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Effect of hypersaline aniline-containing pharmaceutical wastewater on the structure of activated sludge-derived bacterial community

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ABSTRACT

In order to explore the effect of hypersaline aniline-containing pharmaceutical wastewater on the structure of activated sludge-derived bacterial community, RAPD-PCR and PCR-DGGE techniques were used to analyze the change of bacterial community diversity and structure during the acclimation to different concentrations of two types of wastewater. Different bacterial community structures and significant shift in the bacterial community diversity were observed during the acclimation of wastewater. The number of bacterial species and the abundance of bacteria acclimated to different concentration of wastewater decreased when the concentration increased. The trends of bacterial community diversity indices revealed by RAPD or DGGE profiles were different. The change of bacterial community diversity might be due to the adaptation and mutation under selection issues in the acclimation of wastewater. The relationship between diversity indices and the content of chemical oxygen demand (COD), chloride, salinity and aniline based on RAPD and DGGE data indicated that the change of the bacterial community diversity might not be due to a single factor but might be due to the integrated effects of all the contaminants in the wastewater. Phylogenic trees based on RAPD and DGGE fingerprints were different, indicating that the effect of wastewaters A and B on the bacterial community structure was different, which might be due to the characteristics of wastewater.

1. Introduction

The pharmaceutical manufacturing industry produces a wide range of products to be used as human and animal medications [1]. Many pharmaceutical wastewaters contain high salinity [2]. Special attention currently focuses on pharmaceutical wastewaters [3–5]. For pharmaceuticals wastewater, microbial degradation is believed to be the most important removal process in activated sludge wastewater treatment [6]. These wastewaters contain relatively high level of suspended solids and soluble organics, many of which are recalcitrant [7,8], and pose several problems for successful biological treatment. The successful biological treatment of pharmaceutical wastewater presents a significant challenge [9].

Activated sludge of aerobic wastewater treatment plants consists of a complex mixture of microorganisms that are either generalists or specialists [10]. The bacteria in activated sludge are responsible for converting the harmful pollutants into harmless compounds before releasing the wastewater into the environment. For years, researchers have examined the microbial populations of these activated sludge communities in order to understand their specific biological processes [11–15]. Boon et al. evaluated the bacterial communities of activated sludge samples that received wastewater from four different types of industries [10]. Moura et al. studied the bacterial communities in the activated sludge in aerated lagoons of a wastewater treatment plant [16].

In spite of the importance of activated sludge for wastewater treatment, information on microbial ecology in biological wastewater treatment has been quite limited. LaPara et al. revealed that the microbial community in the bioreactor of wastewater treatment system could adapt to changing environmental conditions, such as the influent wastewater characteristics [9]. LaPara et al. studied the effect of temperature on the bacterial community structure during the aerobic biological treatment of a pharmaceutical wastewater and revealed that temperature served as a selective pressure and elevated temperature corresponded to fewer number of bacterial populations [9].

A broad bacterial consortium is required to achieve the desired biological conversions and the performance of wastewater treatment largely depends on the bacterial diversity present [17]. Therefore, a fundamental understanding of the microbial community structure and stability as well as its response to different chemicals entering the wastewater, are desirable for stable and efficient wastewater treatment plant operation. But there are few experimental studies investigating the effects of hypersaline

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aniline-containing wastewater on the microbial community diversity in wastewater treatment plants and the mechanism explaining the influence of hypersaline wastewater on the microbial community. The objective of the paper was to explore the effect of hypersaline aniline-containing pharmaceutical wastewater on the diversity and structure of activated sludge-derived microbial community and the relationship between the microbial community diversity and the chemical oxygen demand (COD), salinity, chloride and aniline content were analyzed to elucidate the mechanism. Random amplified polymorphic DNA (RAPD)-PCR, which has been successfully applied to epidemiological studies with bacteria, in resolving interspecific relationships in other genera [18–20], in bacteria typing [21], and in genetic diversity of bacteria [22–25], has been used as tool for the analysis of diversity. PCR-denaturing gradient gel electrophoresis (DGGE), which has already been successfully used to explore bacterial diversity in various environmental samples and populations shifts in response to environmental changes in bioreactors [26–28], has been used to analyze the structure of bacterial communities.

