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# Exploitation of molecular genetics in microbial degradation and decolorization of industrial waste water effluent

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**Activated sludge samples were used to analyse microbial community structures from the anoxic and aerobic zones of a laboratory-scale modified Ludzack-Ettinger system. Fluorescent *in situ* hybridization and denaturing gradient gel electrophoresis were applied for analysis. With the help of DNA specific fluorochrome DAPI, approximately 75 to 80% of total cells were detected, hybridised with a specific eubacterial probe for the anoxic and aerobic zones. Results corroborate the dominance of the alpha and gamma subclasses of the *Proteobacteria* in the anoxic zone whilst the aerobic zone was dominated with the beta subclass of the *Proteobacteria*. Genetic diversity of the microbial community present in each of the anoxic and aerobic zones was employed by the DGGE technique. Results were obtained from the application of fluorescent *in situ* hybridisation (FISH) and PCR-DGGE yields a more precise understanding of the microbial community structure and genetic diversity present in domestic wastewater of a laboratory scale treatment process. Nitrogen mass balances indicated an upset in the nitrogen levels for wastewater batches two and seven. The carbon mass balance fell in the range of 92.4 and 105.9% and the nitrogen mass balance fell in the range of 98.4 and 160.0%.**

**Key words:** Fluorescent *in situ* hybridisation (FISH), denaturing gradient gel electrophoresis (DGGE), COD and nitrogen mass balances.

## INTRODUCTION

The growth of the world population, the development of various industries and the use of fertilizers and pesticides in modern agriculture has overloaded not only the water resources but also the atmosphere and the soil with pollutants (Shah et al., 2013). Treatment of activated sludge within the modified Ludzack-Ettinger (MLE) system involves the removal of biodegradable organics, unsettleable suspended solids and other constituents. These biodegradable organic compounds are degraded by bacteria in an aerated reactor and the biomass is allowed to settle and concentrate in a clarifier (Muyima et al., 1997). The system is either a continuous or semi-continuous aerobic method for wastewater treatment involving carbon oxidation and nitrification. This process has been developed for the

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removal of carbon, nitrogen and phosphate and it is well known that prokaryotic micro-organisms catalyses these main biological processes in wastewater treatments (Juretschko et al., 2002); nutrients and oxygen present the microbial population in the wastewater achieves optimal growth and respiration (Muyima et al., 1997). The dynamics and diversity of the microbial populations in activated sludge have been analysed by culture-dependent methods, however many members of the natural bacterial communities are still unculturable (Wagner et al., 1994). Hence, microscopic identification based on morphological characteristics was researched and developed in a culture-independent manner by direct rRNA sequence retrieval, where nucleic acid probes which are complementary to the rRNA are used as tools to monitor population dynamics amongst bacteria (Amann, 1995). FISH makes use of rRNA targeted probes which are frequently applied in order to quantify the composition of microbial communities present. This procedure is based on the comparative analysis of macromolecules, mostly ribosomal RNA molecules and fluorescent derivatives of such probes. These probes have been applied successfully for *in situ* enumeration of defined groups of micro-organisms present in activated sludge (Manz et al., 1994).

Fluorescently monolabelled, rRNA targeted oligonucleotide probes detect individual cells, allowing whole-cell hybridisation with rRNA targeted probes to be a suitable tool inferring phylogenetic evolution hence the cell morphology of an uncultured microbe and its abundance can be determined *in situ* (Wagner and Amann, 1997). Cell numbers of the bacteria can be obtained by enumeration under an epi-fluorescent microscope. Enumeration procedures involve the use of a semi-automated digital image analysis tools in order to quantify the fluorescently labelled bacteria in samples (Daims et al., 2001). The molecular technique used for analysing the structure and providing a profile of the microbial population present in wastewaters is the denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993). DGGE that is performed on 16S rRNA genes has been used to produce a genetic fingerprint of mixed microbial communities (Kaewpipat and Grady, 2002). The numbers of bands that are obtained from DGGE profiles provide an estimate of the different microbial species present. The intensity of each band provides a reflection of the relative abundance of each species (Nasu, 2000) because the primers used to amplify a fragment of the 16S rRNA produces a quantitative relationship between the gene copy number and the PCR-DGGE band intensity (Kaewpipat and Grady, 2002). However, many factors can prevent the formation of the number and intensity of the bands in the DGGE gel, therefore representing the exact number and abundance of species in a microbial community can be difficult however DGGE is a sensitive and a rapid technique that detects most singe-base variations when a G-C clamp is added to one of the primers in the PCR process. This provides a profile of changes that occur within a microbial community or between microbial communities (Kaewpipat and Grady, 2002). In this study, a combination of molecular techniques, FISH and PCR-DGGE was used to monitor the microbial composition and examine the microbial community population shifts within a steady state laboratory-scale parent anoxic and aerobic activated sludge system.

**Table 1.** Process parameters recorded at the time of sampling.

| Parameters | Value |
|---|---|
| pH of the digester | 7.1 |
| Total Solid g/L | 22 |
| Organic loading rate applied kgCOD/$m^3$ day | 4.5 |
| Influent COD mg/L | 41,300 |
| VFA mg/L | 517 |
| Acetic acid mg/L | 275 |
| COD removal efficiency % | 93 |
| Methane % v/v | 56 |
| Biogas rate $m^3$/day | 1200 |

## MATERIALS AND METHODS

### Sampling and bioreactor set-up

Activated sludge was collected from a 500 $m^3$ closed digester tank (CDT) for the anaerobic treatment. The CDT was operated under mesophilic condition (32 to 39°C) for 120 days. The system was equipped with a closed digester tank, settling tank, pumps and flow meters for biogas and the effluent. There were three sampling ports at the top, in the middle and at the bottom of the CDT. The sludge sample was obtained from middle sampling port. The process parameters recorded at the time of sampling are shown in Table 1.

