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Integrated lysis procedures reduce extraction biases of microbial DNA from mangrove sediment

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Sufficient lysis of soil or sediment microbes is a critical step for analyzing microbial community structures and for preparing metagenomic DNA libraries. The present study compared lysis methods for recovering archaeal, bacterial, actinomycete, and fungal DNAs from a mangrove sediment sample. PCR results showed that individual procedures using SDS, lysozyme, sonication, freeze–thaw, microwave, and vigorous shaking could extract archaeal or bacterial DNA but failed for actinomycetes or fungi cells. In comparison, an integrated lysis procedure using SDS, lysozyme, and vigorous shaking successfully obtained fungal DNA, and a combination of SDS, lysozyme, vigorous shaking, and microwave treatments recovered DNA from actinomycetes. Denaturing gradient gel electrophoresis (DGGE) results showed that although single lysis procedures can lyse bacterial DNA, all of them assessed the indigenous bacterial community structure with significant biases. The integrated lysis protocols described in the present study could be useful for extracting DNA from various types of sediments.

[**Key words:** DNA extraction; DNA quality; Denaturing gradient gel electrophoresis (DGGE); Microbial community structure]

Metagenome of soil or sediment DNA has become one of the richest resources of novel enzymes and other bioactive natural products (1). In addition, a lot of nucleic acid-based techniques, such as denaturing or temperature gradient gel electrophoresis (DGGE/TGGE), automated rRNA intergenic spacer analysis (ARISA), terminal-restriction fragment length polymorphism (T-RFLP), and 16S rRNA pyrosequencing, are widely applied to assess the microbial diversity in soil or other complex environmental samples (1–4). Nevertheless, these methods could be biased due to the limitations of DNA extraction procedures, and some fractions of valuable DNA, like actinomycetes, may be lost during the process (5,6).

In the DNA extraction procedure, the lysis step is critical to determine the type and amount of released DNA from different phylogenetic groups of soil or sediment microbes (5). The autochthonous microbial cells in soil and sediment were hard to be lysed because they were adsorbed with soil colloids or located in inner soil compartments (7). A mild lysis procedure only extracted Gram-negative, but not Gram-positive, bacterial DNA; however, a harsher procedure extracted DNA from both types but sheared severely, which was unsuitable for constructing metagenomic libraries (4). Even though many DNA extraction methods have been evaluated, the lysis step has not been extensively investigated, and no lysis procedure has been available to extract a broad range of the microbial DNA within the community or to obtain long DNA from various indigenous microbial cells.

The present study therefore aimed to determine the effects of lysis procedures on the quality of the isolated DNA. Both chemical methods including SDS and lysozyme and mechanical measures including sonication, microwave, freeze–thaw, and vigorous shaking were evaluated. We selected mangrove sediment, a typical acidic and organic-rich environment, as a model because most difficulties have been attributed to acidic and high organic content sediment types to obtain high-quality metagenomic DNA. The effectiveness of the lysis protocols was assessed using PCR and DGGE on the resulting profile of indigenous archaeal, bacterial, actinomycete, and fungal cells. In addition, the influence of each procedure on DNA yields, DNA purity, and co-extracted humic compound yields was evaluated.

MATERIALS AND METHODS

Sediment sampling Sediment samples (organic matter content, 5.47 ± 0.06%; pH, 6.33 ± 0.02) were collected from a 1- to 50-cm layer, after removal of the litter layer, from Zhangjiang Estuary Mangrove National Nature Reserve in Fujian province, PR China. All visible roots were removed, and sediment samples were homogenized manually in sealed and sterilized plastic bags. The sediment was taken back to the laboratory on ice and stored at −70°C until use. All sampling instruments were sterile.

DNA extraction The extraction buffer consisted of 100 mM Tris–HCl (pH 8.0), 100 mM sodium EDTA (pH 8.0), 100 mM sodium phosphate (pH 8.0), 1.5 M NaCl, and 1% CTAB (8). Extracellular DNA, which can remain adsorbed to sediment particles for a long period and may be co-extracted with nucleic acids released from recently lysed cells, normally leads to overevaluation of the lysis capability of the tested procedure

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(9). To avoid this bias, extracellular material was removed as described (10) before DNA extraction. The following six procedures and their combinations were compared.

