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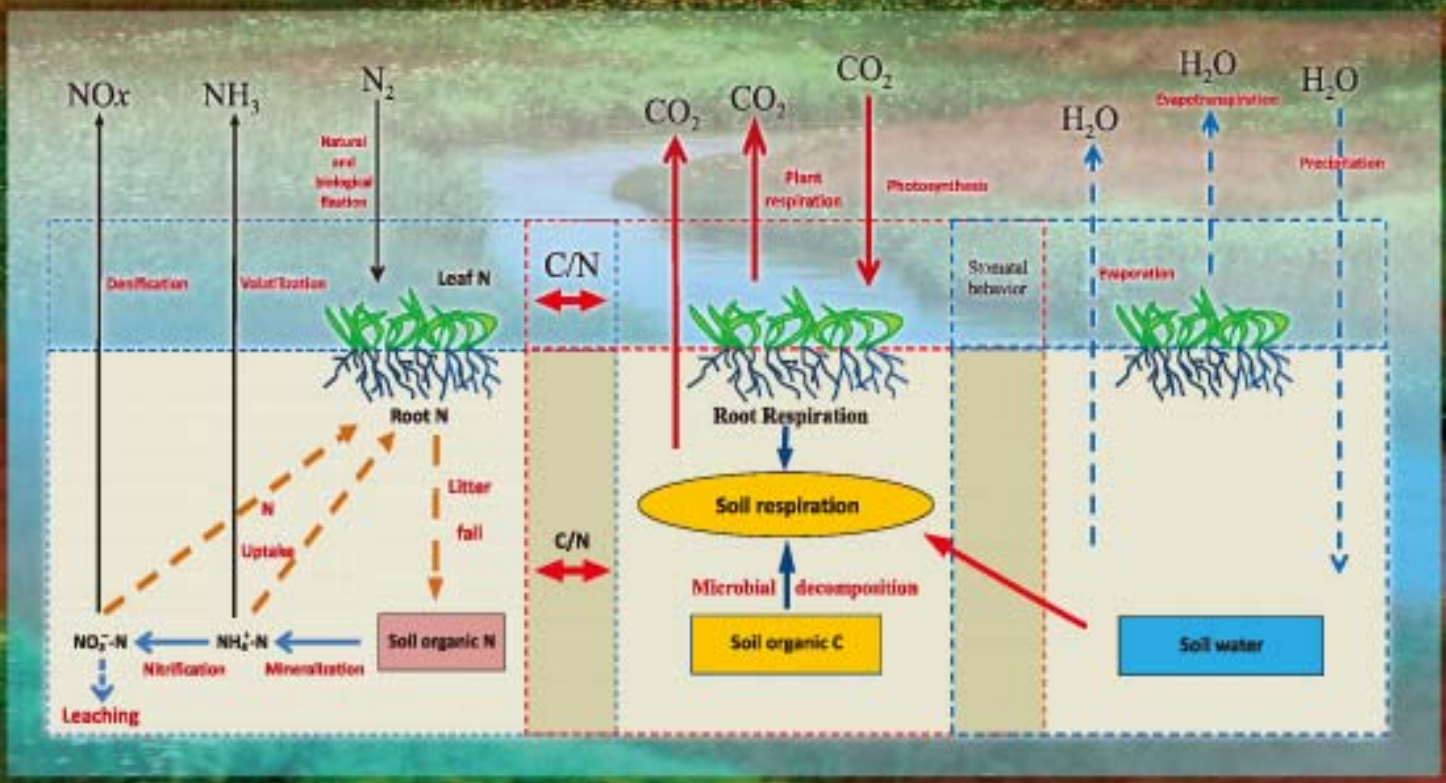

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Effect of a high strength chemical industry wastewater on microbial community dynamics and mesophilic methane generation

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ABSTRACT

A high strength chemical industry wastewater was assessed for its impact on anaerobic microbial community dynamics and consequently mesophilic methane generation. Cumulative methane production was 251 mL/g total chemical oxygen demand removed at standard temperature and pressure at the end of 30 days experimental period with a highest recorded methane percentage of 80.6% of total biogas volume. Volatile fatty acids (VFAs) analysis revealed that acetic acid was the major intermediate VFAs produced with propionic acid accumulating over the experimental period. Quantitative analysis of microbial communities in the test and control groups with quantitative real time polymerase chain reaction highlighted that in the test group, Eubacteria (96.3%) was dominant in comparison with methanogens (3.7%). The latter were dominated by Methanomicrobiales and Methanobacteriales while Methanosarcinaceae in test groups increased over the experimental period, reaching a maximum on day 30. Denaturing gradient gel electrophoresis profile was performed, targeting the 16S rRNA gene of Eubacteria and Archaea, with the DNA samples extracted at 3 different time points from the test groups. A phylogenetic tree was constructed for the sequences using the neighborhood joining method. The analysis revealed that the presence of organisms resembling Syntrophomonadaceae could have contributed to increased production of acetic and propionic acid intermediates while decrease of organisms resembling *Pelotomaculum* sp. could have most likely contributed to accumulation of propionic acid. This study suggested that the degradation of organic components within the high strength industrial wastewater is closely linked with the activity of certain niche microbial communities within eubacteria and methanogens.

