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Exploring effects of chemical structure on azo dye decolorization characteristics by *Pseudomonas luteola*

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Abstract

This follow-up study tended to provide a systematic comparison for how the variation of functional groups and molecular structures present in model azo dyes affects color removal capability of *Pseudomonas luteola*. As sulfo group at methyl orange (p-MO) or carboxyl group at 4-(4′-dimethylaminophenylazobenzoic acid) sodium salt (denoted p-MR) were both *para* to azo bond, the ranking of decolorization rate was p-MO > p-MR due to the stronger electron-withdrawing effect of the sulfo group. For isomers, when the functional groups (sulfo group at 2-(4′-dimethylamino-phenylazo) benzenesulfonic acid sodium salt (o-MO) or carboxyl group at methyl red (o-MR)) were *ortho* to azo bond, the decolorization rate significantly decreased (e.g., p-MO > o-MO or p-MR ≫ o-MR) likely due to steric hindrance near azo linkage(s). Similarly, for phenolic azo dyes the series of decolorization rate was 3-(4′-dimethylaminophenylazo) phenol (m-OH) > 2-(4′-dimethylaminophenylazo) phenol (o-OH). Apparently, azo dyes with different properties of substituent on aromatic ring could affect the efficiency of biodecolorization of *P. luteola*. Moreover, the relative position (e.g., *ortho*, *meta*, *para*) of the substituent to azo bond could also influence the capability of biodecolorization of *P. luteola*. Regarding the electronic effect, azo dyes with stronger electron-withdrawing group (e.g., sulfo group) at specific positions (e.g., at *para*) could be more easily biodecolored than those with a carboxyl group.

Keywords: Bacterial decolorization; *Pseudomonas luteola*; Azo dye; Chemical structure; Monod kinetics

1. Introduction

Azo dyes are the largest chemical class of synthetic dyes, and widely used as colorants with 50% of commercial synthetic dyes in USA [1]. Inevitably, the residual azo dyes in wastewater from the dyestuff or textile industry would be a significant threat to public and environmental health. Azo dyes are originally designed to be recalcitrant for long-term use and thus resistant to aerobic wastewater treatment [2,3]. Moreover, azo dyes are electron-deficient xenobiotics [4] and thus are capable to be degradable via azo reduction. However, due to diverse structures present in the synthetic dye, changes in the chemical structures (e.g., isomers or the presence of different functional groups) would significantly affect the decolorization capability (e.g., biodegradability and reduction). For example, Zimmermann et al. [5] used purified Orange II azoreductase from a *Pseudomonas* strain KF46 to assess decolorization efficiency of various Orange dyes. Evidently, the specificity of Orange II azoreductase toward azo dye is strongly dependent upon the properties (e.g., electron-withdrawing ability) of functional groups in the proximity of azo linkage(s) and thus determines whether the dye is susceptible to biodecolorization. On the other hand, the hydroxy group on the 2-position of the naphthol ring of the azo dye (e.g., 1-(4′-sulfophenylazo)-2-naphthol) is a prerequisite to assist dye decolorization. In contrast, charged groups near azo bond (e.g., 1-(2′-sulfophenylazo)-2-naphthol) significantly hinder the decolorization efficiency. Zimmermann et al. [5] also mentioned that the slope of the correlation between Hammet's substituent constant, σ (an experimental value of the electronegativity of a substituent on a phenyl ring [6]) of various substrates and the logarithm of the decolorization rate for them was positive (i.e., the electron-withdrawing groups present on the phenyl ring accelerate this color removal). To have conclusive remarks, Suzuki et al. [7] also provided a correlation of aerobic biodegradability of 25 sulfonated azo dyes with their chemical structures. Although there are many structure-based

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methods to reveal biodegradation as well as biodegradibility [8], they always need significant database from experiments to obtain highly adequate model predictions.

The model decolorizer stated herein, *Pseudomonas luteola* was predominantly isolated from activated sludge of dyeing wastewater treatment in central Taiwan [9,10]. It was found that the intracellular azoreductase in *P. luteola* is an inducible enzyme to specifically deal with azo reduction. Chang et al. [11] also pointed out some crucial parameters (e.g., pH, temperature, dissolved oxygen) to affect azo dye removal performance of *P. luteola*. They also suggested that mass transfer resistance of azo dyes across cell membrane might be the rate-determining step for color removal. Moreover, both partial reduction and complete cleavage of the azo bond could contribute to decolorization of monoazo dye reactive red 22 by *P. luteola*. Chen [12] mentioned that biodecolorization of *P. luteola* was non-growth associated. In addition, Chen [13] also first adopted a toxicological assessment (e.g., pulse injection technique, dose–response analysis) to quantitatively disclose factors to control decolorization (e.g., toxicity of intermediates). For example, relatively high toxicity of two azo-bonds present on reactive black B (BB) significantly reduced decolorization efficiency of *P. luteola*. In particular, a longer time for the persistence of monoazo intermediary metabolites might enhance synergistic toxicity of BB to the bacterial population. Recently, Hsueh and Chen [14] presented a comparative study to determine the possible reasons on reaction selectivity of azo dye decolorization by *P. luteola*. However, detailed figures of chemical structures related to their reaction selectivity in a systematic analysis were still remained open for discussion. Thus, this study is not only to clarify suspected causes, but also to discuss the difference of biodecolorization between enzymatic and microbial levels.

