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Exploring new strains of dye-decolorizing bacteria

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This study unveiled a new strategy to explore new indigenous strains with excellent decolorization capabilities from freshwaters and seawaters. Two new bacterial decolorizers DX2b and SH7b, which have the capability to decolorize textile dyes, were isolated from Cross-Strait Taiwan and China. According to PCR-augmented 16S rRNA gene analyses for strain identification, >99% of nucleotide sequences in isolated strains were identical to type strains *Rahnella aquatilis, Acinetobacter guillouiae, Microvirgula aerodenitrificans,* and *Pseudomonas* sp. Time-series inspection upon azoreductase activity assay and generation of decolorized intermediates all confirmed in parallel with reductive decolorization of new decolorizers DX2b and SH7b. The result also showed that bacterial decolorization of these new strains was mainly catalyzed via the enzymatic expression of azoreductase and riboflavin reductase, and biosorption seemed not to play a crucial role color removal (approximately <10%).

[**Key words:** Bacterial decolorization; Indigenous decolorizers; Growth association; Azoreductase; Microbiological identification]

Azo dyes are the largest chemical class of dyes frequently used for textile dyeing and paper printing (approximately >60% of worldwide use). These industrial effluents often contain a significant amount of residual dye due to the inefficiency in dyeing processes, inevitably affecting the water quality. Thus, they become a threat to public health and natural ecology since certain azo dyes or their metabolites (e.g., aromatic amines) are highly toxic and potentially carcinogenic (1,2). The color of azo dyes is attributed to the azo bond (i.e., $-N=N-$), the associated auxochromes and a system of conjugated double bonds. In a natural environment, azo dye can be transformed or degraded by a variety of microorganisms, including aerobic and anaerobic bacteria and fungi (3).

This study was to explore new indigenous dye-decolorizing strains from the most biodiverse regions in Cross-Strait Taiwan and Mainland China for sustainable applications (e.g., simultaneous wastewater decolorization and biomass energy uses) (4). The island of Taiwan lies in a complex tectonic area formed by the forced collision of the forerunners of the Eurasian Plate and the Philippine Sea Plate. Although Taiwan Strait separates Taiwan from Mainland China, most of western Taiwan is geologically associated to China (e.g., Cross-Strait Fujian Province). That was why this study selected China and Taiwan as sites of strain isolation for comparison. As prior studies (4,5) have been undertaken to seek for naturally-occurring microbes solely for azo dye biodecolorization, this study tended to conduct in depth survey of bacterial decolorizers with potency to decolorize both azo and non-azo dyes for uses in more environmentally-friendly biodegradation. With dye decolorization as the selection characteristics to isolate bacteria from indigenous microbiota, four bacterial strains with promising decolorization capabilities for non-azo dye RBu198 and several azo dyes were resulted. According to protein expression profiles and PCR-augmented 16S rRNA analyses in comparison with gene sequences available in the NCBI BLAST GenBank, these isolated decolorizers were identified to be *Rahnella aquatilis* DX2b, *Acinetobacter guillouiae* Ax-9, *Microvirgula aerodenitrificans* SH7b, *Pseudomonas* sp. SH98 (> 99% of nucleotide identity). *Pseudomonas* spp. (e.g., *P. aeruginosa*, *P. putida*) are well-characterized bacteria with capabilities for bioremediation of petroleum-contaminated soil (6,7). *Acinetobacter* spp. (e.g., *A. calcoaceticus* and *A. baylyi*) are capable of degradation of myriads of organics (e.g., diesel and heating oil, chloroaniline) (8,9). *R. aquatilis* is capable to produce exopolysaccharide and solubilize hydroxyapatite (10,11). *M. aerodenitrificans* can perform biological nitrogen removal (12). As a matter of fact, strains DX2b and SH7b were new bacterial decolorizers predominantly isolated from natural environment (3,13). They were new dye-decolorizing bacteria with capabilities to decolorize non-azo dyes (e.g., reactive blue 198 and reactive blue 19) and azo dyes (e.g., orange I and reactive blue 160). To the best of our knowledge, there are no reports in literature revealing dye decolorization characteristics of strains in the *Rahnella* and *Microvirgula* genera.

