Continuous treatment of Acid Red B with activated sludge bioaugmented by a yeast *Candida tropicalis* TL-F1 and microbial community dynamics

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**ABSTRACT**

Continuous treatment of Acid Red B (ARB) with activated sludge (AS) bioaugmented by an azo-degrading yeast *Candida tropicalis* TL-F1 under aerobic conditions was investigated in the form of sequencing batch tests. Dynamics of both bacterial and fungal communities were analyzed using polymerase chain reaction followed by denaturing gradient gel electrophoresis (PCR-DGGE) method. The results showed that bioaugmentation with the yeast TL-F1 improved the performance of AS for continuously decolorizing, degrading and detoxifying ARB. Meanwhile, the AS systems bioaugmented by the yeast TL-F1 showed higher sludge concentration and better AS settleability. The result of PCR-DGGE suggested that microbial communities of both bacteria and fungi shifted due to treatment of ARB and bioaugmentation. Some dominant bacteria and fungi were identified as probably efficient degraders of ARB or its decolorization byproducts. Furthermore, the yeast TL-F1 was found as one of the dominant fungi in all the three bioaugmented systems, suggesting that bioaugmentation was successful due to the colonization of the yeast TL-F1 in AS systems.

**Key words** | Acid Red b, bioaugmentation, *Candida tropicalis* TL-F1, continuous treatment, PCR-DGGE

**INTRODUCTION**

Azo dyes, with one or more azo groups (N=N), represent the largest class of synthetic dyes, which are widely applied in various industries such as textile, food, paper and leather (Tan et al. 2009). A large amount of azo dyes are discharged into the environment during production and utilization processes every year. The dyes themselves and their breakdown byproducts (e.g. aromatic amines) are toxic to aquatic organisms or even mutagenic and carcinogenic to humans (Qu et al. 2010). Additionally, azo dyes significantly affect the photosynthetic activity of hydrophytes by reducing light penetration (Gou et al. 2009). Therefore, it is necessary to remove azo dyes from wastewaters before discharge to the environment.

Many azo dyes are difficult to remove due to their physical and chemical properties, such as high water solubility and low exhaustion. Physicochemical methods are applied for treatment of azo dyes; however, they are generally costly and may produce secondary pollution (Waghmode et al. 2012). By contrast, biological processes are environment-friendly and cost-effective alternatives. Many microorganisms, including bacteria and fungi, have been confirmed as capable of decolorizing or even mineralizing azo dyes (McMullan et al. 2001). Among them, bacteria are widely used for azo dyes treatment due to their high metabolic activity and environmental adaptability. For example, Franciscon et al. (2012) found that *Brevibacterium* sp. VN-15 could decolorize and detoxify various azo dyes including Reactive Yellow 107, Reactive Black 5, Reactive Red 198 and Direct Blue 71. However, most of the bacteria would be inhibited by the recalcitrant and toxic intermediates (e.g. some aromatic amines), which limits their large-scale application. In comparison, fungi display higher resistance to recalcitrant and toxic organics due to their extracellular, nonspecific and nonstereoselective ligninolytic enzymes including lignin peroxidase, manganese peroxidase and laccase (Kües 2015). Furthermore, some studies indicated that the co-culture of fungi and bacteria would be more efficient and stable for treating recalcitrant organics than pure cultures of bacteria or fungi due to their synergistic effects. For example, Gou et al. (2009) reported that the co-culture...
of *Penicillium* sp. QQ and *Sphingomonas xenophaga* QYY showed higher efficiency for decolorizing Reactive Brilliant Red X-3B than any single strain. Besides, Su & Lin (2013) found that another bacterium–fungus co-culture consisting of *Bacillus* and *Aspergillus niger* also displayed higher efficiency for decolorization of Reactive Red 120 than single *A. niger* or *Bacillus*. It was indicated that fungi can degrade the mixture of many kinds of macromolecule organics into small molecule substances in order to improve the biodegradability of wastewater, and then the residual simple organics can be further degraded or even mineralized by bacteria in the co-cultures. Such synergistic effects of fungi and bacteria in their co-cultures lead to efficient degradation and even detoxification of azo dyes, thus provide a potentially useful alternate for field application.

