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Full Length Research Paper

Characterization of spore laccase from *Bacillus subtilis* WD23 and its use in dye decolorization

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A bacterial strain, designated as WD23, was isolated from the forest soil using M9 medium supplemented with 0.4 mmol/L Cu^{2+}. The spores from this strain showed laccase-like activity, oxidizing syringaldazine, 2,6-dimethoxyphenol and 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonate acid) (ABTS). The strain was identified as *Bacillus subtilis* based on its morphological and physiological properties, and 16S rDNA sequence analysis. The optimum pH and temperature for the spore-bound laccase were 6.8 and 60°C, respectively. The temperature half-life of the laccase was 2.5 h at 80°C and 68 h at 60°C. It also showed high stabilities over a broad pH range. The pH half-life of the laccase was more than 6 months at pH 6.8. The sensitivities of the laccase to organic solvents and inhibitors were tested and it was found that laccase activity was strongly inhibited by methanol, EDTA and dithiothreitol, but only slightly affected by NaN_3 and xylene. The spore enzyme efficiently decolorized anthraquinone and azo dyes in 24 h.

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

INTRODUCTION

Laccases (EC 1.10.3.2; benzenediol: oxygen oxido-reductase) are a family of multicopper oxidases that require O_2 to oxidize organic compounds, particularly phenols (Piontek et al., 2002) and different non-phenolic substrates by one-electron transfer, resulting in the formation of reactive radicals, while reducing molecular oxygen to water. The enzyme possesses great biotechnological potential because of its wide reaction capabilities as well as broad substrate specificity. The laccases may be applied in drug analysis, clarification of juices and wines, biobleaching of kraft pulp (Srebotnik and Hammel, 2000), decolorization of synthetic dyes (Baldrian, 2006), organic synthesis (Pilz et al., 2003), laundry cleaning (Gouka et al., 2001), bioremediation (Mayer and Staples, 2002) and biosensors (Vianello et al., 2006).

The first laccase was reported in 1883 from *Rhus vernicifera*, the Japanese lacquer tree, from which the designation of laccase was derived and subsequently, laccases were discovered largely in plants and fungi (Mayer and Staples, 2002). However, bacterial laccase was first reported in 1993 (Givaudan et al., 1993). Since that time, more and more studies have shown that laccases are widespread among bacteria, based on homology searches in protein databases and bacterial genomes (Alexandre and Zhulin, 2000) and experimental data. Actual laccase-like activity has been found in *Escherichia coli* (Grass and Rensing, 2001; Kim et al., 2001), *Streptomycetes* (Endo et al., 2002; Suzuki et al., 2003), *Azospirillum lipoferum* (Diamantidis et al., 2000), *Marinomonas mediterranea* (Sanchez-Amat et al., 2001), *Bacillus sphaericus* (Claus and Filip, 1997) and *Bacillus subtilis* (Hullo et al., 2001; Martins et al., 2002). Bacterial laccases contain the canonical four copper-binding domains, but show little overall sequence similarities with fungal laccases. Also, the kinetics and reaction conditions may differ from those of classical laccases. The oxidation rate may be slow, Cu^{2+} supplementation may be required, and the enzyme may be inactivated during turnover (Solano et al., 2001). CotA, which is the endospore coat component of *Bacillus subtilis*, is the most-studied bacterial laccase (Hullo et al., 2001). The spore laccases from *Bacillus* SF, which are active in the alkaline pH

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range, could be used for bioremediation or application in membrane reactors (Held et al., 2005).

In recent years, close attention has been paid on studying classical laccase producing organisms, however, little work has been done on bacterial laccases with respect to industrial applications. In this work, the use of a spore laccase from *B. subtilis* for dye decolorization was reported. The spore-bound laccase, which has a much higher thermostability and pH-stability than fungal laccases, may have advantageous properties when compared to classical laccases in industrial applications.

MATERIALS AND METHODS

Chemicals

Syringaldazine, 2,6-dimethoxyphenol and ABTS were purchased from Sigma (St. Louis, MO, USA). All other chemicals were from Guangfu (Tianjin, China) and were of analytical grade.

