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Full Length Research Paper

Study on microbial community and diversity of an abscisic acid wastewater anaerobic granular sludge system

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The microbial community and diversity in an abscisic acid wastewater anaerobic granular sludge system were characterized using molecular techniques. 16S rRNA clone library and sequence analysis revealed that about 99% of 161 bacterial clones belonged to the *Firmicutes* and *Proteobacteria*, the remaining 2 clones were assigned to *Bacteroidetes*. *Clostridia* (34.8%) and *Bacilli* (32.3%) belonged to *Firmicutes*; in the class of *Proteobacteria*, the clones were assigned to *Betaproteobacteria* (15.5%), *Gammaproteobacteria* (13.7%), *Alphaproteobacteria* (1.2%) and *Epsilonproteobacteria* (1.2%). For the domain of Archaea, 37.35% of 83 archaeal clones were affiliated with *Methanospirillum* and *Methanocorpusculu*m respectively; the other of 25.3% belonged to *Methanosphaerula*.

Key words: Abscisic acid wastewater, microbial community and diversity, 16s rRNA, up-flow anaerobic sludge blanket.

INTRODUCTION

Industrial wastewater contains organic matters, most of which are poisonous to various life forms and would be a potential hazard to natural water system. Anaerobic wastewater treatment is considered as the most cost-effective solution for organically polluted industrial waste-streams (Van et al., 2001). Anaerobic wastewater reactor is known for the unique ability to eliminate highly object-tionable waste, first proposed as a treatment process by Young and McCarty (1969). In recent years, the Up-flow Anaerobic Sludge Blanket (UASB) has been greatly developed, and become one of the most commonly used anaerobic reactors, with great tolerance of organic pollutant load, high efficiency due to short hydraulic retention time (HRT) and low demand of energy (Cintoli et al., 1995; Najafpour et al., 2006). It is widely applied in food, paper, chemical and pharmaceutical industries (Macarie, 2000; Shreeshivadasan and Paul, 2011). The composition and activity of microbial community of anaerobic treatment bioreactor are very important to wipe off organic pollutant of wastewater (Tang et al., 2005; Akarsubasi et al., 2006; Ping et al., 2011), not only helping us to understand the biotreatment mechanism of waste organic matters, but also providing theoretical foundation for large-scale processing.

Abscisi acid, a sesquiterpenoid (15-carbon), is an important signal chemical in plants (Knetsch et al., 1996). Currently, the industrial abscisi acid is produced by microbial fermentation; only one company produces it in China. No study has been reported on biotreatment of the abscisic acid wastewater and their microbial community structure. In order to characterize the mechanism of abscisic acid wastewater anaerobic treatment, a full UASB reactor was processed and its microbial community struc-

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ture and diversity were studied.

MATERIALS AND METHODS

Wastewater introduction and granular sludge reactor

The wastewater was supplied by the abscisic acid production company. Ethyl acetate, petroleum ether and residua of fermentation were the dominant pollutants. The characteristics of wastewater were as follow: soluble COD 162,763 ± 1000 mg/L; NH_3-N 2,454 ± 100 mg/L; and pH 0.6 to 0.8.

A 20 L UASB reactor was inoculated with anaerobic sludge from a full-scale USAB reactor treating abscisic acid wastewater (Pereboom et al., 1994). The reactor was maintained under mesophilic conditions by circulating water at 33 to 35°C through the outer water jacket of the double-layer Plexiglas reactor. To start up the bioreactor, raw anaerobic sludge form a local sewage plants was used as seed, abscisic acid wastewater was injected after adjusting to neutral, and supplemented with sodium lactate (5 g/L), K_2HPO_4 (100 mg/L) and trace elements (1 mL/L), the hydrolic retention time (HRT) was set at 5 days. After one years' operation, the anaerobic granular sludge was used as inoculums to inoculate a 10 L UASB reactor treating abscisic acid wastewater. The average soluble COD feeding into the 10 L reactor was 8000 mg/L and gradually increased to 15,000 mg/L at day 180. The HRT was controlled at 3.5 days. A further 60 days of operation was processed, after a stable removal efficiency of COD and NH_3-N, while the microbial community and diversity of the reactor was analyzed.

