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Bioaugmentation of a sequencing batch reactor with *Pseudomonas putida* ONBA-17, and its impact on reactor bacterial communities

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ABSTRACT

This study demonstrates the feasibility of using *Pseudomonas putida* ONBA-17 to bioaugment a sequencing batch reactor (SBR) treating *o*-nitrobenzaldehyde (ONBA) synthetic wastewater. To monitor its survival, the strain was chromosomally marked with *gfp* gene. After a transient adaptation, almost 100% degradation of ONBA was obtained within 8 days as compared with 23.47% of the non-inoculated control. The bioaugmented reactor has a better chemical oxygen demand (COD) removal performance (96.28%) than that (79.26%) of the control. The bioaugmentation not only enhanced the removal capability of target compound, but shortened system start-up time. After the increase in ONBA load, performance fluctuation of two reactors was observed, and the final treating effects of them were comparable. What is more, denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA genes via a combination of pattern comparison and sequence phylogenetic analysis was performed to uncover changes in sludge microbial communities. Only the members of alpha, beta and gamma subdivisions of Proteobacteria were identified. To isolate ONBA-degrading relevant microorganisms, spread plate was used and four bacterial strains were obtained. Subsequent systematic studies on these bacteria characterized their traits which to some extent explained why such bacteria could be kept in the system. This study will help future research in better understanding of the bioreactor bioaugmentation.

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1. Introduction

ONBA is used as an important intermediate for the synthesis of a number of chemicals such as nifedipine (Adalat, Procardia), dyes, agrochemicals and other organic compounds. In the southeast of China, at least 730 000 tons of ONBA are being produced annually [1]. Further, a vast quantity of ONBA contaminated wastewaters is generated during the manufacturing process. Because of its toxic and recalcitrant nature, ONBA creates pollution hazards. Although, no specific acceptable limit of ONBA has been formulated, it is necessary to treat such wastewater prior to its discharge [1].

Though integrated chemical methods seem to be feasible for the treatment of such wastewater, biological methods should be used preferably considering cost and technical advantages [2]. Bioaugmentation is recognized as a promising and attractive operational means to strengthen wastewater treatment performance [3,4] and has been demonstrated to successfully enhance the degradation and removal of specific pollutants such as phenols [5], 3-chloroaniline [6], phosphate [7], and aromatic hydrocarbons [4,8]. However, it does not always work, and the treating effects of bioaugmentation have been reported to be less predictable and controllable [3]. To achieve successful bioaugmentation, it is necessary to select suitable microorganisms and adopt a proper strategy.

Recently, developed molecular techniques such as denaturing gel electrophoresis (DGE), including DGGE, temperature gradient gel electrophoresis (TGGE), and temporal temperature gradient gel electrophoresis (TTGE), analysis of the polymerase chain reaction (PCR)-amplified ribosomal DNA fragments and *in situ* green fluorescent protein (GFP) fluorescence detection have been employed as powerful tools for monitoring specific microorganisms in complex microbial communities such as biofilms and microbial aggregates [9–11]. The combination of these techniques apparently provides reliable and direct evidence for the fate and *in situ* metabolic activities of the added microorganisms, which can be used to evaluate the effectiveness of bioaugmentation.

Functional dissection of microbial communities based on structure information represents an ongoing effort in the field of microbial ecology [12]. The analysis of population shifts accompanied by changes in the community function yielded information useful for identifying functionally dominant populations [13–15]. In this study, we investigated the enhanced degradation of ONBA by

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activated sludge inoculated with an ONBA-degrading strain, ONBA-17*gfp*. *Gfp*-gene-mark and DGGE techniques were respectively used to examine its survival and effect on the community structure. Besides, four pure cultured strains were obtained through spread plate, which was initially used to isolate ONBA-degrading functionally relevant strains, and their characteristics were systematically investigated.

