Full Length Research Paper

Comparative study of methods for extraction and purification of environmental DNA from high-strength wastewater sludge

Meisam Tabatabaei[1,4]*, Mohd Rafein Zakaria[1], Raha Abdul Rahim[2], Norhani Abdullah[3] andré-Denis G. Wright[5], Yoshihito Shirai[6], Mehdi Shamsara[7], Kenji Sakai[8] and Mohd Ali Hassan[1]

[1]Department of Bioprocess Technology, Universiti Putra Malaysia 43400 Serdang, Selangor, Malaysia.
[2]Department of Cell and Molecular Biology, Universiti Putra Malaysia 43400 Serdang, Selangor, Malaysia.
[3]Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia 43400 Serdang, Selangor, Malaysia.
[4]Microbial Biotechnology and Biosafety Department, Agricultural Biotechnology Research Institute of Iran (ABRII), Seed and Plant Improvement Institute's Campus, 31535-1897, Mahdasht Road, Karaj, Iran.
[5]Department of Animal Science, University of Vermont, Burlington, VT, USA.
[6]Life Science and Systems Engineering, Kyushu Institute of Technology, 2-4 Hibikino, Wakamatsu-ku, Kitakyushu 808-0196, Japan.
[7]National Institute for Genetic Engineering and Biotechnology, 14155-6343, Tehran, Iran.
[8]Labrovatory of Soil Microorganisms, Department of Plant Resources, Graduate School of Bioresources and Bioenvironmental Sciences, Kyushu University, 6-10-10Hakozaki, Higashi-ku, Fukuoka, Japan, 812-8581.

DNA extraction from wastewater sludge (COD 50000 and BOD 25000 mg/l) was conducted using nine different methods normally used for environmental samples including a procedure used in this study and the results obtained were compared. The quality of the differently extracted DNAs was subsequently assessed by measuring humic acid concentration, cell lysis efficiency, polymerase chain reaction (PCR) amplification of methanogenic and eubacterial 16S rDNA. The protocol developed in this study was further evaluated by extracting DNA from various high-strength wastewater sludge samples, denaturing gradient gel electrophoresis (DGGE) and fluorescent *in situ* hybridization (FISH) analyses. The results revealed that great differences existed among the nine procedures and only a few produced satisfactory results when applied to high-strength wastewater sludge. Thermal shock alone was shown inefficient to disrupt the methanogenic cell wall to release the DNA. The method presented in this study (Procedure 9) is generally recommended because of the low concentration of contaminants and its high efficiency despite its simplicity.

Key words: High-strength wastewater sludge, DNA extraction, environmental samples, humic acids, denaturing gradient gel electrophoresis, fluorescent *in situ* hybridization

INTRODUCTION

Accurately determining the presence of microorganisms in wastewater sludge is imperative for its efficient treatment. There are numerous strategies for the detection of specific archaea and bacteria from environmental samples (Amann et al., 1995). It has been shown that conventional methods for studying microbial diversity, such as plating on selective media, are unreliable because only a small fraction of the bacterial species present in the natural habitat will grow on synthetic media (Amann et al., 1995). A newer approach is to estimate the microbial diversity by characterizing the DNA or RNA from a sample without

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*Corresponding author. E-mail: meisam_tab@yahoo.com. Tel: +603-89467590. Fax: +603-89467593.

Table 1. Process parameters recorded at the time of sampling, that is, after 80 days of operation at the steady-state condition.

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

cultivation procedures (Bourrain et al., 1999). This approach has been successfully applied on soil (Kozdroj and van Elsas, 2001), coastal lagoons (LaMontagne and Holden, 2003), biofilm (Hu et al., 2003) and sludges (Tabatabaei et al., 2009). However, the availability of effective DNA extraction methods is essential to obtain purified DNA to achieve this goal. As a result, a large number of protocols have been suggested for the extraction of total microbial DNA from soil (Jacobsen and Rasmussen, 1992; Picard et al., 1992; Tien et al., 1999), sediments, activated sludges (Ogram et al., 1987; Tsai and Olson, 1991; Miller et al., 1999; Yu and Mohn, 1999) and water samples (England et al., 2001; Rivera et al., 2003). These methods are generally based on three steps: physical, chemical and or enzymatic lysis for direct or indirect extraction of DNA followed by purification.

Unlike pure culture samples, factors such as the complex microbial flora and the composition of wastewater sludge could result in low DNA extraction rates or purity levels. On the other hand, an effective DNA extraction method and consequently successful description of archaeal and bacterial species and their diversity in wastewater sludges plays a key role in the characterization of populations favoring floc or granule structuration resulting in efficient waste water treatment.

The efficiency of the different extraction methods has not been completely tested when applied to high-strength wastewater sludge samples. Therefore, the necessity of comparing the commonly used methods as well as striving to develop faster, simpler and more economically reasonable methods which result in relatively higher yield and purity seems inevitable. In addition, the applicability of various methods used for extracting environmental DNA in order to study different domains of life that is, prokaryotes and archaea should be tested. The aim of this study was to compare the relative efficacy of nine microbial DNA extraction methods applied to the high-strength wastewater sludge in order to determine the best DNA extraction protocol to use for subsequent polymerase chain reaction (PCR) and studying the microbial diversity of wastewater sludge.

MATERIALS AND METHODS

Sampling

High-strength wastewater sludge (COD, 50000 and BOD, 25,000 mg/l) was collected from a 500 m^3 closed digester tank (CDT) for the anaerobic treatment of palm oil mill effluent (POME) located in FELDA Serting Hilir Palm Oil Mill, Negri Sembilan, Malaysia. The CDT was operated under mesophilic condition (32 - 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 the middle sampling port. The process parameters recorded at the time of sampling are shown in Table 1. The wastewater sludge samples were transported to the laboratory in sterile 50 ml falcon tubes placed in crushed ice and stored at -20°C until DNA extractions were made.

Sample preparation and DNA extraction

For the comparative study, the wastewater sludge samples were prepared and DNA was extracted based on nine different methods.

Procedure 1

The wastewater sludge sample was washed and lysed based on the protocol adopted from Orsini and Romano-Spica (2001). Sample preparation was carried out by low speed centrifugation and homogenization in buffer (ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), polyvinylpyrrolidone (PVP). Samples were heated in a microwave oven at 600 - 700 W for 1 min to disrupt the cell walls and proteins were removed by phenol : chloroform : isoamylalcohol (25:24:1). The extracted DNA was then purified with an Elutip-d column (Schleicher and Schuell, Keene, N.H.) fitted with cellulose acetate membrane filter (0.45 μm pore size). DNA was recovered from the membrane according to the protocol given by the manufacturer.

Procedure 2

As described by Yeates et al. (1997), wastewater sludge sample was prepared by low speed centrifugation and homogenization in buffer (EDTA) and cell walls were lysed by bead-beating and chemical lysis (SDS, 65°C, 1h). Proteins were removed by phenol : chloroform: isoamylalcohol (25:24:1). DNA purification was conducted as described in procedure 1.

Procedure 3

This procedure was based on the technique described by Bourrain et al. (1999) with modifications (Lemarchand et al., 2005) and applies freeze-thaw and lysozyme-SDS lysis technique. The waste-water sludge pellet was prepared by low speed centrifugation and homogenization in TENP buffer (Tris, EDTA, Nacl and PVP). Cell wall was then disrupted by freezing and thawing, lysozyme treatment and chemical lysis (SDS, 37°C, 2h). Proteins were removed by phenol : chloroform : isoamylalcohol (25:24:1) and DNA purification was conducted as described in procedure 1.