The co-culture of selected microbes can be constructed through bioaugmentation, which is a promising method to improve the performance of biodegradation (Herrero & Stuckey 2015). The microbes used for bioaugmentation were often focused on bacteria or bacterial consortiums (Wang et al. 2014; Chen et al. 2016). However, the bacteria-based bioaugmentation might be ineffective due to the specificity of bacterial metabolic enzymes. By contrast, fungi could be effective for bioaugmentation due to their nonspecific and nonstereoselective ligninolytic enzymes, as mentioned above. For instance, as a group of single-celled fungi, yeasts could produce some important enzymes including lipase, phytase, protease and some oxidases or peroxidases that could degrade various aromatic substances (Yang et al. 2013). Li et al. (2015) reported that an activated sludge (AS) system bioaugmented by a yeast *Candida tropicalis* TL-F1 exhibited high efficiency for treating wastewater containing Acid Red B (ARB) in a membrane bioreactor (MBR). The yeast TL-F1 finally survived and became the dominant fungal species in the MBR. Additionally, the fungi-based bioaugmentation methods were also used for treating other pollutants such as heavy metal ions (Bahafid et al. 2015). Although the corresponding studies are still limited, this method has shown the potential for improving traditional bioprocesses aiming at treating recalcitrant industrial pollutants.

In this study, the effect of bioaugmentation with an azo-degrading yeast, *Candida tropicalis* TL-F1, on continuous treatment of synthetic ARB wastewater was estimated in the form of sequencing batch tests in Erlenmeyer flasks. Effects of the inoculum size of strain TL-F1 on decolorization, organics removal and detoxification processes, as well as the growth and settling property of AS, were investigated. Additionally, shifts of microbial (both of bacterial and fungal) community structures were monitored using polymerase chain reaction followed by denaturing gradient gel electrophoresis (PCR-DGGE) method. The corresponding results were expected to expand the knowledge of azo dyes treatment by fungi-bioaugmented bioreactors and provide useful clues for the bioremediation of other recalcitrant organic pollutants.

### MATERIALS AND METHODS

#### Reagents

Chemical reagents including the target dye ARB used in this study are analytical grade. Biochemical reagents were purchased from TaKaRa Biotechnology Co., Ltd, Dalian, China.

#### Preparation of microbes

The previously isolated azo-degrading yeast *C. tropicalis* TL-F1 was used for bioaugmentation. The yeast TL-F1 was cultured and enriched in the medium containing (g/L): K$_2$HPO$_4$ 1.0, MgSO$_4$ 7H$_2$O 0.5, urea 5.0 and sucrose 10.0. Spores of the yeast TL-F1 were firstly gathered through centrifugation at 10,000 × g for 10 min, and then washed with 0.1 mol/L phosphate buffer solution (pH = 7.0) three times. Finally, the cells were re-suspended in phosphate buffer solution for bioaugmentation. On the other hand, the AS was purchased from the secondary clarifier of Chunliu sewage treatment plant, Dalian, China. Before inoculation, the sludge was activated and enriched for 30 d in the same medium shown above.

#### Decolorization experiments

Biodegradation of ARB were performed in the form of sequencing batch tests in four 250 mL Erlenmeyer flasks with 100 mL medium containing (g/L): sucrose 4.0, (NH$_4$)$_2$SO$_4$ 0.2, K$_2$HPO$_4$ 0.04, MgSO$_4$·7H$_2$O 0.25 and AS 2.8 (mixed liquid suspended solids, MLSS). For bioaugmentation, the four Erlenmeyer flasks were also inoculated with 0, 2, 4 and 6 g/L (concentration of wet cells after centrifugation at 10,000 g for 10 min and 4 °C) of the yeast TL-F1, respectively. The whole operation process was divided into two stages, with initial ARB concentrations of 50 mg/L and 100 mg/L, respectively. Each stage included seven cycles of decolorization, which lasted 72 h (for the first cycle of each stage) or 48 h (for the rest of the cycles of each stage). In each cycle, the sludge and suspended...
microbial cells were separated and maintained in the flasks through settling and centrifugation, respectively. At the end of each stage, microbial samples were gathered and preserved at −80 °C for further analysis of microbial community structures with PCR-DGGE.