2. Methods

2.1. Marking strain Pseudomonas putida ONBA-17 with gfp

Strain ONBA-17 was isolated from an enrichment culture as previously reported [16]. Basic cloning, transformation and other standard recombinant DNA techniques were carried out by published protocols [17]. To obtain *gfp* gene and its relevant constitutive promoter from pBBRGFP-45 [18], the primers 5′-ccgcggGCTCTAGAACTAGTGGATC-3′ and 5′-ccgcggGCGCAATTAACCCTCACTA-3′ were used. BamHI-digested 16S rRNA genes were ligated into the BamHI site of pEX19Gm [19]. The BamHI site flanking 16S rRNA gene was also introduced by PCR amplification. This construct, designated pEX17, was maintained in *Escherichia coli* SM10 lambda *pir* [20]. Then, SacII-*gfp*-promoter-SacII fragment was ligated into the 16S rRNA gene of pEX17. Tri-parental mating among the donor strain *E. coli* SM10 lambda *pir*, the helper strain *E. coli* HB101 (pRK2013) [21] and the recipient strain ONBA-17 were performed. Transconjugants were obtained by first spreading the mixture on Luria-Bertani (LB) medium with gentamicin (20 μg ml^{-1}) and ampicillin (200 μg ml^{-1}), and then counter selected with 5% sucrose.

2.2. Reactor operation

The experiment was conducted with freshly collected sludge from a local municipal wastewater treatment plant. The total direct count of bacteria, 8.9×10^8 cells ml^{-1}, was determined by a fluorescent microscopy method [22]. Two 4 l Plexiglas SBRs (12 cm diameter) with 3.5 l working volume were built up and maintained at 28 °C. The initial biomass concentration in each reactor was ca. 2.3 g VSS l $^{-1}$ (VSS stands for volatile suspended solids). ONBA (100 mg l^{-1}) was fed with a synthetic wastewater [23]. Effluent was discharged at a volumetric exchange ratio of ca. 70%, and the sludge retention time was 10 days. To avoid dissolved oxygen (DO) limitation, DO concentration was maintained higher than 3.0 mg l^{-1} during aeration. The reactors were firstly operated without ONBA for 10 days to achieve stable COD removal performance. Then, reactor A was inoculated with the strain to a final concentration of 6.4×10^6 cells ml^{-1}.

The operation was carried out in two phases. In phase 1, two reactors were operated on a 24 h cycle consisting of the following stages: a 10 min filling phase, a 22 h aeration period, a 1 h 40 min settling phase, and a 10 min draw phase to give a hydraulic residence time (HRT) of 33.6 h. By day 9, the HRT was reduced to 24 h (phase 2).

2.3. Analytical methods

VSS, DO, pH, chemical oxygen demand (COD), and sludge volume index (SVI) were measured periodically using standard methods [24]. A gas chromatography method as described by Yu et al. [1] was used to determine ONBA concentrations.

2.4. Bacterial count and online observation

To count the marked cells, the method reported by Boon and colleagues [6] was used. With a Leica TCS SP2 spectral confocal microscope (Leica, Microsystems, Germany), GFP was imaged using the 488 nm line of an argon ion laser.

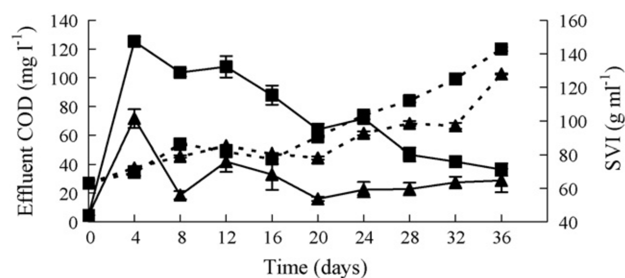

Fig. 1. Time profiles of the effluent COD (solid line) and SVI (dashed line). (▲) Reactor A; (■) Reactor B.

