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Comparative assessment upon dye removal capability of indigenous bacterial strains from Lanyang Plain in northeast Taiwan

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ABSTRACT

This study provides a first attempt from a geological and ecological perspective to look forward isolations of indigenous strains with the decolorization capability from the most biodiverse region in Taiwan for dye-laden wastewater treatment. Serial selections were conducted by a specific use of the fungicide nystatin and model azo dye C.I. reactive red 141 (RR141) during isolation. Several bacterial strains with the excellent capability of azo dye decolorization were predominantly isolated from river water and mud samples of Lanyang River Basin. Phase-curve profiles indicated that azo dye decolorization was found to be non-growth associated for both mixed cultures and isolated pure strains. The color removal efficiency of the mixed culture was nearly 10-fold to that of *Pseudomonas luteola* at ca. 600 mg L^{-1} RR141, indicating a promising feasibility of isolated cultures to be applicable for practical treatments. The decolorization performance of unacclimated and acclimated pure cultures was at most 20% and 70–80% to that of the mixed cultures, respectively. It might suggest that combined interactions among decolorizers were crucial for the optimal color removal. According to the results of physiological and 16S rRNA gene sequence examinations, the isolated strains should belong to *Aeromonas* species (very likely *A. hydrophila*).

1. Introduction

Since the first synthetic dye, mauevin, was developed in 1856 for practical uses, azo dyes have been the largest chemical class of dyes frequently used for textile dyeing and paper printing. These industrial effluents often contain a significant amount of residual dye due to the inefficiency in dyeing processes. It affects the water quality, and may become a profound impact to public health since certain azo dyes or their metabolites (e.g., aromatic amines) may be highly toxic and potentially carcinogenic [1,2]. Textile dyes were originally synthesized to be recalcitrant to biodegradation (ca. $BOD_5/COD < 0.2–0.3$). The color of azo dyes is attributed to the azo-bond, the associated auxochromes and a system of conjugated double bonds. The azo group, –N=N–, is a chromophoric group, that is, a color-producing group. Up to 50% of reactive dyes is lost through the hydrolysis of industrial dyeing processes and at least 15% of azo dyes are released as pollutants into the environment [3–5]. These synthetic dyestuffs are usually recalcitrant to conventional wastewater treatment (e.g., activated sludges). In the natural environment, azo dye can be transformed or degraded by a variety of microorganisms, including aerobic and anaerobic bacteria and fungi [6–9]. For example, bacterial degradation of azo dye is often initiated under anaerobic conditions by an enzymic biotransformation step that involves cleavage of azo linkages with the aid of azoreductase utilizing reduced coenzyme (i.e., NADH) as the electron donor [10]. Azo compounds can also be reduced to amines through cometabolism [11]. The resulting aromatic amines are further degraded by multiple-step bioconversion taking place aerobically or anaerobically [12,13]. Fungal species (e.g., *Phanerochaete chrysosporium*) use lignin peroxidase to degrade azo dyes under aerobic conditions [14]. Pure fungal culture have been used to develop bioprocesses for mineralization of azo dyes [15–17]. However, the long growth cycle and moderate decolorization rates limit the performance of the fungal decolorization system [7]. In contrast, bacterial decolorization is normally faster, but it may require a mixed microbial community to mineralize azo dyes completely through a combined metabolism with an anaerobic–aerobic sequence [11,18].

The goal of this study was to look out for indigenous strains with the specific characteristics from the most biodiverse region in Taiwan for applications (e.g., wastewater treatment, pharmaceutical uses). Taiwan, like most industrialized countries in the world, is facing an environmental crisis. One factor contributing to the crisis is inordinate deforestation for industrial developments, leading to significant losses of habitats for various species. In the past two decades, the inhabitants on Taiwan have raised public aware-

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Nomenclature

ODU	Absorbance units
q_p	specific decolorization rate (mg L^{-1} h^{-1} ODU^{-1})
RR141	C.I. Reactive red 141
SC	stock culture
t	time in aerobic growth ($t < 0^-$) or anaerobic decolorization ($t > 0^+$)
X	cell concentration (ODU)

Greek letter

μ	specific growth rate, SGR (h^{-1})

ness to live in hormony with their highly populated environment to save "Formosa" for sustainable development. In particular, bioremediation of contaminated environments in Taiwan is considered as a priority "green technology", as contaminants can be completely destructed at a lower cost and faster rate with no lingering liability. According to Taiwan EPA's drafted guidelines for incorporating biodiversity conservation, indigenous rather than foreign microbiota should be first considered for bioremediation. Incidentally, to rescue this possibly last "Shangri-La" in Taiwan, an effective strategy to protect vanishing the biodiversity in northeast Taiwan would be inevitably required.

With the deliberate exclusion of polluted industries to be settled in I-Lan County and the geological separation of Lanyang Plain from Central Mountain Range, the abundant microbiota in such a geologically specific and ecologically diverse environment were saved to be explored in Taiwan. This first-attempt feasibility study tended to isolate the strains with the biodecolorization capability of a model diazo dye reactive red 141 (RR141) from indigenous microorganisms in the Lanyang Plain. This study indicated that after serial acclimation to achieve stable color removal activities the color removal efficiency of isolated strains was approximately 4–5-fold better than that of *Pseudomonas luteola* [19–21]. In addition, the color removal efficiency of the mixed culture was nearly 10-fold to that of *P. luteola* at ca. 600 mg L^{-1} RR141, indicating a promising feasibility of isolated cultures to be applicable for practical treatments [22].

