Bacterial diversity in antibiotic wastewater treatment

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ABSTRACT

The bacterial diversity of an antibiotic industrial wastewater treatment system was analyzed to provide the information required for further optimization of this process and for identification of bacterial strains that perform improved degradation of antibiotic industrial wastewater. The total bacterial DNA of samples collected at three stages (aeration, precipitation, and idle) during the sequencing batch reactor (SBR) process were analyzed by polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE) of the 16 s rDNA V3 regions. Community analysis was conducted in terms of the richness value (S), the dominance degree and the Shannon–Wiener diversity index (H). Rich bacterial diversity was apparent in the aeration stage of the SBR process, and the number of bands in the aeration stage was more abundant than that in the precipitation and idle stages. The DGGE analysis showed 15 bands, six of which were uncultured bacteria, and included one anaerobic and five aerobic bacteria. The microbial community in the aeration stage was the most complex of the whole SBR process, while the dominant bacteria differed in each reaction stage. These results demonstrate the cyclical dynamic changes in the bacterial population during the SBR process for the treatment of antibiotic industrial wastewater.

Key words | antibiotic industrial wastewater, bacterial group, PCR-DGGE, SBR approach analysis

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INTRODUCTION

Antibiotics are chemicals produced by some microorganisms that inhibit the growth of, or even destroy, other pathogenic microorganisms (Hiramatsua *et al*. 2012). Penicillin was the first antibiotic to be discovered (Bentley 2005). In China, the main raw materials for antibiotic production are natural compounds such as food and molasses. The production procedure includes processes such as microbial fermentation, filtration, extraction, crystallization, chemical extraction, and refining (Planson *et al*. 2011). During antibiotic production, a large amount of wastewater is generated, which is characterized by high concentrations of organic matter and biological toxic substances, a more volatile pH value, and high chromaticity. Detoxification of such organic wastewater is difficult (Akiyama & Savin 2010); the possibility of techniques such as oxidative degradation, photodegradation, electrocoagulation, biochemical degradation, and adsorption (Mittal *et al*. 2008, 2010; Gupta *et al*. 2009, 2010, 2011) have been exploited. At present, the sequencing batch reactor (SBR) process is widely used. This system is not only simple with broad applications, high sludge activity, and rapid removal of organic matter (Thanikal *et al*. 2007), but also facilitates effective denitrification and phosphorus removal. Furthermore, this process is less prone to sludge expansion (Morling 2010).

Combined molecular and conventional analyses in research on bacterial diversity in activated sludge have been done (Juretschko *et al*. 1998) and several groups have reported on biodegradation and adsorption of antibiotics in the activated sludge process (Li & Zhang 2010). In this study, we exploited polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE) to analyze the changes in bacterial diversity during three different stages (aeration, precipitation, and idle stages) of the complete SBR process (Wang *et al*. 2012) applied to the treatment of antibiotic industrial wastewater treatment. The corresponding bacterial species were identified by sequencing, providing a more informed reference for the investigation of strains suitable for antibiotic wastewater treatment. The micro-ecological study of activated sludge is of great value in determining optimal scientific regulation of the SBR system for improvement of the

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effectiveness of wastewater treatment (Kumar *et al.* 2008). Furthermore, the identification of the dominant strains present during the SBR process provides a theoretical basis for the use of the biologically enhanced technology represented by the addition of specific microorganisms to improve industrial wastewater treatment (Blatchley *et al.* 2007). This information can also be applied to the generation of genetically engineered bacteria for optimal sewage treatment (Chen & Li 2001).

MATERIALS AND METHODS

Samples

Antibiotic wastewater was collected from the activated sludge in the SBR treatment tanks of the Harbin General Pharmaceutical factory of Harbin Pharmaceutical Group Co. Ltd (Harbin, Heilongjiang Province, China). Water samples were collected from two independent reaction cycles conducted at a 1-month interval. On each occasion, samples were collected from the three different stages (aeration (AE), precipitation (PR) and idle stages (ID)) of the same reaction cycle. All samples, comprising a mixture of sludge and water, were collected at the start of sludge disposal, sealed in plastic bottles and stored at 4 °C.

Isolation of total mixed bacterial genomes in samples of antibiotic wastewater

The total mixed bacterial genomes in the samples were isolated using Tianwei Bacterial Genomic DNA Extraction kits (Tianwei Biotech, China) according to the manufacturer's instructions. The extracted DNA was isolated by 1% agarose gel electrophoresis, excised and stored at −20 °C.

