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Effects of gene augmentation on the removal of 2,4-dichlorophenoxyacetic acid in a biofilm reactor under different scales and substrate conditions

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ABSTRACT

With a conjugative plasmid pJP4 carrying strain as the donor, two bioaugmentation experiments were conducted in a microcosm biofilm reactor with 2,4-D as the sole carbon source operated in fed-batch mode, and an enlarged lab-scale sequence batch biofilm reactor with mixed carbon sources of 2,4-D and other easily biodegradable compounds, respectively. In the microcosm study under sole carbon source condition, bioaugmentation led to a persistently increased 2,4-D degradation rate in the five operation cycles with enhancement of 13–64%. For the enlarged lab-scale bioaugmentation experiment under mixed carbon source conditions, no enhancement in 2,4-D removal could be observed during start-up period. After a period of operation, biofilm samples from the bioaugmented reactor demonstrated a stronger degradation capacity than the control and showed the presence of a large number of transconjugants. This study indicates that bioaugmentation based on plasmid horizontal transfer is a feasible strategy to establish functional microbial community in a biofilm reactor, and the strong selective pressure of 2,4-D existing alone and persistently was more favorable for the success of gene augmentation.

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1. Introduction

Horizontal transfer of moving genetic elements (MGEs) is considered to be an important mechanism for a microbial community to adapt to a changing environment and establish new genetic traits. Gene augmentation is defined as the introduction of some special culture containing functional MGEs to a contaminated environment or biotreatment system to enhance the removal of undesired compounds via gene horizontal transfer to indigenous bacteria. Compared with the traditional bioaugmentation method, cell augmentation, gene augmentation shows the advantage of lower requirement for the ability of the inoculated strain to grow and survive in a bioaugmented system [1]. Therefore, gene augmentation can be regarded as an approach of in situ genetic modification of indigenous bacteria.

Plasmid conjugation is the most popular mechanism of gene horizontal transfer. Controlled dissemination of a conjugative plasmid carrying special bacteria in a polluted site or bioreactor has become a promising method of bioaugmentation and bioremediation [2–6].

Biofilm is believed to be the “hot spot” of gene horizontal transfer due to the dense microbial population and the close contact among microorganisms in biofilms. Studies on gene augmentation in biofilm systems have attracted extensive attention recently [7–11]. However, most of these studies were conducted in microcosm biofilm systems such as flow chamber systems, or modeled with filter mating experiments, only a few were done in real biofilm reactors. Their results show that a conjugative plasmid mediated bioaugmentation does not always work in real biofilm reactors, especially in an enlarged lab-scale or pilot scale system [12–16]. For example, Bathe et al. [10,11] once investigated the spread of a plasmid pJP4 (responsible for 2,4-dichlorophenoxyacetic acid (2,4-D) degradation) and a plasmid pNB2 (responsible for 3-chloroaniline degradation) in lab-scale sequence batch biofilm reactors (SBBRs) with glass beads and wood chips as carriers, respectively, but failed to observe positive effects at the beginning of operation. Mohan et al. [17] supplemented a TOL plasmid donor strain to a pilot-scale SBBR and a lab-scale SBBR, respectively, to enhance the removal of benzyl alcohol (BA), but only succeeded in the lab-scale experiment. These failure cases indicate that factors influencing gene horizontal transfer in biofilm reactors are much complicated and far from understanding.

Selective pressure is generally considered to be an important factor influencing catabolic gene horizontal transfer [16,17]. Most of the previous researches on plasmid bioaugmentation were conducted under a strong selective pressure with the target compounds existing alone or persistently [5,10,11,17]. For practical wastewater, recalcitrant pollutants often appear occasionally

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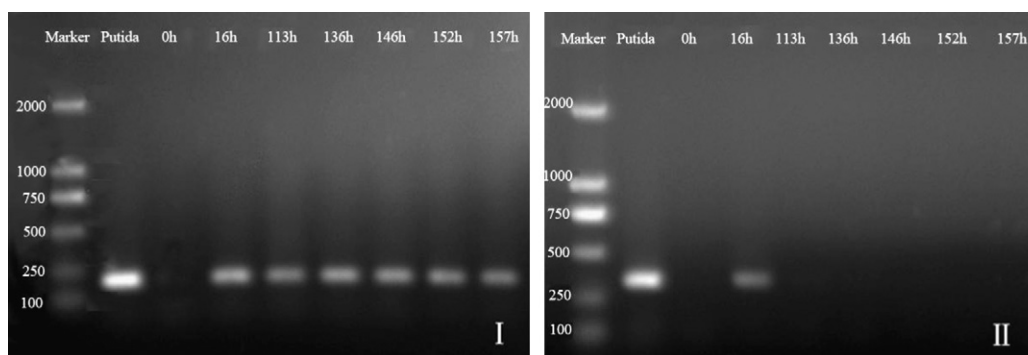

Fig. 1. Agarose gel detecting tfdB gene on the plasmid DNA and gfp on the genomic DNA in the biofilm samples from gene-augmented system. (I) tfdB-PCR and (II) gfp-PCR. Numbers indicated sampling time.

and co-exist with other easily biodegradable compounds. Hence, understanding the effects of gene augmentation under such conditions is of great significance.

