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Effects of gene-augmentation on the formation, characteristics and microbial community of 2,4-dichlorophenoxyacetic acid degrading aerobic microbial granules

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ABSTRACT

Development of 2,4-dichlorophenoxyacetic acid (2,4-D) degrading aerobic granular sludge was conducted in two sequencing batch reactors (SBR) with one bioaugmented with a plasmid pJP4 donor strain *Pseudomonas putida* SM1443 and the other as a control. Half-matured aerobic granules pre-grown on glucose were used as the starting seeds and a two-stage operation strategy was applied. Granules capable of utilizing 2,4-D (about 500 mg/L) as the sole carbon source was successfully cultivated in both reactors. Gene-augmentation resulted in the enhancement of 2,4-D degradation rates by the percentage of 65–135% for the granules on Day 18, and 6–24% for the granules on Day 105. Transconjugants receiving plasmid pJP4 were established in the granule microbial community after bioaugmentation and persisted till the end of operation. Compared with the control granules, the granules in the bioaugmented reactor demonstrated a better settling ability, larger size, more abundant microbial diversity and stronger tolerance to 2,4-D. The finally obtained granules in the bioaugmented and control reactor had a granule size of around 600 μm and 500 μm, a Shannon–Weaver diversity index (*H*) of 0.96 and 0.55, respectively. A shift in microbial community was found during the granulation process.

1. Introduction

Aerobic sludge granulation is an innovative cell immobilization technology in biological wastewater treatment. Compared with conventional activated sludge, aerobic granular sludge demonstrates the advantages like good settling ability, high biomass retention, and high tolerance to medium toxicity. Aerobic granules are formed through the process of microbial self-aggregation under specific selective pressures such as hydraulic shearing forces, short settling time and rich-famine alternative nutrition status. Aerobic granules are generally easily formed in high strength wastewater comprising easily biodegradable substrates like glucose, acetate, sucrose and ethanol [1–4]. In recent years, more efforts have been directed at cultivation of sludge granules for recalcitrant pollutants removal. Several researches have explored the cultivation of aerobic granules with toxic organic compounds such as chlorophenol, phenol, chloroanilines and tert-butyl alcohol [5–9]. For most of these studies, the toxic compounds degrading aerobic granules were cultivated with a mixture of the target compounds and some easily degradable compounds. Up to date, only a few studies have reported the successful cultivation of aerobic granules with hazardous compounds as the sole carbon source [6].

Gene-augmentation, as an important mean of bioaugmentation, has often been used at polluted sites or in biotreatment systems aimed to achieve a microbial community with desired functions. Gene-augmentation works through mobile genetic elements (MGEs) horizontal transfer from inoculated donor strains to recipient microorganisms. This technology has been well investigated in biofilm reactors because gene horizontal transfer generally occurs with a high frequency at the site having a dense microbial population [10–14]. Aerobic granules, as an important form of microbial aggregation, have the potential to become another “hot spot” of gene horizontal transfer. A few studies have explored the feasibility of cultivation of aerobic granules through gene-augmentation. Nancharaiah et al. [15] first studied the bioaugmentation of aerobic granules with a strain *Pseudomonas putida* carrying a TOL plasmid, and found a significant increase in the degradation of benzyl alcohol in the bioaugmented system. Our previous research explored the possibility of cultivating 2,4-dichlorophenoxyacetic acid (2,4-D) degrading aerobic granules through inoculating a plasmid pJP4 donor strain. Results showed that bioaugmentation accelerated the establishment of 2,4-D degradation ability and the finally obtained granules were able to degrade 2,4-D in the presence of glucose [16]. Although the aforementioned researches have demonstrated

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the possibility of developing aerobic granules for toxic compounds degradation through MGEs based gene-augmentation, the effects of gene-augmentation on the formation, characteristics and microbial community of microbial granules under long term operation has not been fully investigated.

2,4-D, as one of the most commonly used phenoxy acid herbicides in agriculture and gardening, has created potential risks on public health and ecosystem. In this study, 2,4-D was chosen as the target compound. A genetically engineered microorganism *P. putida* SM1443, which carries a conjugative plasmid pJP4 responsible for 2,4-D degradation, was used as the donor strain. Development of 2,4-D degrading granules was investigated in two sequencing batch reactors (SBR) with one bioaugmented with the pJP4 donor strain *P. putida* SM1443 and the other not bioaugmented as control. Half-matured granules pre-grown on glucose were used as the starting seeds. This research aimed to reveal the effects of gene-augmentation on aerobic granules formation, pollutants removal, 2,4-D biodegradation kinetics, granule morphology, microbial communities and diversities during the granulation process. This work will be helpful for further understanding the effects of gene-augmentation and promoting its application in the development of specific aerobic granules.

2. Materials and methods

2.1. Reactor operation

Two column type reactors each having a working volume of 1.7 L (5 cm internal diameter and 87 cm total height) were established. Both reactors were seeded by half-matured aerobic granular sludge, which was previously cultivated with glucose as the main substrate and had a mean diameter of approximately 500 μm. A culture of the strain *P. putida* SM1443 carrying conjugative plasmid pJP4 was inoculated to one reactor at an inoculation ratio of 10%. The other reactor was not bioaugmented as a control. As the strain *P. putida* SM1443 carries a *dsRed* tagged pJP4 plasmid and a chromosomally labeled *gfp* gene (donated by Prof. Stephan Bathe), it expresses a constitutive *gfp* fluorescence but no *dsRed* fluorescence due to repression by a chromosomally encoded *lac*-repressor [17]. The transconjugants receiving plasmids could express *dsRed* fluorescence. The donor strain was able to grow in mineral salt medium containing 5 mM 2,4-D and 1 mM NH_4Cl [17]. Both reactors had an initial biomass concentration of 9 g mixed liquor suspended solid (MLSS)/L. The whole granulation process could be divided into two stages (Stage I and Stage II). Stage I (Days 1–107) was operated with mixed substrates of glucose and 2,4-D, with 2,4-D stepwise increased from 14 to 496 mg/L and Chemical Oxygen Demand (COD) ranged 597–1171 mg/L. Stage II (Days 108–220) was operated with wastewater containing the sole carbon source of 2,4-D (approximately 500 mg/L). The two reactors were operated in a sequencing batch mode with a cycle duration of 6 h (Days 1–121) and 4 h (Days 122–220). Each cycle consisted of 5 min fill, 345 or 225 min aeration, 5 min settle and 5 min draw. Fine air bubbles for aeration were supplied through a dispenser installed at the reactor bottom at an air-flow rate of 3 L/min. The volume exchange ratio was set at 50%. Temperature was maintained at about 20 °C and pH was controlled at 7.0–8.0. The other components of the synthetic wastewater were described by Quan et al. [16].