2. Materials and methods

2.1. Screening protocol for decolorizers

To probe the presence of high-performance bacterial decolorizers, diazo reactive red 141 (RR141; chemical structure refer to Fig. 2 in Chen [22]) was used for comparative selection upon feasible dye-degrading isolates. In addition, to exclude fungi during screening 0.1 g L^{-1} (ca. 8200 units L^{-1}) fungicide nystatin (powderized from effervescent vaginal tablets— Mycostatin™; [23]) was added via sterilization through 0.2 μm membrane filter. River and mud samples were obtained from various sources (e.g., Lanyang Hsi and Dongshan River in Lanyang River Basin (site to obtain isolates herein), hot and cold springs and fountain springs sources near the Central Mountain range such as Chiao-Hsi, Su-Ao) in the Lanyang Plain. With a selection pressure of nystatin and RR141, the bacterial strains with color removal capability were predominantly separated from the microbial communities in samples. Serial streak selections (at least 3 cycles) were then applied to guarantee the purity of individual isolated strains. At least 10 bacterial strains with the excellent capability of azo dye decolorization were predominantly isolated from river water and mud samples of Lanyang River Basin, hot and cold springs and fountain springs water sources on the Lanyang Plain in the northeast Taiwan. The bacteria-isolating procedures were conducted in a screening medium 1/5XLB (in g L^{-1}: casein peptone 2.0, yeast extract 1.0, sodium chloride 10.0) and RR141 0.2 g L^{-1}. To reveal the presence or absence of bacterial decolorizers, all of the samples inoculated with isolates were cultured in 5.0 mL RR141-containing 1/5XLB medium using a water-bath incubator (SHINKWANG, SKW-12) at 30 °C for 18–20 h. Note that to prevent any unintentional exclusion of possible decolorizers in samples, only fresh samples (<4 h storage in 4 °C after sampling) were taken for a representative screening among viable populations on site. A loopful of seed from sampled vials in the presence of nystatin was then taken to streak onto a LB-streak agar plate (per

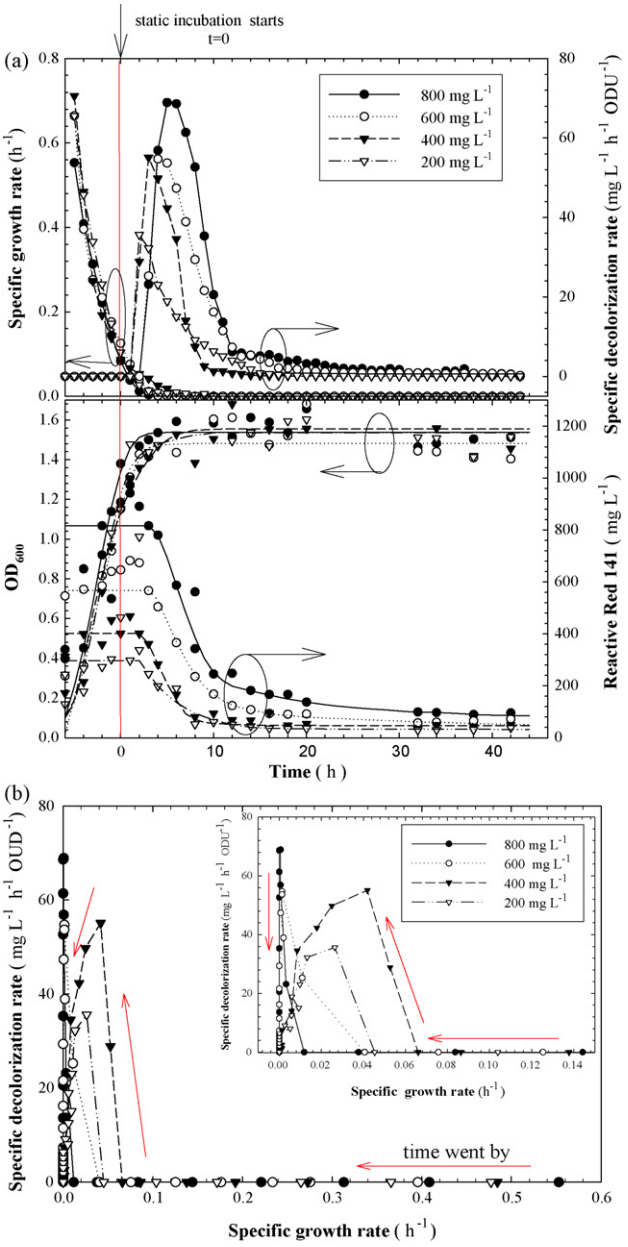

Fig. 1. (a) Time courses of cell growth, color removal, specific decolorization rate and specific growth rate for a mixed culture. Cell growth (i.e., $t < 0$) via shaking was undertaken at various initial RR141 concentrations. Decolorization started at $t = 0$ by static incubation. (b) Phase-curve profiles of specific decolorization rate versus specific growth rate for a mixed culture at various initial RR141 concentrations. The arrows indicated the orientation of all trajectories of profiles (or the joint vector of movement) as time increased.

L: 10.0 g casein peptone, 5.0 g yeast extract, 10.0 g sodium chloride, 30.0 g Bacto agar). After 12 h incubation, colonies with a significant growth (i.e., colonies in larger radii on the agar-plate) on plates were chosen for 5 mL liquid cultures in RR141-containing 1/5XLB media to probe the existence of mixed cultures with decolorization capability (only 1 colony present such a bioactivity in 30 colonies). First assessment of color removal performance upon this mixed culture (Fig. 1) was carried out according to the following routine decolorization steps (RDSs). Prior to the decolorization step, the 1% (v/v) decolorizer-present precultured broth was inoculated into fresh 1/5XLB broth for 12 h culture at 30 °C, pH 7.0, 125 rpm using a water-bath shaker. Subsequently, the culture (controlled at the inoculum $(2.9 \pm 0.3) \times 10^5$ cfu/mL) was switched to a 50 mL RR141-containing 1/5XLB medium for 6 h in aerobic cultures (30 °C, 125 rpm) and then conducted static incubation for color removal [21].

