The decolorisation capacity and mechanism of *Shewanella oneidensis* MR-1 for Methyl Orange and Acid Yellow 199 under microaerophilic conditions

Y. Y. Yang, L. N. Du, G. Wang, X. M. Jia and Y. H. Zhao

**ABSTRACT**

*Shewanella oneidensis* MR-1 was found to reach 99.36% and 78.25% decolorisation for Methyl Orange and Acid Yellow 199 in solutions, respectively. The suitable pH range for decolorisation of Methyl Orange and Acid Yellow 199 by *S. oneidensis* MR-1 was 4.0–7.0 and 6.0–8.0, respectively, The azo dyes' removal by *S. oneidensis* MR-1 was slightly enhanced by addition of Mg$^{2+}$, but inhibited by Pb$^{2+}$, Cd$^{2+}$, Cu$^{2+}$, Fe$^{3+}$ and Fe$^{2+}$. The enzyme activities of NADH-DCIP reductase and azoreductase were 2.67 and 3.0 times higher, and 1.92 and 2.48 times higher, respectively, in the Methyl Orange treatment and in the Acid Yellow 199 treatment as compared to the control treatment. These findings indicated that the azo dyes' decolorisation by *S. oneidensis* MR-1 was via reduction mechanism.

**Key words** | Acid Yellow 199, decolorisation, Methyl Orange, reduction mechanism, *Shewanella oneidensis* MR-1

**INTRODUCTION**

Large amounts of dyes are produced annually and extensively used in the textile, cosmetic, plastic, food, and pharmaceutical industries (McMullan et al. 2001; Esther Forgacs & Gyula 2004). Dyes can be classified into azo, triphenylmethane and anthraquinone dyes based on the chemical structures, in which azo dyes are most widely used. Azo dyes are characterised by the presence of one or more azo groups substituted with aromatic amines. They are produced with stable molecule structure to be recalcitrant during use. Due to the mutagenicity of azo dyes, they have been implicated in pollution and known to be toxic to humans (Banat et al. 1996; Pearce et al. 2003; Olaganathan & Patterson 2009).

Therefore, removal of azo dyes from environment using different methods has become a focus for environmental scientists. Physical and chemical methods are not suitable to treat dye effluent due to their operational costs or secondary sludge disposal problems (Kaushik & Malik 2009; Rodriguez et al. 2009). Even the new developments in the physical and chemical approaches, such as electrocoagulation and electrochemical treatment, have faced some difficulties. However, bioremediation has become a promising way for treatment of pollution from various dyes, including azo dyes, due to its eco-friendly nature and low cost.

*Shewanella* are metabolically versatile and effective to degrade recalcitrant organics and reduce heavy metal ions. Some *Shewanella* strains were found to be able to decolorise anthraquinone dye (Xu et al. 2006), azo dye (Xu et al. 2007a; Khalid et al. 2008; Wu et al. 2009), and triphenylmethane dye (Chen et al. 2008). *S. oneidensis* MR-1, which is currently being used as a model organism, has been extensively studied on its bioremediation ability of heavy metal ions and humic substances (Mugerfeld et al. 2009). However, relatively little work has been done on the dye decolorisation ability of *S. oneidensis* MR-1. In this study, the main objective was to investigate the decolorisation capacity and mechanism of *S. oneidensis* MR-1 for two azo dyes, Methyl Orange and Acid Yellow 199.

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MATERIALS AND METHODS

Microorganism and the culture medium

S. oneidensis MR-1 (ATCC 700550), which is a facultative anaerobic bacterium, was granted by Professor Haichun Gao. It was maintained and grown on a Luria-Bertani medium. The experimental medium consisted of 2.0 g/L lactate, 1.5 g/L KH₂PO₄, 1.0 g/L yeast extract, 0.5 g/L NaCl, 0.1 g/L NH₄Cl and dyes were added.

Dyes and chemicals

One anthraquinone dye, two triphenylmethane dyes, four azo dyes and two other dyes were selected for decolorising tests. Acid Blue 25, Acid Yellow 199 and Acid Red 337 were kindly provided by Heng Sheng chemicals Ltd, China; the others were purchased from Sinopharm Chemical Reagent Co., Ltd, China. All the information of each dye is shown in Table 1.