2.5. DNA extraction, PCR amplification and DGGE analysis

Genomic DNA was extracted from 0.1 g of activated sludge as described by Griffiths et al. [25]. The variable V3 regions of eubacterial 16S rRNA genes were amplified by PCR as suggested by Zhang et al. [26] with a PTC-200 thermal cycler (Bio-Rad Laboratories, USA), using bacterial universal primers F341-GC and R518 [10]. To separate double-stranded DNA (dsDNA) from single-stranded DNA (ssDNA), polyacrylamide gel electrophoresis was performed [26]. The purified fragments were separated by DGGE using a D-Code Universal Mutation Detection System (Bio-Rad Laboratories, USA). In brief, 10% polyacrylamide gels containing a gradient of 30–60% denaturant were prepared, with 100% denaturing solution defined as 8 M urea and 40% formamide. Electrophoresis was performed in $0.5 \times$ TAE buffer at 200 V for 10 min and then at 85 V for 12 h at a constant temperature of 60 °C. After electrophoresis, the gel was silver-stained and scanned as described by McCaig et al. [27]. Representative bands were excised, re-amplified and sequenced [13]. The partial sequences were checked for chimera formation with the CHIMERA CHECK program (version 2.7) as described by Cole et al. [28]. The BLAST search tool [29] was used to find sequence homology.

2.6. Bacterial isolates

Four bacterial strains were isolated by ultrasonic treatment of final sludge samples, and further incubating on synthetic wastewater agar at 25 °C. Strains were characterized by Gram staining and standard biochemical tests, and identified based on the 16S rDNA partial sequences. The ONBA-degrading potential of them were tested according to the reported method [16]. Surface hydrophobicities and flocculation characteristics of the isolates were examined as described before [30,31].

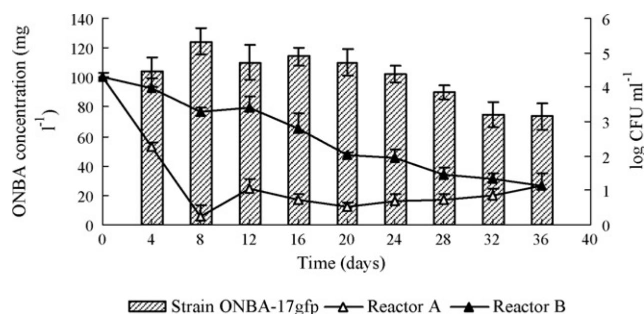

Fig. 2. Concentration of ONBA in two reactors, and survival of strain ONBA-17*gfp* in the reactor A (bars).

3. Results

3.1. Reactor performance

Two reactors were initially seeded with fresh activated sludge and operated without supplement of ONBA for 10 days to minimize the discrepancy. The final sludge in both reactors had a similar biomass concentration of 2.4 g VSS l^{-1}. The SVI values were about 63 ml g^{-1}. By day 8, after inoculation, the effluent COD removal rate of reactor A was 85.1% (Fig. 1), which was significantly (two-tailed *t*-test; $a = 0.05$) higher than the control's (data not shown). Similar trend was also found in the degradation of ONBA, the ONBA-degrading rate of reactor A was 94.39% significantly higher than that (23.47%) of the control (Fig. 2).

In phase 1, initially the sludge settling abilities of two reactors were gradually deteriorated. Then, however, from day 9 to 20 increases in biomass (data not shown) and settling ability became visible (Fig. 1). After reactor A could completely remove the input ONBA within operating cycle, increase in ONBA load was introduced. From day 9, the effluent COD transient increased to a peak value of 42 mg l^{-1}, and then dropped to 16.03 mg l^{-1} by day 20. Subsequently, it steadily climbed and eventually maintained at a level corresponding to ca. 94% removal (Figs. 1 and 2). Similar pattern was also observed with the ONBA concentration. In reactor B, microbial consortium was able to sustain the sudden ONBA loading shock without any obvious difference in effluent quality. The fluctuation of above two items was less than that of reactor A. From day 9 to 12 both of them declined steadily, and became com-