Isolation and characterization of the organism

Soil samples were collected from Liangshui National Nature Reserve in Heilongjiang, China (128° 53′ E and 47° 10′ N). The samples (10 g) were added to 100 ml M9 and $CuSO_4 \cdot 5H_2O$ (0.01%) in Erlenmeyer flasks. To isolate various strains, 1 ml aliquots were added to 9 ml sterile water and a serial dilution (10^{-1} to 10^{-9}) were prepared. About 0.1 ml of each dilution was distributed on LB plates that contained 0.01 mmol/L guaiacol and 5 mmol/L α-naphthol (screening medium) incubated at 37°C for 24 to 48 h, and 46 colonies which showed color change were chosen and purified.

Laccase-like production of isolated strains was tested by dropping syringaldazine (in ethanol), 2,6-dimethoxyphenol and ABTS on the screening medium (Claus, 2003). A forest soil bacterium was selected as the best potential producer of laccase.

Preparation of spore suspension

On solid agar media plus 0.4 mmol/L Cu^{2+}, the sporulation rate of the isolated strain was more than 90% with incubation at 37°C after 7 days. The spores were removed from the agar plate with 1 mol/L KCl, washed with 0.5 mol/L NaCl, and suspended in 0.1 mmol/L citrate-phosphate buffer (pH 6.8) or 0.1 mol/L sodium phosphate buffer (pH 6.8) (Hirose et al., 2003). Finally, 1 ml spore suspension contained 100 mg wet cell.

Laccase assay

Laccase activity of the spore suspension was determined at 30°C using syringaldazine as the substrate. The oxidation of syringaldazine was detected by measuring the absorbance increase at 525 nm (ε_{525} = 65,000 L/(mol ·cm)) using a spectrophotometer (U-2800, Hitachi, Japan). The reaction mixture (3 ml) contained 100 µl of spore suspension sample, 2.4 ml of citrate-phosphate buffer (0.1 mol/L, pH 6.8) and 500 µl of 0.5 mmol/L syringaldazine. Spore sedimentation was not observed during incubation. One unit of enzyme activities 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. The standard deviation did not exceed 5% of the average values.

Effect of pH and temperature on laccase activity and stability

The effect of pH on laccase activity was determined within a pH range of 4.0 and 8.0 using syringaldazine as the substrate in 0.1 mol/L citrate-phosphate buffer. Optimum temperature of the spore laccase was examined over the temperature range of 0 and 100°C with syringaldazine as the substrate at its optimal pH value.

The thermal stability of the laccase was examined by preincubation of 0.1 mol/L citrate-phosphate buffer (optimum pH) of spores at various temperatures and measuring the remaining activity as described earlier. The pH-stability was determined similarly by incubating the spores at 30°C in different buffers ranging from pH 4.0 to 9.0. All assays were carried out in triplicate.

The effects of organic solvents and inhibitors on laccase activity

The effects of organic solvents and potential inhibitors on the laccase were investigated with 1 mmol/L syringaldazine as the substrate in 0.1 mol/L citrate-phosphate buffer at optimum pH.

10 ml of petroleum ether, xylene, aether, chloroform, acetone, ethyl acetate, formaldehyde and methanol were added to 10 ml spore suspension in Erlenmeyer flasks, respectively, and mixed for 30 min. The laccase activities were determined after removing the organic solvents by incubation in a hot water bath at 50°C. The effects of L-cysteine, sodium azide, dithiothreitol (DTT) and EDTA on laccase activity were determined after 3 min of incubation of the enzyme with the various inhibitors at 25°C. All experiments were carried out in triplicate.

Resistance of the strain to ultraviolet C radiation and H_2O_2

The spores of *B. subtilis* WD23 were washed with saline after grew on LB plate for 7 days and the suspension was adjusted to 10^6 CFU/ml and was spread on LB and LB plus Cu^{2+} plates, respectively (Hullo et al., 2001). Subsequently, the strains were irradiated with ultraviolet C radiation (UVC) source illuminator (HD-1360, HDL, China), and survival was determined according to the procedure used by Saxena and coworkers (2002). Percent survival was determined by comparing the number of colonies on irradiated plates to those on non-irradiated plates. For H_2O_2 assays, 10^6 CFU/ml were suspended in 1 ml of 0.1 mol/L citrate-phosphate buffer (pH 6.8) containing 5 mmol/L H_2O_2. At 10 min intervals, aliquots (0.1 ml) were taken and plated on LB and LB plus Cu^{2+} plates (Lee et al., 1995).

