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Effect of seed sludge on characteristics and microbial community of aerobic granular sludge

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Abstract

Aerobic granular sludge was cultivated by using different kinds of seed sludge in sequencing batch airlift reactor. The influence of seed sludge on physical and chemical properties of granular sludge was studied; the microbial community structure was probed by using scanning electron microscope and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). The results showed that seed sludge played an important role on the formation of aerobic granules. Seed sludge taken from beer wastewater treatment plant (inoculum A) was more suitable for cultivating aerobic granules than that of sludge from municipal wastewater treatment plant (inoculum B). Cultivated with inoculum A, large amount of mature granules formed after 35 days operation, its SVI reached 32.75 mL/g, and SOUR of granular sludge was beyond 1.10 mg/(g·min). By contrast, it needed 56 days obtaining mature granules using inoculum B. DGGE profiles indicated that the dominant microbial species in mature granules were 18 and 11 OTU when inoculum A and B were respectively employed as seed sludge. The sequencing results suggested that dominant species in mature granules cultivated by inoculum A were *Paracoccus* sp., *Devosia hwasunensi*, *Pseudoxanthomonas* sp., while the dominant species were *Lactococcus raffinolactis* and *Pseudomonas* sp. in granules developed from inoculum B.

Key words: aerobic granular sludge; seed sludge; microbial community; PCR-DGGE
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Introduction

Aerobic granulation is a novel biotechnology for wastewater treatment. Compared to conventional activated sludge, granular sludge offer several advantages, including denser and stronger microbial aggregate structure, excellent sludge settle-ability, higher biomass concentration, and ability to withstand shock loadings. In addition, aerobic granular sludge has a better removal effect to nitrogen and phosphorus pollution. In recent years, researchers have given more interests in the development of aerobic granular sludge.

Until now, two kinds of seed sludge have been applied to cultivate aerobic granules, which is traditional activated sludge taken from municipal wastewater treatment plant and anaerobic granular sludge respectively. When anaerobic granular sludge was used as seed sludge, aerobic granules could be successfully cultivated, moreover, the formation of anaerobic granules also need three months at least (Sun et al., 2007; Wang et al., 2008; Xia et al., 2007). The formation process of aerobic granular sludge is difficult to control when traditional activated sludge was employed as inoculum, but it might help to understand aerobic granulation process, and further establish theoretical foundation for its practical application, therefore, activated sludge from municipal wastewater treatment plant was often used as seeding inoculum (Advan et al., 2008; Belen et al., 2004; Claudio et al., 2006; Ivanov et al., 2006; Li et al., 2006; Liu et al., 2004, 2005a; Qin et al., 2004a; Su and Yu, 2005).

Aerobic granulation was affected by a number of operational parameters, such as aeration intensity, organic loading rate, feeding strategy, settling time, substrate composition (Advan et al., 2007; Liu et al., 2002, 2005b; Moy et al., 2002; Morogenroth et al., 2000; Mahoney et al., 1987; Pringle and Fletchter, 1983; Qin et al., 2004b; Shin et al., 1992; Tay et al., 2004; Zhu and Wilderer, 2003). Being a complicated ecological system, the bacterial community residing in seed sludge was important for aerobic granulation process. However, there is little information available in literature about the role of seed sludge on aerobic granulation.

This study is focused on investigating the effect of seed sludge on the properties and microbial community of aerobic granules. Aerobic granules was achieved by using different kinds of seed sludge in sequencing batch airlift reactor (SBAR), and their physical and chemical properties were determined, polymerase chain reaction-denaturing

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gradient gel electrophoresis (PCR-DGGE) was employed for revealing the microbial community structure and succession corresponding. The objective of this work is to strengthen the cultivation of aerobic granulation and to accelerate the development of aerobic granulation systems for full-scale application.

1 Materials and methods

1.1 Reactor and operation

The laboratory SBAR were two identical hermetic cylindrical plexiglass vessels, with a working volume of 5.0 L each. The riser had an internal diameter of 8.0 cm and a height of 120 cm, while the down-comer was 6.0 cm in diameter and 90 cm in height. Air was introduced through the bottom by a fine bubble aerator at 4.0 cm/sec controlled by a gas-flow controller, while the operation and temperature of reactor are respectively controlled by PLC and water bath sleeves (Song et al., 2009).