Introduction

Anaerobic degradation has been used as a high strength organic waste and wastewater treatment process for several decades (Ahring, 2003). The degradation process is complex and includes sub-processes like hydrolysis, acidogenesis and methanogenesis, each of which is delicately balanced with each other for optimum substrate degradation. A consortium of Eubacteria converts organic carbon into acetic acid and/or carbon dioxide, which is then further converted to methane by a group of specialized microbes, the methanogens (Ueno et al., 2001). The con-

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version of organics to methane under anaerobic conditions is reliant on the actions of microbial communities on the substrate. Therefore, it is useful to study the microbial flora and its members which are involved not only in order to improve the overall anaerobic process but also to track process changes which may lead to failure of system (Fernández et al., 1999).

Anaerobic degradation has widespread applications in today's pursuit of renewable energy sources. But before determining its effective potential, it would be better served if a key element of the process, i.e., the ultimate biogas generation potential of a given substrate can be better understood. In the past and up until a few years ago, research was focused more on solving the issue of determining the biogas generation potential. The biochemical methane potential (BMP) test does indeed provide a sufficient base for determining the potential of a particular substrate to generate methane. Past research on the BMP test had often focused on either optimizing the substrate to inoculums ratio (Fernandez et al., 2001; Neves et al., 2004; Raposo et al., 2006) or had used specific substrates (Raposo et al., 2006). Some research has also been dedicated to understanding the various factors which may affect the degradation process like temperature, pH and particle size of substrate (Pabon-Pereira et al., 2012). However, substantial research has not been reported on understanding the microbial community dynamics during the process of a BMP test.

Microbial community shifts occur over a period of time during the anaerobic degradation process with niche members possibly growing to dominate over the other members. However, tracking these changes may be difficult with the standard practices of culturing organisms. This may arise from the niche organism's inability to propagate ex-situ and hence leading to false results. Recent developments in molecular techniques targeting the 16S rRNA gene can possibly provide greater insight into such anaerobic community shifts in response to different process settings (Lee et al., 2009). Monitoring microbial communities in an anaerobic degradation process can possibly provide information for process optimization and system configuration. The quantitative real time polymerase chain reaction or q-PCR test allows for targeting microorganisms in an anaerobic degradation process and so can facilitate tracking of community dynamics and shifts as the process undergoes change (Yu et al., 2005). The changes in microbial community structure can be monitored using denaturing gradient gel electrophoresis (DGGE) in combination with investigation of formation and degradation of certain reaction products. The DGGE technique has proven effective in detecting microbial community shifts and also identifying the phylogenetic affiliates of microbial populations in mixed culture systems (Ueno et al., 2001; Calli et al., 2005). Lee et al. (2008) also elucidated that DGGE and qPCR are ideal techniques to study microbial transitions in batch systems operating with mixed microbial cultures. The phylogenetic analysis would help in the in-depth better understanding of how the communities at the species or strain level react to the presence of a particular substrate. This would prove especially helpful in the study of acidogens, whose community dynamics are relatively unknown.

This article describes a study which used the BMP test coupled with microbial community analysis, to investigate anaerobic degradation process challenged with a high strength chemical industry wastewater at mesophilic (35°C) temperature. Process performance was investigated in terms of chemical oxygen demand (COD) reduction, biogas production, and microbial community responses. Duration of the BMP test in this study was 30 days. Community shifts of individual methanogen families were monitored with the q-PCR technique and DGGE combined with phylogenetic analysis provided the community structure within the systems. The research also provided an insight on impact of wastewater on methane yields with changes in community structure.

1 Materials and methods

1.1 Wastewater characteristics

The chemical industry wastewater was initially profiled in terms of parameters shown in **Table 1**. The wastewater was defined as high strength in terms of its high total chemical oxygen demand (TCOD) and soluble chemical oxygen demand (SCOD) value.

1.2 Seed biomass

Anaerobic sludge was collected from a mesophilic anaerobic digester at a local municipal sewage treatment plant treating primary and secondary sludges. This seed sludge, identified as AnSL, was profiled as shown in **Table 2**.

Table 1 Chemical industry wastewater properties

Parameter	Value
Total chemical oxygen demand (TCOD)	(343.12 ± 3.56) g/L
Soluble chemical oxygen demand (SCOD)	(294.35 ± 2.78) g/L
Volatile fatty acids (VFA's)	(3.6 ± 0.02) g/L
Acetic acid	0.83 g/L
Valeric acid	1.23 g/L
Other organic components present	Glutarate, adipate, and succinate
pH	9.23
Total dissolved solids (TDS)	(9914 ± 46) mg/L
Sodium	44.1 g/L
Colour (visual)	Deep red

Table 2 Properties of the anaerobic seed sludge AnSL

Parameter	Value
TCOD	(23.58 ± 0.11) g/L
SCOD	(4.32 ± 0.17) g/L
Total solids (TS)	(22.40 ± 0.97) g/L
Volatile solids (VS)	(18.53 ± 1.02) g/L
Total suspended solids (TSS)	(19.49 ± 0.73) g/L
Volatile suspended solids (VSS)	(16.64 ± 1.15) g/L
Total VFA*	0.18 g/L

*Only acetic acid detected.

1.3 Pre-experimental phase

AnSL was "degassed" at 35°C in a manner as described in a previous study (Angelidaki et al., 2009). This preparatory stage was performed for 10 days for depletion of residual substrate originally present in the sludge.