MATERIALS AND METHODS

Analytical methods Sixteen textile dyes classified in three categories (purchased from Everlight Chemical Ltd., Taipei, Taiwan) were used for study including (i) non-azo

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dye—oxazine: reactive blue 198 (RBu198), anthraquinone: reactive blue 19 (RBu19) and acid blue 264 (AB264), (ii) monoazo dyes—direct orange 39 (DO39), reactive blue 160 (RB160), reactive red 198 (RR198), orange I (O I) and orange II (O II) and (c) diazo dyes—acid yellow 42 (AY42), direct blue 22 (DB22), direct yellow 86 (DY86), reactive blue 171 (RB171), reactive blue 222 (RB222), reactive blue 5 (RB5), reactive green 19 (RG19), reactive red 141 (RR141), reactive yellow 84 (RY84), acid black 172 (AB172) (14). Here, O I (λ_{max} = 476 nm) was selected for quantitative comparison upon decolorization of bacterial isolates. The dye and cell concentrations were determined as described elsewhere (4). The growth and decolorization performance could be calculated by specific growth rate (SGR) μ, and specific decolorization rate (SDR) q_P (7) as follows (4):

$$SGR = \mu = d\ln X/dt \quad (1/X)(\Delta X/\Delta t) \tag{1}$$

and

$$SDR = q_P = (1/X)(d[Dye]/dt) = (1/X)(\Delta[Dye]/\Delta t) \tag{2}$$

respectively; where X, $[Dye]$, and t were cell concentration, dye concentration, and time, respectively.

Screening protocol for decolorizers Freshwater or seawater samples obtained from 19 sites in Cross-Strait—Taiwan and China (i.e., freshwater samples: Alancheng-Xiyigou (AX), Alancheng-Youyongcheng (AY), Baoshi-Shan quanshui (BS), Datong-Xiao miji (DX), Jiu-Liao xishui (JL), Jing-Yidaxue xingzhenglou (JY), Lan-Hua xi (LH), Si-Makusi (SM), Song-Huajiang (SH), Tou-Cheng nongchang (TC), Wu-Shi xi (WS), Xi-Men guojia kuaiji xueyuan (XM); seawater samples: Bo-Hai (BH), Bei-Gan ginbicun (BG), California (CA), Lv-Dao (LD), Mission Bay (MB), Zhuang-Wei (ZW), Qi-Gu yanshui (QG); Table S1) were used to isolate high-performance dye-decolorizing bacteria. Samples CA, MB were obtained from Southern California as a cross-Pacific Ocean control for comparison. Aqueous solutions of 10, 40 g L^{-1} NaCl were used for regular freshwater and seawater sample LB cultures, respectively. The 60 g L^{-1} NaCl was used for cultures of QG samples since this sample was originally selected from highly-salty environment in southern Taiwan. First, 100 mL fresh samples were mixed with the fungicide nystatin 0.1 g L^{-1} (approximately 8200 units L^{-1}) to remove fungi during isolation. Then, samples were well agitated in a water-bath shaker (SHINKWANG, SKW-12) at 125 rpm for 30 min to achieve complete mixing. Then, 1.0 mL-mixed samples were inoculated in 5 mL LB broth containing 200 mg L^{-1} RBu198 or RBu19 for 24 h preculture. LB broth (Luria–Bertani) (in g L^{-1}) contained casein peptone 10.0, yeast extract 5.0, sodium chloride 10.0 and 200 mg L^{-1} test non-azo dye (i.e., RBu198 or RBu19). After 24 h preculture, static incubation for color removal was carried out and decolorization capability of mixed cultures was evaluated over time. To obtain strains with board-spectrum decolorization capabilities, only ranked top 4 mixed cultures (i.e., AX, DX, JL, SH) of decolorization capabilities to both non-azo and azo dyes were selected for bacterial isolation afterwards. Then, strain isolation on four mixed cultures AX, DX, JL and SH was carried out via at least three cycles of serial streak selections to ensure the purity of individual isolates from microbial communities. These four mixed cultures were then isolated via three-step serial purification as follows: (i) Approximately >5 colonies in different radii, color or morphology from each mixed culture were taken for at least three cycles of colony purification and isolation; (ii) approximately 200 colonies obtained 3-serial isolation were taken as seeds in 5.0 mL test-tube vial cultures to quantify their decolorization capabilities of RBu198 and RBu19; (iii) since only RBu198 could be decolorized by most of pure isolates and RBu19 was totally not decolorized by pure isolates, 19 colonies with top-rank color removal performance of RBu198 were then selected for pre-screening strain identification through comparison of protein expression profiles in SDS-PAGE.