Sample collection, DNA extraction and microbial community analysis

2 g (fresh weight) sludge sample was taken from the reactor, centrifuged at 10,000 rpm for 10 min, and the precipitation was collected. DNA of microbial biomass was extracted by Soil DNA Fast Extraction Kit (Bioteke, China). Nucleic acids were stored at -20°C.

Construction of 16S rRNA gene libraries

The DNA was used to construct the clone libraries of bacteria and archaea. 16S rRNA was amplified by PCR using bacterial (27f and 1492r) or archaeal (46f and 1072r) specific primers. The reaction mixture (50 µl) contained 1.25 U Taq DNA polymerase (Takara, Japan), 5 µl of 10 × buffer, 1.5 mM $MgCl_2$, 200 µM each dNTP, 0.5 µM of each primer, and 10 ng of genomic DNA. The amplification program consisted of a 2 min denaturing step at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 60 s at 72°C, and finally 10 min of extension at 72°C. PCR amplified products were purified and cloned with a pMD19-T vector kit (Takara, Japan), then transformed into *E. coli* DH5α for sequencing analysis.

16S rRNA gene sequences and phylogenetic analysis

Sequence analysis and phylogenetic trees construction were performed using the software of ClustalX and MEGA 4.1. The similarity of sequences than 97% was regarded as one phylotype. A total number of 35 partial 16S rRNA sequences had been deposited in the GenBank sequence database with accession numbers JX000026 to JX000060.

RESULTS

Reactor performance

The overall removal efficiency of COD and NH_3-N in the 10 L UASB reactor during the course of 240 days operation is shown in Figure 1. After the reactor was stable, the removal efficiency of COD and NH_3-N were approximately 85 to 90 and 90 to 93% respectively and consistently.

Phylogenetic affiliation of bacterial 16S rRNA gene sequences

161 clones were sequenced and categorized on the basis of their sequence similarity (97% identity). 30 phylotypes were found, among which 13 phylotypes had high levels of similarities with their closest counterparts in public databases, and 17 sequence types showed less than 97% sequence similarity to their nearest database entries (Figure 2). The result suggested that most of them may belong to hitherto unknown phylotypes (Stackebrandt and Goebel, 1994). The dominant phyla in the wastewater treating equipment were representatives of the following divisions: *Firmicutes* (*Clostridia* and *Bacilli*), *Proteobacteria* (*Alpha-, Beta-, Gamma-* and *Epsilon-*) and *Bacteroidetes*. The phylotypes within these groups are shown in Table 1.

25 clones were affiliated with the genus *Thauera*. *Thauera* spp. has been reported as deniftrifying bacteria in anaerobic wastewater treatment processes (Tarlera and Denner, 2003) and can be abundant phenol degrading members of reactor communities (Manefield et al., 2002). 22 clones belonged to the *Clostridium* and *Enterococcus* respectively. *Clostridium* are obligate anaerobic microorganisms, fermenting a wide variety of carbon sources and produced ethanol, hydrogen and volatile fatty acids such as formic, acetic and lactic acids. *Enterococcus* is a facultative anaerobe and lactic acid bacteria, and can be tolerant of a high concentration of sodium chloride (Gilmore et al., 2002). *Morrella*, *Citrobacter*, and *Planococcus* are the other main genus of the bioreactor. *Citrobacter* can use citrate, fermenting lactose and malonate (Lipsky et al., 1980). The genus of *Morrella* is a thermophilic and homoacetogenic bacterium. *Planococcus* (Engelhardt et al., 2001) can use alkanes of crude oil as carbon-source; *Eubacterium* can ferment demethoxylates O-methoxylated aromatic acids to volatile fatty acids (Mountfort et al., 1988).

Phylogenetic affiliation of archaeal 16S rRNA gene sequences

83 archaeal clones were obtained and sequenced. They were categorized on the basis of their sequence similarity (97% identity), 5 phylotypes were found (Table 2 and Figure 3).