1.4 Experimental phase

1.4.1 BMP tests

The test was performed in a manner similar to Angelidaki et al. (2009) but with modifications. Volume of the serum bottle was 150 mL, with 90 mL working volume and 60 mL headspace. A sterile mineral salts medium (MSM) was prepared with the following components (g/L): NH_4Cl 1.5, $KH_2PO_4{\cdot}2H_2O$ 0.8, $NaHCO_3$ 1.5, $MgSO_4{\cdot}7H_2O$ 0.3, NaCl 3.0 and 5 mL trace elements solution. The trace elements solution contains the following components (g/L): $CaCl_2{\cdot}2H_2O$ 1.6, H_3BO_3 0.38, $CoCl_2{\cdot}6H_2O$ 0.20, $(NH_4)_2MoO_4{\cdot}2H_2O$ 0.10, $MnSO_4{\cdot}4H_2O$ 0.10, $CuCl_2{\cdot}2H_2O$ 0.10, $ZnSO_4{\cdot}7H_2O$ 0.23, $FeCl_3{\cdot}6H_2O$ 0.3, and $NiCl_2{\cdot}6H_2O$ 0.05.

The 750 mL of seed biomass was mixed well with 75 mL wastewater and 175 mL MSM to maintain a substrate to inoculum ratio of 2 g SCOD:1 g volatile suspended solids (VSS). For the blank controls, sludge was mixed with MSM to maintain the final VSS equal to the test group. Then 90 mL of homogenous test mixture was added to serum bottles, sealed with rubber septa, crimped and the headspace flushed with N_2 gas for 2 min. After flushing with N_2 gas, the serum bottles were incubated in shaker incubators at 150 r/min, and 35°C. Serum bottles containing the wastewater were identified as AD/IND-WW and blank control bottles named AD/Control. All the analyses were performed in triplicate. The experimental period was considered as 30 days due to no further methane production and VFA degradation between day 30 and day 35 (data for day 35 not illustrated).

1.4.2 Physicochemical characterization

For initial testing of total solids (TS), volatile solids (VS), total suspended solids (TSS), VSS, TCOD, SCOD and VFAs, samples were drawn from the homogenous bulk mixture. Later during the experimental stages, for TCOD, SCOD and VFA analyses, 1 mL samples were withdrawn from the serum bottles and diluted suitably for analysis. The total volume changes during the sampling period were taken into consideration for calculation purposes. VSS (taken to determine microbial growth) and pH measurement were performed only on day 0 and day 30 due to limited sample volume. Tests for TCOD, SCOD, TS, VS, TSS, and VSS were performed in accordance with standard methods (APHA, 1998). For VFA analysis, the liquid part of diluted samples was filtered through a 0.2 μm syringe filter and 900 μL of the filtered sample was mixed with 100 μL of 10% formic acid for testing the VFA's. Samples were analyzed using a gas chromatograph equipped with flame ionization detector (GC-FID) (Agilent Systems, model 7890A, Palo Alto, California, United States of America).

1.4.3 Gas measurement

Individual biogas components (CH_4, CO_2, and H_2) were analyzed using a gas chromatograph equipped with thermal conductivity detector (GC-TCD) (Agilent Systems, model 7890A, Palo Alto, California, United States of America). An airtight pressure lock syringe was used to draw 5 mL of gas from the headspace in each serum bottle. Withdrawal of gas was done such that the headspace pressure did not fall below atmospheric pressure (pressure was measured using Dwyer Series 475 Mark III manometer).

1.4.4 DNA extraction and q-PCR analysis

DNA was extracted from diluted sludge samples using Roche MagNA Pure DNA extraction kits. The extraction procedure was performed in a Roche MagNA Pure DNA extractor (Roche Diagnostics, Mannheim, Germany). Sample volume for the extraction was 100 μL with the same eluted volume of DNA. The probes and primers (Yu et al., 2005) used for q-PCR were obtained from TIB MolBiol (Berlin, Germany) and are listed in **Table 3**. q-PCR reaction was performed in a Roche Lightcycler 480-II (Roche Diagnostics, Mannheim, Germany). The reaction volume for q-PCR was set at 20 μL with each reaction mixture containing 2 μL of template DNA (concentrations not shown), 1 μL (final concentration, 5×10^{-7} mol/L) of the forward and reverse primers along with 2 μL (final concentration 2×10^{-7} mol/L) of the TaqMan probe corresponding to each primer and probe set, 10 μL of Roche Lightcycler Mastermix and PCR-grade sterile water, to a final volume of 20 μL.