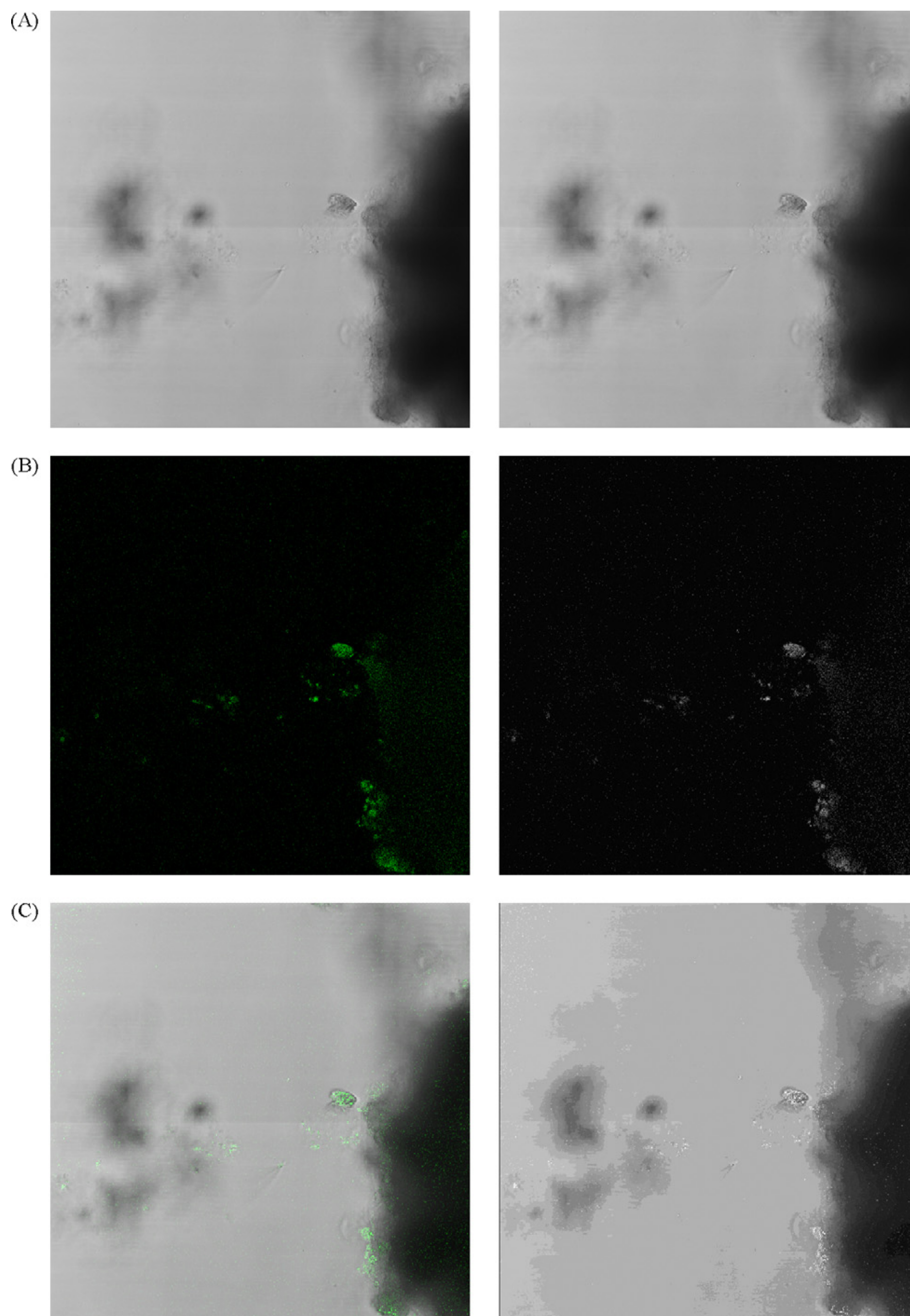

Fig. 3. Detection of ONBA-17*gfp* in activated sludge by confocal laser scanning microscopy. (A) Phase-contrast. (B) Epifluorescence micrography. (C) True color overlay images of a and b. Note the protozoa (*Aspidisca* sp.) in the middle-right part of the picture.