Decolorisation analysis of each dye by Shewanella oneidensis MR-1 under different decolorisation parameter conditions

Stock solutions of dyes were prepared and diluted to the desired concentration. Strain S. oneidensis MR-1 was first cultivated under aerobic conditions at 30 °C overnight and cell suspension was obtained as reported previously (Chen et al. 2008). In the decolorisation parameter experiments, 25 mL of dye solution with various medium components was added into an Erlenmeyer flask (100 mL) and then cell suspension was transferred to reach an initial cell mass of 0.4–0.5 g/L and was kept in static conditions for 24 h. After then, samples were collected from the flask, centrifuged and supernatant was analysed spectrophotometrically for the residual dye concentration at absorption peak of each dye. Controls without inoculation were kept under the same conditions.

The dye decolorisation, an indicator of the dye bioremediation ability of S. oneidensis MR-1, was calculated as follows:

\[
\text{Decolorisation percentage} = \left(1 - \frac{A_f}{A_c}\right) \times 100\% 
\]

where \(A_c\) is the final absorbance value of aqueous solution of controls and \(A_f\) is the final absorbance value of aqueous solution of samples at fixed time.

The decolorisation parameter experiments included the following treatments:

1. **pH**: The pH of the experimental medium for culturing the strain was adjusted to 3, 4, 5, 6, 7, 8, and 9 with 0.1 mol/L HCl or 0.1 mol/L NaOH.
2. **Carbon sources**: 2% (w/v) formate, sucrose, maltose, lactose, D-fructose, D-mannose, and galactose were added to a modified experimental medium, which included 1.5 g/L KH₂PO₄, 0.5 g/L NaCl, 0.5 g/L NaCl, 0.5 g/L yeast extract and dyes.
3. **Nitrogen sources**: 0.3% (m/v) NH₄Cl, NaNO₃, beef extract, peptone, glycine, glutamic acid, proline were added to a modified experimental medium, which included 1.5 g/L KH₂PO₄, 0.5 g/L NaCl, 0.5 g/L yeast extract and dyes.
4. **Metal ions**: 0.1 mM CuCl₂, FeCl₃, FeSO₄, NiCl₂, MgCl₂, PbCl₂, MnCl₂ were added to the experimental medium for culturing the strain.
5. **Initial dye concentrations**: Dyes of different initial concentrations, ranging from 50 to 500 mg/L, were added to the experimental medium for culturing the strain of optimum pH.

Each experiment was carried out in triplicate.

Preparation of cell free extract and enzyme assays

The bacterial cells grown in the LB medium at 30 °C for 24 h at aerobic conditions were considered as control. The medium was centrifuged at 10,000 rpm for 10 min and the biomass was suspended in a potassium phosphate buffer (50 mM, pH 7.4). Then the suspension was sonicated (5S, 100 amplitude, 50 strokes) at 4 °C. The homogenate was centrifuged at 7,000 rpm for 20 min and supernatant was used as a source of crude enzyme. Similar procedure was used to quantify enzyme activities for the dye decolorisation of Methyl Orange and Acid Yellow 199 by S. oneidensis MR-1.

Laccase was measured by monitoring the oxidation of 1 mM ABTS in 100 mM sodium acetate buffer (pH 4.5) at 420 nm (Saratale et al. 2009). NADH-DCIP reductase activity was assayed as Bhosale reported (Bhosale et al. 2006). The azoreductase activity was determined by monitoring the decrease in the Methyl Orange concentration at 480 nm in a reaction mixture of 3 mL containing 100 μM Methyl Orange, 50 mM sodium phosphate buffer (pH 5.5) and 20 μM NADH (Jadhav et al. 2008). One unit of enzyme activity was defined as a microgram of Methyl red reduced min⁻¹ mg⁻¹ of protein. All enzyme assays were run in triplicate.
Table 1: Dye removal of each dye (100 mg/L) used in this study by Shewanella oneidensis MR-1 after 24 h.

