Study of microbial community structures in UASB sludge treating municipal wastewater by denaturing gradient gel electrophoresis of 16S rDNA

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Abstract The structures of microbial communities in lab-scale upflow anaerobic sludge blanket (UASB) reactors for treating municipal wastewater with different ratios of $COD_{soluble}/COD_{total}$ were studied using denaturing gradient gel electrophoresis (DGGE) of 16S rRNA genes. The microbial structure of the inoculum sludge obtained from a full-scale UASB reactor of treating potato processing wastewater was compared with the structures of sludges collected from three lab-scale UASB reactors after eight months feeding with raw municipal wastewater, with CEPS (chemically enhanced primary sedimentation) pretreated municipal wastewater, and with a synthetic municipal sewage, respectively. Computer-aided numerical analysis of the DGGE fingerprints showed that the bacterial community underwent major changes. The sludges for treating raw and CEPS pretreated wastewater had very similar bacterial and archaeal communities (82% and 96% similarity) but were different from that for treating the synthetic sewage. Hence, despite similar % COD in the particulate form in the synthetic and the real wastewater, the two wastewaters were selected for different microbial communities. Prominent DGGE bands of Bacteria and Archaea were purified and sequenced. The 16S rRNA gene sequences of the dominant archaeal bands found in the inoculum, and UASB sludge fed with raw sewage, CEPS pretreated wastewater, and synthetic sewage were closely associated with *Methanosaeta concilii.* In the UASB sludge fed with synthetic sewage, another dominant band associated with an uncultured archaeon 39-2 was found together with *M. concilii.*

Keywords: **UASB, municipal wastewater, anaerobic sludge, community analysis, DGGE.**

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The upflow anaerobic sludge blanket (UASB) technology has been widely used for industrial wastewater treatment in the last two decades [1-3]. Yet, its application to municipal wastewater treatment is still a main challenge[4 − 6]. Municipal wastewater is a low-strength complex type of wastewater, characterized by (i) low COD concentrations (200—700mg/L), (ii) high fractions of suspended solids (50% of the COD_t), (iii) relatively low temperatures (4—20℃) in temperate region, and (iv) strong fluctuations in hydraulic and organic loading rates[3, 7, 8]. The high concentration of suspended solids is a major problem when using UASB technology. Particulate material is hydrolysed very slowly, and tends to accumulate in the

reactor, leading to low COD conversion efficiencies and extra sludge to be removed. Recently, pre-treatment with chemically enhanced primary sedimentation (CEPS) to remove the suspended solid was suggested by Kalogo and Verstraete[7]. Lab-scale studies have shown that the use of $FeCl_3$ or a water extract of Moringa oleifera seeds (WEMOS), a natural coagulant, can remove a significant fraction of the suspended solid from raw sewage[8, 9]. The combined CEPS-UASB approach increases the possibility of treating municipal wastewater anaerobically. Lab-scale studies showed that the sedimentology of the sludge bed in an UASB reactor treating low-strength wastewater was quite different from the sludge for treating high-strength industrial wastewater [9]. The original granular sludge experienced serious instability and disintegration, leading to a much finer grain assemblage. The UASB design relies on the granulation of bacterial biomass with enhanced settling properties, and the formation of microstructure of UASB granules depend on the physicochemical condition and feeding composition [10-15]. For a better design and control of the anaerobic systems for treating municipal sewage, a detailed knowledge of the microbial communities involved in the processes is important. Till now, no microbial communities in a UASB reactor for treating municipal wastewater have been described.

For testing purposes, different synthetic wastewaters are commonly used in lab-scale studies. Most of them are not very representative of actual domestic sewage in terms of composition and important parameters [16-19]. Recently, a new kind of synthetic sewage, SYNTHES [9] was proposed, which mimics real wastewater well in some important parameters, particularly the ratio of $COD_{soluble}/COD_{total}$ and COD/ N/ P. In this study, we evaluate whether or not the synthetic sewage can simulate normal sewage in terms of microbial community structures of the UASB reactor.

During the last decade, advanced molecular methods based on direct PCR amplification and analysis of ribosomal RNA genes were developed providing a rapid overview of complex microbial communities. The analysis of amplified 16S rRNA genes by denaturing gradient gel electrophoresis (DGGE) has been frequently used to examine the microbial structures of environmental samples and to monitor changes in microbial communities [20-23], including some UASB granular sludges[24, 25]. In this study, microbial communities in the sludges of lab-scale UASB reactors for treating raw, CEPS pretreated, and synthetic wastewater were analyzed with PCR-DGGE methods using bacterial and archaeal primer sets.