One reactor (R_A) was inoculated using 1.5 L fresh activated sludge taken from beer wastewater treatment plant, the other reactor (R_B) was inoculated using 3 L fresh activated sludge taken from municipal wastewater treatment plant. Two reactors were operated in successive cycles of 6 hr, including 10 min of influent filling, 320–330 min of aeration, 5–15 min of settling and 15 min of effluent discharge. Supplied with an influent loading rate of 3.0 kg COD/(m^3·day), two reactors were operated simultaneously at (30 ± 1)°C over 60 days without excess sludge discharge.

1.2 Experimental materials

The composition of synthetic wastewater is shown in Table 1.

Seeding inoculums were respectively taken from aeration tank of Wen-Chang Municipal Wastewater Treatment Plant (inoculum A) and Song-Jiang Beer Wastewater Treatment Plant (inoculum B) in Harbin, China. The features of two kinds of activated sludge are summarized in Table 2.

1.3 Analytical methods

Chemical oxygen demand (COD), NH_3-N, TP, mixed-liquor suspended solid (MLSS), and sludge volumetric index (SVI) were measured using standard methods (SEPAC, 2002). Cell surface hydrophobicity was determined by measuring zeta potential using micro electrophoresis (JS94J3, Shanghai, China).

Table 1 Components of synthetic wastewater

Nutrition component	Concentration (mg/L)	Trace element	Concentration (mg/L)
Glucose	2000	H_3BO_4	150
Peptone	300	$ZnSO_4·7H_2O$	120
Beef extract	200	$MnCl_2·7H_2O$	120
NH_4Cl	400	$CuSO_4·5H_2O$	30
$CaCl_2$	200	$NaMoO_4$	65
$FeSO_4·7H_2O$	40	$NiCl_2$	50
$MgSO_4·7H_2O$	30	$CoCl_2·6H_2O$	210
K_2HPO_4	70	KI	30
KH_2PO_4	30		
Trace element	1.0 mL/L		

Table 2 Characteristics of seeding inoculum

Source	MLSS (g/L)	SVI (mL/g)	v (m/hr)	Zeta potential (mV)	SOUR (mg/g·min)
Song-Jiang (A)	10.0	80	21.19	–2.841	0.591
Wen-Chang (B)	4.50	106.12	13.89	–18.376	0.481

MLSS: mixed liquor suspended solids; SVI: sludge volume index; v: settling speed; SOUR: specific oxygen utilization rate.

Specific oxygen utilization rate (SOUR) was determined using the method developed by Anna and Malte (1997). Microstructure was probed by using an electronic microscope (DM1000, Leica, Germany).

For settling speed (υ), after being sieved, five granules were taken out from each size gradation, then a granule sample was added into 1 L cylinder filled with tap-water. After recording the time that the granule settle down to bottom, the settling speed of each granule can be calculated according to following equation:

$$v = H/t$$

where, H (m) and t (hr) represent the settling height and settling time, respectively. The arithmetic average speed of fifty granules was reported.

1.4 Microbial community analysis

The community characteristics of granular sludge are analyzed using denaturing gradient gel electrophoresis method (DGGE).

(1) Preparation of sludge sample and genomic DNA extraction. Firstly, the frozen sludge samples are melted by warm bath and 100 mg (wet weight) sample are taken into a clean centrifuge tube (1.5 mL), and centrifugate at 12,000 r/min for 5 min. Secondly, wash the samples for 2 times with PBS buffer and after centrifugations, extract the genomic DNA of sludge samples with the Small Amount of Bacterial Genome DNA Rapid Extraction and Purification Kit (product number W6511, Shanghai Huashun Biological Engineering Co., Ltd., China). Finally, detect the extraction effect by 0.8% agarose gel electrophoresis.

