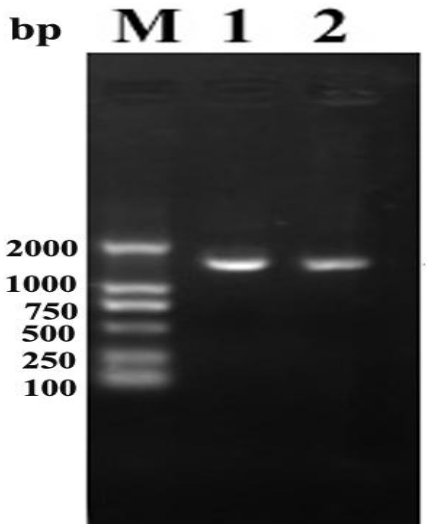

Figure 1. PCR products of 16S rDNA of *B. subtilis* strain WD23. Lane M: Molecular weight marker (DL2000); lanes 1 and 2: *B. subtilis* strain.

density of the growing cultures was observed at 600 nm using U-2800 spectrophotometer (Hitachi, Japan) to determine the optimum growth conditions. All assays were carried out in triplicate.

Effect of metals and saline solution on bacterial growth

To study the effect of metals on growth, 200 µg/ml Zn^{2+}, Fe^{3+}, Ca^{2+}, Mn^{2+}, Mg^{2+} or Cu^{2+} was supplemented in LB culture medium, respectively. Cultures were grown in 25 ml medium in 100 ml conical flasks at 37°C for 24 h. Culture grown in absence of metal was used as a control. Growth was determined by measuring the absorbance at 600 nm against blank.

The strain was inoculated in LB medium supplemented with 1, 2, 4, 6, 8, 10 or 12% NaCl. The turbidity of the growing cultures was observed at 600 nm using U-2800 spectrophotometer (Hitachi, Japan) to determine the growth status. All assays were carried out in triplicate.

The relationship of sporulation rate and laccase activity

B. subtilis WD23 was inoculated on LB plates containing 0.2 mmol/L Cu^{2+} and incubated at 30°C. The quantities of the spore were calculated every day and the laccase activity was determined at the same time. The sporulation rate was determined by the percentage of the quantities of spore opposite to all cells. The spores were removed from the agar with 1 mol/L KCl, washed with 0.5 mol/L NaCl, and re-suspended in 0.1 mol/L citrate–phosphate buffer (pH 6.8). The spore suspension was prepared for laccase activity determination. All assays were carried out in triplicate for each sample.

Assay of spore laccase activity

Spore laccase activity was determined at 40°C using syringaldazine (dissolved in absolute ethanol, Sigma) as the substrate. The oxidation of syringaldazine was detected by measuring the absorbance increase at 525 nm (ε_{525} = 65 $mmol^{-1}$ L cm^{-1}) after 3 min using a spectrophotometer (U-2800, Hitachi, Japan). The reaction mixture (3 ml) contained 100 µl of spore suspension (10 mg wet spores), 2.4 ml of citrate–phosphate buffer (0.1 mol/L, pH 6.8), and 0.5 ml of 0.5 mmol/L syringaldazine. 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 for each sample. Standard deviation did not exceed 5% of the average values.

Effect of pH and temperature on the activity and stability of spore laccase

Determination of the optimum pH was conducted in 0.1 mol/L citrate–phosphate buffer in the range of pH 4.0 – 8.0 using syringaldazine as the substrate. The optimum temperatures of the spore laccase were determined over the range of 0 – 100°C with syringaldazine as the substrate at their optimum pH values. All assays were carried out in triplicate.

Thermal stability of the spore laccase was determined by preincubation of 0.1 mol/L citrate-phosphate buffer (optimum pH) of spores at 60 and 80°C and the remaining activity was measured with the assay described above. The pH-stability was examined similarly at 30°C in different buffers ranging from pH 4.0 to 9.0. All assays were carried out in triplicate.