Raymond et al. [8] mentioned that biodegradation of an organic chemical could be classified as primary (changes of molecular integrity), ultimate (complete mineralization), acceptable (toxicity reduction); and thus characterized as primary, ultimate or aerobic degradation, respectively. Although thousands of organic compounds are developed for use, most are still remained unexplored for their biodegradation [15]. This is the reason why we experimentally conducted this study in order to reveal whether replacements of chemical structure(s) (e.g., sulfo group, carboxyl group –COOH, and hydroxyl group –OH) in the proximity of azo bond affect dye decolorization capability of *P. luteola*. The purpose of this study was to compare anaerobic decolorization performance of three model dyes (sulfonated azo dyes, caboxylated azo dyes and phenolic azo dyes) using *P. luteola* and to disclose how the steric hindrance and electronic effect in dyes affect biodegradability using the Monod's kinetics model.

2. Materials and method

2.1. Microorganism and culture conditions

P. luteola [9] predominantly isolated from indigenous activated sludge of a dye-bearing wastewater treatment plant in Taichung, Taiwan, was used as a model strain to indicate decolorization performance [9–11]. To obtain synchrony in the growth phase (i.e., late exponential or early stationary growth phase) of cultures, a loopful of *P. luteola* seed taken from a single colony on a LB-streak plate was precultured in 50-mL Bacto LB broth, Miller (Luria-Bertani (per liter); 10 g Bacto tryptone, 5 g Bacto yeast extract, 10 g sodium chloride) for 24 h at 30 °C, pH 7.0, 125 rpm using a water-bath shaker (SHINKWANG, SKW-12). The 10% (v/v) precultured broth was then inoculated into fresh sterile LB broth for culture. In flask cultures, the pH was not controlled. The initial pH effect was initially adjusted by adding 0.1 N $HCl_{(aq)}$ or $NaOH_{(aq)}$. Decolorization experiments (e.g., Figs. 3–5 and 8) at various initial dye concentrations were then carried out by using 7 day-old bacteria. Experiments were carried out in duplicate for data reproducibility.

2.2. Chemical and analytical methods

Azo-dyes (Fig. 1) used for decolorization were synthesized according to the protocol indicated in [16] except methyl orange (p-MO), and methyl red (o-MR) were purchased from Santoku Chemical Company Ltd. (Osaka, Japan) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively. Synthesized chemicals were purified chromatographically and recrystallized as suggested in [5,16]. The chemicals used for preparation were *N*,*N*-dimethylaniline (ACROS ORGANICS), aniline-2-sulfonic acid, 2-amino phenol, and 3-amino phenol (ALDRICH Chem.), 4-aminobenzoic acid (Lancaster Synthesic), and 2-nitroaniline-4-sulfonic acid sodium salt (Tokyo Chemical industry Co. Ltd.). Dye solutions were sterilized by filtration (Millipore Millex®-GS 0.22 μm filter unit), as these dyes may be unstable in moist-heat sterilization. With appropriate calibrations at maximal absorption wavelengths (i.e., λ_{max}), concentrations of biomass and dyes were determined using an UV–visible spectrophotometer (HITACHI Spectrophotometer, model UV-2001). The dye concentration was determined by measuring the absorbance (Abs) of the cell-free supernatant of the sample at λ_{max} after centrifugation for 2 min at $700 \times g$ (HSIANGTAI Centrifuge MCD-2000). The λ_{max} for methyl orange (p-MO), 2-(4′-dimethylaminophenylazo)benzenesulfonic acid sodium salt (o-MO), 4-(4′-dimethylaminophenylazo)-2-nitrobenzenesulfonic acid sodium salt (o-NO_2-p-MO), methyl red (o-MR), 4-(4′-dimethylaminophenylazobenzoic acid sodium salt (p-MR), 2-(4′-dimethylaminophenylazo) phenol (o-OH) and 3-(4′-dimethylamino-phenylazo)phenol (m-OH) are 465, 440, 440, 540, 465, 435 and 435 nm, respectively. A sterile cell-free medium was chosen as the control. Since all samples contained *P. luteola* and dye to be studied, concentrations of bacterial cells and dye could then be determined as described elsewhere [12,13]. Since cell concentrations were shown in parentheses of figures in different experiments, Abs_{600nm} values might be considered as time-invariant in the entire decolorization phase. Samples were diluted to an optical density of less than 0.6 when absorbance was not within the linear range (0.1–0.7). The relationship between the cell concentration and Abs_{600nm} is 1.0 $Abs_{600nm} \cong 0.32$ g dry cell weight l^{-1}.

