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Microbial community structures in mixed bacterial consortia for azo dye treatment under aerobic and anaerobic conditions

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ABSTRACT

Thirteen pure strains that possessed high methyl red (MR)-decolorizing ability were isolated from dye-contaminated water. Each isolate was identified by 16S rDNA sequencing. The results reveal that all of the isolated strains were facultative anaerobic bacteria. Two novel bacterial consortia (AE and AN), which could decolorize MR under aerobic and anaerobic conditions, respectively, were developed. Azo dye decolorization rate was significantly higher with the use of consortia compared to that with the use of individual strains. Both of the consortia can decolorize different azo dyes effectively in a short time, and tolerate MR with high concentrations. To provide further insight into the microbial diversity of the bacteria consortia under aerobic and anaerobic conditions, polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analyses were performed. PCR-DGGE profiles revealed that the microbial community had changed significantly with varying initial concentrations of MR. Phylogenetic analysis indicated that microbial populations in the aerobic compartment belong to *Klebsiella*, *Buttiauxella* and *Bacillus*, whereas *Klebsiella*, *Escherichia*, *Bacillus* and *Clostridium* were present in the anaerobic compartment. *Klebsiella*, which was the majority genus in both of the consortia, may play an important role in azo dye removal.

1. Introduction

Azo dyes, which are the largest and most versatile class of dyes, have been widely used in textile, food, printing and cosmetic industries [1]. However, insufficient treatment of waste from the dyestuff industries leads to dye contamination in the environment [2]. Moreover, most azo dyes are toxic and their biotransformation products are carcinogenic and mutagenic. Therefore, dye waste must be treated before it is released into the natural environment [3].

Most physicochemical dye removal methods, including adsorption, coagulation, precipitation and chemical oxidation, have not been widely applied because they have low efficiency, high costs and intensive energy requirements [4]. However, biodegradation of azo dyes is considered an environment friendly and cost-effective option [5]. In recent years, many studies have focused on various microorganisms that degrade azo dyes under anaerobic and aerobic conditions. Rafii et al. [6] have reported that the new strain *Clostridium paraputrificum* decolorizes Direct Blue 15 under anaerobic conditions. Yu et al. have shown the *Klebsiella oxytoca* strain GS-4-08 decolorizes Methyl Orange effectively under anaerobic conditions. Moreover, the strain exhibits a good capacity of simultaneous azo dye decolorization and hydrogen production in the presence of electron donor [7]. Liu et al. [8] have reported that a strain, which belongs to *Shigella*, can decolorize different azo dyes under static conditions, and the addition of reduction products of acid azo dyes can effectively accelerate dye decolorization efficiency. A *Escherichia coli* strain, which can decolorize Congo Red and Direct Black 38, has been reported by Işik et al. The efficiency of color removal with this strain in anaerobic incubations is faster than that obtained in aerobic incubations [9]. The *Kerstersia* strain VKY1 decolorizes Amaranth, Fast Red E, Congo Red and Ponceau S by 100% (100 mg L^{-1}) within 24 h under aerobic conditions [10]. The *Bacillus cereus* strain M1 decolorizes sulfonated azo dyes under aerobic conditions [11].

Different taxonomic groups of bacteria have been reported for their ability to decolorize azo dyes [12]. It has been reported that the treatment systems with mixed microbial populations are more effective to decolorize azo dyes than that of pure cultures [13]. Therefore, an acclimated microbial community is a more appropriate and efficient approach for the decolorization of azo dyes. A number of studies have been performed on reliable biological community processes in azo dye decolorization. Joshi et al. have reported that the novel bacterial consortium TJ-1, which consists of *Aeromonas caviae*, *Proteus mirabilis* and *Rhodococcus globerulus*,

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decolorizes many azo dyes under microaerophilic conditions [14]. The aerobic bacterial consortium SKB-II, which consists of many species of *Bacillus*, possesses a high rate of azo dye decolorization [15]. Many studies have focused on the efficiency of dye decolorization and strain isolation. However, little information concerning microbial population structures and dynamic changes in azo dye decolorization systems is available.

PCR-DGGE is generally used to determine the existing and dominant members of the complex microbial system [16]. The strength of DGGE as a screening method for diversity lies in its ability to monitor the community structure in response to the changes of experimental parameters by overcoming some of the limitations in cultural techniques [17].

This paper presents comparative studies on the ability of two consortia (AE and AN) to decolorize different azo dyes under aerobic and anaerobic conditions, respectively. The microbial community diversity of two bacterial consortia was evaluated. PCR-DGGE was used to monitor the succession of the microbial community, and predominant bands on DGGE gels were sequenced to determine the microbial community composition.

2. Materials and methods

2.1. Dyes and chemicals

The dyes used in this study were of industrial grade and were procured from the Guangfu Fine Chemical Research Institute (Tianjin, China). The dyes tested were MR (λ_{max} = 430 nm), Congo Red (λ_{max} = 500 nm), Orange I (λ_{max} = 476 nm), Methyl Orange (λ_{max} = 464 nm), Eriochrome Red B (λ_{max} = 466 nm) and Eriochrome Black T (λ_{max} = 529 nm). The rTaq DNA polymerase, PCR purification kit, pMD-18T vector and *E. coli* JM109 strain were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). All other chemicals were of analytical grade or the highest quality.

2.2. Sample

An inoculum of dye-contaminated water from the disposal site of a textile-dyeing industry located in Haicheng, China was used to develop the bacterial consortia for the decolorization.