### Analyses

The laboratory-scale modified Ludzack-Ettinger process was fed with 30 ℓ of mainly domestic wastewater on a 10 day sludge age process. After reaching steady state, the MLE system was run for duration of 18 batches. The wastewater was diluted with tap water to give an influent feed of approximately 500 COD mg /L from the original 800 COD mg /L. The process was maintained by wasting 1.5 L/day of the mixed liquor from the aerobic reactor. Samples from the influent, anoxic, aerobic and effluent were analysed daily for the following: chemical oxygen demand (COD), total Kjeldahl nitrogen (TKN), mixed liquor suspended solids (MLSS), volatile suspended solids (VSS) and nitrate analysis (nitrite tests were not performed because the nitrite concentration was 2% less than nitrates). Microbial community analyses were performed on samples obtained from the aerobic and anoxic reactors. The oxygen utilisation rate (OUR) was monitored within the mixed liquor with an online dissolved oxygen (DO) controller (hi-tech micro-system) according to Randall et al. (1991). The pH was kept constant at 7.5 and the temperature was kept constant at 20°C.

### Total cell counts

Membrane filtration was performed according to Hicks et al. (1992). Fixed samples were sonicated and diluted (dilution factor of 200) with phosphate-buffered saline (PBS) and 1% nonidet. Nucleopore

filters with a pore size of 0.2 µm were pretreated with 0.3% Sudan black and placed on top of a 0.45 µm backing filter. The samples were stained with DAPI (4,6-diamidino-2-phenylindole) (Sigma, Deisenhofen, Germany) at an end concentration of 1.25 µg/mℓ. The Zeiss Axiolab microscope (50 W high-pressure mercury bulb and Zeiss filter set 01) fitted for epifluorescence microscopy was used with the Zeiss image analysis software in order to quantify the fluorescing cells.

**Hybridization and pretreatment**

The fixed and sonicated samples were immobilised onto pretreated slides and dehydrated with 60, 80% and absolute ethanol to prepare for whole-cell hybridisation. Samples were hybridised with appropriate hybridisation buffers and probed *in situ* with oligonucleotide probes listed in Table 1 according to Amann (1995). The probes were either labelled with tetramethylrhodamine-5-isothiocyanate or 5(6)-carboxyfluorescein-*N*-hydroxy sccinimide-ester. The probe-conferred fluorescence was detected with a Zeiss Axiolab microscope (50 W high-pressure mercury bulb and Zeiss filter sets 09 and 15). Dual staining of samples with DAPI and fluorescent rRNA probes was performed according to Hicks et al. (1992).

**DNA extraction and PCR amplification**

DNA was extracted from activated sludge following the method of Bourrain et al. (1999) and 16S rDNA was amplified using the Wright and Pimm (2003) methanogenic specific primers Met86F (5'-GCTCAGTAACACGTGG-3') and Met1340R (5'-CGGTGTGTGCAAG GAG-3'). PCR reactions (25 µL) were set up in 0.2 mL thin-walled Eppendorf® tubes, containing 100 ng sludge DNA, 10 X Taq DNA polymerase buffer, 0.5 µL of 10 mM dNTP mix (Fermentas, Maryland, USA), 0.5 µl of 10 mM dNTPs, 2.5 µl of 25 mM $MgCl_2$, 0.5 µl of each methanogen primer and 0.2 µl of 5 U AmpliTaq DNA polymerase (Fermentas, Maryland, USA). DNA templates from three sludge samples obtained from the bottom and middle of the bioreactor, and from the recycling tank were amplified separately for cloning. Samples were amplified using a Perkin Elmer Gene Amp system 9600. Each PCR cycle consisted of 94°C for 40 s, 54°C for 50 s, and 72°C for 90 s. On the 35th cycle, the elongation step was increased to 10 min at 72°C.

**Cloning 16S rDNA**

PCR products were purified from a 1% agarose gel using a gel extraction kit according to the manufacturer's instructions (Qiagen, Germany). The recovered PCR fragment was cloned into pTZ57R vector (Fermentas, Maryland, USA), and transformed into *Escherichia coli* TOP10 competent cells (Invitrogen, Carlsbad, CA). White colonies were randomly selected from plates and recombinant plasmids identified by size selection on a 1% agarose gel prior to restriction fragment length polymorphism (RFLP) analysis.

**RFLP and sequences analysis**

Cloned PCR products were digested separately with *Hae*III (Fermentas, Maryland, USA) in 20 µl reaction volumes containing 10 µl of PCR product, 2 µl x 10 reaction buffer and 10 units of restriction endonuclease in sterile ultrapure water. Digested PCR products were incubated for 1 h at 37°C and digested fragments were separated using a 10% polyacrylamide gel (w/v). The resulting bands were visualized by silver-staining and each clone was assigned to an RFLP group based upon the banding patterns. Two groups (groups I and II) were assigned. For each RFLP type, the 16S rRNA insert of a few representative clones were sequenced. The recombinant plasmids were extracted using the Qiagen plasmid DNA extraction kit (Qiagen, Germany) according to the manufacturer's instructions. Approximately, 27 nucleotides were sequenced using an ABI 3730 XL DNA Sequencer. Chromatograms were edited using Chromas software (Technelysium, Australia), while CHECK_CHIMERA software (Maidak et al., 1997) was used to scan for potential chimeric sequences. The sequences were compared to known 16S rRNA sequences in the GenBank™ database, using the basic logical alignment search tool (BLAST) to locate nearly exact matches in the GenBank database. DNA sequences were aligned using the program CLUSTAL W (Thompson et al., 1994) and further edited manually. Phylogenetic analyses were performed using the neighbor-joining (NJ) method using the MEGA ver. 3.1 (Kumar et al., 2004).

**PCR-based DGGE fingerprinting of methanogens**

For each RFLP group, one representative clone was selected for further study. DGGE-PCR reactions were performed on sludge DNA, as well as on the extracted plasmids from representative clones. The aim was to obtain the DGGE profiles for the archaeal and methanogenic species in the POME anaerobic bioreactor. Therefore, universal DGGE methanonenic and archaeal primers were used in the amplification reactions. The universal archaeal primers PARCH340f and PARCH519r based on the *E. coli* 16S rRNA gene sequence and methanogenic primers 0357F and 0691R were used to amplify 179 and 334 bp fragments (Watanabe et al., 2004), respectively. The GC clamp, 5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG-3' as described by Chan et al. (2001), was included on the 5 end of the forward primer PARCH340f and 0357F to enable the separation of the fragments using DGGE. PCR reactions for both primer sets were performed in 50 µl reaction volumes containing 0.5 µl of *Taq* DNA polymerase (5 U/µl) (Fermentas, Maryland, USA), 5 µl of 10 x PCR reaction buffer, 0.5 µl of each of the primers (10 mM), 5 µl dNTPs (10 mM), 4 µl of $MgCl_2$ (Fermentas, Maryland, USA) and 1 µl of the extracted DNA or plasmid. The PCR amplification conditions for the primer sets were as follows: initial denaturation was performed at 94°C for 3 min; followed by denaturation at 94°C for 1 min; annealing at 60°C for archaeal primers and 58°C for methanogenic primers for 1 min; and chain elongation at 72°C for 2 min. On the 35th cycles, the final elongation step was performed at 72°C for 8 min. The amplified products were checked on 1% agarose gels and visualized under UV light.