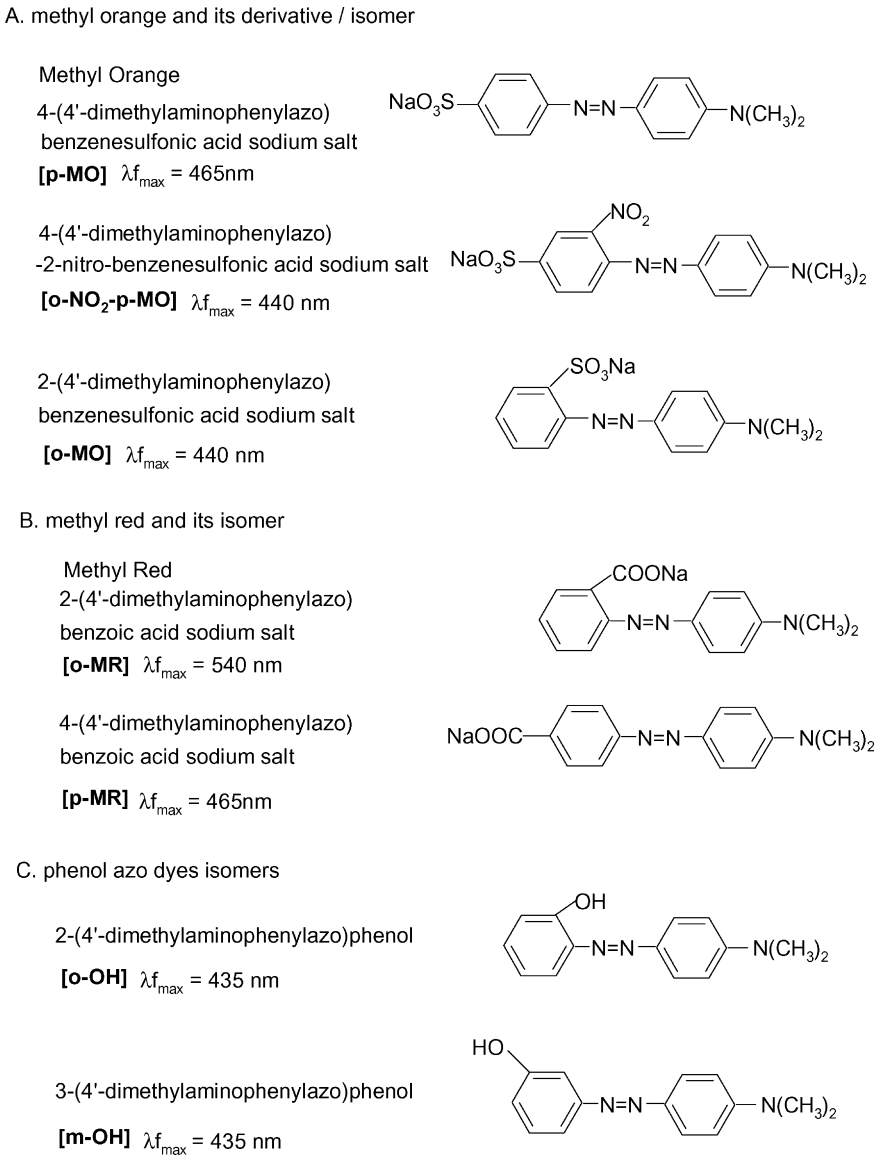


Fig. 1. Chemical structures of azo dyes for study.

3. Results and discussion

As indicated in UV/VIS spectra (Fig. 2A and B) of p-MR (*para*-position isomer of methyl red; $\lambda_{max} = 465$ nm) and o-MO (*ortho*-position isomer of methyl orange; $\lambda_{max} = 440$ nm), respectively, the maximal adsorption tended to shift from visible range (e.g., $\lambda_{max} = 465$ nm for parent azo dye p-MR) towards UV range (e.g., $\lambda_{max} = 280$ nm for daughter intermediate 4-amino benzoic acid (or sodium 4-aminobenzoate), and *N*,*N*-dimethyl-aniline). As known in organic chemistry, p-MR can be biodecolorized via the following reaction [11,14]:

4-(4′-Dimethylaminophenylazobenzoic acid sodium salt (p-MR) $\xrightarrow[P.\,luteola]{}$ *N*, *N*-dimethyl aniline + 4-aminobenzoic acid (or sodium 4-aminobenzoate)

When the π bonds of characteristic conjugated chromophores in an azo dye molecule (e.g., p-MR or o-MO) were broken, the maximal absorption wavelength was shifted towards a shorter wavelength [17]. As the absorption of the shorter-wavelength was represented the higher energy of electronic transition, the azo dye with the characteristic conjugated system of the π bonds has the lower energy of electronic transition than aromatic amines (i.e., their intermediates). Similar shifting phenomena could also be observed for decolorization of different dyes (Fig. 2B).

Apparently, the steric effect of chemical structure strongly affected the efficiency of color removal. For example, compared to p-MO the introduction of sulfo group (i.e., o-MO) and nitro group to *ortho* position (i.e., o-NO_2-p-MO) decreased rate of break-down of azo-linkage during decolorization (Fig. 3). In addition, the introduction of these functional groups might significantly delay an initiation for dye decolorization (ca. 7.5 and 12 h lag times for o-NO_2-p-MO and o-MO, respectively, in Fig. 3). This resulted in specific decolorization rate of 3.13, 1.08, and 1.54 μM/g cell/min (Table 1) at initial dye concentration at

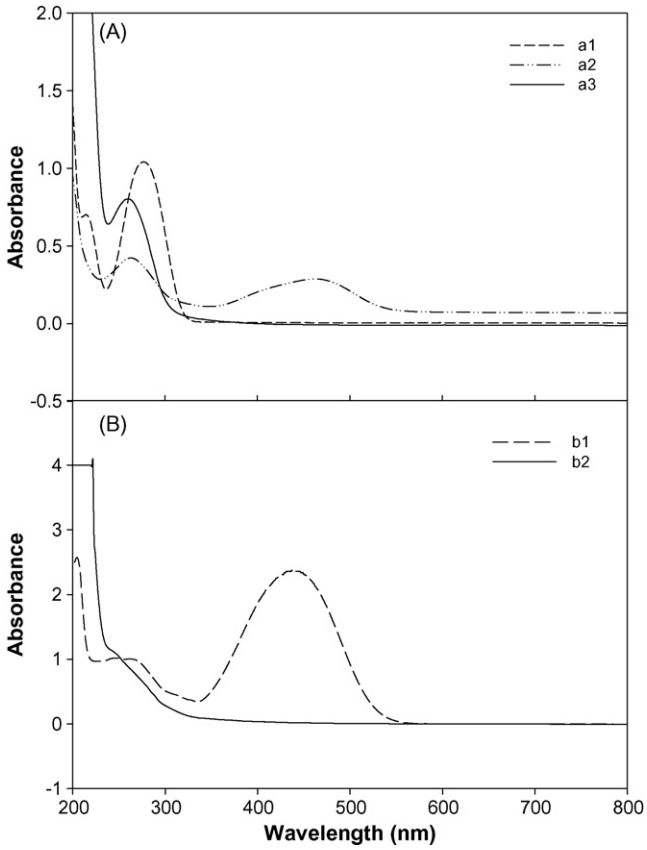


Fig. 2. UV–VIS spectra of (A) p-MR (a1 = 4-aminobenzoic acid; a2 = intact p-MR; a3 = residual p-MR); and (B) o-MO (b1 = intact o-MO; b2 = residual o-MO) before and after decolorization.