PCR amplification of 16 s rDNA V3 regions

The V3 region of the 16S rDNA gene was amplified using primers 341F (5′-CCTAC GCGAG GCAGC AG-3′) and 518R (5′-ATTAC CGCGG CTGCT GG-3′) to amplify bacterial 16S rDNA-V3 segments corresponding to nucleotides 341–534 of the *Escherichia coli* sequence. A 40 bp GC-clamp CGCCC GCCGC GCGCG GCGGG CGGGG CGGGG GCACG GGGGG was added to primer 341F to increase the separation of DNA bands in DGGE gel (Muyzer *et al.* 1993). The PCR amplification was performed in a reaction mixture (50 μL) containing 4 μL DNA as a template, 5 μL 10× Ex Taq Buffer (Mg^{2+} free), 4 μL 2.5 mM dNTP mixture, 4 μL 25 mM $MgCl_2$, 5 U Taq polymerase, and 5 μL 0.1 pM primers. The PCR conditions were as follows: initial denaturation at 95 °C for 5 min followed by 35 cycles comprising denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, and polymerization at 72 °C for 40 s, with a final extension at 72 °C for 7 min. PCR products were separated by 2% agarose gel electrophoresis followed by ethidium bromide staining.

DGGE analysis

DGGE analysis for V3-16S rDNA variable region products (230 bp) was performed using gels containing a gradient of 40–70% denaturant over 9 h at 150 V followed by silver staining. Representative bands were excised from DGGE gels, and DNA was recovered and inserted into the pGM-T Easy vector. The recombinant was transformed into competent *E. coli* DH5α. The positive clones were amplified for sequencing of clone inserts (by Sangon Biotech, Shanghai, China). The sequencing results were compared with those available in the GenBank nucleotide database using the Basic Local Alignment Search Tool (BLAST) program to identify the predicted genus, followed by phylogenetic analyses (Omar & Ampe 2000).

Diversity analysis

Community analysis is commonly expressed in terms of the richness value (S), the dominance degree, and the Shannon–Wiener diversity index. The richness value and dominance degree were determined using a gel imaging system; the Shannon–Wiener diversity index (H) was calculated according to the following formula:

$$H = -\sum_{i=1}^{s} (p_i)(\log_2 P_i)$$

According to the unweighted pair group method with arithmetic averages calculation method, the places where bands appeared in the DGGE profile were recorded as 1, and those without bands were recorded as 0. The quadratic matrix matching the DGGE bands was then obtained and used as the basis for clustering analysis with the SPSS16.0 software. The phylogenetic tree of the DGGE bands was drawn.

RESULTS AND DISCUSSION

The total bacterial DNA extraction of antibiotic wastewater in different reaction stages and PCR amplification of the 16S rDNA V3 region

Analysis of the total extracted bacterial DNA obtained during the complete SBR process by 1% agarose gel electrophoresis revealed that the total genomic DNA from the three different reaction stages was consistent at approximately 15,000 bp.

The 16S rDNA V3 region was amplified from the total genome isolated from the mixture, using the F518 and R338 universal primers. The products were then analyzed by 1% agarose gel electrophoresis, which revealed that the length of target fragments after PCR amplification was approximately 230 bp.

Sequencing of the DGGE bands and the phylogenetic tree

The bacterial 16S rDNA V3 region products of the activated sludge samples from the three stages of the SBR process were analyzed by DGGE (Figure 1). The profiles obtained showed specific differences in the number and position of bands in every sample. Fifteen bands were recovered for sequencing (Table 1). Analysis of the DGGE profiles allowed identification of the 15 bands as *Flavobacterium* sp., "Benzene-decomposing" bacterium, *Frigoribacterium* sp., two types of *Desulfobacterium anilini*, *Chryseobacterium* sp., actinobacterium, *Bacteroidetes* sp., *Paracoccus* sp., "Iron-reducing" bacterium, *Acidobacteria* sp., *Bacillus subtilis*, two types of *Syntrophus aciditrophicus*, and *Chloroflexi* sp. Among these, we identified one species of anaerobic bacteria and five species of aerobic bacteria that were uncultured bacteria and difficult to separate and identify by traditional methods.