2.2. Biodegradation kinetics of 2,4-D by the aerobic granules

Some aerobic granule samples were withdrawn from the two reactors on Days 18 and 105. Degradation to 2,4-D by those granules at different initial 2,4-D concentrations (41–713 mg/L) were investigated in a small reaction volume of 100 mL. Reactions were performed at 25 °C and 180 rpm. The specific 2,4-D degradation rates were calculated from 2,4-D degradation curves. Kinetic analysis of the degradation data was performed on the basis of Haldane kinetics model for an inhibitory substrate, $V = V_{max}S/[K_s + S + (S^2/K_i)]$, where V and V_{max} are the specific and maximum specific substrate degradation rates (mg 2,4-D/(g of volatile suspended solid (VSS)·h), respectively; and S, K_s and K_i are the substrate concentration, half-saturation constant and inhibition constant (mg of 2,4-D/L), respectively.

2.3. Microbial community analysis by denaturing gradient gel electrophoresis (DGGE)

Genomic DNA was extracted from the granule samples withdrawn at different operation times using the EZ-10 Spin Column Bacterial Genomic DNA MiniPreps Kits (Bio Basic Inc., Canada). Bacterial 16S rRNA fragments of the granule samples were amplified by Polymerase Chain Reaction (PCR) using the primers 341F-GC and 907 R [18,19]. 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 (10 μM), 2 μL; primer 907R (10 μM), 2 μL; and sterilized MilliQ water, 15.75 μL. Amplification was performed with touchdown PCR under the following conditions: an initial denaturing step at 95 °C for 7 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; followed by 25 cycles of 94 °C for 30 s, 56 °C for 1 min, 72 °C for 90 s; final extension at 72 °C for 7 min and then kept at 4 °C. The DGGE was performed using a DCode universal mutation detection system (Bio-Rad, USA). PCR products (15 μL) were run on 6% acrylamide gels with a denaturing gradient of 35–55%. Electrophoresis was performed at 120 V for 10 h at 60 °C. Gels were stained with SYBR Green I and photographed using a GEL imaging system (VILBER INFINITY 3000, France). Bands of interest were excised from the gels and DNA was recovered from the target bands for sequence determination. The isolated sequences were compared with 16S rRNA sequences obtained via BLAST searches of the National Center for Biotechnology Information database (http://blast.ncbi.nlm.nih.gov). The scanned gels containing DNA band profiles were analyzed using quantity One 1-D analysis software. The Shannon–Weaver index of species diversity (H) was calculated to evaluate the microbial diversity according to the following equation:

$$H = -\sum_{i=1}^{S} p_i \log(p_i) \quad (1)$$

where p_i is the proportion of band i in the DGGE profile and s is the total number of the bands.

2.4. Analytical methods

The effluent water samples withdrawn from the reactors were filtered with 0.22 μm filter prior to determining the concentrations of 2,4-D and COD. 2,4-D was analyzed with a high performance liquid chromatograph (HPLC, Waters 1525, USA) equipped with a UV–vis detector and a C18 reverse-phase column (250 × 4.6 mm). The detection wavelength used was 285 nm and the mobile phase was a mixture of methanol, water and acetic acid (85:13:2). The detection limit of 2,4-D was 0.01 mg/L. COD, MLSS, volatile suspended solids (VSS) and the sludge volume index (SVI) were measured according to Standard Methods [20].

Granule size was measured by a laser particle size analysis system with a range of 0.02–2000 μm (MasterSizer 2000, Malvern Instruments, UK). Granule morphology was observed by a microscope and the microbial composition was observed with a scanning

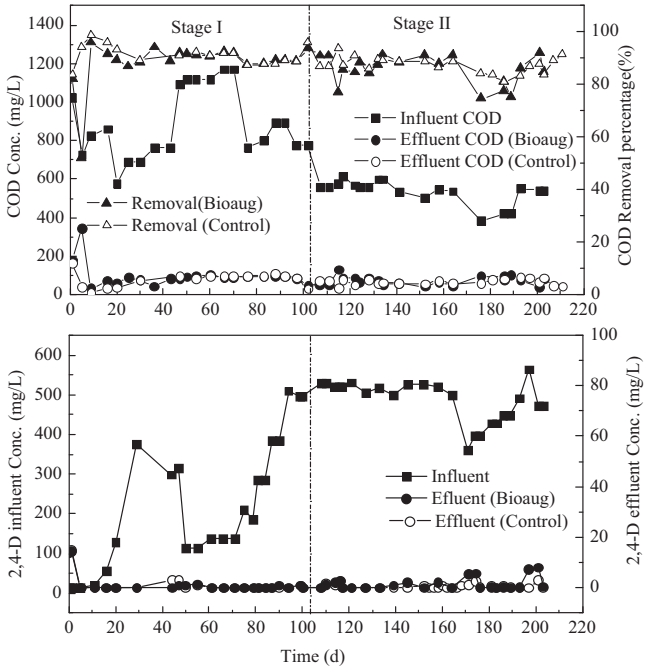

Fig. 1. Removal of COD and 2,4-D in the granule reactors during the whole operation.

electron microscope (Quanta200, FEI, Netherland). The granule samples were gently fixed with 5% glutaraldehyde and 1% OsO_4, and then dehydrated by a graded series of ethanol solutions (50%, 70%, 80%, 90%, 95% and 100%). The dehydrated granules were dried with a liquid CO_2 critical point dryer and observed in SEM. The distribution of transconjugants in the granules was examined by a confocal laser scanning microscope (CLSM) (Carl Zeiss LSM 510, Jena, Germany). A 488 nm laser line with a 505 nm long-pass emission filter and a 543 nm laser line with a 560–615 band-pass emission filter were used to detect fluorescence emitted by *gfp* and *dsRed*, respectively.