(2) PCR amplification of sludge sample DNA. The universal primer in 16s rDNA V6–V8 District for most bacteria are used. The primers are BSF968GC (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGAACGCGAAGAACCTTAC-3′) and BSR 1401 (5′-CGGTGTGTACAAGACCC-3′) which are corresponding to *Escherichia coli* 16S rDNA 968–984 district and 1385–1401 district nucleotides respectively. The length of amplified fragment is about 470 bp. PCR reaction system is as follows: the materials include of 10× Ex Taq buffer (Mg^{2+}) 2 μL, 2.5 mmol/L dNTP 1 μL, 20 pmol/L *pros* and *cons* primer each 0.5 μL, template DNA 100 ng, Ex Taq 0.2 μL and then add water to make up 20 μL. The study utilizes TD-PCR: pre-deformation at 94°C for 5 min, then at the same temperature deformation for 40 sec, further to annealing at 55°C for 40 sec, follow by extension at 72°C for 30 sec. Every cycle of the 35

cycles decrease annealing temperature by 0.1°C until to 51.5°C and then the final extension is at 72°C for 7 min. Finally, detect the extraction effect by 0.8% agarose gel electrophoresis.

(3) Optimize the condition of DGGE gel electrophoresis and cloning sequencing for the purpose strip. DGGE gel electrophoresis of PCR is carried out by the gene mutation detection system (Dcode^{TM} of Bio-Rad company). The parameters are as follows: the concentration of polyacrylamide gel is 6%, denaturing gradient is from 30% to 60% (100% denaturant with 7 mol/L urea and 40% deionized formamide mixture), after the denatured gradient gel completely polymerized, the gelatin plate should be installed into the electrophoresis which is full of electrophoretic buffer, then mix 5 μL buffer with 5 μL PCR sample and trickle the mixture in the sample holes. PAGE lasts for 10 hr under the voltage 70 V, followed by PAGE is silver staining and finally to obtain the gelatin map by scanning. DNA is recovered by cutting the objective band of the gelatin map, then re-amplified and cloned by the pMD19-T vector, finally sent to the Shanghai Public Health and Biological Engineering Co., Ltd. (China) for sequencing.

2 Results and discussions

2.1 Effect of seed sludge on properties of granules

A certain amount of granular sludge was taken out periodically from sample connections on SBAR during operation. After being mixed up, the aerobic granular sludge was observed under electronic microscope. The results are shown in Fig. 1.

It can be seen from Fig. 1 that the color of seed sludge taken from beer wastewater treatment plant was black, and it was gray from municipal sewage treatment plant. Some tiny regular granules appeared after 7 days operation, but flocculent sludge are predominant in both reactors, which are irregular aggregation due to self-clotting of microbes. At day 14, more larger and regular granules covered by gelatinous matter appeared in the reactors, and the color of granular sludge gradually changed from brown to yellow, granules in R_A were obvious larger than those in R_B, SVI decreased from 80 to 50.31 mL/g in R_A, from 106.12 to 54.00 mL/g in R_B (Table 3). The amount of granular sludge increased gradually along with the SBAR running, round-shaped granules were superior in R_A at the day 35, the granular sludge had a compact structure, its SVI reached 32.75 mL/g and then maintained around 30 ml/g, the removal rate of COD, NH_3-N, and TP was 94%, 93.7%, and 89.15% respectively. By contrast, granules in R_B showed clear outline until day 56, meanwhile its SVI reached 30.67 mL/g and tend to stabilize, while the removal rate of COD, NH_3-N, and TP was 95%, 92.6%, and 67% respectively. It can be concluded that the cultivation time of aerobic