Comparative analysis of the retrieved sequences showed that all sequences were representatives of *Methanobacteria*, phylogenetically related to representatives of the genera *Methanospirillum*, *Methanocorpusculum* and *Methanosphaerula*. All of them belonged to hydrogenotrophic methanogen.

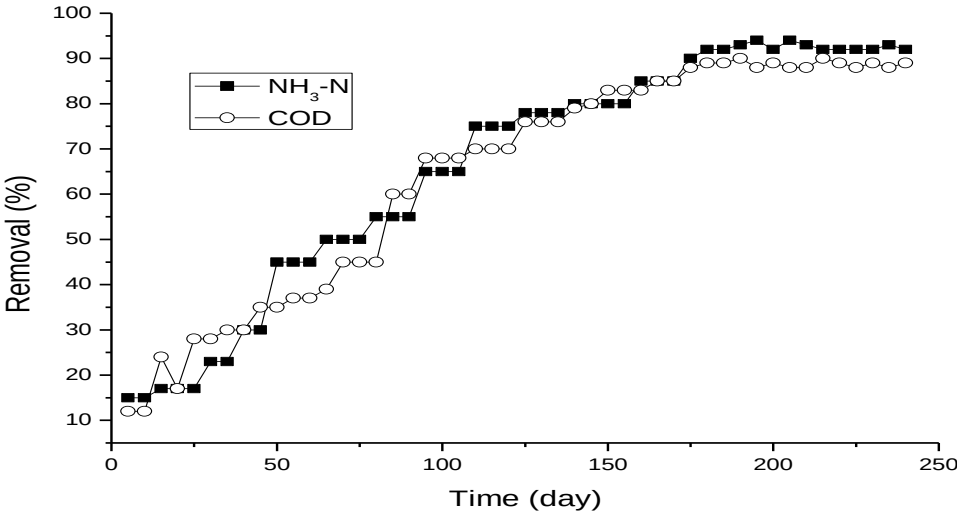

Figure 1. COD and NH_3-N reduction profile of the 10 L UASB reactor.