This study aimed to investigate enhancement of the removal of a specific pollutant in biofilm reactors using gene augmentation under different scales and substrate conditions. 2,4-D was chosen as the target compound due to its wide application as a herbicide in agriculture and serious ecological effects. A plasmid pJP4 carrying genetic microorganism *Pseudomonas putida* SM1443, capable of degrading 2,4-D, was used as the donor strain. Two bioaugmentation experiments were conducted in a microcosm and an enlarged lab-scale biofilm reactor, respectively, with 2,4-D present as the sole carbon source or mixed with other easily degradable compounds. The event of plasmid horizontal transfer to biofilm community and its effects on original biofilm microbial community were also examined.

2. Methods and materials

2.1. Donor strain and plasmid

The donor strain used in this study was a genetically modified *P. putida* SM1443, which carried a dsRed tagged pJP4 plasmid and was chromosomally labeled with a gfp gene (donated by Stephan Bathe). The strain showed constitutive gfp fluorescence, but no dsRed fluorescence due to its repression by a chromosomally encoded lac-repressor [10]. Transconjugants receiving plasmid pJP4 can express dsRed fluorescence. The donor strain is able to grow in MMN medium containing 5 mM 2,4-D and 1 mM NH_4Cl, but not in the synthetic wastewater of the bioaugmentation experiment I described below [10].

2.2. Isolation of transconjugants

Transconjugants were isolated according to the method described by Bathe et al. [9]. Briefly, biofilm samples taken from the studied reactor were mixed with distilled MilliQ water in a centrifuge tube through a vortex to make a homogenized cell suspension. This cell suspension was plated on R2A agar containing $HgCl_2$ (10 μg/ml). The developing colonies were streaked on gentamycin (25 μg/ml) containing R2A and microscopically checked for red fluorescence. Mercury and gentamycin-resistant, red-fluorescent isolates were considered to be transconjugants. R2A medium consisted of (g/l): yeast extract 0.5; bacto peptone 0.5; casamino acid 0.5; glucose 0.5; soluble starch 0.5; Na-pyruvate 0.3; K_2HPO_4 0.3; and $MgSO_4{\cdot}7H_2O$ 0.05.

2.3. Pre-development of biofilm in an off-line bioreactor

As a large quantity of biofilm carrier samples were required for the following bioaugmentation experiments, biofilms were grown in an off-line bioreactor containing suspended carriers. The bioreactor had a working volume of 15 l and operated in a sequence batch mode with an operation cycle consisting of fill 60 min, react 240 min, settle 55 min and draw 5 min. The volume exchange ratio in each cycle was set at 65%, and sludge retention time (SRT) was controlled at about 25 days. The carrier particles used in this study was tube-like, 2 cm-long and 1 cm internal diameter with micropores on the surface. The reactor was seeded with activated sludge taken from a municipal wastewater treatment plant and was fed with synthetic wastewater I for about two months until biofilm was successfully established on the carriers. Synthetic wastewater I contained the following components (mg/l): glucose 300, starch 150, peptone 45, urea 33, KH_2PO_4 66, $MgSO_4{\cdot}7H_2O$ 15; and $NaHCO_3$ 141.

2.4. Bioaugmentation experiment I

Bioaugmentation experiment I was conducted in a microcosm biofilm reactor fed with synthetic wastewater containing 2,4-D as the sole carbon source. Two 500 ml Erlenmeyer flasks each with a working volume of 200 ml were used as the modeled biofilm reactor. Ten carrier particles were withdrawn from the foregoing off-line bioreactor after biofilm establishment and were added

Table 1
The removal of 2,4-D by the bioaugmented system and the control in the five fed-batch runs.

Run number	Run time	Removal percentage[a]		Average removal rate (mg/l h)	
		Control reactor (%)	Bioaugmented reactor (%)	Control reactor	Bioaugmented reactor
Run 1	0–113	61	99	1.12	1.57
Run 2	120–136	74	100	9.82	11.10
Run 3	140–146	60	96	17.74	29.00
Run 4	147–152	69	99	25.36	36.66
Run 5	153–156.5	65	95	35.95	49.25

[a] Removal percentage at the end of this run.

