

Short Communication

Isolation and characterization of *Shewanella oneidensis* WL-7 capable of decolorizing azo dye Reactive Black 5

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Azo dyes used widely in textile, paper, leather, cosmetics, and food industries are generally xenobiotic compounds which are rather recalcitrant to conventional sewage treatment systems (Banat et al., 1996). Since most of them have mutagenic or carcinogenic effects, industrial effluent containing azo dyes must be treated before it is discharged into the environment. The biological processes by microorganisms have received considerable attention for the treatment of dye-containing wastewater, because they are cost-effective and environmentally friendly, and don't produce large amount of sludge, compared with physical and chemical methods (Banat et al., 1996).

To date, many bacterial strains capable of decolorizing azo dyes have been reported and their characteristics have been reviewed (Banat et al., 1996; Stolz, 2001). The bacterial metabolism of azo dyes is initiated in most cases by a reductive cleavage of the azo bond, which results in the formation of colorless amines (Stolz, 2001). Bacterial degradation of azo dyes is generally considered a specific reaction by azore-

ductase under aerobic conditions or a nonspecific reduction process mediated by redox mediators shuttling electrons from bacteria to azo dyes under anaerobic conditions (Hong et al., 2007; Stolz, 2001).

Very recently, it has been reported that some *Shewanella* species, such as *Shewanella* strain J18 143 (Pearce et al., 2006), *S. decolorationis* S12 (Xu et al., 2006) and *S. putrefaciens* AS96 (Khalid et al., 2008), were efficiently able to decolorize azo dyes and they showed higher decolorization ability under anaerobic than aerobic conditions. Although biodegradation mechanisms of azo dye by *S. decolorationis* S12 have been studied (Hong et al., 2007; Xu et al., 2007a, b), the enzyme involved in decolorization of azo dyes by *Shewanella* strains is unknown. In this paper, we report the isolation and characterization of a new potent bacterium, *S. oneidensis* WL-7 capable of efficiently decolorizing azo dyes including Reactive Black 5 (RB-5). Enzymatic activities responsible for dye decolorization were first investigated. This is the first report showing the presence of laccase and NADH-2,6-dichlorophenol indophenol (DCIP) reductase activities that might be involved in azo dye decolorization by a *Shewanella* strain.

Activated sludge was collected from the effluent treatment plant of a textile and dyeing company in Busan, South Korea, and screened for dye decoloriza-

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tion microorganisms. The screening of the strains for dye decolorization was firstly carried out on NM9 medium (beef extract, 0.3 g/L; peptone, 0.5 g/L; Na_2HPO_4 , 6 g/L; KH_2PO_4 , 3 g/L; NH_4Cl , 1 g/L; NaCl , 0.5 g/L, pH 7.0) and then performed on Luria-Bertani (LB) agar plates (Tryptone, 10 g/L; yeast extract, 5 g/L; NaCl , 10 g/L; agar, 1.2 g/L, pH 7.0) containing $100 \mu\text{M}$ of RB-5. Microorganisms were selected on the basis of clear zone on the agar plates. The strains growing on the plates and decolorizing the dye were selected and grown in the same liquid medium. A bacterial strain with high RB-5 decolorization ability was identified by carbon source utilization patterns (Konopka et al., 1998) using Biolog GN2 microplates (Biolog, CA, USA) and analysis of 16S rDNA sequences (positions 27–1492) as described previously (Pearce et al., 2006).

Four bacterial strains capable of decolorizing RB-5 were isolated and among them, one strain, WL-7, which had the highest decolorization ability against RB-5, was selected for further study. This isolate was a motile gram-negative, straight rod, and physiological tests indicated that the strain WL-7 was a member of the genus *S. oneidensis*. Moreover, the phylogenetic analysis of strain WL-7 using its 16S rDNA sequence data showed this strain had the highest homology (98%) with *S. oneidensis* (AF387347). Therefore, this strain was named *S. oneidensis* WL-7.