Determination of dye decolorization efficiency

The general dyes, remazol brilliant blue R (RBBR), alizarin red, congo red, methyl orange and methyl violet, were prepared individually with the concentration of 25 mg/L in sterilized distilled water. The prepared dye solution was supplemented with 100 g/L spores and incubated at 37°C under mild shaking conditions for 5 days. Dye samples without spores were given the same treatment as the control. The absorption spectrum of each dye between 200 and 800 nm was measured with a U-2800 spectrophotometer (Hitachi, Japan). Dye decolorization was assessed by the decrease in absorbance under the maximum wavelength of the dye. All assays were carried out in triplicate.

RESULTS

Isolation of bacterial strain with high laccase activity

Four hundred colonies were screened from cultures in M9 medium supplemented with 0.2 mmol/L Cu^{2+}. After secondary screening, 46 bacterial strains were picked out by color development reaction to syringaldazine. One of the potential strains showing a high level of laccase activity was named WD23 and selected for further studies. The strain, WD23, that formed pinkish colonies on LB agar plate, was a gram-positive, spore forming, rod shaped, 1 - 2 µm long, motile bacterium, and formed white colonies on LB agar supplemented 0.2 mmol/L Cu^{2+}. The optimum pH was 7.0 and the optimum temperature was observed at 25°C, though they were able to grow at temperatures ranging from 20 - 55°C. The obtained 16S rDNA PCR product was about 1.5 kb in length (Figure 1).

The morphological, physiological, biochemical characteristics (Table 1) and the comparative analysis of DNA sequence with available database showed that WD23 was close to the members of *B. subtilis*. The highest

Table 1. The morphological and biochemical characteristics of bacterial isolate (WD23).

Characteristics	*Bacillus subtilis* WD23
Colony diameter	1 – 3 mm
Colony color	White
Cell morphology	Rod
Motility	+
Gelatin hydrolysis	+
Urase	+
Lipase	–
Oxidase	+
Catalase	+
Casein protease	+
Amylase	+
NO_3^- reduction to NO_2^-	+
M-R reaction	–
V-P reaction	+
Utilization of:	
Mannite	+
Phaseomannite	+
Sorbierite	+
L-rhamnose	–
Melibiose	+
Lactose	–
Glucose	+
Maltose	+
Xylose	–
Sucrose	+
Gum sugar	–
Fructose	+

sequence similarity (100%) and phylogeny based on Clustal X indicated that the strain WD23 was *B. subtilis* (Figure 2). A 1513 nucleotides-long 16S rDNA sequence was submitted to the National Center for Biotechnology Information (NCBI) databases under the accession number EU780682.

Effect of metals and saline solution on bacterial growth

Metal cations Zn^{2+}, Fe^{3+}, Ca^{2+}, Mn^{2+}, Mg^{2+} and Cu^{2+} (200 µg/ml) all showed a certain extent of inhibition to the growth of the strain. Among them all, Zn^{2+} showed the highest degree of inhibition. The strain, WD23, showed strength of tolerance to 10% NaCl.

The relationship between sporulation rate and laccase activity

The positive correlation of laccase activity and sporulation percentage was observed during the 10 day's cultivation as shown in Figure 3. The result demonstrated that the laccase activity was derived from the spores.

Effect of pH and temperature on the activity and stability of spore laccase

The optimum pH of the spore laccase activity was 6.8 and the optimum temperature was observed at 60°C. The spore laccase exhibited a higher stability in high temperature and alkaline conditions than most fungal laccases. The temperature half-life of the laccase was 2.5 h at 80°C. The pH half life time of the spore laccase was 15 days at pH 9.0.

Efficiency of dye decolorization

In order to prove the potential application of this bacterium to the treatment of wastewater containing dyestuff, the spore laccase was used for the decolorization of RBBR, alizarin red, congo red, methyl orange and methyl violet. The decolorization rates were 90% in the treatments of RBBR and alizarin red, and 50 - 70% in the treatments of other dyes (Figure 4). These results indicated that the spore laccase could decolorize most dyes efficiently without additional redox mediators.