1 Materials and methods

1.1 Anaerobic digesters and inoculum

Three 2.1-L mesophilic (33℃) lab-scale glass UASB reactors were seeded with 250 mL granular sludge from a full-scale UASB reactor (30—34℃) treating potato processing wastewater (PRIMEUR, Belgium) and operated for 8 months before sampling. The influents and the performance of the three reactors were as follows:

Reactor 1 (Raw sewage) was fed with raw domestic sewage from the city of Gent, Belgium. The sewage was taken from a 250000 p.e. domestic sewage treatment plant after the primary grit chamber (Ossemeersen, Belgium). Some of the characteristics are given in table 1. The average removal efficiency in term of total COD was 84% at a hydraulic retention time (HRT) of 10 hours and a loading rate of 1.2 g COD/L.d.

Reactor 2 (CEPS) was fed with the same raw sewage but after chemically enhanced primary sedimentation (CEPS). Some characteristics of this wastewater are given in table 1. The pretreatment comprised of chemical precipitation with $FeCl_3$ and an anionic polymer (Allied colloids E 10, Bradford, England) dosed at 50 mg/L and 10 mg/L, respectively. After pretreatment, about 80% COD_t, 55% NH_4^+, and 90% PO_4-P were removed from the sewage [26]. This resulted in a very low UASB influent COD concentration of approximately 140mg/L, which consisted mainly of soluble COD. The reactor achieved 60% removal of residual COD_t at an HRT of 10 hours and a volumetric loading rate of 0.4 g COD/L.d.

Reactor 3 (SYNTHES) was fed with synthetic sewage, for which the composition is shown in table 1.

Table 1 Overview of some parameters of the potato processing wastewater on which the inoculum sludge was grown, the raw domestic sewage, raw domestic sewage after CEPS, and the synthetic sewage. The composition of the synthetic wastewater was described by Aiyuk, S[9]

Treatments	Potato processing wastewater	Raw sewage	CEPS pretreated wastewater	Synthetic sewage
COD_t (mg/L)	9000	522 ± 300	140 ± 50	570 ± 50
% soluble COD	46	30 ± 11	82 ± 5	30± 12
COD_t / N / P	100/25/1	65/5/1	200/43/1	30/3/1

Table 2 Primers used in this study

Primer	Target	Sequence	Reference
P63f	Bacteria	5'CAGGCCTAACACATGCAAGTC	[50]
P1378r	Bacteria	5'CGGTGTGTACAAGGCCCGGGAACG	[51]
PRBA338f-GC	Bacteria	5'ACTCCTACGGGAGGCAGCAG	[52]
PRUN518r	Universal	5'ATTACCGCGGCTGCTGG	[29]
PRA46f	Archaea	5'(C/T)TAAGCCATGC(G/A)AGT	[53]
PREA1100r	Archaea	5'(T/C)GGGTCTCGCTCGTT(G/A)CC	[53]
PARCH340f-GC	Archaea	5'CCCTACGGGG(C/T)GCA(G/C)CAG	[53]
ARCH519r	Archaea	5'TTACCGCGGC(G/T)GCTG	[53]
GC-clamp*		5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG	[29]

The GC clamp was attached to the 5' end of the PRBA 338f and PARCH340f primers.

The $COD_{soluble}$ /COD_{total} ratio of the synthetic sewage simulated that of the raw sewage. Average removal efficiency of total COD was 89% at an HRT of 10 hours and a loading rate of 1.2 g COD/L.d.

The performance of the reactors has been described in detail by Aiyuk and Verstraete[9].

Four mL of sludge from the middle of the three reactors and the inoculum sludge were collected when the system got stabler after 8 months of operation, and stored at –20℃ before analysis.

1.2 DNA extraction, PCR-DGGE analysis

Two mL of each sludge sample were ground with a mortar on ice with 4 mL 10 mM Tris-HCl (pH 8) followed by DNA extraction and purification[27]. 16S rDNA genes of Bacteria and Archaea from the purified DNA were amplified by nested-PCR. The primers used in this study are listed in table 2.