To determine the effect of aeration on the decolorization of RB-5, the cells were aerobically grown at 35°C for 15 h in LB medium. Precultured cells were inoculated at 1% (v/v) into 500 ml flasks containing 100 ml LB medium with RB-5 ($50 \mu\text{M}$) and cultured at 35°C in rotary shakers running at 200 rpm (shaking condition) or without shaking (static condition). After incubation for 12 h, the cells were centrifuged at $10,000 \times g$ for 20 min and the precipitated cells, after being washed twice with sterile water, were dried at 80°C for 24 h to measure dry weight of the cells. The supernatants were used as samples for decolorization assay. Decolorization assay was performed using a scanning spectrophotometer (Ultrospec 3000 UV/VIS, Pharmacia, Sweden) and expressed in terms of the percentage decolorization by the same method as described previously (An et al., 2002). All experiments were conducted in triplicate. As shown in Fig. 1, *S. oneidensis* WL-7 showed a higher dye decolorization under static conditions although cell growth was comparatively faster under shaking conditions. This is consistent with the recent reports describing decoloriza-

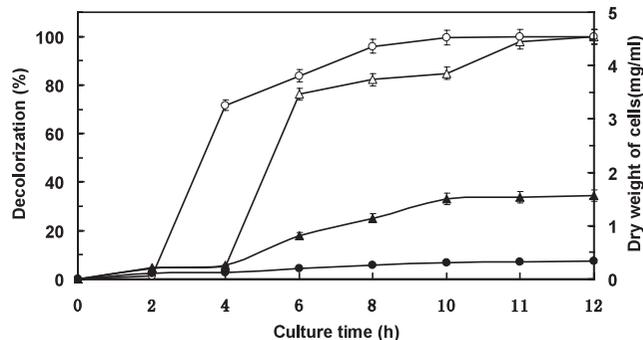


Fig. 1. Effect of aeration on the cell growth and the decolorization of RB-5 ($50 \mu\text{M}$).

S. oneidensis WL-7 was incubated at 35°C under shaking conditions (\triangle , \blacktriangle) or under static conditions (\circ , \bullet). The cell growth and the decolorization are shown as closed and open symbols, respectively. Each point represents mean \pm SD of triplicates.

tion of azo dye by *S. decolorationis* S12 (Xu et al., 2006), *Shewanella* strain J18 143 (Pearce et al., 2006), and *S. putrefaciens* AS96 (Khalid et al., 2008).

To investigate the effects of temperature, pH and carbon or nitrogen sources on the decolorization of RB-5, precultured cells were inoculated at 1% (v/v) into 500 ml flasks containing 100 ml LB medium with RB-5 ($50 \mu\text{M}$), and incubated at various temperatures or in the same medium adjusted to different pH values under static conditions. The cultures were also incubated under static conditions in M9 medium (Na_2HPO_4 , 6 g/L; KH_2PO_4 , 3 g/L; NH_4Cl , 1 g/L; NaCl , 0.5 g/L, pH 7.0) containing various carbon or nitrogen sources at 35°C for 48 h. At temperatures of 30 – 35°C , the decolorization rate was two times faster than that at a range of 20 – 25°C and the optimal pH for decolorization ranged from 6 to 8. *S. oneidensis* WL-7 showed the most effective decolorization rate in the presence of yeast extract or peptone, like *S. putrefaciens* AS96 (Khalid et al., 2008), while glucose resulted in lower decolorization activity, like *S. decolorationis* S12 (Xu et al., 2006) (data not shown).

To investigate the decolorization of various azo dyes by *S. oneidensis* WL-7, precultured cells were inoculated at 1% (v/v) into 500 ml flasks containing 100 ml LB medium with appropriate concentrations of dyes and cultured at 35°C for 12 h under static conditions. Using culture supernatants after centrifugation at $10,000 \times g$ for 20 min, decolorization of dyes was determined by monitoring the decrease in absorbance at the maximum wavelength of each dye. The results are shown in Table 1. The cell mass of *S. oneidensis* WL-7

Table 1. Decolorization of azo dyes by the *S. oneidensis* WL-7 under static conditions.

Dye conc. (μM)	Decolorization (%) after 12 h ^a				
	RB-5	MR	CR	RB-19	CV
50	100	100	100	36	8
100	95	97	98	32	13
200	86	95	97	26	13
300	73	95	93	25	12
500	36	93	76	21	10

RB-5, Reactive Black 5; MR, Methyl Red; CR, Congo Red; RB-19, Reactive Blue 19; CV, Crystal Violet; ND, not decolorized.

