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Original Research

Freeze- and Thaw-Based Procedures for Extracting DNA from Activated Sludge

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Abstract

Activated sludge treatment is one of the most popular biological processes for wastewater treatment. Detection of activated sludge structures from complex substrates such as soil and water is not easy. A rapid and effective method for extracting high molecular weight amplifiable DNA from activated sludge was modified. Five nanogram quantities of DNA per milligram activated sludge were recovered with SDS-based and freeze-and-thaw procedures. The ratio of absorbance at 260 nm to absorbance at 280 nm varies between 1.6 and 2, which is an indicator of the purity of DNA. Gel electrophoresis of the isolated DNA further showed intact genomic DNA bands of high molecular weight (greater than 12,000 base pairs) with no RNA contamination. The quality of obtained DNA was verified by PCR amplification reactions and restriction enzyme digestion. A comparison of the optimized protocol with commercial dBioZol DNA isolation kit suggested that the method described in this report would be more efficient in removing PCR inhibitors from activated sludge samples. The random amplified polymorphic DNA (RAPD) patterns demonstrated the genetic diversity of activated sludge samples. A 542-bp fragment of the 16S rRNA gene of the bacterial isolates was amplified. PCR using primers targeting 16S rDNA shows promise in the enumeration of gram-positive bacteria in activated sludge samples.

The current protocol can be used to efficiently monitor the presence of microorganisms in sludge from effluent treatment plants.

Keywords: microbial ecology, activated sludge, purification, DNA

Introduction

Wastewater treatment based on activated sludge is known to be one of the most effective and popular wastewater purification methods. Activated sludge processes with cyclic changes of anaerobic and aerobic conditions have been used to remove phosphate from wastewater and are becoming more important for reducing eutrophication from aquatic water [1].

The analysis of complex microbiota present in activated sludge is important for the understanding and possible control of severe separation problems in sewage treatment, such as sludge bulking or sludge scumming [2, 3].

DNA isolation directly from bacterial cells in a bacterial community accelerates the identification procedures, assessment of composition and variableness of individual

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microbial groups without previous pure culture isolation [4]. The inability to culture most microorganisms from environmental samples is a fundamental obstacle to understanding microbial ecology and diversity [5, 6]. Conventionally, investigations on the soil microbial community were based mainly on isolation and laboratory cultivation. This method is both time- and labor-consuming and applicable to less than 1% of microorganisms present in soil that are readily culturable [7]. Enrichment media used to culture microorganisms in the laboratory are inherently selective for PCR-based methods that have been used for studies of microbial ecology in activated sludge [8-11].

Extraction of nucleic acids from activated sludge has been reported by various groups [12-18]. However, the DNA extracts still contained considerable amounts of humic impurities sufficient to inhibit PCR. Several approaches were used to reduce interference with the PCR (use of CTAB in extraction step, Elutip-d column purification, addition of BSA to PCR buffer) to accomplish PCR with DNA extract as a template [19, 20]. Activated sludge contains organic compounds such as humic substances that inhibit restriction endonucleases and Taq DNA polymerase, the key enzyme of PCR [19]. Many methods have been used to eliminate the humic substances from environmental DNA, such as hexade-cyltrimethylammonium bromide (CTAB) [20]. Of these methods, some are limited in their application by being extremely time-consuming or expensive. The objective of our study was to optimize a rapid and simple method for the direct extraction and purification of DNA from activated sludge. Also, to compare the performance of one commercially available DNA extraction kit with optimized protocol for their ability to recover DNA from activated sludge.

Materials and Methods

Activated Sludge Samples

Four activated sludge samples were collected from an aeration tank of a coking wastewater treatment plant in Saudi Arabia. Samples were transported to the laboratory at 4ºC and subsequently refrigerated until analysis.

Extraction of DNA from Activated Sludge

Commercial Kit

DNA extractions were performed using a dBioZol kit (Bioer Technology Co., Ltd., China) according to the manufacturers' instructions. The supernatant was then ready for PCR. All extracted DNA was stored at -20ºC until use.