3. Results

3.1. Reactor performance

The performance of the two reactors based on the removal of 2,4-D and COD is presented in Fig. 1. When a mixture of glucose and 2,4-D was fed to the reactor during Stage I, 2,4-D at the influent concentration of 14 mg/L was degraded to below detection limit within 5 days for both reactors, and a nearly complete removal of 2,4-D and more than 90% removal of COD were maintained till the end of this operation stage even under the enhancement of 2,4-D loading. During Stage II, when glucose was not added and 2,4-D (at about 500 mg/L) served as the sole carbon source in influent, both reactors maintained a stable removal of pollutants with 2,4-D and COD average removal percentages at 99% and 87%, respectively. On the whole, the bioaugmented reactor and the control demonstrated a similar performance on pollutants removal during the granulation process (Fig. 1). Pollutants removal profiles for a typical cycle period showed that nearly 100% removal of 2,4-D and about 90% removal of COD (data not shown) could be achieved at the end of a cycle time, indicating that 2,4-D could be mineralized by the microbial granules.

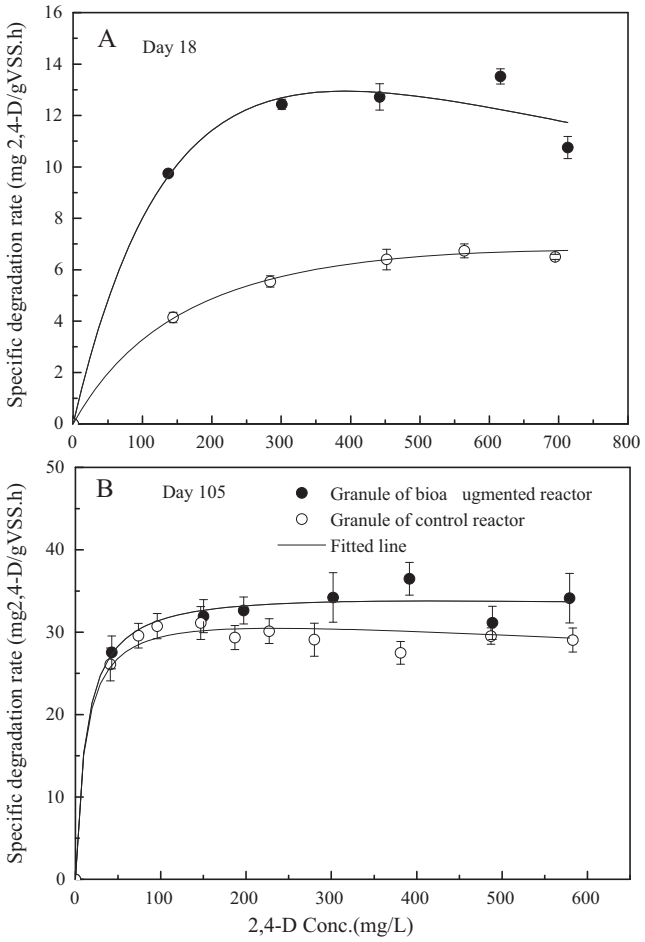

Fig. 2. Specific 2,4-D degradation rates for the granules from the bioaugmented reactor and its control (a) Day 18; (b) Day 105.

3.2. Biodegradation kinetics of 2,4-D

Biodegradation kinetics of 2,4-D by the granules sampled on Days 18 and 105 were investigated through batch degradation experiments. Specific degradation rates achieved at different 2,4-D initial concentrations are presented in Fig. 2. The granules in the bioaugmented reactor demonstrated higher 2,4-D degradation rates than that in the control reactor. The difference in degradation rates was more significant for the granules sampled on Day 18 than that on Day 105, indicating that bioaugmentation exhibited more benefits for the granules at the initial operation stage. The maximum specific degradation rates demonstrated by the aerobic granules in the bioaugmented reactor and the control were 13.52 and 6.63 mg 2,4-D/gVSS·d for Day 18, 36.49 and 30.46 mg 2,4-

Table 1
Degradation kinetic parameters obtained through fitting data to Haldane equation.

Granule type	Day	V_{max} (mg2,4-D/gVSS·h)	K_s (mg2,4-D/L)	K_i (mg2,4-D/L)	R^2
Bioaugmented reactor	18	29.9	256.7	600.5	0.9784
	105	35.9	13.2	13462.9	0.9841
Control reactor	18	10.4	213.3	2986.6	0.9993
	105	33.6	11.9	4612.5	0.9977

