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Direct Extraction of DNA from Soil for Amplification of 16S rRNA Gene Sequences by Polymerase Chain Reaction

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Microgram quantities of DNA per gram soil were recovered with SDS-based and freeze-and-thaw procedures. The average DNA fragment size was > 23 Kb. This method generated minimal shearing of extracted DNA. However, the DNA extracts still contained considerable amounts of humic impurities sufficient to inhibit PCR. Several approaches were used to reduce the interferences 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. Most of the DNA extracts were not digested completely by restriction endonuclease, and CTAB-treated and Elutip-d column purified DNA extracts were partially digested. Regarding as restriction enzyme digestion, all PCRs failed to amplify 16S rRNA gene fragments in the DNA extracts. In the case of DNA extracts only where BSA was added to PCR buffer, PCR was successfully conducted whether the DNA extracts were treated with CTAB or purified with columns. However, these two treatments were indispensable for humic impurity-rich DNA extracts to generate the PCR-compatible DNA samples. Direct extraction of DNA, coupled with these procedures to remove and relieve interferences by humic impurities and followed by the PCR, can be a rapid and simple method for molecular microbiological study on soil microorganisms.

Key words: Soil, DNA extraction, PCR

Total bacterial counts in the environments are usually more than one order of magnitude higher than plate counts, and most of them cannot be cultured on the laboratory media (15). Consequently, molecular biological techniques have been used by a lot of microbial ecologists to investigate the ecological functions of certain characterized genes that encode important metabolic pathways and reveal microbial community structure and diversity. Newly developed method for researching the structure and diversity of bacterial community is the PCR amplification of specific variable regions in the base sequence and size of 16S rRNA genes followed by the single strand conformational polymorphism (SSCP)/heteroduplex pattern analysis (9). To apply such molecular techniques to ecological studies, extraction of DNA from the natural environment is inevitable. However, there are only a few reports concerned with the extraction of DNA from soil, although several techniques have been described for the extraction of DNA from aquatic ecosystems (2, 5, 13, 17).

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Two approaches for the extraction of DNA from soil have been proposed. The first approach is the cell extraction method (6, 19). This approach involves separation of bacterial cells from soil particles by differential centrifugation followed by cell lysis and DNA recovery. But this method is laborious and requires a large sample size (ca. 100 g). The other newly developed approach is the direct lysis method (12, 18, 20). This method involves the release of DNA from the cell without separating the cells from the soil particles, followed by direct SDS-based DNA extraction and purification of DNA extract. This second approach is relatively simple and yielding. However, the purity of DNA obtained by the direct method is too low to be applied to molecular biological analysis. DNA extract by this method contains such high amounts of enzyme inhibitors, like humic materials, that it cannot be used for precise reactions as PCR.

In this study we developed a direct DNA extraction method which gives high purity of DNA extract. We were particularly concerned with the effect of the use of CTAB in the lysis step, elutip-d column purification of

DNA extract, and the addition of BSA to PCR buffer on the elimination of PCR inhibitors. The proposed procedure for extracting DNA from the soil environment will be a useful tool for molecular ecological study on the soil ecosystem.

Materials and Methods

Soils

Soil samples from the hillside of Kwanak mountain (KA) and organic farming land (OF) in Kyounggi and Chungnam province, respectively, were taken from a depth of 0~15 cm after the surface litter was cleared. After sieving (mesh size 2 mm) the soil samples, all physicochemical and microbiological analysis was performed by the method of ASA-SSSA (1). The texture of both soil samples was sandy loam. Water content and pHw was 12% and 6.0 for soil KA, and 27% and 6.0 for soil OF, respectively. Soil OF contained higher organic matter content (6.68%) than soil KA (1.44%). Since a sample OF was collected from the organic farming land which had been cultivated with organic fertilizer, mainly composed of animal excreta and plant straw for several years, it was expected that soil OF contained high amounts of humic acid-like substances. Viable bacterial counts of soil KA and OF were 7.1×10^6 and 6.9×10^8 cfu/g dry soil.

Effects of CTAB, Elutip-d column purification, BSA

To accomplish PCR with DNA extract from soil as a template DNA, the effects of adding hexadecylmethylammonium bromide (CTAB) in the extraction step and bovine serum albumin (BSA) to the PCR buffer, and purification of crude extracts with commercial Elutip-d column (Schleicher & Schuell, Keene, N.H.) were investigated.