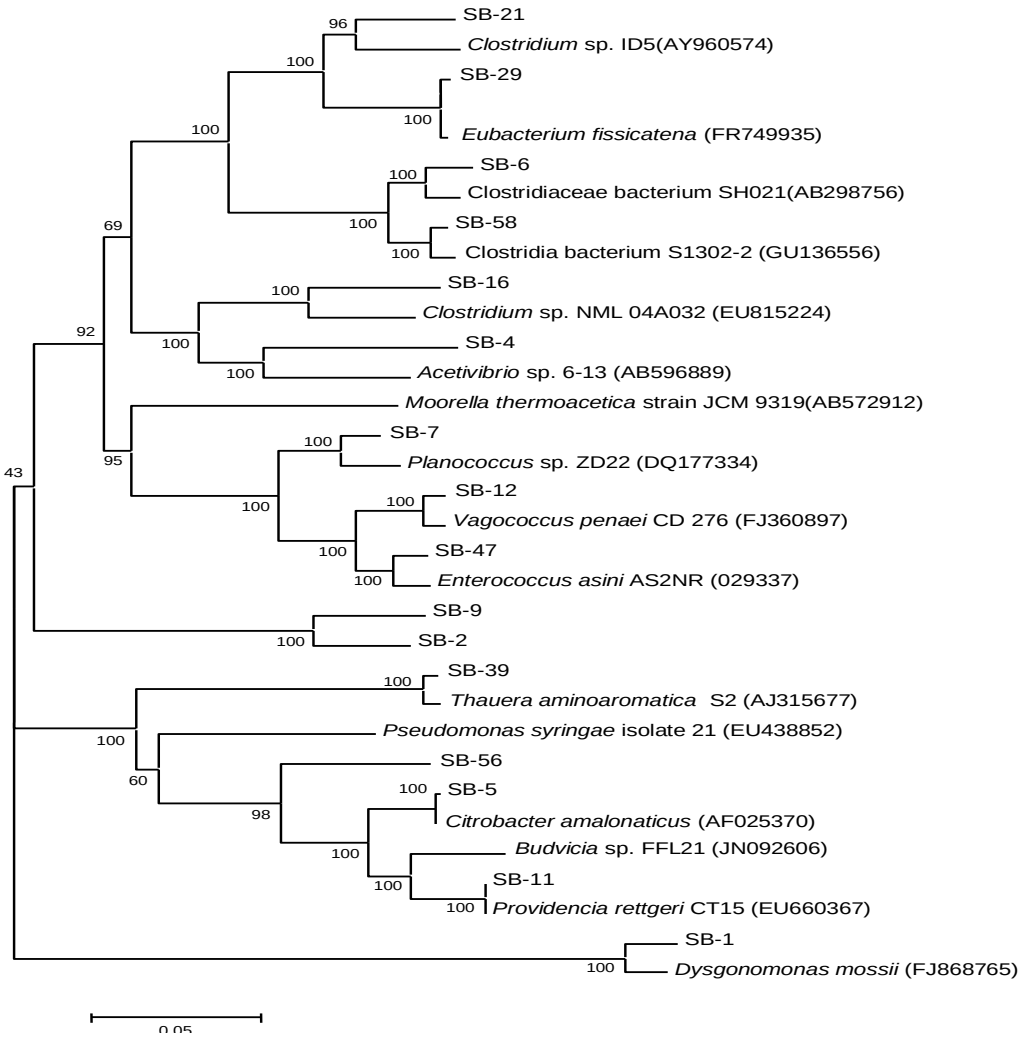

Figure 2. Phylogenetic tree of the 16S rRNA gene phylotypes of the bacterial (SB) sequence types and closely related sequences from the GenBank database. Only the major clades of clones (≥2) were used to construct the phylogenetic tree.

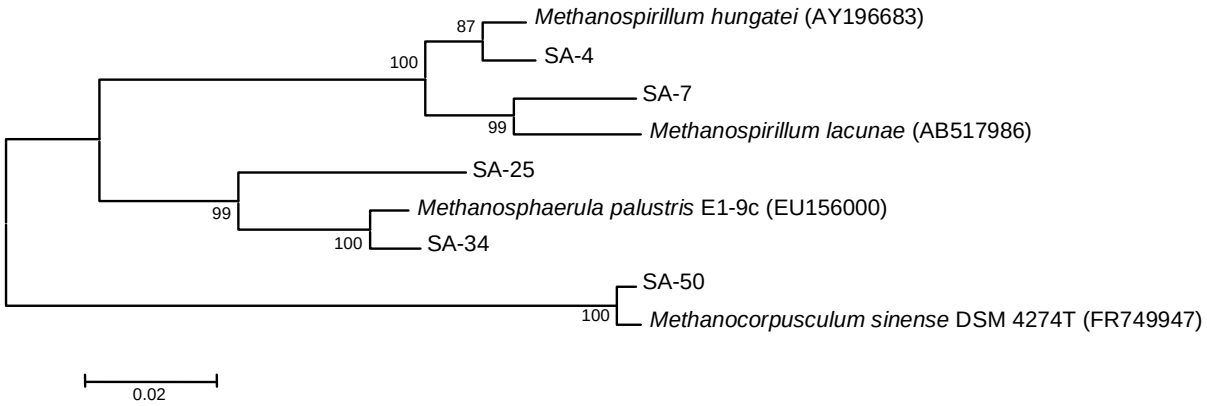

Figure 3. Phylogenetic tree of the 16S rRNA gene phylotypes of the archaeal (SA) sequence types and closely related sequences from the GenBank database.

Table 1. Distribution of dominant sequence types from the bacterial 16S rRNA gene libraries.