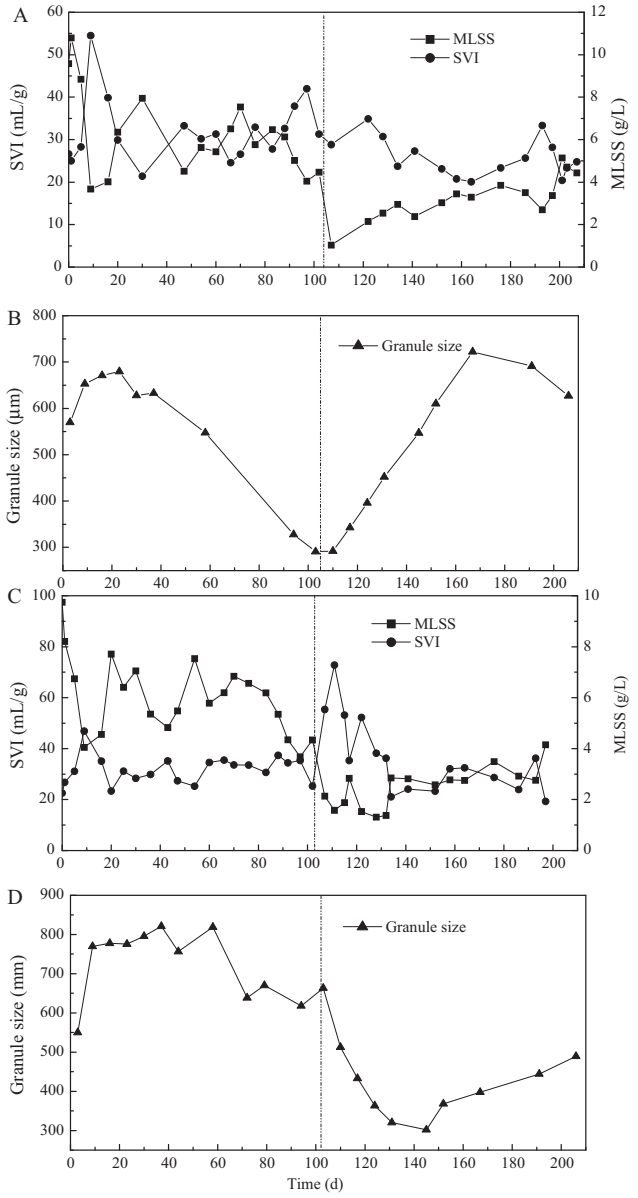

Fig. 3. Variation of biomass, SVI and granule size during the granulation process for the bioaugmented reactor (A and B) and its control (C and D).

D/gVSS·d for Day 105, respectively. Gene-augmentation resulted in the enhancement of 2,4-D degradation rates by the percentage of 65–135% for the granules on Day 18, and 6–24% for the granules on Day 105.

The Haldane equation was used to model the degradation data and the obtained kinetic parameters are presented in Table 1.

3.3. Evolution of biomass and formation of granules

The evolution of biomass, granule size and sludge settling ability during the granulation process are presented in Fig. 3. For the granules in the bioaugmented reactor, sludge settling ability deteriorated due to the feeding of 2,4-D at the beginning of operation, with the SVI values sharply increased from 25 mL/g to 55 mL/g accompanied by a fast drop of biomass from 11 g MLSS/L to 4 g MLSS/L. Granule size increased first from 560 μm to 700 μm, and then declined gradually to 300 μm on Day 105. When the influent switched to the 2,4-D sole carbon source wastewater, the granules recovered settling ability gradually with SVI finally stabilized at 20–30 mL/g and biomass reached around 6 g MLSS/L at end of operation; meanwhile, granule size also increased linearly to a peak value of 721 μm on Day 167 and then decreased slightly and finally stabilized at about 600 μm.

For the granules in the control reactor, granules settling ability remained relatively stable with SVI at about 30 mL/g during Stage I except an ephemeral deterioration at the beginning of operation (Days 1–10); meanwhile, biomass concentration varied in the range of 4–8 g MLSS/L. Different to the granules in the bioaugmented reactor, granule size for the granules in the control reactor was not significantly influenced by the feeding of 2,4-D during Stage I, as it first increased to 770–820 μm during Days 10–60 and then decreased and stabilized at 620–660 μm during Days 70–105. However, at the beginning of Stage II, sludge settling ability deteriorated with a sharp increase of SVI to 73 mL/g and decrease of biomass to below 2 g MLSS/L on Day 110; sludge granule size also dropped down correspondingly to 300 μm on Day 145. After a period of adaptation, the granules recovered settling ability and granule size began to increase. The matured granules had a granule size of about 500 μm and a SVI value of about 30 mL/g.

3.4. Granule morphology

Microscopic observation revealed the dynamic changes of granule morphology in the bioaugmented reactor during the whole operation (Fig. 4). The half-matured granular sludge used as the starting seeds had an irregular shape and loose structure with mean diameter of about 550 μm (Fig. 4(a)). Some black dots or areas appeared within the granules at the beginning of operation due to the toxicity of 2,4-D (Fig. 4(b)). Those sludge granules further broke into small particles of about 300 μm on Day 105 (Fig. 4(c)). When the reactor was operated with the wastewater of 2,4-D as sole carbon source (Stage II), small granular particles began to aggregate and grew; meanwhile, some "super" sludge flakes with a diameter of 3–4 mm and an obvious granule color change from yellow to black was observed (Fig. 4(d and e)). Those "super" sludge flakes involved several sludge particles embedded by a dense layer of extracellular polymeric substances (EPS), and finally disintegrated under the function of hydraulic washing and formed matured aerobic granules (Fig. 4(f)).

Detailed microstructures of the granules were examined using a SEM (Fig. 5). Both types of aerobic granules showed a compact structure and the domination of non-filamentous bacteria that were tightly linked and covered with extrapolysaccarides. The granules in the bioaugmented reactor were composed of rod, coccus bacteria and a few filamentous bacteria, while that in the control reactor showed the predominance of coccus. Filamentous bacteria are generally regarded as the backbone of sludge granules [21], whereas, only a few filamentous bacteria was found in the granules of this study, which indicated that filamentous structure in not necessary for the formation of the 2,4-D degrading granule. The strong toxicity of 2,4-D and a high DO condition applied in this study may inhibit the growth of the growth filamentous bacteria.