The mixed culture was then used for the isolation of pure strains via three-step serial purification as follows: (1) all colonies streaked onto LB-agar plates from mixed cultures were taken as seeds in 5.0 mL test-tube vial cultures for probing decolorization capability. (2) Only cultures with color removal activities were then streaked onto LB-agar plates and only 2–3 representative colonies (e.g., colonies with large sizes and different morphology) were selected for further purity tests. (3) All representative colonies were chosen as seeds in 5.0 mL test-tube vial cultures to evaluate decolorization capability. After three-step serial purification, only 55 test-tube vials were confirmed as pure isolates with decolorization potential and then mixed in a glycerol-containing medium as stock cultures (SCs; Figs. 2 and 3) for a long-term storage at −80 °C. After four times three-step serial purification upon stock cultures (e.g., KB15 in Fig. 4), the most metabolically stable decolorizers with the maximal color removal capability were obtained (Figs. 5 and 6).

2.2. Microorganism and culture conditions

The 1% (v/v) seed culture was taken for a preculture in 50-mL 1/5XLB broth at 30 °C, pH 7.0, 125 rpm for 24 h using a water-bath shaker. Then, all precultured broths in appropriate volume ratios were inoculated into fresh 1/5XLB broth for culture. In these flask cultures, the pH was not controlled. Once the cells had grown to late exponential or early stationary phase (i.e., 12–15 h), shaking was switched off and the culture was kept in static incubation (i.e., $\forall t \geq 0$) for decolorization. Acclimated cultures were also carried out in the same procedures for comparison. Samples were diluted to an optical density less than 0.6 when absorbance was not in the linear range (ca. 0.1–0.7). The relationship between the biomass concentration, viable cell density and $OD_{600\,nm}$ is 1.0 ODU ≅ 0.373 g L^{-1} dry cell weight ≅ $(1.7 \pm 0.2) \times 10^8$ cfu/mL.

2.3. Analytical methods

The model azo dye used for decolorization was C.I. reactive red 141 (RR141; λ_{max} = 544 nm; [22]). Dye solutions were sterilized by filtration (Millipore Millex®-GS 0.22 μm filter unit), since these dyes may be unstable in moist-heat sterilization. With appropriate calibrations at specific wavelengths, concentrations of biomass and dyes were determined using an UV–vis spectrophotometer (HITACHI Spectrophotometer, model UV-2001). The concentration of dye was primarily determined by measuring the optical density (OD) of the supernatant of the sample after centrifugation for 2 min at 700 × *g* (HSIANGTAI Centrifuge MCD-2000). In addition, a sterile cell-free medium was chosen as the control. As all samples contained biomass and dye, concentrations of biomass (i.e., (1) and

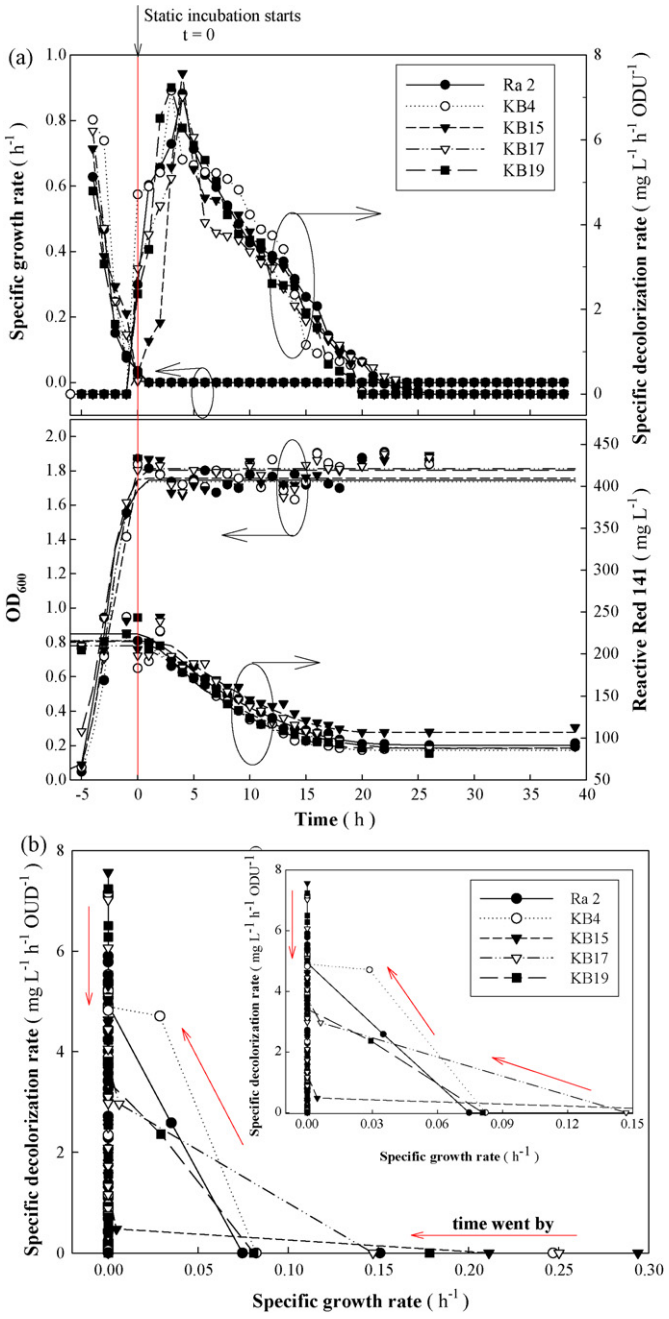

Fig. 2. (a) Time courses of cell growth, color removal, specific decolorization rate and specific growth rate for unacclimated cultures Ra2, KB4, KB15, KB17, KB19. Cell growth (i.e., $t < 0$) via shaking was undertaken at various initial RR141 concentrations. Decolorization started at $t = 0$ by static incubation. (b) Phase-curve profiles of specific decolorization rate versus specific growth rate for unacclimated cultures Ra2, KB4, KB15, KB17, KB19 at various initial RR141 concentrations. The arrows indicated the orientation of all trajectories of profiles (or the joint vector of movement) as time increased.