<table>
<thead>
<tr>
<th>Chemical structure class</th>
<th>Dye</th>
<th>Chemical Structure</th>
<th>C.I. name</th>
<th>$\lambda_{max}$ (nm)</th>
<th>Decolorisation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthraquinone</td>
<td>Acid blue 25</td>
<td><img src="image" alt="Anthraquinone" /></td>
<td>C.I. 62055</td>
<td>598</td>
<td>49.15 ± 8.60</td>
</tr>
<tr>
<td>Triphenylmethane</td>
<td>Malachite Green</td>
<td><img src="image" alt="Triphenylmethane" /></td>
<td>C.I. Basic Green 4</td>
<td>618</td>
<td>56.32 ± 10.42</td>
</tr>
<tr>
<td></td>
<td>Crystal Violet</td>
<td><img src="image" alt="Crystal Violet" /></td>
<td>C.I. Basic Violet 3</td>
<td>584</td>
<td>58.51 ± 6.77</td>
</tr>
<tr>
<td>Azo</td>
<td>Methyl Orange</td>
<td><img src="image" alt="Methyl Orange" /></td>
<td>C.I. 13025</td>
<td>470</td>
<td>97.64 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>Acid Yellow 199</td>
<td><img src="image" alt="Acid Yellow 199" /></td>
<td>C.I. Acid yellow 199</td>
<td>455</td>
<td>77.92 ± 3.76</td>
</tr>
<tr>
<td></td>
<td>Acid Red 337</td>
<td><img src="image" alt="Acid Red 337" /></td>
<td>C.I. 17102</td>
<td>494</td>
<td>78.02 ± 0.26</td>
</tr>
<tr>
<td>Congo Red</td>
<td><img src="image" alt="Congo Red" /></td>
<td>C.I. Direct red 28</td>
<td></td>
<td>497</td>
<td>85.86 ± 0.32</td>
</tr>
<tr>
<td>Other</td>
<td>Methylene Blue</td>
<td><img src="image" alt="Methylene Blue" /></td>
<td>C.I. Basic blue 9</td>
<td>662</td>
<td>35.14 ± 6.97</td>
</tr>
<tr>
<td></td>
<td>Safranine T</td>
<td><img src="image" alt="Safranine T" /></td>
<td>C.I. basic red 2</td>
<td>532</td>
<td>55.36 ± 9.34</td>
</tr>
</tbody>
</table>
The decolorisation manner of azo dyes, decolourisation by *Shewanella oneidensis* MR-1

Each dye (100 M) was inoculated with *S. oneidensis* MR-1 for 10 h at optimum pH and temperature. After incubation for 10 h, samples from the culture were centrifuged at 12,000 rpm for 10 min. Then the supernatant was scanned from 400 nm to 800 nm using UV-visible spectrophotometer to detect any transformation of each dye in the system. After that, the supernatant was used to detect the products of decolourisation using HPLC analysis. The RP-HPLC column was C-18 reverse phase column (150.00 mm × 4.60 mm) and at the temperature of 25 °C. The mobile phase was methanol: water (90:10, v/v). The flow rate was 0.8 mL/min and 5 μL was injected in the sampler. The metabolites were detected at 480 nm and 254 nm for Methyl Orange, and 455 nm and 280 nm for Acid Yellow 199, respectively.

**Effect of initial pH on decolourisation**

As an important factor affecting the decolourisation process, optimum pH value is not only determined by the optimum growth pH of bacterium, but also by the dye state in the tested system. In this research, we identified that the suitable pH for decolourisation of Methyl Orange by *S. oneidensis* MR-1 was 4.0–7.0, and the optimum pH for Acid Yellow 199 was about 6.0–8.0 (Figure 1(a)). This was a little different from that of *S. decolorationis* S12, *Shewanella* strain J18 143 and *Shewanella* sp. NTOU1, for which pH 6.0–8.0 was the optimum pH value for decolourisation (Pearce *et al*. 2006; Xu *et al*. 2007; Chen *et al*. 2008). The situation of *S. oneidensis* MR-1 for Methyl Orange may be similar to that of yeast reported (Ramalho *et al*. 2004). The protonisation of N, N-dimethylamino-based dyes at an azo nitrogen occurred under lower pH, which was easier to degrade (formulas 2 and 3). At pH 7 and afterwards, the decolourisation of Methyl Orange decreased consistently whereas the decolourisation of Acid Yellow 199 decreased little. Electron-withdrawing groups (SO₄²⁻) of Methyl Orange might lead to a decreased electron density at the N = N double bond, especially with para position (Maier *et al*. 2004), which enhanced the decolourisation rate for Methyl Orange. This indicated that pH 4.0 not only had a positive effect on shifting the following equilibrium toward decreasing decolourisation of Methyl Orange (formulas 2 and 3), but also decreased the electron density at the N = N double bond. Methyl group in the ortho-position and three benzene rings structure of Acid Yellow 199, which enhanced the electron cloud density at N = N double bond, attenuated the decolourisation rate(Maier *et al*. 2004). Thus, pH has little effect on the dye state of Acid Yellow 199 in the tested system. Extreme acid environment had a negative effect on dye decolourisation by *S. oneidensis* MR-1 due to its being poisonous to cell growth.