All the PCR amplifications were performed in a 2400 thermal cycler (Perkin-Elmer, Norwalk, Conn.), using the following program: 94℃ for 5 min; 30 cycles of denaturation at 95℃ for 1 min, annealing at 53 ℃ for 1 min, and extension at 72℃ for 2 min; and a single final extension at 72℃ for 10 min. In the first round PCR amplification for archaeal DNA, 5% (wt/vol) of acetamide was added to the PCR mixture to facilitate denaturation[28], but in the second round PCR, acetamide was not included. The GC clamps[29] were attached to the 5' end of the second-round PCR forward primers for both the Bacteria and Archaea (PRBA338f and PARCH340f). The presence of PCR products was examined on ethidium bromide-stained agarose gels, and the second round PCR products were used for DGGE analysis.

DGGE was performed with Bio-Rad D Gene System (Bio-Rad, Hercules, CA, USA). Ten μL of second-round PCR products were loaded onto 8% (wt/vol) polyacrylamide gels in 1×TAE (20 mM Tris, 10 mM acetate, and 0.5 mM EDTA pH 7.4). The 8% (wt/vol) polyacrylamide gels were made by denaturing gradients ranging from 45% to 70% for bacterial DNA fragments, and from 55% to 70% for archaeal DNA fragments (where 100% denaturant contains 7 M urea and 40% formamide). The electrophoresis was run at 60℃ for 16 hours at 38 V. After electrophoresis, the gels were stained for 20 min in SYBR Green I nucleic acid gel stain (1:10,000 dilution; Molecular Probes, Eugene, Oreg.). The stained gels were immediately photographed on a UV transillumination table with a Video Camera Module (Vibert Lourmat, Marne-la Vallé, France). The DGGE patterns were clustered using the Bionumerics software 2.0 (Applied Maths,

Kortrijk Belgium). Then, a matrix of similarities between the densitometric curves of the band patterns was calculated.

1.3 Sequence analysis of DGGE bands

Prominent DGGE bands were excised for nucleotide sequence determination. For each band selected, only the middle portion was excised with a sterile razor, and slices were placed in PCR tubes containing 40 μL of PCR water. The DNA was allowed to diffuse into the water at 4℃ overnight. Five μL of the eluate was used as template DNA in a PCR with the primers of the second-round PCR and the same conditions as described above. Following amplification, the PCR products were analyzed by DGGE to confirm their electrophoretic mobility relative to the fragment from which they were excised.

The PCR products were then sequenced by IIT Biotech-Bioservice (Bielefeld, Germany). The partial 16S rRNA sequences obtained were compared with those in the GenBank by means of National Centre for Biotechnology Information (NCBI) Blast program.

2 Results

The community structure fingerprints of the bacterial and archaeal DNA populations in the four sludges samples were analyzed by DGGE. DNA extraction and PCR-DGGE analyses were performed in duplicate for each sample and resulted in 96-99% similiarities (data not shown). Therefore only one DGGE pattern was used per sample for the analysis (fig. 1). The similarity values of the microbial communities were analyzed with Bionumerics Software 2.0. Fig. 1(a) shows that the bacterial community structures changed from the inoculum to the other three sludges. The bacterial community structures in the reactor 3 fed with synthetic sewage showed very low similarity with that of CEPS (48%) and raw sewage (37%), but the bacterial community structures in the reactors 1 and 2 fed with raw sewage and CEPS were quite similar (82%). Moreover, Fig. 1(b) shows that the archaeal community structures in the inoculum were very similar with raw sewage (93%) and CEPS (97%), but that the synthetic sewage resulted in a different pattern (55%), suggesting that the microbial community structure in the reactor fed with raw sewage or CEPS cannot be simulated by synthetic sewage. Since the microbial community structures were very similar in the reactors fed with raw sewage and CEPS, suspended solid apparently had little influence on the prokaryotic community. The DGGE fingerprints also indicate that about 30 numerically dominant bacterial populations and 5—7 dominant archaeal populations were present in each of the UASB sludges. Thus, the bacterial population profiles are much more complex than those of the archaeal populations.