Dye decolorization by laccase

The decolorization of Remazol brilliant blue R (RBBR), Alizarin red, Congo red, methyl orange and methyl violet were investigated by the spore laccase. Stock solutions of the dyes were prepared in sterilized distilled water and diluted to 25 mg/L. The reactions were initiated with wet spore suspensions (100 g/L) and incubated at 37°C under mild shaking conditions. Control samples were done in parallel without spores under identical conditions. All measurements were done in triplicate.

The absorption spectrum of dye between 200 and 800 nm was measured with a U-2800 spectrophotometer (Hitachi, Japan). The effect of dye decolorization was determined by the decrease in absorbance under the maximum wavelength of the dye, respectively. The efficiency of decolorization was expressed in terms of percentage.

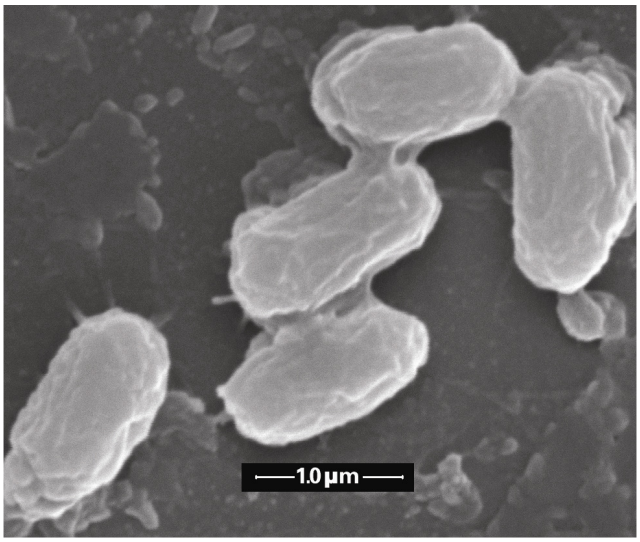

Figure 1. Scanning electron micrograph of spores from *B. subtilis* WD23.

RESULTS

Identification of microorganism and 16S rDNA analysis

All 46 strains were screened for laccase-like activity and the strain WD23 was selected as the best potential producer of laccase. The strain WD23 was gram-positive, aerobic, motile, sporulating and straight rod-shaped organisms. Sporidia were swollen and centralterminal (Figure 1). The cell of WD23 was found to have numerous flagella and was actively motile. The cells of WD23 were encapsulated by the observation of the transmission electron microscope (TEM). The optimum pH for growth was pH 7.0 to 9.0. The strain was able to grow in media containing different concentrations of NaCl up to 10% (w/v), which indicated that it was alkali-tolerant and halotolerant. The growing temperature range was from 25 to 55°C. The optimum growth temperature was around 37°C. Red pigmentation was secreted to solid media by the strain after incubation at 37°C for 72 h. The sequenced 16S rDNA of the strain was 1513 bp in length. There was 99.9% sequence similarity between the strain WD23 and *B. subtilis* according to GenBank database. The strain was named *B. subtilis* WD23 based on morphological and physiological properties, and 16S rDNA sequence analysis. The GenBank accession number for 16S rDNA sequence of the strain was EU780682.

Laccase activity

The activity of the spore-bound laccase clearly correlated well with the spore count and was dependent on the age of the spore. The maximum activity was 0.96 U/mg wet weight of spores achieved after 7 days of growth on solid agar media. The spore-bound laccase could be reused and the residual activity remained about 50% after 5 consecutive cycles.

Effect of pH and temperature on laccase activity and stability

The pH profile for laccase activity against syringaldazine showed a peak of maximum activity at pH 6.8. The optimum temperature of the spore-bound laccase was determined at pH 6.8, and the maximum activity was observed at 60°C. It showed higher activity within a temperature range from 40 to 70°C. Therefore, laccase activity was observed at 0 and 100°C (Figure 2).