Procedure 1 (SDS): 5 g of sediment (wet weight) sample was mixed with 13.5 ml DNA extraction buffer and shaken on a horizontal shaker at 37°C for 45 min. After shaking, 1.5 ml of 20% SDS was added and the samples were incubated in a 65°C water bath for 3 h, with gentle end-over-end inversions every 20 min. Treated samples were centrifuged (8000×*g*, 5810 R, Eppendorf) for 15 min at room temperature, and the supernatant was extracted with chloroform–isoamyl alcohol (24:1, vol./vol.) and precipitated with 0.6 volumes of ice-cold isopropanol at room temperature for 1 h. Pellets of crude nucleic acid were obtained by centrifugation at 11,000×*g* for 20 min at room temperature.

Procedure 2 (lysozyme): the first step was performed as described in procedure 1. After shaking, lysozyme (Sigma) was added to final concentrations of 5, 15, and 45 mg ml^{-1}. The samples were then incubated in a 37°C water bath for 1 h with agitation every 20 min. The remainder of the extraction protocol was continued as described in procedure 1.

Procedure 3 (sonication): the first step was performed as described in procedure 1. After shaking, the sample mixture was sonicated with a titanium microtip operated at 100, 200, and 400 W for 15 s (JY92-II ultrasonicator, Xinzhi, PR China). Samples were then cooled on ice, and the sonication was repeated 6 times. The remainder of the extraction protocol was continued as described in procedure 1.

Procedure 4 (freeze–thaw): 5 g of sediment sample was repeatedly treated for 3 cycles (frozen in liquid nitrogen for 5 min and thawed in a 65°C water bath for 5 min) and then suspended in the extraction buffer. The remainder of the extraction protocol was continued as described in procedure 1.

Procedure 5 (microwave): 5 g of sediment sample was placed in a microwave oven and repeatedly heated for 1 min (5 times) at 200, 400, and 800 W. Samples were then suspended in the extraction buffer. The remainder of the extraction protocol was continued as described in the procedure 1.

Procedure 6 (vigorous shaking): 5 g of sediment sample and 5 g of glass beads (Dia. 0.71–1.18 and 0.10–0.11 mm) were added to 50-ml sterile tubes and mixed with 13.5 ml of DNA extraction buffer by vigorous vortexing for 10 min. The remainder of the extraction protocol was continued as described in procedure 1.

Different integrated procedures, including lysozyme/SDS, vigorous shaking/SDS, vigorous shaking/lysozyme/SDS, and vigorous shaking/lysozyme/SDS/microwave, were used to extract microbial DNA from sediment. The lysozyme treatment was performed before the SDS lysis. The microwave treatment was performed after SDS lysis. The vigorous shaking was performed before, during, and after cell lysis. The elapsed time of 3 vigorous shaking steps was 10 min.

DNA quality assay The size of the extracted DNA was determined by electrophoresis on a 1% (wt./vol.) agarose gel, and DNA yields were determined from gel images relative to a molecular weight marker of known concentration with UN-SCAN-IT software (Silk Scientific, Orem, UT, USA). The purity of the crude DNA extracts was assessed by *A*260/*A*280 and *A*260/*A*230 ratios measured with a spectrophotometer (UV310, Thermo Fisher Scientific, USA).

Co-extracted humic compounds were determined by comparison of the *A*230 of crude DNA extracts to the *A*230 (0.5) of a 19 μg ml^{-1} humic acid sodium salt solution (11).

Diversity assay of extracted DNA To determine the microbial diversity of the extracted DNA, PCR amplification of rRNA gene fragments from bacteria, archaea, fungi, and actinomycetes, representing different components of the indigenous sediment microbial community, was performed (Table 1). In our present analysis, we used a 100 bp domain III of the 23S rDNA as a phylogenetic marker for Gram-positive bacteria with a high G + C content (actinomycete), as other eubacteria do not contain this insertion (16,17).

All of the crude DNA extracts were purified with GENECLEAN Turbo Kit (Qbiogene, USA) as recommended by the manufacturer before PCR. The reaction mixture consisted of 10 μl template DNA, 5 μl 10× PCR buffer (Takara), 1 μl dNTP (10 mM) mix (Takara), 1 μl each primer (10 pM, Invitrogen), 2 U Taq DNA Polymerase (TaKaRa), 2 μl $MgCl_2$ (50 mM), 5 μl bovine serum albumin (BSA, 1 mg ml^{-1}, Promega), and sterile distilled water (18.2 Mcm, Milli-Q 185 Plus, Millipore) to make a final reaction volume of 50 μl. PCR amplification was performed with an initial denaturation step (94°C, 5 min), followed by 30 cycles of denaturation (94°C, 1 min) and annealing ((a) 60°C, 1 min for archaeal 16S rDNA genes; (b) 55°C, 1 min for the detection of bacterial 16S rRNA genes; (c) 46°C, 1 min for the detection of actinomycete domain III of 23S rRNA genes; and (d) 55°C, 1 min for the detection of fungal 18S rRNA and ITS genes and extension (72°C, 1 min)). A final extension step of 10 min at 72°C completed the reaction.