Procedure 4

This protocol was originally used by Ogram et al. (1987). The cells
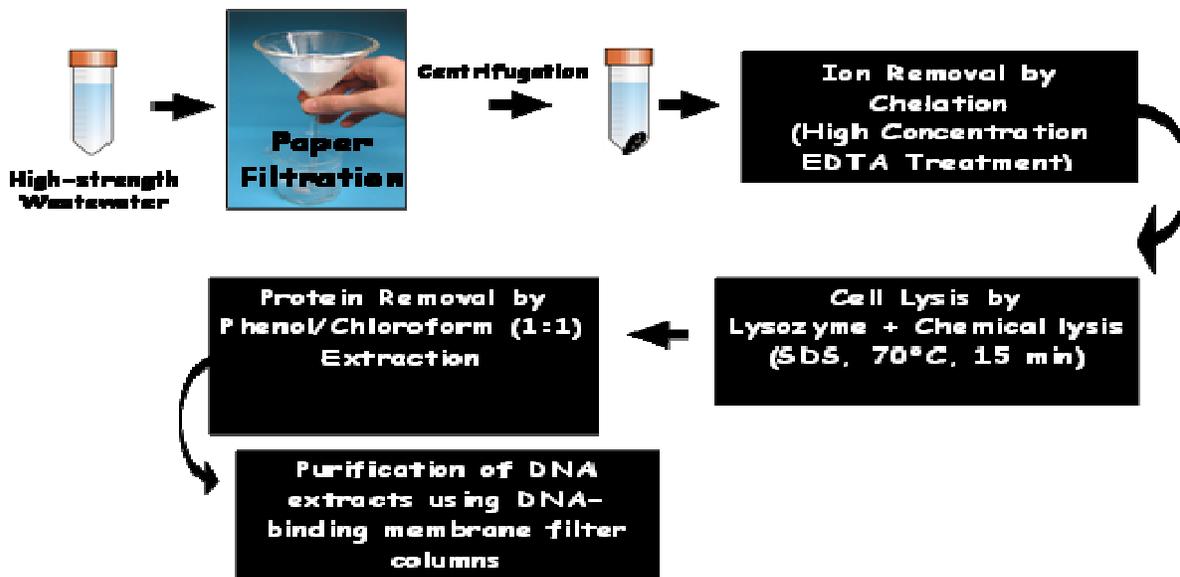
Figure 1. Schematic diagram of procedure 9.

were disrupted by bead-beating and chemical lysis (SDS, 70°C, 1h) and proteins were removed by phenol: chloroform: isoamylalcohol (25:24:1) extraction. Purification step was carried out by adding 3 M potassium acetate to the concentrated samples to the final concentration of 0.5 M and stored on ice for 2 h to precipitate the organic carbon in the sample in the particular humic acids. The resulting brown precipitate was removed by centrifugation and the supernatant was collected for further purification. The supernatants were purified by CsCl-EtBr (cesium chloride-ethidium bromide) gradient centrifugation and DNA was stored at 4°C.

Procedure 5

This procedure was adopted from Bourrain et al. (1999) on activated sludge samples with modifications (Lemarchand et al., 2005) and was based on sample preparation by low speed centrifugation, sonication and homogenization in buffer (EDTA). Cell wall lysis was carried out by lysozyme, proteinase K treatment and chemical lysis (SDS, 37°C, 2h). Finally phenol/chloroform/isoamyl alcohol DNA extraction was used for proteins removal and DNA purification was conducted as described in procedure 1.

Procedure 6

This procedure was based on the technique previously described by Yu and Mohn (1999) with slight modification and is based on bead beating and chemical lysis (SDS) to disrupt the cell walls. Ammonium acetate purification method was used to remove the protein impurities and additional DNA purification was conducted as described in procedure 1.

Procedure 7

This procedure was first described by Jacobsen and Rasmussen (1992). The sample preparation step is based on shaking in buffer (4°C 1 h), cation- exchange resin and low speed centrifugation. Cell wall disruption was conducted by lysozyme, proponase treatments

and chemical lysis (SDS, 65°C, 10 min). Proteins were removed by phenol: chloroform: isoamylalcohol (25:24:1) and DNA purification was carried out as described in procedure 4.

Procedure 8

This protocol was adopted from the method of Tsai and Olson (1991). Wastewater sludge sample was prepared by low speed centrifugation and washing with phosphate buffer followed by cell wall disruption step by using lysozyme treatment, chemical lysis (SDS) and freezing/thawing. Finally, DNA purification was conducted as described in procedure 1.

Procedure 9

This protocol was first developed in this study (Tabatabaei et al., 2008; Malaysian Patent PI20082842 and PCT/MY2009/000143) (Figure 1). 25 ml of the wastewater sludge sample was diluted with sterile water (1:1), vortexed vigorously and filtered using ultra-violet (UV)-sterilized 125 mm filter paper (Whatman® No. 541). The samples were thoroughly homogenized by vortexing vigorously in order to make sure that the attachments between the microbes and particles have been removed and that the microbial fraction has been released into the aqueous phase. The papers were also sterilized prior to use by UV radiation to prevent the risk of introducing any source of contamination. The effluent was then centrifuged at 8,200 × g for 15 min and the pellet was resuspended in 10 ml of 0.5 M EDTA–Na, pH 8.0 and left at room temperature for 10 min before the enzymatic lysis. Ten ml of the lysis buffer (10 mM Tris, 1 mM EDTA with 2 mg/ml lysozyme, pH 8.0) was added to the slurry and was mixed and incubated in a 37°C water bath for at least 30 min. A 10 % (w/v) SDS was added to a final concentration of 0.5 % and the sample was incubated at 70°C for 15 min. The sample was mixed very gently with an equal volume of phenol/chloroform (1:1) and centrifuged at 2,000 × g for 10 min. The upper phase was collected and the phenol/chloroform step was repeated to remove traces of proteins. Then sodium acetate (3 M, pH 5.2) at 10% of the total volume was added to the supernatant and nucleic acids in the aqueous phase (≈22 ml) were precipitated

Table 2. PCR primers and *FISH* probes targeted 16S rDNA/rRNA regions used in this study.

Primer[a]/ Probe	Positions (bases)[b]	Target	Sequence	Reference
Met86F	86 - 101	Methanogens 16S rDNA	5' GCT CAG TAA CAC GTG G	Wright, 2003
Met1340R	1340 - 1325	Methanogens 16S rDNA	5' CGG TGT GTG CAA GGA G	Wright, 2003
pAf	8 - 28	Eubacteria 16S rDNA	5' AGA GTT TGA TCC TGG CTC AG	Whitby, 2001
pHr	1542 - 1522	Eubacrteria 16S rDNA	5' AAG GAG GTG ATC CAG CCG CA	Whitby, 2001
518R	518 - 534	Eubacteria V3 region	5' ATT ACC GCG GCT GCT GG	Yu, 2004
357F	341 - 357	Eubacteria V3 region	5' CCT ACG GGA GGC AGC AG	Yu, 2004
PARCH340f	340 - 357	Archaea, V3 region	5' CCC TAC GGG G(C/T)G CA(G/C) CAG	Nicol, 2003
PARCH519r	519 - 533	Archaea, V3 region	5' TTA CCG CGG C(G/T)G CTG	Nicol, 2003
GC clamp[c]			5`-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG	Chan, 2001
MSMX860	860 - 880	Methanogens 16S rDNA	5'- GGC TCG CTT CAC GGC TTC CCT	Crocetti, 2006
EUB338	338 - 355	Eubacteria 16S rDNA	5'- GCT GCC TCC CGT AGG AGT	Daims, 1999
EUB338-II	338 - 355	Eubacteria 16S rDNA	5'- GCA GCC ACC CGT AGG TGT	Daims, 1999
EUB338-III	338 - 355	Eubacteria 16S rDNA	5'- GCT GCC ACC CGT AGG TGT	Daims, 1999