2. Materials and methods

2.1. Sources of wastewater and activated sludge

Two types of hypersaline aniline-containing wastewater were collected from different production lines in a pharmaceutical chemicals and intermediates industry. pH, salinity and chloride of the wastewater were analyzed according to the standard methods for the analysis of wastewater [29]. COD was analyzed in a high dose of $HgSO_4$ (at the ratio of $HgSO_4$:Cl^- = 10:1) added to the samples to eradicate the chloride interference. The aniline compounds existing in the wastewater were determined by N-(1-naphthyl) ethylene diamine dihydrochloride spectrophotometric method (GB/T 15502-1995 http://english.mep.gov.cn/standards_reports/standards/Air_Environment/air_method/200809/t20080923_129249.htm). Wastewater neutralized with NaOH was used for the further study.

Activated sludge was used as starting material. The activated sludge was collected from effluent treatment plant treating wastewater generated by pharmaceutical formulation industry. A liter of sludge sample was collected from three different points in the aerobic biological reactor. The supernatant was discarded and the biomass was pooled, mixed thoroughly and transported to the laboratory immediately.

2.2. Wastewater domestication assay

Aerobic cultures (20 ml culture media) were grown in 50 ml conical flasks with cotton wool bungs. Initial enrichment cultures were set up with LB medium receiving 10% (v/v) activated sludge. After 48 h shaking at 150 rpm at 30 °C, acclimatization assays were set up using hypersaline aniline-containing wastewater added into LB medium at concentrations ranging from 5% to 40% at 5% interval. After 48 h, 1% of this inoculum was transferred into subsequent subcultures and the acclimation process was continued till the time at which OD_{540} was less than 0.2.

Table 1
Sequences of 9 primers successfully used in the RAPD analysis.

Primer	Sequence (5′–3′)	Primer	Sequence (5′–3′)
S303	TGGCGCAGTG	S308	CAGGGGTGGA
S370	GTGCAACGTG	S313	ACGGGAGCAA
S220	GACCAATGCC	S26	GGTCCCTGAC
S180	AAAGTGCGGC	S178	TGCCCAGCCT
S321	TCTGTGCCAC		

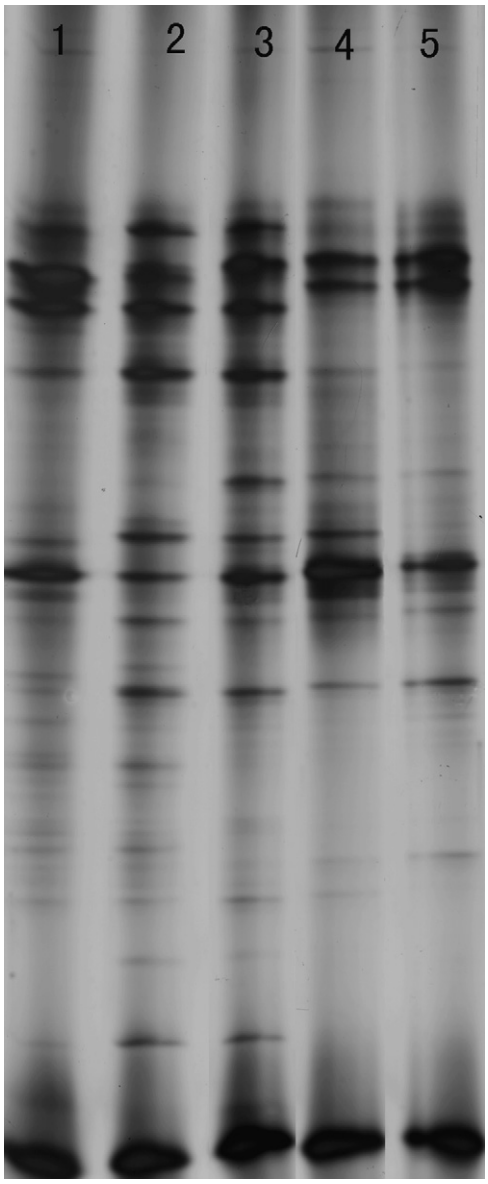

Fig. 1. PCR-DGGE patterns of bacterial community acclimated to different concentration of wastewater A. Lane 1, bacteria acclimated to 0% wastewater; Lane 2, bacteria acclimated to 5% wastewater; Lane 3, bacteria acclimated to 10% wastewater; Lane 4, bacteria acclimated to 20% wastewater A; Lane 5, bacteria acclimated to 40% wastewater.