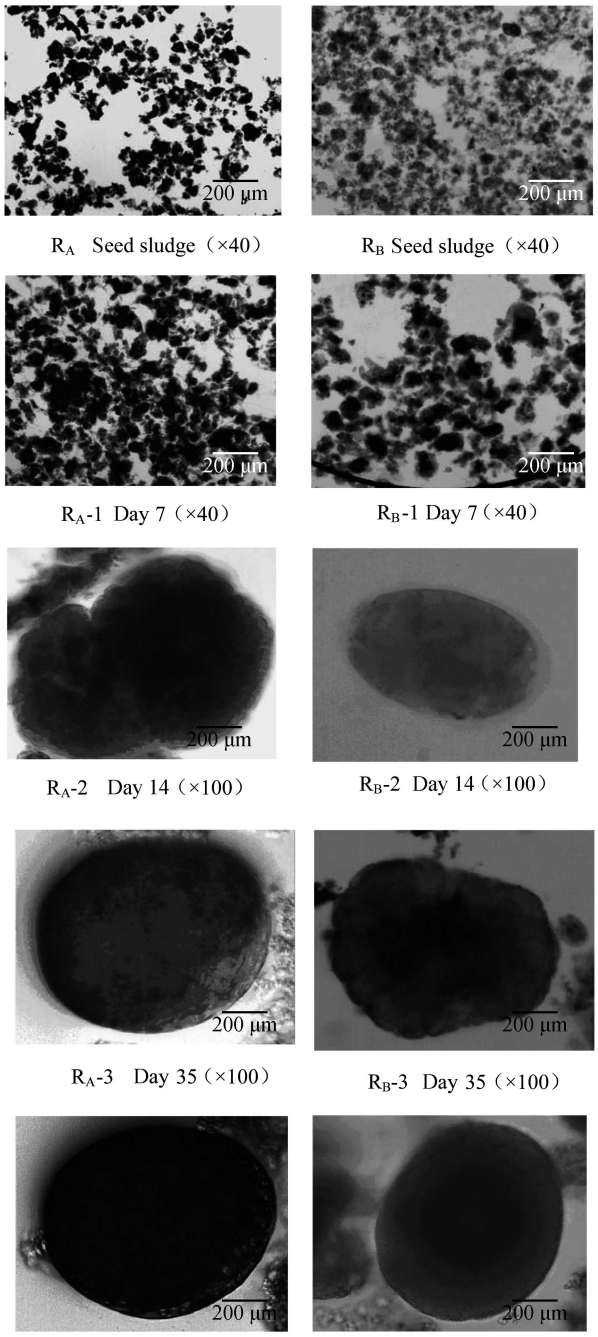

Fig. 1 Morphology of granules.

Table 3 Properties of granules at different phases

Property	Reactor	7 days	14 days	21 days	35 days	42 days	49 days	56 days
SVI (mL/g)	R_A	50.31	38.90	37.98	32.75	30.36	31.91	29.38
	R_B	54.00	49.26	46.70	45.17	39.46	35.81	30.67
Zeta potential (mV)	R_A	–5.46	–4.06	–1.66	–1.59	–1.43	–1.10	–1.02
	R_B	–15.77	–14.74	–10.34	–8.05	–5.38	–5.06	–4.86
SOUR (mg/(g·min))	R_A	0.96	0.95	1.00	1.18	1.13	1.16	1.14
	R_B	0.56	0.70	0.93	1.07	1.04	1.12	1.13

granules is about 35 days in R_A, which is faster than that of 56 days in R_B.

As shown in Table 3, the hydrophobicity of aerobic granular sludge in R_A was greatly improved, zeta potential raised from –5.46 at day 7 to –1.66 mV at day 21 with a subsequent slow increase. Meanwhile, the value of SOUR slightly increased to 1.14 mg/(g·min) at day 56. By contrast, zeta potential in R_B was shown a gradual increase from the beginning to day 56, finally maintained at –4.86 mV, the value of SOUR raised from 0.56 to 1.13 mg/(g·min). It seems that aerobic granulation is not sensitive to the microbial activity of seed sludge, but the effect of hydrophobicity of seed sludge on the aerobic is found to be significant, the variation of granular hydrophobicity is found in conformity with granulation process, this may be explained by cell surface hydrophobicity hypothesis. According to the thermodynamic theory, increasing cell surface hydrophobicity would cause a corresponding decrease in the excess Gibbs energy of the surface and promote cell-cell interaction to further drive the self-aggregation of bacteria (Pringle and Fletchter, 1983; Bos et al., 1999; Liu et al., 2003), therefore, excellent hydrophobicity of seed sludge favors the formation of aerobic granules.