The aerobic granules in the bioaugmented reactor were also examined with a CLSM to investigate the survival status of the transconjugants produced through receiving the pJP4 plasmid. A large quantity of bacteria emitting red fluorescence was found in the granules throughout the experiment (data not shown), which indicated that pJP4 successfully transferred to granule microbes and transconjugants had become an important member of the granule microbial community through subsequent growth and proliferation. As the fluorescent cells existed both on surface and inner part of the granules,

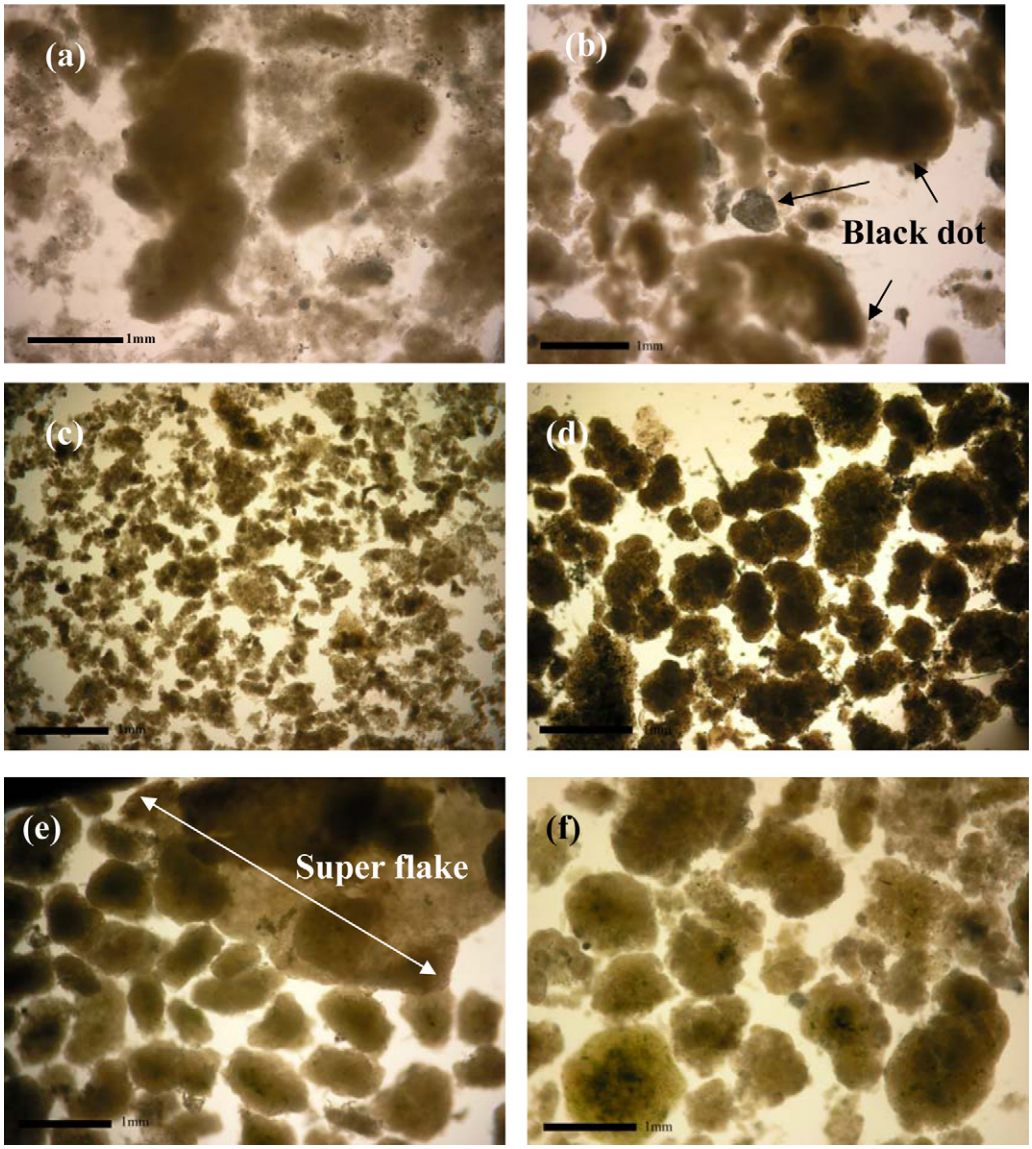

Fig. 4. Images of the sludge in the bioaugmented reactor at different operation times: (a) seeded half-matured granular sludge on Day 0, (b) Day 44, (c) Day 105, (d) Day 134, (e) Day 167, (f) Day 220.

their number was hard to measure accurately. Other molecular technologies like Real-Time PCR are required for further investigation the dynamic changes of transconjugants number.

3.5. Dynamic changes of microbial community

Well-resolved DGGE band profiles were obtained for both types of granules during the granulation process (Fig. 6). A gradual succession in granule microbial community was observed for both types of granules. The granule seeds showed four dominant bands (bands 1–4), with two belong to the member of *Actinobacteria* and two belong to *β-proteobacteria*. With the feeding a mixture of glucose and 2,4-D (Days 1–107), bands 1–4 disappeared, some new bands like bands 5–9 appeared. When the reactors were operated with the 2,4-D sole carbon source wastewater, bands 5, 6, 7, 9 disappeared and bands 10–15 appeared. Both reactors showed an altered microbial community during different operation stages. Some dominant genes bands from the DGGE profile were sequenced and their Blast results were presented in Table 2. The finally obtained granules in the bioaugmented reactor and the control shared four dominant species of *Pseudoxanthomonas taiwanensis* (band10), Uncultured *Sphingobium* sp. (band 11), *Novosphingobium* sp. TYA-1(band 14) and uncultured *β-proteobacteria* bacterium QEDR3DA12 (band 13). Besides the four common strains, the granules in the bioaugmented reactor also showed the presence of *Aquincola tertiaricarbonis* L108 (band 8) and *Sphingobium* sp. JW16.2a (band 12), and the granules in the control reactor found the existence of uncultured *Xanthomonas* sp. (band 15).