2.3. Strain isolation and identification

The Luria-Bertani culture medium (LB) that was supplemented with 50 mg L^{-1} MR was used as the isolation medium (pH 7.0). For enrichment of the azo dye-decolorizing bacteria under aerobic and anaerobic conditions, 50 mL of dye-containing media in 100-mL flasks were inoculated with 1 mL of sample (2%, v/v) and incubated in orbital shaker or under static conditions at 37 °C for 12 h. Repeated transfers were performed using fresh dye-containing media until stable dye-decolorizing cultures were obtained. The LB plates containing MR (100 mg L^{-1}) were inoculated with the cultures under aerobic or anaerobic conditions. The different isolates with high potential for dye degradation were isolated and used for further study.

Identification of the isolated strains was performed using 16S rDNA gene sequence analysis. Total DNA was extracted in 1.5-mL tubes containing 500 μL of sample and 500 μL of lysis buffer (100 mM Tris–HCl pH 8.0, 1.4 mM NaCl, 2% (w/v) CTAB and 2 mM EDTA). The mixture was incubated at 65 °C for 30 min. Then 1 mL of phenol-chloroform (1:1) was added to each tube, and the samples were shaken vigorously. After centrifugation at 6000 × *g* for 10 min, the supernatant was transferred to a new tube and extracted again with phenol-chloroform. The DNA was precipitated with ethanol for 1 h, and the final pellets were re-suspended in 1 × TE buffer (1 mM Tris–HCl pH 8.0 and 0.1 mM EDTA). The 16S rDNA gene was amplified using PCR with the specific primers 27f (5′-GAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′). The PCR amplifications were performed using an initial denaturation step of 3 min at 94 °C; followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 1.5 min. A finally extension was performed for 10 min at 72 °C. The PCR product was purified using a PCR purification kit and cloned using the pMD18-T plasmid vector system. The DNA sequences were determined using the chain-termination method with an ABI 3730 DNA sequencer by a commercial service provided by Shanghai Shengon Biological Technology Company (Shanghai, China).

2.4. Enrichment of the dye-decolorizing consortium

For enrichment of the dye-decolorizing consortium under aerobic conditions, the 100-mL flasks containing 50 mL LB culture medium (pH 7.0) were inoculated with 1 mL of contaminated water. The flasks were incubated at 37 °C at 120 rpm for another 12 h. Then 1 mL of cultures was transferred to 50 mL of LB culture medium containing 50 mg L^{-1} MR and incubated at 37 °C at 120 rpm for 12 h. Repeated transfers were performed using fresh media with different initial dye concentrations (50–300 mg L^{-1} MR, increasing 50 mg L^{-1} of dye for each transfer). Each sample was withdrawn periodically for future use.

For enrichment of the dye-decolorizing consortium under anaerobic conditions, the serum bottles containing 50 mL of LB culture medium (pH 7.0) were inoculated with 1 mL of contaminated water. Oxygen was removed by repeated evacuation and flushing with nitrogen gas. The serum bottles were transferred to an anaerobic incubation chamber (YQX-II, CIMO Medical Instrument Manufacturing Co., Ltd., Shanghai, China). After 16 h incubation at 37 °C, 1-mL cultures were transferred to 50 mL of fresh dye-containing media (50 mg L^{-1} MR, pH 7.0), and were incubated for another 16 h. Repeated transfers were performed using fresh media with different initial dye concentrations (50–500 mg L^{-1} MR, increasing 50 mg L^{-1} of dye for each transfer). Each sample was withdrawn periodically for future use.

2.5. Decolorization assay

The individual strains and developed consortia were used to monitor decolorization of different azo dyes under static anoxic and shaking conditions. The dye decolorization was estimated by measuring the absorbance at the respective λ_{max} of the different dyes individually in a UV–vis spectrophotometer (UV-2800, Unico Instruments Co., Ltd., Shanghai, China). The samples were centrifuged at 6000 × *g* for 3 min, and the supernatant was used for the analysis. The uninoculated dye was used as blank. Rate of decolorization was calculated from the difference between the initial and the final absorption values of the supernatant at the λ_{max} for each dye. All of the experiments were performed in triplicate.

2.6. Phytotoxicity studies

Phytotoxicity tests were performed in order to assess the toxicity of the untreated and treated dye. Distilled water contained 50 mg L^{-1} of MR was incubated with the two consortia under aerobic and anaerobic conditions, respectively, for 6 h. After treatment, the samples were then centrifuged at 6000 × *g* for 5 min and filtered through a 0.45 μm pore size filter. The phytotoxicity study was carried out at 25 °C in relation to *Brassica pekinensis* (10 seeds of each) by watering separately 3 mL sample of control MR (50 mg L^{-1}) and metabolites obtained after its decolorization per day. Control set was carried out by using distilled water at the

same time. Germination (%) as well as the length of plumule and radical (cm) was recorded after 3 days.

2.7. PCR-DGGE analysis

2.7.1. DNA extraction of the microbial communities and PCR amplification of the 16S rDNA V3 region

Genomic DNA was extracted used the aforementioned method. The 16S rDNA V3 region was amplified using the forward primers F357-GC (5′-CGCCCGCCGCGCGCGGCGGGCGGGGC-GGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3′) and reverse primer R518 (5′-ATTACCGCGGCTGCTGG-3′). The PCR amplification used the following protocol: initial denaturation of DNA for 5 min at 94 °C; 10 cycles of 1 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C and 20 cycles of 1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C followed by a final extension for 10 min at 72 °C. After amplification, PCR products were electrophoresed in 0.8% (w/v) agarose gel to evaluate the extent of amplification.