[a]f, Forward primer; r, reverse primer; [b]the numbering of positions is based on *E. coli* 16S rRNA except for Met86F and Met1340R which is based of *M. mobile* 16S rRNA (GenBank accession number M59142); [c] the GC clamp was attached to the 5' end of the 357F and PARCH340f primers.

an equal volume of cold isopropanol at -20°C for 15 min. Bulk nucleic acids were obtained by centrifugation at 18,500 × g for 10 min at 4°C and the pellet was washed with 70% ethanol and recentrifuged at 18,500 × g for 6 min at 4°C. The DNA pellet was vacuum dried for 1 h at room temperature (25°C ± 2), resuspended in 1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). Innovatively, for further purification of DNA extracts, DNA-binding membrane filter columns which were provided with the plasmid extraction kit (Yeastern Biotech, Taiwan) were used and binding, washing and elution steps were carried out based on the manufacturer's manual.

Gel electrophoresis

In all procedures described, RNA was eliminated from the sample by a RNaseA treatment (1 mg/ml, 20 min at 37°C). Then, the DNA extracts (5 µl) were run on 1% agarose gel at 130 V for 1 min followed by 45 min at 70 V, then stained with GelRed (Invitrogen, CA, USA) or ethidium bromide and transilluminated. The resulting PCR products were run on 1% agarose gel at 70 V for 45 min. DNA markers, Lambda DNA/HindIII Marker and GeneRuler™ DNA Ladder

Mix (Fermentas, Hanover, Maryland, USA) were used.

Comparison of extraction efficiencies

Nine different protocols were used for isolating nucleic acids from wastewater sludge. For each procedure, four replicates were analyzed and six parameters (that is, quantity, purity, humic acid content, fragmentation level of DNA, lysis efficiency and time) were compared to evaluate the performance of the different methods. The amount and purity of extracted DNA were assessed by absorbance at 260 nm and the ratio of absorbance at 260 and 280 nm, respectively. Although DNA quality is basically marked by the ratio of A_{260} to A_{280} higher than 1.8. However, for environmental DNA, the DNA quality was considered reasonable when the ratio was >1.50 (Lemarchand et al., 2005). The occurrence of fragmentation of the extracted DNA was determined by electrophoresis of each DNA through a 1% (w/v) agarose gel. Humic acid level was measured by absorbance reading at 340 nm (Howeler et al., 2003). Triplicate standard curves were created by making serial dilutions (0.1 - 100 ng/µl) of commercial humic acid mixture (Aldrich Chemical). Lysis efficiency was

checked by 4′, 6-diamidino-2-phenylindole (DAPI) staining as described by Howeler et al. (2003).

PCR primers and amplification conditions

In order to evaluate the protocols, the extracted DNAs from wastewater sludge in a 500 m³ bioreactor were used directly in PCR reactions to amplify the 16S rDNA gene from methanogenic archaea and eubacteria. Each PCR mixture (25 µl) contained 2.5 µl PCR buffer (Fermentas, Hanover, Maryland, USA), 0.5 µl of 10 mM dNTPs, 2.5 µl of 25 mM MgCl₂, 0.5 µl of each methanogen primer Met86F and Met1340R (Wright and Pimm, 2003), or eubacterial primers pAf and pHr (Whitby et al., 2001) (Table 2) and 0.2 µl (1 unit) of *AmpliTaq* DNA polymerase (Fermentas, Hanover, Maryland, USA). This mixture was completed to 25 with 18.3 µl of sterile distilled water containing 100 ng of genomic DNA extracted from wastewater sludge samples. A positive control, consisting of 50 ng of *Escherichia coli* and *Methanosaeta concilii* DNA for eubacterial primers pAf/pHr and methanogen primer Met86F/Met1340R, respectively and a negative control (no DNA) were added to each amplification run. An aliquot (5 µl) of each amplification

reaction was analyzed on 1% (w/v) agarose gel in 1 × TBE buffer (pH 8). PCR was performed in a Perkin Elmer Gene Amp system 9600 and the reaction parameters for methanogenic and eubacterial primers were 35 cycles of 94°C for 40 s, 54°C for 50 s and 72°C for 90 s and 25 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, respectively.

Further validation of the patented protocol (Procedure 9)

DNA extraction from various wastewater sludge samples

The patented protocol for DNA extraction in this study was further validated using various high-strength wastewater sludge samples obtained from the palm oil mill anaerobic pond (COD > 40,000 mg/l and BOD > 20,000 mg/l), recycling tank (COD > 70,000 mg/l and BOD > 34,000 mg/l) and municipal wastewater treatment facilities (COD > 55,000 mg/l and BOD > 30,000 mg/l) and pure cultures of methanogens. The methanogens were pure culture of *Methanothrix concilii* (ATCC, 35696) and *Methanosaeta thermophila* (ATCC, BAA-1166). They were grown in 1-l crimp-top culture bottles using the roll tube technique. *M. thermophila* was grown at 61°C and pH 6.5 in defined mineral salts (MS) medium as described by Valentine et al. (2000). *M. concilii* was grown using MS medium but at 35°C and pH 7.6 (Steinhaus et al., 2007). The initial gas phase composition of both *M. thermophila* and *M. concilii* was 5 × 10⁴ Pa for CO_2 and 5 × 10⁴ Pa for N_2 (Valentine et al., 2000).

PCR amplification and DGGE analysis

In order to investigate the reproducibility of the patented method (Procedure 9), DGGE-PCR amplification of archaeal and eubacterial 16S rDNA was performed. The two PCR products of 16S rDNA region were both analyzed by DGGE. The universal archaeal primers PARCH340f and PARCH519r (Nicol et al., 2003) based on the *E. coli* 16S rRNA gene sequence and eubacterial primers 357F and 518R were used (Yu and Morrison, 2004) (Table 2). The guanine-cytosine (GC) clamp as described by Chan et al. (2001), was included on the 5' end of the forward primer PARCH340f and 357F. The PCR products of 16S rDNA region were performed by a DGGE apparatus (Dcode™ system, Bio-Rad, USA). The gradient extended from 30 to 70% of denaturants consisting of 7 M urea and 40% formamide. DGGE was operated in 1 × TAE buffer at 60°C and 100 V.

The sharpest bands from the archaeal profile were punched out with a sterile pasture pipette and used as a template in a re-amplification using the archaeal primers PARCH340f and PARCH 519r. The resultant PCR products were purified using the Mag Extractor-PCR and Gel Clean up-kit (Toyobo, Japan) according to the manufacturer's instructions, cloned and sequenced on both strands using an ABI 3730 XL DNA sequencer. The sequences obtained by PCR were compared to 16S rRNA gene sequences database using center for biotechnology information's basic local alignment search tool (NCBI's BLAST) interface.