Domain, subclass	Type sequence	Closest cultivated species or sequences	Similarity (%)	Number of clones (%)
Clostridia	SB-21	*Clostridium* sp. ID5 (AY960574)	92	8 (5.0)
	SB-25	*Clostridium propionicum* JCM 1430 (AB649276)	90	1 (0.6)
	SB-16	*Clostridium* sp. NML 04A032 (EU815224)	91	13 (8.1)
	SB-29	*Eubacterium fissicatena* DSM 3598T (FR749935)	99	9 (5.6)
	SB-46	*Ruminococcus* sp CE2 (AY960567)	87	1 (0.6)
	SB-10	*Acidaminococcus fermentans* DSM20731 (CP00189)	96	1 (0.6)
	SB-9	*Moorella thermoacetica* JCM 9319 (AB572912)	82	15 (9.3)
	SB-6	*Clostridiaceae bacterium* SH021 (AB298756)	97	2 (1.2)
	SB-38	*Clostridiaceae bacterium* SN021 (AB298755)	93	1 (0.6)
	SB-58	*Clostridia bacterium* S130(2)-2 (GU136556)	99	2 (1.2)
	SB-4	*Acetivibrio* sp. 6-13 (AB596889)	98	2 (1.2)
	SB-10	*Acidaminococcus* sp. DJF_RP55 (EU728758)	96	1 (0.6)
Bacilli	SB-7	*Planococcus* sp. ZD22 (DQ177334)	94	12 (7.5)
	SB-12	*Vagococcus penaei* CD 276 (FJ360897)	98	18 (11.1)
	SB-41	*Vagococcus carniphilus* 12J (AY669387)	91	1 (0.6)
	SB-47	*Enterococcus asini* AS2 (Y11621)	97	19 (11.8)
	SB-13	*Enterococcus gallinarum* 22B (EF025908)	92	1 (0.6)
	SB-40	*Enterococcus* sp. CSL 7544-3 (GU905013)	99	1 (0.6)
Gammaproteobacteria	SB-28	*Proteus* sp. K107 (EU710747)	99	1 (0.6)
	SB-48	*Methylocaldum* sp. 05J-I-7 (EU275146）	90	1 (0.6)
	SB-20	*Proteus* sp. L2 (EF426446)	99	1 (0.6)
	SB-15	*Enterobacter* sp. R4M-B (GQ478257)	93	1 (0.6)
	SB-5	*Citrobacter amalonaticus* (AF025370)	99	14 (8.7)
	SB-11	*Providencia rettgeri* CT15 (EU660367)	99	2 (1.2)
	SB-56	*Budvicia* sp. FFL21 (JN092606)	95	2 (1.2)
Alphaproteobacteria	SB-2	*Pseudomonas syringae* 21(EU438852)	85	2 (1.2)
Betaproteobacteria	SB-39	*Thauera aminoaromatica* S2 (AJ315677)	98	25 (15.5)
Epsilonproteobacteria	SB-51	*Sulfurospirillum deleyianum* DSM 6946 (Y13761)	97	1 (0.6)
	SB-62	Uncultured clone BXHA95 (Q480041)	95	1 (0.6)
Bacteroidetes	SB-1	*Dysgonomonas mossii* JCM 16699 (FJ868765)	96	2 (1.2)

Table 2. Distribution of dominant sequence types from the archaeal 16S rRNA gene libraries.

Domain, subclass	Type sequence	Closest cultivated species or sequences	Similarity (%)	Number of clones (%)
Methanobacteria	SA-4	*Methanospirillum hungatei* JF-1 (AY196683)	98	28 (34)
	SA-7	*Methanospirillum lacunae* Ki8-1 (AB517986)	98	3 (4)
	SA-50	*Methanocorpusculum sinense* DSM 4274T (FR749947)	99	31 (37)
	SA-34	*Methanosphaerula palustris* E1-9c (EU156000)	98	19 (23)
	SA-25	*Methanosphaerula palustris* E1-9c (EU156000)	93	2 (2)