2.7.2. DGGE analysis

DGGE was performed using the Dcode universal mutation detection system (Bio-Red Co., Ltd., Hercules, CA, USA). The PCR products were electrophoresed in a 10% poly-acrylamide gel in 1 × TAE buffer containing a linear gradient ranging from 30% to 60% of denaturant (denaturation of 100% corresponded to 7 M urea and 40% (v/v) formamide). The gradient gel was cast using a gradient delivery system (Model 475, Bio-Red Co., Ltd., Hercules, CA, USA). Electrophoresis was run at 60 °C with a constant voltage of 160 V for 6 h. The gel was stained with $AgNO_3$, and an image of the gel was captured using a bio-imaging system (Bio-Red Co., Ltd., Hercules, CA, USA).

DGGE profiles were analyzed using the software Quantity One 4.6.2. Dendrograms relating band pattern similarities were automatically calculated using the unweighted pair group method with the arithmetic average (UPGMA) clustering algorithm, which was included in the Quantity One software. Relative band densities, which were necessary to determine the Shannon diversity index (SDI), were quantified, and the statistical data was exported for further SDI analyses.

2.7.3. Sequencing and phylogenetic analysis

Prominent bands were excised from the DGGE gels for nucleotide sequence determination. The gel of each selected band was crushed in 12 μL of ddH_2O and incubated at 50 °C for 30 min, after centrifugation at 7200 × *g* for 1 min, the supernatant was collected and used as a PCR template. The DNA fragment was reamplified using the primers F357/R518, and the PCR amplification was conducted as follows: initial denaturation at 94 °C for 3 min and 30 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 1 min and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. PCR products were determined using 0.8% agarose gel electrophoresis. The samples were purified using the PCR purification kit and cloned using the pMD18-T plasmid vector system. The DNA sequences were determined using the chain-termination method with an ABI 3730 DNA sequencer by a commercial service provided by Shanghai Shengon Biological Technology Company (Shanghai, China). DNA sequences were compared with the 16S rDNA sequences in the GenBank database using BLASTN algorithm for species identification.

2.8. Statistical analysis

All the values are the means of three independent observations and ± indicate the standard deviation (SD).

2.9. Nucleotide sequence accession numbers

The sequences determined in this study have been deposited into the GenBank database under the accession numbers JN049565-JN049566, JN049570-JN049588, JN0499591-JN049596 and JN049598-JN049604.

3. Results and discussion

3.1. Isolation and identification of bacterial culture

The isolates were selected based on their ability to form a high dye decolorization zone on agar plates under aerobic or anaerobic conditions. Each isolate was identified using 16S rDNA sequencing. These sequences were compared to those in GenBank to obtain the most similar 16S rDNA fragments, which are shown in Table 1. Six isolates with a high efficiency rates to decolorize azo dyes under aerobic conditions were identified. The sequences of five isolates (H1, H2, H3, H8, H11) showed 99% similarity with *Klebsiella pneumoniae*, whereas H7 showed 99% similarity with *Shigella dysenteriae*. Seven strains that decolorized azo dyes under anaerobic conditions were also identified. The sequences of most isolates (Y1, Y3, Y6, Y9, Y10, Y12) showed maximum similarity with *Klebsiella* sp. However, the isolate Y11 had the closest relationship with *E. coli*.

The 13 strains that were isolated under aerobic and anaerobic conditions were all Gram-negative, facultative anaerobic bacteria of the family Enterobacteriaceae. Many strains in this family were abundant in the samples of dye-contaminated water in this study. Among these strains, 11 of 13 isolates showed high sequence similarity to *Klebsiella* sp., which indicated that *Klebsiella* might be the major genus in decolorization consortia. These strains decolorized MR under aerobic and anaerobic conditions. Previous studies have shown that the *Klebsiella* strains have the ability to decolorize azo dyes. Wong and Yuen have reported that the strain *K. pneumoniae* RS-13, which had been isolated from dye-contaminated sludge, completely degrades MR under aerobic conditions [18]. Franciscon et al. [19] showed that a facultative *Klebsiella* sp. strain VN-31 decolorizes four different azo dyes under microaerophilic conditions. Ghosh et al. [20] have reported two types of azoreductases in *S. dysenteriae*, which are responsible for Ponceau SX decolorization. However, there are few published studies regarding dye decolorization using *Shigella* sp. under aerobic conditions. In the present study, the isolate H7 showed 99% similarity with *S. dysenteriae*, and could efficiently decolorize MR which was indicated by degradation of 97% of MR (50 mg L^{-1}) within 10 h under aerobic conditions. In previous study, a newly isolated *E. coli* strain CD-2, which decolorized different azo dyes under aerobic conditions, have been reported. The strain decolorizes azo dyes at high salt concentration and over a wide range of pH, even without a carbon source [21]. In the present study, the *E. coli* strain Y11, which decolorized MR under anaerobic conditions, was also isolated. The results indicate that *E. coli* could decolorize azo dyes under both aerobic and anaerobic conditions.

3.2. Decolorization studies

3.2.1. Individual strains vs. consortia under aerobic and anaerobic conditions

In this study, two bacterial consortia (AE and AN), which were capable of decolorizing azo dyes under aerobic and anaerobic conditions, were enriched. Decolorization of MR (100 mg L^{-1}) with individual strains which could produce the largest decolorized zone on the agar plate (H3 and Y10) as well as with two consortia was studied and the results were shown in Fig. 1. Although individual

Table 1
Sequence analysis and species identification of pure strains isolated from wastewater under aerobic and anaerobic conditions.