Two-step amplification of the target DNA, combining the annealing and the extension steps, was performed as described in a previous study (Yu et al., 2005) with modification and applying the following conditions: an initial 10 min incubation at 95°C for Taq DNA polymerase activation; 55 cycles of denaturation at 95°C for 10 sec; and simultaneous annealing and extension at 60°C for 30 sec. The fluorescence response data obtained during annealing and extension period was in the "single" mode

Table 3 List of primers and probes used for the 16S rRNA gene copy number quantification

Target group	Primer name	Primer sequence (5′→3′)
Eubacteria	BAC338F^{+}	ACTCC TACGG GAGGC AG
	BAC516F*	TGCCA GCAGC CGCGG TAATA C
	BAC805R$^{\#}$	GACTA CCAGG GTATC TAATC C
Methanobacteriales	MBT857F^{+}	CGWAG GGAAG CTGTT AAGT
	MBT929F*	AGCAC CACAA CGCGT GGA
	MBT1196R$^{\#}$	TACCG TCGTC CACTC CTT
Methanomicrobiales	MMB282F^{+}	ATCGR TACGG GTTGT GGG
	MMB749F*	TYCGA CAGTG AGGRA CGAAA GCTG
	MMB832R$^{\#}$	CACCT AACGC RCATH GTTTA C
Methanosaetaceae	MST702F^{+}	TAATC CTYGA RGGAC CACCA
	MST753F*	ACGGC AAGGG ACGAA AGCTA GG
	MST862R$^{\#}$	CCTAC GGCAC CRACM AC
Methanosarcinaceae	MSC380F^{+}	GAAAC CGYGA TAAGG GGA
	MSC492F*	TTAGC AAGGG CCGGG CAA
	MSC828R$^{\#}$	TAGCG ARCAT CGTTT ACG

$^{+}$ Forward primer, * TaqMan probe, $^{\#}$ reverse primer.

with the channel setting at F1. The fluorescent signal data was then processed using Roche Lightcycler software (version 4.0).

1.4.5 Denaturing gradient gel electrophoresis (DGGE) profiling and phylogenetic analysis

The DGGE test was conducted to investigate Eubacterial and Archaeal community structures targeting the 16S rRNA gene, in a Biorad D-Code Universal Mutation Detection System (Biorad, Hercules, California, United States of America). Time points chosen for the DGGE profile were at day 0, 15 and 30. The analysis was performed for the test groups alone to investigate the effect of the high strength industrial wastewater on the microbial communities present in anaerobic biomass.

A conventional PCR was performed with domain-level universal primers: BAC338F (5′-ACTCCTACGGGAG GCAG-3′) and BAC805R (5′-GACTACCAGGGTATCT AATCC-3′) (Lee et al., 2008) for Eubacteria; ARC787F (5′-ATTAGATACCCSBGTAGTCC-3′) and ARC1059R (5′-GCCATGCACCWCCTCT-3′) for Archaea (Shin et al., 2010). The 5′ ends of forward primers were capped with 40-bp GC-clamps, 5′-CGCCCGCCGCGCGCGGC GGGCGGGGCGGGGGCACGGGGGG-3′ for Eubacteria and 5′-CGCCCGCCGCGCCCCGCGCCCGTCCCG CCGCCCCCGCCCG-3’ for Archaea, to stabilize the melting behaviour of PCR products (Muyzer et al., 1993). The PCR mixture of 20 μL was prepared by GoTaq Mastermix (Promega, Madison, Wisconsin, United States of America) 10 μL, forward primer 1 μL, reverse primer 1 μL, template DNA 2 μL and PCR grade water 6 μL. The mastermix contained dNTP's, $MgCl_2$, Taq polymerase enzyme and buffer solution. The working concentrations of the forward and reverse primers were 5×10^{-7} mol/L. A touch-down PCR was conducted according to the protocol described by Shin et al. (2010). A mixture of the triplicate touchdown PCR products were used as template (1:1:1, $V/V/V$, final volume 30 μL) which were loaded onto an 8% (W/V) acrylamide gel with a denaturing gradient of 30%–70%. Here the gradient of 100% was defined as 7 mol/L urea with 40% formamide. The gel was subjected to electrophoresis at 85 V for 13 hr in 1× TAE buffer. After staining with ethidium bromide, the bands visible to naked eye were excised and eluted in distilled water. The eluted DNA samples were further amplified using primers without the corresponding GC clamps. The PCR product was then purified from 1% agarose gel and cloned onto p-GEMT Easy vector (Promega, Madison, Wisconsin, United States of America).

The cloned 16S rRNA gene fragments were then sequenced by capillary sequencing (AIT biotech, Singapore) and the sequenced results were compared with reference sequences generated in the GenBank database using the BLAST program (BLAST: Basic Local Alignment Search Tool at http://blast.ncbi.nlm.nih.gov). Sequences were deposited in the GenBank database with the accession numbers from KF511593 to KF511611. Neighbour joining trees were constructed for phylogenetic analysis using the MEGA-5.1 software (Tamura et al., 2011).

2 Results

2.1 Physicochemical characterization

The changes in VSS, pH, TCOD and SCOD are listed in **Table 4**. The relatively unchanged pH levels in the test group on day 30 indicated a likely self buffering system, with possibly no need for external pH control. The VSS

Table 4 Physicochemical changes during the experimental period

Group	Day 0	Day 15	Day 30
TCOD (AD/Control)	(15.68 ± 0.12) g/L	(13.70 ± 0.22) g/L	(9.05 ± 0.07) g/L
TCOD (AD/INDWW)	(39.22 ± 0.07) g/L	(38.96 ± 0.08) g/L	(34.94 ± 0.10) g/L
SCOD (AD/Control)	ND	0.23 g/L	ND
SCOD (AD/INDWW)	(21.26 ± 0.15) g/L	(23.43 ± 0.32) g/L	(17.09 ± 0.11) g/L
VSS (AD/Control)	(10.32 ± 0.17) g/L	NA	(9.18 ± 0.24) g/L
VSS (AD/INDWW)	(10.29 ± 0.84) g/L	NA	(11.56 ± 0.45) g/L
pH (AD/Control)	7.45	NA	7.11
pH (AD/INDWW)	7.34	NA	7.41