parable to those of reactor A at the end of operation. As to the item of biomass concentration, it firstly reached the maximum of 2.6 VSS l^{-1} (reactor A, day 20), and then decreased to 2.5 VSS l^{-1} by the end of the experiment, while the counterpart was steadily increased to 2.8 VSS l^{-1}. Moreover, the loading shock was accompanied with deterioration in settling ability. Finally, the SVIs declined to 128 ml g^{-1} and 142.9 ml g^{-1}, respectively (Fig. 1).

3.2. Activity and survival of strain ONBA-17gfp in SBR

In order to monitor the survival of strain ONBA-17, it was chromosomally marked with the *gfp* gene. The insertion of the gene in several transconjugants was confirmed by PCR with *gfp*-specific primers. The *gfp*-marked bacteria showed same degradation capability as the original strain in the selective minimal salts medium containing ONBA as the sole carbon source [16]. Figs. 1 and 2 show that the inoculation could initially (in phase 1) significantly enhances the capabilities of COD removal and ONBA degradation. After ONBA load increased from day 9, however, these capabilities were gradually degenerated. The marked bacteria were recognized and counted through antibiotic selection and GFP autofluorescence. In the first 4 days, after inoculation, the cell numbers of GFP-tagged strain sharply decreased by two orders of magnitude and dropped to ca. 2.8×10^4 cells ml^{-1} (Fig. 2). Then, it reached to a higher level of 2.2×10^5 cells ml^{-1} on day 8. Subsequently, however, an obvious decline was observed, the number declined to 1.4×10^3 cells ml^{-1} at the end of the phase 2. In control reactor, no GFP autofluorescence was detected on selective plates. The distribution of ONBA-17*gfp* in the system was studied through combination of phase contrast and epifluorescence microscopies. After inoculation, marked cells were visible as green cells under UV light. The inoculated bacteria were initially found swimming freely among sludge flocs. Later, some of them were appeared within various protozoa. As observation prolongs, protozoa containing many food vacuoles fully filled with green fluorescent cells were found (Fig. 3). Besides, food vacuoles highly fluorescent without any visible bacteria were also observed. Theses indicate cell lyses due to digestion of the predators. In agreement with plate counting, there were only a few detectable fluorescent cells after 28 days of inoculation, and most of them were found within flocs.

3.3. DGGE analysis

DGGE was employed to produce genetic fingerprint that could provide information on the composition and diversity of microbial communities of the SBRs. Every 3 days sludge samples were collected, and then PCR amplification and DGGE analysis were performed. Fig. 4 shows DGGE profiles of amplified 16S rRNA genes. At least 15 different bands were detected on the polyacrylamide gel. Generally, there are two DGGE patterns, corresponding to phases 1 and 2, respectively, during the operation with very few differences between reactors, indicating microbial communities were stable and the inoculation did not bring any noticeable difference. The intensities of some bands, corresponding to the densities of specific microbial populations, were enhanced, while others were not. At the end of the operation (day 36), changed pattern of the reactor A appeared for unknown reasons. Partial 16S rRNA gene sequences of close to 200 nucleotides were successfully obtained from five interesting bands (a–e in Fig. 4 and Table 1). The subsequent phylogenetic analysis showed that they could be largely clustered as

