Journal of the Taiwan Institute of Chemical Engineers 64 (2016) 180–188

Contents lists available at ScienceDirect

Journal of the Taiwan Institute of Chemical Engineers

journal homepage: www.elsevier.com/locate/jtice

Feasibility study of reduction of nitroaromatic compounds using indigenous azo dye-decolorizers

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ARTICLE INFO

Article history:
Received 14 July 2015
Revised 8 April 2016
Accepted 15 April 2016
Available online 4 May 2016

Keywords:
Anaerobic processes
Bioconversion
Bio-kinetics
Bioremediation
Azo-decolorizer
Nitro reduction

ABSTRACT

This first-attempt study assessed and compared eight model indigenous dye-decolorizing bacteria strains (*e.g.*, *Aeromonas hydrophila* NIU01, *Aeromonas salmonicida* 741,760 and 829, *A. tecta* 644, *Enterobacter cancerogenus* BYm30, *Exiguobacterium acetylicum* NIU-K2, and *Ex. indicum* NIU-K4) for reduction of nitroaromatic compounds (NACs) to aminoaromatic compounds in "cell pellet" (CP) cultures. Possibly due to identical reductases originated from azoreductases and nitroreductases, indigenous decolorizing bacteria also owned promising nitro reduction characteristics. UV–vis spectroscopy and HPLC chromatography studies revealed that the strains have significant azo dye-decolorizing capabilities, and also can reduce 4-nitrobenzoic acid (4-NBA) to 4-aminobenzoic acid (4-ABA). Nitro reduction performance of indigenous bacterial strains were revealed via determination of specific reduction rate (SRR) of 4-NBA to 4-ABA. The ranking of SRRs of eight indigenous bacterial strains to reduce 4-NBA revealed that NIU01 had the greatest reduction potential; however, NIU-K4 had the least SRR in LB-removed cultures. The multiple-spectrum reduction properties of these indigenous decolorizer strains may be applicable to the treatment of various organic pollutants (*e.g.*, azo dyes and NACs).

1. Introduction

As known, nitroaromatic compound(s) (NACs) may be potentially toxic, mutagenic, and possibly carcinogenic [1–3]. They have been widely used in pharmaceuticals, pesticides, explosives, and dyes, and also used as chemical feedstock in various chemical industries [4–7]. Apparently, natural environment is potentially at risk in face of industrial development involving the popular use of NACs, and some have been listed as priority pollutants by US Environmental Protection Agency [8]. As NACs contained strong electron-withdrawing groups in common with azo dyes, these compounds are also electron deficient. That is, they are susceptible to reduction, and not easy to be oxidatively catabolized in aerobic environments [5,6,9]. As literature [10,11] mentioned, nitroreductases and azoreductases may originally derive from similar enzymatic systems. Attentions on nitroreductases have been paid recently not only due to biotechnological potential for environmental bioremediation, but also clinical significance for human health of chemotherapeutic tumor treatment [7]. Although wastewater treatment of activated sludge is well reported in literature, they were not pure cultures and dealt with complicated interaction in mixed consortia. In contrast, when pure cultures were used to treat wastewater, detailed characteristics of such cultures could be confirmed without interference of other microbial species. Thus, this study is to clearly disclose whether different azo dye—decolorizing bacteria also owned characteristics of nitro reduction (*i.e.*, using 4-nitrobenzoic acid (4-NBA) as indicator reaction shown by Rafii and Cerniglia) [10]. Therefore, indigenous bacterial strains (*e.g.*, *A. hydrophila* NIU01, *A. salmonicida* 741,760 and 829, *A. tecta* 644, *E. cancerogenus* BYm30, *E. acetylicum* NIU-K2, and *Ex. indicum* NIU-K4) investigated in this study, were capable of reductively decolorizing azo dyes [12–14], and thus very likely to reduce NACs. This study investigated the interactive association of the reduction of azo compounds with nitro reduction processes using indigenous dye-decolorizing bacteria under anaerobic conditions. Meanwhile, comparative analyses upon the efficiencies of bio-reduction 4-nitrobenzoic acid (4-NAB) by these strains in "cell pellet" (CP) cultures were also assessed for conclusive remarks.