**DGGE**

DGGE was used to separate the 179 and 334 bp PCR products. The initial parameters for the denaturing gradient were optimized empirically. The optimized gradient extended from 40 to 60% and 50 to 60% of denaturant consisting of 7 M urea and 40% formamide for methanogens and archaeal PCR products, respectively; this was established in 8% acrylamide gels. Electrophoresis was performed at 100 V for 14 h at a constant temperature of 60°C. The DNA was stained with cyber green and visualized under UV light.

**Band selection, DNA purification and sequencing**

All DGGE bands from the methanogenic profile were punched out with a sterile pasture pipette and used as a template in a re-amplification using the methanogens` primers 0357F and 0691R (Watanabe et al., 2004). The resultant PCR products were purified using the Mag Extractor-PCR and Gel Clean up-kit. (Toyobo, Japan) according to the manufacturer's instructions and sequenced using an ABI 3730 XL DNA Sequencer at the DNA Sequencing Facility at Kyushu Institute of Technology, Japan. The sequences

**Table 2.** Steady state results for the MLE anoxic and aerobic activated sludge system

| WW Batches | COD (in ppm) | | TKN (in ppm) | | Nitrate (in ppm) | | | OUR (in ppm) | Mixed Liquor (in ppm) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | INF | EFF | INF | EFF | Anoxic | Aerobic | Effluent | | VSS | COD | TKN |
| 1 | 502.6 | 46 | 38.8 | 5.3 | 0.6 | 3.9 | 7.9 | 30.6 | 2352.4 | 3720 | 234.6 |
| | (48.8) | (10.1) | (6.3) | (2.1) | (1.0) | (1.9) | (1.5) | (0.4) | (153.1) | (553.2) | (20.7) |
| 9 | 506 | 46.8 | 41.9 | 3.5 | 1.2 | 6.9 | 10.0 | 26.7 | 2341.6 | 3652.8 | 223.7 |
| | (16.1) | (7.8) | (3.4) | (0.9) | (1.0) | (1.6) | (1.3) | (1.9) | (251.9) | (426.3) | (21.9) |
| 18 | 449.2 | 39.1 | 42.1 | 4.4 | 0.9 | 7.2 | 8.9 | 30.7 | 1914.5 | 2903.7 | 201.3 |
| | (40.0) | (8.2) | (5.4) | (0.8) | (0.5) | (2.3) | (2.5) | (0.7) | (239.2) | (372.1) | (49.3) |

obtained were compared to 16S rRNA gene sequences in the National Center for Biotechnology Information (NCBI) database using the BLAST.

#### FISH

To directly analyze methanogens populations in an anaerobic digester sludge sample, the probe MSMX860, complementary to the 16S rRNA of some methanogens including *Methanosarcina spp., Methanococcoides* spp., *Methanolobus* spp., *Methanohalophilus* sp. and *Methanosaeta* spp. was used (Crocetti et al., 2006). To target the sludge bacteria, the 16S rRNA probe EUB338 for the domain bacteria was used (Amann et al., 1990).

#### Fixation and permeabilization of the cells

Cells were fixed and hybridized using the protocol reported from Amann (1995) with some modification (Sakai et al., 2004). The sludge sample was fixed in 3% paraformaldehyde/phosphate buffer saline (PBS) (PBS; 130 mM NaCl, 10 mM sodium phosphate buffer, pH 7.0) for 1 to 3 h at 4°C, pelleted by centrifugation (3500 x *g*, 15 min, 4°C) and then stored in a 1 : 1 mixture of ethanol and PBS. Fixed cell suspensions were diluted using an ethanol/PBS mixture (8 µl) and were spotted on coated glass slides, dried at 46°C for 30 min and immersed for 3 min each in 50, 80, and 99% (v/v) ethanol. Cell smears were covered with 20 µl of lysozyme (50 mg/mL PBS; 37°C, 30 min). Enzymatic digestion was terminated by thoroughly rinsing the slides with distilled water followed by air-drying at room temperature (25 ± 2°C).

## RESULTS AND DISCUSSION

### Modified Ludzack-Ettinger system

The reliability of the experimental data obtained from the steady state analyses which included COD, TKN, OUR, nitrates and mixed liquor analyses is represented in Table 2. The chemical oxygen demand concentration calculated for the MLE process was 90.9%, thus indicating that a high amount of oxygen (as shown with the OUR concentrations) was required for the proliferating bacteria and their biological reactions. The nitrate concentrations of the anoxic, aerobic and effluent zones are as follows: the anoxic zone supported high rates of denitrification with an average of 1.5 N mg/L present whilst the aerobic zone had a higher level of concentration with an average of 7 N mg/L present, indicating an increase in nitrification; however, the highest concentration of nitrates was present in the effluent with an average of 11.3 N mg/L. These results suggest that a high denitrification potential of the MLE process is prominent; however, complete denitrification is not possible due to the absence of secondary reactors, which is clearly shown in the effluent results. The mixed liquor was analysed by performing volatile suspended solids (VSS), chemical oxygen demand (COD) and total Kjeldahl nitrogen (TKN). VSS analyses showed a high amount of biomass present in the mixed liquor, which accounted for the increased levels of chemical oxygen demand and total Kjeldahl nitrogen concentrations (results for only 3 wastewater batches are shown).

### Screening the 16S rRNA clone library by RFLP

16S rRNA was PCR amplified from the DNA extract of sludge taken from an anaerobic bioreactor. From 237 clones screened (84 from the bottom sludge library, 75 from the middle sludge library and 78 from the recycling sludge), all had a DNA insert of the correct size (1260 bp). Two different *Hae*III RFLP-patterns were observed from the clone libraries and assigned as groups I and II (Figure 1). Representative examples from RFLP group I and II (SamaliEB and Samali15) are shown in Figure 1. RFLP group I contained 97.1% of clones and 2.9% fell in group II.