2.3. Total genomic DNA extraction method

Total genomic DNA extraction was performed according to the cetyltrimethyl ammonium bromide (CTAB)–lysozyme–proteinase K-freezing thaw lysing method reported earlier [30]. The sludge was harvested by centrifugation at 10,000 rpm for 10 min. DNA was extracted from 1 g (wet weight) sludge pellet. The harvested cells were pre-washed by polyvinylpyrrolidone (PVP) buffer (2% sodium hexametaphosphate, 1% PVP K30). The pellet was collected by centrifugation at 10,000 rpm for 10 min. The pellet was resuspended in CTAB–lysozyme buffer [100 mmol/L Tris·HCl (pH 8.0), 100 mmol/L EDTA·2Na (pH 8.0), 100 mmol/L phosphate sodium buffer (pH 8.0), 1.5 mmol/L NaCl, 1% CTAB, 2% $CaCl_2$, 1 μg/mL bovine serum albumin (BSA)] and incubated at 37 °C for 1 h and sonicated for 30 min followed by incubation at 65 °C. This was followed by incubation at 65 °C in the presence of 100 μg/mL protease K with alternating freezing (−20 °C) and thawing (65 °C) for three times. DNA was

Table 2
The content of COD_{Cr}, chloride, salinity and aniline in hypersaline aniline-containing wastewaters A and B with different concentration.

Concentration	COD_{Cr} (mg/L)		Chloride (g/L)		Salinity (g/L)		Aniline (mg/L)	
	A	B	A	B	A	B	A	B
0%	0	0	0	0	0	0	0	0
5%	970	2,910	0.6845	0.5590	1.9950	3.6258	124	68.5
10%	1,940	5,820	1.3691	1.1180	3.9900	7.2515	248	137
20%	3,880	11,640	2.7382	2.2360	7.9800	14.5130	596	274
40%	7,760	23,280	5.4764	4.4720	15.9600	29.0260	1,192	548

purified using a chloroform/isoamyl alcohol (1/1) solution, and precipitated by the addition of 50% (v/v) 25% PEG 8000 (w/v) and stored at 4 °C overnight.

2.4. RAPD-PCR amplification

The effects of Mg^{2+}, dNTP, DNA templates, primers and DNA polymerase on the amplification were tested, and the determined optimal reaction system of RAPD was as follows: 1× Taq polymerase buffer [10 mmol/LTris·HCl (pH 9.0), 50 mmol/L KCl, 0.1% Triton X-100, 1.5 mmol/L $MgCl_2$], 1 U Taq DNA polymerase (Dingguo Inc., Beijing, China), 10 ng template DNA, 20 pmol primer, 2 mg/ml bovine serum albumin (BSA), and 0.15 mmol/L each of dATP, dCTP, dGTP or dTTP in the total 10 μl reaction volume. Amplification reaction was performed in a PTC 220 Thermal Cycler (Bio-Rad, Inc.) The touchdown cycle program included an initial 5 min denaturation at