Strains no.	GeneBank no.	Closest sequences	Closest sequences GeneBank no.	Similarity (%)
H1	JN049594	*Klebsiella pneumoniae* strain HDDMM07	EU723828	99
H2	JN049595	*Klebsiella pneumoniae* strain K30	EU661377	99
H3	JN049596	*Klebsiella pneumoniae* strain HR16	EU078621	99
H7	JN049593	*Shigella dysenteriae* strain FBD015	EU009186	99
H8	JN049598	*Klebsiella pneumoniae* strain TCCC11046	EU231611	99
H11	JN049599	*Klebsiella* sp. enrichment culture clone SRC_DSB7	GU374025	99
Y1	JN049600	*Klebsiella* sp. enrichment culture clone SRC_DSA24	GU374009	99
Y3	JN049592	*Klebsiella pneumoniae* strain NK 2.bp-1	EU352755	99
Y6	JN049601	*Klebsiella pneumoniae* strain HR16	EU078621	99
Y9	JN049602	*Klebsiella pneumoniae* strain HR9	EU086097	99
Y10	JN049603	*Klebsiella pneumoniae* strain 2	DQ444287	99
Y11	JN049591	*Escherichia coli* strain Acj 213	AB480777	99
Y12	JN049604	*Klebsiella pneumoniae* strain HR16	EU078621	99

strains could decolorize MR, on comparison, the consortia had more efficiency to decolorize azo dyes. After the 12 h incubation under aerobic conditions, about 75% of MR was degraded by the strain H3, however, more than 95% of MR was decolorize by the consortium AE. Similar results were observed under anaerobic conditions. The decolorization rate of the consortium AN was significantly higher by comparison with the strain Y10 at any given time. We observed that 100 mg L^{-1} of MR could be decolorized completely within 10 h by consortium AN. However, only about 78% of MR was decolorized after 10 h incubation with strain Y3. Enhanced rate of decolorization of various azo dyes by using bacterial consortia was also reported by other studies. Joshi et al. reported that Acid Orange 7 decolorization was significantly higher with the use of consortium as compared to the use of individual strains. The consortium TJ-1 could decolorize Acid Orange 7 (120 mg L^{-1}) completely within 10 h, whereas the pure cultures required more time [14]. Saratale et al. [22] found that the consortium GR could completely decolorize Scarlet R with an average decolorization rate of 16.67 mg h^{-1}, which was about 6 times faster than that of the pure strains. The results suggested that the complementary role of the different bacterial isolates in the consortium led to an increase in decolorization efficiency.

3.2.2. Decolorization of different azo dyes by consortia

The two consortia were tested for their ability to decolorize different azo dyes under aerobic and anaerobic conditions, respectively. The results showed that both of the consortia could decolorize most of azo dyes in a short time (Fig. 2). Under aerobic conditions, MR, Congo Red, Orange I were decolorized almost completely by the consortium AE, and over 70% of Eriochrome Black T and Eriochrome Red B was degraded within 16 h. However, for Methyl Orange, only 40% decolorization rate was attained within 16 h. Under anaerobic conditions, the consortium AN presented even higher decolorizing efficiency. All the six dyes could be decolorized effectively in a short time. Almost 100% decolorization rate of MR, Congo Red, Orange I and Methyl Orange could be attained within 14 h, and more than 80% of Eriochrome Black T and Eriochrome Red B was degraded after 16 h. The results indicated that the two consortia showed non-specificity of azo bond reduction, and they could decolorize azo dyes with different chemical structures. Moreover, it showed that the consortium that was enriched under anaerobic conditions showed an increased decolorization efficiency than the consortium that was enriched under aerobic conditions. It was likely because that the presence of oxygen may not be conducive for bacteria to cleave "N=N" bonds and prevented the strains from using azo dyes as the source of carbon and energy for their growth.

3.2.3. Effect of initial dye concentration

The decolorization rate of MR by the two microbial consortia under aerobic and anaerobic conditions at different initial concentrations (200–500 mg L^{-1}) was studied (Table 2). The results showed that both of the two microbial consortia could decolorize MR effectively at varying initial dye concentrations. With consortium AE under aerobic conditions, more than 85% of MR was decolorized within 30 h when the dye concentration was below 400 mg L^{-1}. However, further increase in dye concentration resulted in slight reduction in decolorization rates, as the concentration of dyes was increased to 500 mg L^{-1}, MR decolorization rate decreased to 70%. Under anaerobic conditions, over 90% decolorization of MR was observed within 10, 12, 16 and 20 h for the dye concentrations of 200, 300, 400 and 500 mg L^{-1}, respectively.