Modified Method

10 µl protinase K (20 µg/µl) and 500 µl TESN buffer [100 mmol/L tris-HCl (pH 8.0), 100 mmol/L sodium EDTA (pH 8.0), 100 mmol/L sodium phosphate (pH 8.0) and 100 mmol/L NaCl] were added to 100 mg activated sludge homogenized with liquid Nitrogen. The mix was incubated for 14 hours at -20ºC. 50 µl 20% SDS and 5 µl RNAse A (20 µg/µl) were added and the sample was gently vortexed to get an emulsion. Centrifugation was carried out at 13,000 rpm for 15 min to separate the organic and aqueous phases. The aqueous DNA-containing top layer was transferred to a new microtube, and 0.15 ml 3 M sodium acetate (pH 5.2) was added and incubated at -20ºC for 10 min. Tubes were centrifuged in a microfuge at 13,000 rpm and 0.3 ml of the supernatant transferred to another tube. An equal volume of isopropanol was added and incubated at room temperature for 15 min. The aqueous phase was precipitated by centrifugation at 13,000 rpm for 1 min. DNA pellets were washed twice in 70% (v/v) ethanol, vacuum dried for some minutes and resuspended in 50 µl TE. The obtained DNA was purified according to Moreira [21].

DNA Fragmentation and Quantification

DNA was electrophoresced through 1% agarose gels (Agarose MP, Roche Diagnostics Nederland BV) containing 1 µg ethidium bromide per ml of 1× Tris-acetate-EDTA (TAE) buffer. The Gel Doc 1000 system (Gel Documentation and Analysis Systems, Uvitec, Cambridge, UK) was used for image capturing under UVillumination and the graphic files were exported as 8-bit TIFF images. The fragment size distribution was determined using a 1-kb ladder (Jena Bioscience, Germany). The quantity and quality of the DNA obtained was evaluated using a NanoDrop spectrophotometer, ND-1000 (NanoDrop, Technologies, USA) with absorbance of 260/280 nm.

Restriction Endonuclease Digestion

Restriction endonuclease CfoI (Roche-Germany) was used to test digestibility of DNA obtained by the proposed method. The digestions were done under conditions specified by the manufacturer. The restriction fragments were run in 1.5% agarose gels and stained with ethidium bromide (0.1 mg/mL) for 10-15 min. Fragment size was estimated by comparison to electrophoretic mobility of the 1 kp DNA ladder.

PCR Amplification of Template DNA

PCR reaction mixtures of 20 µl contained 20 ng template DNA, PCR buffer (JenaBioscience, Germany), 0.2 mM of each deoxynucleiotide (dATP, dCTP, dGTP and dTTP), 2.5 mM $MgCl_2$, 1.0 UTaq polymerase (JenaBioscience, Germany), 15 pmol of TubeQ-08, and a single 10-mer primer (5'-CTCCAGCGGA-3') purchased from MWG Biotech, Germany. Amplification was performed in a DNA Thermal cycler (Techne TC-312, Techne, Stone, UK). The PCR program consisted of 30 cycles of denaturation at 94ºC for 1 min, annealing at 36ºC for 1 min and extension at 72ºC for 2 min. The final cycle had a 3-min extension step at 72ºC. The PCR product (5 µl) was electrophoresced through 1.5% (w/v) agarose gel in TAE buffer and stained with ethidium bromide. The band inten-

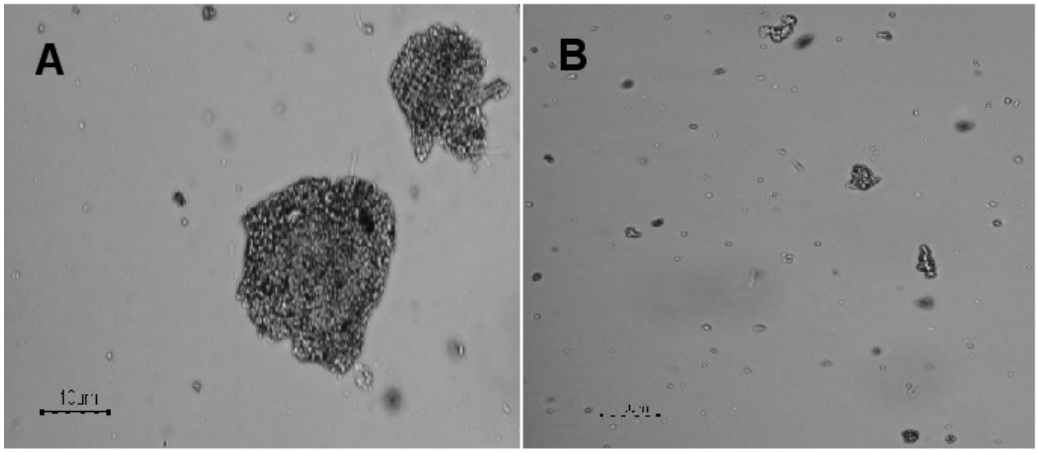

Fig. 1. Activated sludge non-homogenized (A) and homogenized (B) using N_2 grinding and freeze-thawing procedure.