Fluorescent in situ hybridization (FISH)

In order to confirm the efficiency of the patented procedure, the microbial diversity in the same sample used for DGGE was analyzed using the probe MSMX860, complementary to the 16S rRNA of some methanogens including *Methanosarcina* spp., *Methanococcoides* spp., *Methanolobus* spp., *Methanohalophilus* spp. and *Methanosaeta* spp. (Crocetti et al., 2006). To target the sludge bacteria, the 16S rRNA probe EUB338, EUB338-II and EUB338-III were used (Daims et al., 1999). Oligonucleotides and their fluorescent derivatives (5'-labelled with either FITC or rhodamine)

were purchased from Hokkaido System Science (Sapporo). Cells were fixed, hybridized and visualized as described by Tabatabaei et al. (2009). Fluorescence was observed using an epifluorescence microscope (BX50; Olympus, Tokyo, Japan) and photomicrographs were taken using a chilled 3-CCD color camera (640 x 483 pixels, C5810; Hamamatsu Photonics, Shizuoka, Japan).

RESULTS AND DISCUSSION

Nine different extraction procedures were examined to check their relative efficiencies in extracting and purifying nucleic acids from wastewater sample (Table 3). All of the protocols successfully extracted DNA from the samples but with varying efficiency (Figure 2 and Table 4). As indicated by cells bright DNA fluorescence after staining with DAPI, all procedures except Procedure 1 had an efficiency of > 95%. Thermal shock alone as the lysis step in Procedure 1, produced the weakest result of 60% in disrupting the cell wall.

The DNA generated by all the procedures prior to the purification step, possessed a brownish color due to contaminants (humic acids) resistant to the extraction process (Table 4). Procedure 9 resulted in the purest extracted DNA both after and before purification step, 480 and 12 ng humic acid/µl DNA, respectively. Moreover, this method even without undergoing the final purification step resulted in approximately 50% less contamination load which facilitated the final purification step and ultimately led to a comparatively higher purity. This also stressed the efficiency of the method as it well out competed the commercially available DNA purification columns in this case Elutip-d column (Schleicher and Schuell, Keene, N.H.) used in Procedures 1, 2, 3, 5, 6 and 8 in this study. Of the nine protocols, Procedure 2 resulted in the highest yield of DNA (5.9 µg DNA/g of sample) but had a medium degradation level and a lower than average purity level (coefficient of 1.3). Although the purity coefficient is not adequate (Lemarchand et al., 2005), the quality of the extracted sample appears suitable since the resuspended DNA was successfully amplified (Figures 3 and 4). In addition, by comparing the results obtained for DNA quantification by spectrophotometry and the quantity of DNA observed on agarose gel, the absorbance at 260 nm seemed to occasionally overestimate the concentration presumably due to ultra-violet light absorbing contaminants (Lemarchand et al., 2005).

Great variability in extraction efficiencies was observed (from 0.35 to 4.50 µg) among the eight other protocols, as well as between replicates for Procedure 4. Procedures 2 and 4 differed in the sample preparation and DNA purification steps. In the forth procedure which was the most time consuming technique (27 h), the lower purification of DNA could be attributed to the use of CsCl- EtBr gradient centrifugation instead of the Elutip-d® column purification method. These two techniques used a physical and chemical lysis procedure. Due to the presence of aggregates in wastewater influent, mini-bead beating, which is

Table 3. Comparison of DNA extraction methods.

Procedure	Sample preparation	Cell lysis	Protein removal	Purification	Reference
1	Low speed centrifugation and homogenization in buffer (EDTA, SDS, PVP)	Microwave (thermal shock)	P:C:I[a] (25:24:1)	Elutip-d® column	Orsini, 2001
2	Low speed centrifugation and homogenization in buffer (EDTA)	Bead-Beating + Chemical lysis (SDS, 65 °C, 1h)	P:C:I (25:24:1)	Elutip-d® column	Yeates, 1997
3	Low speed centrifugation and homogenization in TENP buffer (Tris, EDTA, Nacl and PVP)	Freezing and thawing + Lysozyme + Chemical lysis (SDS, 37 °C, 2h)	P:C:I (25:24:1)	Elutip-d® column	Bourrain, 1999; Lemarchand, 2005
4	-	Bead-Beating + Chemical lysis (SDS, 70 °C, 1h)	P:C:I (25:24:1)	CsCl-EtBr (cesium chloride-ethidium bromide) gradient centrifugation	Ogram, 1987
5	Low speed centrifugation, sonication and homogenization in buffer (EDTA)	Lysozyme and proteinase K + Chemical lysis (SDS, 37 °C, 2h)	P:C:I (25:24:1)	Elutip-d® column	Bourrain, 1999; Lemarchand, 2005
6	-	Bead-Beating + Chemical lysis (SDS)	Ammonium acetate purification	Elutip-d® column	Yu, 1999
7	Shaking in buffer (4 °C 1 h) + cation-exchange resin + Low speed centrifugation	Lysozyme and Proponase + Chemical lysis (SDS, 65 °C, 10 min)	-	CsCl-EtBr (cesium chloride-ethidium bromide) gradient centrifugation	Jacobsen, 1992
8	Low speed centrifugation and washing with phosphate buffer	Lysozyme + Chemical lysis (SDS) + Freezing and thawing	P:C:I (25:24:1)	Elutip-d® column	Tsai, 1991
9	Paper filtration + Low speed centrifugation and homogenization in buffer (0.5 M EDTA–Na)	Lysozyme + Chemical lysis (SDS, 70 °C, 15 min)	Phenol/Chloroform (1:1)	DNA binding membrane (Plasmid extraction kit, Yeastern Biotech, Taiwan)	This Study

[a]Phenol : chloroform : isoamylalcohol (25:24:1).

most efficient in breaking bacterial aggregate flocs and cells (Yu and Mohn, 1999), was applied to the wastewater pellets prior to a phenol–chloroform extraction of the DNA. The extraction efficiency of Procedure 2 was three-fold higher than that of Procedure 4, with the extracted DNA having a better purity (Table 4).