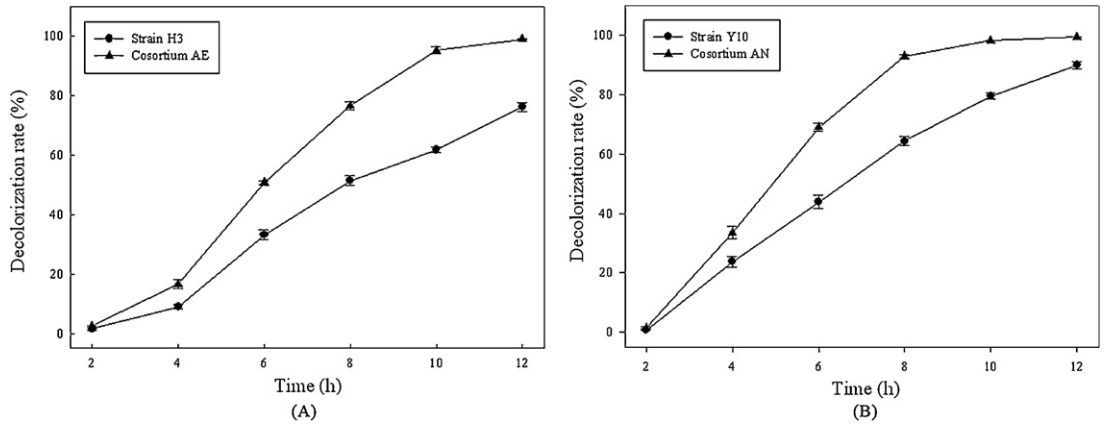

Fig. 1. Decolorization performance of MR (100 mg L^{-1}) by using (A): strain H3 and cosortium AE under aerobic conditions, and (B): strain Y10 and cosortium AN under anaerobic conditions.

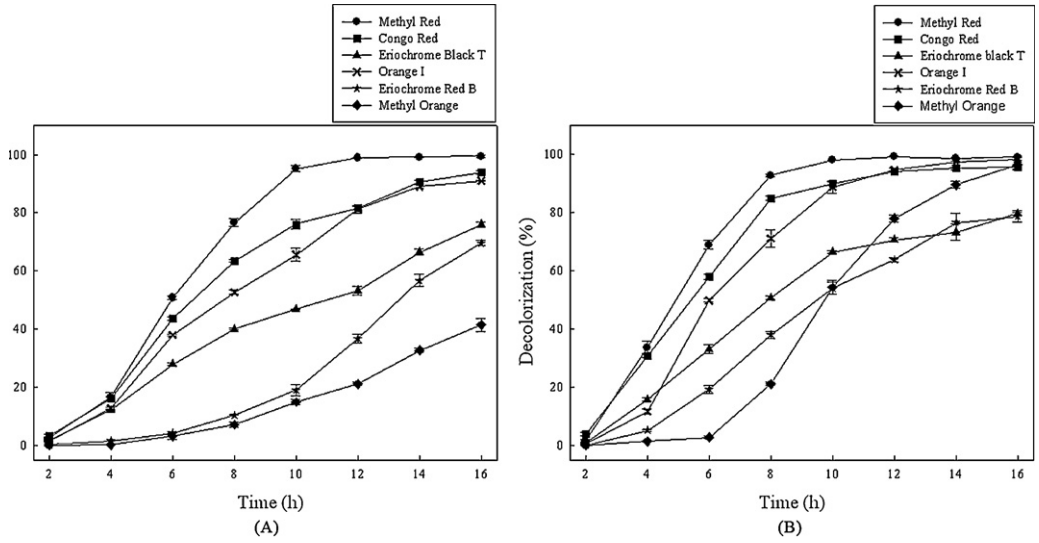

Fig. 2. Decolorization of different azo dyes (100 mg L^{-1}) by (A): consortium AE under aerobic conditions, and (B): consortium AN under anaerobic conditions.

In this study, the two decolorization consortia exhibited much better azo dye-decolorization ability than the pure isolates. Both of the consortia could decolorize various azo dyes with different structures in a short time. Moreover, they could also tolerate high concentrations of azo dyes. The results suggested that the two consortia have a great potential for wastewater treatment.

3.3. Phytotoxicity studies

The phytotoxicity tests revealed that growth inhibition was observed with control MR with *B. pekinensis* whereas its degradation products showed comparatively less growth inhibition (Table 3). We observed that the dye significantly affected the germination rate. The germination of the plant seeds was inhibited 100% when seeds were treated with 50 mg L^{-1} of MR. The result indicated that azo dye had serious hazards on plant seeds, and untreated dyeing effluents could not be used for the agriculture purpose. However, after treated by the bacterial consortia, the degradation products of MR showed comparatively less growth inhibition. When watered by dyeing effluents which were treated under aerobic conditions, the germination rate of *B. pekinensis* seeds was 90% while plumule length and radical length were found to be 0.88 ± 0.07 and 1.03 ± 0.12 cm. When watered by dyeing effluents which were treated under anaerobic conditions, the germination rate of *B. pekinensis* was 70% while plumule length and radical length were found to be 0.61 ± 0.04 and 0.73 ± 0.05 cm. These findings suggested that the degradation products were less toxic to plant seeds compared with the control MR. This study indicated that MR could be detoxicated by both of the two consortia.

3.4. PCR-DGGE analysis

In recent years, many studies have focused on decolorization of different azo dyes using bacterial consortia [13–15]. The results indicate that mixed bacterial cultures may be more appropriate for decolorization of azo dyes. Although some strains, which were responsible for dye decolorization in these bacterial consortia, were isolated and identified, there was little information to reveal the complete composition of the microbial community. To provide further insight into the microbial diversity, PCR-DGGE analysis of the decolorization consortia under aerobic and anaerobic conditions was performed.

3.4.1. Structure of microbial community under aerobic conditions

PCR-DGGE profiles revealed dynamic changes within the microbial communities that were cultured with different initial concentrations of MR (Fig. 3a). Different banding patterns of the microbial community structure were observed, and each distinguishable band in the separation pattern represented an individual bacterial genus. With increasing dye concentration, the changes of relative quantities of various microbes were detected in the profiles. The quantities of some microbes increased (AE-3, -7, -9, -10, etc.), whereas others decreased (AE-14, -15, -16, etc.). Some microbes maintained their populations, such as AE-1 and AE-2.

