Isolation and characterization of *Pseudomonas otitidis* WL-13 and its capacity to decolorize triphenylmethane dyes

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**Abstract**

*Pseudomonas otitidis* WL-13, which has a high capacity to decolorize triphenylmethane dyes, was isolated from activated sludge obtained from a wastewater treatment plant of a dyeing industry. This strain exhibited a remarkable color-removal capability when tested against several triphenylmethane dyes under both shaking and static conditions at high concentrations of dyes. More than 95% of Malachite Green and Brilliant Green was removed within 12 h at 500 \(\mu\)mol/L dye concentration under shaking conditions. Crystal Violet lost about 13% of its color under the same conditions tested. The rate of decolorization increased when the M9 medium was supplemented with yeast extract. The optimum pH and temperature for color removal were 7–9 and 35–40°C, respectively. The observed changes in the visible spectra and the inspection of bacterial growth indicated the color-removal by the adsorption of dye to the cells during incubation with strains.

**Key words**: adsorption; decolorization; *Pseudomonas otitidis* WL-13; triphenylmethane dye

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**Introduction**

Triphenylmethane dyes, used extensively in the textile industry as well as in human and veterinary medicine, are resistant to conventional wastewater treatment systems (Azmi et al., 1998; Robinson et al., 2001). Some of these dyes are mutagenic, mitotic poisons and clastogens (Au et al., 1978; Cho et al., 2003). Several physicochemical methods have been used to eliminate the colored effluents from wastewater (Banat et al., 1996), but these methods are expensive, of limited applicability and produce large amounts of sludge. As a better alternative, therefore, the development of biological processes using microorganisms for the treatment of dye-containing wastewater has become increasingly important (Azmi et al., 1998; Robinson et al., 2001). Microbial decolorization processes have the advantage of being environmentally friendly and low in cost compared to conventional treatments.

Several bacteria capable of decolorizing triphenylmethane dyes have been reported, which include *Pseudomonas pseudomallei* 13NA, *Pseudomonas mendocina* MCM B-402, *Pseudomonas putida*, *Bacillus subtilis*, *Karthia* sp., *Aeromonas hydrophila*, etc. (Azmi et al., 1998; Sani and Banerjee, 1999; Sarnaik and Kanekar, 1999; Sharma et al., 2004; Ren et al., 2006; Chen et al., 2007). A new bacterium, *Citrobacter* sp. strain KCTC 18061P, has been isolated recently, which has a higher decolorization capability compared to all the other microorganisms reported to date, even at high concentrations of triphenylmethane dyes (An et al., 2002). Furthermore, our research group has, for the first time, biochemically purified and characterized an enzyme designated triphenylmethane reductase (TMR), capable of decolorizing triphenylmethane dyes from *Citrobacter* sp. strain KCTC 18061P (Jang et al., 2005).

In the course of screening processes for dye-decolorizing bacteria, a bacterium identified as *Pseudomonas otitidis*, was isolated, which is a novel species of *Pseudomonas*, and is capable of decolorizing triphenylmethane dyes. This article describes the isolation and characterization of *Pseudomonas otitidis* WL-13, which demonstrated a high capacity to decolorize triphenylmethane dyes.

**1 Materials and methods**

**1.1 Chemicals**

Crystal Violet, Malachite Green, Basic Fuchsin, Brilliant Green, Congo Red, Methyl Red, and 1-butanol were purchased from Sigma-Aldrich Inc. (Seoul, Korea). Stock
solutions of each dye were prepared by membrane filtration. All other chemicals used were of analytical grade.

1.2 Isolation and identification of dye-decolorizing bacteria

The dye-decolorizing bacterial strain was isolated from activated-sludge samples collected from wastewater treatment plants of dyeing industries in Busan, Korea. Screening of the strains for dye decolorization was performed by enrichment culture technique using NM9 and DNM9 media as described previously (Xu et al., 2005). After purification by successive single colony isolation on a DNM9 agar plate, strain WL-13 was identified by carbon source utilization patterns using Biolog GN2 microplate (Biolog, USA) and the analysis of 16S rDNA sequences.