ND: not detected, NA: not analyzed.

profiles of the test group on day 30 (11.56 ± 0.45 g/L) indicated a slight increase in the biomass content (day 0: 10.29 ± 0.84 g/L). This was a likely indicator that the seed biomass was able to propagate in the presence of the wastewater. However, other parameters such as VFA production and degradation profile, COD reduction and methane generation need to be considered for studying biomass activity and efficacy. The results of VFA analysis for the test groups are illustrated in **Fig. 1**. VFA concentration for the test groups was 3.6 g/L on day 0 and reached a maximum of 7.46 g/L on day 20 but subsequently decreased till day 30 (3.93 g/L). Acetic acid was the major intermediate VFA with a highest concentration of 3.34 g/L on day 20. Propionic acid was also found to increase throughout the experimental period with a maximum of 2.51 g/L on day 30. VFA values in control groups were below detectable limits and hence not illustrated.

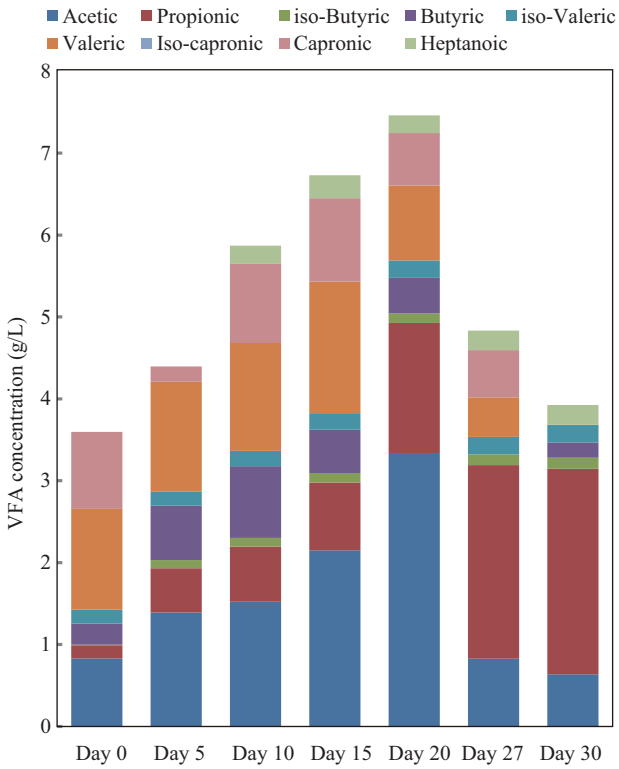

Fig. 1 Volatile fatty acid profile of AD/INDWW.

2.2 Biogas measurement and profile

Biogas measurement was done every 5 days except between day 20 and 30 where measurements were made on day 27 and day 30. Gas production was calculated as volume produced over every 5 days after pressure in headspace was equalized to 1 atm at room temperature following each measurement. The cumulative methane production in test group was 233 mL at standard temperature and pressure (STP) while the control group produced a cumulative methane volume of 98 mL STP (highlighted in **Fig. 2**). Maximum methane production in the mesophilic test group occurred between day 27 and day 30 where it had produced 60.5 mL within 3 days. The highest recorded methane percentage of total biogas in the test group was 80.6% while those in control recorded 66.8%.

2.3 q-PCR analysis

The shifts in the numbers of 16S rRNA gene copies of Eubacteria and different Methanogenic communities are illustrated in **Fig. 3**. It was observed in the test groups that, Eubacterial populations gradually decreased till day 20 after which their numbers became more stable. However, the Eubacteria still accounted for 96.3% of the total microbial population with the Archaea contributing only

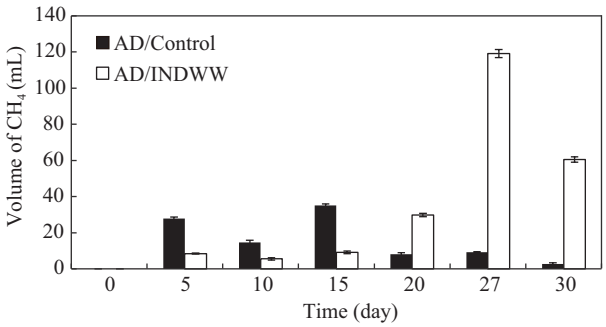

Fig. 2 Volume of methane produced over 30 days period. The error bars represent the standard deviation of the average of triplicate measurements.