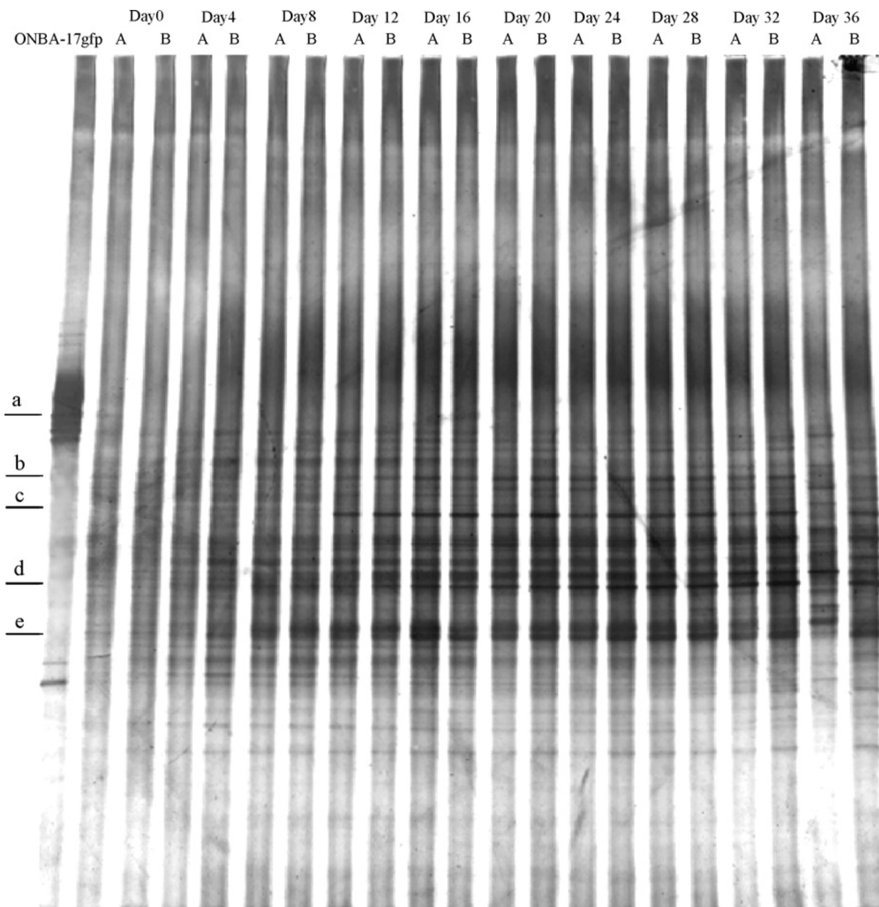

Fig. 4. DGGE profiles of two reactors using partial bacterial 16S rRNA gene fragments from ONBA-degrading sludge samples.

Table 1
Sequence analysis of DGGE bands from ONBA-degrading sludge samples.

Band	Length (bases)	Closest relatives (accession no.)	Identity (%)	Phylogenetic division
a	194	*Pseudomonas* sp. S12 (AY752921) *P. putida* ONBA-17 (DQ079062)	100	γ-Proteobacteria
b	194	Uncultured beta proteobacterium clone SM1E11 (AF445688) Uncultured *Hydrogenophaga* sp. clone J-11 (AF523049)	99	β-Proteobacteria
	169	Uncultured alpha proteobacterium clone SA-B16 (DQ295442)	100	α-Proteobacteria
c	194	*Thauera* sp. (AJ315677)	99	β-Proteobacteria
	169	Uncultured alpha proteobacterium clone WN-HSB-23 (DQ432265)	100	α-Proteobacteria
d	169	Uncultured bacterium clone A1-3-2 (AY675970)	97	Unknown
e	195	Uncultured bacterium clone SBR-Sludge-04 (AB231402)	99	Unknown
	169	Uncultured alpha proteobacterium clone WN-HSB-23 (DQ432265)	99	α-Proteobacteria

members of Proteobacteria. Besides, the sequence of band a is 100% homology with the corresponding part of ONBA-17 (accession no. DQ079062).

3.4. Characterization and identification of the isolates

In order to isolate ONBA-degrading functionally dominant microorganisms, the method of spread plate was used. Totally, there were four kinds of bacteria appeared and were picked up. The identity and flocculation characteristics of them are summarized in Table 2. All these bacteria were good floc formers with aggregation index more than 75%. A further survey of adsorption of the isolates to *p*-xylene indicated that some of them were hydrophobic (Table 2). What is more, positive correlation between cell surface hydrophobicities and aggregation indices was observed. However, neither of them owns ONBA-degrading capability.