(2)) and dye (i.e., (3)) were evaluated as follows:

$OD_{600\,nm}$ of sample mixtures without centrifugation : $OD^{X+dye}_{600\,nm}$

$$= OD^{dye}_{600\,nm} + OD^{X}_{600\,nm}, \quad (1)$$

$OD_{600\,nm}$ of sample supernatant (sup) after centrifugation :

$$OD^{sup}_{600\,nm} = OD^{dye}_{600\,nm}, \quad (2)$$

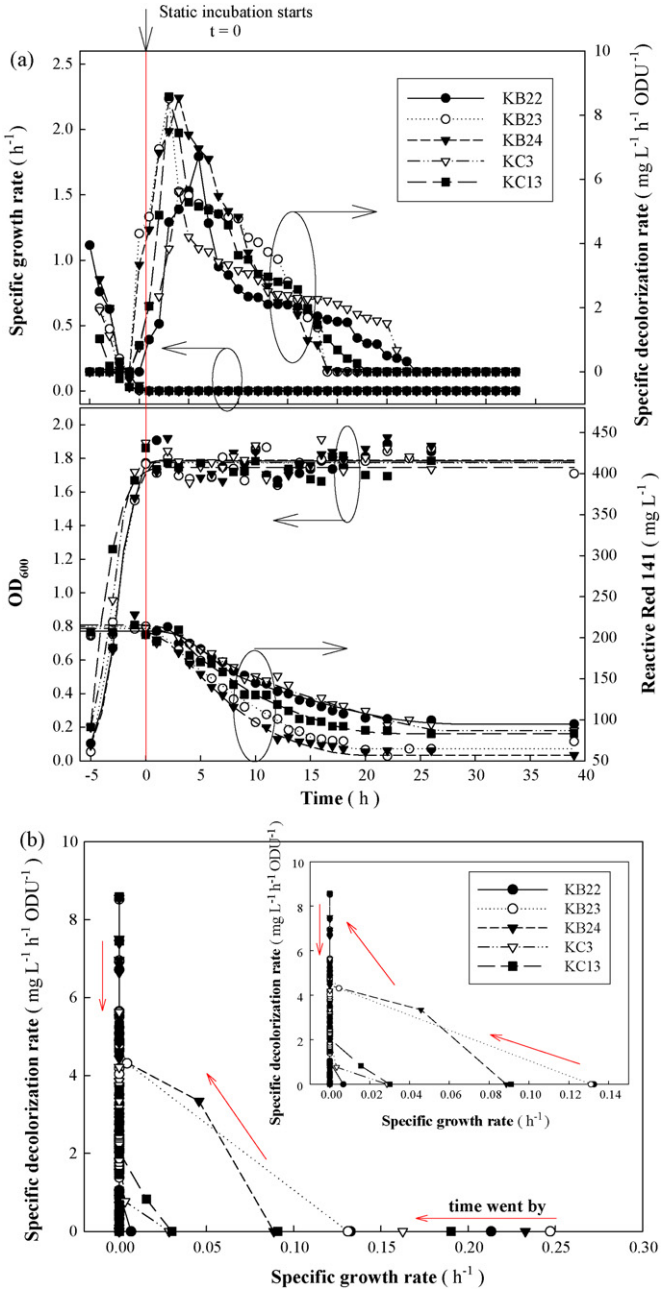

Fig. 3. (a) Time courses of cell growth, color removal, specific decolorization rate and specific growth rate for unacclimated cultures KB22, KB23, KB24, KC3, KC13. Cell growth (i.e., $t < 0$) via shaking was undertaken at various initial RR141 concentrations. Decolorization started at $t = 0$ by static incubation. (b) Phase-curve profiles of specific decolorization rate versus specific growth rate for unacclimated cultures KB22, KB23, KB24, KC3, KC13 at various initial RR141 concentrations. The arrows indicated the orientation of all trajectories of profiles (or the joint vector of movement) as time increased.

and

$$\mathrm{OD}_{\lambda_{\max}} \text{ of sample supernatant after centrifugation} : \mathrm{OD}^{\mathrm{sup}}_{\lambda_{\max}} = \mathrm{OD}^{\mathrm{dye}}_{\lambda_{\max}}. \quad (3)$$

Samples were diluted to an optical density of less than 0.6 when absorbance was not within the linear range (ca. 0.1–0.7). As the function of cell density (X) and dye concentration ([Dye]) are continuous, strictly monotonic and differentiable