As presented in Table 3, Procedure 1 was the fastest, generated high yields of intact DNA (4.5 µg) but with just above the acceptable purity level (OD 260/280 ratio of 1.51). This low purity co-efficient could be due to insufficiency of thermal shock as the efficiency of cell disruption affects the purity of the extracted DNA (Shan et al., 2008). However, this contradicts the findings of Shan et al. (2008) that the combination of thermal shock and SDS produced the purest DNA. A high quantity of DNA (2.74 µg) with the highest purity level (coefficient of 1.63) among all tested method and a low degradation level was extracted by using Procedure 9. The main difference between Procedure 9 and the other methods is the sample preparation step which involves paper filtration and high concentration of EDTA (0.5 M). This procedure was further tested and successfully extracted quality DNA form various wastewater sludge samples and two cultivated methanogens, M. concilii and M. thermophila, were used as positive controls (Figure 5). In addition, DGGE-PCR amplification of 16S rDNA region using archaeal and eubacterial primers proved the reproducibility of this DNA extraction and purification procedure (Figure 6). The double bands observed
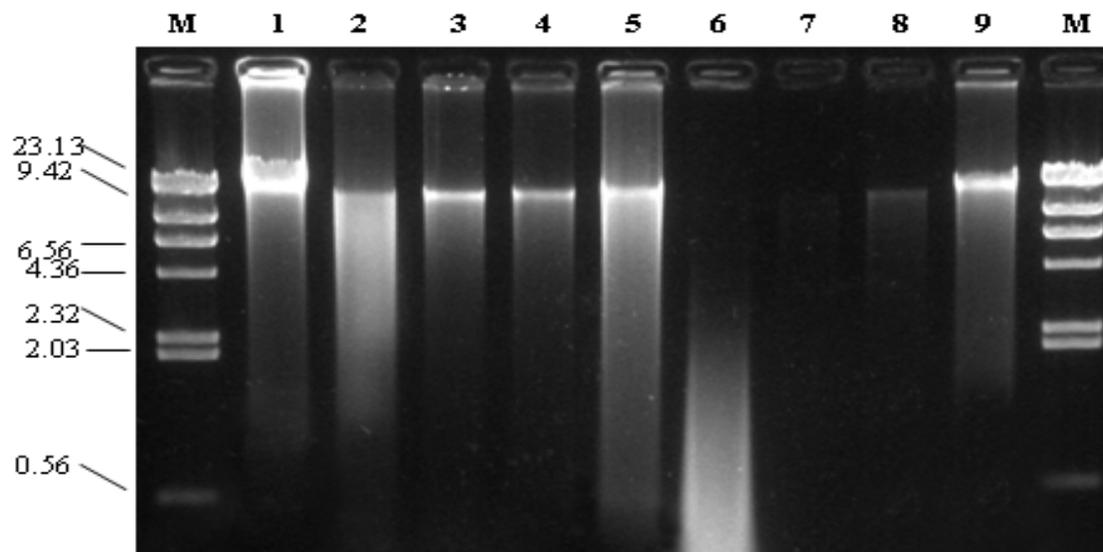
Figure 2. Agarose gel electrophoresis of DNA extracted from wastewater sludge using nine methods. M, Marker lambda digested with *Hin*dIII (marked in kbp); Lanes 1 to 9, the tested procedures. The DNA extracts were run on 1% agarose gel at 70 V for 45 min, stained with GelRed and visualized under UV transillumination

Table 4. Comparison of processing times, yields, purities and humic acid concentrations of DNA for different methods.

Procedure	Time [a] (Hours)	DNA yield (µg DNA/(g of fresh sample) [b]	Purity [c]	Successful amplification without final purification step	Humic acid concentration (ng/µl unpurified DNA) [d]	Humic acid concentration (ng/µl purified DNA)	Reference
1	3.0	4.50 ± 0.07	1.51 ± 0.05	No	800 ± 110	28 ± 4.3	Orsini, 2001
2	7.5	5.90 ± 0.08	1.3 ± 0.03	No	820 ± 125	32 ± 3.9	Yeates, 1997
3	7.0	2.00 ± 0.08	1.48 ± 0.03	No	830 ± 120	33 ± 4.7	Bourrain, 1999; Lemarchand, 2005
4	27	1.80 ± 0.22	1.14 ± 0.05	No	820 ± 110	50 ± 4.9	Ogram, 1987
5	5.0	2.10 ± 0.08	1.50 ± 0.03	No	780 ± 100	29 ± 4.2	Bourrain, 1999; Lemarchand, 2005
6	5.0	1.80 ± 0.08	1.53 ± 0.03	No	800 ± 120	31 ± 3.8	Yu, 1999
7	22	0.35 ± 0.05	1.37 ± 0.03	No	790 ± 110	27 ± 5.1	Jacobsen, 1992
8	11	0.70 ± 0.03	1.15 ± 0.05	No	850 ± 120	33 ± 4.4	Tsai, 1991
9	5.0	2.74 ± 0.03	1.63 ± 0.05	No	480 ± 70	12 ± 1.7	This Study

[a]Time required for extracting and purifying DNA from one wastewater sludge sample; [b]values are means of four independently extracted samples with standard error; [c]ratio of A_{260} to A_{280}; [d]humic acid concentrations were determined using absorbance reading at 340 nm.
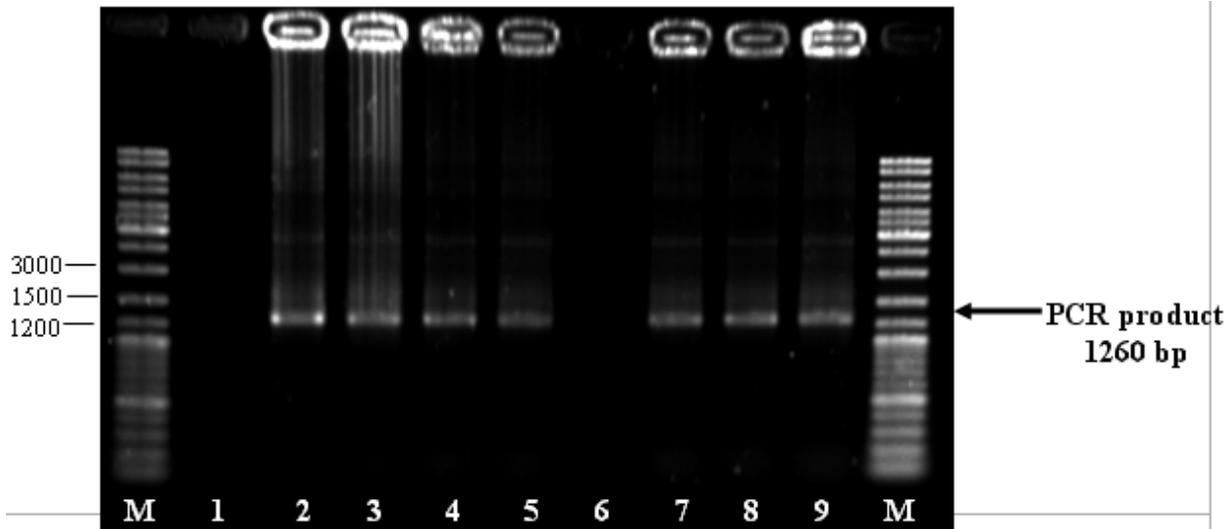
Figure 3. PCR amplification of methanogen 16S rRNA gene from DNA extracted using the nine different methods from wastewater sludge. M, Generuller DNA ladder Mix (marked in bp); Lanes 1 to 9, the tested procedures. The PCR products were run on 1% agarose gel at 70 V for 45 min, stained with GelRed and visualized under UV transillumination.
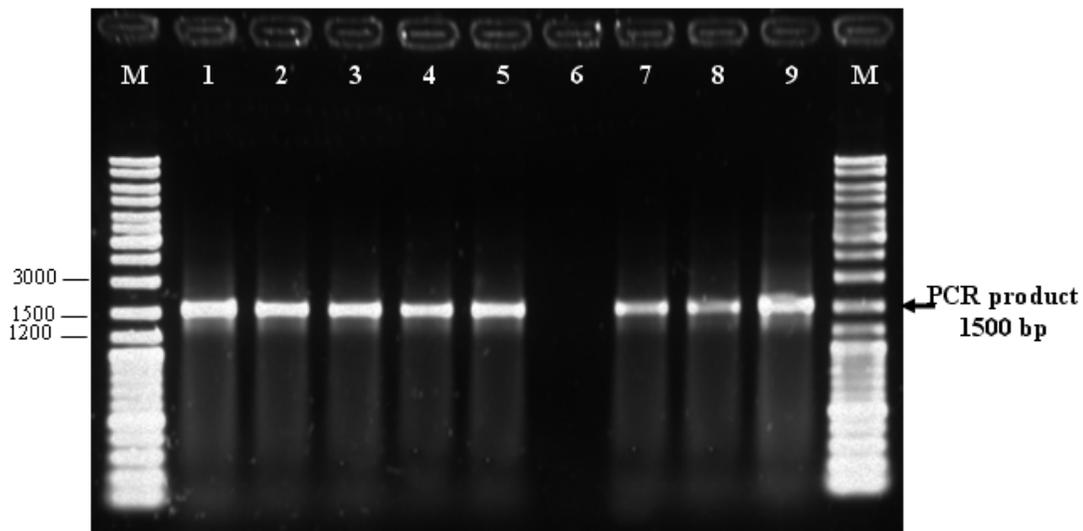
Figure 4. PCR amplification of bacterial 16S rRNA gene from DNA extracted using the nine different methods from wastewater sludge. M, Generuller DNA ladder Mix (marked in bp); Lanes 1 to 9, the tested procedures. The PCR products were run on 1% agarose gel at 70 V for 45 min, stained with GelRed and visualized under UV transillumination.