For the 16S rDNA sequence analysis, bacterial genomic DNA was extracted and purified using a Wizard Genomic DNA Prep. Kit (Promega Corp., Madison). Two primers annealing to the 5' and 3' end of the 16S rRNA gene were 5'-GAGTTTGATCCTGGCTCAG-3' (positions 9 to 27 (Escherichia coli 16S rDNA numbering)) and 5'-AGAAGGAGG TGATCCAGCC-3' (positions 1542 to 1525 (E. coli 16S rDNA numbering), respectively. Polymerase chain reaction (PCR) was performed as follows: pre-denaturation at 95°C for 5 min, 30 cycles at 95°C for 40 s, 55°C for 40 s and 72°C for 2 min. The PCR product was subcloned into pGEM-T easy vector (Promega, Madison, USA) and its nucleotide sequence was determined by GenoTec Co., Ltd. (Taejon, Korea). The partial rDNA sequences were analyzed using a BLAST search algorithm to estimate the degree of similarity to other rDNA sequences obtained from the NCBI/GenBank. Phylogenetic trees were constructed by the ClustalX program (Thomson et al., 1997).

1.3 Effects of carbon and nitrogen sources on decolorization of dye

M9 media supplemented with 5 g/L yeast extract, 10 g/L peptone, 3 g/L beef extract, 10 g/L glucose, a combination of 10 g/L peptone and 5 g/L yeast extract, or a combination of 5 g/L peptone and 3 g/L beef extract were used to detect the utilization of carbon and nitrogen sources. The pH of M9 medium was adjusted to 7.5 before sterilization and the cells were cultured at 37°C with shaking at 200 r/min.

1.4 Effects of physicochemical parameters on decolorization of dye

Pre-cultured cells (1% seeding) were inoculated in 500 mL flasks containing 100 mL LB medium with 100 µg/mL of ampicillin and 30 µmol/L of Crystal Violet. The cultures were incubated at different pH (5–10) and temperatures (25–50°C) in rotary shakers at 200 r/min (shaking condition) or without shaking (static condition). The pH of the LB medium was adjusted with 1 mol/L HCl or 1 mol/L NaOH. A 10 mL of each culture was taken out at selected intervals and the cells were centrifuged at 10000 × g for 20 min. The precipitated cells, after being washed twice with sterile water, were dried at 80°C for 24 h to measure the dry weight of the cells. The supernatant was then applied to the decolorization assay.

1.5 Decolorization of dyes by growing cells

The cells were aerobically grown at 37°C overnight in LB medium containing 100 µg/mL of ampicillin. Pre-cultured cells (1% seeding) were inoculated in 500 mL flasks containing 100 mL LB medium supplemented with 100 µg/mL of ampicillin and cultured at 37°C at 250 r/min. The pH of LB medium was adjusted to 7.5 before sterilization. Different dyes were added aseptically to separate flasks at the peak growth phase of the bacteria. After incubation for 12 h, the cells were centrifuged at 10000 × 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 the dry weight of the cells. The supernatants were used as samples for the decolorization assay. In order to detect the relative effects of adsorption and biodegradation of cells on color removal, after incubation for 12 h in LB medium containing 100 µg/mL of ampicillin and 30 µmol/L of Crystal Violet, Basic Fuchsin, Brilliant Green or Malachite Green, the cultures were divided into the culture medium and the cell pellet by centrifugation at 10000 × g for 20 min. The cell pellet was subjected to ultrasonification in 1-butanol and then centrifuged at 10000 × g for 20 min. The supernatants containing butanol extract and the culture medium were used as samples for the decolorization assay. Two kinds of controls were used as an un-inoculated sterile control and a heat-killed control. The first control contained only the components of the medium and indicated the effect of medium components on decolorization, while the latter demonstrated the effect of cell-adsorption on the decolorization process.

1.6 Decolorization assay

Decolorizing activity was expressed in terms of the percentage of decolorization by the modified method described previously (Yatome et al., 1991; Sani and Banerjee, 1999). The residual dye in the sample (2 mL) was extracted with an equal volume of 1-butanol. Decolorization of dyes was determined by monitoring the decrease in absorbance at the visible absorbance maximum of each dye. A scanning spectrophotometer (Ultrspec 3000 UV/Vis, Pharmacia, Sweden) was used for absorbance measurement and recording visible absorption spectra. Decolorization activity was calculated as follows: decolorization = (initial absorbance–observed absorbance)/initial absorbance × 100%. All assays were done in triplicates.