Microorganism and culture conditions Decolorization performance of the identified decolorizer isolates was carried out as follows: First, 1% (v/v) seed culture was inoculated for 12 h preculture in 100-mL LB broth at 30°C, 125 rpm using a water-bath shaker. Then, all precultured broths in appropriate volume ratios were inoculated into fresh LB broth with 200 mg L^{-1} dye for color removal experiments. Once cells had been grown to late exponential or early stationary phase (approximately 12 and 24 h for pure and mixed cultures, respectively), shaking was switched off and the culture was kept in static incubation for decolorization (i.e., $\forall t \geq 0$). Then, time courses of decolorization at various initial dye concentrations were determined.

Morphological and biochemical test Morphological examination was observed by a light microscope (Zeiss Axioskop). BD Gram stain kits and reagents (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and the Ryu non-staining KOH method (15) were used to ascertain the Gram reaction. Biochemical identification presented in the Microgen ID microtests was determined by Microgen ID software Version 1.1.16.19 according to as recommended by the manufacturer (Microgen Bioproducts, UK).

16S rRNA gene sequencing and phylogenetic analysis Amplification and sequence analysis of the 16S rRNA genes was performed as described previously (16). The sequence was compared with others available in GenBank. The multiple-sequence alignment including closest relatives of isolates was performed using the BioEdit program (17). The phylogenetic reconstruction was inferred by using the neighbor-joining method in the BioEdit software. A bootstrap analysis (confidence values estimated from 1000 replications of each sequence) was performed for the neighbor-joining analysis using the CLUSTAL W 1.7 program (18). A phylogenetic tree was drawn using the TREEVIEW program (19). Sequence identities were calculated using the BioEdit program.

Assay for azoreductase activity Bacterial cultures sampled at various time of dye decolorization were taken for azoreductase activity assay. According to Maier et al. (20), assays were carried out in cuvette (path length = 1 cm) with total volume of 1 mL. First, cultivated cells were separated by centrifuge at 13,000 rpm for 5 min and supernatants were removed. Next, precipitated cells were twice washed with sterile phosphate-buffer saline (PBS 0.05 M) solution to remove residual dye and metabolites in cultures. Then, cells concentrated in 5 mL were mixed with 5 mL 0.05 M PBS solution as mix-vials. The mix-vial was well mixed with 8 μL 2-mercaptoethanol, 100 μL chloroform, and 100 μL of 0.1% SDS for 1 min as the reaction pre-mix. This pre-mix was precipitated via centrifuge at 13,000 rpm, 4°C. Next, 200 μL supernatant was degassed with nitrogen purge and mixed with 400 μL 0.05 M PBS solution, 200 μL reactive black 5 (RB5) 100 mg L^{-1}, 200 μL degassed NADH (7.09 g L^{-1}) as reaction mix. Then, time course of dye decolorization was carried out for azoreductase activity assay using cell-free cases as zero control. The assay reaction was initiated by the addition of 200 μL NADH and was monitored photometrically at 597 nm (20). The slope of initial linear decrease of absorption ($\Delta A\ min^{-1}$) was used to calculate azoreductase activity based on the molar absorption coefficient of RB5 (ε = 35.5 $mmol^{-1}\ cm^{-1}$). One nanokatal was defined as 1 nmol dye-reduced s^{-1}.