2.2 Effect of seed sludge on microstructure of aerobic granules

The scanning electron microscope photographs of mature granules taken from two reactors at the end of running are shown in Fig. 2. It can be seen that granules in R_A has a compact structure with uniform pores, microbes are closely connected, and brevibacterium as well as cocci are predominant. While granules in R_B display a loose netty framework, some spherical and oval organisms adhered on the frame, this framework was mainly formed by filamentous fungus, This means that seed sludge play an important role in the formation of aerobic granule.

2.3 Microbial community structure and succession

Sludge samples of day 0 (seed sludge), 7, 14, 21, 28, 35, and 42 during aerobic granulation were taken from the reactor, which inoculated by inoculum A. For the other reactor inoculated by inoculum B, sludge samples of day 0 (seed sludge), 21, and 42 were collected. PCR-DGGE analysis was performed to reveal the microbial community structure of chosen sludge samples (Fig. 3).

The DGGE profile indicated abundant microbial diversity for the seed sludge of both inoculum A and B. Since each band can be considered as an operational taxonomy unit (OTU), the dominant microbial species were 16 and 12 OTU for the seed sludge of inoculum A and B respectively. However, microbial community changed in different trends during aerobic granulation. For inoculum A, microbial community structure tended to be stable even at the beginning phase with slight changing along with granulating progress. Dominant microbial species in seed sludge were always found in the sludge sample of day 42, whereas their relative quantities varied more or less. For inoculum B, microbial community structure of the sludge sample at day 42 changed a lot compared with the seed sludge. Most dominant species were found in less amount or even disappeared in the system, such as band 13, 18, 26, 29, and 30. At the end of aerobic granulation, the microbial community diversity was abundant for inoculum A with its dominant microbial species reached 18 OTU, and even simple for inoculum B with the dominant species of 11 OTU.

The obvious differential of microbial community

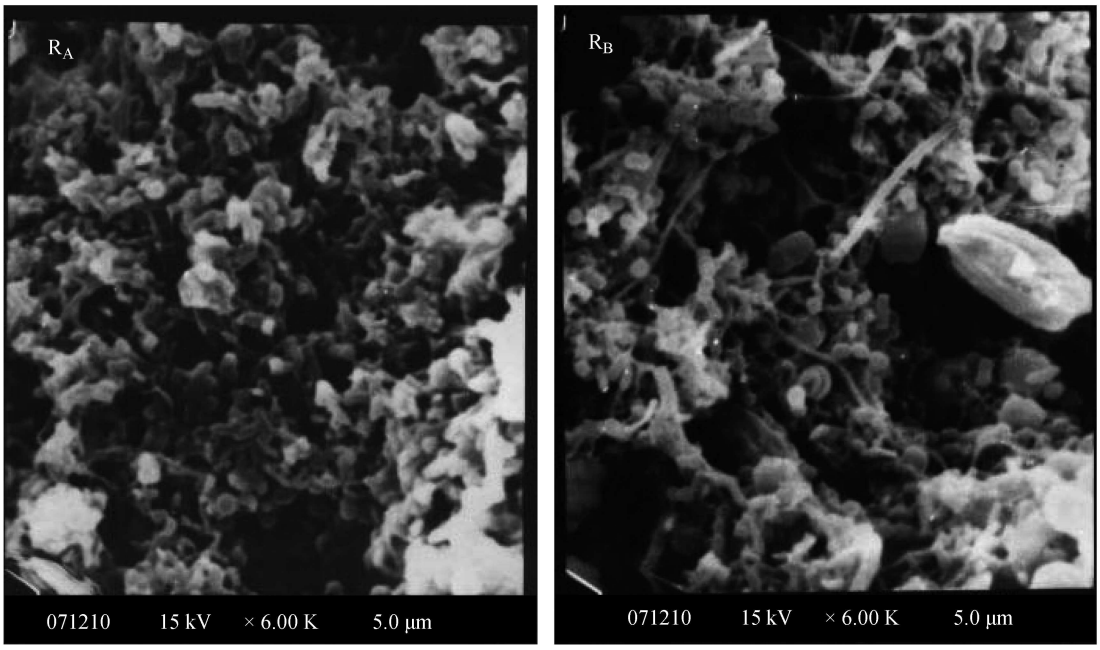

Fig. 2 Scanning electron microscope photographs of the aerobic granules.