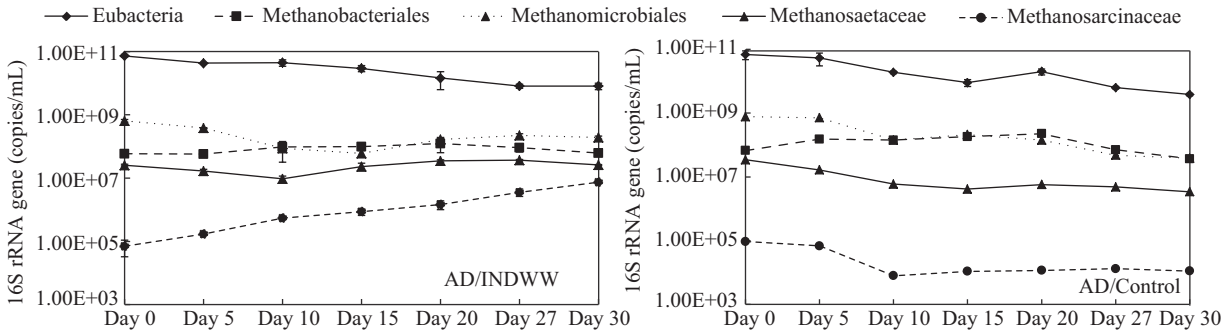

Fig. 3 The shifts in microbial populations during the 30 days period in AD/INDWW and AD/Control systems. The error bars indicate standard deviation of the average of triplicate measurements.

3.7%. Eubacteria in the control groups decreased continuously during the test period. Within the Methanogenic populations of both test and control groups, Methanomicrobiales and Methanobacteriales were most dominant. While Methanosarcinaceae increased over the 30-day period in the test groups, it decreased in copy numbers over the 30 days period in the control groups.

2.4 DGGE profile and phylogenetic analysis

DGGE profile and phylogenetic analysis was conducted to characterize the microbial community structure in response to the presence of wastewater. Both Archaeal and Eubacterial PCR products were subjected to DGGE profiling and phylogenetic analysis. This was performed with DNA samples collected during day 0, day 15 and day 30 from the test groups. The DGGE profile is illustrated in **Fig. 4**. The profile indicates certain DNA bands of the Eubacteria faded in intensity over the 30 days period and so suggesting the wastewater may have stressed the microbes, resulting in a decrease of copy numbers of DNA. However, the Archaeal profile had remained relatively stable through the 30 days period, suggesting Archaea were more resistant to the stress. The phylogenetic trees constructed are illustrated in **Fig. 5a** for Eubacteria and **Fig. 5b** for Archaea.

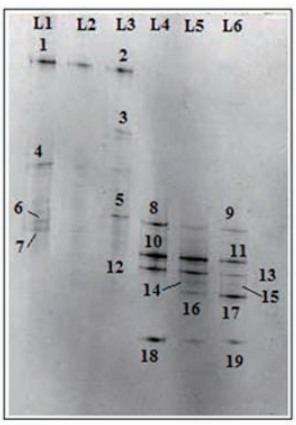

Fig. 4 DGGE profile of microbial DNA of test groups. L1, L2 and L3 represent the Eubacterial DNA isolated on day 0, day 15 and day 30 while L4, L5 and L6 represent Archaeal DNA isolated on day 0, day 15 and day 30 respectively. Bands numbered represent the bands analyzed.

In the DGGE profile, band 1 of Eubacteria was the most prominent band which did not visibly decrease in intensity (refer to band 2). When the sequences were analyzed, it resembled closely (98%–99%) to the genera *Pelomonas*, belonging to the phylum Proteobacteria. Another interesting band development was band 4 in the Eubacteria which closely resembled (95%–97%) the genera *Pelotomaculum* belonging to the phylum Firmicutes. This band had decreased in its visual intensity as evidenced in lanes 2 and 3. Another band belonging to the phylum Firmicutes was band 3 which was present only on day 30 which closely resembled (99%–100%) the genus *Sedimentibacter* as well as the thiosulfate reducer, *Dethiosulfatibacter*. Bands 5, 7 and 6 closely resembled (96%–99%) the genera *Syntrophomonas* and *Pelospora* respectively which belong to the phylum Firmicutes as well.

The Archaeal bands fell within three main orders: Methanomicrobiales, Methanosarcinales and Methanobacteriales. The Methanomicrobiales had 4 band profiles with bands 8 and 9 similar (98%–100%) to *Methanospirillum hungatei* JF-1 strain while bands 10 and 11 closely resembled (99%–100%) *Methanolinea tarda* NOBI-1. The Methanosarcinales had 6 bands out of which band 12 and band 13 were similar to the genus *Methanosaeta*. Bands 14 and 15 resembled closely (97%–99%) to *Methanosarcina barkeri* strain DSM-800 while bands 16 and 17 closely resembled (96%–99%) *Methanosarcina mazei* strain DSM-2053 and OCM-26. The Methanobacteriales had just two bands, band 18 and band 19 which closely resembled (97%) *Methanobacterium formicicum* strain DSMZ-1535.

3 Discussion

There have been many studies reporting use of BMP test for various organic compounds. Studies by Owens and Chynoweth (1993) and Hansen et al. (2004) proposed protocols for determination of BMP while recent studies by Angelidaki et al. (2009) have refined the method-