The Shannon–Weaver index of species diversity (H) value was calculated based on DGGE band patterns to evaluate the apparent diversity of a microbial community (Fig. 7). The H value is influenced by both the number and abundance of species. A high H value signifies high species diversity [22]. For the granules in the bioaugmented reactor, the H index increased slightly from 0.75 to 0.91 during Days 1–105, and then fluctuated in the range of 0.78–0.96 for the subsequent operation days. For the granules in the control reactor, index H increased from 0.78 to 0.88 first and then declined gradually and finally stabilized at about 0.55. These data indicated that the granules in the bioaugmented reactor showed more microbial species than that in the control.

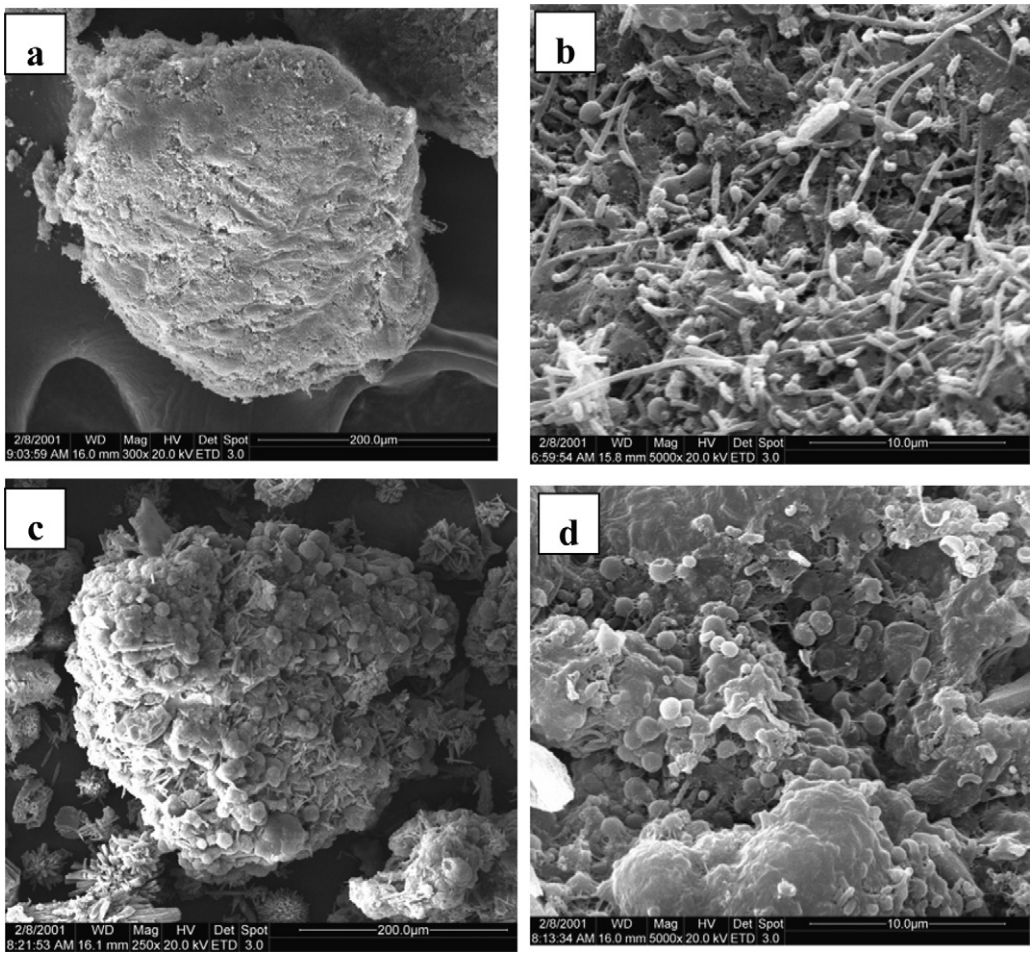

Fig. 5. SEM images of granules in the bioaugmented reactor (a and b) and the control reactor(c and d) on Day 160 (a × 300, b × 5000, c × 250, d × 5000).

4. Discussion

Chlorophenoxy herbicides are reported to have toxic and metabolic uncoupling effects on microbial cell growth [23,24]. Therefore, cultivation of sludge granules degrading these compounds is much more difficult than cultivation of sludge granules degrading common pollutants. To promote the fast formation of 2,4-D degrading sludge granules, half-matured sludge granules pre-cultured with glucose were used as the starting seeds, and a specific strain *P. putida* SM1443 carrying a conjugative plasmid pJP4 was bioaugmented. Half-matured granules were selected as the starting seeds because of its strong tolerance to inhibitory compounds. In addition, half-matured granules are more suitable than completely matured granules to incorporate bioaugmented strains because of their less compact structure. To promote the steadily transform of glucose-fed granules to 2,4-D degrading granules, glucose was added as a benign substrate and 2,4-D was increased gradually at the initial operation stage.

Bioaugmentation is often applied in biotreatment systems to promote system start-up. In this study, it took 5 days for both the

Table 2
Sequence analysis and species identification of selected DGGE bands for the aerobic granules (the band numbers are shown in the schematic DGGE profiles in Fig. 7).