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http://dx.doi.org/10.1016/j.jtice.2016.04.015
1876-1070/

2. Materials and methods

2.1. Microorganism and culture conditions

Indigenous decolorizers (*i.e.*, *A. hydrophila* NIU01, *A. salmonicida* 741,760 and 829, *A. tecta* 644, *E. cancerogenus* BYm30, *E. acetylicum* NIU-K2, and *Ex. indicum* NIU-K4) selected to reduce nitroaromatic compounds were isolated from Taiwan (except BYm30 obtained from California) and identified via 16S rRNA phylogenetic-tree analysis [12–14]. All of the strains were first cultured in LB (Luria-Bertani) broth medium (per liter: 10 g Bacto tryptone, 5 g Bacto yeast extract, 10 g sodium chloride) for 12 h at 30 °C, 125 rpm in water-bath shaker (SHINKWANG, SKW-12). Then, the precultured broth with appropriate volume ratio was inoculated into fresh LB broth medium (1% (v/v)) for shake flask cultures [12–14]. Once cells had grown to late exponential or early stationary phase (*ca.* 6 h), shaking for aeration was switched off and the culture was kept in static incubation for treatability testing to reduce 4-nitrobenzoic acid (4-NBA, purchased from Alfa Aesar Chemicals).

2.2. Reduction of 4-nitrobenzoic acid in "cell pellet" culture

To assess capabilities of nitro reduction of indigenous bacterial strains in LB-removed cultures, all of the strains were cultured as aforementioned in Section 2.1. Then, they were centrifuged and rinsed by using 0.1 M PBS (containing: sodium chloride, potassium chloride, disodium hydrogen phosphate, and potassium dihydrogen phosphate obtained from Aldrich), pH 7.00. Cell cultures were centrifuged and rinsed three-times to guarantee no residues from prior cultures to obtain "cell pellet"(CP) culture. Then, CP cultures were added to 4-NBA solution in deionized water (100 mg/L) for static incubation for reduction. The 4-NBA solutions were sterilized by filtration (Millipore Millex-GS 0.22 lm filter unit).

2.3. Analytical methods

2.3.1. UV detection of the reduction of 4-NBA

Pure chemicals 4-nitrobenzoic acid (4-NBA), 4-aminobenzoic acid (4-ABA), 3,4-dihydroxybenzoic acid (3-4-dHBA, protocatechuate), 4-hydroxybenzoic acid (4-HBA), and 3-hydroxy-4-aminobenzoic acid (3-H-4-ABA) obtained from Alfa Aesar Chemicals were scanned by UV–vis spectrophotometer (HITACHI Spectrophotometer, model UV-2001) to distinguish the wavelength of maximal absorption from each other (Fig. 2a). In addition, UV–vis spectra of 4-ABA and 4-NBA would be chosen as standards for reference (Fig. 2b) to compare with the spectra of eight 4-NBA—containing CP cultures during 0–4 day(s) (Figs. 1 and 2 as detailed figure of NIU01 in Fig. 1).

2.3.2. Identification and quantification of 4-nitrobenzoate and 4-aminobenzoate by HPLC-UV and HPLC-MS

Samples were analyzed by HPLC-UV (HITACHI) on an SUPELCO 516 C-18 column (5 μm; 4.6 × 250 mm) and compared with pure 4-nitrobenzoate (4-NBA) and 4-aminobenzoate (4-ABA) for qualitative and quantitative determination. The mobile phase was an isocratic solvent system consisting of phosphate buffer (3.4×10^{-2} mM, pH 3.37): acetonitrile 75:25 (v/v) at 1 ml/min [15]. Compounds were monitored at UV OD230 nm with a diode array detector L-2455(HITACHI). Samples were derived from CP culture of strains containing 4-NBA at initial time and after incubating for 30 h or 4 days to convert 4-NBA to 4-ABA for nitro reduction (Fig. 4).

The LC-ESI-MS/MS system consisted of two model LC-10 AD pumps (Shimadzu, Kyoto, Japan), 5 μl sample loop, two six-port switching valves (Rheodyne, Cotati, CA, USA), a precolumn (100 μm i.d. × 1.5 cm C18 fused-silica capillary) and a separation column (75 μm i.d. × 10 cm C18 fused-silica capillary). The separation column was tapered and also served as the ESI emitter. The analysis methods of HPLC-MS-MS were described elsewhere [27].