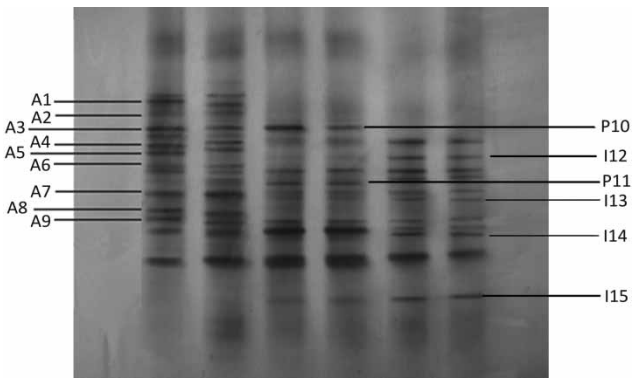

Figure 1 | Denaturing gradient gel electrophoresis profiles of sequencing batch reactor treatment samples of antibiotic wastewater. Three water samples were collected on each of two occasions (at a 1-month interval) from three different stages (aeration (AE), precipitation (PR) and idle stages (ID)) during the same reaction cycle. Lanes 1 and 2: samples collected in the aeration stage (AE1 and AE2, respectively). Lanes 3 and 4: samples collected in the precipitation stage (PR1 and PR2, respectively). Lanes 5 and 6: samples collected in the idle stage (ID1 and ID2, respectively).

Table 1 | BLAST analysis of 15 sequences from the DGGE profiles of samples obtained during the SBR process

Clone number	Sequence size (bp)	Strains with the highest identity from the NCBI database (Accession No.)	Similarity
A1	233 bp	*Bacillus subtilis* BSn5 (CP002468.1)	233/234 (99%)
A2	236 bp	*Flavobacterium* sp. (EU430699.1)	236/238 (99%)
A3	239 bp	"Benzene-decomposing" bacterium (AJ279493.1)	239/239 (100%)
A4	231 bp	Uncultured *Frigoribacterium* sp. (JF269114.1)	231/235 (98%)
A5	228 bp	*Desulfobacterium anilini* culture collection (HQ170544.1)	228/235 (97%)
A6	209 bp	*Chryseobacterium* sp. (EF208922.1)	209/221 (95%)
A7	217 bp	Uncultured actinobacterium (EF188800.1)	217/220 (99%)
A8	230 bp	Uncultured *Bacteroidetes* sp. (DQ811910.1)	230/231 (99%)
A9	233 bp	*Desulfocapsa thiozymogenes* (AF418166.1)	233/233 (100%)
P10	235 bp	*Paracoccus* sp. (FR744799.1)	235/237 (99%)
P11	219 bp	"Iron-reducing" bacterium (FJ802357.1)	219/230 (95%)
I12	228 bp	Uncultured *Synergistetes* sp. (GQ324231.1)	228/230 (99%)
I13	237 bp	Uncultured *Acidobacteria* sp. (FJ037346.1)	237/238 (99%)
I14	239 bp	*Syntrophus aciditrophicus* SB (CP000252.1)	239/239 (100%)
I15	233 bp	Uncultured *Chloroflexi* sp. (JF508351.1)	233/237 (98%)

The sequencing results showed that the length of most fragments was approximately 235 bp, and exhibited comparatively high similarity with the known strains in the GenBank database; most were above 98% except for A5 (97%) and A6 and P11 (95%). Comparison of the DGGE bands showed the following levels of similarity: A1 $\leq$99%

with *Bacillus subtilis* (CP002468.1); A2 ≤99% with *Flavobacterium* sp. (EU430699.1); A3 ≤100% with "Benzene-decomposing" bacterium (AJ279493.1); A4 ≤ 98% with *Frigoribacterium* sp. (JF269114.1); A5 ≤ 97% with *Desulfobacterium anilini* (HQ170544.1); A6 ≤ 95% with *Chryseobacterium* sp. (EF208922.1); A7 ≤ 99% with actinobacterium (EF188800.1); A8 ≤ 99% with *Bacteroidetes* sp. (DQ811910.1); A9 and P10 ≤ 99% with *Paracoccus* sp. (FR744799.1); P11 ≤ 95% with "Iron-reducing" bacterium (FJ802357.1); I12 and I13 ≤ 99% with *Acidobacteria* sp. (FJ037346.1); I14 ≤ 100% with *Syntrophus aciditrophicus* SB (CP000252.1); I15 ≤ 98% with *Chloroflexi* sp. (EF208922.1).