Band no.	Closest relatives	Ref. accession no.	Identity	Phylogenetic division	Accession no.
1	*Leifsonia* sp.	HQ222274.1	97%	Actinobacteria	JF804658
2	*Microbacterium pumilum*	AB234027.1	99%	Actinobacteria	JF804659
3	Uncultured *Alicycliphilus* sp.	GQ891858.1	99%	β-proteobacteria	JF804660
4	Uncultured *Comamonas* sp.	FJ439050.1	98%	β-proteobacteria	JF804661
5	Uncultured *Alphaproteobacteria* bacterium 16S rRNA gene from clone QEDN1CD03	CU927486.1	99%	α-proteobacteria	JF804662
6	*Hydrogenophaga* sp. Gsoil 1545	AB271047.1	99%	β-proteobacteria	JF804663
7	Uncultured bacterium partial 16S rRNA gene, clone H2SRC126X	FM174360.1	99%	unknown	JF804664
8	*Aquincola tertiaricarbonis* L108	DQ656489.1	99%	β-proteobacteria	JF804673
9	*Lysobacter* sp. T-15	AB490175.1	99%	γ-proteobacteria	JF804665
10	*Pseudoxanthomonas taiwanensis*	AB210278.1	98%	γ-proteobacteria	JF804666
11	Uncultured *Sphingobium* sp.	HM438584.1	96%	α-proteobacteria	JF804672
12	*Sphingobium* sp. JW16.2a	FN556564.1	97%	α-proteobacteria	JF804667
13	Uncultured *Betaproteobacteria* bacterium from clone QEDR3DA12	CU922449.1	96%	β-proteobacteria	JF804669
14	*Novosphingobium* sp. TYA-1	AB491194.1	98%	α-proteobacteria	JF804670
15	Uncultured *Xanthomonas* sp.	EU381114.1	98%	γ-proteobacteria	JF804674

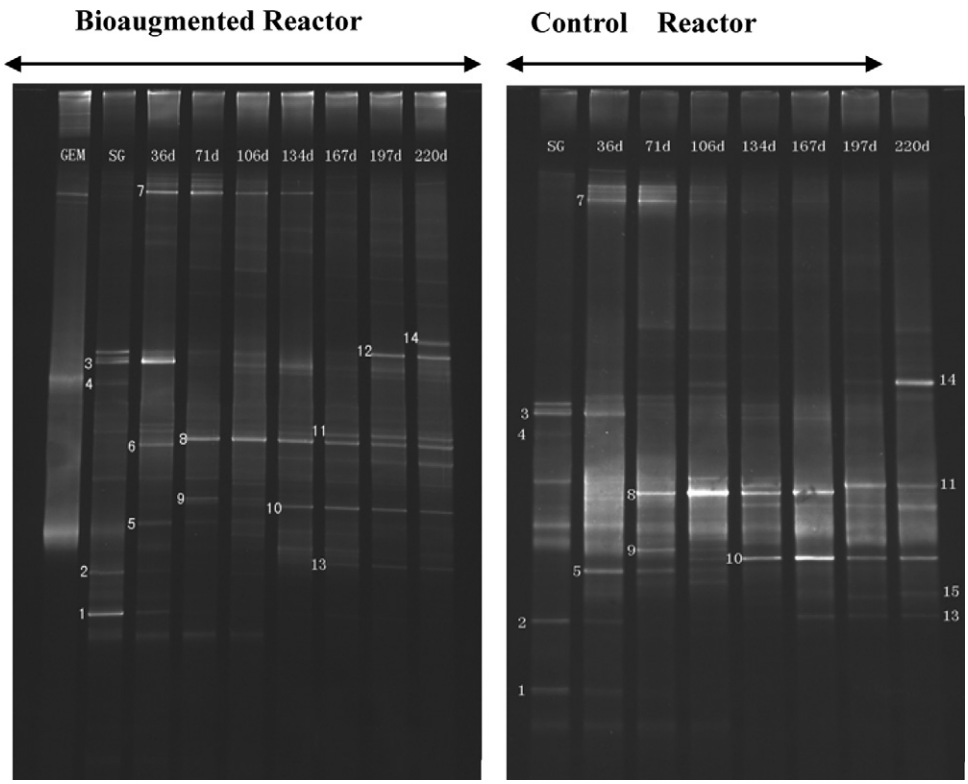

Fig. 6. DGGE fingerprints for the granule samples in the bioaugmented reactor and the control. Lanes are labeled with letter SG (seed granule), GEM (genetically engineered microorganism *Pseudomonas putida* SM1443) and the number indicates sampling period in days.

bioaugmented and control reactors to achieve a stable removal of 2,4-D and COD. Bioaugmentation failed to reduce the start-up period. This may be attributed to the low concentration of 2,4-D (14 mg/L) applied at the beginning of operation and a strong tolerance of sludge granules to toxic organic compounds [7,25]. When influent 2,4-D increased to 375 mg/L on Day 25, both granule reactors maintained a nearly complete removal of 2,4-D and 90% removal of COD. The start-up time for the two granule reactors was much less than that for activated sludge based reactors reported previously. Mangat and Elefsiniotis [26] investigated the degradation of 2,4-D in three SBR reactors seeded with phenol degrading microorganisms, activated sludge and their mixture. A long acclimation period (about 4 months) was required for those reactors to establish a stable degradation ability for 2,4-D at the concentration of about 100 mg/L. Orhon et al. [27] indicated that activated sludge needed a acclimation period of 35–45 days to degrade 2,4-D at about 100–400 mg/L.

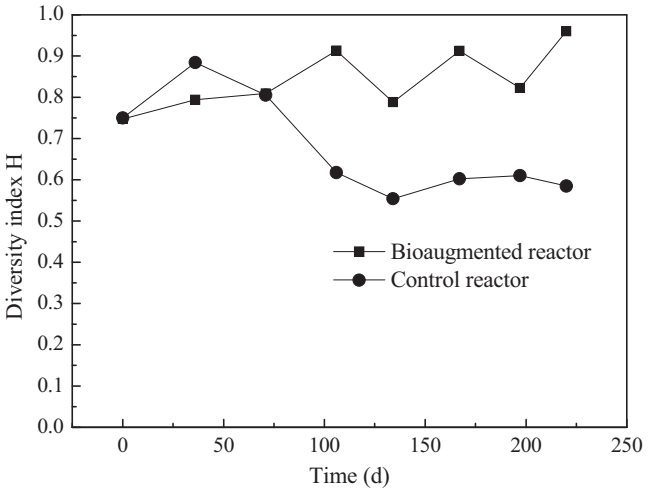

Fig. 7. Bacterial species diversity using the Shannon–weaver diversity (*H*) calculated from the DGGE band profiles for the granules from the bioaugmented reactor and its control.