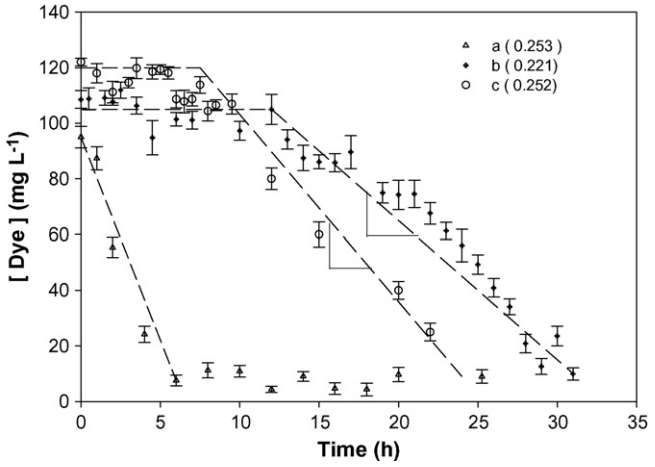


Fig. 3. Time courses of dye decolorization for azo dyes (a: 100 mg L^{-1} p-MO; b: 110 mg L^{-1} o-MO; c: 120 mg L^{-1} o-NO_2-p-MO). Parenthesis denoted the initial cell density OD_{600} of *P. luteola* for all cases.

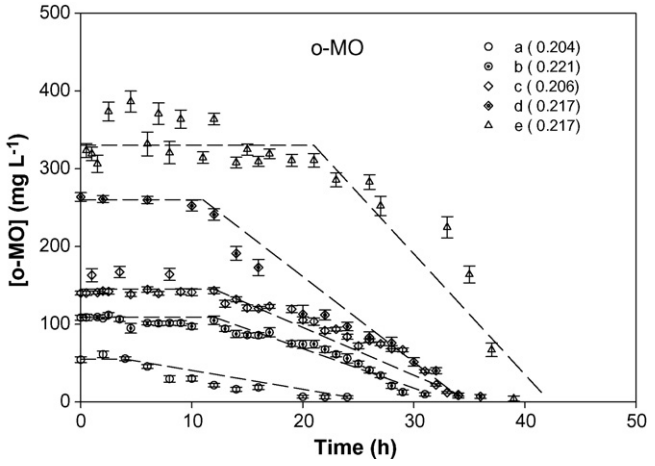


Fig. 4. Time courses of dye decolorization at various initial o-MO concentrations (a: 55 mg L^{-1}; b: 110 mg L^{-1}; c: 150 mg L^{-1}; d: 260 mg L^{-1}; e: 320 mg L^{-1}). Parenthesis denoted the initial cell density OD_{600} of *P. luteola* (ca. 0.204–0.217).

100–120 mg L^{-1}, respectively. Evidently, the steric hindrance of subsituents at *ortho* to azo bond in o-MO, and o-NO_2-p-MO decreased the decolorization performance of *P. luteola*. In addition, more substituents in the proximity of azo linkages led to less efficient biodecolorization (e.g. o-NO_2-p-MO). This was in consistent with findings of Zimmermann et al. [5]: "a second polar subsituent on the dye molecule impedes (color removal)". Similar profiles were also shown in Figs. 4 and 5 for o-MO, and p-MR at various initial concentrations, respectively. As the dye concentration gradually increased, the duration of lag phase for decolorization became longer (Fig. 6). It might be explained by the biochemical mechanism [18]. When cells were placed in dye-containing environments, intracellular levels of essential factors (e.g., vitamins, Mg^{2+} and Ca^{2+}) to express the activity of azo-reductase for color removal might be diluted due to transport across the cell membrane. Cells must then metabolize the available nutrient sources to replenish the intracellular pools prior to initiating bacterial decolorization. In addition, relatively higher concentrations of azo dyes might be easier moved across the membrane from the region of high concentration to low concentration via passive diffusion [13] due to relatively higher concentration gradient as a driving force. According to thermodynamics (i.e., the free energy change $\Delta G^{\circ} = RT \times \ln(c_{in}/c_{ex})$),

Table 1
Comparative list of color removal rate for various azo decolorization (parenthesis denoted the initial concentrations of azo dyes)

	MW (g/mol)	V (mg L^{-1} h^{-1} g^{-1} cell)	V (μM min^{-1} g^{-1} cell)
p-MO (100 mg L^{-1})	327	61.4	3.13
o-MO (110 mg L^{-1})	327	30.2	1.54
o-NO_2-p-MO (120 mg L^{-1})	372	24.05	1.08
p-MR (110 mg L^{-1})	291	36.83	2.11
m-OH (100 mg L^{-1})	241	0.68	4.7×10^{-2}
m-OH (150 mg L^{-1})	241	0.11	7.3×10^{-3}
o-OH (50 mg L^{-1})	241	0.55	3.8×10^{-2}