Analytical methods

Concentration of ARB (characteristic absorption wavelength $\lambda_{\text{max}}$ was 516 nm) was analyzed using the spectrophotometric method. The decolorization ratio was calculated using the following equation: Decolorization ($\%$) = $(A_0 - A_t)/A_0 \times 100$, where $A_0$ and $A_t$ represented the initial and final absorbance of the dye, respectively. Chemical oxygen demand (COD) was analyzed according to the standard method for Water and Wastewater Examination (Shi et al. 2015). MLSS was analyzed through an amended standard methods. Firstly, we established a linear relation curve between the wet weight concentration (after centrifugation) and MLSS: \[ \text{MLSS} = 0.0857C_w - 0.055 \] ($R^2 = 0.9985$), where $C_w$ represents the wet weight concentration of AS (with and without the yeast TL-F1). Then wet sludge samples were separated from 10 mL sludge through centrifugation and weighed for calculating the wet weight concentration. Finally, MLSS could be calculated through the curve mentioned above. SVI were calculated using the following equation: \[ \text{SVI} = \frac{\text{SV}_30}{\text{MLSS}}, \] where $\text{SV}_30$ represented settling velocity within 30 min (also analyzed through the standard method). Additionally, the acute toxicity of ARB before and after decolorization were assessed by Microtox bioassays using the luminescent bacteria *Vibrio fischeri* (NRRL B-11777). *Vibrio fischeri* suspension at 5 to 30 min exposure was determined by Berthold LB960, which was used to calculate the inhibition ratio (IR) of luminescence by: \[ \text{IR} = (S_0 \times C_t - S_i)/(S_0 \times C_i), \] where $S_0$ and $S_i$ were the luminescence intensities of the samples at 0 and t min, respectively. $C_i$ was the relative luminescence intensity variations of a negative sample at 0 to t min. All the analytical experiments were performed in triplicate and the average values were used in calculations.

DNA extraction, PCR amplification and DGGE

Genomic DNA was extracted with the FastDNA SPIN Kit for Soil (MP Biomedicals Co., Ltd, Shanghai, China). GC541F–(CGCCCGGCAGCGCCGCGCCGCGCGCGCGCGCGCGCGCGGGCTCAATTCGAGGCAGCAG) – 907R (CCGTCGAATTCTTTTGAGTT) and GCFR1 (CGCCCGGCGCGCGCGCCG CGGCGGGCGGGGCGCGCGGGAICCATATGCTC1) – FF590 (CGATAACGAACGAGACCT) were chosen as the PCR primer pairs for bacterial and fungal communities, respectively (Tan et al. 2009; Prévost-Bouré et al. 2011). PCR was performed using a PCR thermal cycler Dice (BioRad Co., Ltd, USA) with a program consisting of an initial pre-degeneration at 94 °C for 2 min, 30 cycles of 1 min at 94 °C, 1 min at 53 °C, 1 min at 72 °C, and a final 2 min extension at 72 °C for the bacterial community and a program consisting of an initial pre-degeneration at 95 °C for 30 s, 40 cycles of 10 s at 95 °C, 30 s at 50 °C, 45 s at 72 °C, and a final 5 min extension at 72 °C for the fungal community, respectively. DNA concentration was tested by the QuantiFluor™-ST blue fluorescent quantitative system (Promega, USA). Denatured gradient gel electrophoresis (DGGE) was performed with a BioRad Dcode system (BioRad Co., Ltd, USA). The concentrations of polyacrylamide gel were 6% and 8% (w/v) and denaturing gradient ranged from 30% to 60% and 30% to 55% (100% was 7 mol L$^{-1}$ urea and 40% (v/v) formamide) for bacterial and fungal communities, respectively. Other electrophoresis conditions were all 60 °C, 60 V and 16 h. Then the gel was stained with ‘GeneFinder’ (BIO-V Co., Ltd, Xiamen, China) at 10,000-fold dilution, and the image was captured using a gel imaging instrument (BioRad Co., Ltd, USA).