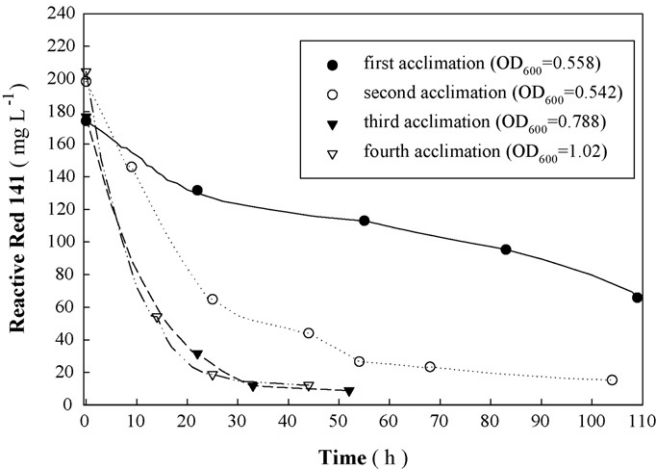

Fig. 4. Decolorization profiles for cells of KB 15 in different acclimation cycles.

for all time, their differential terms dX and d[Dye] can be denoted by forward-difference formula (e.g., $\mathrm{d}X \cong X|_{t+\Delta t} - X|_t = \Delta X$, $\mathrm{d[Dye]} \cong [\mathrm{Dye}]|_{t+\Delta t} - [\mathrm{Dye}]|_t = \Delta[\mathrm{Dye}]$) for specific rate determination. To ensure the step size Δt sufficiently small enough for convergence, numerical differentiations were compared with differentiations by reducing step size as $\Delta t/2$ (i.e., $\|(\mathrm{d}f|_{\Delta t} - \mathrm{d}f|_{\Delta t/2})\|/\|\mathrm{d}f|_{\Delta t}\|$). Only the error term less than 1% was defined as calculated tolerance to achieve convergent criteria. Otherwise, the step size Δt was reduced by half for approximations until convergence was attained. Therefore, specific growth rate (SGR) μ, and specific decolorization rate (SDR) q_{P} could be calculated as

$$\mathrm{SGR} = \mu = \frac{\mathrm{d}\ln X}{\mathrm{d}t} \cong \left(\frac{1}{X}\right)\left(\frac{\Delta X}{\Delta t}\right) \quad \text{and}$$

$$\mathrm{SDR} = q_{\mathrm{P}} = -\left(\frac{1}{X}\right)\left(\frac{\mathrm{d[Dye]}}{\mathrm{d}t}\right) \cong -\left(\frac{1}{X}\right)\left(\frac{\Delta[\mathrm{Dye}]}{\Delta t}\right),$$

respectively; where X, [Dye], and t stand for cell concentration, RR141 concentration, and time, respectively.

2.4. Morphological and biochemical test

Morphological examination was observed by a light microscope (Zeiss Axioskop). Gram Stain Set S (BD, USA) and the Ryu non-staining KOH method [24] 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 the recommendation of the manufacturer (Microgen Bioproducts, UK).

2.5. 16S rRNA gene sequencing and phylogenetic analysis

Amplification and sequence analysis of the 16S rRNA gene was performed as described previously [25]. The sequence was compared with others available in GenBank. The multiple-sequence alignment including six hydrogen-producing strains and their closest relatives were performed using the BioEdit program [26]. 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 w1.7 program [27]. A phylogenetic tree was drawn using the TREEVIEW program [28]. Sequence identities were calculated using the BioEdit program.

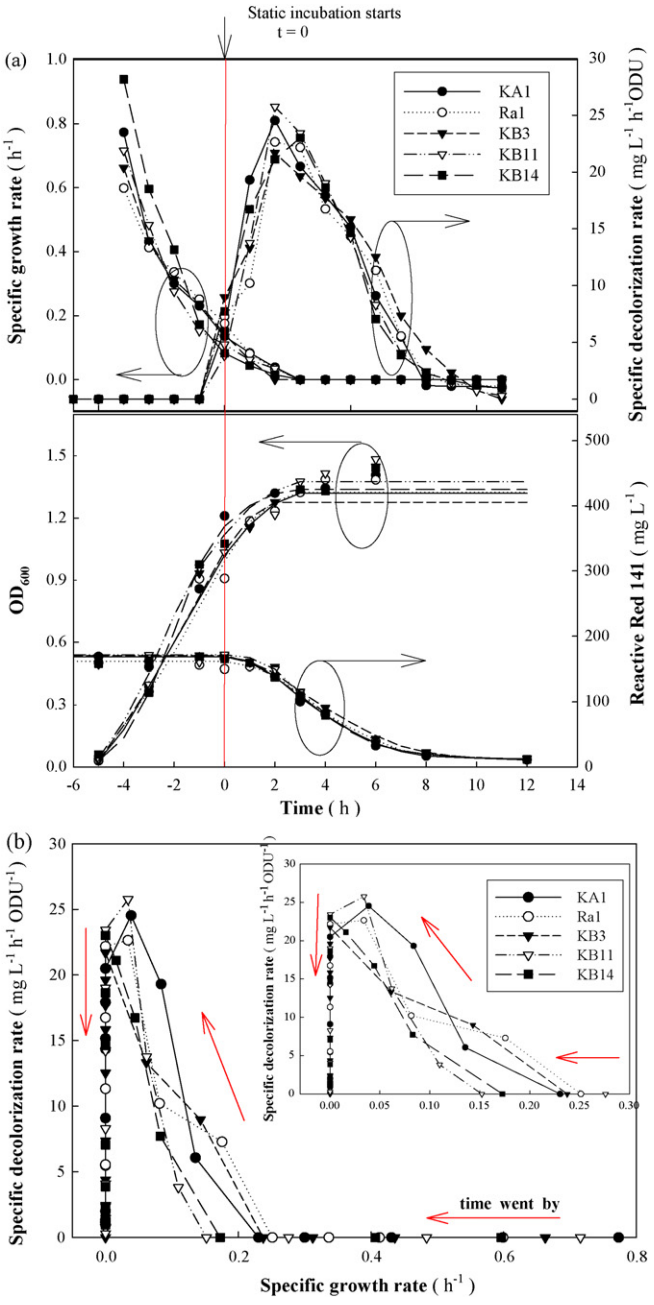

Fig. 5. (a) Time courses of cell growth, color removal, specific decolorization rate and specific growth rate for unacclimated cultures KA1, Ra1, KB3, KB11, KB14. Cell growth (i.e., $t < 0$) via shaking was undertaken at various initial RR141 concentrations. Decolorization started at $t = 0$ by static incubation. (b) Phase-curve profiles of specific decolorization rate versus specific growth rate for unacclimated cultures KA1, Ra1, KB3, KB11, KB14 at various initial RR141 concentrations. The arrows indicated the orientation of all trajectories of profiles (or the joint vector of movement) as time increased.

