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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}.