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Contributed Paper

A Simple Method for DNA Extraction from Activated Sludge

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ABSTRACT

A protocol for DNA extraction directly from activated sludge is described. Sludge samples were lysed by glass bead and CTAB. The extracted DNA was purified with phenol: chloroform: isoamyl alcohol mixture and precipitated in sodium acetate and isopropanol. The proposed protocol is simple, rapid and required small sample size, yielding about 40.5 ± 1.8 μg DNA g^{-1} sludge. The quality of DNA is good enough for successful PCR amplifications of 16S rDNA, 16S rDNA V3 region and denaturing gradient gel electrophoresis (DGGE) analysis of archaeal population. This described method can also be applied to other environmental samples such as whey and soil.

Keywords: Activated sludge; DNA extraction; DGGE; Archaeal population.

1. INTRODUCTION

The efficiency of wastewater treatment mainly relies on the formation and the biological activities of anaerobic sludge [1]. Activated sludge harbors highly diverse microorganisms that organized as cellular aggregates or flocs [2]. The investigation of microbial population and their diversity in activated sludges are important for control and maintenance of wastewater treatment system. Moreover, the obtained information provided new insight in microbiology which helped to improve the design and performance of new generation reactors [3]. However, conventional methods such as plate or liquid culture are inadequate for microbial diversity study because of limited knowledge on the real growth conditions of most microorganisms in their habitats and the difficulty of media development for cultivation [4]. In recent year, bacterial communities in activated sludge have been studied using culture independent method [3]. The key success of this approach lies in the quality of nucleic acids isolated from the environmental samples. However, most currently available DNA extraction methods

are either costly or time-consuming. An increased yield may also lead to a decrease in its purity [5]. DNA isolation from activated sludge is difficult to achieve complete cell lysis as microorganisms are heavily encapsulated [6]. Since a quality and quantity of DNA are affected by DNA extraction methods so an improvement of an extraction method is essential to get high quality nucleic acids which represent entire microbial population inside in the activated sludge.

In this study, we report the DNA extraction method from activated sludge. This method is simple, rapid and required small amount of sample. It can be used to study microbial diversity in activated sludge. DGGE analysis of the archaeal population in activated sludge sample was presented to demonstrate the efficiency of this extraction method.

2. MATERIALS AND METHODS

2.1 Sample Collection

Activated sludge samples were collected from a 1000 m^3 channel digester up-flow anaerobic sludge blanket (CD-UASB) reactor digesting pig manure with Napier grass. The reactor was operated under mesophilic condition for 66 days. Samples were preserved at -20 °C until the DNA extraction was performed.

2.2 DNA Extraction Protocol

A 500 µl of sludge sample (0.2 g [wet weight]) was added to a 1.5 ml micro-centrifuge tube and centrifuged at 14,000 rpm for 10 min. Sludge pellet was lysed by adding an equal volume of sterile glass beads (≤ 106 µm diameter ; Sigma) and 500 µl of hexadecyltrimethyl ammonium bromide (CTAB) (Fluka) extraction buffer [7]. The suspension was mixed by vortexing for 1 min and 500 µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the mixture. The resulting suspension was vortexed for 1 min and frozen on ice for 1 min. This step was carried out 3 times to release individual cells from the flocs. This step is crucial for effective lysis, however, heat may occur during vortex for a long time so keeping the mixture on ice can help prevent DNA degradation. The mixture was centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was transferred to a new microcentrifuge tube. Phenol extraction was repeated with 500 µl of phenol: chloroform: isoamyl alcohol (25:24:1). The aqueous phase was transferred to a new microcentrifuge tube and phenol removed by mixing with 500 µl of chloroform: isoamyl alcohol (24:1), followed by centrifugation at 14,000 rpm for 10 min at 4°C. The DNA was precipitated with 0.1 volume of 3M sodium acetate (NaOAc) pH 5.5 and 0.6 volume of isopropanol at -20°C for 30 min. DNA pellet was washed with 70% (v/v) ice cold ethanol, air-dried and resuspended in 30 µl of TE buffer containing 0.002% (w/v) RNase.