DISCUSSION

In the present study, a novel *B. subtilis* strain WD23 was isolated from forest soil. This strain was unable to utilize xylose and gum sugar, while the classical strain of *B. subtilis* does according to Bergey`s manual. Unlike other *B. subtilis* strains conserved in our laboratory which show little laccase activity, the strain WD23 exhibits high laccase activity. Therefore, strain WD23 is more valuable for further research.

Laccases as biocatalysts have received lots of attention because of their high capacity of oxidizing phenolic and other aromatic compounds. This advantage makes laccases suitable for some biotechnological applications, such as biodegradation of xenobiotic compounds including methoxyphenols, anilines, and benzenethiols (Solano et al., 1997; Xu, 1996). In contrast to fungal laccases, bacterial laccases are highly active and much more stable at high temperatures and high pH values. As stated above, most wastewaters from textile industries are characterized by a neutral to alkaline pH (around 7 – 11) (Manu and Chaudhari, 2002; Jahmeerbacus et al., 2004). For many industrial applications, it is necessary that the catalysts such as laccases are kept active in the whole process via immobilization or membrane reactors (Sharma et al., 2007). Spore laccase of *B. subtilis* WD23 has a high thermostability and a high stability at alkaline

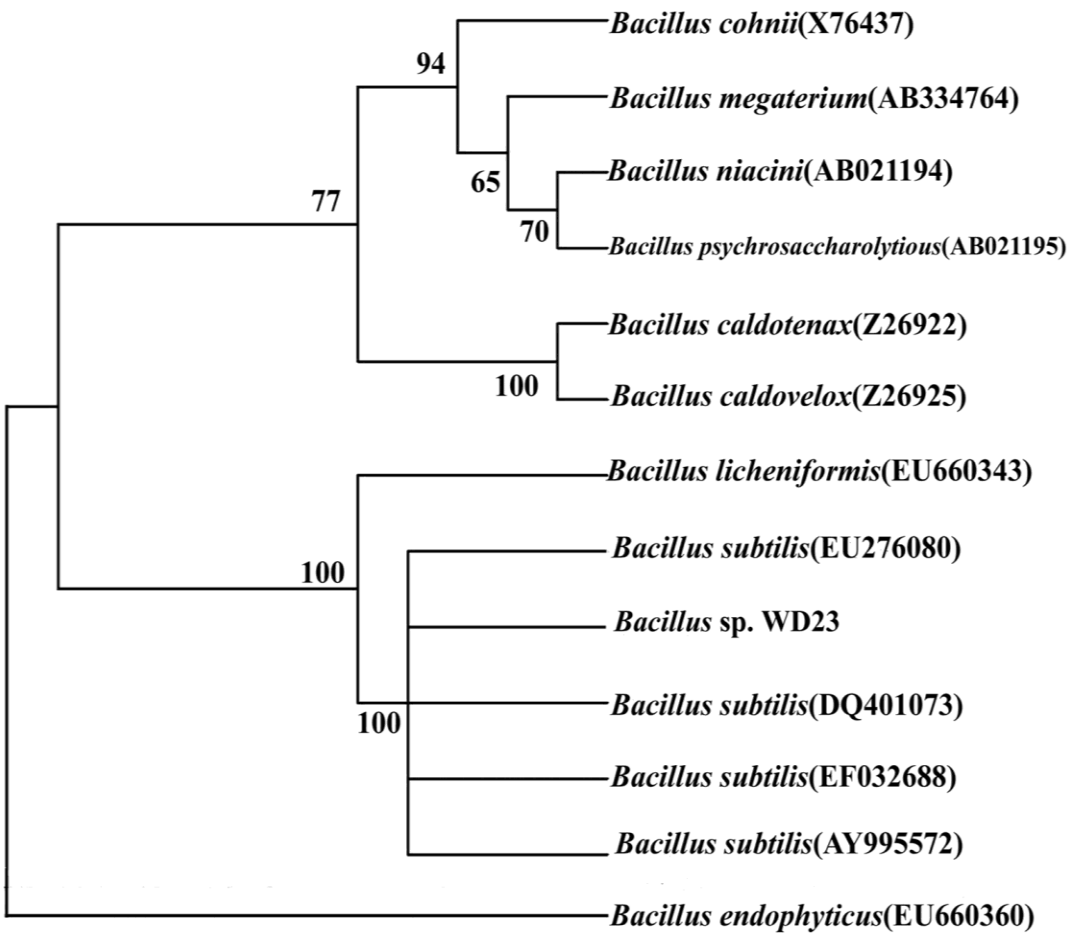

Figure 2. Phylogenetic analysis of 16S rDNA sequences of *B. subtilis* and related taxa.

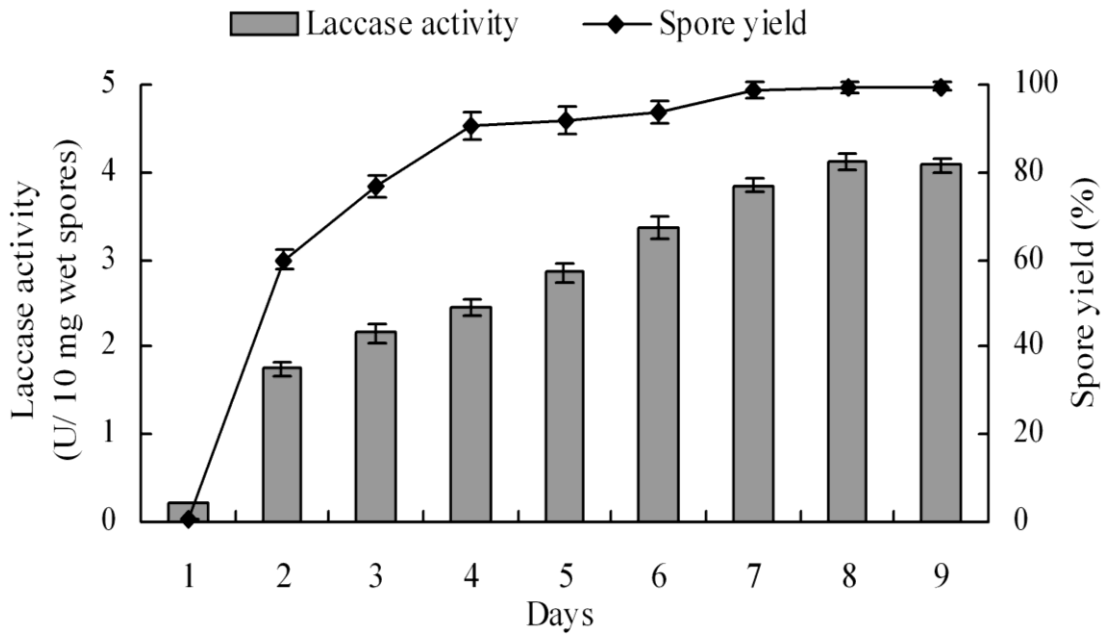

Figure 3. The relationship between sporulation rate and laccase activity of *B. subtilis* WD23.