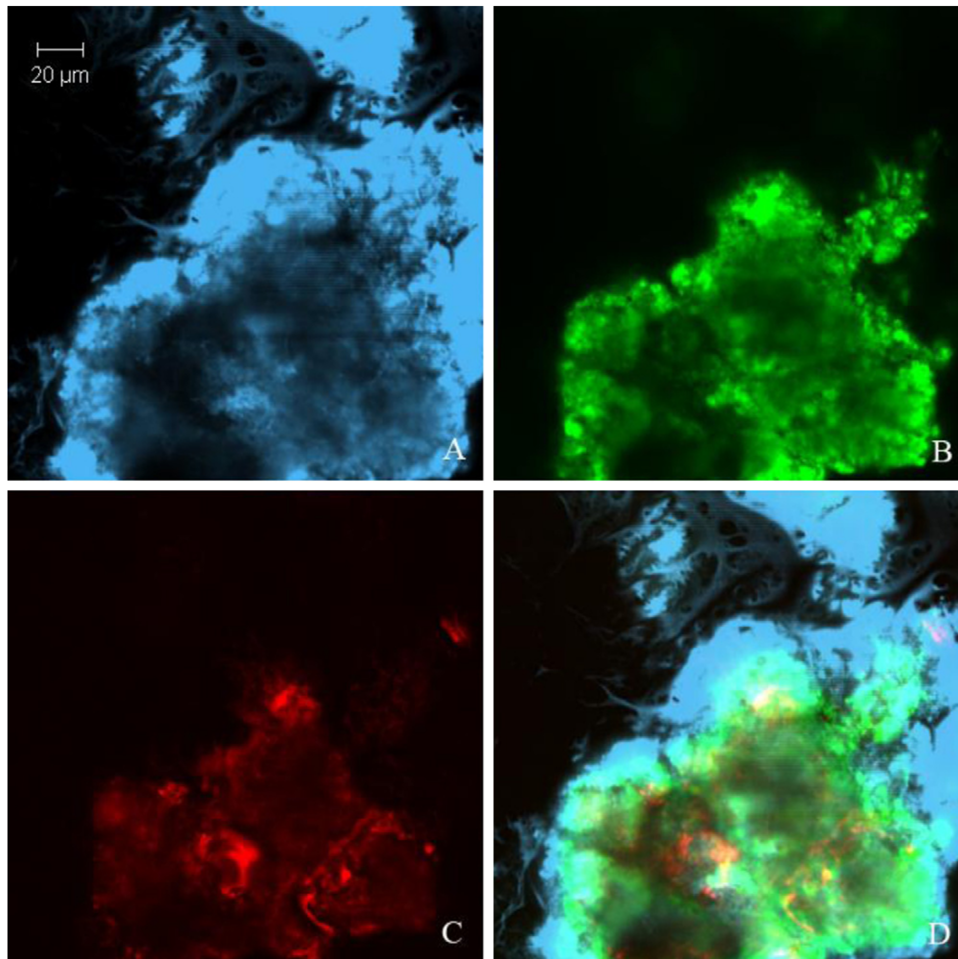

Fig. 2. In situ visualization pJP4 plasmid transfer on the biofilm carrier by CLSM (A) total microbes on the biofilm carrier stained by SYTO 45; (B) pJP4 donor strain *Pseudomonas putida* SM1443 showing gfp fluorescence; (C) transconjugants surface of biofilm carrier expressing *dsRed* fluorescence; (D) combined images of (A), (B) and (C). Scale bar = 20 μm.

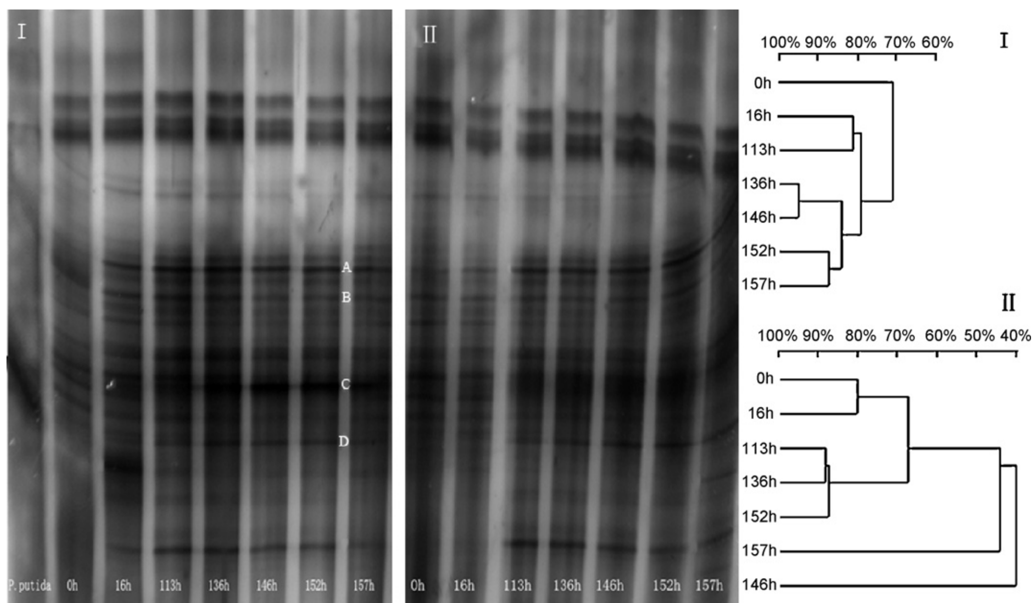

Fig. 3. DGGE fingerprint and cluster analysis by UPGMA (I) and Bioaugmented reactor (II) control reactor. Numbers indicated sampling time.