4. Discussion

Nowadays, bioaugmentation is being used as a powerful tool to accelerate the removal of undesired compounds in polluted sites and bioreactors. So far researches have mostly been focused on soil bioaugmentation, though the activated sludge process could also be particularly conducive with it. Due to the fact that bioaugmentation is less predictable and controllable than the direct physical or chemical removal of pollutants, it is not applied widely despite of the low cost and several small-scale successes [4,6,8].

In this study, a previously isolated ONBA-degrading bacterium *P. putida* ONBA-17 was used to accelerate the removal of ONBA from synthetic wastewater. After a transient adaptation, complete degradation of ONBA was achieved within 8 days, while only a 23.47% removal was obtained in the non-inoculated reactor. In the case of the COD removal rate, the bioaugmented one also owned a significantly higher rate (96.28%). Bioaugmentation indeed shortened the start-up time of the system. The loading shock dramatically influenced the performance of two reactors, and the final treatment trends of two reactors were opposite. The decrease in biomass concentration from day 20 and accelerated deterioration in settling ability from day 32 (reactor A) further corroborated this. On one hand, it seems that there is a positive correlation between the population density of GFP-tagged strain and the ONBA removal rate, suggesting that the bioaugmentation was effective but ephemeral that is often experienced in the bioreactors, and biological supplement for such systems have to be added on a regular basis in order to assure continuous treatment efficacy. On the other hand, the result support the idea that natural uncontaminated systems contain sufficient genetic diversity to make them valid choices for the removal of xenobiotics, either by metabolism or co-metabolism, after an adequate exposure time [8,32,33]. Moreover, the acclimatized non-bioaugmented system owns more stable sludge floc structure and operation performance than the bioaugmented one, which is especially true under shocks.

The origin and type of inoculated strain have been considered as important factors in the survival and activity of the inoculum. Boon et al. [6] inoculated an indigenous 3-chloroaniline-degrading *Comamonas testosteroni* strain I2*gfp* into a lab-scale semicontinuous bioreactor. The inoculated strain successfully maintained itself in the sludge for at least 45 days. After a 6-day adaptation, complete degradation of 3-chloroaniline was obtained within 2 weeks. In the case of the experiment done by Tchelet et al. [34], a strain P51 originally isolated from sediments was used both in soil column and sewage sludge for bioaugmentation study. The survival and activity of the bacterium in the soil column were successful, but the strain was not able to maintain itself in the bioreactor and thus no degradation was observed. Our work further confirmed this. The inoculum initially isolated from municipal wastewater treatment system works well within the SBR.

With spread plate and GFP autofluorescence techniques, reliable and sensitive monitoring was achieved. But just as Boon et al. [6] have pointed out that it is not known whether this culturable part resembles the total viable count of the GFP-tagged strain in the system. Moreover, our work strongly support previous study suggesting that predation by protozoa is a major cause for the disappearance of the introduced bacteria [35]. We further considered that the fate of the inoculum mainly depends on three factors: (i) the adaptation and retention capabilities; (ii) the competition between allochthonous bacteria and indigenes; (iii) and the capability to survive against predatory protozoa. In our experiment, an equilibrium seems to be reached between growth rate, predation, and washout during day 4 and 24 (Fig. 2). In this period, the reactor performance was quite well as compared with the control (Figs. 1 and 2). The marked bacteria were incorporated into activated sludge, which

Table 2
Characterization and identification of bacterial isolates.

Isolate	Accession no.	Proposed identity	Identity (%)	Result of Gram staining	Aggregation index[a] (%)	Adsorption to *p*-xylene[a] (%)
M	EF108297	*P. aeruginosa*	99	–	81.2	38.2
R	EF108298	*Exiguobacterium* sp.	99	+	79.5	36.9
W	EF108299	*Bacillus cereus*	98	+	90.0	53.5
S	EF108300	*Pseudomonas* sp.	96	–	83.1	46.2

[a] The values are the average of three independent experiments. The maximum S.D. is within ±0.5.

was helpful for their maintaining and could efficiently avoid the predation [36]. Theoretically, the incorporation could also lead to a higher probability of gene transfer, yielding diverse transconjugants with possibly better performance than the introduced donor strain. However, at the end of the experiment the equilibrium was broken and reactor performance was deteriorated. Further research will be focused on prolonging the efficient degradation and elucidating the imbalance mechanism.