sity was quantified using UVIsoft analysis (Gel Documentation and Analysis Systems, Uvitec, Cambridge, UK) equipped with the BandMap Software. Resulting RAPD patterns were normalized and analyzed with the BandMap software. This program calculated the genetic similarities (GS) with the Pearson's product moment correlation coefficient (r) between samples to construct a matrix. The samples were then clustered using the unweighted pair-wise grouping method with mathematical averages (UPGMA).

Specific-PCR for Microbial Targets

Detection of microbial groups in activated sludge using 16S rRNA gene primers (ActinoF 5'-GGC-CTTCGGGTTGTAAACC-'3) and ActinoR (5'-CTTTGAGTTTTAGCCTTGCGGC-'3) [22]. The PCR mixture consisted of 5 µl of 10 × PCR buffer (final concentrations: 100 mM KCl, 20 mM Tris-HCl pH 8.0), 2.5 mM of $MgCl_2$, 2.5 mM of each dNTP, 1 µl of each primer, 1 µl of the template DNA, and 5.0 units of Taq polymerase (Jena BioScienc, Germany) for a total volume of 50 µl. The program parameters are as follows: initial denaturation at 95ºC for 3 min; 40 cycles consisting of 94ºC for 1 min, 63ºC for 1 min, and 72ºC for 1 min; and a final extension step was 72ºC for 7 min.

Results

DNA Yield and Fragmentation

A total of 100 mg of activated sludge was homogenized with a mortar and pestle in liquid nitrogen. Frozen in liquid nitrogen and ground in a mortar and pestle, readily disintegrated into a fine powder, thus exposing the embedded activated sludge cells (Fig. 1). However, the difference in the yield may be related to the method used for the quantification of the DNA, since absorbance at 260 nm can easily overestimate the DNA concentration due to its inability to distinguish between DNA and RNA. The UV absorption ratio was 260 nm/280 nm of DNA extracted by dBioZol, and the proposed method was between 1.7 and 2, showing that the two DNAs were relatively free of protein contamination. Isolated DNA examined by gel electrophoresis (1.0% agarose in TAE buffer) showed no visible DNA degradation or RNA contamination (Fig. 2). The proposed protocol could achieve a high yield of about 500 ng (total

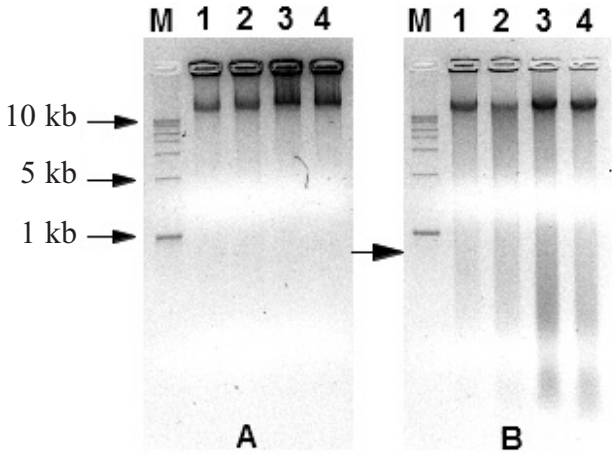

Fig. 2. Comparison of fragmentation of activated sludge genomic DNA by extraction using two methods: (A) freeze-thaw method (B) dBioZol method.

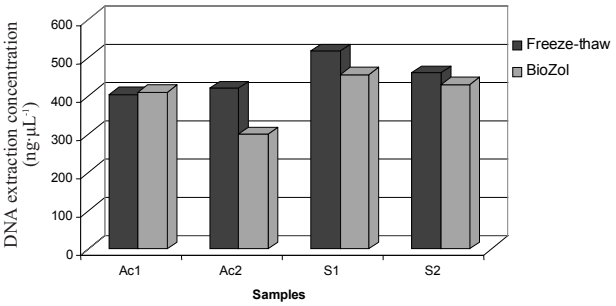

Fig. 3. Mean values and standard deviations (bars) of DNA extraction concentration ($ng \cdot \mu L^{-1}$) obtained from activated sludge samples by using freeze-thaw and dBioZol procedures.