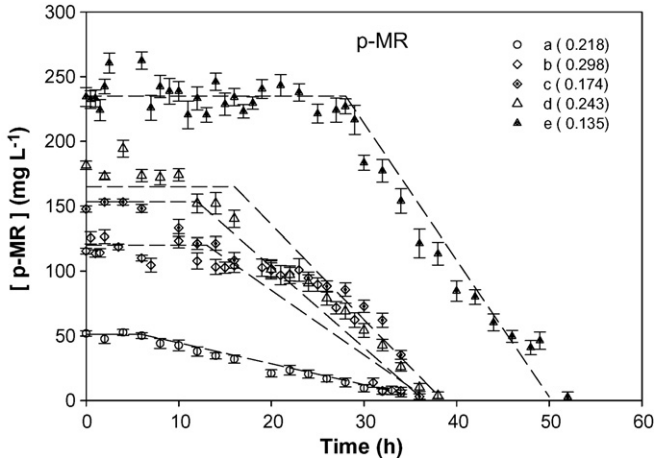


Fig. 5. Time courses of dye decolorization at various initial p-MR concentrations (a: 50 mg L^{-1}; b: 110 mg L^{-1}; c: 150 mg L^{-1}; d: 170 mg L^{-1}; e: 250 mg L^{-1}). Parenthesis denoted the initial cell density OD_{600} of *P. luteola*.

passive diffusion is spontaneous (i.e., $\Delta G^{\circ} < 0$), where c_{in} and c_{ex} denoted intracellular and extracellular dye concentration, respectively [13]. This point implied that higher dye concentration was more favored to move across the membrane. Owing to this reason, azo dye might still appreciably penetrate into the plasma membrane to establish significant toxicity to cells. As phospholipids in many biological membranes have polar head groups, their transfer across the hydrophobic interior of the bilayer as well as their dissociation from water at the bilayer surface would require a positive change in free energy (i.e., ATP requirement) to make the process a spontaneous one. Evidently, this significant toxicity caused a longer lag time to trigger decolorization as membrane transport and toxicity tolerance are energy-driven.

To have detailed profile of concentration effect, decolorization of o-MO at various concentrations were carried out (Fig. 4). When o-MO concentration increased, lag time and decolorization rate increased progressively. As dye decolorization required a high-level expression of intracellular azo reductase, these significant delays and increases in rate for decolorization were likely due to gradually increased energy requirement for effective diffusion into intracellular compartment. For example, if substrate transport to the cell is limited by the activity of a permease, dye decolorization might well be expected to follow Monod kinetics [18]. It was thus postulated that only one substrate (dye) is important in determining the decolorization rate for Monod kinetics. As the Monod kinetics indicated (Table 2, Fig. 7), the maximal rate of decolorization V_{max} of o-MO was less than that of p-MO. Higher value of K_m for o-MO revealed higher dissociation affinity of cell–dye complex than of p-MO. Similar monotonically increasing profiles for lag time and rate of decolorization with respect to initial dye concentration were also seen for p-MR (Fig. 5). Previous study [14] indicated that o-MR was not feasible to be decolorized by *P. luteola*. However, p-MR could be decolorized as indicated in Fig. 5. As Zimmermann et al. [5] revealed, for enzymatic decolorization "charged group in proximity to the azo group hinder the reaction (i.e., color removal)". This point was still appropriate for bacterial decolorization (e.g., *P. luteola*) as shown here. In addition, different substitutes at *ortho* to azo bond apparently resulted in diverse rates of decolorization. As known, the formal charges of o-MR and o-MO were −1/2 and −1/3, respectively [19] as follows:

Table 2
List of Monod kinetic parameters for o-MO, p-MO and p-MR (V_m and K_m)

	o-MO[a]	p-MO[b]	p-MR[a]
r^2	0.985	0.996	0.972
Slope	3.41	0.8263	2.38
Intercept	0.0115	−0.0188	0.0066
V_m (mg L^{-1} h^{-1} g^{-1} cell)	86.2	278	152
V_m (mg L^{-1} min^{-1} g^{-1} cell)	1.44	4.64	2.53
V_m (μM min^{-1} g^{-1} cell)	4.39	14.2	8.68
K_m (mg L^{-1})	294	53.2	360
K_m (μM)	900	163	1240

[a] Determined by Linweaver–Burk plot: $1/V$ (mg L^{-1} h^{-1} ODU^{-1}) versus $1/S_0$ (mg L^{-1}).
[b] Determined by $\ln(S_0/S)/(S_0 - S)$ versus $t/(S_0 - S)$ [14].

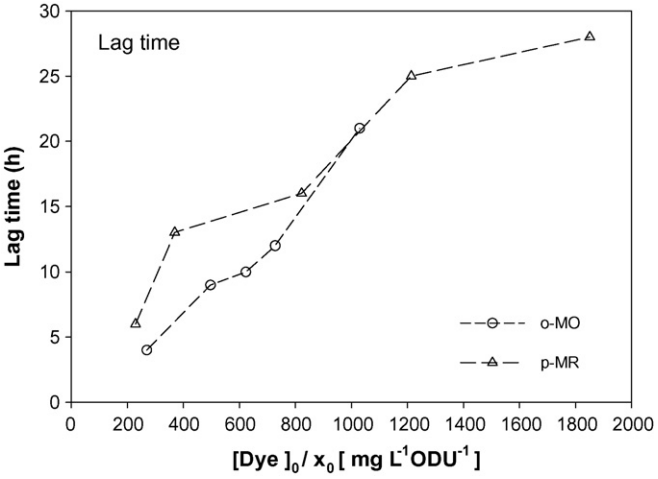


Fig. 6. Correlation of lag time with dye loading per unit cell $[Dye]_0/X_0$ (mg L^{-1} ODU^{-1}) for o-MO, and p-MR.