DGGE profiles were analyzed with the software ‘Quantity One’ for cluster analysis. And then dominant DGGE bands were excised and re-amplified by PCR with the primer pairs 541F-907R and FRI-FF590. The sequencing of PCR products was performed by Sangon Biotech Co., Ltd (Shanghai, China), and was compared with the nucleotide sequences in the GenBank database using the BLAST program.

RESULTS AND DISCUSSION

Decolorization of ARB and COD removal

Results of decolorization and COD removal are displayed in Figure 1(a) and 1(b), respectively. It was shown that the decolorization efficiency of all the four sets was almost the same (more than 95% decolorization ratio within 3 d) during the first cycle (Figure 1(a)). The reason was probably that part of the dye was absorbed by AS at the beginning, which was faster than biodegradation (Qu et al. 2009). In the second cycle, less than 90% of 50 mg/L ARB was decolorized by the non-augmented system within 2 d. However, three bioaugmented systems still achieved more than 95% color removal percentages; meanwhile, there was little difference between any two of them. When the
influent ARB concentration increased to 100 mg/L, the color removal percentage of the non-augmented system decreased to less than 80%. In comparison, decolorization percentages finally achieved about 92%, 94% and 95% for the three bioaugmented systems with 2 g/L, 4 g/L and 6 g/L of the yeast TL-F1, respectively. These results suggested that the yeast-augmented AS systems showed better performance for decolorization of ARB due to the synergistic effects of the fungi and bacteria (Li et al. 2015). However, higher inoculation size of the yeast TL-F1 did not obviously further improve the decolorization efficiency.

On the other hand, it was obvious that the COD removal processes of the four sets were also almost the same at the beginning. However, the COD removal percentage of the non-augmented system started to be lower than the other three from the second cycle. The effluent COD of the non-augmented system was about 320 mg/L and 540 mg/L (corresponding to removal ratios of 92.4% and 87.6%, respectively) at the ends of the first and second stages, with the influent ones of about 4,200 mg/L and 4,350 mg/L, respectively. In comparison, the corresponding ones for the other three groups were 38–58 mg/L and 70–93 mg/L (corresponding to removal ratios of 98.6–99.1% and 97.8–98.3%, respectively) with an influent COD of about 4,150 mg/L and 4,250 mg/L, respectively. It was reported that fungi–bacteria co-cultures were always more efficient and stable for treatment of recalcitrant organics than any of them alone. It had also been confirmed that the fungi-bacteria co-cultures were effective for degrading azo dyes as described by Gou et al. (2009) and Qu et al. (2010). Furthermore, Lade et al. (2012) reported that the co-culture of fungus Aspergillus ochraceus NCIM-1146 and bacterium Pseudomonas sp. SUK1 achieved higher efficiency for decolorizing and even detoxifying an actual textile effluent containing azo dyes than either the bacterium SUK1 or the fungus NCIM-1146. Dhouib et al. (2006) also indicated that the AS system bioaugmented with fungi would lead to a decrease of COD/BOD5 ratio and the toxicity of organic wastewaters. Therefore, it was also suggested that fungi could obviously improve biological treatment efficiency through detoxifying the recalcitrant and toxic organic pollutants that might inhibit bacteria.

**Figure 1** Performance of AS systems bioaugmented with different concentrations of the yeast TL-F1 for continuous treatment of 50–100 mg/L ARB in the form of batch tests: (a) decolorization and (b) COD removal.
Characteristics of AS systems

Sludge concentration and settling property of four systems are displayed in Figure 2(a) and 2(b), respectively. It is shown that the initial MLSS of four systems with different concentration of the yeast TL-F1 are almost the same. The reason is that the inoculation size of the yeast was wet weight solid concentration, which corresponded to a very low MLSS (dry weight solid concentration). At the first stage, the sludge concentration of all four sets increased because they were fed with synthetic wastewater containing 4.0 g/L sucrose (Figure 2(a)). The growth rate of AS in the non-augmented system was slower than that in the three bioaugmented ones. When the influent ARB concentration increased to 100 mg/L, the biomass (MLSS) in the non-augmented set sharply decreased from about 3,200 mg/L to 2,400 mg/L at the beginning of the second stage. Although it stopped decreasing and recovered from the

Figure 2 | Characteristics of sludge in four systems: (a) sludge concentration (MLSS) and (b) sludge settling performance (SVI).