2 Results and discussion

2.1 Isolation and characterization of triphenylmethane dye-decolorizing bacteria

A bacterial strain capable of decolorizing Crystal Violet, named strain WL-13, was isolated after screening and purification by successive single colony isolation. The strain was a Gram-negative, motile rod and was positive for oxidase and catalase. It showed the highest similarity to
\textit{Pseudomonas otitidis} in the carbon-source utilization test. Moreover, a comparison of the 16S rRNA gene sequence of WL-13 with that of other strains using the BLAST(N) program of the National Center for Biotechnology Information (NCBI) also showed the highest homology to \textit{P. otitidis} with 99\% similarity (Fig. 1). The 16S rRNA gene sequence of WL-13 was submitted to GenBank under the accession number EF687744. Accordingly, the isolated bacterium WL-13 was named \textit{P. otitidis} WL-13. Cell growth and decolorization ability of \textit{P. otitidis} WL-13 were not affected by ampicillin (100 \(\mu\)g/mL), but other antibiotics (tetracycline, kanamycin, chloramphenicol, streptomycin) inhibited cell growth. \textit{P. otitidis} WL-13 showed high dye decolorization properties under static and shaking conditions although the decolorization was comparatively slower under static conditions (Fig. 2). The rate of decolorization decreased with decreasing temperature. In temperatures range 35–40\(^\circ\)C, the rate of decolorization was two times faster than that within 20–25\(^\circ\)C, but almost the same amount of dye was removed at both temperature ranges. The optimal pH for decolorization ranged from 7 to 8.5 (data not shown). Consequently, subsequent experiments were performed at 37\(^\circ\)C, pH 7.5 with agitation.

The effects of carbon and nitrogen sources on color removal of Crystal Violet were investigated by adding glucose or different nitrogen sources at 0.5\% or 1\% to the M9 medium. The rate of decolorization was about 75\% after 24 h of cultivation in the presence of 0.5\% yeast extract, whereas less than 30\% decolorization was observed after adding 0.5\% beef extract. Moreover, the addition of 1\% peptone accelerated the speed of decolorization after 32 h incubation (Fig. 3). On the other hand, the addition of 1\% glucose had no significant effect on cell growth and color removal.

2.2 Decolorization of triphenylmethane and azo dyes by \textit{P. otitidis} WL-13

The decolorization of various triphenylmethane and azo dyes by the growing cells of \textit{P. otitidis} WL-13 is shown in Table 1. In case of the triphenylmethane dyes tested, \textit{P. otitidis} WL-13 showed the highest decolorization capability against Malachite Green and Brilliant Green. It is remarkable that \textit{P. otitidis} WL-13 was able to decolorize Malachite Green and Brilliant Green by more than 95\% at dye concentrations as high as 500 \(\mu\)mol/L. Compared

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**Fig. 1** Phylogenetic location of the isolated strain WL-13 based on the partial 16S rRNA sequences. The phylogenetic tree was constructed using the Clustal X program. Accession numbers of reference sequences retrieved from GenBank/EMBL/DDBJ are given in parentheses.
to Malachite Green and Brilliant Green, Crystal Violet showed 100% decolorization up to 20 ìmol/L, while the degree of decolorization was decreased by 65% at a dye concentration of 200 ìmol/L. Sani and Banerjee (1999) reported that the decolorization of both Crystal Violet and Malachite Green at concentrations of 5 ìmol/L reached 100% after incubation for 30 min with Kurthia sp. while only 36% of Malachite Green was removed when the dye concentration was increased to 105 ìmol/L, and decolorization of Crystal Violet was not detected in the same concentration. Our group also reported that Malachite Green and Brilliant Green lost over 50% of their colors within 1 h at 500 ìmol/L dye concentration after incubation with Citrobacter sp. strain KCTC 18061P (An et al., 2002). It was recently reported that the bacterial consortium decolorized more than 80% of Malachite Green and Brilliant Green within 24 h at 32.3 and 62.2 ìmol/L dye concentrations, respectively (Sharma et al., 2004). Therefore, the results of the present study indicate that the rates of decolorization by Pseudomonas otitidis WL-13 are much higher than those shown in previous articles (Sani and Banerjee, 1999; An et al., 2002; Sharma et al., 2004; Ren et al., 2006).

In the case of Basic Fuchsin with three amino groups, the rate of decolorization was less than other triphenylmethane dyes used. This may be due to different chemical structures of the dyes. In the case of azo dyes, only 32.6% decolorization of Methyl Red (mono azo group) was observed in 12 h experimental period at dye concentration of 20 ìmol/L, and 38.4% of Congo Red (diazot group) was removed at dye concentrations as low as 10 ìmol/L. This may be due to the complex structure of the azo group (–N=N–), compared to the triphenylmethane dyes tested.