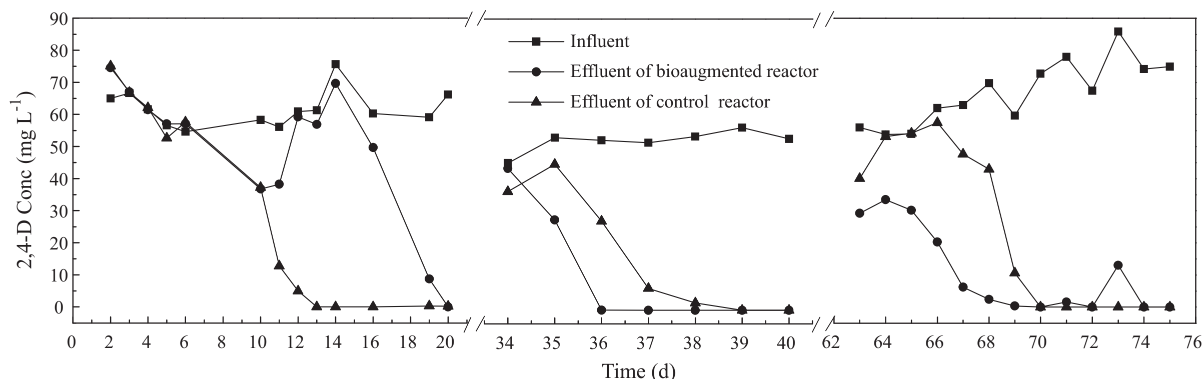

Fig. 4. Performance of the bioaugmented and control SBBR with respect to 2,4-D degradation under long-term operation. (□) Influent 2,4-D concentration; (●) effluent 2,4-D concentration of the bioaugmented reactor; (▲) effluent 2,4-D concentration of the control reactor.

to each flask with initial biomass concentration of 3.63 g MLSS/l (MLSS, mixed liquor suspended solids). The pJP4 donor strain *P. putida* SM1443 was pre-cultured overnight and supplemented to one flask by an inoculation ratio of 3%, while the other flask was not inoculated with the donor strain and was used as a control. The two flasks were conducted in a rotary shaker under the condition of 30 °C and 120 rpm. Synthetic wastewater II as described in Bathe's study with about 170 mg/l 2,4-D as the sole carbon source was used as feed [10]. The two reactors were operated in a fed-batch mode for five cycles: when more than 95% of the initial 2,4-D concentration was removed, the medium in the flasks was completely removed and replaced with fresh synthetic wastewater for the next cycle.

2.5. Bioaugmentation experiment II

Bioaugmentation experiment II was conducted in two enlarged lab-scale SBBRs treating synthetic wastewater containing carbon sources of 2,4-D and other easily biodegradable compounds. Each reactor had a working volume of 4 l with an internal diameter of 10 cm and a height of 25 cm. Pre-established biofilm carriers taken from the off-line bioreactor were added to each reactor with initial biomass concentration controlled at 1.75 g MLSS/l. Overnight pre-cultured donor strain *P. putida* SM1443 was collected and added to the SBBR reactor at an inoculation ratio of 13%. The control reactor was not inoculated. To ensure adequate plasmid transfer to biofilm bacteria, the donor strain and biofilm carriers were kept contact in the bioaugmented reactor for 24 h, then the SBBRs were operated with normal SBR operation mode, with a cycle (6 h) consisting of 4 h react, 0.5 h draw, 0.5 h fill and 1 h idle periods. The volume exchange ratio was set at 75% and sludge retention time (SRT) was controlled at about 20 days. Synthetic wastewater I added with 45–85 mg/l 2,4-D was fed to the SBBRs, except in days 21–33 and 41–62 when 2,4-D was omitted in the synthetic wastewater. Influent chemical oxygen demand (COD) concentration in the synthetic wastewater ranged 406–968 mg/l.

2.6. DNA extraction from biofilm samples

Some carrier particles sampled from the SBBRs were added to a centrifuge tube containing 2 ml MilliQ water. Biomass was removed from the carriers by agitation (5 min) and vortex at 3500 rpm for about 10 min. Then, the detached biomass was collected through centrifugation, washed twice with 0.85% KCl solution, and stored as a pellet at −20 °C. DNA was extracted from the biomass with an EZ-10 Spin Column Genomic DNA Isolation Kit (Bio Basic Inc., Canada).