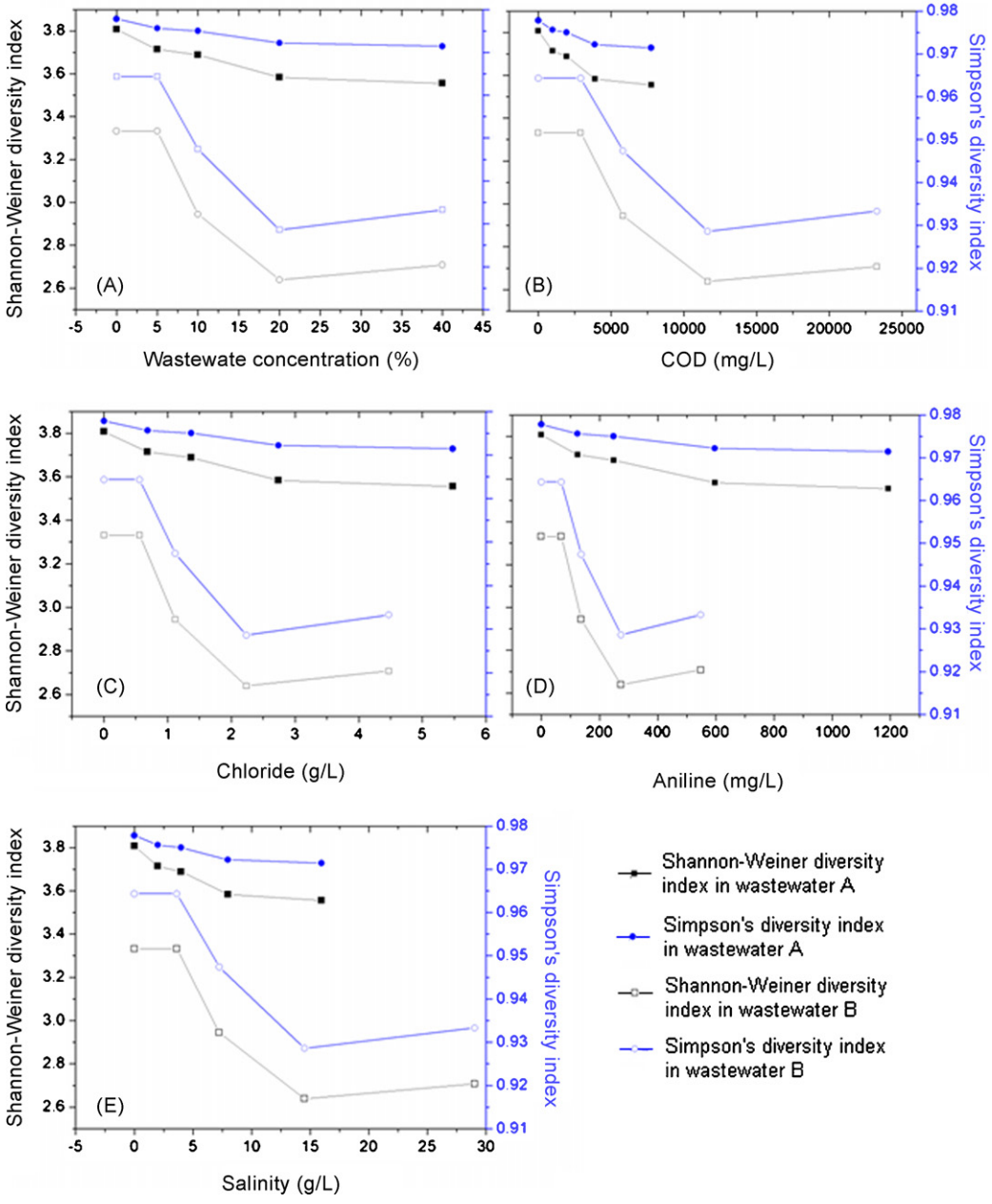

Fig. 2. Change of bacterial diversity indices based on DGGE profiles over concentration, COD, aniline, salinity and chloride of wastewater.

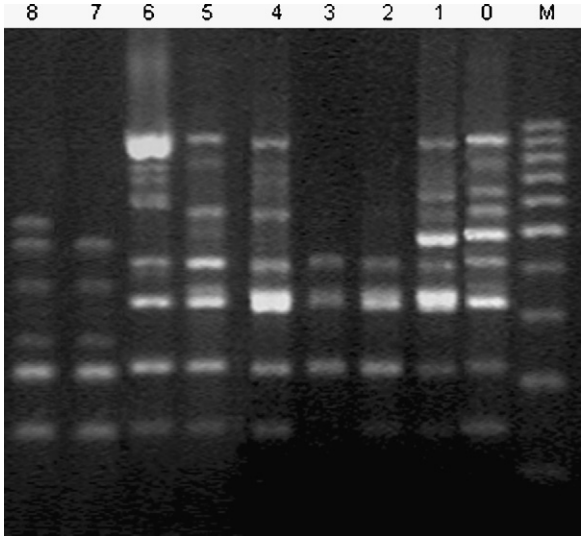

Fig. 3. RAPD-PCR patterns of bacterial community acclimated to different concentration of wastewater with primer S308. Lane M, 200 bp DNA ladder; Lane 0, bacteria acclimated to 0% wastewater; Lane 1, bacteria acclimated to 5% wastewater A; Lane 2, bacteria acclimated to 10% wastewater A; Lane 3, bacteria acclimated to 20% wastewater A; Lane 4, bacteria acclimated to 40% wastewater A; Lane 5, bacteria acclimated to 5% wastewater B; Lane 6, bacteria acclimated to 10% wastewater B; Lane 7, bacteria acclimated to 20% wastewater B; Lane 8, bacteria acclimated to 40% wastewater B.

94 °C, followed by 10 cycles of 1 min at 94 °C, 1 min at 42 °C (touchdown for 0.5 °C every cycle) and 1.5 min at 72 °C, followed by 25 cycles of 1 min at 94 °C, 1 min at 37 °C and 1.5 min at 72 °C, and 5 min final extension at 72 °C. PCR product was electrophoresed in 1.6% agarose gel at 100 V for 2 h, stained with ethidium bromide. The electrophoresis buffer was 0.5× TBE. Images were photographed with GIS-2008 gel imaging and analysis system (Shanghai Tannon Science & Technology Co., Ltd.). The negative control was run as well by replacing template DNA with ddH_2O. To every primer, triplicate amplification was conducted. A subset of 9 primers (Table 1) from 150 primers (purchased from Sangon Inc., Shanghai, China) for further analysis was chosen based on the following criteria: (i) consistent, strong amplification products, (ii) product of uniform and reproducible fragments between replicate PCRs, and (iii) no amplification in negative control.