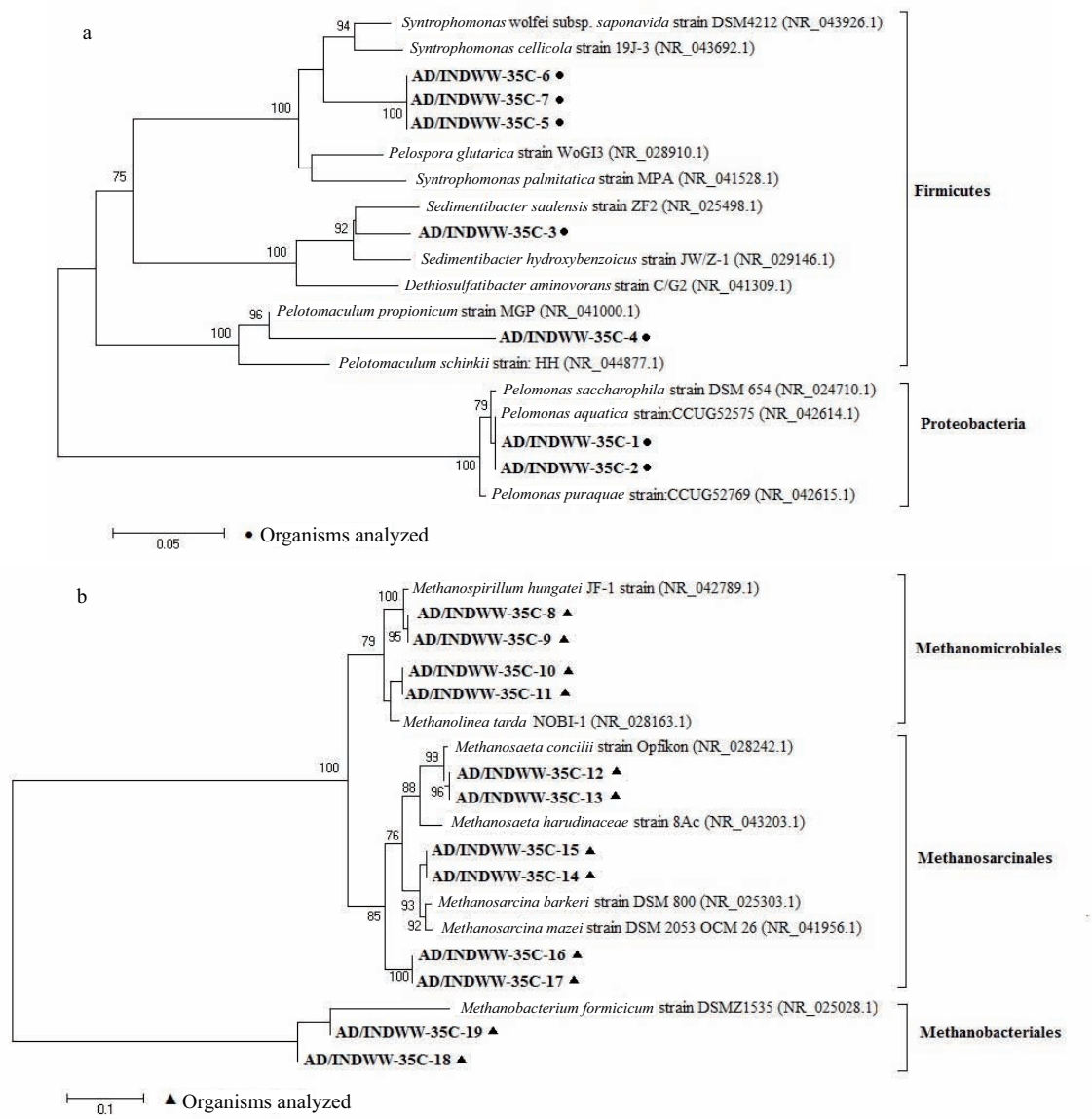

Fig. 5 Neighbor-joining method used for constructing phylogenetic tree, highlighting the identities of 16S rRNA genes of Eubacteria (a) and Archaea (b). Numbers at nodes are bootstrap values derived from 100 analyses.

ology involved. However, past studies have not given due importance to understanding the microbiology behind the degradation process involved during the BMP test. As mentioned earlier, the degradation process would be underpinned by microbial communities involved and it would only be prudent to study the microbial community dynamics in tandem with the process parameters to get a better picture of the degradation process. Therefore, this study focused on the relation between the microbial communities, COD reduction, VFAs generation and the CH_4 produced during the anaerobic degradation process of a high strength chemical industry wastewater at mesophilic (35°C) temperature.

VFA intermediates are generated in majority of anaerobic degradation processes. They serve as precursors for the production of methane, especially acetic acid. The wastewater investigated in this study was inherently rich in VFA's. Valeric acid along with glutaric and acetic acid formed its major components. Hence it was initially assumed that anaerobic process (especially methanogenesis) would perform better due to readily available VFA for degradation. According to Speece (1996), acetate is the most important VFA intermediate, contributing to more than 70% of the methane produced during an anaerobic degradation process. However, acetate can be used by only a small group of organisms, the "aceticlastic methanogens", belonging to the order Methanosarcinales which comprises the families Methanosaetaceae and Methanosarcinaceae. These use acetate as a substrate to generate methane as the end-product. Considering the VFA data from the test group, the acetate levels had increased till day 20 after which it rapidly decreased till day 27. This was concomitant with the

q-PCR results which highlight that Methanosarcinaceae had increased till day 30 although Methanosaetaceae remained relatively constant. Previous studies have shown Methanosaetaceae have a relatively low acetate K_s of ca. 5×10^{-6}–70×10^{-6} mol/L for conversion to methane while Methanosarcinaceae have a higher acetate K_s of ca. 1×10^{-3} mol/L (Hori et al., 2006; Sekiguchi et al., 1999; Jetten et al., 1992). Thus Methanosaetaceae would likely predominate under low acetate concentration while Methanosarcinaceae would prevail under higher acetate concentration conditions, with the latter also having a higher growth rate compared to Methanosaetaceae. The results of this study are in correlation with previous findings wherein organisms resembling Methanosarcina had been found more prominent from day 15; the point where acetate was on the verge of reaching its highest concentration. However, from day 20 there was rapid drop in acetate concentration which was likely caused by the effective aceticlastic action of Methanosarcinaceae. The most logical hypothesis would be that Methanosarcinaceae need a desired acetate concentration combined with a particular population level for effective aceticlastic methanogenesis.