The UPGMA clustering analysis was used to analyze the community similarity among different initial dye concentrations under aerobic conditions (Fig. 3b). The results show that the microbial community structure in enrichment cultures had the lowest similarity with that in other cultures. The rapid change of community structure resulted from dye addition, which imposed

Table 2
Decolorization of MR at different concentrations by the two consortia.

Microbial consortium AE (under aerobic conditions)			Microbial consortium AN (under anaerobic conditions)		
Dye concentration (mg L^{-1})	Incubation time (h)	Decolorization rate (%)	Dye concentration (mg L^{-1})	Incubation time (h)	Decolorization rate (%)
200	14	95 ± 0.9	200	10	98 ± 0.7
300	22	92 ± 1.2	300	12	96 ± 1.2
400	30	86 ± 1.5	400	16	97 ± 0.7
500	48	70 ± 1.5	500	20	94 ± 1.9

Table 3
Toxicity of MR and its degradation products for *Brassica pekinensis*.

Parameters	*Brassica pekinensis*			
	Distilled water	Methyl Red ($50\,mg\,L^{-1}$)	Extracted metabolites (under aerobic conditions)	Extracted metabolites (under anaerobic conditions)
Germination (%)	100	0	90	70
Plumule (cm)	1.17 ± 0.08	0	0.88 ± 0.07	0.61 ± 0.04
Radical (cm)	1.42 ± 0.13	0	1.03 ± 0.12	0.73 ± 0.05

selective pressure on the microbial community. The microbial community reached a relatively stable stage when the dye concentration reached $150\,mg\,L^{-1}$, which was indicated by the high similarity of microbial community structures among these lanes in DGGE gel. The results show that the microbial community was stable and revealed the high microbial adaptability and dye decolorization ability during culture in the dye-containing media.

The SDI described the change in the species dominance of a bacterial community. The SDI for lanes I and lane II decreased from 4.16 to 3.68. The result suggests that some microorganisms might be inhibited by azo dye. Therefore, these microorganisms cannot grow in dye-containing media, thus, which decreases the diversity of the bacterial community. However, the SDI did not significantly change among lanes III to VII, indicating that these bacterial consortia adapted to the azo dye and that the microbial community was steady.

To gain further insight into the microbial diversity, eleven predominant bands were extracted from the DGGE gel were sequenced and compared with the published species in NCBI (Table 4). Dominant taxonomic units observed in the gel were divided into four groups. The majority of the bacterial 16S rDNA sequences (6 out of 11) grouped with *Klebsiella* sp. These results suggest that most of bacteria in dye-containing media belonged to *Klebsiella*, and they were responsible for dye decolorization. In the second group, the bacteria clones AE-4 and AE-7 clustered with *Buttiauxella izardii*. In the third group, the clone AE-10 presented high sequence similarity to *Bacillus* sp. In the fourth group, two clones clustered with uncultured bacterium clones.

3.4.2. Structure of microbial community under anaerobic conditions

A significant shift in the microbial community structure was observed under anaerobic conditions with different initial dye concentrations (Fig. 4a). With increasing dye concentration, some bands (AN-3, -4, -5, etc.) that were initially present in the media faded away, whereas few bands (AN-6 and AN-7) remained. Some bands emerged and even became more prominent (AN-8, -9, -10, etc.).

The UPGMA clustering analysis was used to analyze the community similarity among different initial dye concentrations under anaerobic conditions (Fig. 4b). Populations in different media were categorized into two separate groups. The first group represented the enrichment sample, and the second group represented the dye-containing culture. The second group was sub-categorized into two separate groups that contained low ($50–200\,mg\,L^{-1}$) or high ($250–500\,mg\,L^{-1}$) concentrations of dye. The results indicate that the microbial community structure in enrichment cultures had the lowest similarity with that in other cultures. With increasing MR concentration, the community structure was dramatically altered. When the dye concentration reached $250\,mg\,L^{-1}$, the microbial community exhibited a high microbial adaptability and the structure of bacterial populations was maintained.

The SDI for lanes I and II decreased from 3.45 to 2.57, which suggested that some microorganisms were unable to grow in dye-containing media. However, the SDI did not change much in lane V and XI. It was likely because that once the microbial populations adapted to the azo dyes, the predominant population structure was developed and did not dramatically change over time.