using archaeal primers; PARCH340f and PARCH519r (Figure 6, lane 2) was a result of degeneracy of the primers used (Piceno et al., 1999). The sharpest bands from the archaeal profile (Figure 6, Lane 2; bands A and B) were analyzed and found to be *Methanosarcina* sp. and *M. concilii,* respectively. This was in line with *FISH* results (Figure 7) which found *Methanosarcina* sp. and *M. concilii* as the only and dominant methanogens in the studied wastewater sludge.

Therefore, the experimental reproducibility was satisfying.

The smallest DNA yields hardly detected on the gel (Figure 2) were obtained by Procedure 7 (0.33 µg) due to DNA loss at cation-exchange resin step required in this procedure or in another word because of the strong attachment of the cells to the sludge particles (Leff et al., 1995). Therefore, recovery of cells by this technique varies depending on the strength and nature of the attachment of the cell to the particles. In addition the purity obtained (coefficient of 1.37) and the time taken (22h) were not favorable either. Procedure 6, the average
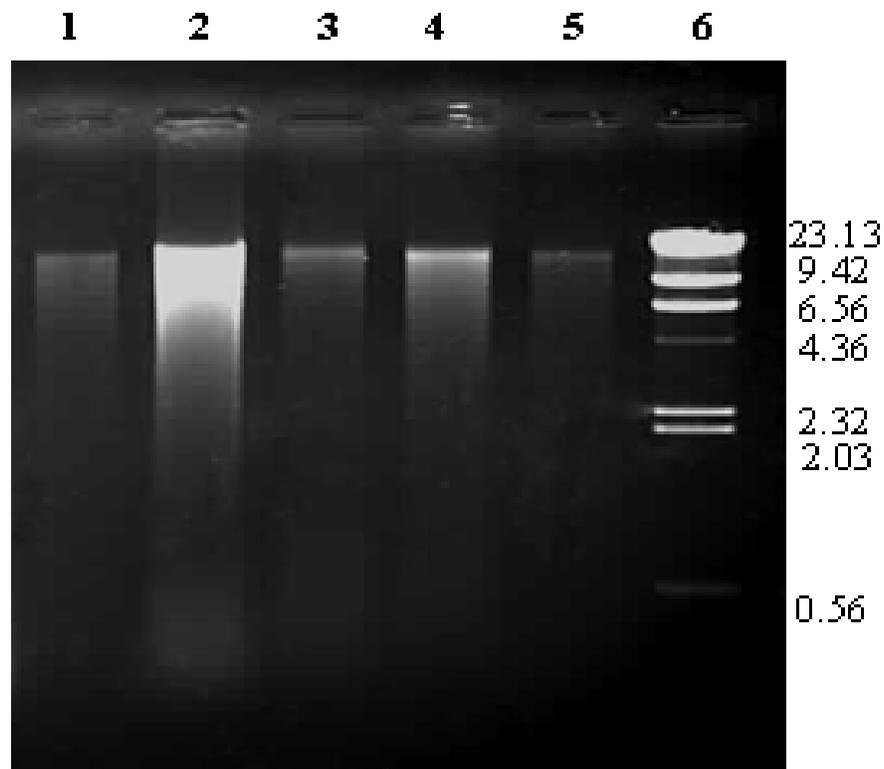
Figure 5. Agarose gel electrophoresis of DNA extracted from different various wastewater sludge samples using the patented method (Procedure 9). Lane 1, 2 and 3; various wastewater sludge samples obtained from the palm oil mill anaerobic pond, recycling tank and municipal wastewater treatment facilities, respectively; Lane 4, culture of *M. concilii*; Lane 5, culture of *M. thermophila* and Lanes 6, marker lambda digested with *Hind*III (marked in kbp). The DNA extract were run on 1% agarose gel at 70 V for 45 min, stained with ethidium bromide and visualized under UV transillumination.

technique tested in this study, was quite similar to Procedure 2 except that ammonium acetate purification replaced phenol–chloroform purification. It produced a lower quantity of DNA (1.8 µg) with an appropriate purity level (co-efficient of 1.53) but a high degradation level (Figure 2).

Approximately, 2.0 µg of good integrity DNA with a purity coefficient just below the passable level (1.48) was extracted by Procedure 3 which used a combination of physical, enzymatic and chemical lysis. By using Procedure 8, a low quantity of DNA was obtained (0.7 µg) demonstrating lower than acceptable purity at the same time (coefficient of 1.15). On the average, procedures which applied bead beating (Procedures 2 and 4) were more successful than those which include freezing and thawing (Procedures 3 and 8). Miller et al. (1999) compared the efficiency of bead-beating cell lysis with that of freeze-thaw lysis and came to the same result that bead-beating is superior to the freeze-thaw technique. Procedure 5 produced acceptable quantity and purity (coefficient of 1.50) of DNA but rather fragmented. This high level of degradation is presumably a consequence of sonication which can disrupt DNA molecules (Picard et al., 1992).

Despite these differences of extraction efficiencies, a few differences in PCR outcome were observed between the different protocols.