2.5. PCR-DGGE analysis

Bacterial 16S rRNA gene from total bacterial genomic DNA were amplified by PCR in a reaction mixture containing (as final concentration): 1× Taq polymerase buffer [10 mmol/LTris·HCl (pH 9.0), 50 mmol/L KCl, 0.1% Triton X-100], 2.0 mmol/L $MgCl_2$, 1 U Taq DNA polymerase (Dingguo Inc., Beijing, China), 20 ng template DNA, 20 pmol forward primer F338GC (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCT ACGGGAGGCAGCAG-3′) and reverse primer R518 (5′-ATTACCGCGGCTCGTGG-3′), 2 mg/ml bovine serum albumin (BSA), and 0.25 mmol/L each of dATP, dCTP, dGTP or dTTP in the total 20 μl reaction volume. Amplification reaction was performed in a PTC 220 Thermal Cycler (Bio-Rad, Inc.). The touchdown cycle program included an initial 5 min denaturation at 94 °C, followed by 10 cycles of 1 min at 94 °C, 1 min at 65 °C (touchdown for 1 °C every cycle) and 1.5 min at 72 °C, followed by 25 cycles of 1 min at 94 °C, 1 min at 55 °C and 1.5 min at 72 °C, and 5 min final extension at 72 °C. DGGE was performed with a Dcode system (Bio-Rad, Hercules, California, USA) in accordance with the manufacturer's instructions. The PCR amplification products were loaded onto 4–10% (w/v) polyacrylamide gels in 1× TAE. The polyacrylamide gels were made with a denaturing gradient ranging from 30% to 50% (where 100% denaturant contains 7 M urea and 40% formamide). Electrophoresis was carried out at 60 °C for 9 h at 100 V. After electrophoresis, the gels were stained with silver and scanned at Epson 1680 Scanner. To every sample, triplicate amplification was conducted.

2.6. Data analysis

Amplified bands were scored in a size range from 0.2 to 2 kb. A data matrix of RAPD and DGGE profiles was obtained based on the locations of the fragments. The presence of the fragment was represented with "1" and the absence was represented with "0". Based on the presence (1) or absence (0) of individual bands in each lane, a binary matrix was constructed. The binary data representing the banding patterns were used to generate a pairwise Dice distance matrix. Biological ToolBox version 0.10 Add-In For Excel was used to calculate Shannon–Weiner diversity index (H) and Simpson's diversity index (D). Nei and Li genetic distance was calculated and unweighted pair group method arithmetic average (UPGMA) cluster analysis was performed to explore the relationship among bacterial community using DPS 3.01 software [31].

3. Results and discussion

3.1. Characteristics of wastewater

Wastewater A had pH lower than 1, with COD 19,400 mg/L, chloride 13.69 g/L, aniline 2480 mg/L and salinity 39.900 g/L. Wastewater B had pH lower than 1, with COD 58,200 mg/L, chloride 11.18 g/L, aniline 1370 mg/L and salinity 72.515 g/L. The characteristics of wastewater in different concentrations (0–40%) are listed in Table 2.

3.2. Effect of wastewater on bacterial community structure

DGGE fingerprint profile of bacterial community acclimated by different concentrations of wastewater A and wastewater B is shown in Fig. 1. 16S rDNA bands in a DGGE gel might link to different bacteria species and the intensity of the bands might correspond at least semiquantitatively with the abundance of the corresponding species [32]. The number of the bacterial species and the abundance of bacteria acclimated to different concentrations of wastewater decreased when the concentration of wastewater increased. Different bacterial community structures were found during acclimation of wastewater by analyzing DGGE profiles. This indicated that the wastewater had an inhibitory effect on the microorganism during the acclimation, and subsequently affected the bacterial community structure [33]. In acclimation experiments, the bacteria would adapt to a specific condition and shift towards those organism best suited to metabolize the available organic nutrient in wastewater [5]. The selection pressure posed by wastewater, such as COD, salinity, chloride and aniline, could inhibit the growth of unsuitable bacteria and promote the growth of the tolerant bacteria. After proper adaptation, many bacteria have proved capable of removing efficiently the organic matter from hypersaline aniline-containing wastewater [34].