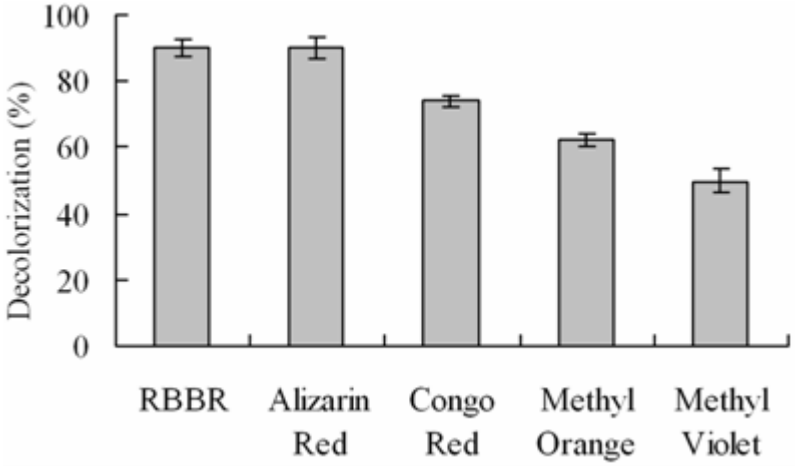

Figure 4. Decolorization of dyes with spore-bound laccase from *B. subtilis* WD23.

conditions. These characteristics of *B. subtilis* WD23 may be of significant importance for biotechnological applications.

Copper ions were added to the medium during enrichment culture. It is well known that copper ions are toxic even at low concentrations to lots of bacteria. However some bacterial laccases, such as CueO and PcoA play a role in copper tolerance (Rensing and Grass, 2003). The regulation of copper homeostasis of *E. coli* has been analyzed, although the mechanism is still unclear (Nakamura and Go, 2005). The main mechanism of CueO in copper resistance has been postulated to be the oxidation of the Cu^{+} to $Cu2^{+}$ (Singh et al., 2004). This process is effective for copper resistance because the Cu^{+} is more harmful than $Cu2^{+}$ (Outten et al., 2001). The present study is the first report that demonstrates copper resistance of spore protein. *B. subtilis* WD23 can survive in copper-containing medium. However, the strain WD23 is unable to form melanin-like pigment in the medium containing copper ions though CotA of *B. subtilis* was associated with the formation of a brownish pigment (Endo et al., 2002; Sanchez-Amat and Solano, 1997). The strain, WD23, also showed a strong endurance to high concentrations of NaCl; it can survive in 10% NaCl. This advantage makes it potentially useful to deal with wastewater containing saline solution and to reduce the time of pretreatment.

To date, bacterial laccases have only been found in *A. lipoferum*, *Alteromonas* sp. MMB-1, *Pseudomonas* sp. KU03, *E. coli*, and a few species of *Streptomyces* and *Bacillus*. There is limited information on methods of enrichment culture and sampling bacterial laccases from forest soil. In this study, soil samples were collected from the forest rich in deadwood and humus, which were the nutrients of bacteria possessing laccase activity. Other reports on the isolation of bacterial species showing laccase activity was carried out from rice rhizosphere (Givaudan et al., 1993), seawater (Solano et al., 1997), river sludge or top-soil containing organic litter (Ruijssenaars and Hartmans, 2004), soil contaminated with dye and textile industry effluent and lignocellulosic wastes (Senan and Abraham, 2004).

In our study, the spore laccase was used for the decolorization of anthraquinone and azo dyes without nutrition or redox mediators. Our results indicated that the spore laccase could decolorize the dyes efficiently within 5 days. This result is similar to that of the spore laccase from *Bacillus* SF (Held et al., 2005). However, few spore laccases could be reused, because it is difficult to separate spore laccase from the decolorized solution. The limitation reduces their utilization rate. Immobilized enzyme has high efficiency in dye decolorization, because immobilization can improve the utilization rate of enzyme despite the reducing enzyme activity. Our next efforts are to immobilize the spore laccases. The results demonstrate that spore laccase has potential application in dyestuff treatment.

In summary, *B. subtilis* WD23 exhibiting laccase activity was screened from forest soil and is well characterized. The strain showed activity of catalyzing canonical laccase substrates, syringaldazine, good growth state at 55°C, and its spore laccase could decolorize the most regular dyes efficiently without additional redox mediators.

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