Most of the amplifications of 1260 and 1500 bp of methanogenic and eubacterial 16S rDNA gene, respectively, were successful even when DNA purity was below the acceptable limit of 1.5 (Procedures 2, 3, 4, 7 and 8) (Figures 3 and 4). Procedure 6 failed to produce any amplifiable DNA. This could be attributed to the high level of fragmentation induced by beat beating step used in this method (Figure 2). Methanogenic 16S rDNA was not amplified when DNA extract by Procedure 1 was used in the reaction (Figure 3). This shows that thermal shock alone is probably not sufficient for disrupting archaeal strong cells such as methanogens in order to release the archaeal DNA. Vijayaraghavan et al. (2006) found heat treatment for a period of 2 h at 105°C not sufficient to kill or inhibit the methanogenic archaea. For all nine procedures, when the DNA extract did not undergo the final purification step, no amplification was generated by any primer sets. This was due to the presence of PCR inhibitors such as humic acids and some metal ions found in crude
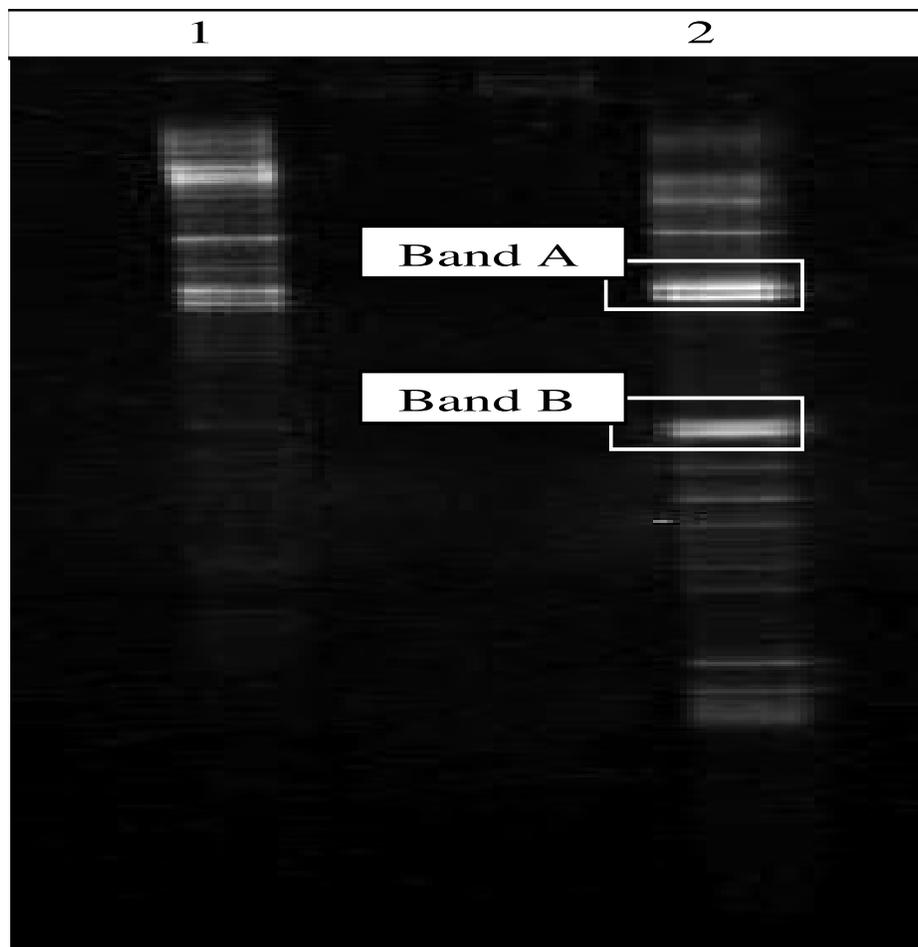
Figure 6. DGGE analysis of the PCR products of DNA extracted from wastewater sludge by Procedure 9. DGGE pattern using eubacterial primers (Lane 1) and archaeal primers (Lane 2). The PCR products were run on 8% acrylamide gel at 100 V for 14 h, stained with cyber green and visualized under UV transillumination.

DNA extracts as a small amount of humic-acid-like sub-stances (e.g. 27 µg) or pure humic acid as low as 10 ng is sufficient to inhibit PCR (Tsai and Olson, 1992). In Procedure 9, when the initial paper filtration and washing step by high concentration of EDTA solution (0.5 M) was absent, no PCR amplification was observed. It is due to the fact that EDTA is a molecule to chelate or complex 2 and 3 valent cations such as Fe^{3+} which are PCR-inhibitors (Akane et al., 1994) in 1:1 metal-to-EDTA complexes and therefore, plays a key role in removing DNA impurities (Khosravinia and Ramesha, 2007).

Conclusion

If DNA purity is of paramount concern, the method presented in this study (Procedure 9) is recommended because of the low concentration of contaminants. If DNA is to be used directly e.g. in DNA-DNA hybridizations, Procedure 2 is recommended since it gives maximal yields.

However, if the level of DNA shearing is of concern, the methods that produced low fragmentation are recom-mended. If time is of concern for studying bacterial population (non-methanogen communities), Procedure 1 is recommended as it was the fastest and resulted in acceptable quantity and purity, however, it had a draw-back of failing to extract DNA from methanogenic cells. These observations suggested that the extraction method needs to be carefully selected to produce desired results when dealing with high-strength wastewater sludge samples. Moreover, Procedure 9 was shown to be a very efficient method overall in spite of its simplicity.

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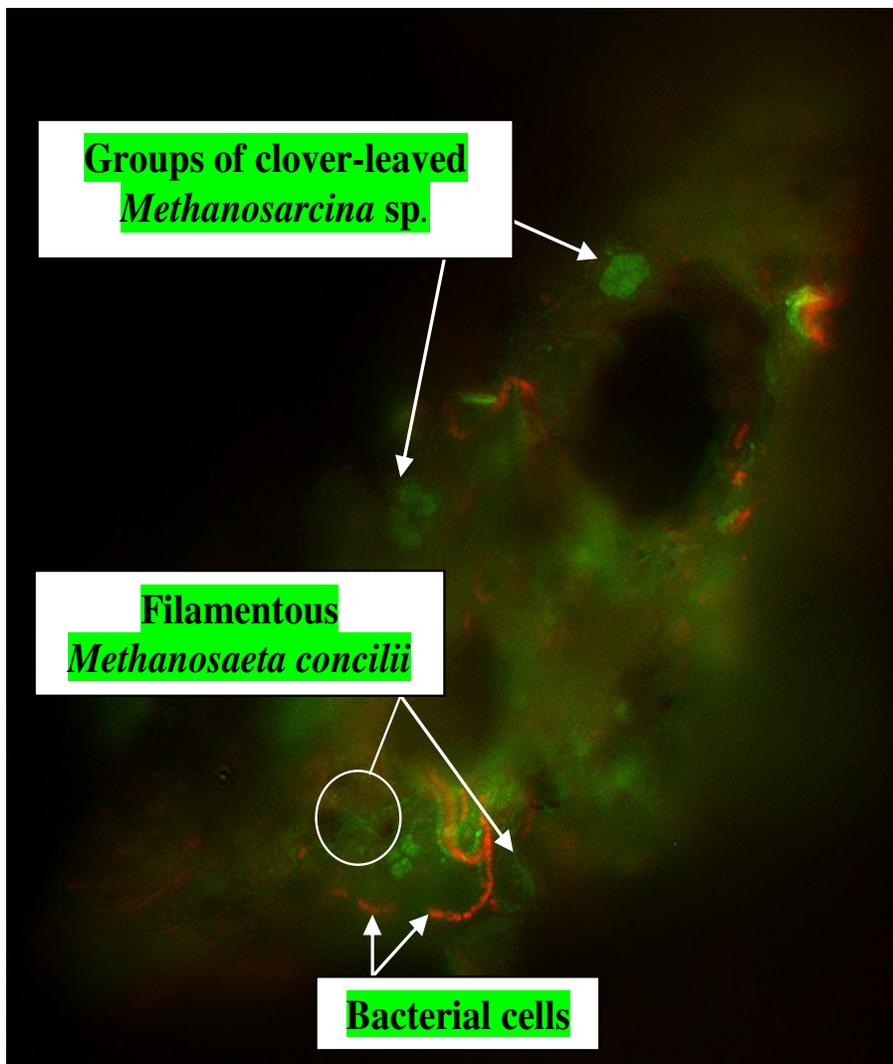
Figure 7. *FISH* staining of the sludge samples analyzed by confocal laser microscopy of fluorescent *in situ*-hybridized cells. A flocs simultaneously hybridized with rhodamine-labeled bacterial-domain probe (EUB338, EUB338-II and EUB338-III) (red) and FITC-labeled methanogens probe (MSMX860) (green) showing the distribution of the only two methanogens (*Methanosarcina* sp. and *M. concilii*) and bacterial cells at 400X magnification.

used in this study.