### Phylogenetic analysis of representatives from each RFLP group

Partial DNA sequences were obtained from representative amplicons for each RFLP group.

Approximately 27 clones were sequenced (20 from group I and 7 from group II), and were used to search the GenBank nucleotide database with the BLAST search tool. One representative for each RFLP group was included in the phylogenetic tree. The sequence data set contained 20 sequences, including *Methanopyrus kandleri*

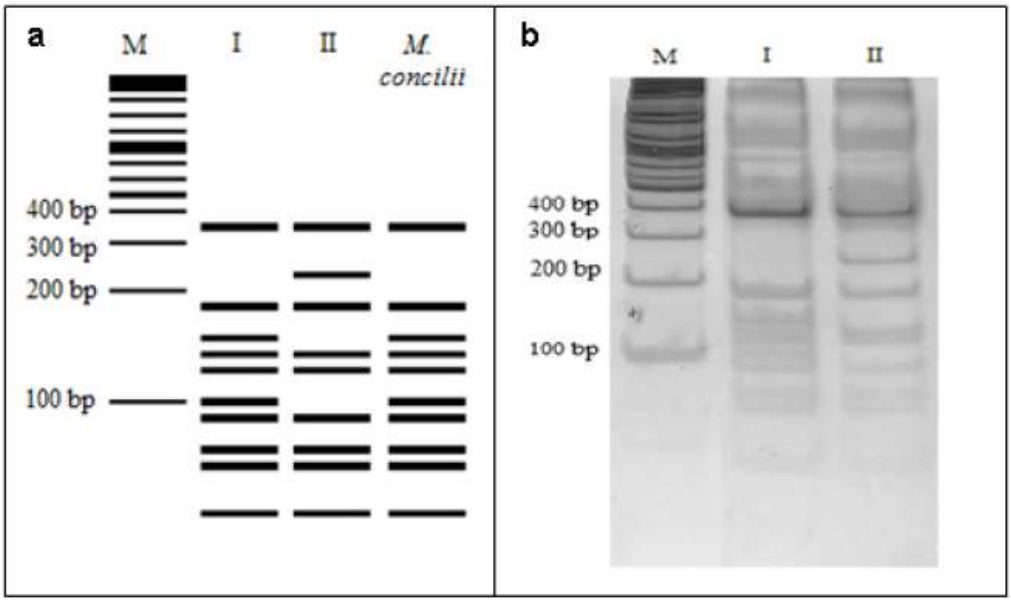


**Figure 1.** Calculated (a) and observed (b) *Hae*III RFLP patterns that occurred among 237 cloned archaeal 16S rRNA fragments from sludge taken from bottom and middle of the bioreactor and the recycling tank. Fragment sizes were inferred from DNA sequences of representative clones. M: 100 kb molecular mass marker (Fermentas, Hanover, Maryland, USA); RFLP patterns were labeled 'I'; Samali EB, II; Samali15' and *M. concilii* (M59146).

that was used as the outgroup (Figure 2).

Matches obtained for each clone sequenced had a similarity of ≥ 99% with those contained in the GenBank nucleotide database. All RFLP groups I and II clones sequenced were *Methanosaeta concilii*. Although, the RFLP group II clones had a unique *Hae*III restriction profile, partial sequencing of the clones revealed a 99% identity with *M. Concilii* (M59146), a sequence shared by all of the samples in RFLP group I. This distribution of the same species sequence in two different RFLP groups illustrates the limitation of relying on partial DNA sequences as well as RFLP only for representing the diversity of samples contained in a library.

## PCR-based DGGE analysis

A total of five methanogenic bands (EBM1, EBM2, EBM3, EBM4 and EBM5) (Figure 3a) were excised from the methanogenic DGGE fingerprints, re-amplified, purified and sequenced. Yielded sequences (334 bp) were analyzed by CHECK_CHIMERA software (Maidak et al., 1997) to scan for potential chimeric sequences and three were found chimeric. The remaining two (EBM2 and EBM4) were analyzed using the BLAST program. The RFLP groups' representatives (SamaliEB and Samali15) were in line with EBM2. Band EBM4 was found to be *Methanosarcina*. Although, band EBM2 correlated with the reference clones SamaliEB and Samali15 (*M. concilii)*, it was also excised and sequenced to confirm the identification of the band as *M. concilii*. Band EBM2 showed a 99% sequence similarity to the uncultured *Methanosaeta* sp. clone I2 that had been previously found in environmental samples taken from anaerobic biofilms. Band EBM4 showed a 97% sequence similarity to *Methanosarcina* sp. 48. The methanogenic DGGE pattern showed that the band which belonged to *Methanosaetaceae* appeared to be denser and sharper than the one of *Methanosarcina*. The double bands observed using archaeal primers; PARCH340f and PARCH519r (Figure 3b) was a result of degeneracy of the primers used (Piceno et al., 1999). The archeal PCR products using clones SamaliEB and Samali15 as DNA template did not align on the DGGE gel with the band related to SamaliEB lying lower which was due to a single nucleotide difference between the two amplicons (Figure 4). As shown in the Figure 4, one base pair G/C in SamaliEB is replaced with A/T in Samali15. As a matter of fact, DNA fragments richer in G/C are more stable and remain double-stranded until it reached higher denaturant concentrations (lower levels of the gel). Double-stranded DNA fragments migrate better in the acrylamide gel, while denatured DNA molecules become effectively larger and stop in the gel. In this study, a DNA fragment of higher G/C content stoped at a lower level of gel and by providing a narrow range of optimized gradient (50 to 60%), a subtle difference between the DNA fragments known as single nucleotide polymorphism (SNP) was detected. This confirmed the RFLP results which found clone SamaliEB to be a different strain of *M. concilii* in comparison with