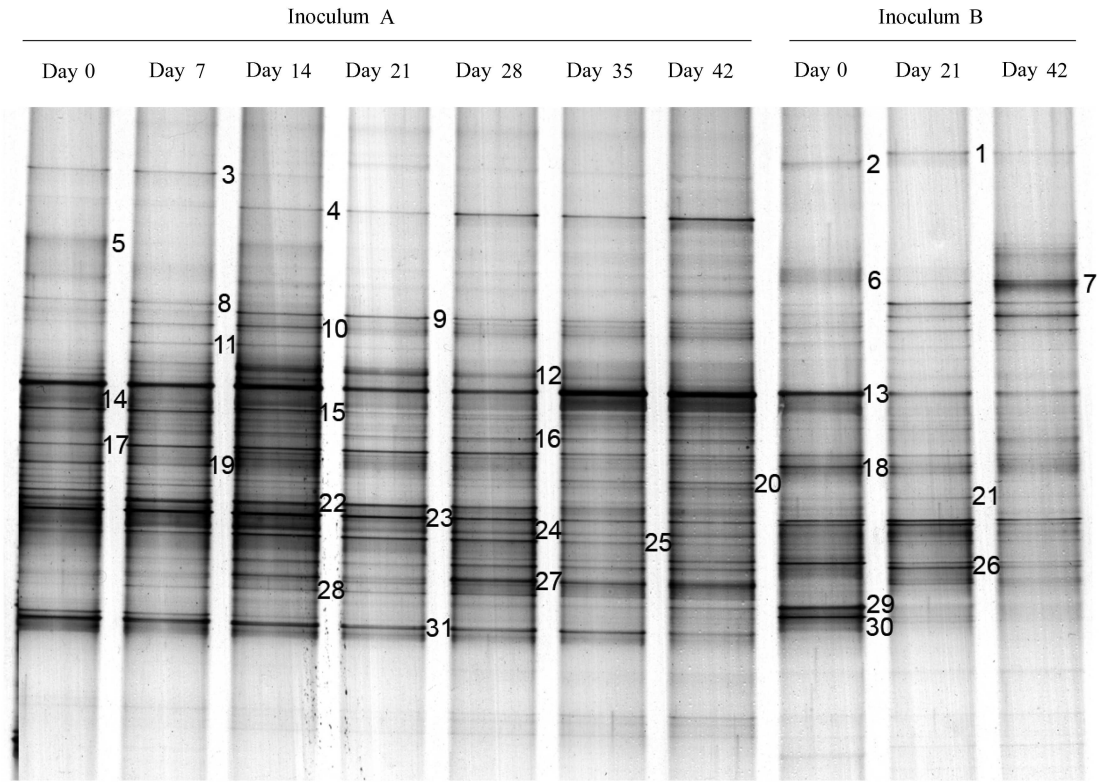

Fig. 3 DGGE profile of sludge samples of aerobic granulation under different seed sludge.

succession for inoculum A and B, as we observed, might be attributed to the properties and microbial components of seed sludge. The sludge of beer wastewater treatment plant was proved to be more suitable for inoculation. As the seed sludge, the sludge of beer wastewater treatment plant was acclimated rapidly in the reactor. The stable microbial community was then associated with a better performance of aerobic granulation. For the sludge of wastewater treatment plant as the inoculum B, microbial community structure was affected largely by operational conditions. Although the aerobic granulation was also processed, the formed granular sludge did not show perfect characteristics.