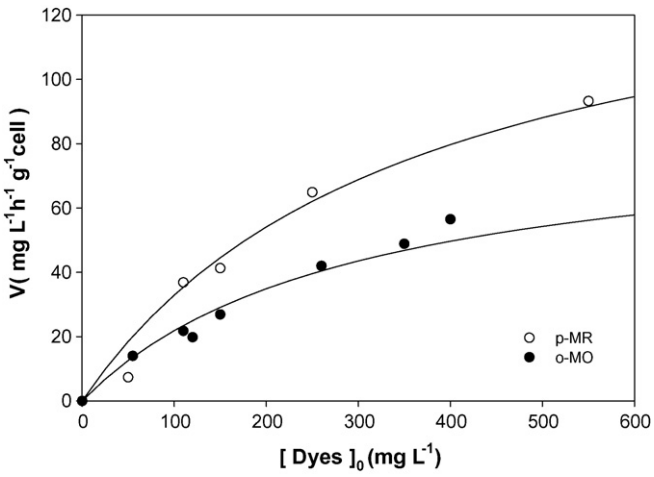
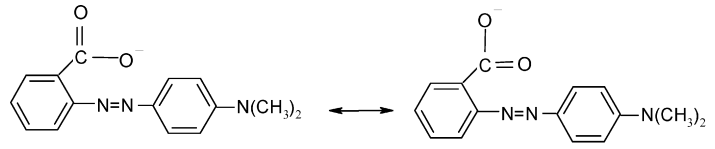
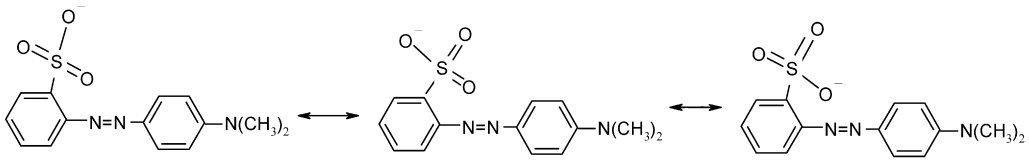


Fig. 7. Predicted Monod kinetics for specific decolorization rates of p-MR and o-MO at various initial dye concentrations.



resonance forms of o-MR



resonance forms of o-MO

Thus, it was anticipated that the electronic density (ED) on carboxyl group was higher than that on sulfo group. This difference in ED might result in negligible color removal for o-MR [14] and feasible decolorization for o-MO (this study). According to Pearcea et al. [20], "for the reduction of azo dyes, reduction to the anion radical occurs by a fast one-electron transfer reaction, followed by a second, slower electron transfer event to produce the stable dianion [5,21–23]." Thus, the functional group in high ED might be not favorable to this second electron transfer to form the dianion, leading to low or no capability for decolorization.

Regarding the effect of chemical structure on decolorization, as indicated in [14] the lag time for color removal of p-MO was insignificant. However, the lag times for decolorization of its isomer o-MO (ca. 12 h) and its derivative o-NO_2-p-MO (ca. 7.5 h) (Fig. 3) were considerably large. According to Shuler and Kargi [24], the lag phase occurs immediately after inoculation and is a period of adaptation of cells to a new environment. Microorganisms reorganize their molecular constituents when they are transferred to a new medium (e.g., dye-laden wastewater). Depending on the composition of nutrients, new enzymes are synthesized and the syntheses of some other enzymes are repressed. Thus, the internal machinery of cells is adapted to new environment conditions. However, there was no lag time for those azo dyes with the smaller-ED or "size" substituents (e.g., hydroxyl in o-OH azo dye and Erichrome black T [14]; and amino group in Congo red [14]) at *ortho* to azo bond. Thus, the large-sized substituent in the proximity of azo linkages very likely resist bacterial decolorization by *P. luteola* because higher levels of essential enzyme expression might be required.

Similar to Orange II azoreductase from *Pseudomonas* KF46 [5], azo dye decolorization by *P. luteola* also strongly dependent upon the relative strength of the electron-withdrawing group(s) and the replacement of chemical groups near the azo-linkage as well. The group substituent closer to azo bond tends to hinder biodecolorization of *P. luteola* as indicated from both isomers of MO and MR (refer to [14] and Figs. 4 and 5 in this study). Moreover, the stronger electron-withdrawing group might enhance color removal of *P. luteola* (e.g., p-MO was more effective to be decolorized than p-MR in Tables 1 and 2). This is because that the sulfo group of p-MO is a stronger electron-withdrawing group than carboxyl group in p-MR [25]. These similar results in azo dye decolorization by *P. luteola* to Orange II azoreductase from *Pseudomonas* KF46 [5] simply suggest that both bacterial and enzymatic decolorization possibly took place through a parallel biochemical mechanism. Kulla et al. [26,27] also supported that microbial cultures could be adapted to produce azoreductase enzymes that had very high specificity towards particular dye structures. It was also shown that sulphonated dyes (e.g., 1-(4′-sulfophenylazo)-4-naphthol (Orange I) and 1-(4′-sulfophenylazo)-2-naphthol (Orange II), stronger e-withdrawing) were reduced faster than carboxylated dyes (e.g., 1-(4′-carboxyphenylazo)-4-naphthol (carboxy-Orange I) and 1-(4′-carboxyphenylazo)-2-naphthol (carboxy-Orange II), relatively weaker e-withdrawing) due to the higher electronegativity of the sulpho group, which renders the azo group more accessible to electrons."