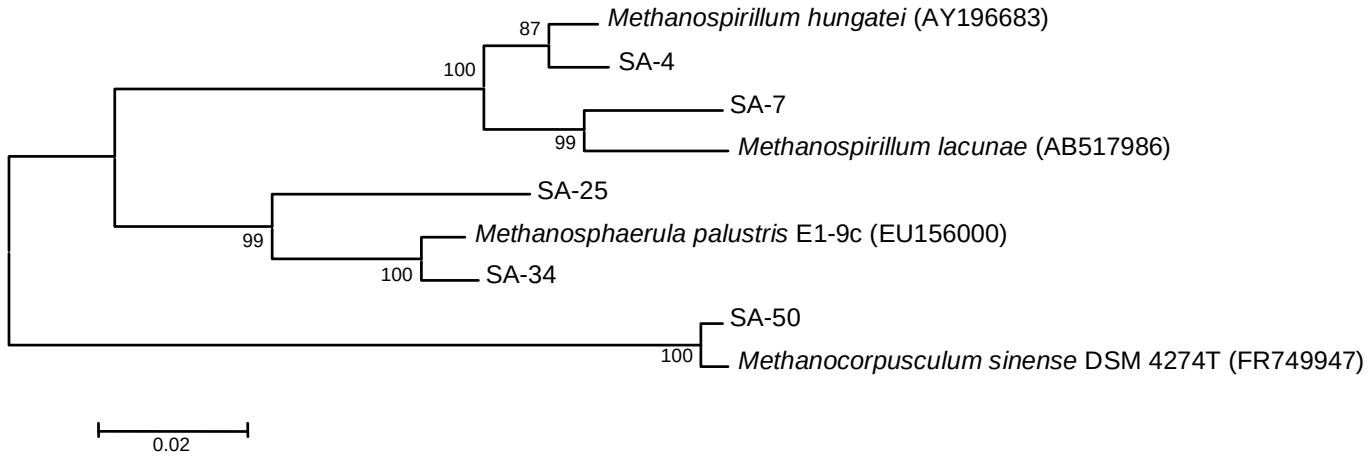

Figure 3. Phylogenetic tree of the 16S rRNA gene phylotypes of the archaeal (SA) sequence types and closely related sequences from the GenBank database.

DISCUSSION

Currently, the industrial abscisi acid is produced by microbial fermentation; only one company produces it in China. The residua of fermentation and extraction solvent are the dominant pollutants of abscisic acid wastewater. The results of the UASB reactor show that anaerobic treatment system is suitable for treating the abscisic acid wastewater, wiping off 85 to 90% of COD and 90 to 93% of NH_3-N respectively.

To determine the microbial diversity and community, the molecular analysis of 16S rRNA sequence analysis was used. 244 colonies were detected, involving in 30 bacterial and 5 archaeal phylotypes. We concluded that the number of colonies might not be sufficient to exhaustively sample microbial diversity, however, it was successful in characterizing a large fraction of the biotreatment system. The phyla *Firmicutes* and *Proteobacteria* dominated the bacterial clone library, similar with Satoh's report (Satoh et al., 2007); while different from Chan's (Chan et al., 2001) study. In Chan's report, more bacterial clones were detected, such as *Acidobacteria, Bacteroidetes, Nitrospira* and *Chloroflexi*. The predominant clones in the archaeal clone library were affiliated with *Methanospirillum*, *Methanocorpusculu*m and *Methanosphaerula*, which was different from the *Methanosaeta* of Chan and Satoh's study. The microbial community may be due to components of abscisic acid wastewater, with complex residue of fermentation and organic extraction solvent.

Fermentative residue and residue of extraction agent are the main components of wastewater, which may the main reason to produce the complex microbial community and diversity of our reactor. UASB is one effective anaerobic digestion for high-concentration organic wastewater (Madsen et al., 2011). The microbial community suggested that the degradation of abscisi acid wastewater to acetate and hydrogen was performed by a fermentative population, and the methanogenesis step was mainly performed by hydrogenotrophic methanogens. And, the acetate should be converted into methane by syntrophic acetate oxidation, since no aceticlastic methanogen was found. The data from this study indicated that 17 phylotypes were less than 97% sequence similarity to any database entry, some of which should be acetate-oxidizing bacteria.

In this research, we found and studied a microbial community structure and diversity in one stable and efficient abscisi acid wastewater bioreactor, which might be very important for the future large-scale process.

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