^aThe variation in decolorization between three replicas was 0.01 to 0.18%. Dry cell weight after 12 h of growth under static conditions was almost similar about 0.48–0.52 mg/ml and the variation in dry cell weight of triplicates was 0.01 to 0.05 mg/ml.

cultures grown for 12 h after addition of each dye was almost similar about 0.48–0.52 mg/ml (dry weight). In the case of azo dyes tested, *S. oneidensis* WL-7 showed the highest decolorization capability against Methyl Red (mono azo group): even at 500 μM , more than 90% of color was removed in 12 h. In addition, *S. oneidensis* WL-7 was able to decolorize Congo Red and RB-5 (diazo group) by more than 93% and 73%, respectively, at 300 μM . Khalid et al. (2008) reported that *S. putrefaciens* AS96 completely decolorized 100 μM of RB-5 within 6 h under static conditions at 35°C. On the other hand, only 36% of Reactive Blue 19 (anthraquinone group) was removed at 50 μM and less than 10% of Crystal Violet (triphenylmethane group) was decolorized at the same concentration. This may be due to differences in the chemical structure of the dyes, as reported in other bacteria (An et al., 2002; Banat et al., 1996; Stolz, 2001).

Decolorization of the dye solution may take place in two ways, either adsorption on the microbial biomass or biodegradation of the dyes by the cells (Zhou and Zimmermann, 1993). Dye adsorption may be evident from inspection of the bacterial growth; those adsorbing dyes will be deeply colored, whereas those causing degradation will remain colorless. While the *S. oneidensis* WL-7 cells cultured under static conditions for 12 h with Reactive Blue 19 and Crystal Violet were dark blue in color, none was found to be colored with any one of the other dyes tested after decolorization. This result indicates that decolorization of these 2 dyes was mainly due to adsorption to cells. UV-visible spectral analyses showed the shifts in λ_{max} values of RB-5 (597 nm), Methyl Red (415 nm), and Congo Red (507 nm) to the UV region with lower wavelengths,

with prolonged incubation times (data not shown), suggesting *S. oneidensis* WL-7 effectively removed the colors of these dyes through biodegradation, as reported previously (Pearce et al., 2006).

Previous studies on bacterial decolorization of azo dyes have primarily focused on the biodegradation of dyes by azoreductase, which catalyzes reductive cleavage of azo bonds ($-\text{N}=\text{N}-$) (Banat et al., 1996; Stolz, 2001). Very recently, several reports have shown that oxidative enzymes (lignin peroxidase and laccase), tyrosinase and NADH-DCIP reductase are responsible for dye decolorization in bacteria (Dawkar et al., 2008; Kalme et al., 2007; Kalyani et al., 2008; Parshetti et al., 2006). To investigate whether these enzymes are responsible for the decolorization of RB-5 by *S. oneidensis* WL-7, the cells were grown at 35°C under aerobic conditions until the optical density at 600 nm was approximately 0.6, after which RB-5 (50 μM) was added to the culture medium. After cultivation for a further 5 h at 35°C under static conditions, cells collected from LB medium without and with RB-5 (50 μM) were used as the control and induced samples, respectively. After centrifugation at $10,000 \times g$ for 20 min, the supernatants were used as the extracellular enzyme sources. The harvested cells were suspended in 0.1 M potassium phosphate buffer (pH 7.4) and disrupted by ultrasonication. The debris from the disrupted cells was removed by centrifugation at $10,000 \times g$ for 10 min and the cell-free extract containing intracellular enzymes, together with extracellular enzyme sources, were used for enzyme assay.

Lignin peroxidase activity was determined by monitoring the formation of propanaldehyde at 300 nm containing 100 mM 1-propanol, 250 mM tartaric acid,

and 10 mM H₂O₂ in 2.5 ml reaction mixture (Shanmugam et al., 1999). Laccase activity was determined spectrophotometrically as the absorbance increase at 420 nm of 10% 2,2'-azino-bis (3-ethyl benzothiazoline-6-sulfonic acid) (ABTS) in 100 mM sodium acetate buffer, pH 4.9 (Hatvani and Meecs, 2001). Tyrosinase activity was determined by the formation of catechol quinone at 410 nm in a reaction mixture containing 0.01% catechol in 0.1 M potassium phosphate buffer (pH 7.4) in 2 ml reaction mixture (Zhang and Flurkey, 1997). Azoreductase activity was determined spectrophotometrically by monitoring NADH disappearance at 340 nm as described previously (Zimmerman et al., 1982). NADH-DCIP reductase activity was determined by the procedure reported previously (Salokhe and Govindwar, 1999). As shown in Table 2, the cell free extract of *S. oneidensis* WL-7 also showed the presence of the enzymes (laccase and NADH-DCIP reductase) responsible for dye decolorization. Intracellular laccase and NADH-DCIP reductase were found to be present in the control cells without dye addition. A significant increase in the laccase (227%) activity was observed in the cells after decolorization, whereas induction of NADH-DCIP reductase activity was not found. On the other hand, other enzyme activities including azoreductase, lignin peroxidase, and tyrosinase were not detected in either intracellular or extracellular fractions. This indicates that intracellular laccase is involved in the decolorization process by *S. oneidensis* WL-7. Laccases belonging to a diverse group of multicopper oxidases catalyze the oxidation of a variety of aromatic compounds and their catalytic centers are composed of three structurally and functionally distinct copper centers (Martins et al., 2002).

