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Toxicity assessment of aromatic amines to *Pseudomonas luteola*: Chemostat pulse technique and dose–response analysis

Bor-Yann Chen *

Department of Chemical and Materials Engineering, National I-Lan University, I-Lan 260, Taiwan, ROC

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Abstract

This study demonstrated a first-attempt of combining chemostat pulse technique (CPT) and dose–response analysis in pursuit of quantitative rankings of toxicity of model aromatic amines (MAAs) in the presence of diazo dye Reactive Red 141 (or Evercion Red H-E7B; RR141) upon *Pseudomonas luteola*. As known, bacterial decolorization performance of azo dyes is directly dependent upon both the characteristics of biochemical reactivity and biotoxicity of dyes and related aromatic amines towards color removal. Thus, the findings herein indicated that the relative toxicity series of MAAs were (1) *ortho* > *meta* > MAA-free control > *para* position (for isomeric aminophenols); (2) $-OH > -SO_3H >$ MAA-free control ($-NH_2$ at *ortho* position); (3) $-COOH > -OH >$ MAA-free control ($-NH_2$ at *meta* position) through the CPT at 200 mg/L MAAs. Comparison on results in higher levels of MAAs at 1000 mg/L almost showed similar relative rankings except 4-aminophenol. Quantification using traditional plate-count method also confirmed nearly similar trends for corresponding MAAs, revealing the promising feasibility of CPT for the toxicity assessment in practical applications. In addition, dose–response analysis regularly used in toxicology was used to quantitatively determine toxicity rankings of MAAs. In conclusion, this feasibility study directly provided a kinetic model to quantify the relative toxicity ranking of MAAs in the presence of RR141. It could provide a viable guideline for assessment on the toxicity or treatability of azo dyes and MAAs to *P. luteola* in wastewater treatment.

Keywords: *Pseudomonas luteola*; Chemostat pulse technique; Biotoxicity; Dose–response analysis; Reactive azo dye; Aromatic amine

1. Introduction

The reactive azo dyes regularly used for textile dyeing and paper printing are characterized by the presence of the azo group, –N=N–, a chromophoric group, that is, a color-producing group. As textile dyes are originally synthesized to be recalcitrant to biodegradation, industrial effluents often contain residual dyes which significantly affect water quality. Usually 30–70% of reactive azo dyes are hydrolyzed and eliminated into wastewater for dyeing processes [1–4]. In addition, inappropriate disposal of dyes in wastewater can cause a threat to public health, as certain azo dyes or their metabolites (e.g., aromatic amines or amino-azo compounds) are highly toxic and potentially carcinogenic [5]. Basically, cytotoxicity of typical azo dyes may be relatively low, but the toxicity of related aromatic amine intermediates are very likely still significantly high due to their carcinogenicity or mutagenicity [1]. For example, large ‘‘clusters’’ of bladder cancer cases were reported among dye workers in 1890s due to aromatic amines as the culprits [6]. Azo compounds can be reduced to amines through cometabolism and the aid of azoreductase [7,8] for decolorization. As aromatic amines are difficult to be removed via traditional wastewater treatment and inevitably tend to be persistent, the toxicity evaluation upon these amines will be apparently crucial to operation success or failure in dye decolorization and biodegradation afterwards. This study was thus intentionally designed to investigate toxic impacts of aromatic amines to decolorizer *P. luteola* for risk assessment in operation.

As indicated previously, biotoxicity of dyes and their intermediary products to biodecolorizers directly determines the performance of dye decolorization and biodegradation. For example, Mechsner and Wuhrmann [9] pointed out that one of the limiting steps for bacterial degradation of azo dyes is microbial uptake into intracellular compartments. Donlon et al. [10] also reported that mordant orange 1 (MO1; an azo dye) was reductively cleaved to aromatic amines which were relatively less toxic towards methanogens. Since aromatic amines

* Tel: +886 3 9357400x711; Fax: +886 3 9357025.
E-mail address: bychen@niu.edu.tw.

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generated by degradation of azo dyes were cytotoxic, respiration-inhibition tests eventually showed increased toxicity in anaerobic treatment [11]. In addition, *Daphnia magna* [12] was used to determine the toxicity of azo dyes in aerobic treatment of textile wastewater. Wang et al. [13] also performed bioluminescence bacteria (Microtox®) to reveal the toxicity of Ramazol Black. Lumistox bacteria were used in evaluation of toxicity of Reactive Black 5 decolorized in a baffled reactor. Moreover, ATA-anaerobic toxicity, respiration/inhibition and *Daphnia magna* tests [14,15] were used to assess the toxicity of Direct Black 38 in an anaerobic/aerobic sequential reactor system. Although aromatic amines may be toxic to decolorizers and in feedback repression to decolorization, quantitative evaluation of toxicity of aromatic amines compared to azo dyes still remained open to be discussed due to the specific characteristics of different decolorizers (e.g., *P. luteola*). Thus, this first-attempt study tended to combine transient reaction kinetics with biotoxicity assessment in pursuit of explicit toxicity rankings among aromatic amines compared to the model diazo dye (i.e., Reactive Red 141 or Evercion Red H-E7B; RR141 for short). As intermediate amines of RR141 cannot be easily purified and identified, this pioneer study thus intentionally selected certain simple model amines to disclose the technical feasibility of the chemostat pulse technique (CPT) for toxicity assessment. Furthermore, to verify the practicality of the proposed CPT method plate-count method was also adopted with aid of dose–response assessment to compare with the results. As known, in the exponential growth phase for batch cultures, cells have adapted to their new environment to multiply in a maximum rate as they can. Hence, the growth rate [16–18] was used as an equilibrium outcome of metabolic status in cells in response to a hostile environment (e.g., metal or phenol-bearing wastewater). Such a core perspective was extended herein to steady-state bacterial cultures in CSTR for toxicity assessment. Kuhn et al. [19] and Goldberg and Er-el [20] mentioned that the CPT via an instantaneous injection could be used to identify growth limiting nutrients for medium optimization [20]. Similarly, this CPT not only was applicable to define the toxic sources, but also quantify toxic level of suspected pollutants present in the culture. After a steady-state growth in LB-bearing continuous culture was achieved, the process of testing chemicals (e.g., aromatic amine and/or RR141) toxic to growth was carried out. First of all, the model aromatic amine and/or RR141 were intentionally injected individually into the culture broth as substrate(s) of either growth-stimulation or inhibition. After a single impulse injection, allowing for a period of ca. 1/3 mean residence time to elapse the cell and dye concentration were continuously determined [20]. So long as the injected substrate stimulated the growth characteristics, an increase in injected substrate concentration should apparently yield a proportional increase in cell concentration. In contrast, if the injected source contained toxic or inhibitory compositions, an increase in injected-source concentration should yield a marked decrease of cell concentration [20].