2.7. Polymerase chain reaction (PCR)-based qualitative analysis of pJP4 transfer in the bioaugmented reactor

To understand the fate of the donor strain in the bioaugmented system and pJP4 transfer to indigenous bacteria, the tfdB gene, a key functional gene on pJP4, and the gfp gene, a reporter gene marked on the chromosome of the donor strain, were amplified from biofilm DNA samples through PCR according to the same method described by Bathe et al. [10] Briefly, one PCR reaction contained: Taq-DNA-polymerase(5 U/μl), 0.25 μl; 10 × PCR buffer (Mg^{2+} Plus), 25 μl; dNTP mixture (each 2.5 mM), 4 μl; template DNA, 1 μl; primer tfdBf and tfdBr (or gfpf and gfpr), each 1 μl; and sterilized MilliQ water, 38.75 μl. The PCR program consisted of an initial denaturation step at 94 °C for 10 min, followed by 25 cycles of 94 °C for 30 s, 65 °C (tfdB-PCR) or 62 °C (gfp-PCR) for 60 s, 72 °C for 90 s and final extension at 72 °C for 7 min. The DNA was then separated by electrophoresis in a 1% agarose gel and visualized by staining with gold view.

2.8. Observation of pJP4 transfer in the biofilm community by confocal laser scanning microscopy (CLSM)

Some biofilm carriers were taken from the flasks of Bioaugmentaiton experiment I 12 h after the donor strain inoculation and were stored in a phosphorus buffer solution (PBS) at 4 °C for one week until the mature of the dsRed fluorescence. Biomass was detached from the carrier particles and stained with 20 mM SYTO 45 for 30 min before CLSM observation. SYTO 45 is a general nucleic acid stain and used as a counter stain to determine total cells in biofilms. CLSM was performed with a LSM 510 instrument (Carl Zeiss, Jena, Germany) equipped with an Axiovert inverted microscope to visualize cells expressing green or red fluorescent proteins. A 488-nm laser line and a 505 nm long-pass emission filter, a 543-nm laser line and a 560–615 band-pass emission filter were used to detect gfp and dsRed, respectively. The 458-nm laser line was used to detect SYTO 45.

2.9. Microbial community analysis by PCR-denaturing gradient gel electrophoresis (DGGE)

Bacterial 16S rDNA fragments were extracted from biofilm samples and amplified by PCR using primers 341F-GC and 907R. One PCR reaction (50 μl) contained: Taq-DNA-polymerase (5 U/μl), 0.25 μl; GC Buffer I (Mg^{2+} Plus), 25 μl; dNTP mixture (each 2.5 mM), 4 μl; DNA template, 1 μl; primer 341F-GC, 1 μl; primer 907R, 1 μl; and sterilized MilliQ water, 18.75 μl. Amplification was performed with touchdown PCR according to the program as follows: an initial denaturing step at 94 °C for 5 min; then 8 cycles of denaturing at

94 °C for 30 s, annealing at 63–56 °C for 1 min (decreasing by 1 °C each cycle) and extension at 72 °C for 90 s; 20 cycles of 94 °C for 30 s, 55 °C for 1 min, 72 °C for 90 s; final extension at 72 °C for 7 min and then held at 4 °C. The DGGE was performed using a DeCode Universal Mutation Detection System (Bio-Rad). PCR products (20 μl) were run on 6% acrylamide gels with a denaturing gradient of 30–65%. Gels were run at 150 V for 12 h at 60 °C. To visualize the DNA, the gel was stained with silver. Bands of interests were excised from the gel. DNA collected from the targeted band was used as template and amplified through a PCR reaction as described above. The amplified PCR products were further sequenced.

2.10. Determination of 2,4-D and COD concentration

Water samples withdrawn from reactors were filtered (0.22 μm) prior to determining 2,4-D and COD concentration. 2,4-D was analyzed by a high pressure liquid chromatograph (Waters 1525) equipped with a UV–vis detector and a C18 reverse-phase column (250mm × 4.6 mm). The detection wavelength used was 285 nm. The mobile phase was methanol, water and acetic acid in the ratio of 85:13:2. COD was measured according to standard methods [18].

3. Results

3.1. Bioaugmentation experiment I-a microcosm study with 2,4-D as the sole carbon source

3.1.1. Effects of bioaugmentation on 2,4-D removal

The removal of 2,4-D in the five fed-batch runs was presented in Table 1. For the first run, both the bioaugmented reactor and the control experienced a lag period of 76 h before starting degradation. At the end of this run (113 h), 2,4-D at the initial concentration of about 180 mg/l was completely removed by the bioaugmented system, while only 61% was removed by the control. With the increasing of run number, 2,4-D degradation rates increased sharply, and the bioaugmented system still maintained higher degradzation rates than the control. The gaps of 2,4-D average degradation rates between the two reactors in the five operation cycles were calculated to be 0.5, 1.3, 11.3, 11.3 and 13.3 mg/(l h), respectively, and the enhancement of 2,4-D degradation rates ranged 13–64%. These data indicated that the effectiveness of bioaugmentation was slight at the beginning of operation, but it became significant with increasing of operation time.