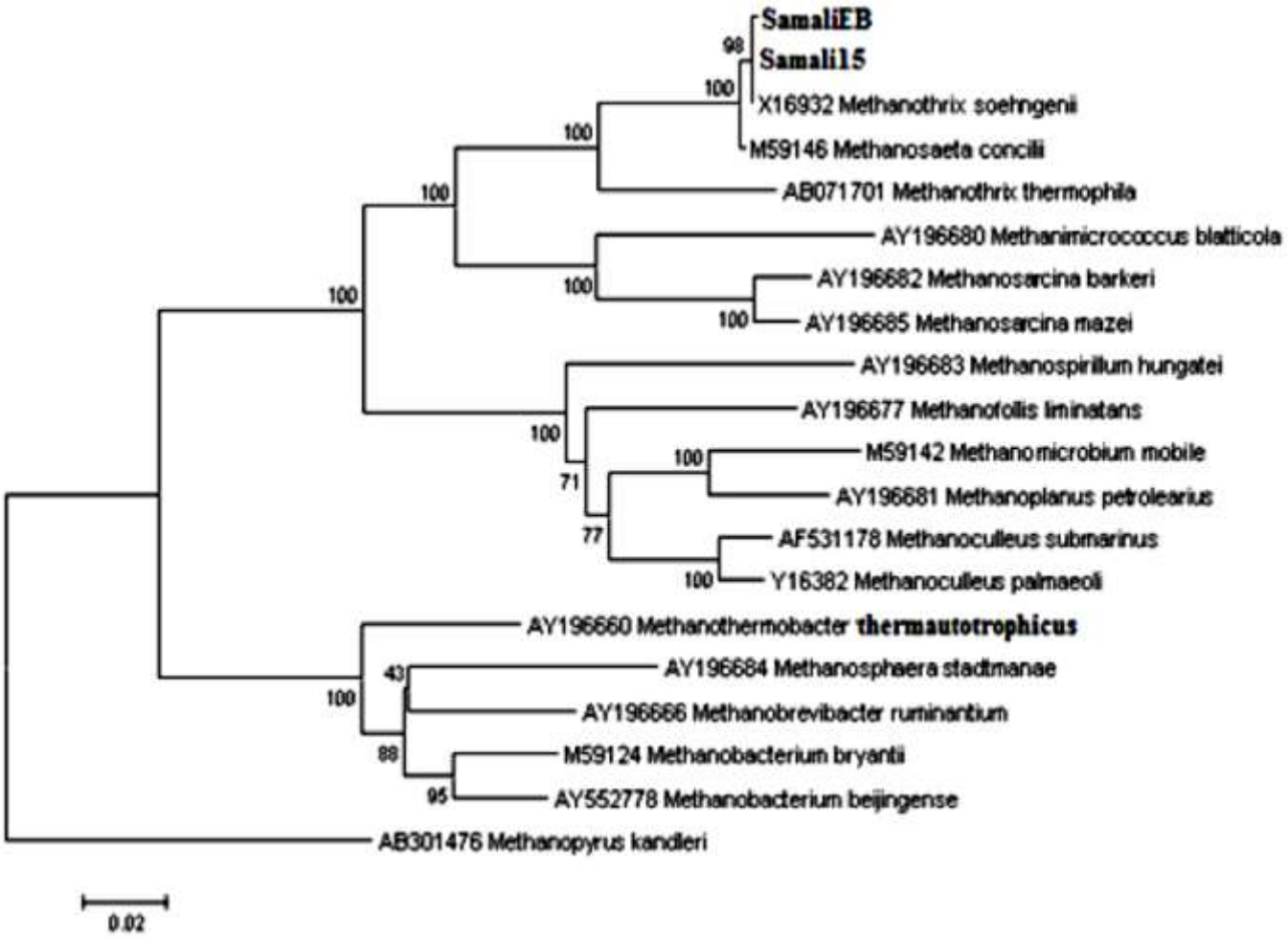


**Figure 2.** A phylogenetic tree, showing how clones SamaliEB and Samali15 are related to other methanogens.

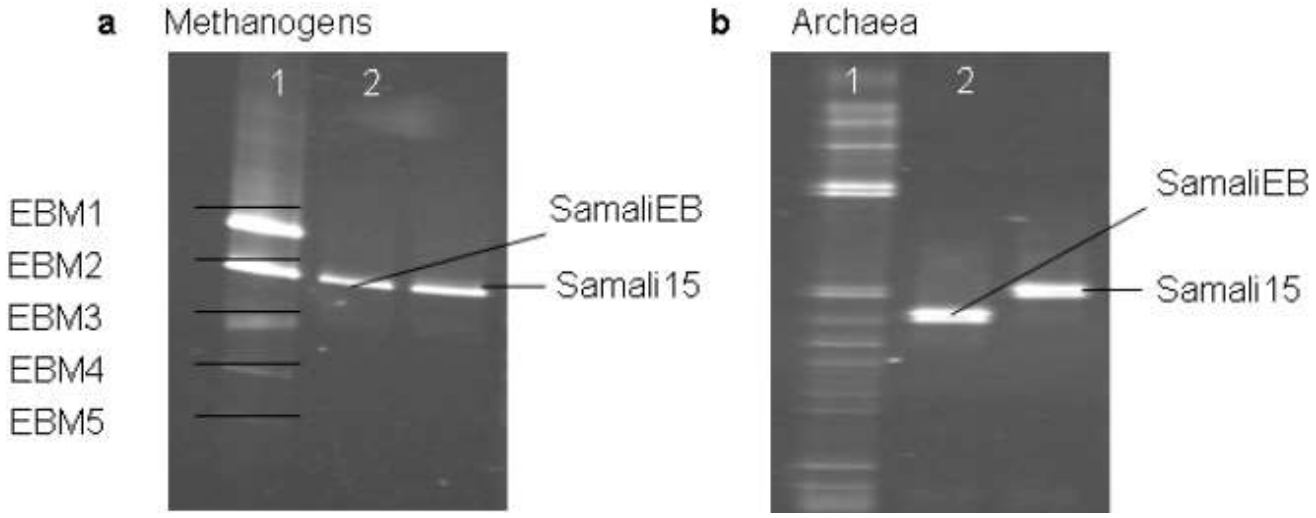


**Figure 3.** PCR-based DGGE fingerprints of the methanogens-denaturants gradient from 40 to 60% (a), and archaea denaturants gradient from 50 to 60% (b) present in the CDT treating POME using methanogenc and archaeal primers. Lane 1 (a): Methanogens present in the anaerobic bioreactor; Lane 1 (b) Archaea present in the anaerobic bioreactor; Lane 2 and 3: *Methanosaeta concilii* (SamaliEB and Samali15).