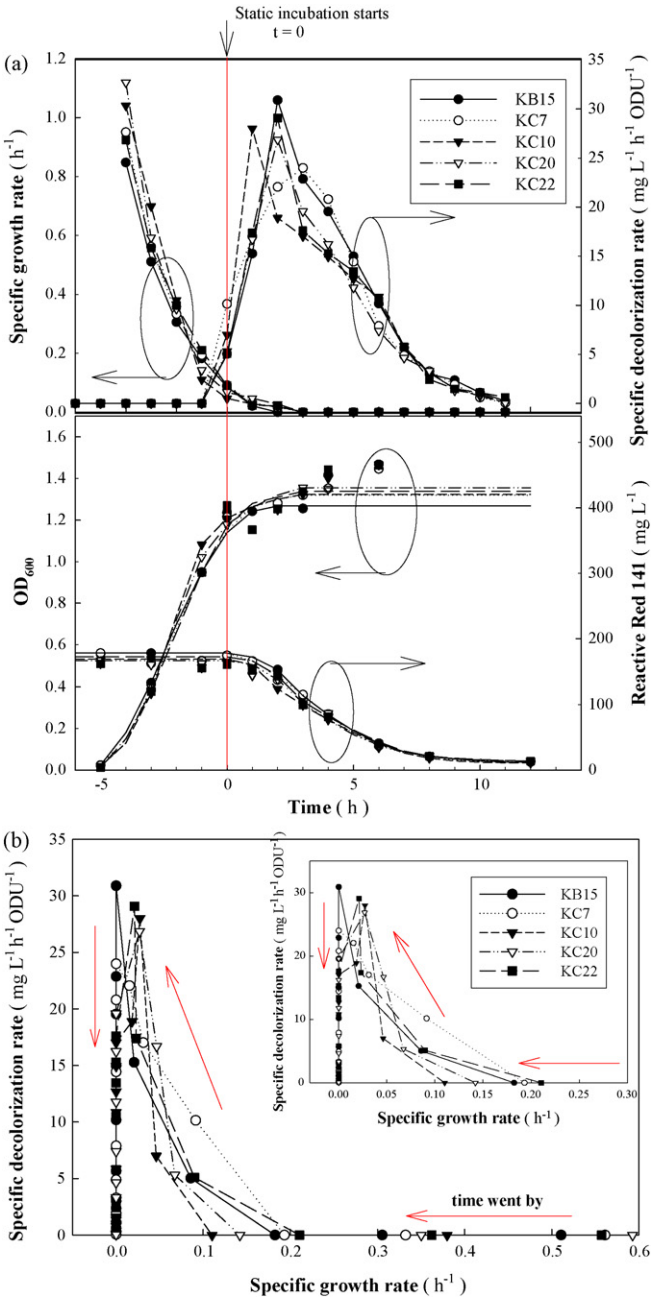

Fig. 6. (a) Time courses of cell growth, color removal, specific decolorization rate and specific growth rate for unacclimated cultures KB15, KC7, KC10, KC20, KC22. Cell growth (i.e., $t < 0$) via shaking was undertaken at various initial RR141 concentrations. Decolorization started at $t = 0$ by static incubation. (b) Phase-curve profiles of specific decolorization rate versus specific growth rate for unacclimated cultures KB15, KC7, KC10, KC20, KC22 at various initial RR141 concentrations. The arrows indicated the orientation of all trajectories of profiles (or the joint vector of movement) as time increased.

3. Results and discussions

3.1. Mixed culture

After screening upon river and mud samples from various sources, one mixed culture (only one positive from total 30 tested vials) from the Lanyang Hsi and Dongshan River in Lanyang River Basin was found to reveal a significant color removal capability of the model dye RR141. As indicated in Fig. 1, the decolorization of RR141 took place with static incubation (i.e., anaerobic condition; $t > 0^+$) and was repressed in aerobic cultures during aerobic growth ($t < 0^-$). As literature indicated [14–16], only fungal culture was feasible to develop aerobic mineralization of azo dyes. Thus, our result simply suggested the absence of any aerobic decolorizers in this mixed culture due to the successful exclusion of fungi by use of nystatin during screening. However, in this mixed consortium perhaps due to the presence of anaerobic microorganisms (e.g., facultative strains) for growth, cell growth was still not completely inhibited in early static incubation (ca. $t = 0$–6 h). Combined interactions (e.g., competition, mutualism) among species in the

mixed culture also slightly delayed the biodecolorization capability to be expressed (i.e., early incubation at $t = 0$–6 h). As indicated in Fig. 1(a), dye decolorization reached a maximal value when cell growth had nearly stopped at the stationary phase (ca. 1–2 h delay after static incubation). Such a non-growth-associated phenomenon suggested that anaerobic decolorizers were dominated in the mixed culture. That is, SGR progressively decreased to approximately zero and SDR gradually increased (Fig. 1(b)). However, SDR reached a maximum value at nearly 3–5 h after static incubation and then gradually decreased. This decrease in decolorization might be due to a significant accumulation of toxic intermediates (i.e., aromatic amines), which were formed by a cleavage of azo-bonds. In addition, the exhaustion of essential nutrients (e.g., basic growth factors and energy sources) and formations of inhibitory products limited cells to regenerate NADH, which is a required electron donor for bacterial decolorization. In addition, as the initial RR141 concentration increased, the maximal SDR was also increased in parallel. This point might suggest that RR141 and its related intermediates were not so toxic to this mixed culture compared to *P. luteola* [22]. This might explain why the maximal SDR (ca. 60 mg L^{-1} h^{-1} ODU^{-1}) at 600 mg L^{-1} RR141 was nearly 10-fold to maximal SDR of *P. luteola* [21,22], showing a more promising color removal capability taking place in the mixed culture.