3.1.2. Qualitative analysis of pJP4 horizontal transfer in the bioaugmented biofilm reactor

A PCR-based method was used to validate pJP4 horizontal transfer in the bioaugmented reactor through amplification and detection of the tfdB gene and gfp gene from biofilm samples, respectively. The results are shown in the agarose gel in Fig. 1. Unsurprisingly, the tfdB gene was detected in the plasmid DNA samples of the donor strain *P. putida* SM1443 due to the presence of pJP4. The tfdB gene was also persistently detected in biofilm samples from the bioaugmented reactor after bioaugmentation, but not in the control biofilm samples (0 h). The gfp gene, as the reporter gene of the donor strain, only appeared in the first 16 h after bioaugmentation, indicating the donor strain just survived for a short period and died soon. From foregoing results, it could be deduced that the existence of the tfdB gene in the bioaugmented biofilm samples could be attributed to the transfer of pJP4 from the donor strain to the indigenous biofilm culture.

3.1.3. CLSM observation of pJP4 transfer in biofilm culture

As the donor strain was a gfp and dsRed dual-labeled genetic microorganism, the event of pJP4 horizontal transfer to biofilm microbes can be observed with CLSM by detecting green or red fluorescence. One biofilm sample taken from the bioaugmented reactor 12 h after bioaugmentation was visualized by CLSM (Fig. 2). All the living cells on the biofilm carrier were stained by SYTO 45 and emitted blue fluorescence (Fig. 2(A)). The green fluorescence and red fluorescence in Fig. 2(B) and Fig. 2(C) reveals the presence of the donor strain *P. putida* SM1443 and transconjugants in the biofilm community, respectively. Fig. 2(D) is a combined image of image (A)–(C) showing the spatial distribution of the donor strain and transconjugants in the biofilm. These images further validated the occurrence of pJP4 transfer in biofilm culture.

3.1.4. Effects of gene augmentation on the changes of microbial community

The dynamic changes of the microbial community of the bioaugmented and control system were investigated by PCR-DGGE and analyzed by UPGMA (Fig. 3). Both reactors showed a gradual change in the microbial community during the whole operation due to the feeding of the toxic compound 2,4-D. For the bioaugmented system, DNA spectrum of the samples taken at 16, 113, 136, 146, 152 and 157 h clustered with a similarity of 80%, which remained 70% similarity to that of the original sample (before adding the plasmid donor strain, 0 h). For the control system, DNA spectrum changed greatly; samples taken at the end of operation (146 and 157 h) showed less than 50% similarity to the samples of other operation times. These data indicated that a more stable microbial community was maintained by the bioaugmented biofilm reactor than the control reactor due to the introduction of 2,4-D degradative gene to some indigenous bacteria which were originally unable to degrade 2,4-D and quick establishment of functional microbial populations.

Four bands (A–D) with a relatively high intensity were excised from the DGGE spectrum of the bioaugmented sample of 157 h, they were sequenced and identified as *Brevundimonas* sp. (accession no. DQ413173, 89% similarity), *Comamonas* sp. (accession no. DQ453128, 96% similarity), uncultured *Gamma proteobacterium* (accession no. CU466843, 99% similarity) and *Paracoccus* sp. (accession no. AM990798, 96% similarity), respectively.

3.2. Bioaugmentation experiment II—an enlarged-lab scale study with multi carbon sources

3.2.1. Performance of the bioaugmented SBBR under long-term operation

Reactor performance was evaluated from the removal of COD and 2,4-D. For the first 10 days, both reactors showed similar degradation rates with a slight reduction of 2,4-D (Fig. 4). During days 10–20, effluent 2,4-D from the bioaugmented system increased unexpectedly and attained a peak value of 70 mg/l, then it declined and reached a complete removal by day 20. This sharp increased effluent might be attributed to the losing of the donor strain due to hydraulic washing since they played a primary role in degrading 2,4-D at the beginning of operation, which was also evidenced by CLSM observation. 2,4-D from the control reactor dropped down directly to detection limit by day 13. On the whole, bioaugmentation did not cause an increased 2,4-D degradation rate during start-up period.

To investigate the effect of selective pressure lacking on reactor performance, 2,4-D was omitted from influent during days 21–33. When 2,4-D was re-added from day 34, the bioaugmented reactor and the control successfully re-started up within 2 and 5 days, respectively. Similarly, 2,4-D feeding was interrupted again during days 41–62 and continued from day 63. The bioaugmented reactor still showed better performance on 2,4-D removal than the con-