In this study, the samples for biodiversity analysis were collected from the Harbin General Pharmaceutical factory of Harbin Pharmaceutical Group Co. Ltd, which mainly produces penicillin and cephalosporin antibiotics. Benzene rings are prevalent in the structural formulae of these drugs, which accounts for the dominance of the "Benzene-decomposing" bacterium during the aeration stage of the SBR process (Moussavi *et al.* 2010). Some research has shown that *Desulfobacterium anilini* exhibits tolerance to the antibiotic stress (Kraigher *et al.* 2008).

The level of dissolved oxygen also has an effect on this bacterium; when the SBR process enters into the precipitation and idle stages, the microbial composition of the environment is transformed from aerobic to anaerobic and the amount of *Desulfobacterium anilini* gradually reduces (Ahn *et al.* 2009); this species was absent in the corresponding position in DGGE profiles. The bands representing I12 (uncultured *Synergistetes* sp.) and I14 (*Syntrophus aciditrophicus* SB) appeared when the process entered into the idle stage, whereas these bands were absent in the two former stages. These bacteria represent two types of obligate anaerobe, and were detected as the dominant bacteria only in the idle stage. In general, the changes of bacterial diversity in the different stages of the SBR process were closely related to the variations in wastewater composition and conditions.

In order to better understand the relationship between the amplified target sequences and the known bacteria as well as their systematic positions, the phylogenetic tree was constructed according to the bacteria with the closest relative of each sequence in the GenBank database and the identified bacteria with the closest relative (Figure 2).

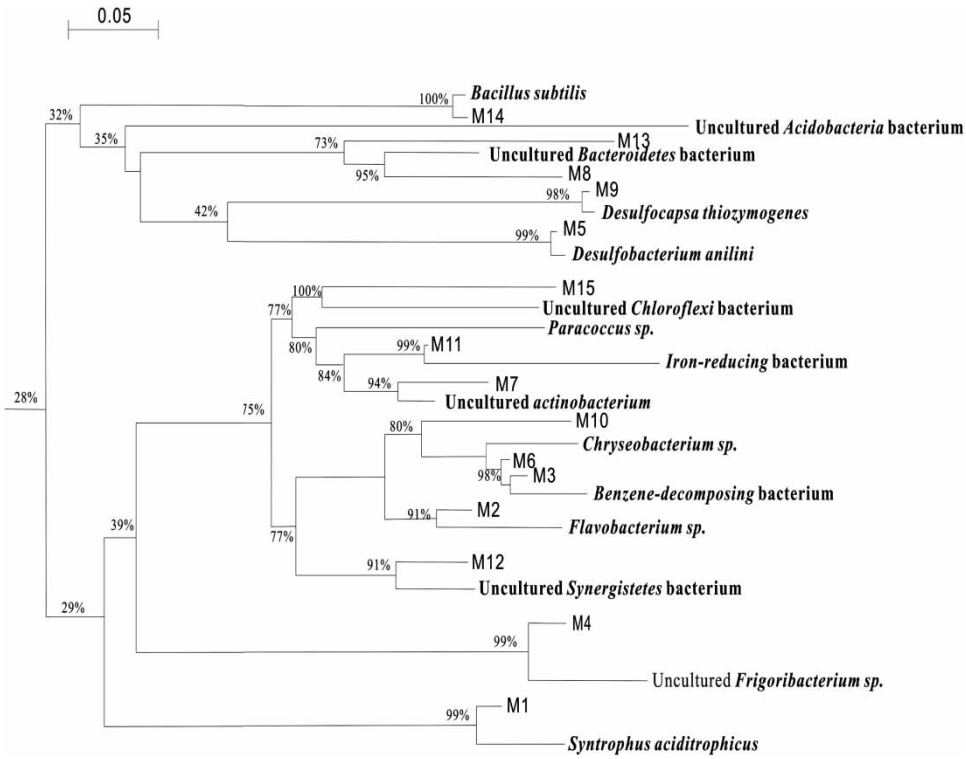

Figure 2 | Phylogenetic tree constructed according to the bacteria with the closest relative of each of 15 DGGE sequences identified in the GenBank database and the identified bacteria with the closest relative.

DGGE analysis

The richness value of DGGE profiles

The Gel-Pro Analyzer 4.5 software was used to analyze the DGGE profiles, and the richness value of the DGGE profiles at the three SBR stages were calculated (Figure 3). The intensity of 20 bands in every sample was assessed (Table 2).

In the analysis of the microbial diversity of DGGE profiles, the richness value indicated the sum of sample bands and therefore, reflected to some extent the dominance degree of microbial diversity in the whole community.