Plane-curve profiles of SDR versus SGR at various time (Fig. 1(b)) also showed the trajectories of decolorization at various initial RR141 concentrations. By increasing values of time, the orientation of all profiles started at certain points (e.g., 0.24 h^{-1} for 800 mg L^{-1}) in $+x$-axis (i.e., +SGR-axis) and headed to negative x-direction to the points (e.g., 0.01–0.02 h^{-1}) near the origin, and then turned along $+y$-axis (i.e., +SDR-axis). Once the trajectories reached a maximal value of ca. 70 mg L^{-1} h^{-1} ODU^{-1}, they asymptotically decayed back to the origin along negative y-direction. The resulting trajectories along x and y-axes clearly illustrated the growth-independent characteristics of bacterial decolorization.

3.2. Unacclimated cultures

To reveal the existence of possible predominant "hero" decolorizers in the mixed cultures, decolorization experiments at 200 mg L^{-1} using 55 SCs as seed cultures were carried out (e.g., only rank top 10 in maximal SDR were plotted in Figs. 2 and 3). As shown in Figs. 2(a) and 3(a), apparently none of any maximal SDR (the best for KC13 at 8.6 mg L^{-1} ODU^{-1} h^{-1}; Fig. 2(a)) in unacclimated seed cultures could achieve the maximal SDR in the mixed culture (36.5 mg L^{-1} ODU^{-1} h^{-1}; Fig. 1(a)). According to the maximal SDR (unit in mg L^{-1} ODU^{-1} h^{-1}), the top 10 rankings in a decreasing order are KC13(8.6) > KB24(8.51) > KB15(7.6) > KB19(7.23) > KB4(7.2) > Ra2 (7.1) > KB17(7.0) > KB22(6.7) > KC3(5.6). Comparison upon Figs. 1–3 simply suggested that symbiotic and cooperative interactions (e.g., the aluruistic behavior of some strain released extracellular metabolites as "stimulators" for an enhancement in decolorization; [29]) among species in mixed cultures for decolorization might be still significant to provide higher efficiency in color removal compared to unacclimated single strains. It might suggest that decolorization performance could be significantly increased after serial acclimation. With serial adaptations in dye-laden environments, bacterial cells should provide a more efficient machinery for color removal in order to achieve maximal and stable decolorization capability. As indicated in Figs. 2(b) and 3(b), dye decolorization performance of the top 10 strains was confirmed to be non-growth associated. Although RR141 is a diazo dye, the biphasic phenomena of decolorization for diazo reactive black B taking place in *P. luteola* [21] were not observed in these isolated strains (Figs. 2(a) and 3(a)). This explained why all the trajectories in Figs. 2(b) and 3(b) were almost not located within the first quadrant, but located in +SDR and +SGR axes. This might indirectly suggest that RR141 was not toxic to our isolated strains compared to *P. luteola*.

3.3. Acclimated cultures

As the unacclimated pure cultures could not perform efficient color removal efficiencies, serial acclimation upon these unacclimated cultures was conducted in order to obtain metabolically stable cultures with maximal decolorization capabilities. For example, for the culture KB15, the initial SDR (in mg L^{-1} h^{-1} ODU^{-1}) in the serial acclimation gradually increased (i.e., 3.62 (first) < 11.43 (second) < 13.96 (third) < 17.65 (fourth)). After the fourth cycle in acclimation, the most metabolically stable dye decolorization was achieved as SDR was nearly invariant (<∼5–10% variation). The monotonic increases in initial SDR simply revealed a promising feasibility of this acclimation protocol to attain maximal color removal performance. After this serial acclimation to obtain metabolically stable decolorizers, the top 10 rankings of maximal SDR (in mg L^{-1} h^{-1} ODU^{-1}) among isolated strains in a decreasing order are KB15(30.9) > KC22(29.0) > KC10(28.0) > KC20(26.9) > KB11(25.8) > KA1(24.5) > KC7(24.0) > KB14(23.0) > Ra1(22.6) > KB3(21.7) (Figs. 5(a) and 6(a)). However, the maximal SDR for the best decolorizer KB15 was still ca. 20% less than the maximal SDR in mixed culture (36.5 mg L^{-1} h^{-1} ODU^{-1}) at initial RR141 concentration 200 mg L^{-1}. This might point out combined cooperative interactions among decolorizers played a crucial role for optimal color removal. Since almost all of unacclimated cultures could tolerate in the presence of RR141 for decolorization (Figs. 2(a) and 3(a)), evidently fully acclimated cultures could perform much better decolorization performance without any toxic stress (Figs. 5(a) and 6(a)). This also confirmed that the trajectories in phase-plane plots in Figs. 5(b) and 6(b) were located in +SDR and +SGR axes for non-growth-association of color removal.