DNA)/100 mg of initial sample (Fig. 3), the size of the DNA fragment was about 12 kb. Slightly less DNA was recovered with dBioZol reagent than with the proposed method. Further DNA purification of the extracted DNA did not show a significant difference between both methods. The suitability of extracted DNA for downstream molecular processes was further verified by restriction endonuclease digestion and RAPD-PCR amplification. As shown in Fig. 4, the isolated DNA was amenable to restriction digestion using CfoI. The genomic DNA of activated sludge samples were highly amplifiable by PCR, as indicated by the amplification products resolved on 1.5% agarose gel. PCR amplification with tubeQ-08 primer was performed using suggested DNA isolation protocol. The RAPD-PCR amplification was successful, and the same banding pattern was seen when we repeated the amplification (production of a relatively large DNA fragment of 1,220 bp). Negative control without DNA as a template also resulted in non-PCR amplification (data not shown). An unweighted pair group method with the arithmetic averages (UPGMA) tree was produced by using the RAPD analysis

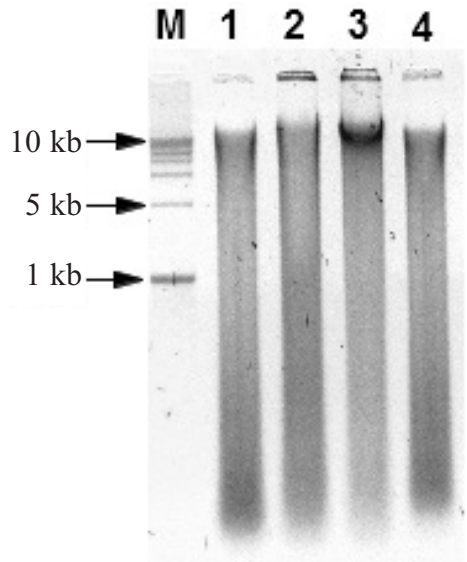

Fig. 4. Restriction analysis of total genomic DNA of activated sludge samples isolated by freeze-thaw method and resolved on 1% agarose gels after restriction with CfoI. Column No. 3 is the partly restricted DNA.

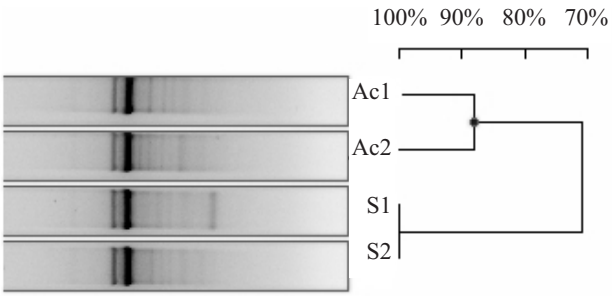

Fig. 5. Digitized profiles and dendrogram showing clustering analyses of activated sludge, obtained with polymerase chain reaction RAPD from the target DNA extracted by the freeze and thaw method.

of four activated sludge samples (Fig. 5). The dendrogram obtained after cluster analysis showed that at genetic similarity of 70%, UPGMA analysis of RAPD banding patterns separated isolates into two distinct clusters. The first group included 2 samples with genetic similarity of 88%. Also, the second group included two samples with genetic similarity of 100%. Using universal primers to most eubacterial 16S rDNA, we detected gram-positive bacteria with high DNA G+C content in activated sludge samples. The PCR products of the expected size were detected from all samples from all sample groups, indicating that bacterial 16S rDNA molecules existed in activated sludge samples (Fig. 6).

Discussion

Activated sludge processes are widely employed to treat domestic and industrial wastewaters. The microbial community in activated sludge contains a diverse microbial community of bacteria and protozoa, and much information is now available on their ecology and *in situ* physiology [23]. The general aim was to achieve high-quality DNA in amounts suitable for molecular testing combined, if possible, with a rapid, inexpensive, reliable preparation method.

Freeze-thaw cycles combined with gel patch electrophoresis and dBioZol commercial kit were tested for their ability to release genomic DNA from activated sludge. Although two variations of the DNA extraction procedure were used, there was a slight difference between two protocols. DNA yield from activated sludge was 500 ng/100 mg for tested protocol, while 420 ng/100 mg was for dBioZol protocol. DNA yields isolated from activated sludge by commercial kit ranged from 443 to 5,633 μg/liter [24].