As indicated in time courses of decolorization of phenolic azo dyes isomers 2-(4′-dimethylaminophenylazo) phenol (o-OH), and 3-(4′-dimethylaminophenylazo) phenol (m-OH) (Fig. 8), the rank of biodecolorization rates were m-OH (100 mg L^{-1}) > o-

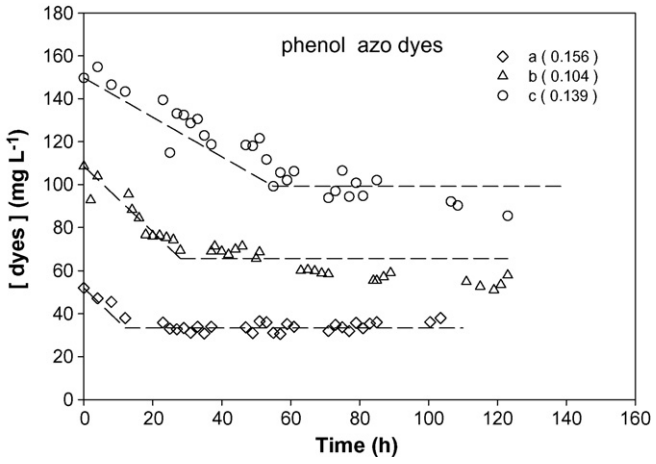


Fig. 8. Time courses of dye "decolorization" for –OH azo dyes (a: 50 mg L^{-1} o-OH; b: 100 mg L^{-1} m-OH; c: 150 mg L^{-1} m-OH). Parathesis is denoted the initial cell density OD_{600} of *P. luteola*.

OH (50 mg L^{-1}) > m-OH (150 mg L^{-1}) (Table 1). Note that phenolic azo dyes are slightly soluble and thus only some concentrations were inspected. The decolorization rates of phenolic azo dyes were less than the rest of azo dyes in Table 1. Because hydroxyl group is electron-releasing group [25], it is probably not biochemically favorable for anaerobic decolorization of *P. luteola*. Hydrogen bonding, in addition to the electron density in the region of the azo bond (may also have) a significant effect on the rate of reduction [20,28]. For example, likely due to the contribution of hydrogen bonding, the position and the nature of substituents on the dye molecule influence the azo-hydrazone tautomerism (i.e., –N=N– → –NH–N=) of hydroxyazo compounds. The hydroxy proton of phenylazo-naphthol derivatives is labile and can bond with a nitrogen atom of the azo group, causing a rapidly formed tautomeric equilibrium between the azo and hydrozone forms. Zimmerman et al. [29] found that, "with certain azoreductases, a decreased rate of reduction was observed when the enzyme system catalyzed the reduction of a dye substrate that was stabilized in the hydrazone form via hydrogen bonding". Due to the steric hindrance of hydroxy group, o-OH was less susceptible to decolorization than m-OH. Apparently, lag times for decolorization of both azo dyes were negligible, significant portion of residual azo dyes were still remained to be intact at least for 140 h. In particular, color removal efficiency of o-OH and m-OH were <40% (ca. >16 h). As the concentration of m-OH slightly increased, the decolorization rate was decreased as well. As Chen [12] mentioned, significant percentages of residual azo dyes after "decolorization" and cell debris after centrifugation were in dye-like color might suggest that the biosorption rather than decolorization was taking place. Similar example of Eriochrome Black T (also contained hydroxyl group in the proximity to azo bond) also showed decreases in color removal at higher concentration [14]. Recently, Chen [13] quantitatively evaluated the toxicity of three amino-phenol isomers in the ranking of *o*-aminophenol > *m*-aminophenol > *p*-aminophenol, indicating that the formation of amine intermediates after decolorization might still feedback to repress color removal.

Evidently, our findings of effects in chemical structure for decolorization by *P. luteola* (i.e., bacterial decolorization) was still in parallel with Knackmuss' predictions [4] and results of enzymatic decolorization [5]. As azo dyes (i.e., xenobiotic compound) could be reduced by biodegradation under anaerobic conditions, these compounds with strong electron-withdrawing group was more feasible to be decolorized. In addition, compared to *ortho*, *meta* positions, the substituent in the *para* position to azo linkage was most viable to be decolorized. Moreover, azo dyes with the substituent(s) (e.g., high ED substituents) right next to azo bond significantly resisted color removal. In particular, the presence of a higher electron-density substituent in azo dye even switched the biodegradability to be recalcitrance. Compared with the decolorization of enzymes which were exposed to environments, microbial biodecolorizers were packaged and apparently resistant to cell lysis of lysozymes in the cultures, performed better than enzymatic decolorization, in particular for a long term. However, the toxicity of pollutants (e.g., dyes or related intermediates) may be lethal to the microbial cellular activity (e.g., color removal), causing the limitation for microbial decolorization.

The follow-up study will be focused on a systematic comparison upon the decolorization versus toxicity of a common monoazo or diazo dye with a variety of functional groups (e.g., hydroxyl groups on benzene or naphthalene; [13,14]). In addition, which conditions (e.g., acclimation) for the growth or decolorization of *P. luteola* could significantly enhance the decolorization performance for heterocyclic azo dyes (e.g., Y3GP CI Reactive Yellow 2; [10]) would be studied.

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References

[1] S.V. Kulkarni, C.D. Blackwell, A.L. Blackard, C.W. Stackhocese, M.W. Alexander, Textile dyes and dyeing equipment, classification, properties and environmental aspects, U.S. Environmental Protection Agency, Research Triangle Park, N.C., EPA-600/2-85/010 (1985).

[2] K.T. Chung, S.E. Stevens Jr., Degradation of azodyes by environmental microorganisms and helminths, Environ. Toxicol. Chem. 12 (1993) 2121–2132.