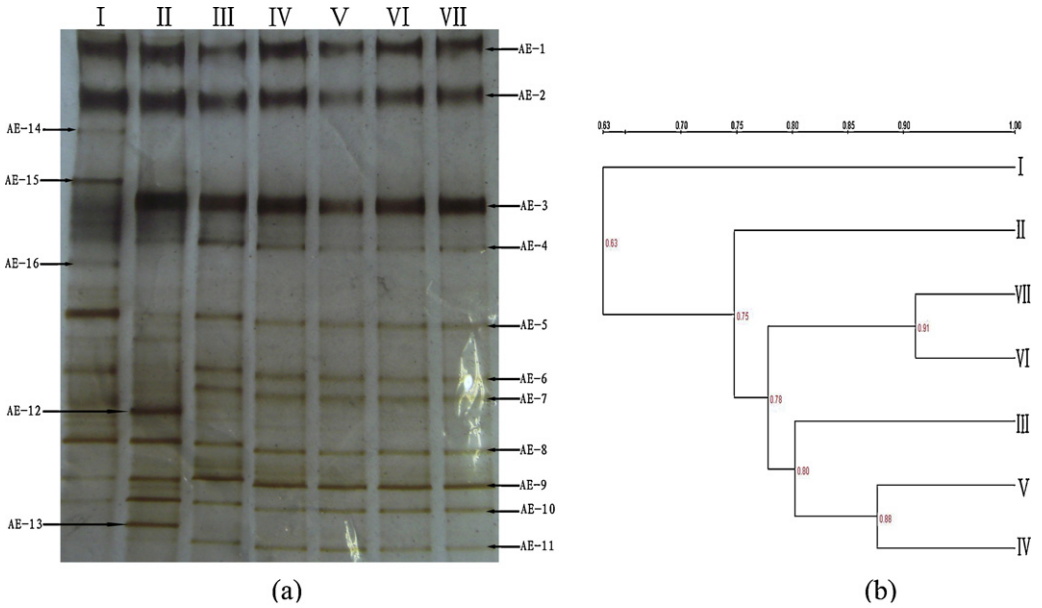

Fig. 3. DGGE fingerprints of microbial communities (a) and cluster analysis based on "UPGMA" method (b) with different initial concentration of MR under aerobic conditions. Lane designations: I: original bacterial consortium without MR; II–VII: bacterial consortia with $50–300\,mg\,L^{-1}$ of MR (increasing $50\,mg\,L^{-1}$ of dye for each transfer).

Table 4
16S rDNA sequence analysis and species identification of selected dominant DGGE bands for the aerobic consortium.

Band no.	GeneBank no.	Closest sequences	Closest sequences GeneBank no.	Similarity
AE-1	JN049577	*Klebsiella pneumoniae* strain RSN19	HM751200	100
AE-2	JN049578	*Klebsiella pneumoniae* strain Ned	HQ200415	100
AE-3	JN049579	*Klebsiella* sp. strain ICB390	HM748059	99
AE-4	JN049580	*Buttiauxella izardii* strain MHF ENV 19	HM625745	100
AE-5	JN049581	Uncultured bacterium clone TIGU1260	HM558964	100
AE-6	JN049582	Uncultured bacterium clone 16slp112-1a03.p1k	GQ158223	100
AE-7	JN049583	*Buttiauxella izardii* strain MHF ENV 19	HM625745	100
AE-8	JN049584	*Klebsiella* sp. enrichment culture clone SRC_DSD23	HM195208	99
AE-9	JN049585	*Klebsiella* sp. clone SL13	HQ264073	100
AE-10	JN049586	*Bacillus* sp. strain JSM 099021	HM460884	99
AE-11	JN049587	*Klebsiella* sp. enrichment culture clone SRC_DSD23	HM195208	99

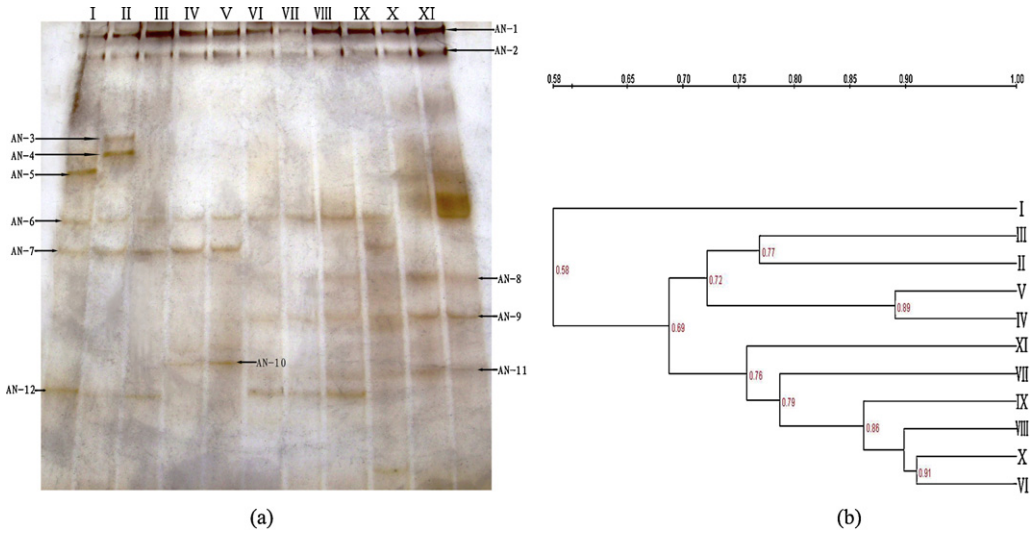

Fig. 4. DGGE fingerprints of microbial communities (a) and cluster analysis based on "UPGMA" method (b) with different initial concentration of MR under anaerobic conditions. Lane designations: I: original bacterial consortium without MR; II–XI: bacterial consortia with 50–500 mg L^{-1} of MR (increasing 50 mg L^{-1} of dye for each transfer).

The 16S rDNA fragments that were extracted from DGGE bands were sequenced. The closest relative strains that are available in the GenBank were obtained and compared (Table 5). The 9 bacteria clones were divided into 5 groups. In the first group, three bacteria clones (AN-2, -8, -10) belonged to *Klebsiella*. In the second group, two bacteria clones (AN-6 and AN-9) were most similar to *Bacillus* sp. In the third group, the clone AN-7 showed 99% sequence similarity to *Clostridium* sp. In the fourth group, the clone AN-12 presented 98% sequence similarity to *E. coli*. In the fifth group, two bacteria clones (AN-1 and AN-11) were associated with uncultured bacterium clones.