It is known that dye concentrations can influence the efficiency of dye removal by bacteria through the combination of factors including the toxicity of the dye at higher concentrations (Pearce et al., 2003). Michaels and Lewis (1986) reported that the inability of many bacteria to degrade Crystal Violet is due to its cytotoxicity. A previous report (Sani and Banerjee, 1999) showed that the decolorization of Crystal Violet by Kurthia sp. was severely inhibited at a dye concentration of 30 ìmol/L. Yatome et al. (1993) reported that the cell growth of Nocardia corallina was completely inhibited by Crystal Violet at a concentration as low as 7 ìmol/L. On the contrary, the results of this study showed that the decolorization of Crystal Violet by P. otitidis WL-13 was severely inhibited at dye concentrations as high as 500 ìmol/L (Table 1), which was attributed to the cytotoxicity of dyes.

### 2.3 Mechanism of decolorization by Pseudomonas otitidis WL-13

Decolorization of dye solutions by bacteria are known to take place in two ways, either adsorption on the microbial biomass or biodegradation of the dyes by cells (Banat et al., 1996; Azmi et al., 1998). Dye adsorption may be evident from the inspection of the bacterial growth; those adsorbing dyes will be deeply colored, whereas those causing degradation will remain colorless. In addition, dye decolorization can also be judged clearly by observing the spectrum changes of dyes. The removal of a triphenylmethane dye by biodegradation shows the complete reduction of the major peaks and the production of new peaks at the same time (Jang et al., 2005; Chen et al., 2007).

When the UV-Visible spectra of Crystal Violet-containing culture fluid after incubation for defined intervals, were scanned in the range of 300–800 nm, a significant decrease in optical density at 590 nm was observed after 12 h of incubation and the peak almost completely disappeared after 24 h incubation. This indicated the complete decolorization of Crystal Violet (Fig. 4). However, the productions of new peaks at wavelengths other than 590 nm were not detected and cells became deeply colored along with cell growth. Moreover, P. otitidis WL-13 cells cultured for 24 h with Crystal Violet remained dark blue in color (Fig. 5), compared with Citrobacter sp. strain KCTC 18061P showing color removal by biodegradation through enzymatic reaction (Jang et al., 2005). These results indicated that the decolorization by P. otitidis WL-13 was most likely caused by the adsorption of dyes to cells rather than the degradation through enzymatic reaction, like decolorization of Crystal Violet by Aeromonas sp. B5 (Nobuki et al., 2000).

![UV-Visible spectral scans (300–800 nm) showing color removal of Crystal Violet by P. otitidis WL-13.](image-url)

**Table 1** Decolorization of triphenylmethane and azo dyes by the growing cells of P. otitidis WL-13

<table>
<thead>
<tr>
<th>Dye conc. (ìmol/L)</th>
<th>CV</th>
<th>BF</th>
<th>MG</th>
<th>BG</th>
<th>MR</th>
<th>CR</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>43.2</td>
<td>38.4</td>
</tr>
<tr>
<td>20</td>
<td>100</td>
<td>87.2</td>
<td>100</td>
<td>100</td>
<td>32.6</td>
<td>27.6</td>
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<tr>
<td>50</td>
<td>98.6</td>
<td>83.4</td>
<td>98.3</td>
<td>100</td>
<td>21.4</td>
<td>13.4</td>
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<tr>
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</tr>
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<td>200</td>
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<td>23.1</td>
<td>96.8</td>
<td>97.3</td>
<td>2.2</td>
<td>7.4</td>
</tr>
<tr>
<td>500</td>
<td>13.2</td>
<td>12.6</td>
<td>95.7</td>
<td>96.6</td>
<td>1.6</td>
<td>2.1</td>
</tr>
</tbody>
</table>

CV: Crystal Violet; BF: Basic Fuchsin; MG: Malachite Green; BG: Brilliant Green; MR: Methyl Red; CR: Congo Red.

* The variation in decolorization among three replications was ranged in 0.01%–0.18%.

**Fig. 4** UV-Visible spectral scans (300–800 nm) showing color removal of Crystal Violet by P. otitidis WL-13. After cultivation for defined intervals in LB medium containing 30 ìmol/L of Crystal Violet under shaking condition, samples were centrifuged at 6000 r/min for 10 min and then decolorization of supernatants was monitored using a scanning spectrophotometer.
3 Conclusions

*Pseudomonas otitidis* WL-13 was isolated and the ability of this organism to decolorize triphenylmethane dyes with high adsorption capacities was demonstrated. To our knowledge, this strain is the most efficient among bacteria that are capable to decolorize triphenylmethane dyes by biomass adsorption. Further studies, including the detailed mechanism for dye adsorption and desorption, are needed to provide useful information for the development of biosorbents available in the treatment of dye wastewater.

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References