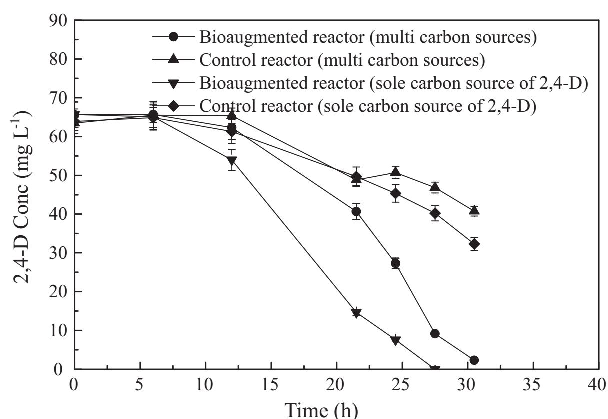

Fig. 5. Profiles of 2,4-D biodegradation by biofilm samples from the bioaugmented and control reactors under different substrates conditions. (●) Bioaugmented reactor under multi carbon sources condition; (▲) control reactor under multi carbon sources condition; (▼) bioaugmented reactor under 2,4-D sole carbon source condition; (♦) control reactor under 2,4-D sole carbon source condition.

trol. It deserves noting that advantages of bioaugmentation were exhibited during these two re-startup periods. During the whole stable operation period, both reactors showed a stable removal of COD above 90%.

3.2.2. Batch study of 2,4-D degradation ability of biofilm samples

To exactly evaluate 2,4-D biodegradation ability possessed by biofilm samples from different systems, some biofilm carriers were withdrawn from each reactor at the end of operation and their degradation capacities were tested in batch degradation experiments (Fig. 5). Results showed that biofilm carriers from the bioaugmented reactor demonstrated a stronger 2,4-D degradation capacity than the control no matter 2,4-D existed alone or mixed with other carbon sources. When present alone, 2,4-D at the initial concentration of 65 mg/l was completely degraded by the bioaugmented biofilm samples within 27.5 h, while 86% was removed by the control one. When 2,4-D co-existed with other easily biodegradable compounds (glucose and starch), biodegradation of 2,4-D was inhibited due to the preferable utilization of these easily utilized compounds by the biofilm bacteria.

4. Discussion

Successful application of gene augmentation in real wastewater treatment system depends on many factors including influent components, reactor configuration, operation mode, selective pressure, etc. Our study investigated the performance of gene augmented reactors under different scales and substrate conditions.

First of all, the two bioaugmentation experiments failed to promote system start-up greatly, for the startup time required by the bioaugmented reactor was comparable or even longer than that of the control one. This might be attributed to the low abundance of 2,4-D degrading bacteria and transconjugants at the beginning of bioaugmentation. In bioaugmentation experiment I, 2,4-D degrading bacteria number were measured to be 1.3×10^8 cfu/ml 24 h after the donor strain inoculation, which was just slightly larger than the control reactor of 6.0×10^7 cfu/ml, with 6.1×10^6 cfu/ml degrader as transconjugants. This “lag effectiveness” of gene augmentation is somewhat different to the “traditional” bioaugmentation method, cell augmentation, as it was reported to be an efficient way to reduce system startup time through adding special culture itself having specific degradation ability directly to a polluted system [19–21]. Similar phenomena were also observed in other studies [10,11]. Bathe et al. [11] investigated plasmid pNB2 mediated bioaugmentation in a sequencing batch moving bed reactor receiving 3-chloroaniline (3-CA). During the first 12 days, both the bioaugmented reactor and the control one were not able to degrade 3-CA and no effectiveness of bioaugmentation could be observed, but advantages of bioaugmentation was observed after the first operation cycle. Another research was done in a SBBR inoculated with a plasmid pJP4 carrying *P. putida* SM1443 aimed to enhance the removal of 2,4-D. During the first 8 days of operation with 8 h cycles, both the bioaugmented and control reactor showed no degradation of the targeted compound, but effectiveness of bioaugmentation was seen when the cycle time was switched from 8 to 90 h [10].

Bioaugmentation experiment I was conducted in a microcosm biofilm reactor with 2,4-D as the sole carbon source in influent and operated in a fed-batch mode. Therefore, the selective pressure from 2,4-D was strong and persistently available throughout the experiment. Bioaugmentation led to an increased 2,4-D degradation rate in all the five operation cycles. This enhancement could be attributed to the presence of a larger number of 2,4-D degrading bacteria and transconjugants in the bioaugmented reactor, because the total 2,4-D degrading bacteria number in the bioaugmented reactor was measured to be approximately 2–10 times of the control with transconjugants accounting for 10–30%. The condition of 2,4-D as the sole carbon source and a fed-batch operation mode created a persistent and strong selective pressure which promoted the production and proliferation of transconjugants via plasmid horizontal transfer and vertical transfer.