The prominent bacterial and archaeal DGGE bands were purified and sequenced in order to further determine the composition of the microbial communities. Nine dominant bacterial populations corresponding to DGGE bands B1-B9 from the inoculum were identified. Two other bands (B10 and B11), which distinctly appeared in the SYNTHES and CEPS, were also identified. Band B1, which only existed in the inoculum and disappeared in the other three sludges was phylogenetically most closely related (87% similarity) to an uncultured bacterium clone UASB_brew_B36, reported to be found in granular sludge treating brewery wastewater [30]. The sequence of band B2 was most similar (98 % similarity) to the 16S rRNA gene of an uncultured bacterium clone F13.41 [31]. B3, which was more prominent in the inoculum than the other three, was most closely affiliated (89% similarity) with an uncultured bacterium SHA-25 16S rRNA gene [32]. Band B4 was also found in the other three sludges affiliated (99%) with the uncultured bacterium DCE29 [33]. Band B5, affiliated (98%) with uncultured bacterium a2b029 [34], was not observed in the SYNTHES sample. Band B6, affiliated (95%) with the uncultured bacterium clone TDC-S1:14, capable of degrading tetrachloroethene[35], was found in the four sludges but was more dominated in the raw sewage and CEPS. Band B7, affiliated (99%) with uncultured bacterium mle1-42, was not included in the sludge of SYNTHES, and was reported to be found in bioreactors treating pharmaceutical wastewater [36]. Band B8 and band B9 were affiliated (92% and 95%) with *Desulforhabdus amnigena*, iso

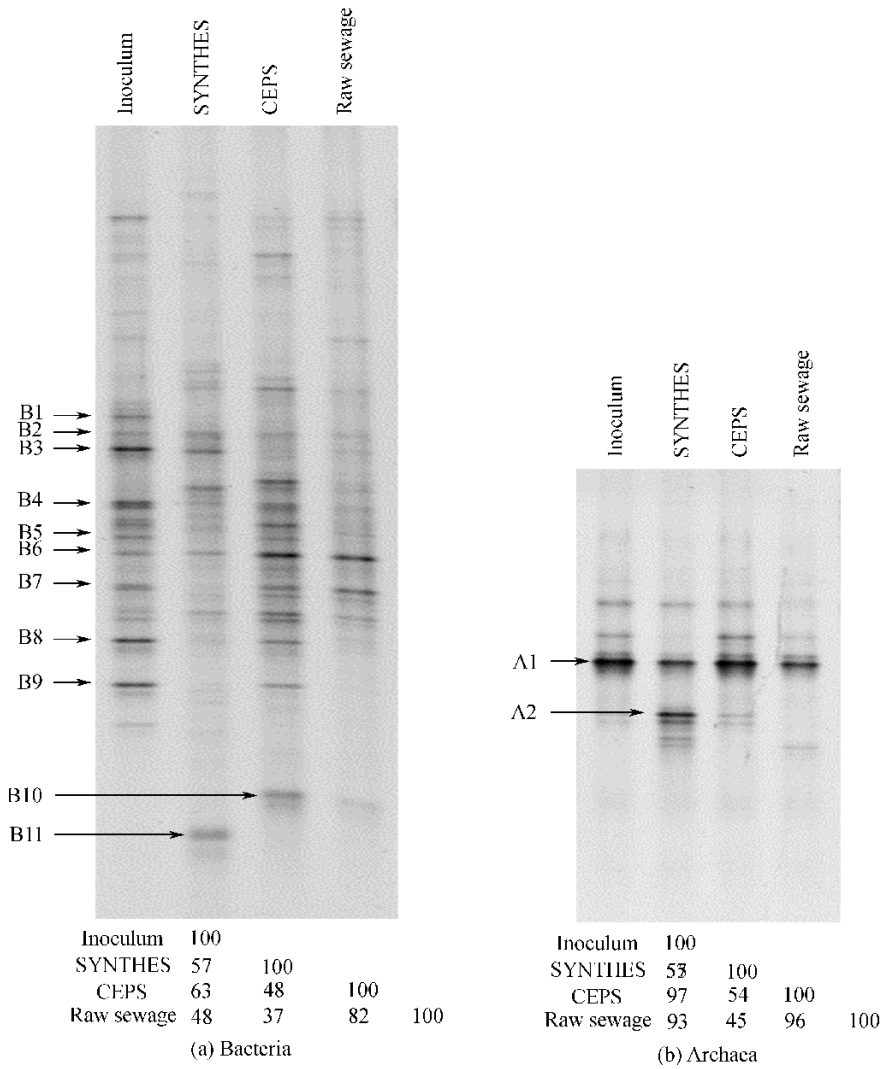

Fig. 1. DGGE fingerprints and similarity values of Bacterial and Archaeal 16S rDNA populations.

lated from anaerobic granular sludge[37], and an uncultured bacterium clone TDC-S5:21[20]. These two bands were dominated only in the sludge of the inoculum and CEPS. Band B10 only appeared in raw sewage and CEPS, affiliated (97%) with an unidentified bacterium clone Qui4P2-29, which was found in sediments of water reservoirs [38]. Band B11, found only in SYNTHES, was affiliated (89%) with an unidentified bacterium wb1_C17, reported by Holmes[39]. These populations underwent quite different shifts after 8 months adaptation to the new influents, with some becoming more dominant and some less dominant, even though invisible. In addition some new populations were detected. While it is thus possible to use some DGGE bands as markers to monitor the performance of UASB reactors, further studies are needed to understand the importance of each of these populations in the performance of the reactor..