\[
\text{Ar} - \text{N} \equiv \text{N} - \text{Ar} + 4 \text{H}^+ + 2 \text{NAD}(P)
\]

\[
\text{Ar} - \text{N} \equiv \text{N} - \text{Ar} + \text{H}^+ \rightarrow \text{Ar} - \text{N} \equiv \text{N} - \text{Ar} + \text{H}^+ + 2 \text{NAD}(P)
\]

**Effect of medium components on decolourisation**

**Effect of nitrogen and carbon sources on decolourisation**

Nitrogen and carbon sources were required to promote the degradation of azo dyes. Peptone was the best nitrogen source...
while NaNO₃ was the worst. Organic nitrogen was more effective for degradation of azo dyes than inorganic nitrogen. Glycine was the best amino acid tested as an energy source for dye removal of Methyl Orange. However, no significant differences were found among the tested amino acids for the dye removal of Acid Yellow 199. It was observed that nitrogen sources had greater effect on dye removal of Methyl Orange than that of Acid Yellow 199 (Figure 1(b)). This study also revealed that yeast extract was the best carbon source for dye removal, with 99.36% of Methyl Orange and 78.25% of Acid Yellow to be decolorised, respectively (Figure 1(c)). Yeast extract and peptone may be the best choice in practice with regard to costs and dye removal efficiency.

**Effect of metal ions on decolorisation**

Several metal ions were chosen to study their effects on decolorisation because many metal ions could affect the activities of enzymes. It was observed that dye removal was slightly enhanced by addition of Mg²⁺ (Figure 1(d)). The dye removal was 97.21% in the presence of Mg²⁺ and 96.82% without addition of metal ions. The presence of Mn²⁺ had a greater effect on the dye removal of Methyl Orange than that of Acid Yellow 199 (Figure 1(d)). Heavy metal ions (e.g. Pb²⁺, Cd²⁺ and Cu²⁺) which influenced the cell growth and viability had a significant (P<0.05) negative influence on the dye removal (Figure 1(d)). It was reported that the activity of azoreductase, which can decompose azo dyes, appears to be unaffected by Mg²⁺, Mn²⁺, Ca²⁺ and Zn²⁺ but is almost completely inhibited by Fe²⁺ and moderately inhibited by Cu²⁺ and Hg²⁺ (Nachiyar & Rajakumar 2005). Xu reported that addition of external Fe³⁺ enhanced azo reduction by *S. decolorationis* S12 under anaerobic conditions, but that chemically or bacterially produced Fe²⁺ did not accelerate the decolorisation rates (Xu et al. 2007a). However, both Fe³⁻ and Fe²⁺ inhibited the dye removal by *S. oneidensis*
MR-1 in this report. Further study was needed to understand the mechanisms associated with the effects of the metal ions on the decolourisation of the azo dyes by *S. oneidensis* MR-1 in this study.

**Effect of initial dye concentration on decolourisation**

The dye removal by *S. oneidensis* MR-1 generally decreased with increasing initial concentrations of Methyl Orange and Acid Yellow 199. This could be due to a combination of factors including the toxicity of the dyes to cell growth at higher concentrations, and the ability of the enzyme to recognise the substrate efficiently at very low concentrations (Pearce *et al.* 2005). Dye removal decreased gradually with increase in the initial dye concentrations after 24 h. It indicated that the decolourisation of Methyl Orange and Acid Yellow 199 were still kept at a high level at moderate concentration (200 mg/L) (data not shown). However, high concentration was more recalcitrant and difficult to decolourise. The negative relationship between the dye removal and initial dye concentration could be well described with a linear model for Methyl Orange (*y* = 106.51–0.14x, *R*² = 0.98) and Acid Yellow 199 (*y* = 80.34–0.09x, *R*² = 0.83). From the equation, we may conclude that Acid Yellow 199 could not be decolourised completely (*y*max = 80.34). Acid Yellow 199 was more recalcitrant than Methyl Orange.