Abbreviations

PCR, Polymerase chain reaction; **DGGE,** denaturing gradient gel electrophoresis; **FISH,** fluorescent *in situ* hybridization; **CDT,** closed digester tank; **POME,** palm oil mill effluent; **EDTA,** ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; **PVP,** polyvinylpyrrolidone; **CsCl-EtBr,** cesium chloride-ethidium bromide; **NCBI's BLAST,** national center for biotechnology information's basic local alignment search tool; **DAPI,** 4′,6-diamidino-2-phenylindole.

REFERENCES

Akane A, Matsubara K, Nakamura H, Takahashi S, Kimura K (1994). Identification of the heme compound copurified with deoxyribonucleic acid (DNA) from bloodstains, a major inhibitor of polymerase chain reaction (PCR) amplification. J. Forensic Sci. 39: 362-372.

Amann RI, Ludwig W, Schleiffer KH (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59: 143-169.

Bourrain M, Achouak W, Urbain V, Heulin T (1999). DNA extraction from activated sludges. Curr. Microbiol. 38: 315-319.

Chan O-C, Liu WT, Fang HHP (2001). Study of microbial community of brewery-treating granular sludge by denaturing gradient gel electrophoresis of 16S rRNA gene. Water Sci. Technol. 43: 77-82.

Crocetti G. Murto M, Björnsson L (2006). An update and optimisation of oligonucleotide probes targeting methanogenic Archaea for use in fluorescence *in situ* hybridisation (*FISH*). J. Microbiol. Methods, 65: 194-201.

Daims H, Bruhl A, Amann R, Schleifer KH, Wagner M (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. 22: 434-444.

England LS, Trevors JT, Holmes SB (2001). Extraction and detection of baculoviral DNA from lake water, detritus and forest litter. J. Appl. Microbiol. 90: 630-636.

Howeler M, Ghiorse WC, Walker LP (2003). A quantitative analysis of DNA extraction and purification from compost. J. Microbiol. Methods, 54: 37-45.

Hu JY, Yang F, Lin YH, Zhang HB, Ong SL, Dong N, Xu JL, Ng WJ, Zhang LH (2003). Microbial diversity and prevalence of virulent pathogens in biofilms developed in a water reclamation system. Res. Microbiol. 154: 623-629.

Jacobsen CS, Rasmussen OF (1992). Development and application of a new method to extract bacterial DNA from soil based on separation of bacteria from soil with cation-exchange resin. Appl. Environ. Microbiol. 58: 2458-2462.

Khosravinia H, Ramesha KP (2007). Influence of EDTA and magnesium on DNA extraction from blood samples and specificity of polymerase chain reaction. Afr. J. Biotechnol. 6: 184-187.

Kozdroj J, Van Elsas JD (2001). Structural diversity of microorganisms in chemically perturbed soil assessed by molecular and cytochemical approaches. J. Microbiol. Methods, 43: 197-212.

LaMontagne MG, Holden PA (2003). Comparison of free-living and particle-associated bacterial communities in a coastal lagoon. Microb. Ecol. 46: 228-237.

Leff LG, Dana JR, Mcarthur JV, Shimkets LJ (1995). Comparison of methods of DNA extraction from stream sediments. Appl. Environ. Microbiol. 61: 1141-1143.

Lemarchand K, Berthiaume F, Maynard C, Harel J, Payment P, Bayardelle P, Masson L, Brousseau R (2005). Optimization of microbial DNA extraction and purification from raw wastewater samples for downstream pathogen detection by microarrays. J. Microbiol. Methods, 63: 115-126.

Miller DN, Bryant JE, Madsen EL, Ghiorse WC (1999). Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. Appl. Environ. Microbiol. 65: 4715-4724.

Nicol GW, Glover LA, Prosser JI (2003). Spatial analysis of archaeal community structure in grassland soil. Appl. Environ. Microbiol. 69: 7420-7429.

Ogram A, Sayler GS, Barkay T (1987). The extraction and purification of microbial DNA from sediments. J. Microbiol. Methods, 7: 57-66.

Orsini M, Romano-Spica V (2001). A microwave-based method for nucleic acid isolation from environmental samples. Lett. Appl. Microbiol. 33: 17-20.

Picard C, Ponsonnet C, Paget E, Nesme X, Simonet P (1992). Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction. Appl. Environ. Microbiol. 58: 2717-2722.

Piceno YM, Noble PA, Lovell, CR (1999). Spatial and temporal assessment of diazotroph assemblage composition in vegetated salt marsh Sediments using denaturing gradient gel electrophoresis analysis. Microb. Ecol. 38: 157-167.

Rivera IN, Lipp EK, Gil A, Choopun N, Huq A, Colwell RR (2003). Method of DNA extraction and application of multiplex polymerase chain reaction to detect toxigenic Vibrio cholerae O1 and O139 from aquatic ecosystems. Environ. Microbiol. 5: 599-606.

Shan G, Jin W, Kh Lam E, Xing X (2008). Purification of total DNA extracted from activated sludge. J. Environ. Sci. 20: 80-87.

Steinhaus B, Garcia ML, Shen AQ, Angenent LT (2007). A Portable Anaerobic Microbioreactor Reveals Optimum Growth Conditions for the Methanogen Methanosaeta concilii. Appl. Environ. Microbiol. 73: 1653-1658.

Tabatabaei M, Zakaria MR, Rahim RA, Wright ADG, Shirai Y, Abdullah N, Shamsara M., Sakai K, Hassan MA (2008). Method for isolating DNA. Malaysian Patent PI20082842; PCT/MY2009/000143.

Tien CC, Chao CC, Chao WL (1999). Methods for DNA extraction from various soils: a comparison. J. Appl. Microbiol. 86: 937-943.

Tsai YL, Olson BH (1991). Rapid method for direct extraction of DNA from soil and sediments. Appl. Environ. Microbiol. 57: 1070-1074.

Tsai YL, Olson BH (1992). Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. Appl. Environ. Microbiol. 58: 2292-2295.

Valentine DL, Blanton DC, Reeburgh WS (2000). Hydrogen production by methanogens under low-hydrogen conditions. Arch. Microbiol. 174: 415-421.

Vijayaraghavan K, Ahmad D, Ibrahim MKB, Herman HNB (2006). Isolation of hydrogen generating microflora from cow dung for seeding anaerobic digester. Int. J. Hyd. Energy, 31: 708-720.

Whitby C, Saunders JR, Pickup RW, McCarthy AJ (2001). A comparison of ammonia-oxidiser populations in eutrophic and oligotrophic basins of a large freshwater lake. Anton. Leeuw. Int. J. G. 79: 179-201.

Wright ADG, Pimm C (2003). Improved strategy for presumptive identification of methanogens using 16S riboprinting. J. Microbiol. Methods. 55: 337-349.

Yeates C, Gillings MR, Davison AD, Altavilla N, Veal DA (1997). PCR amplification of crude microbial DNA extracted from soil. Lett. Appl. Microbiol. 25: 303-307.

Yu Z, Mohn WW (1999). Killing two birds with one stone: simultaneous extraction of DNA and RNA from activated sludge biomass. Can. J. Microbiol. 45: 269-272.

Yu Z, Morrison M (2004). Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. 70: 4800-4806.