The dominant archaeal DGGE band A1 found in the four sludges was closely affiliated (98% similarity) with *Methanosaeta concilii*[40]. It was also similar to the uncultured *euryarchaeote EHB 109*[41] and the uncultured *archaeon MUAHR 2*[42] with the same similarity. The partial sequence retrieved (~200 bp) was not sufficient to resolve the phylogenetic affiliation with closely related sequence. In the SYNTHES, an-

other dominant band A2 was identified as an uncultured *archaeon 39-2* 16S rRNA gene with 98% similarity value [43], which also existed at very low level in the inoculum and CEPS.

3 Discussion

In this study, the microbial diversities and community structures within the inoculum, and anaerobic sludges grown for eight months on raw domestic sewage, CEPS pretreated domestic sewage, and SYNTHES were estimated. The microbial community structures of bacteria and archaea are similar for the raw sewage and CEPS sludges. This indicates that the presence of suspended solids in the wastewater is not a predominant factor in selection of microbial community composition and that the pretreatment technology applied had very little effect on the microbial community in the sludge. The components of soluble COD are probably the major factor determining the bacterial populations. This aspect desires further confirmation.

Many of the synthetic sewage found so far in the literature have not been very representative of actual domestic sewage. A new type of synthetic sewage, SYNTHES, was proposed recently [9], which mimics real wastewater well in some important parameters. It is interesting to know that the bacterial and archaeal community structures in the reactor fed with SYNTHES were quite different relative to those grown on real raw sewage. This might be due to the fact that i) real domestic sewage provides a constant influx of bacteria and spores which can act as an inoculum for the anaerobic sludge, and/or ii) the different kinds of substrates in the real sewage select for different microbial populations. This gives us a hint that the testing using synthetic wastewater should be evaluated with caution. If synthetic wastewater only affects community composition but not reactor function, it may still be OK to perform several functional tests with such wastewater. But, if the wastewater contains complex and difficult to be degraded into compositions, the microbial community may be important for the functioning of the reactors.

A nested PCR approach was used to increase sensitivity, which allows visualizing those populations that are present in lower numbers and may play an important role in the anaerobic digestion process. More complex DGGE fingerprints of the bacterial community were obtained compared to a previous study with granular sludge used to treat brewery wastewater [25]. Although the number and intensity of bands in a DGGE gel do not necessarily reflect the number and abundance of the corresponding species within the microbial community [44, 45], the nested-PCR-DGGE fingerprints provide indications that the bacterial community structures in the UASB sludge are quite complex and difficult to evaluate. Design and application of primers which allow the amplification of the 16S rDNA fragments of certain bacterial groups from mixed communities will be needed to dissect complex communities and specifically track the shifts of certain groups. On the other hand, metabolically active bacteria contain more rRNA than resting or starved cells [46, 47]. Hence, the analysis of 16S rRNA reverse transcription PCR (RT-PCR) products will provide more accurate method to estimate the shifts of active Bacteria and Archaea.

Of the eleven bacterial DGGE bands, only B8 was affiliated (92% similarity) to a previously described, cultured bacterium, i.e. *Desulforhabdus amnigen*[37]. All other bands corresponded to unknown or uncultured strains. The most predominant bacterial partial 16S rDNA sequences found in the inoculum sludge did not match any of those found in sludge treating winery effluent[48], brewery effluent[25], and a mixture of sucrose, propionate, and acetate[24], likely due to the different composition of the wastewater. Some populations became less dominant or not detectable, and some became more dominant after 8 months adaptation to the new wastewater. Also, some new populations were detected. The predominance of *M. concilii* in UASB reactors has been reported frequently [25, 12, 49, 24]. Our study indicates that *Methanosaeta* is also a major methanogen in UASB sludge treating municipal wastewater.

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