2.3 Qualitative and Quantitative Analysis of DNA

The size of DNA was determined on 1% (w/v) agarose gel in 1× TAE buffer, using 1 kb DNA ladder (Fermentas) as molecular weight marker and electrophoresed at 100 V for 1 h. The gel was stained with 0.5 µg/ml ethidium bromide (EtBr) and visualized under UV light (Syngene). The concentration of extracted DNA was determined by spectrophotometer at 260 nm with GENESYS 10 (Thermo Fisher Scientific). The experiments were carried out in duplicate. The DNA purity was estimated spectrophotometrically by calculating an A_{260}/A_{280} and A_{260}/A_{230} ratio [8]. The extracted DNA was used as a template for PCR amplification of the 16S

rDNA and 16S rDNA V3 region. The universal archaeal primers PRA 46f and PREA 1100r [9] were used in a PCR amplification to produce a 1,072 bp fragment of PCR product. The amplified fragments were then used as a template for the PCR amplification of 179 bp fragment using a set of the primer PARCH 340f and PARCH 519r [9]. The GC clamp, 5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG-3' was attached on the 5' end of the forward primer PARCH 340f to enable fragment separation by DGGE [9].

The reaction mixtures of both primer sets were prepared in a total volume of 25 µl containing 1× PCR buffer, 1.5 U of *Taq* DNA polymerase (Fermentas), 0.4 µM of both the primers, 0.4 mM of dNTPs, 3 mM $MgCl_2$, 3.96% (v/v) of dimethyl sulphoxide (DMSO) and 1 µl of the extracted DNA. PCR amplifications were performed by initial denaturation step at 92°C for 2 min, followed by 30 cycles of denaturation at 92°C for 1 min, annealing at 55°C for 30s, elongation at 72°C for 1 min. Final extension was performed at 72°C for 6 min before cooling down to 4°C. All PCR amplifications were performed in a MyCycler™ thermal cycler (Bio-Rad). PCR products were separated on 1.4% (w/v) agarose gel, using 100 bp DNA ladder (Fermentas) as molecular weight marker and electrophoresed at 100 V for 1 h.

The 179 bp PCR fragments were performed using DGGE apparatus (Dcode™ Universal Mutation Detection System ; Bio-Rad). PCR products (10 µl) were loaded on 8% (w/v) polyacrylamide gel (40% acrylamide/bis solution, 37:1) in 1× TAE buffer. Polyacrylamide gel with a gradient between 20%-45% (100% denaturant contains 7M urea and 40% (v/v) formamide) was run at 130 V for 4 h at a constant temperature of 60°C [10].

3. RESULTS AND DISCUSSION

3.1 DNA Extraction

The activated sludge samples from UASB reactor digesting pig manure and Napier grass were used in this study. The appearance was viscous and deep brown in color. Microscopic observations showed the presence of few filamentous bacteria which are good characters of sludge. High numbers of filamentous bacteria were reported as major cause of bulking problem in wastewater treatment [11]. Sludge samples were collected at different times from the same reactor and independently extracted. Similar results were obtained thus indicated reproducibility of our proposed method. Currently, available DNA extraction methods from activated sludge are complicated, time-consuming or based on expensive equipments [12]. Our developed DNA extraction method was simple using a combination of mechanical and chemical lysis. During mechanical lysis step, sludge was dispersed to allow good penetration of an extraction buffer and cells were disrupted by vortexing with glass beads. For chemical lysis, CTAB was added to improve the purity of the DNA by precipitation of polysaccharides and complex humic compounds in sludge [13]. Humic acid contamination can inhibit PCR amplification because the phenolic groups can denature DNA by bonding with amide or form quinone which covalently binds to DNA. This interaction may inhibit *Taq* polymerase, the key enzyme in PCR amplification [14]. In this study, sludge samples are rich in organic materials and contaminants so phenol: chloroform extraction step was repeated to purify DNA (for clean samples, this procedure may be omitted). DNA was precipitated with 0.1 volume of 3M

NaOAc pH 5.5 and 0.6 volume of isopropanol at -20°C for 30 min or overnight to obtain the maximum yield. The appearance of DNA pellet was clear which indicated that there was no presence of contaminants. DNA was of good quality under agarose gel electrophoresis (Figure 1).