Methanospirillum, Methanolinea and Methanobacterium-like organisms were also detected in the test group. These methanogens are obligately linked with the Syntrophomonadaceae (also present in the system) to produce methane through the hydrogenotrophic pathway. However, the hydrolytic and acidogenic action of Syntrophomonadaceae could have occurred at a faster rate than the consumption rate of acetate or hydrogen to produce methane by the Methanosarcina or the hydrogenotrophic methanogens. This could have led to the initial accumulation of acetate and then followed by the decrease and could also explain the slow initial rate of methane generation.

Aside from the above discussed phenomena, there was also accumulation of propionic acid in the test group. Propionate is an important part of the anaerobic metabolism and contributes to 6%–35% of the carbon balance in the anaerobic degradation system (Scholten and Conrad, 2000). In the test group, propionic acid accumulation was continuous and reached a maximum on day 30. Past research has shown that when *n*-valerate was present in the substrate, the acidogenic degradation produced propionate as the major intermediate, rather than acetate, thereby leading to propionate accumulation (Gallert and Winter, 2008). It had also been reported that if odd number of carbon atoms were to be present in VFAs or organics in the substrate, propionate would be the final end product of the acidogenic degradation pathways instead of butyrate or acetate (Weng and Jeris, 1976). The wastewater used in this study has *n*-valerate as the major VFA and sodium salts of glutaric acid which contains odd number of carbon atoms and hence these may have caused the propionic acid accumulation. However, band 4 in the DGGE profile has corresponding similarity to the genus *Pelotomaculum* which as reported in earlier studies is an important genus in propionate oxidation (Imachi et al., 2007) along with species of the genera *Desulfotomaculum*. However, propionate oxidizers (including *Pelotomaculum* and *Desulfotomaculum*) are slow growers and highly sensitive organisms and therefore are easily susceptible to even low levels of high strength organics (Gavala et al., 2003). The DGGE profile shows that band 4, showing similarity to propionate oxidizers, had decreased in intensity on day 15 and day 30, indicating a probable reduction in their copy numbers. This reduction of population numbers could have been one of the major factors leading to the accumulation of propionic acid.

From the various parameters analysed, it is clear that the degradation process was not comprehensive and a significant amount of residual COD was observed at the end of day 30. This may have been due to a partial inhibition of the anaerobic degradation process (e.g., accumulation of propionic acid possibly due to reduction in copy numbers of *Pelotomaculum* sp.). This inhibition could have been caused by the presence of certain organic compounds present in the wastewater not amenable to biological transformation (recalcitrants) and which could have been toxic for certain microbial genera (Duran and Speece, 1999). They also showed that mass transfer limitations influence the degradation of a particular biodegradable compound. This could have been an underlying factor for incomplete propionic acid degradation. The propionic acid may have been generated at a faster rate by organisms closely resembling *Syntrophomonas* and *Pelospora* which might have led to propionate itself inhibiting the propionate degraders, possibly through a feedback mechanism. This build up of propionate could have also adversely affected other microbial communities as well. In support of this argument, it has been reported earlier that imbalance of microbial populations causes build-up of intermediates unfavourable to the methanogenic populations (McMahon et al., 2001), which could very well be the case in this study. Leclerc et al. (2001) also state that the imbalance between population members could very well be the underlying reason behind the inhibition of anaerobic processes.

This study demonstrated the changes in microbial community dynamics as well as diversity during over a period of time during the batch operation of BMP test. The study was conducted with no changes in operating conditions over the experimental time period. However, it is impossible to determine which specific component of the wastewater would have actually affected the microbial community dynamics and in turn, the bioprocess, due to the complex nature of the wastewater involved. It is also highly unlikely that the microbial community dynamics could be completely explained as a function of one or a few factors since the seed used in this study consisted of a complex set of microorganisms. Hence a much more

profound study is needed in order to completely understand the role that the microbial communities play in the anaerobic degradation process.

4 Conclusions

The anaerobic degradation process at mesophilic temperatures is closely linked to its microbial community dynamics. In the test group, VFAs production was marked by the presence of *Syntrophomonas*, *Pelospora* and *Sedimentibacter* like Eubacterial species. Propionic acid accumulation was observed throughout the experimental period which could have stemmed from reduction in the population of *Pelotomaculum* sp. in combination with a possible feedback inhibition mechanism. Methanogens remained relatively unaffected by the presence of wastewater. Methanosarcinaceae population increase could have contributed to effective aceticlastic methanogenesis. Complex nature of wastewater in conjunction with complex microbial community structure in seed biomass makes it difficult to explain process as function of one or more physical parameters involved.

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