[3] P.G. Rieger, H.M. Meier, M. Gerle, U. Vogt, T. Groth, H.J. Knackmuss, Xenobiotics in the environment: present and future strategies to obviate the problem of biological persistence, J. Biotechnol. 94 (2002) 101–123.

[4] H.J. Knackmuss, Basic knowledge and perspectives of bioelimination of xenobiotic compounds, J. Biotechnol. 51 (1996) 287–295.

[5] T. Zimmermann, H.G. Kulla, T. Leisinger, Properties of purified orange II azoreductase, the enzyme initiating azo dye degradation by *Pseudomonas* KF46, Eur. J. Biochem. 129 (1982) 197–203.

[6] L.P. Hammett, The effect of structure upon the reactions of organic compounds benzene derivatives, J. Am. Chem. Soc. 59 (1937) 96–103.

[7] T. Suzuki, S. Timofei, L. Kurunczi, U. Dietze, G. Schuurmann, Correlation of aerobic biodegradability of sulfonated azo dyes with the chemical structure, Chemosphere 45 (2001) 1–9.

[8] J.W. Raymond, T.N. Rogers, D.R. Shonnard, A.A. Kline, A review of structure-based biodegradation estimation methods, J. Hazard. Mater. B84 (2001) 189–215.

[9] T.L. Hu, Decolourization of reactive azo dyes by transformation with *Pseudomonas luteola*, Bioresour. Technol. 49 (1994) 47–51.

[10] T.L. Hu, Degradation of azo dye RP_2B by *Pseudomonas luteola*, Water Sci. Technol. 38 (1998) 299–306.

[11] J.S. Chang, C. Chou, Y.C. Lin, P.J. Lin, J.Y. Ho, T.L. Hu, Kinetic characteristics of bacterial azo-dye decolorization by *Pseudomonas luteola*, Water Res. 35 (2001) 2841–2850.

[12] B.Y. Chen, Understanding decolourization characteristics of reactive azo dyes by *Pseudomonas luteola*: toxicity and kinetics, Process Biochem. 38 (2002) 437–446.

[13] B.Y. Chen, Toxicity assessment of aromatic amines to *Pseudomonas luteola*: chemostat pulse technique and dose-response analysis, Process Biochem. 41 (2006) 1529–1538.

[14] C.C. Hsueh, B.Y. Chen, Comparative study on reaction selectivity of azo dye decolorization by *Pseudomonas luteola*, J. Hazard. Mater. 141 (2007) 842–849.
[15] L.P. Wackett, L.B.M. Ellis, Predicting biodegradation, Environ. Microbiol. 1 (2) (1999) 119–124.
[16] B.S. Furniss, A.J. Hannaford, V. Rogers, P.W.G. Smith, A.R. Tatchell, Vogel's Textbook of Practical Organic Chemistry including Qualitative Organic Analysis, fourth ed., Longman, London and New York, 1978, pp. 687–721.
[17] D.A. Skoog, J.J. Leary, Principles of Instrumental Analysis, fourth ed., Saunders College Publishing HBJ, U.S., 1992, pp. 150–155.
[18] H.W. Blanch, D.S. Clark, Microbial growth, in: Biochemical Engineering, Marcel Dekker Inc., New York, 1997, Chapter 3, pp. 181–185.
[19] K.P.C. Vollhardt, N.E. Schore, Organic Chemistry: structure and function, fifth ed., Freeman, U.S., 2007, pp. 14–23.
[20] C.I. Pearcea, J.R. Lloydb, J.T. Guthriea, The removal of colour from textile wastewater using whole bacterial cells: a review, Dyes Pigments 58 (2003) 179–196.
[21] J. Rau, H.-J. Knackmuss, A. Stolz, Effects of different quinoid redox mediators on the anaerobic reduction of azo dyes by bacteria, Environ. Sci. Technol. 36 (2002) 1497–1504.
[22] G.H. Aylward, J.C. Garnett, J.C. Sharp, Alternating and direct current polarography of azobenzene in indifferent electrolyte in dimethylformamide, Anal. Chem. 39 (1967) 457.
[23] J.L. Sadler, A.J. Bard, The electrochemical reduction of aromatic azo compounds, J. Am. Chem. Soc. 90 (1968) 1979–1989.
[24] M.L. Shuler, F. Kargi, How cells grow, in: Bioprocess Engineering: Basic Concepts, second ed., Prentice Hall PTR, New York, 2001, Chapter 6, pp. 154–157.
[25] J. McMurry, Organic Chemistry, 6th ed., Brooks/Cole, Belmont, CA, USA, 2004, pp. 539–543.
[26] H.G. Kulla, Biodegradation of synthetic organic colorants, in: T. Leisinger, R. Hutter, A.M. Cook, J. Nuesch (Eds.), Microbial degradation of xenobiotics and recalcitrant compounds, FEMS Symposium no. 12. London, UK, Academic Press, 1981.
[27] H.G. Kulla, F. Klausener, U. Meyer, B. Lüdeke, T. Leisinger, Interference of aromatic sulfo groups in the microbial degradation of the azo dyes Orange I and Orange II, Arch. Microbiol. 135 (1) (1983) 1–7.
[28] M.I. Beydilli, S.G. Pavlostathis, W.C. Tincher, Biological decolorization of the azo dye reactive Red 2 under various oxidation–reduction conditions, Water Environ. Res. 72 (6) (2000) 698–705.
[29] T. Zimmerman, F. Gasser, H.G. Kulla, T. Leisinger, Comparison of two bacterial azoreductases acquired duringadaptation to growth on azo dyes, Arch. Microbiol. 138 (1984) 37–43.