Most of previously published methods involved complicated procedures such as sample preparation step, cell lysis and DNA purification. The comparison of

Table 1. Comparison of each DNA extraction methods from activated sludge

Method	Sample preparation	Mechanical lysis	Chemical lysis	Protein removal	Purification
Globin *et al.* [1]	homogenize in TENP buffer (Tris-base, EDTA, NaCl, PVPP)	freeze/thaw in liquid nitrogen	SDS	P:C[b] + CTAB + KAc	-
Roh *et al.* [12]	homogenize in TENC buffer (Tris-HCl, EDTA, NaCl, CTAB, proteinase K)	freeze/thaw in liquid nitrogen	SDS	P:C:I[a]	In-gel patch electrophoresis + gel extraction kit (Qiagen)
Bourrain *et al.* [15]	cation exchange resin	vortex	Lysozyme + SDS	P:C:I[a]	-
Tabatabaei *et al.* [16]	paper filtration homogenize in buffer (EDTA-Na)	-	Lysozyme + SDS	P:C[b]	DNA binding membrane (Yeastern Biotech)
Orsini and Romano-Spica [17]	resuspend in washing solution (Tris-HCl, EDTA, SDS, PVP)	microwave	Lysis solution (Tris-HCl, EDTA, SDS, PVP) + Extraction solution (Tris-HCl, EDTA, NaOAc, PVP)	P:C[b]	-
This study	-	glass bead	CTAB	P:C:I[a]	-

the procedure, chemical and equipment differences in each DNA extraction methods from activated sludge was summarized in Table 1. Guobin *et al.* [1] used freeze/thaw in liquid nitrogen combine with TENP buffer (Tris-base, EDTA, NaCl, polyvinyl-pyrrolidone [PVP]), sodium dodecyl sulfate (SDS), CTAB, potassium acetate (KAc) and phenol: chloroform. Roh *et al.* [12] used freeze/thaw in liquid nitrogen combine with TENC buffer (Tris-HCl, EDTA, NaCl, CTAB and proteinase K), SDS and phenol: chloroform: isoamyl alcohol and followed by DNA purification. Bourrain *et al*. [15] used vortex in combination with SDS and phenol: chloroform and sludge was dispersed with cation exchange resin prior to cell lysis. Tabatabaei *et al.* [16] used lysozyme, SDS, phenol: chloroform which sludge was filtered and homogenized with vortex in EDTA-Na buffer prior to cell lysis and extracted DNA was further purified by DNA binding membrane. Our proposed method only used vortex with glass bead, CTAB, phenol: chloroform: isoamyl alcohol and chloroform: isoamyl alcohol. It is evident that our proposed procedure is simple and required fewer steps compared to other available methods. Our method is also cheaper as only small amounts of chemical are employed and expensive enzymes are not required. Furthermore, expensive tools such as bead beater or liquid nitrogen were not used and all reagents are easily available. This method is fast because there is no requirement for such preprocessing step prior to cell lysis such as isolation of microbial cells from their matrix or washing sludge with distilled water or organic solvent. There is also no incubation step with enzyme. Moreover, purification steps were not required; as a result the entire procedure could be completed within 1 h and 30 min. The obtained DNA was larger than 10 kb in size with high band intensity which suggested that this method caused less DNA shearing (Figure 1).

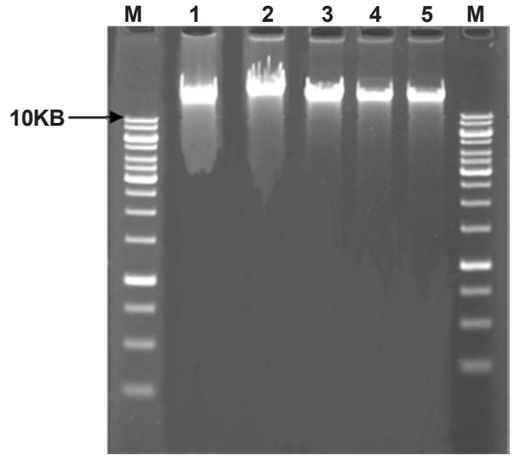

Figure 1. Agarose gel electrophoresis of DNA extracted obtained from sludge samples. Lanes M = 1 kb DNA Ladder. Lane 1-5 = activated sludge samples from Day 1-5.