Different to bioaugmentation experiment I, bioaugmentation experiment II was operated in a sequence batch mode with a constant cycle time and fed with synthetic wastewater containing multi carbon sources of 2,4-D, glucose and starch. Therefore, the system selective pressure was more complicated. Inoculation of the donor strain did not enhance the removal of 2,4-D during start-up period. Besides the reasons of low number of transconjugants produced during this period, an inadequate selective pressure resulting from the co-existence of easily degradable compounds and 2,4-D in one operation cycle might be another important reason for this failure, as the biofilm community preferred to utilize easily biodegradable compound than 2,4-D. However, when the two reactors were operated with no 2,4-D containing wastewater for a period and restart up with 2,4-D influent again, the bioaugmented system showed significant enhancement in degradation. This enhancement could be attributed to the increased selective pressure resulting from increased biomass and degradation ability, which created a relatively low food/microbe ratio and forced more microbes to utilize 2,4-D in one operation cycle. Microscopic observation of biofilm samples during this period by CLSM showed the presence of a large abundance of transconjugants, which was about 4–50 times of the donor bacteria. These results indicated that a specific microbial population having a strong 2,4-D degradation ability had been established in bioaugmented biofilm reactor and transconjugants played a more important role in degrading 2,4-D than the donor strain. However, the methodology employed in this study failed to exactly discriminate the relative contribution of the donor cells, transconjugant cells and indigenous bacteria in degradation, so further researches based on advanced molecular biotechnology such as real-time PCR are required.

Forgoing research results showed that a strong selective pressure of target recalcitrant compounds existing alone and persistently was more beneficial for exhibiting effectiveness of gene augmentation. Other researches also proved this point [10,15]. Nancharaiah et al. [15] observed persistently enhanced degradation rates in an aerobic granular bioreactor bioaugmented with a TOL plasmid donor strain under benzyl alcohol sole carbon source condition. Bathe et al. [10] also did a successful experiment in a sequencing batch reactor bioaugmentated with a pJP4 donor strain and fed with 2,4-D sole carbon source wastewater. However,

results from those plasmid bioaugmentation studies under mixed substrates condition are more complicated [11,17]. We failed to observed effectiveness in bioaugmentation experiment II during start-up period due to an inadequate selective pressure. Similar results were also reported by Bathe et al. and Mohan et al. [11,17]. Mohan et al. did a full scale bioaugmentation experiment in a SBBR reactor using a TOL plasmid donor under the mixed substrates of BA and other easily degradable compounds but found no positive effects. Therefore, controlling a proper selective pressure is necessary for a gene bioaugmentation study under mixed substrates condition and it deserves further research.

Laboratory scale is another important factor influencing effectiveness of gene bioaugmenation. Most of previous researches were conducted in laboratory scale bioreactors with working volume of 200–600 ml [10,11,15,17]. To the best of our knowledge, no successful case of full scale gene augmentation in biotreatment system has been reported till now. Mohan et al. once tried a plasmid mediated bioaugmentaiton in a pilot scale SBBR with a total volume of 38 l but failed at last [17]. We tried an enlarged laboratory scale bioaugmentation experiment with a working volume of 4 l in this study but failed to find positive effects during start-up period. These results indicate that it is much harder to gain success for gene augmentation in a large or full scale bioreactor, because lots of factors might influence horizontal gene transfer and microbes survival status. Therefore, we should pay more efforts to promote the application of gene augmentation in practical wastewater treatment system.

pJP4 is a well characterized plasmid, and knowledge about its possible distribution in a microbial community has been extensively studied [9,10,22,23]. Bathe et al. studied phylogenetic diversity of the transconjugants receiving the plasmid pJP4 in an activated sludge derived microbial community after inoculation of the donor strain *P. putida* SM 1443. The isolated tranconjugants were found to belong to a variety of genera, but mainly attributed to the families *Rhizobiaceae* and *Comamonadaceae* and the genus *Stenotrophomonas* [9]. Bathe et al. [10] also investigated pJP4 mediated bioaugmentation in a SBBR and found 13 transconjugants showing high similarity (98%) to *Ralstonia* sp. AU3369. In our study, 17 transconjuants were obtained from the bioaugmented SBBR reactor and their partial 16S rDNA sequences were determined. All of these transconjugants showed 98% identical to the sequence of *Alcaligenes* sp. Together with other's research on pJP4, we could conclude that as an Inc-p plasmid pJP4 can spread to a wide host of microorganisms, but the predominant transconjugants isolated from different bioaugmented system varied greatly in phylogenetic groups.

5. Conclusions

In our study, the addition of the pJP4 donor strain *P. putida* SM1443 to a microcosm biofilm system under 2,4-D sole carbon source condition not only promoted the degradation of 2,4-D successfully, but also helped to maintained a more stable microbial community than the control. When adding the donor strain to an enlarged lab-scale biofilm rector under multi carbons source condition, more complicated bioaugmentation results were obtained: bioaugmentation failed to promote systems start up greatly, but established a more efficient 2,4-D degrading biofilm community which showed a stronger degradation ability than the control. Plasmid mediated bioaugmentation was proved to be a feasible strategy to establish a special microbial community in a biofilm reactor. A strong selective pressure was more favorable for the demonstration of this advantage. Few successful cases of gene augmentation in an enlarged lab-scale or pilot scale biofilm reactor imply further researches are required.

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