The spore-bound laccase exhibited high thermal and pH-stability. The temperature half-life of the laccase was 2.5 h at 80°C and the laccase activity disappeared after 18 h. In addition, the laccase had a high stability at the optimum temperature ($t_{1/2}$ = 68 h at 60 °C). The pH half-life of the spore-bound laccase was more than 6 months at pH 6.8. The laccase activity of strain WD23 showed higher stability over a broad pH range. Within a pH range from 5.0 to 7.0, the half-life was more than 240 h. In contrast to fungal laccases, spore-bound laccase activity of *B. subtilis* WD23 showed a very high stability at alkaline pH values ($t_{1/2}$ = 15 d at pH 9.0).

The effects of organic solvents and inhibitors on laccase activity

The effects of several organic solvents and putative inhibitors on laccase activity were tested with syringaldazine as the substrate (Table 1). Methanol and formaldehyde strongly inhibited the spore-bound laccase activity, and the activity also was inhibited by 0.1 mmol/L EDTA, as well as by 1 mmol/L L-cysteine, sodium azide and DTT. 0.1 mmol/L DTT, NaN_3 and other organic solvents only showed a slight inhibitory effect.

Resistance of the strain to UVC light and H_2O_2

The UVC band was susceptible to being absorbed by the DNA of organisms, so the UVC radiation played the sterilization among UV radiation; particularly the wave length around 253.7 nm was the best. UVC light exposure indicated that the LD_{50} was 3.6 KJ/m^2 for strain WD23 on LB medium and 5.4 KJ/ m^2 for the strain on LB medium plus Cu^{2+}. The LD_{50} with H_2O_2 on LB medium was 30 and 40 min on LB medium plus Cu^{2+} (Figure 3).

Dye decolorization experiments

The spore laccase was used for the decolorization of

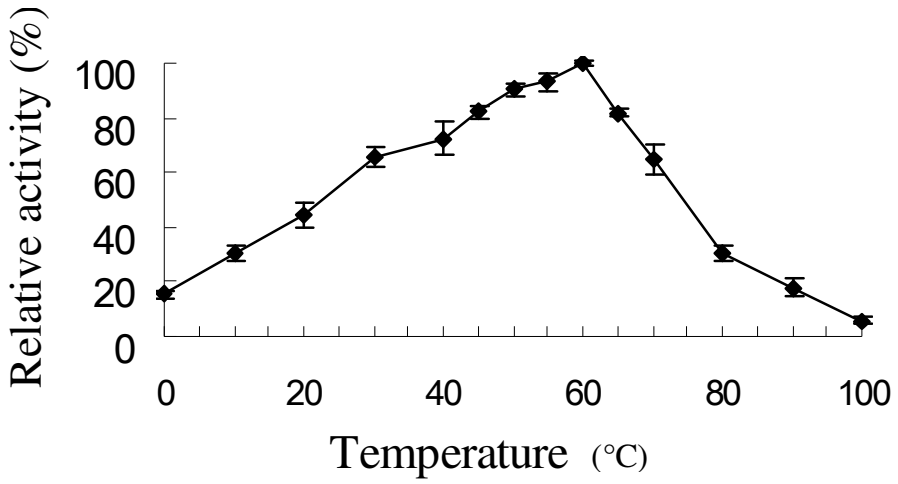

Figure 2. Effect of temperature on spore-bound laccase activity.

Table 1. Effects of organic solvents and inhibitors on laccase activity.

Organic solvent	Relative activity (%)	Inhibitor	Concentration (mmol/L)	Relative activity (%)
None	100	None	-	100
Petroleum Ether	96.55	Dithiothreitol	0.1	94.84
Xylene	94.28		1	0
Aether	91.05	NaN_3	0.1	77.52
Acetone	84.93		1	23.57
Chloroform	83.05	L-Cysteine	0.1	60.39
Ethyl Acetate	81.98		1	22.62
Formaldehyde	15.83	EDTA	0.1	34.72
Methanol	2.53		1	2.72

RBBR, Alizarin red, Congo red, methyl orange and methyl violet in order to demonstrate their potential in the treatment of dyestuff wastewater. 90% of the RBBR and Alizarin red were removed in the first day, while the final percentage of degradation of other dyes was more than 50% (Figure 4). The spore-bound laccase could efficiently decolorize the dyes without additional redox mediators.