2.3.3. Calculation of specific reduction rate

The relationships between biomass concentrations and $OD_{600\,nm}$ for strains NIU01, 740, 760, 829, 644, BYm30, K2, and K4 are 1.0 $OD_{600\,nm} \cong 0.3730$ [16], 0.6328 ± 0.0757, 0.4143 ± 0.0339, 0.3910 ± 0.0140, 0.5828 ± 0.0462, 0.4320 ± 0.0060, 0.5379 ± 0.0209 and 0.4635 ± 0.0299 g/L dry cell weight, respectively.

The concentration of reduced-4-ABA was measured by HPLC in Section 2.3.2 upon sample supernatants after centrifugation. Samples were diluted with appropriate volume of phosphate-saline solution (PBS) to an optical density of less than 0.6 when absorbance was not within the linear range (*ca.* 0.1–0.7). Since the function of cell density (X) and 4-ABA concentration ([4-ABA]) are continuous, strictly monotonic and differentiable for all time, their differential terms dX and d[4–ABA] could thus be denoted by forward-difference formula (e.g., $dX \cong X|_{t+\triangle t} - X|_t = \triangle X, d[4-\mathrm{ABA}] \cong [4-\mathrm{ABA}]|_{t+\triangle t} - [4-\mathrm{ABA}]|_t = \triangle [4-\mathrm{ABA}]$) for specific rate of reduction determination (*e.g.*, SRR). To ensure the step size $\triangle$ t sufficiently small for convergence, numerical differentiations were compared with differentiations by reducing step size as $\triangle t/2$(*i.e.*, $||(df|_{\triangle t} - df|_{\triangle t/2})||/||df|_{\triangle t}||$). Only the error term less than 1% was then defined within the calculated tolerance for convergence. Otherwise, the step size $\triangle$ t was reduced by half for approximations until convergence was achieved. Therefore, specific reduction rate SRR could be calculated [16,17].

3. Results and discussion

3.1. Identification of 4-aminobenzoic acid via UV–vis and LC-MS-MS spectra

Bioremediation of 4-NBA might occur either by bioconversion or cell adsorption. To clarify this point, UV–vis spectra of the 4-NBA supernatants were inspected before and after reduction individually using eight indigenous bacterial strain (*e.g.*, *A. hydrophila* NIU01, *A. salmonicida* 741,760 and 829, *A. tecta* 644, *E. cancerogenus* BYm30, *E. acetylicum* NIU-K2, and *Ex. indicum* NIU-K4) cultures, and qualitatively confirmed 4-NBA at day 0 and 1–4 days after bacterial reduction of 4-NBA to 4-ABA. (Fig. 1(a–h)). Strains NIU01, 741, 760, BYm30, 829, and 644 displayed significant capabilities of reducing 4-NBA to 4-ABA from day 1 to 4 (UV–vis spectra Fig. 1(a–f)). Additionally, products of bio-converting 4-NBA into 4-ABA using strain 760 was analyzed by LC-MS-MS (Fig. S1–3). Comparing LC-MS chromatograms of CP culture of strain 760 for 4-NBA reduction (Fig. S1 (c) and (d)) with the standards of 4-ABA and 4-NBA (Fig. S1 (a) and (b), respectively), strain 760 apparently could bio-reduce 4-NBA to 4-ABA. Moreover, MS-MS spectra of CP culture of strain 760 for 4-NBA reduction (Fig. S2(b) and Fig. S3(b)) were consistent with 4-ABA (Fig. S2 (a)) and 4-NBA (Fig.S3 (a)) standards.

However, NIU-K2 and NIU-K4 showed lower capabilities of reducing 4-NBA (Fig. 1g and h). NIU-K2 and NIU-K4 both were adapted and isolated from conditions of salt-halotolerance with dye-decolorizing capabilities [13]. Nevertheless, other six decolorizer strains (*e.g.*, NIU01, 760, 741, 829, BYm30, and 644) were well adapted and isolated from fresh water and soil by decolorization of azo dyes [12,14]. As literature indicated [10,11], nitro reduction is metabolically-related to biodecolorization. Nitroaromatic compounds are strong electron withdrawing because of strong electronegativity of the nitro group (similar to electron-withdrawing characteristics of azo dyes). Thus, they might be bio-reduced to amino-aromatic compounds under anaerobic conditions