The trend of bacterial community diversity indices (H and E) could be linked to variation during the acclimation. Based on DGGE profiles, minor variation occurred during the acclimation of wastewater A, while major variation occurred during the acclimation of wastewater B (Fig. 2A–E). Analysis of the DGGE patterns of wastewater A indicated that the bacterial diversity H and E decreased slowly with the increased concentration of wastewater A. Analysis of the DGGE patterns of wastewater B indicated that H and E did not vary in bacteria acclimated to 5% concentration of wastewater B and decreased sharply in bacteria acclimated to 10%

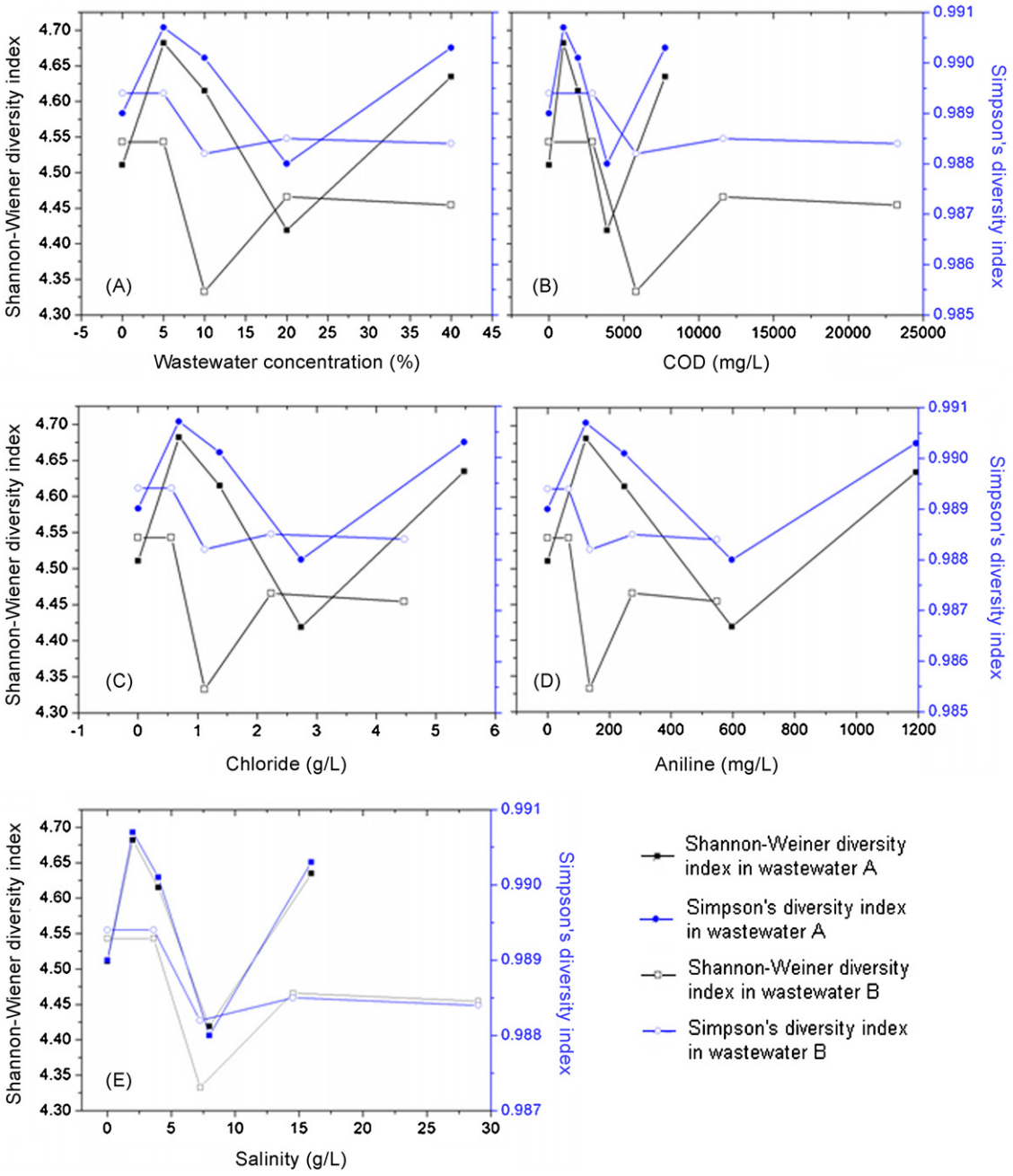

Fig. 4. Change of bacterial diversity indices based on RAPD profiles over concentration, COD, aniline, salinity and chloride of wastewater.

(COD 5820 mg/L, chloride 1.1180 g/L, aniline 137 mg/L and salinity 7.2515 g/L) and 20% concentration of wastewater B. Interestingly, *H* and *E* increased again in bacteria acclimated to 40% concentration of wastewater B (COD 23,280 mg/L, chloride 4.4720 g/L, aniline 548 mg/L and salinity 29.0260 g/L), indicating that new bacteria species with change of V3 region of 16S rDNA might occur during the acclimation.