To investigate whether the copper concentration has an effect on intracellular laccase activity in the cytoplasmic fraction and decolorization by *S. oneidensis* WL-7, the cells were grown at 35°C under aerobic conditions until the optical density at 600 nm was approximately 0.6, after which RB-5 (50 μM) and different concentrations (0.5–2.0 mM) of CuSO₄ were added to the culture medium. After further incubation for 6 h under static conditions, the cells were separated into the cell pellets and the supernatants by centrifugation at 10,000 × *g* for 20 min. The supernatants and the cell-free extracts obtained by sonication of cell pellets were used as samples for decolorization and intracellular laccase assay, respectively. Interestingly, cells grown in LB medium supplemented with copper were dark blue in color, whereas none was found to be colored without copper. Decolorization and laccase activity were also not observed in the fractions obtained by the addition of copper (data not shown). In cell-free extracts from cells grown in LB medium unsupplemented with copper, however, laccase activity for the oxidation of ABTS was gradually increased by addition of copper ions to the assay mixture and decreased at a higher copper concentration than 2 mM (Fig. 2). These results clearly indicate that CuSO₄ supplemented exogenously into culture medium induced the adsorption of dye to cells, which resulted in inhibitory effects on the dye removal through biodegradation and promotion of laccase activity.

In conclusion, the present study has shown here that *S. oneidensis* WL-7 isolated from activated sludge of an effluent treatment plant of a textile and dyeing company is capable of efficiently decolorizing azo dyes including RB-5. Decolorization efficiency was

Table 2. Enzyme activities in control (without RB-5) and induced state (with RB-5) after incubation for 5 h at 35°C under static conditions.

Enzyme	Control	Induced state
Lignin peroxidase ^a	ND	ND
Laccase ^a	0.0102±0.0016	0.0232±0.0050*
Tyrosinase ^a	ND	ND
DCIP reductase ^b	0.0093±0.0012	0.0106±0.0029
Azoreductase ^c	ND	ND

ND, not detected.

Values are mean of three experiments ± SEM, significantly different from the control cells at **p*<0.001.

^aUnits/min/ml.

^bμg of the reduced DICP/min/mg protein.

^cUnits/min/mg protein.

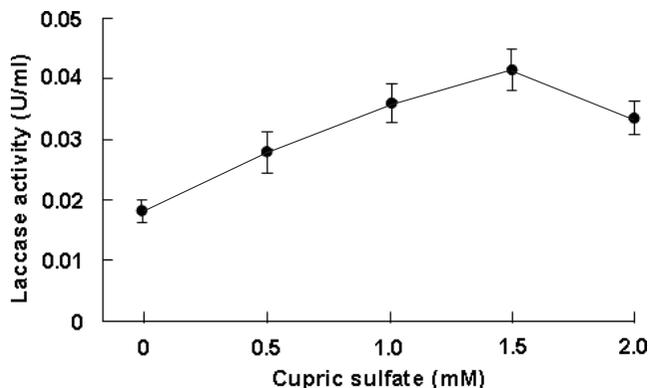


Fig. 2. Effect of copper concentration on the laccase activity. *S. oneidensis* WL-7 was grown at 35°C under aerobic conditions until the optical density at 600 nm was approximately 0.6, after which RB-5 (50 μ M) was added to the culture medium. After cultivation for a further 5 h at 35°C under static conditions, cells were harvested and suspended in 0.1 M potassium phosphate buffer (pH 7.4). The cell-free extracts obtained by sonication were used for laccase assay. Laccase activity for oxidation of ABTS was measured in the presence of different concentrations of CuSO₄. Each point represents mean \pm SD of triplicates.

higher under static conditions. This is the first report showing the presence of laccase activity possibly involved in azo dye decolorization by a *Shewanella* strain.

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