3.4. Microbiological identification

After screening upon the isolates via SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis; data not shown), all protein spectra (i.e., translation profiles) were similar and thus all isolates identified to be phenotypically related (also genetically related if they are prokaryotic). In addition, microscopic examination showed that six strains (27@M11K5, 133@D9E9, 157@G3.5H11, KB23, KB24, 52@D9E15) were in rod shape (1.0–2.5 μm × 0.5–0.8 μm) with the Gram negative reaction. Note that strains 27@M11K5, 133@D9E9, 157@G3.5H11, 52@D9E15 were some indigenous strains also isolated from I-Lan as controls. Biochemical identification by Microgen ID microtest system indicates that the six strains were similar to each other and all belong to genus *Aeromonas*. Two strains (KB23 and 133@D9E9) were randomly selected to determine their sequences of 16S rRNA gene. Further inspections upon gene diversity [30] in samples taken from the Lanyang Plain will be conducted for conclusive remarks. Based on the sequence identity of 16S rRNA gene, KB23 and 133@D9E9 resemble to each other with a 99.9% identity. The highest similarity values of two strains were obtained towards type strains of *Aeromonas hydrophila* ATCC 7966^T (98.1% identity) and *Aeromonas media* ATCC 33907^T (97.8% identity). The similarity levels towards other *Aeromonas* species were below 97.5%. The 16S rRNA gene phylogenic tree was constructed for the two strains and those of the described *Aeromonas* species were shown in Fig. 7. According to the results of physiological and 16S rRNA gene sequence examinations, the strains should belong to *Aeromonas* species.

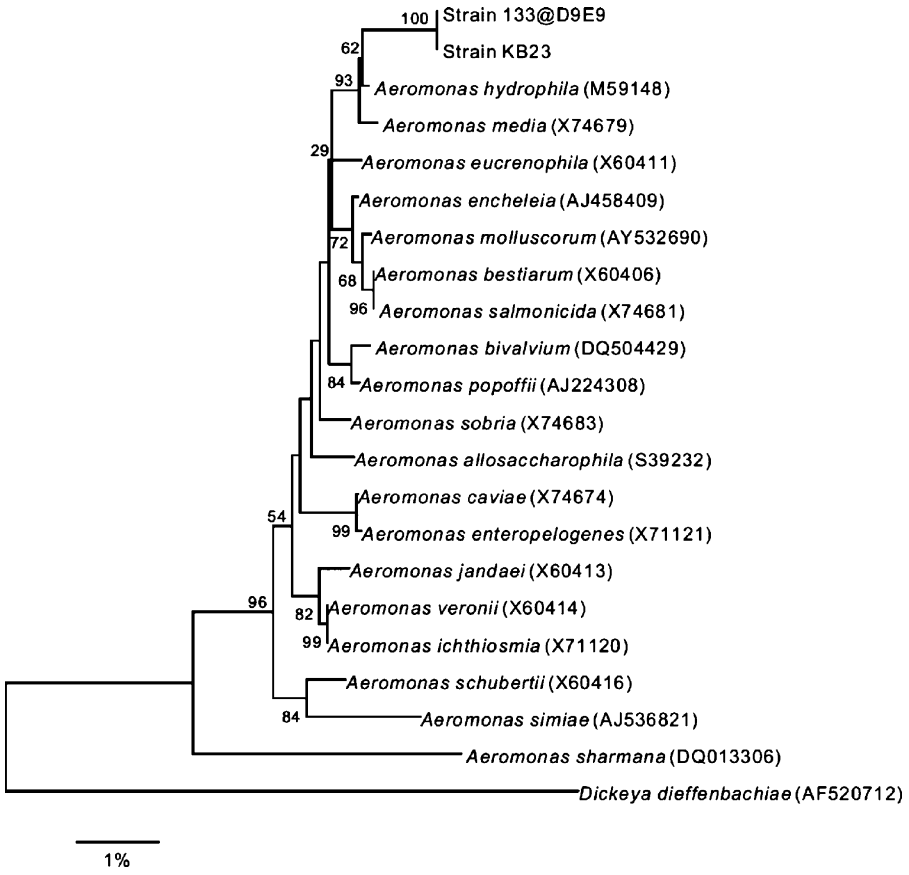

Fig. 7. Phylogenetic tree analysis: neighbor-joining showing phylogenetic positions of two strains and *Aeromonas* species based on 16S rRNA gene sequence comparisons. *Dickeya dieffenbachiae* was used as an outgroup. Bootstrap values are indicated at nodes. Only bootstrap values > 50% are shown. Scale bar denotes 1% sequence dissimilarity (one substitution per 100 nt). Representative sequences in the dendrogram were obtained from GenBank (accession number in parentheses).

4. Conclusion

In summary, the SDRs of unacclimated bacteria were approximately only one-fifth of SDR for mixed culture. The SDRs of metabolically stable biodecolorizers after serial acclimation only increased to ca. 70–80% of SDR for mixed culture. It might suggest that combined cooperative interactions among strains in mixed cultures might play a vital role to maximize color removal performance (refer to [29,31,32] for similar cooperative phenomena). In comparison with color removal performance, our isolated strains were approximately 4–5-fold better than *P. luteola* [19–21] (which was predominantly isolated from activated sludge of a dye-containing wastewater treatment plant). This might suggest that the most feasible bacteria for efficient color removal are not absolutely necessary to be isolated only in contaminated or polluted environments likely due to different tolerance to toxic intermediate aromatic amines [2]. According to the results of physiological and 16S rRNA gene sequence examinations, the isolated strains should belong to *Aeromonas* species (very likely *A. hydrophila*). However, similar to a possible human pathogen, *Pseudomonas aeruginosa* (that is popularly used for bioremediation of petroleum-contaminated soil and groundwater), *Aeromonas* species is probably more appropriate to be cultivated in closed-vessel bioreactor due to its likely pathogenicity.

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