The greatest richness values (15) were detected at the aeration stage, indicating that this stage was the main reaction phase of the wastewater treatment process with the most abundant bacterial species. The bacterial species decreased at the precipitation stage with a richness value of 10. After spoil disposal the reaction device entered the idle stage, with a richness value of 11, which was approximately equal to that at the precipitation stage, although the position of the bands differed. For example, in the precipitation stage, bands 4, 17, and 19 were dominant, while the dominant bands at the idle stage were 6, 10, and 19.

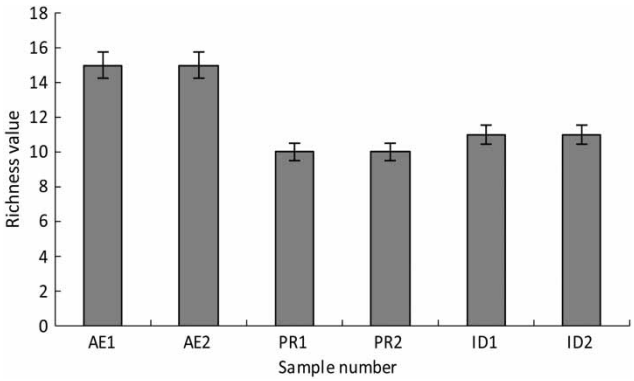

Figure 3 | Richness values of different SBR stages. Two samples were collected at a 1-month interval. AE, aeration stage; PR, precipitation stage; ID, idle stage.

The SBR system involves alternation of aerobic and anaerobic bacterial processes. The aeration stage is an aerobic process in which aerobic bacteria dominate in the population (Wang *et al.* 2011). As the reaction enters into the precipitation stage, which is an anaerobic process (Chen & Qi 2002), the number of aerobic bacteria that were dominant in the aeration stage begins to reduce gradually, while some obligate anaerobes appear and become dominant. The bacterial diversity in the idle stage is roughly the same as that in the precipitation phase, with the exception of changes in a few bacteria.

The dominance degree of DGGE bands

The dominance degree refers to the area of each individual band in the DGGE profile expressed as a percentage of the summed areas of all bands in each sample. The dominance degree of 20 bands in the DGGE profiles was obtained after analyzing every band in Figure 1 (Figure 4). The pattern of dominance differed obviously at each reaction stage, while that of the two samples collected during the same stage were generally similar. For example, in sample AE1, band 13 exhibited a comparatively high dominance degree of 10.07%, while band 19 had the highest dominance degree of 12.28%. Samples AE1 and AE2, which were collected during the same SBR stage, exhibited similar dominance degrees, of which band 19 was the highest at 12.74%, although band 13, which was comparatively high at 8.56% in AE2 was lower than that in AE1. Band 20 in the PR1 sample had the highest dominance degree of 19.75%, while bands 19 and 6 were also comparatively high at 13.63% and 12.43%, respectively. The dominance degree of PR2 was

Table 2 | Intensity of 20 bands of the DGGE profiles of samples obtained during the SBR process

	Band																			
Sample	1	2	3	4	5	6	7	8	9	0	11	12	13	14	15	16	17	18	19	20
AE1	+	++	+	++	+	+	+	–	+	+	–	+	+	–	+	+	+	–	++	–
AE2	+	+	+	+	+	+	+	–	+	+	–	+	++	–	+	+	+	–	++	–
PR3	–	–	–	++	–	+	–	–	–	+	–	+	–	+	–	+	++	+	++	+
PR4	–	–	–	+	–	+	–	–	–	+	–	+	–	+	–	+	++	+	++	+
ID1	–	–	–	–	–	++	–	+	–	++	+	–	+	+	–	+	+	+	++	+
ID2	–	–	–	–	–	+	–	+	–	++	+	–	+	+	–	+	+	+	++	+

Note: + and ++ indicates no band, band and strong, respectively.