Assay for laccase and reductase enzymes All the enzymatic activities were assayed by a spectrophotometer (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA) at kinetic mode and 37°C for the intra-celllular proteins in which cells were washed twice with deionized water and disrupted via sonication. The cell debris was then removed by centrifugation at 12,000×g for 15 min at 4°C and the supernatant was collected. The activity of laccase was determined in a reaction mixture with ABTS [2,2-azinobis(3--ethylbenzathiazoline-6-sulfonic acid)] as the substrate. The 205 μL reaction mixture contained 100 μL of 1 mM ABTS, 100 μL of 50 mM citric acid buffer (pH 3.0), and 5 μL of enzyme solution. The enzymatic activity was monitored kinetically via an increase in A_{420} using ABTS as the substrate. The riboflavin reductase was measured by the modified method of Fontecave et al. (21). The reaction was performed using a solution (final volume, 200 μL) containing 10 μL of 0.4 mM of NADH, 100 μL of 0.4 mM riboflavin, 80 μL of sodium phosphate buffer (50 mM, pH 7) and 10 μL intracellular enzyme. For NADH reductase activity, the reaction was performed using a solution (final volume, 200 μL) containing 100 μL of 0.4 mM of NADH, 90 μL of sodium phosphate buffer (50 mM, pH 7) and 10 μL intracellular enzyme. The decrease in A_{340} was then measured spectrophotometrically. Reaction rates were calculated by using a molar extinction coefficient of 36 mM/cm for ABTS and 6.22 mM/cm for NADH, respectively. One unit of enzyme activity was defined as 1 μmol of substrate reduced or oxidized per minute. To ensure that the finding was not taking place by chance, all enzyme assays were performed in triplicate.

Electrochemical measurements Cyclic voltammetry of azo dyes was performed using an electrochemical workstation (Jiehan 5600, Jiehan Company, Chiung, Taiwan) at 50 mV s^{-1} scan rate. The working, counter, and reference electrodes were, respectively, a glassy carbon electrode (0.07 cm^2), platinum wire electrode (10.8 cm^2), and an Hg/Hg_2Cl_2 electrode filled with saturated KCl. The glassy carbon electrode (GCE, ID = 3 mm; model CHI104, CH Instruments Inc., Austin, TX, USA) was successively polished with 0.05 μm alumina polish and then rinsed with 1 M NaOH (for protein removal) and deionized water before use. The experiments were performed in 0.1 M phosphate-buffered saline (PBS) at dye concentration of 7.52 mM. Prior to analyses all solutions were purged with nitrogen for 15 min. The redox potentials recorded vs. Hg/Hg_2Cl_2 reference electrode were corrected by 0.241 V (i.e., E^0 of Hg/Hg_2Cl_2) to the standard hydrogen electrode (SHE).

RESULTS AND DISCUSSION

Decolorization of pure and mixed cultures As indicated in Table S1, the ranking of decolorization performance of non-azo dyes for mixed cultures was RBu198 > RBu19 > AB264. Regarding the lowest color removal efficiency of AB264, cell precipitate of all samples after high-speed centrifugation (approximately 12,000 rpm) showed in blue color, which was more evident to indicate biosorption primarily as the mechanism of "decolorization" of AB264. A similar example of biosorption was also observed in the blue color of *Desulfovibrio desulfuricans* biosorbed with Cu(II) ions and yellow color of *Pseudomonas luteola* biosorbed with reactive acid yellow 72 (RAY) (22,23). As a matter of fact, dye decolorization might be metabolism-associated, since dye decolorization inevitably required combined interactions of membrane-associated transport, internal compartmentalization of dye into cytoplasm, catalysis of dye-decolorizing enzymes and mediation of electron transport chain for functioning. As inadequate decolorization was likely due to dye toxicity (23), intracellular bioaccumulation of AB264 via biosorption was possibly due to cytotoxicity of AB264 to bacterial cells. When dye decolorization by cells was insufficient, a significant gradient in AB264 concentration would tend to enforce diffusion of this dye into the bacterial cytoplasm for bioaccumulation (i.e., dye storage instead of decolorization). To prevent "pseudo"-decolorization of AB264, only dye RBu198 and RBu19 were selected as indicator dyes for further isolation of decolorizers. Among all cultures, 4 top-ranked samples AX, DX, JL, SH in decolorization performance were obtained as candidates for strain isolation (Table S1). However, pure