To check whether the extracted DNA was suitable for further fungal DGGE analysis, a nested PCR that amplified a fungal partial ITS region was performed as described by Anderson et al. (15).

DGGE analysis of PCR amplicons To investigate the influence of different lysis procedures on sediment bacterial community structure analysis, PCR–DGGE was employed. The bacterial 16S rRNA V3 region fragments were amplified using the primer pair 341F with a GC clamp and 517R (Table 1). The reaction mixture consisted of 10 μl template DNA, 5 μl 10× PCR buffer (Takara), 0.5 μl dNTP (10 mM) mix (Takara), 0.5 μl each primer (10 pM, Invitrogen), 2 U Taq DNA Polymerase (TaKaRa), 2 μl $MgCl_2$ (50 mM), 5 μl bovine serum albumin (BSA, 1 mg ml^{-1}, Promega), and sterile distilled water (18.2 Mcm, Milli-Q 185 Plus, Millipore) to make a final reaction volume of 50 μl. A touchdown PCR protocol was performed according to a reported protocol (13).

Denaturing gradients were performed with the DCode Universal Mutation Detection System (Bio-Rad). Denaturing gradients of 40–60% denaturant (100% denaturant corresponds to 7 M urea and 40% (vol./vol.) of deionized formamide) were prepared. The 8% acrylamide gels were polymerized for 3 h. PCR samples were loaded onto the gel and DGGE was run in 1× TAE buffer. Electrophoresis was performed at constant voltage (100 V) and temperature (60°C) for 11 h. After electrophoresis, the gels were stained with ethidium bromide and photographed. Bandscan 5.0 was used to analyze DGGE profiles by measuring migration distance and intensity of the bands within each lane of the gel.

Statistical analyses All of the experiments were performed at least in triplicate to check the reproducibility. The differences between treatments were analyzed by one-way ANOVA and *t*-test; a *p*-value of <0.05 was considered statistically significant.

RESULTS

Comparison of DNA extraction yields, size, purity, and co-extracted humic compounds The differences in DNA yields, DNA purity, and co-extracted humic compound yields from the six procedures are shown in Table 2. The highest amount of crude DNA (19.83 ± 3.31 μg g^{-1} sediment) was obtained using the SDS procedure, which was 1.84, 3.59, 9.49, 4.81, and 7.75 times higher than that obtained by the lysozyme (45 mg ml^{-1}), sonication (400 W), microwave (800 W), freeze–thaw, and vigorous shaking procedures, respectively. Nevertheless, the SDS method also produced the highest amount of co-extracted humic compounds (83.67 ± 0.37 μg g^{-1} sediment), which was 1.63, 2.22, 2.36, 2.42, and 2.99 times of that obtained by the lysozyme, sonication, microwave, freeze–thaw, and vigorous shaking procedures, respectively. We observed that the higher the concentration of lysozyme and the power of the sonicator, the more the DNA yields ($p<0.05$). Nevertheless, none of the tested procedures obtained a DNA with an *A*260/*A*280 ratio above 1.3 or an *A*260/*A*230 ratio above 0.9, indicating that a further purification step was necessary for downstream molecular applications.

Results from gel electrophoresis showed that all of the test DNA extraction methods yielded high molecular weight DNA from sediments (Fig. 1), among which the sonication method showed the highest amount of sheared sediment DNA (Fig. 1, lanes 1–3).

TABLE 1. PCR primers used for the amplification of extracted soil DNA.

Organism group	Primer pair	Primer sequence(5′–3′)	References
Bacteria	27F	AGAGTTTGATCCTGGCTCAG	(12)
	1492R	GGTTACCTTGTTACGACTT	
	341F	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG	(13)
	517R	ATTACCGCGGCTGCTGG	
Achaea	Arch21F	TTCCGGTTGATCCYGCCGGA	(12)
	Arch915R	GTGCTCCCCCGCCAATTCCT	(14)
Fungi	EF4	GGAAGGG[G/A]TTATTTATTAG	(15)
	ITS4	TCCTCCGCTTATTGATATGC	
	ITS1F	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGCACGGGGGGCTTGGTCATTTAGAGGAAGTAA	
	ITS2	GCT GCG TTC TTC ATC GAT GC	
Actinomycete	1900 V	CCTAAGYYGAGGC	(16)
	1028 R	CCTTCTCCCGAAGTTACGG	

GC clamp: 5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3′.