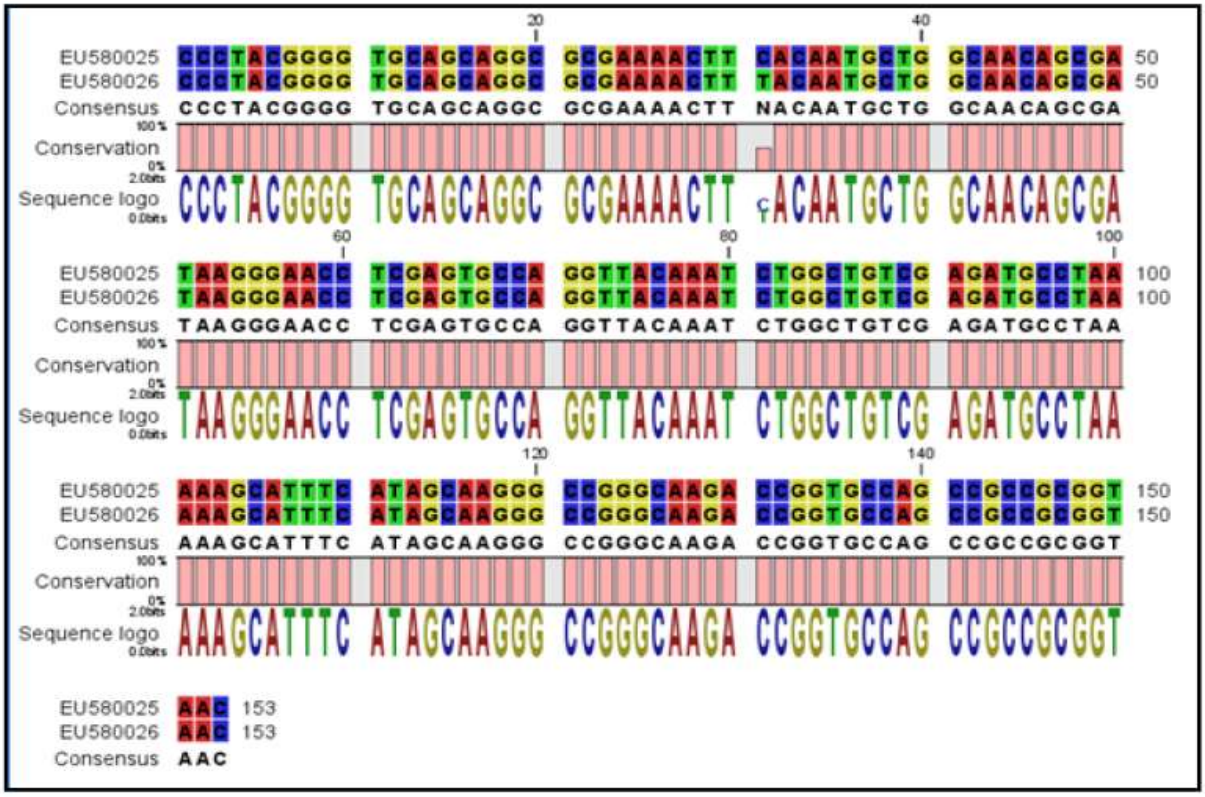


**Figure 4.** Alignment of the *Methanosaeta concilii* clone SamaliEB (EU580025) and *Methanosaeta concilii* clone Samali15 (EU580026) amplified by archaeal primers PARCH340f and PARCH519r using CLC Free Workbench version 4.1.2 (CLC Bio A/S, Denmark).

the other clones including clone Samali15 and the gene bank database. In combination with the DGGE fingerprint using Methanogens primers, just two of the archaeal bands.

## Analysis of sludge sample by FISH

Under optimal hybridization conditions, methanogens and bacteria were specifically visualized and detected using the corresponding probes. Figures 5 and 6 show representative micrographs of the fluorescent methanogenic and bacterial cells in the sludge sample double-stained with rhodamine-EUB338 and FITC-MSMX860. Solid and complex materials in digested sludge showed strong self fluorescence that pictures taken by a chilled CCD color camera with a modified RGB color balance helped distinguish the fluorescence of FITC-probes from the self fluorescence of the refuse materials. The self-fluorescence of refuse materials was yellowish in the fermented sample. The presence of members of the *Methanosaetaceae* and *Methanosarcinaceae* in sludge was also in accordance with DGGE results but, the presence of *Methanosarcinaceae* was not detected by PCR-cloning. All aceticlastic methanogens belong to the

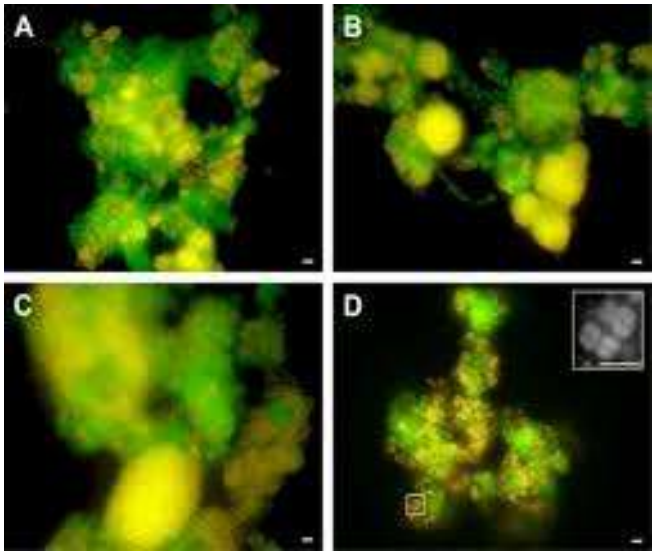


**Figure 5.** FISH staining of the sludge sample analyzed by confocal laser microscopy of *FISH* cells (a, b and c) and light microscopy (d). (a) *Methanosaeta concilii*; (b) bacteria; (c) simultaneously hybridized with rhodamine-labeled bacterial-domain probe (EUB338) (red) and FITC-labeled methanogens probe (MSMX860) (green) showing the consortium between methanogens and bacteria; (d) Light microscopy.