**Analysis of enzymes related to the dye decolourisation and degradation**

Azoreductase, which catalyses reductive cleavage of azo bonds (-N=N-), has been extensively investigated in the biodegradation of azo dyes (Banat *et al.* 1996; Stolz 2001). Recently, oxidative enzymes and NADH-DCIP reductase were found to be responsible for the dye decolourisation in bacteria (Wu *et al.* 2009). Laccase, NADH-DCIP reductase and azoreductase were detected to investigate whether these enzymes were responsible for the decolourisation of Methyl Orange and Acid Yellow 199 by *S. oneidensis* MR-1. In the present study, significant increase (*P* < 0.05) in the enzyme activity of NADH-DCIP reductase and azoreductase was observed over period of azo dyes, decolourisation (Table 2), while laccase activity decreased significantly after decolourisation. Reductive cleavage of azo bonds (-N=N-) with the help of azoreductase was the initial and critical step for the azo dyes, biodegradation (Hrmova *et al.* 1984). The reductase activities of NADH-DCIP reductase and azoreductase increased significantly for Methyl Orange (267% and 300%) than that of Acid Yellow 199 (192% and 248%) compared to control, which indicated that Methyl Orange was more available and easy to decolourise by *S. oneidensis* MR-1. This was in accord with the decolourisation percentage in the experiments. From these results, we proposed that the strain *S. oneidensis* MR-1 decolourised azo dyes via enzymatic reduction mechanism. Wu *et al.* (2009) reported that laccase was responsible for the decolourisation of Reactive Black 5 by *S. oneidensis* WL-7, indicating the azo dyes, decolourisation by *S. oneidensis* WL-7 was via oxidation mechanism. This was different from what we found in *S. oneidensis* MR-1. The enzyme activities were significantly different for Methyl Orange and Acid Yellow 199 decolourisation due to their different chemical structure, indicating substitution was accounting for the degradability of different dyes and interaction between dyes and enzyme system.

**The decolourisation manner of azo dyes, decolourisation by *Shewanella oneidensis* MR-1**

Microbial decolourisation is mainly attributed to biosorption and biodegradation (Knapp & Newby 1995). In the case of adsorption, dyes are only adsorbed onto the surface of cells and the cells become deeply coloured, whereas cells were still kept colourless after degradation. To discover the possible dye removal mechanism by *S. oneidensis* MR-1, we analysed the UV-vis spectral changes and HPLC before and after microbial decolourisation. Firstly, UV-vis scan (300–800 nm) of culture supernatants withdrawn at different time intervals of Methyl Orange and Acid Yellow 199 are shown in Figure 2. For Methyl Orange, peak observed at 470 nm (0 h) was decreased without any shift in *λ*max up to nearly complete

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Intracellular enzyme activities of <em>Shewanella oneidensis</em> MR-1 cells after decolourisation compared to cells in control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme assay</td>
<td>Control</td>
</tr>
<tr>
<td>Laccase</td>
<td>0.037 ± 0.003</td>
</tr>
<tr>
<td>NADH-DCIP</td>
<td>17.72 ± 1.15</td>
</tr>
<tr>
<td>Azoreductase</td>
<td>0.76 ± 0.07</td>
</tr>
</tbody>
</table>

Values are mean of three experiments ± SEM. Significantly different from the control cells at *P* < 0.01, *P* < 0.001 by Dunnett-t test. Significantly different from the decolourisation of two dyes at *P* > 0.05, *P* < 0.01, *P* < 0.001 by one way ANOVA with Tukey-Kramer multiple comparisons test.

*% DCIP reduced, mg·mL⁻¹·min⁻¹.*

*% Methyl red reduced, mg·mL⁻¹·min⁻¹.*
decolorisation of dye (10 h), while, peak observed at 455 nm (0 h) was decreased slowly without any shift in $\lambda_{\text{max}}$ for Acid Yellow 199. The great changes in UV and visible spectra indicated that dyes in medium disappeared and new products could come into being. The colour of cells for the two dyes after 10 h was colourless. Secondly, HPLC was carried out to detect the biotransformation of the azo dyes by S. oneidensis MR-1. The peaks for Methyl Orange and Acid Yellow 199 detected at 480 nm and 455 nm were not found after decolorisation, indicating the two azo dyes decomposed to small molecules. The metabolites of Methyl Orange and Acid Yellow 199 were detected at 254 nm and 280 nm at retention time 3.014 min and 2.567 min, respectively (data were not shown). It indicated that the new products came into being. Hence, the decolorisation of Methyl Orange and Acid Yellow 199 of S. oneidensis MR-1 was mainly due to the microbial degradation, rather than inactive surface adsorption.

CONCLUSIONS

The model microorganism S. oneidensis MR-1 was found to be able to decolorise efficiently the two azo dyes under the experimental conditions. Yeast extract and peptone could be the suitable carbon and N source for culturing the strain in decolorising the dyes. Most of the heavy metal ions appeared to have negative effects on the dye removal, although addition of Mg$^{2+}$ could slightly enhance the dye removal. Significant increase in the enzyme activities of NADH-DCIP reductase and azoreductase were observed after the decolorisation, which implied that the two enzymes were involved in the decolorisation process.

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