Recently, powerful PCR-based approaches such as DGGE and TGGE, using 16S rRNA genes as markers, are frequently used to characterize the complex microbial communities without cultivation and isolation, and monitor their structural shifts in response to environmental changes. Although the biases in genomic DNA extraction and PCR amplification exist [26,37], the advantages of these technologies are obvious. These techniques are convenient in simultaneous analysis of a large number of samples or monitoring the behavior of one community over time or under different conditions, and in comparing the band patterns and finding bands of interest [11,15]. Sequences of these bands could provide important phylogenic information that reflects microbial community shifts caused by environment changes. Moreover, the reliability could be further improved either by methods such as optimization of PCR amplification and purification of PCR product via denatured polyacrylamide gel electrophoresis [26] or by combined analysis with other techniques, including stable-isotope probing, full-cycle rRNA gene sequencing, and fluorescence *in situ* hybridization-microautoradiography [38–41], etc. In this work, DGGE was used to dynamic monitor microbial communities. As mentioned above, the DGGE profiles of two reactors were similar (Fig. 4), indicating that the inoculation could not bring any obvious changes to the composition of microbial consortium, although it indeed enhanced the treating effect. Mahmood et al. [40] have pointed out that changes in DNA-DGGE profiles would only result from bacterial growth or death and could not enable distinction between active and dormant organisms; more sensitive and functional detection needs the utilization of RNA-based means. Therefore, further investigation should be carried out in this way to elucidate the association between microbial diversity and ecosystem function.

Through optimization of amplification and minimization of single-stranded DNA contamination, five clone libraries with high quality were constructed. Further analysis revealed that the phenomenon of sequence heterogeneity, especially common in complex community analysis, exists in about 60% of the sequenced bands. Nucleotide database search indicated that some species, corresponding to bands b to e in Fig. 4 and Table 1, were enriched during the operation. As to why only Proteobacteria was used to cluster them, the degrading recalcitrance of ONBA and known enzymatic pathways for complex organic compounds were almost confined within these groups [14,42–45] might explain it to some extent.

We tried to get functionally dominant strains through a direct and simple way. However, only a few kinds of bacteria appeared, and none of them could utilize ONBA. A systematic study has been conducted to identify the ONBA-degrading pairs among isolates and strain ONBA-17, but none of positive combinations was found. Further we checked their surface hydrophobicities and aggregation abilities; interestingly all of them are good floc formers according to the standard described by Malik et al. [31]. Combined with the operation characteristics of SBR system, why bacteria own such traits could be kept and screened out could be largely explained. More over, the partial 16S rDNA sequences of them were compared with the sequences of dominant populations, which revealed that the phylogenetic positions of strain M and S, and R and W are respectively close to the band c and d. But, no conclusion could be easily drawn before further systematic studies.

In short, this study suggests that bioaugmentation of a SBR for specific pollutant ONBA could be successfully achieved under laboratory conditions, although the enhanced treating effect was transient. The combination of *gfp* expression monitoring and DGGE profile analysis allows mapping of the structure–activity profile. Our work will improve current understandings about bioreactor bioaugmentation. Due to the fact that wastewater generally is a mixture of multiple organic pollutants and the presence of other pollutants might strongly influence the removal efficiency of target compound. Nowadays, the investigation of ONBA-17 mediated bioaugmentation under practical conditions is in progress. Besides, different isolation and incubation methods were used to obtain more ONBA-degrading relevant microorganisms, and their physiological functions and interactions will be examined for better uncovering the mechanisms.

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