Although no obvious effects of bioaugmentation were found in pollutants removal during the granulation process, batch degradation experiment conducted on Days 18 and 105 revealed a considerable difference in 2,4-D degradation rates between the two types of granules. The granules in the bioaugmented reactor demonstrated much higher 2,4-D degradation rates than that in the control. This may be due to the production of transconjugants through plasmid horizontal transfer and subsequently cell proliferation, as a large number of transconjugants emitting red fluorescence were found in the granules from the bioaugmented reactor but not found in the control during the whole operation period. The number of transconjugants in the matured 2,4-D degrading granules was approximately 2×10^5 cell/L. The donor strain *P. putida* SM1443 disappeared 8 days after bioaugmentation as no green fluorescence cells could be detected thereafter. 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.

Biodegradation kinetics of 2,4-D by granules showed that both types granules demonstrated high degradation rates over a wide range of 2,4-D concentration (100–700 mg/L). This strong resistance to high concentrations of 2,4-D could be attributed to the compact granule structure which created a diffusion barrier and made the 2,4-D concentration inside granules lower than that in the bulk liquid. Similar phenomenon was observed for granules degrading other recalcitrant pollutants like phenol, p-Nitrophenol

and 2,4-dichlorophenol [7,8,28]. The 2,4-D specific degradation rates achieved by the granules in this study were higher than that by activated sludge. For example, Mangat and Elefsiniotis [26] investigated the biodegradation of 2,4-D in a SBR seeded with activated sludge and obtained the specific removal rates of 1.46–1.75 mg 2,4-D/g VSS·h.

Selective pressure is an important factor influencing the effectiveness of bioaugmentation. It has been reported that a strong selective pressure of target recalcitrant compounds existing alone and persistently was more beneficial for exhibiting effectiveness of gene augmentation [14,15]. For example, Quan et al. [14] conducted two gene augmentation experiments in a biofilm reactor under different substrates conditions aimed to enhance the removal of 2,4-D. No enhancement in 2,4-D removal was observed during start-up period for the bioreactor operated with mixed carbon sources of glucose and 2,4-D, but a significant enhancement was found for the reactor operated with the sole carbon source of 2,4-D. Similar phenomena were also found in this study. The granule size for the bioaugmented reactor decreased gradually during Stage I (mixed substrates of 2,4-D and glucose) but step increase during Stage II (2,4-D as the sole carbon source), whereas the granule size for the control reactor showed an opposite variation trend.

The granules in the two reactors demonstrated a distinct trend in the changes of granule size. During Stage I, the granule size for the granules in the bioaugmented reactor declined gradually, while that in the control reactor were relatively stable, which indicated that the granules in the bioaugmented reactor was less stable than that in the control when operated with mixed substrates of glucose and 2,4-D. During Stage II, the granule size increased steadily for the bioaugmented reactor while that for the control decreased first and then increased slightly, indicating that the granules in the bioaugmented demonstrated obvious advantages in treating the wastewater with 2,4-D as the sole carbon source. This finding was also corroborated by other researches.

Another important feature for the granules in the bioaugmented reactor was more microbial diversity than that in the control. This may be due to the fact that many indigenous bacteria originally having no 2,4-D degradation ability established this ability through receiving the conjugative plasmid pJP4 and thus survived in the 2,4-D containing wastewater. The dominant bacteria in the granules cultivated through bioaugmentation were found to mainly belong to the member of *Aquincola tertiaricarbonis*, *Pseudoxanthomonas taiwanensis*, *Sphingobium* sp. and *Novosphingobium* sp. *Pseudoxanthomonas taiwanensis* has been reported to be a thermophilic bacteria isolated from the thermophilic aerobic granular biomass and paper mill slime [29,30]. *Novosphingobium* sp. TYA-1 was able to degrade bisphenol A and bisphenol F in the rhizosphere sediment [31]. *Sphingobium* sp. was isolated from soils, sediment and activated sludge and exhibited degradation ability to many aromatic hydrocarbons including herbicides, polyaromatic hydrocarbons and estrogen [32–35]. *A. tertiaricarbonis* L108 was reported to grow on the fuel oxygenates methyl tert-butyl ether (MTBE), ethyl tert-butyl ether (ETBE) and tert-amyl methyl ether (TAME) [36]. Many 2,4-D-degrading microorganisms have reported to be isolated from agricultural, urban, and industrial soils and sediments, which mainly belong to the subdivision of *Proteobacteria* such as *Rhodoferax, Bulkholderia*, *Ralstonia, Alcaligenes, Halomonas, Variovarax* and *Pseudomonas*, *Bradyrhizobium* sp. and *Sphingomonas* [37–41]. All the identified strains in the finally obtained granules have not been reported to be 2,4-D degrading bacteria before except *Sphingobium* sp.

Above research results indicates that gene-augmentation is a promising strategy for the fast establishment granular sludge possessing specific functions. Compared with the traditional bioaugmentation method, cell augmentation, it shows the advantage of lower requirement for the survival of the added strains in a bioaugmented system, and therefore can be regarded as an in situ method of genetic modification [16,42].

In summary, aerobic granules with a mean diameter of 500–600 μm and capable of utilizing 2,4-D of about 500 mg/L as the sole carbon source were successfully established in both the bioaugmented reactor and the control. The granules cultivated in the bioaugmented reactor exhibited larger sizes, better settling ability, stronger 2,4-D degradation ability and richer microbial species than that in the control. More researches are required to understand the dynamic changes of transconjugants number and their contribution in 2,4-D degradation in the complex microbial communities.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2011.09.023.

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