3.3. *Effect of the wastewater on the bacterial community diversity*

RAPD-PCR technique was used to explore the mechanism of effect of hypersaline aniline-containing wastewater on bacterial community diversity. RAPD fingerprint of bacterial community acclimated to different concentrations of wastewaters A and B is shown in Fig. 3. The number of RAPD bands of bacteria acclimated to 5% of wastewater A, 0% or 5% of wastewater B was the highest, while those of bacteria acclimated to 20% of wastewater A and 10% of wastewater B was the lowest. This indicated that a significant shift in microbial community was observed when the wastewater was present.

Major variation of *H* and *E* occurred between 5% and 40% wastewater A, while that occurred between 5% and 20% wastewater B (Fig. 4A–E). Analysis of the RAPD patterns of wastewater A indicated that the diversity of bacteria increased much when acclimated to 5% wastewater A and decreased sharply when acclimated to 20% wastewater A (COD 3880 mg/L, chloride 2.7382 g/L, aniline 596 mg/L and salinity 7.9800 g/L). Analysis of the RAPD patterns of wastewater B indicated that bacterial community shifts were slower when acclimated to 5% wastewater B, decreased sharply when acclimated to 10% wastewater B (COD 5820 mg/L, chloride 1.1180 g/L, aniline 137 mg/L and salinity 7.2515 g/L) and increased again when acclimated to 20% wastewater B. The trends were

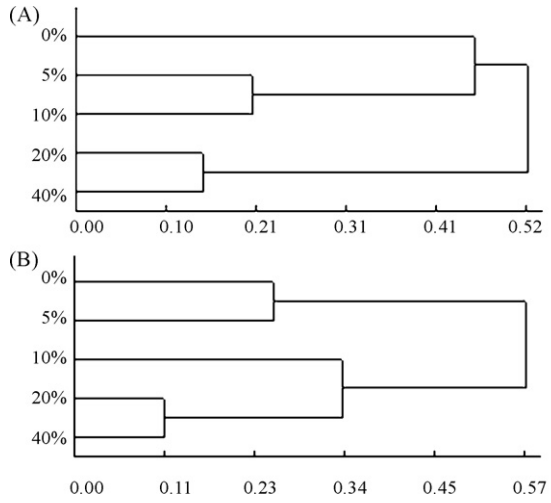

Fig. 5. Dendrogram of DGGE patterns of bacterial community acclimated to different concentration of wastewater A (A) and wastewater B (B). 0% indicated bacteria acclimated by 0% wastewater; 5% indicated bacteria acclimated by 5% wastewater; 10% indicated bacteria acclimated by 10% wastewater; 20% indicated bacteria acclimated by 20% wastewater; 40% indicated bacteria acclimated by 40% wastewater.

different from that revealed by analyzing DGGE profiles (Fig. 2A–E), which indicated that the origin of the bacterial diversity revealed by different techniques might be different. DGGE profiles targeted the highly variable V3 region of 16 S rDNA [35] and could be linked to bacterial compositions during the acclimation, while RAPD technology utilized the whole genome [36] and could detect the genetic variation in the same or different bacteria species.

A change in microbial community diversity may be related to environmental conditions [9]. Eichner et al. found that the microbial diversity in activated sludge decreased after a shock load [37]. The two types of trends revealed by RAPD and DGGE profiles indicated that the change of bacterial community diversity might be due to the adaptation and mutation under selection issues in the acclimation of wastewater. The decrease in bacterial composition based on DGGE data might be due to the adaptation and selection of bacteria to the contaminant components in wastewater and the variation of the bacterial diversity based on RAPD data might be due to the mutation and adaptation of bacteria to the contaminant components in wastewater.

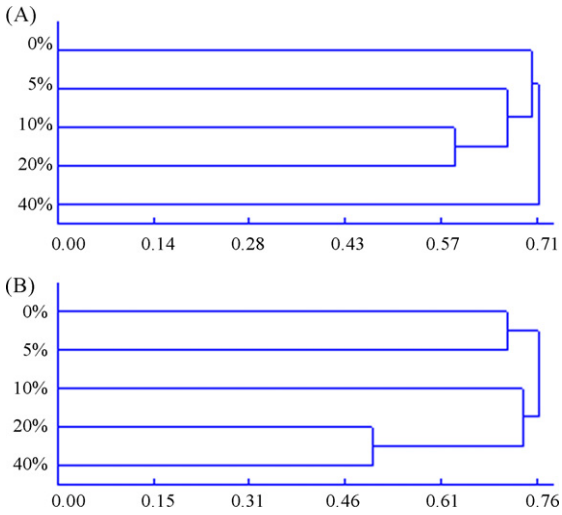

Fig. 6. Dendrogram of RAPD-PCR patterns of bacterial community acclimated by different concentration of wastewater A (A) and wastewater B (B). 0% indicated bacteria acclimated by 0% wastewater; 5% indicated bacteria acclimated by 5% wastewater; 10% indicated bacteria acclimated by 10% wastewater; 20% indicated bacteria acclimated by 20% wastewater; 40% indicated bacteria acclimated by 40% wastewater.