3.2 Quantity and quality analysis of DNA

The result from spectrophotometer showed that yield of the extracted DNA was about 40.5 ± 1.8 μg g^{-1}. However, yield can be varied depending on type and properties of sludge samples. The A_{260}/A_{280} ratio of the extracted DNA was 1.8, indicated that the DNA obtained by this method was free from protein contamination. In addition, the A_{260}/A_{230} ratio of the extracted DNA was 1.6, suggested that it was relatively free from humic acids adherent [1, 8]. McIlroy *et al.* [5] showed that most of extraction methods gave pure DNA without protein contamination but the DNA still contaminated with humic acid which affected its further use in subsequent molecular studies. The quality of the extracted DNA was investigated by amplification of 16S rDNA and 16S rDNA V3 region with universal archaeal primers. In this step, DMSO was added to PCR to minimize formation of secondary structure in DNA template particularly for GC-rich templates by interfering the formation of hydrogen bonds. GC-rich DNA sequences can be folded and decrease product yield [6]. All of the obtained DNA could be directly used as a template to amplify both selected genes by PCR. DNA extracts obtained from other methods were usually not pure enough and must be diluted before amplification to decrease the interference of the impurity in DNA extracts [17]. Figure 2a and b show amplification bands of 16S rDNA and 16S rDNA V3 region, as demonstrated by the presence of the PCR products of 1,072 bp and 179 bp, respectively. It proved that the DNA obtained from this extraction method was of good quality and suitable for PCR amplification. The PCR products of 179 bp were run in DGGE to determine the dominant archaeal population present in the sludge samples (Figure 3). It is evident that the PCR products were separated into 7 bands by DGGE. The DGGE results of the DNA extracts obtained by this method were also reproducible as DGGE profiles from 5 independently extracted sludge samples were similar (Figure 3), suggested that the extracted DNA were suitable for molecular studies of microbial community in activated sludge.

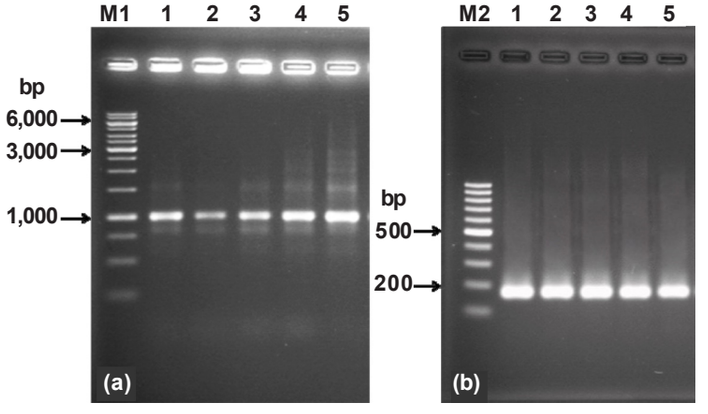

Figure 2. (a) PCR product of 16S rDNA; (b) PCR product of 16S rDNA V3 region. Lane M1 = 1 kb DNA Ladder, Lane M2 = 100 bp DNA Ladder. Lane 1-5 = activated sludge samples from Day 1-5.

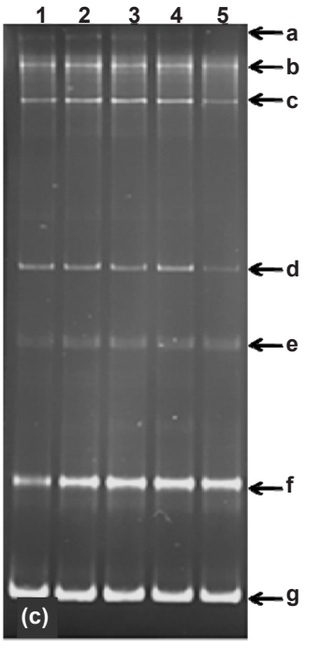

Figure 3. DGGE analysis on PCR product of DNA extracted in the 16S rDNA V3 region. Lane 1-5 = activated sludge samples from Day 1-5. a-g = DGGE bands representing different archaeal population.

In related studies, the proposed method could be applied to other environmental samples such as whey, soil or phenol contaminated activated sludge with similar success (data not shown).

4. CONCLUSIONS

Our proposed DNA extraction method has several advantages such as 1) simple 2) cheap, as only small amounts of chemicals are used 3) no special equipment are employed and 4) high yield (40.5 ± 1.8 μg g^{-1}) from small sample. The obtained DNA was of high quality suitable for PCR amplifications of 16S rDNA and 16S rDNA V3 region. Archaeal population in sludge samples was successfully monitored by DGGE using this extraction method. This protocol could be very useful for molecular microbiological study especially in laboratories with limited resources.

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