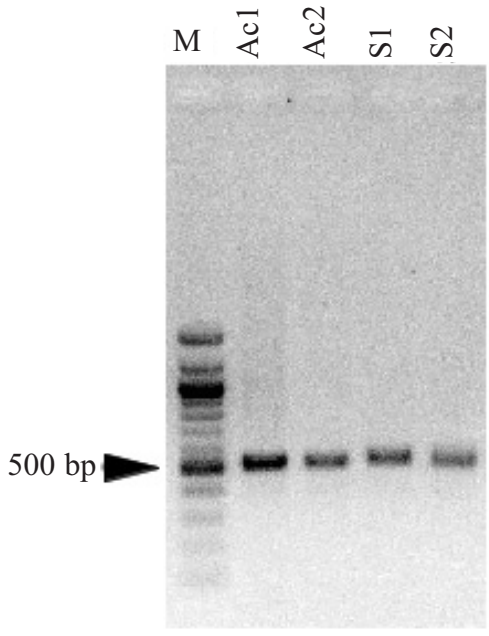

Fig. 6. PCR products derived from specific amplification of 16s rDNA gene fragments of the gram-positive bacteria with high DNA G+C content in activated sludge samples. DNA segments of approximately 542 bp were amplified using ActinoF and ActinoR primers.

These yields were approximately 25-fold lower than those found by Bourrain et al. [25] using phenol-chloroform extraction protocols for extracting DNA from activated sludges, and three-fold lower than those reported by Yu and Mohn [18]. Activated sludge DNA was subjected to restriction endonuclease digestion, and in both cases the DNA was completely digested. These results indicate that the extracted DNA quality was suitable for applications involving restriction digestion, our findings in harmony with results obtained by Roh et al. [26]. The amount and quality of the DNA obtained by this procedure were suitable for PCR amplification and other molecular assays. The DNA extracted by the developed protocol can be used to check the activated sludge diversity by effective RAPD analysis; the entire genomic DNA sample produced a clear, sharp, and reproducible PCR product. Because Taq polymerase is highly sensitive to humic acid and other potential inhibitors found in soil and PCR amplification is a major use of extracted soil DNA, we chose to use PCR-based methods in evaluating the extracted DNA's purity, as was done in other studies [27]. The present method eliminates the need to use phenol or chloroform to obtain high quality gDNA from activated sludge. During the SDS lyses phase, proteins and polysaccharides become trapped in large complexes that are coated with dodecyl sulfate. Also, the use of liquid nitrogen allowed cell disruption under temperature conditions that minimized nucleic acid degradation. Effective cell disruption is achieved by shaking the sample in lysis buffers containing high concentrations of detergent, using freeze-thawing cycles. In addition, it has been demonstrated that when cells were disrupted at the temperatures of liquid nitrogen, nucleic acid degradation was minimized [28]. Thermal shock consists of freezing and thawing samples.

The number of freeze-thaw cycles and the incubation time in liquid nitrogen, or on ice and in a water bath at 50, 65 or 100ºC can vary [29-31]. Thermal shock is less violent than mechanical treatment, but it can be effective. By using three thermal shocks (-70/65ºC) with lysozyme and SDS obtained 90% of cell lysed measured by direct acridine orange cell counts [32]. The use of agarose blocks containing embedded DNA improves the PCR amplification from templates naturally contaminated with humic acids, two powerful PCR inhibitors. Sequencing of the 16S ribosomal RNA (rRNA) gene has provided an important method for determining phylogenetic kinships between bacteria. By using a pair of 16S rDNA primers universal to most eubacteria, we showed in this study that bacterial rDNA exists in activated sludge samples. Many researchers have used electrophoretic methods to remove contaminants, notably humic acids [32, 33]. Humic acids, appearing as a brown band in agarose gels stained with ethidium bromide under UV light, migrate faster than the genomic DNA obtained after lysis. Low melting point (LMP) agarose is preferred to standard agarose [33], because gels containing DNA can be melted at relatively low temperature (30-40ºC) and can be used directly for PCR [34].

Conclusions

Total DNA yields resulting from this procedure were at least as high as those obtained with commercial extraction kits. 16SrDNA-targeted genus-specific PCR primers have been used for the identification of gram-positive bacteria in activated sludge samples. The results of this study will prove valuable for future work examining the environmental sources of activated sludge.

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