The UPGMA clustering analysis was used to analysis microbial community similarity (Fig. 4). It showed that two separate groups existed in the clustering tree. One consisted of sludge samples of inoculum A, and the other included the samples of inoculum B. Within each group, microbial community structure in seed sludge had the lowest similarity with other sludge samples, whereas it tended to be similar in the following time. The UPGMA analysis proved remarkable unlikeness between sludge samples of inoculum A and sludge samples of inoculum B. However, rapid succession was experienced at the beginning phase for both inoculum A and B.

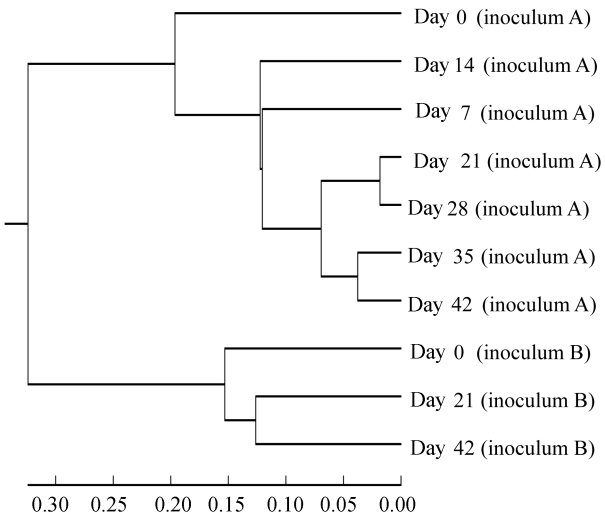

Fig. 4 UPGMA cluster analysis of DGGE profile.

2.4 Identification of dominant microbial species

Sequencing results of bands were aligned in the GenBank database to obtain the closest relative strains (Table 4). The similarities between most bands and corresponding sequences were larger than 94%, which indicated that they may belong to the same genus. The alignment results of 31 OTU indicated that six bacteria clones belonged to Actinobacteria. Seven and three bacteria clones were most similar to γ and α subclass of Proteobacterium respectively. It also contained 2 Clostridiales, 1 Acidobacteria, and 1 Lactobacillales. There were still 11 OTU that can not be affiliated to known species. It can be hypothesized that γ and α subclass of *Proteobacterium* and *Actinobacteria* might act important roles in the formation of aerobic granular sludge.

Corresponding to a better performance of aerobic granulation, a stable climax community was achieved

Table 4 Partial 16S rDNA sequence similarity of bands 1–31 on DGGE profile

Band	Closest sequence (GenBank number)	Similarity (%)	Putative division
1	*Pseudoxanthomonas* sp. D12-26a (AM403237.1)	94	γ-Proteobacterium
2	Uncultured Acidobacteria bacterium clone 500M2_G6 (DQ514191.1)	93	Acidobacteria
3	*Leucobacter* sp. 38 (DQ406732.1)	94	Actinobacteria
4	Uncultured *Paracoccus* sp. clone HKT155 (AY875883.1)	99	α-Proteobacterium
5	Uncultured Actinobacterium clone 7-2 (AY755363.1)	98	Actinobacteria
6	*Clostridium vincentii* (X97432.1)	98	Clostridiales
7	*Lactococcus raffinolactis* strain DSM 20443 (EF694030.1)	93	Lactobacillales
8	Uncultured Cellulomonadaceae bacterium clone HT06Ba18 (EU016444.1)	98	Actinobacteria
9	*Pseudomonas* sp. KN74 (EF469221.1)	99	γ-Proteobacterium
10	Uncultured bacterium clone HB101 (EF648093.1)	92	–
11	Environmental 16S rDNA sequence from Evry wastewater treatment plant anoxic basin (CU466788.1)	97	α-Proteobacterium
12	Uncultured Actinobacterium clone: Dolo_39 (AB257657.1)	90	Actinobacteria
13	*Devosia hwasunensis* strain HST2-16T (AM393883.1)	93	α-Proteobacterium
14	Uncultured *Rhodobacter* sp. clone SH2B-1C (EU073791.1)	99	α-Proteobacterium
15	Uncultured γ-*Proteobacterium* clone F04_MO02 (EF220852.1)	94	γ-Proteobacterium
16	*Acinetobacter junii* (FM208850.1)	98	γ-Proteobacterium
17	Uncultured Acidobacterium clone SBR1013 (AF368180.1)	98	Acidobacteria
18	*Zymophilus raffinosivorans* strain VTT E-90406 (DQ217599.1)	94	Clostridiales
19	Uncultured bacterium clone F002_D12 (AY962315.1)	97	–
20	*Nocardioides dokdonensis* strain FR1436 (EF633986.1)	90	Actinobacteria
21	Uncultured bacterium clone GP_2aaa04h08 (EU775588.1)	94	–
22	Uncultured bacterium clone oc23 (AY491567.1)	94	–
23	*Tetrasphaera elongata* (AB051430.1)	99	Actinobacteria
24	Unidentified soil bacteria clone 100 (AM168230.1)	91	–
25	Uncultured bacterium isolate BF26 (DQ839332.1)	99	–
26	Uncultured High G+C Gram-positive bacterium lpha7 (AF109793.1)	99	Actinobacteria
27	*Pseudoxanthomonas* sp. D12-26a (AM403237.1)	95	γ-Proteobacterium
28	Uncultured γ-Proteobacterium clone AKYG1642 (AY921834.1)	98	γ-Proteobacterium
29	Uncultured Cellulomonadaceae bacterium clone HT06Ba18 (EU016444.1)	98	Actinobacteria
30	*Lactococcus lactis* strain CICC6018 (DQ212977.1)	94	Lactobacillales
31	*Acinetobacter* sp. P-155 (AM412159.1)	94	γ-Proteobacterium