Figure 3 | The acute toxicity of 50 mg/L ARB before and after treatment by different systems assessed by Microtox bioassays using the luminescent bacteria Vibrio fischeri (NRRL B-11177). 70 ≤ IR (%) < 100, high toxicity; 20 ≤ IR (%) < 70, moderate toxicity; 10 ≤ IR (%) < 20, low toxicity.

Figure 4 | Results of PCR-DGGE for bacterial communities: (a) DGGE profiles and (b) cluster analysis basing on ‘UPGMA’ method. AS, activated sludge; B1-1 to B1-4 (B2-1 to B2-4), bacterial communities for treatment of 50 mg/L (100 mg/L) ARB with 0, 2, 4 and 6 g/L of the yeast TL-F1, respectively.
22th day, the final MLSS was only about 2,500 mg/L. In comparison, the biomass in the other three sets slightly decreased, and then recovered after further operation for another 5 d. At the end of the second stage, the biomass of these three sets was 3,200–3,420 mg/L. These results suggested that the yeast TL-F1 might improve the growth of AS through detoxification of toxic intermediates, as mentioned above. On the other hand, shifts of SVI shown in Figure 2(b) suggested that the settling property of the AS in three bioaugmented sets was improved compared with the non-augmented one. The SVI index shifted in the range of 85–110 mL/g for the systems with 4 g/L and 6 g/L of the yeast TL-F1. Meanwhile, the corresponding shift range of SVI for the group with 2 g/L of the yeast TL-F1 was 95–135 mL/g, which was wider and higher than that of the other two bioaugmented groups. In comparison, the range of SVI for the non-augmented system (95–245 mL/g) was much wider than the other three sets. These results suggested that the settling property of AS was more stable with bioaugmentation by a higher concentration of the yeast TL-F1 due to the coagulation effects. It was indicated that better settling property meant higher solids retention time, thus related to higher treatment efficiency (Hailei et al. 2006). Therefore, it could be concluded that bioaugmentation with the yeast TL-F1 also improved the sludge settling property for treatment of the azo dye ARB.

**Toxicity assessment (Microtox test)**

The influent and effluent from four sets at the last cycle of the first stage (the 15th day) were analyzed. The result in Figure 3 shows that 50 mg/L ARB had a moderate toxicity against *V. fischeri*. After treatment, effluent from the non-augmented set displayed a high toxicity against *V. fischeri* as indicated by the IR of more than 90%, suggesting that the toxicity increased after treatment by AS alone. The reason should be that some breakdown byproducts (e.g. aromatic amines) with higher toxicity than ARB were generated and accumulated (Li et al. 2015). By contrast, the effluent from the other three bioaugmented systems displayed obviously lower toxicity against *V. fischeri* as indicated by IRs of less than 20%. Furthermore, as the inoculation size of the yeast increased, the toxicity of

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**Figure 5** | Phylogenetic analysis of partial 16S rDNA gene sequences retrieved from the dominant DGGE bands in Figure 4(a).
the effluent slightly decreased. Therefore, the results of the toxicity assessment reconfirmed that bioaugmentation with fungi would protect some useful bacteria from being inhibited by toxic intermediates and thus improve the treatment efficiency of ARB.