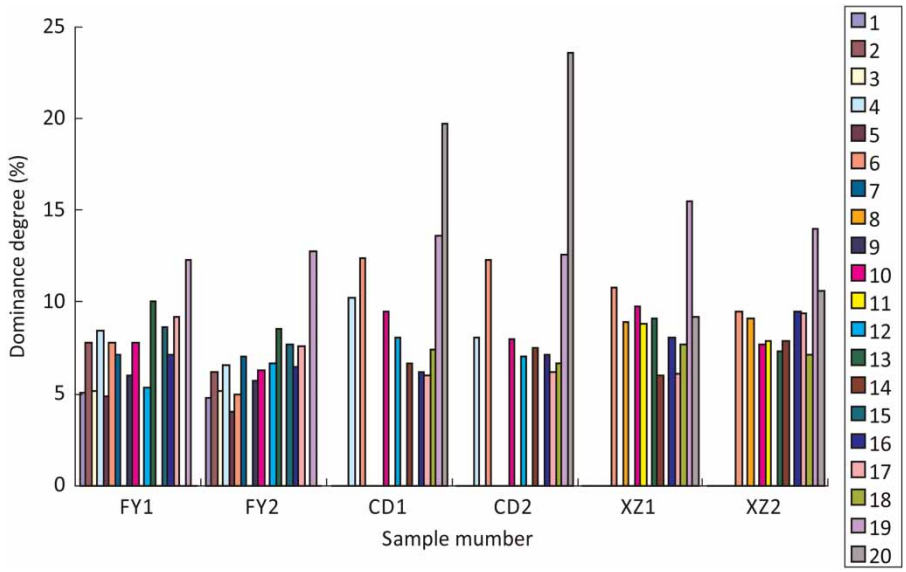

Figure 4 | The dominance degree of 20 bands in the DGGE profiles of samples obtained during the SBR process. The dominance degree refers to the area of each individual band in the DGGE profile expressed as a percentage of the summed areas of all bands in each lane. The dominance degree of 20 bands in the DGGE profiles was obtained after analyzing every band in Figure 1.

approximately the same as that of PR1. Band 19 band in ID1 had the highest dominance degree of 15.54%, followed by band 6 at 10.79%. However, although band 19 also exhibited the highest dominance degree of 13.96% in ID2, this was followed by band 20 at 10.65%.

Analysis of the richness value and dominance degrees of the samples revealed the occurrence of obvious changes in the composition of the bacterial populations during the SBR process, with reductions in these values observed from the aeration stage to the idle stage. Some strains, such as that represented by band 19, remained dominant during the entire process. However, the dominance of some species altered with the progression of the process, even to the point of being completely lost from the population or completely absent until a later stage of the process, for example, bands 13 and 20. These data illustrate the microbial community in the aeration stage was the most complex of the whole process, while the dominant bacteria differed in each reaction stage. Furthermore, these results demonstrate cyclical dynamic changes in the bacterial population during the SBR process for the treatment of antibiotic industrial wastewater.

The diversity indexes of DGGE bands

The diversity index comprehensively indicates the biodiversity of the environment, reflecting the dominant species in the population, as well as the frequency and uniformity of distribution of individuals in a population. The Shannon–Wiener diversity index (H) of each sample collected from the three SBR stages was calculated based on the known dominance degree (Table 3).

The diversity indexes at the aeration stage ranged from 3.859 to 4.147 and were maintained between 3.1 and 3.2 at the precipitation stage, reaching approximately 3.4 at the idle stage.

CONCLUSIONS

In this study, we exploited PCR-DGGE to analyze the bacterial diversity of an antibiotic industrial wastewater SBR treatment system to provide the information required for further optimization of this process and for identification of bacterial strains that perform improved degradation of antibiotic industrial wastewater. With the micro-ecological study of activated sludge, it was possible to identify the dominant strains present during the different stages of the SBR process. This information can also be applied to the generation of genetically engineered bacteria for optimal sewage treatment and to exploit the application of PCR-DGGE technology in the microbial diversity research of the SBR system.

Table 3 | Shannon–Wiener indexes of samples obtained during the SBR process

Sample number	AE1	AE2	PR1	PR2	ID1	ID2
Shannon–Wiener	4.147	3.859	3.213	3.153	3.410	3.431

Samples were collected from two independent reaction cycles conducted at a 1-month interval in order to determine the stability and reproducibility of our results. On each occasion, samples were collected during the aeration, precipitation, and idle stages. DGGE profile analysis of the samples collected at the three different stages during the two independent SBR processes showed good reproducibility in terms of the number and intensity of the bands observed, although some slight differences in the positions of the bands were observed. The bacterial diversity in the idle stage is richest. The dominant bacteria differed in each reaction stage during the SBR process, and were "Benzene-decomposing" bacterium in the idle stage, *Paracoccus* sp. in the precipitation stage, and *Chryseobacterium* sp. in the aeration stage.

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