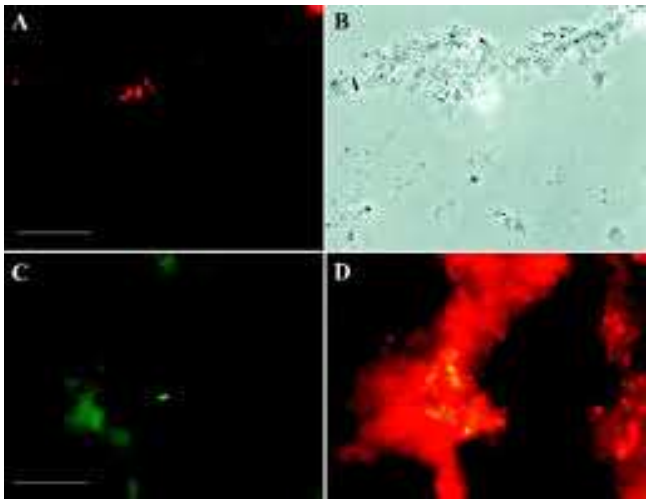


**Figure 6.** *FISH* staining of the sludge samples analyzed by confocal laser microscopy of fluorescent *in situ* -hybridized cells (a, c and d) and light microscopy (b). A fluorescent *in situ* -hybridized floc of *Methanosaeta concilii* (a); light micrograph of a floc of *Methanosaeta concilii* (b); filaments of *Methanosaeta concilii* (c); simultaneously hybridized with rhodamine-labeled bacterial-domain probe (EUB338) (red) and FITC-labeled methanogens probe (MSMX860) (green) showing Methanosarcina (d).

order *Methanosarcinales* comprising two families, *Methanosarcinaceae* and *Methanosaetaceae*. Among six genera belonging to *Methanosarcinaceae*, the genus *Methanosarcina* can only utilize acetate as a substrate. All members of the family *Methanosaetaceae* are aceticlastic (Garrity and Holt, 2001). The *Methanosaetaceae* and *Methanosarcinaceae* families differ in their physiology, biokinetics, and growth environment with respect to acetate concentrations. The family *Methanosaetaceae* has a high affinity for acetate accompanied with a relatively low growth rate, while the family *Methanosarcinaceae* has a much lower substrate affinity but with a higher growth rate, that describes why the POME anaerobic digester used in the present study was dominated by *Methanosaetaceae* as the acetate concentration in the sludge digester favourable to them and acetate is the only substrate that supports the growth of *Methanosaetaceae* (Garrity and Holt, 2001). The presence of members of the *Methanosaetaceae* in anaerobic bioreactors has been widely reported (Chan et al., 2001; Batstone et al., 2004; Hulshoff Pol et al., 2004). It is also generally known that the presence of *Methanosaeta* species leads to an improved granulation process and it results in a more stable bioreactor performance (Hulshoff Pol et al., 2004). The bioreactor used in this study was designed for continuous or intermittent mixing to maximize the contact between POME and the microbes. This differs from the upflow anaerobic sludge blanket (UASB) design in which granules are formed. However, *Methanosaetaceae* is assumed to form the initial nuclei followed by floc formation as well as described in Spaghetti Theory (Wiegant, 1987). Therefore, *Methanosaetaceae* plays an important role in floc formation in CDT and consequently higher performance of the bioreactor in terms of COD removal and methane production. However, this contrasts with the findings of Sallis and Uyanik (2003) indicating that associations between mixed bacterial populations and inert material formed the so-called nuclei at the initial stage of granule formation and the predominance of *Methanosaetaceae* occurred only when the granules had developed. This methanogen is one of the main species responsible for the conversion of acetate to methane. Species within this family use acetate as their sole energy source, which is metabolized into methane and carbon dioxide. These organisms can be found in anaerobic sediments and anaerobic sludge bioreactors (Garrity and Holt, 2001). In order to maximize the efficiency of the bioreactor in terms running operation; for example, organic loading rate (OLR) (kg COD/$m^3$/day), pH and alkalinity is of great importance. In this study, high COD removal 93% was observed which was higher than the findings of Choorit and Wisarnwan (2007) at mesophilic conditions. This was achieved due to a higher retention time (10 days) in comparison with seven days used in their study. However, the methane production of 1.35 L/L[reactor]/day was lower which could be attributed to a different bioreactor design (continuous stirred tank reactor (CSTR)) which enables higher application of OLR. Hence, higher methane production could be achieved.

## Quantification of methanogens by *FISH*

The results of FISH counting showed that the total number of bacteria in the sludge sample was 1.4 x $10^5$ cells/ml sludge. The major group in the treated sludge sample was the *Methanosaeta* sp. with a count of 2 x $10^8$ cells/ml sludge. In contrast, the number of *Methanosarcina* sp. was almost 1000 times lower and was equal to that of bacteria. In other words, for each one thousand cells of *Methanosaeta* sp., there was just one single cell of *Methanosarcina* sp. present in the sludge. These results indicate that, the closed fermentation of POME led to the domination of *Methanosaeta* sp. in particular *M. concilii* emphasizing on the importance of the substrate. The VFAs concentration of 517 mg/L showed high activity of methanogens which was reflected by high methane production (56% v/v methane of 1200 $m^3$ biogas/day) and satisfactory COD removal of 93%. The high number of methanogenic archaea (2 x 108 cells/ml sludge) in comparison with the bacteria cells (1.4 x $10^5$ cells/ml sludge) might be attributed to the stage of sampling at which the highest methane production was recorded. The finding also showed that quantitative FISH using group and species specific rRNA-targeted probes could be applied to the direct analysis of semi-solid POME