for both inoculum A and B at the end of process (day 42). From the DGGE fingerprints, we can determine important participants based on their quantities, and then identify them using sequencing alignment. For inoculum A, dominant species in the stable microbial community (day 42) were uncultured *Paracoccus* sp. clone HKT155 (Band 4, α-Proteobacterium), *Devosia hwasunensi*s strain HST2-16T (Band 13, α-Proteobacterium) and *Pseudoxanthomonas* sp. D12-26a (Band 27, γ-Proteobacterium). For inoculum B, *Lactococcus raffinolactis* strain DSM 20443 (Band 7, Lactobacillales) and *Pseudomonas* sp. KN74 (Band 9, γ-Proteobacterium) were found dominant in the reactor.

From these identified dominant species, previous studies had revealed that uncultured *Paracoccus* sp. clone HKT155 was found in a common effluent treatment plant (Kapley et al., 2007). This specie in inoculum A might be beneficial to the formation of aerobic granules and then became an important functional microbe in the process. For inoculum B, *Lactococcus raffinolactis* strain DSM 20443 was identified as a lactic acid bacterium, which was isolated from activated sludge foam (Cho et al., 2008). *Pseudomonas* sp. KN74 was found as a common participant in soil (Adesina et al., 2007), the abundance of them might not lead to aerobic granulation. In addition, there existed other kinds of unknown microbes. Their function and efforts need further investigations.

3 Conclusions

(1) The properties of seed sludge played important roles on the cultivation of aerobic granules. Compared with activated sludge from municipal wastewater treatment, the sludge of beer wastewater treatment plant was proved to be more suitable for inoculation. The better the hydrophobicity of seed sludge, the faster the aerobic granulation with excellent settling ability.

(2) DGGE profile revealed that microbial community changed in different ways under different inoculations. Microbial community structure slightly changed along with granulating progress in R_A. the relative quantities of dominant species varied more or less. While microbial community structure changed a lot compared with the seed sludge in R_B. It was also observed that the microbial community diversity under inoculum A was much more abundant than that of inoculum B.

(3) Sequencing results of 31OTU suggested that γ and α subclass of Proteobacterium and Actinobacteria were predominant in the system. For inoculum A, dominant species in the stable microbial community were *Paracoccus* sp., *Devosia hwasunensi*, and *Pseudoxanthomonas* sp. For inoculum B, *Lactococcus raffinolactis* and *Pseudomonas* sp. were found dominant in the reactor. Due to their large amounts, the efforts of them on aerobic granulation were important and deserved more investigations.

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