The objective of this study was to provide a first-attempt from a toxicological perspective to put forward, in significant terms and quantitative evaluations, biotoxicity of model aromatic amines (MAAs) to the biodecolorizer *Pseudomonas luteola* using the CPT. Using CPT, quantitative rankings of toxicity of model aromatic amines (MAAs) in the presence of diazo dye RR141 upon *Pseudomonas luteola* were obtained. Previous study [21] proposed that bacterial decolorization performance of azo dyes was likely directly dependent upon the characteristics of biochemical reactivity and biotoxicity of dyes and related aromatic amines towards decolorization. However, lack of adequate toxicity assessment upon contaminants makes the biotreatment unreliable for on-site applications. This study thus tended to clearly reveal a promising viability of CPT as well as dose–response assessment for toxicity evaluation of MAAs to *P. luteola*.

2. Materials and methods

2.1. Microorganism and culture conditions

Pseudomonas luteola (a facultative strain; [22,23]) predominantly isolated from activated sludge of a dye-containing wastewater treatment plant in Taichung, Taiwan, was used as a reporter strain of decolorization performance. To obtain the synchronous growth characteristics of cultures for study, a loopful of *P. luteola* seed taken from an isolated 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). Approximately 200 mL precultured broth was then inoculated into 1300 mL fresh LB broth (ca. 30 μL/L antifoam 204; Sigma) for aerobic continuous culture (1 vvm, 200 rpm, 30 °C) at $t = -48$ h. After 12 h batch cultivation (i.e., $t = -48$ h), the fresh LB broth stream was pumped in 79 mL/h (i.e., $D \approx 0.060 \pm 0.002\ h^{-1}$) to the fermentor to maintain nearly constant working volume at 1300 mL for continuous cultures (refer to Fig. 1 for experimental setup). Once the steady-state (i.e., $X_0 = 1.15 \pm 0.18$ g/L; data not shown) was achieved at 48 h cultivation (i.e., $t = 0$), appropriate amounts of aromatic amine (AA)-bearing dye solutions (i.e., tested cases) were injected into the fermentor to reach the levels at ca. 200 or 1000 mg/L AA using sterile syringe. Moreover, the aromatic amine-free control was simply the case of injected RR141 alone in the absence of AA. In addition, diazo RR141 was intentionally included in chemostat runs with the aromatic amines to reveal whether the combined toxicity of RR141 and MAA still could be disclosed by the proposed CPT. In these continuous cultures, the pH was not controlled to simulate on-site or in situ practical situations.

For plate-count methods, a loopful of *P. luteola* seed taken from an isolated colony in LB-streak plate was precultured in 50 mL Luria–Bertani medium (LB broth, Miller, Difco) for 12 h at 30 °C, pH 7.0, 200 rpm. Approximately 5% (v/v) cultured broth was then inoculated to fresh LB medium and a cell culture was harvested in the mid-exponential growth phase (ca. 4 h) for further toxicity assessment. Then, the 1.0 mL cell culture was serially diluted with 9.0 mL sterile saline solution (SSS; NaCl 10.0 g/L) and only the diluent with appropriate viable cell concentrations (ca. 1500–15000 cells/mL) was selected as the test seed (TS) for later uses.

2.2. Analytical methods

The model diazo dye (Fig. 2) used for study were C.I. Reactive Red 141 (RR 141; $\lambda_{max} = 544$ nm), which is often used in dyestuff plants and was obtained from Sumitomo, Inc. (Tokyo, Japan). The MAAs (Fig. 2) employed for toxicity assessment are 3-aminobenzoic acid (3ABA; >99% Janssen Chimica), aniline-2 sulfonic acid (A2SA; 95% Aldrich), 2-aminophenol (2AP; 99% Acros Organics), 3-aminophenol (3AP; 99% Aldrich), 4-aminophenol (4AP; 97.5% Acros Organics). Prior to experiments, the dye and amine solutions were sterilized by filtration (Millipore Millex®-GS 0.22 μm filter unit), since the dye and amines may be unstable in moist-heat