The adaptation and selection have a threshold which could induce significant shift. Li et al. analyzed the responses of bacterial community structure to different amounts of diesel fuel amendments and the results showed that low-level contamination only had a slight and short time effect on community diversity [38]. However, the significant decrease in bacterial community diversity at the level >10,000 mg/kg diesel fuel suggested that heavy petroleum contamination resulted in the dramatic decrease in community diversity in meadow brown soil [38]. The threshold in this study might be 20% wastewater A (COD 3880 mg/L, chloride 2.7382 g/L, aniline 596 mg/L and salinity 7.9800 g/L) and 10% wastewater B (COD 5820 mg/L, chloride 1.1180 g/L, aniline 137 mg/L and salinity 7.2515 g/L).

As described previously, bacterial biodiversity depends on gradient salinity, with a decrease in bacterial diversity when salinity increased [38,39]. The bacterial community diversity indices H and E based on DGGE data correlated negatively with the concentration of COD, salinity, chloride and aniline of wastewater A (r is -0.9120 and -0.9216, respectively, $P < 0.05$), but not with wastewater B ($P > 0.05$). There was also no significant correlation between diversity indices based on RAPD data and the content of COD, salinity, chloride and aniline ($P > 0.05$). It suggested that the change of the bacterial community diversity might not be due to a single factor but rather due to the integrated effects of all the contaminants in the wastewater. In spite of adaptation, mutation is important during the acclimation and might be the main way to produce new species [40]. The increase of diversity might be the result of mutation and subsequent adaptation to be new dominant bacteria species or strains.

3.4. Phylogenic analysis of bacterial communities

Phylogenic tree based on DGGE profiles placed bacteria acclimated to 0%, 5% and 10% of wastewater A into one group and those acclimated to 20% and 40% wastewater into another group (Fig. 5A), while the clustering analysis based on RAPD fingerprints showed that bacterial community acclimated by 40% wastewater was clustered as one group and the others were clustered into another groups (Fig. 6A). Clustering analysis showed that bacterial community acclimated by different concentrations of wastewater B could be divided into two major groups based on RAPD or DGGE fingerprints: bacterial community acclimated by 0% and 5% wastewater was clustered into one group and the others were clustered into another group (Fig. 5B and Fig. 6B). The influent wastewater characteristics could change the microbial community in the bioreactor of wastewater treatment system [8]. The results indicated that the effect of wastewaters A and B on the bacterial community structure was different, which might be due to the characteristics of wastewater.

4. Conclusion

In this study, RAPD-PCR and PCR-DGGE techniques were used to explore the effect and mechanism of hypersaline aniline-containing pharmaceutical wastewater on the structure and diversity of activated sludge-derived microbial community. The results were as follows:

1. Different bacterial community structure was found during acclimation of wastewater by analyzing DGGE profiles. The number of bacterial species and the abundance of bacteria acclimated to different concentrations of wastewater decreased when the concentration of wastewater increased. Bacterial diversity decreased

with the increased concentration of wastewater except for that of bacteria acclimated to 40% concentration of wastewater B.
2. A significant shift in the microbial community was observed by analysis the RAPD profiles when the wastewater was present. Major variation of *H* occurred between 5% and 40% concentration of wastewater A, while that occurred between 5% and 20% concentration of wastewater B. The diversity of the bacterial population decreased sharply at 20% concentration of wastewater A and 10% concentration of wastewater B. The diversity increased sharply again at 40% concentration of wastewater A and 20% concentration of wastewater B.
3. The change of bacterial community diversity might be due to the adaptation and mutation under selection issues in the acclimation of wastewater. The relationship between diversity indices and the content of COD, chloride, salinity and aniline based on RAPD and DGGE data indicated that the change of the bacterial community diversity might not be due to a single factor but rather due to the integrated effects of all the contaminants in the wastewater.
4. Clustering analysis based on RAPD-PCR and PCR-DGGE fingerprints indicated that the effects of wastewaters A and B on the bacterial community structure were different, which might be due to the characteristics of wastewater.

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