In the aerobic environment, the prevalent genera were *Klebsiella*, *Buttiauxella* and *Bacillus*. In the anaerobic environment, the prevalent genera were *Klebsiella, Escherichia*, *Bacillus* and *Clostridium*. The results show some deviation from other studies. Moosvi et al. [23] have selected the microbial consortium JW-2, which completely decolorizes Reactive Violet 5R (100 mg L^{-1}) within 36 h. The consortium JW-2 consists of three isolates that have been identified as *Paenibacillus polymyxa*, *Micrococcus luteus* and *Micrococcus* sp. Joshi et al. [14] have identified a bacterial consortium that decolorizes Acid Orange 7 and consists of *A. caviae*, *P. mirabilis* and *R. globerulus*. Tony et al. [15] have reported the novel aerobic bacterial consortium SKB-II that consists of five different bacteria, which have been identified as *Bacillus* sp.. The variance in the microbial communities in these consortia might involve different mechanisms for dye decolorization.

In this study, all of the genera that were responsible for dye degradation were categorized into two groups. The first group contained *Klebsiella*, *Escherichia* and *Buttiauxella*, which were all Gram-negative, facultative anaerobic bacteria of the family Enterobacteriaceae. The second group contained *Bacillus* and *Clostridium* which were Gram-positive bacteria of the family Bacillaceae. The

Table 5
16S rDNA sequence analysis and species identification of selected dominant DGGE bands for the anaerobic consortium.

Band no.	GeneBank no.	Closest sequences	Closest sequences GeneBank no.	Similarity
AN-1	JN049565	Uncultured bacterium clone EAS1106_H18	JF501387	99
AN-2	JN049566	*Klebsiella* sp. clone F5feb.15	GQ415984	99
AN-6	JN049570	*Bacillus cereus* strain IARI-CW-45	JF343175	100
AN-7	JN049571	*Clostridium* sp. clone 1	FR717579	99
AN-8	JN049572	*Klebsiella pneumoniae* subsp. rhinoscleromatis strain F3	HQ696467	99
AN-9	JN049573	*Bacillus* sp. LKS-113	JF502710	99
AN-10	JN049574	*Klebsiella pneumoniae* strain JDM-8	JF690978	99
AN-11	JN049575	Uncultured bacterium clone AUS23-1	HQ396013	98
AN-12	JN049576	*Escherichia coli* NA114	CP002797	98

majority of the bacterial sequences grouped with members of *Klebsiella*, which were consistent with the results of the isolated pure strains mentioned above. As a member of intestinal microflora, *Klebsiella* is commonly found in contaminated soil and wastewater. Numerous studies have reported its ability to metabolize toxic compounds [19,24]. These studies have suggested that *Klebsiella* might have a great potential to treat contamination. Moreover, the first group belongs to the family Enterobacteriaceae. In previous studies, many species in this family have been reported to have azo dye-decolorizing abilities. Chen et al. [25] reported a strain which was identified as *P. mirabilis*, could decolorize RED RBN under anoxic conditions, even at a high concentration of azo dye. Moutaouakkil et al. purified an azoreductase from *Enterobacter agglomerans*. The purified enzyme was a monomer with a molecular weight of 28 kDa, and the enzyme could decolorize several azo dyes in a short time [26]. Chang et al. [27] found that *E. coli* strain NO3 could decolorize Reactive Red 22 effectively under anaerobic conditions, and the decolorization rate increased greatly when the biodegradation system contained extracellular metabolites. Based on these, it was likely to conclude that most species in this family have the potential to decolorize azo dyes.

The second group contained *Bacillus* and *Clostridium*, which belong to the family Bacillaceae. In previous studies, several species belonging to *Bacillus* have been reported to have azo dye-decolorizing abilities. Zissi et al. [28] have reported a strain that belongs to *Bacillus subtilis*, which degrades *p*-aminoazobenzene in the presence of glucose as a carbon source. Deng et al. [2] have isolated and characterized a new *B. cereus* strain DC11 that is capable of decolorizing a broad-spectrum of dyes. Modi et al. [11] have reported that *B. cereus* exhibits a high efficiency of decolorizing sulfonated azo dyes under anaerobic and aerobic conditions. All of these results indicated that *Bacillus* may play an important role in developing treatments for azo dye-containing wastewater.

4. Conclusion

In this study, thirteen isolates were selected from dye-contaminated water based on their ability to decolorize MR under aerobic and anaerobic conditions. All of the isolated strains were facultative anaerobic bacteria of the family Enterobacteriaceae. Two bacterial consortia, which were capable of decolorizing azo dyes under aerobic and anaerobic conditions, were enriched. Decolorization studies showed that both of the two consortia had better decolorization characteristics as compared to pure cultures. Phytotoxicity studies indicated that MR could be detoxicated by both of the two consortia. PCR-DGGE analysis showed that dynamic changes of microbial communities were observed in aerobic and anaerobic compartments. Sequencing results indicated that all of the genera in the two consortia were categorized into two groups. The first group were all Gram-negative, facultative anaerobic bacteria of the family Enterobacteriaceae; the second group were Gram-positive bacteria of the family Bacillaceae. The results suggested that strains which belong to the two families might have a great potential for dye-contaminated water treatment.

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