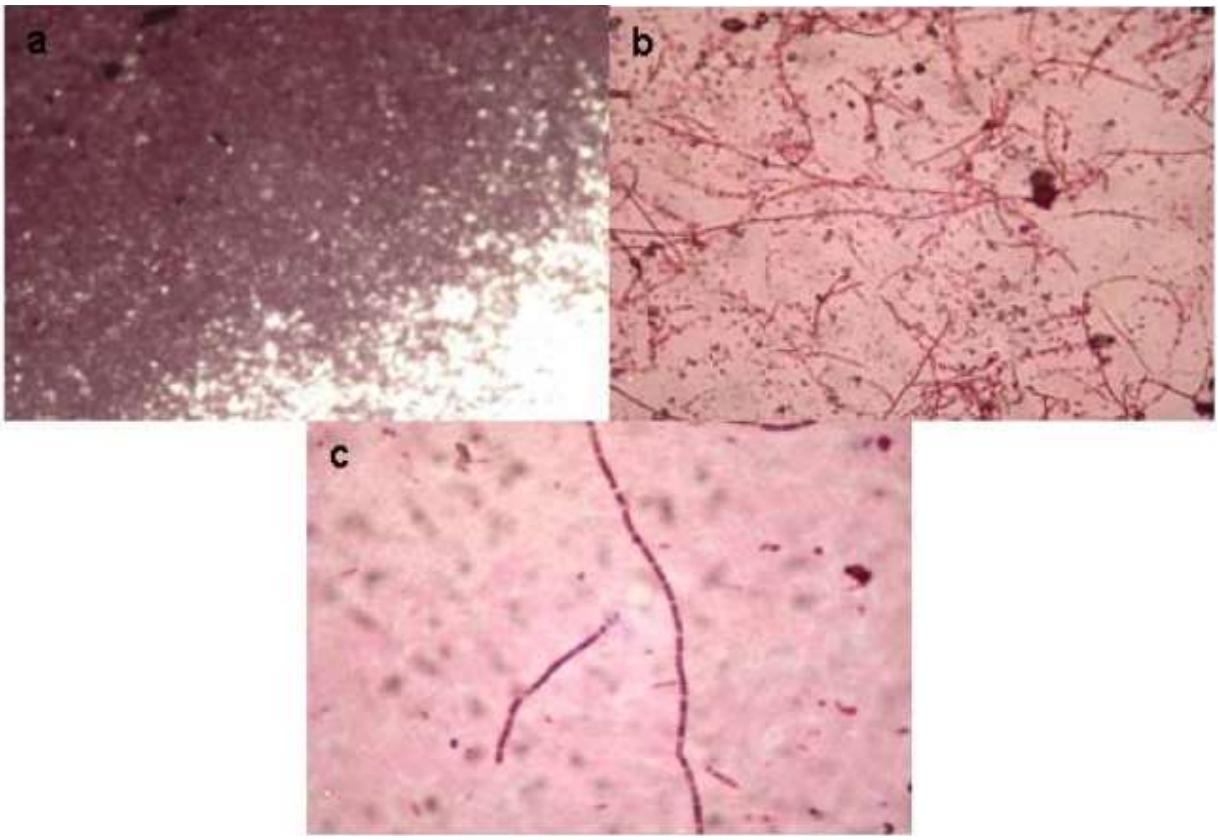


**Figure 7.** Light micrographs of POME sludge at a magnification of: (a) 10x; (b) 40x; (c) 100x. Gram staining test showed that the filamentaous-like Methanosaeta sp. were dominant species in the CDT sludge.

sludge to provide a good estimate of the dominant species of methanogens which plays an important role in optimizing running conditions of the digester for achieving the highest COD removal and methane production. The PCR-cloning, DGGE analysis and species-specific *FISH* confirmed that *M. concilii* was the dominant species during the fermentation of POME.

## SEM

The presence of *Methanosaeta* sp. in the studied industrial activated sludge was corroborated by its morphology using light microscopy (Figure 7). It has been reported that *Methanosaeta* sp. can form filaments of 10 to 300 cells (Garrity and Holt, 2001). The scanning electron microscopy provided a better resolution of *Methanosaeta* sp. than that obtained by conventional light microscopy. Filamentous like *Methanosaeta* sp. was observed and found to be the dominant species in the CDT system (Figure 8a and 8b). It is clearly seen that the microorganisms bend around the organic matters and thus play an important roles in flocs/aggregate formation during POME treatment. Nevertheless, the actual size of the flocs or aggregates formed during the CDT process was not monitored. *Methanosaeta* sp. was normally determined to be at the core of the granules formation in UASB system (Hulshoff Pol et al., 2004). A demand to be strictly anaerobic and consume solely acetate makes them to be located at the core and other archaea-like *Methanosarcina* sp., fermentative and facultative bacteria are placed on the outer granules layer to prevent oxygen contamination (Savant et al., 2002). Some other microbes that coexisted in the digester are shown in Figure 8c. However, *Methanosaeta*-like microorganisms outnumbered short rods and cocci.

## Concluding remarks

The results indicate that filamentous acetate utilizing methanogens detected in anaerobic bioreactor treating POME belong to the genus *Methanosaeta* based on the cell-morphology, and the phenotypic and phylogenetic characteristics described above. The data obtained suggest that *M. concilii* is the most abundant methanogen in POME anaerobic digestion and that it plays an important role in methane production from acetate and the optimum condition for its growth should be considered when an attempt is made to treat POME anaerobically.

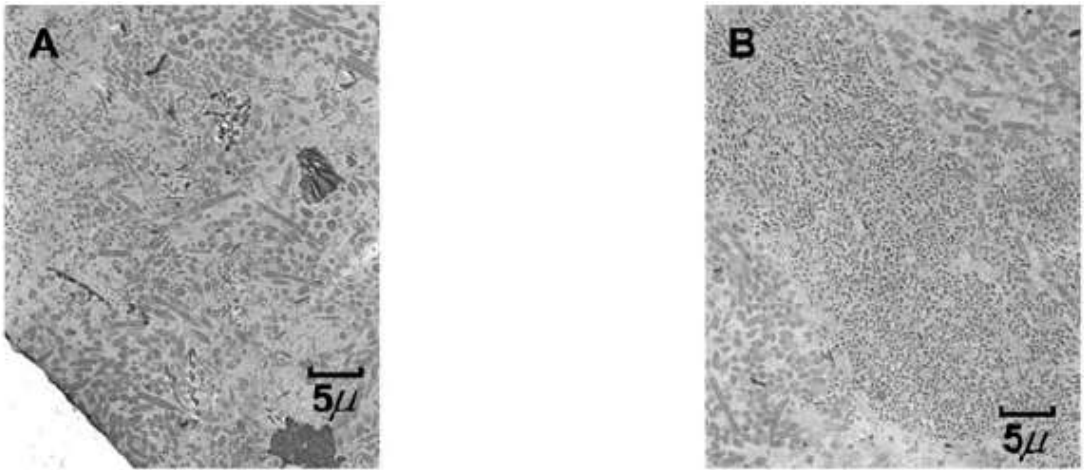


**Figure 8.** Scanning electron micrographs of sludge showing microorganisms in the flocs of POME sludge.

It also revealed the presence of possibly new strain of *Methanosaeta* in the bioreactor for treating POME.

## Conflict of interests

The author did not declare any conflict of interest.

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