**Shifts of microbial community structures**

Shifts of microbial (including bacterial and fungal) community structures revealed by PCR-DGGE are displayed in Figures 4–7. It was shown that the band patterns of bacterial communities after treatment of ARB (B1-1 to B2-4) were obviously different from that of AS (Figure 4(a)), since the similarity of cluster analysis between AS and other eight samples was almost 0 (Figure 4(b)). The number of dominant bacterial species sharply decreased after treatment of ARB, suggesting that some original bacteria were inhibited by ARB or its decolorization intermediates. Besides, the abundance of bacteria in sample B1-2 was more than that in B1-4, which suggested that a higher concentration of the yeast TL-F1 might inhibit the growth of some bacteria. Only one dominant bacterium was simultaneously detected in all four sets with an influent ARB concentration of 50 mg/L. When the influent ARB concentration increased to 100 mg/L, the bacterial community structures significantly shifted compared with those with 50 mg/L influent ARB, which suggested that microbial structures would change with external conditions (Tan et al. 2009). Furthermore, the bacterial abundance of the bioaugmented set with 6 g/L the yeast TL-F1 was more than the other three sets. To further analyse the bacterial community structures, dominant bands were sequenced and identified (shown in Figure 5). Band 3 was dominant in microbial communities B1-1 to B1-4 and identified as Uncultured cyanobacterium clone PS41 (KF951520), which was previously detected in a microbial community capable of degrading linear alkylbenzene sulfonate (Carosia et al. 2014). It was indicated that sulfonates were decolorization intermediates of various azo dyes including ARB (Qu et al. 2012). Thus the dominance of this bacterium probably related to the degradation of sulfonates which were produced during the decolorization of ARB. However, this bacterium was not detected in samples B2-3 and B2-4, suggesting that it might be inhibited due to higher organic loading. Band 4 was only detected from the four sets with 100 mg/L ARB and was identified as Uncultured bacterium clone BioPlate2_E10 (HE587186). This uncultured bacterium was firstly found in a microbial community related to dissolution of HgS, which suggested that it might tolerate high-toxic conditions (Jew et al. 2014). Bands 6 and 8 were identified as the same species, suggesting that the affinity between them was close. Additionally, another two bands 5 and 10 only appeared in the sample B2-4 with 6 g/L of the yeast TL-F1 and 100 mg/L of influent ARB. Among them, band 5 was identified as Dyella sp. FP0 (JX910135), which was confirmed as a novel phenanthrene-degrading bacterium (Muangchinda et al. 2013). Besides, band 10 was identified as Thiomonas sp. NI-1 (HE587300), which was also firstly found by Jew et al. (2014). Therefore, it was suggested that these two bacteria would also tolerate highly toxic pollutants and could co-exist with high concentration of the yeast TL-F1.

On the other hand, fungal community structures also shifted after treatment of ARB, as shown in Figure 6(a) and 6(b). Five dominant fungal species in AS were detected before treatment of ARB, in comparison, only one dominant fungus was detected after treatment. The abundance of fungal communities from the three bioaugmented sets increased with inoculum size of the yeast TL-F1 and was higher than that from the non-augmented one. It was
suggested that fungal-based bioaugmentation improved the growth of some other fungi which might be important for degradation of ARB and its recalcitrant intermediates. Moreover, the yeast TL-F1 survived and became one of the dominant fungal species in the three bioaugmented systems, suggesting that bioaugmentation was successful due to the colonization of the yeast TL-F1. Results of dominant bands sequencing shown in Figure 7 suggested that the affinity between five dominant fungi (labeled as f1 to f5) was close. Bands 1 and 2 were identified as Uncultured fungus clone T3_JI_2a_02 (EF628568) and Uncultured Nectria clone T25 (HQ661374), respectively, both of which were found in marine environments. It was noteworthy that band 5, which was identified as Trichoderma sp. fP6 (KJ467778), was firstly found in hydrocarbon-degrading microbial communities (based on the NCBI database). It was suggested that this fungus might be another important degrader of ARB in the community apart from the yeast TL-F1.

**CONCLUSIONS**

Results of this study demonstrated that decolorization, degradation and detoxification of synthetic ARB wastewater by AS systems was promoted through bioaugmentation by an azo-degrading yeast *C. tropicalis* TL-F1 during continuous treatment processes. The yeast TL-F1 also improved the growth and settling property of AS. PCR-DGGE analysis revealed that both bacterial and fungal communities obviously shifted after treatment of ARB and some potentially useful species finally became the dominant species. Meanwhile, the yeast TL-F1 colonized in the bioaugmented systems and became one of the dominant fungi, suggesting that bioaugmentation was successful.

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