DISCUSSION

Laccase-like activity was detected in a thermoalkaliphilic bacterial strain, which was isolated from forest soil. Based on the morphological, physiological and biochemical characteristics and phylogenetic analysis, the strain was defined as *B. subtili* WD23. To date, there are only few reports on thermoalkaliphilic *Bacillus* species, such as *Bacillus* sp. TAR-1 (Takahashi et al., 2000), *B. subtilis* (Martins et al., 2002), *Bacillus thermoalcaliphilus* (Sarkar and Upadhyay, 1993) and *Bacillus* SF (Gudelj et al., 2001; Held et al., 2005).

Polyphenols, methoxy-substituted phenols, diamines and a considerable range of other compounds can be used as substrates for laccase, but syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde azine) has been considered as the laccase-specific substrate. In this study, syringaldazine was used as the oxidation substrate, and it was found that laccase activity correlated closely with spore formation, which was the same as the laccases from *Bacillus sphaericus* (Claus and Filip, 1997) and *Bacillus* SF (Held et al., 2005).

The spore-bound laccase exhibited a higher thermal stability and pH-stabilitiy. The temperature half-life was 2.5 h at 80°C. The value was about 5 times higher than that of other spore-bound laccases reported at the same temperature and optimum pH values (Held et al., 2005). The pH half-life time was more than 6 months at pH 6.8. Within a pH range from 5.0 to 7.0, the half-life of the spore-bound laccase was more than 240 h, while the half-life of a laccase from *Trametes hirsute* was 13 h (Abadulla et al., 2000). In contrast to fungal laccases, the spore-bound laccase activity of *B. subtilis* WD23 showed a very high stability at alkaline pH values ($t_{1/2}$ = 15 d at pH 9.0). The observation of half-life time indicated that the spore-bound laccase had a high potential in industrial processes where high temperatures and pH values are

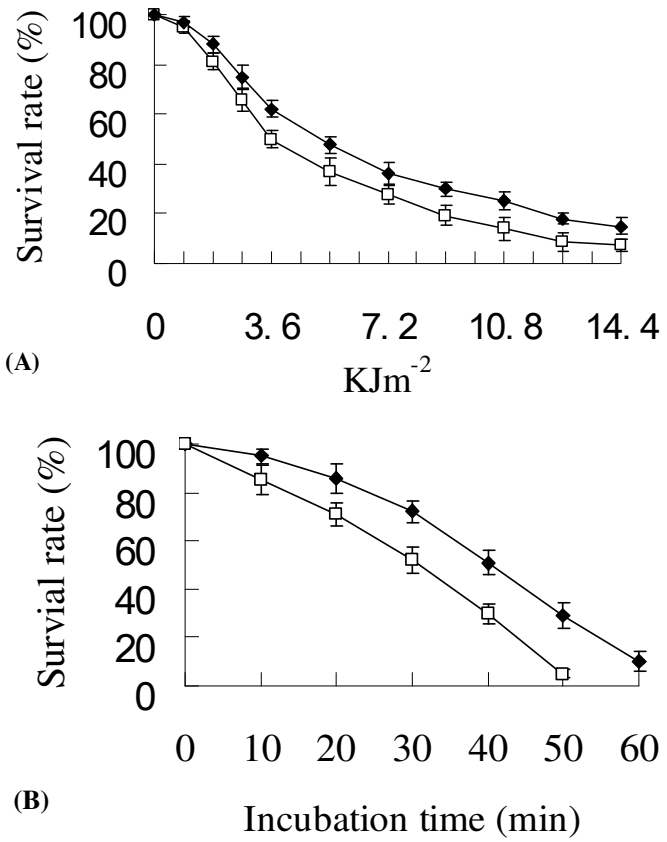

Figure 3. (A) UVC light resistance of *B. subtilis* WD23; (B) H_2O_2 resistance of *B. subtilis* WD23; *B. subtilis* WD23 grown on LB medium without $CuSO_4$ supplementation; □, *B. subtilis* WD23 grown on LB medium plus 0.4 mmol/L $CuSO_4$. Each experiment was done with three different batches of spores.

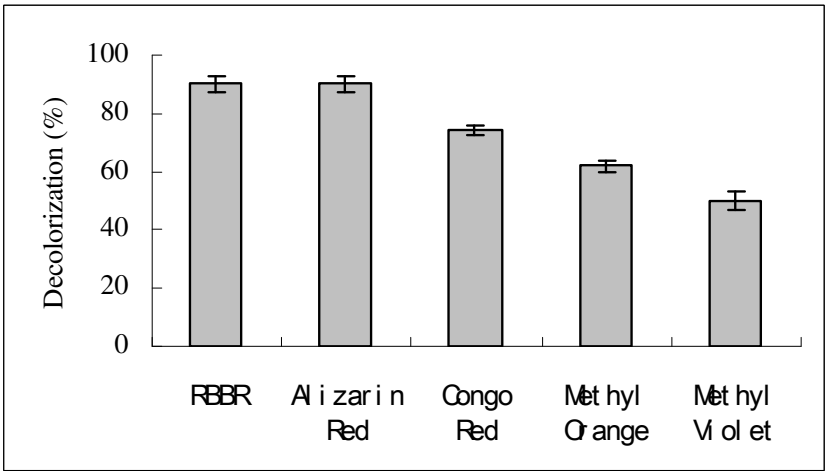

Figure 4. Decolorization of dyes with spore-bound laccase from *B. subtilis* WD23.

common, such as dye decolorization, detoxification and transformation of phenolic and other compounds.

The metal ion chelator EDTA was an efficient inhibitor of the spore-bound laccase. The result was different from

the fungal laccase of *Pycnoporus sanguineus* (Lu et al., 2007). EDTA could deprive copper from the spore laccase which was copper-dependent (Hullo et al., 2001). Similarly, sodium azide, L-cysteine and DTT demonstrated strong inhibition towards the laccase activity and those laccases from *Lentinus edodes* (Nagai et al., 2002) and *Daedalea quercina* (Baldrian, 2004). However, the spore laccase coexisted with many organic solvents. Under the treatment of UVC and H_2O_2, the growth of the strain on LB+Cu^{2+} plate was better than that on LB plate. This suggested a possible correlation between spore formation and Cu^{2+} as well as resistance against UV and H_2O_2 (Riesenman and Nicholson, 2000).

In previous studies, higher decolorization rates were reported for the laccases from a number of fungi, such as *Sclerotium rolfsii*, *Trametes modesta*, *Pleurotus pulmonarius* and *Pycnoporus sanguineus* (Lu et al., 2007). The process of dye decolorization based on laccase was an efficient method and attracted increasing interest (Couto and Herrera, 2006). However, the potential of bacterial laccases for this purpose has rarely been addressed, and no decolorization activity was observed at pH values higher than pH 7 (Kandelbauer et al., 2004). This limited the industrial potential of bacterial laccase since many dyeing processes (such as cotton) were performed in the alkaline pH range. Alternatively, spore laccases which were also active in the alkaline pH range could be used for dye decolorization and did not need to be liberated from the spores. Anthraquinone-based dyes are difficult to decolorization due to their com-plicated aromatic ring structures (Fu and Viraraghavan, 2001). In this study, anthraquinone and azo dyes were used for dye decolorization. Without nutrition and redox mediators, results indicated that the spore laccase was very efficient in the removal of color in less than 5 days of incubation time, which was similar to the spore laccase from *Bacillus* SF (Held et al., 2005).

In terms of resistance to extreme environmental stresses, the bacterial spore represents a pinnacle of evolution. Spores are highly resistant to a wide variety of physical stresses such as wetness, dry heat, UV, gamma radiation, oxidizing agents, chemicals, extremes of both vacuum and hydrostatic pressure. Compared with fungal laccases, spore-bound laccases have no glycosylation and have higher thermal stability and enzyme activity in alkaline pH. The spores could retain their laccase activity in wastewater with no need for the addition of nutritional substances. Due to these unusual properties, the spore-bound laccase from *B. subtilis* WD23 is of significant importance in industrial applications.

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