CTAB and Elutip-d column purification procedure were used to evaluate their effects on removing impurities such as humic acid-like materials in crude extracts. Soil samples (5 g) were mixed with lysis solution (see below) containing i) CTAB (final 1%) or ii) no CTAB, respectively. Then soil suspensions were processed by the extraction methods described below. After DNA was extracted from the soil, crude DNA extracts were further purified i) without, or ii) with a) single or b) double Elutip-d column, respectively. DNA was recovered from the column as suggested by the manufacturer.

PCR was conducted with the final DNA extract as template DNA using PCR buffer containing i) BSA (100 ng/μl) or ii) no BSA. The PCR procedure is described below.

DNA extraction

DNA was extracted from soil samples by directly lysing the cells and extracting the DNA by the modified procedure of Tsai and Olson (20), and Erb and Wagner-Döbler (3). These methods were modified to increase yield and handiness. The soil samples (5 g) were initially suspended in 10 ml of lysis solution (0.15 M NaCl, 0.1 M Na_2EDTA [pH 8.0]) containing 15 mg of lysozyme/ml, and incubated at 37℃ for 1hr, and then 10 ml of SDS solution (0.1 M NaCl, 0.5 M Tris-HCl [pH 8.0], 10% sodium dodecyl sulfate) was added. Four cycles of freezing at -70℃ and thawing at 65℃ were conducted to release DNA from the bacterial cells in the soil. After the freeze-thaw cycles, 10 ml of 0.1 M Tris-HCl (pH 8.0)-saturated phenol was added and the sample was gently vortexed to get an emulsion. The mixture was centrifuged at 12,000×g for 10 min. The top aqueous layer was collected and extracted once with equal volume of phenol-chloroform mixture (phenol: chloroform:isoamyl alcohol, 25 : 24 : 1) and twice with chloroform mixture, and then centrifuged (12,000×g for 10 min). Nucleic acids in the aqueous phase were precipitated with equal volume of cold isopropanol at -20℃ overnight. Nucleic acids were pelleted by centrifugation at 12,000×g for 15 min. The nucleic acid pellet was resuspended in TE buffer (20 mM Tris-HCl, 1mM EDTA [pH 8.0]) containing heat-treated RNase A (final conc., 0.2 μg/μl) and incubated at 37℃ for 2 h. Resulting DNA extracts were stored at -20℃ until analyzed.

Determination of the purity and yield of DNA

For quantitating the amount of DNA, the concentration of DNA in extracts was determined spectrophotometrically by the method of Sambrook *et al.* (16). The purity of the DNA was determined by spectrophotometric readings at 230, 260, and 280 nm wavelengths in TE buffer. A_{260}/A_{280} and A_{260}/A_{230} ratios were calculated to evaluate levels of protein and humic acid impurities, respectively, in the extracts (11, 18).

Restriction endonuclease digestion

The interference of humic impurities in restriction enzyme activity was investigated. Restriction enzyme digestion was performed with approximately 0.5 μg of extracted DNA and 10 U of an endonuclease (*Eco*RI, *Hind*III, and *Eco*RI plus *Hind*III) in 10 μl of the appropriate buffer as provided by the manufacturer. To verify the effects of humic impurities, DNA extracts (0.25 μg) plus undigested bacteriophage lambda DNA (0.25 μg) was digested by endonucleases (*Eco*RI plus *Hind*III). After incubation for 12 hrs, the DNA fragments were resolved in a 1% agarose gel.

Polymerase Chain Reaction

DNA extracts from soil samples were employed as the

template in the PCR. The variable V3 region of 16S rDNA was selectively amplified with oligonucleotide primers (9) designed to anneal to a conserved position in the 3' and 5' regions of eubacterial 16S rRNA genes. The forward primer corresponded to positions 330~348 of *Escherichia coli* 16S rRNA (5'-CGGYCCAGACTCCTACGGG-3'), and the reverse primer corresponded to the complement of positions 515~533 (5'-TTACCGCGGCTGCTGGCAC-3').

The final reaction concentrations were 1 ng/μl for template DNA, 0.1 M for each primer, 50 M for each deoxynucleoside triphosphate, and 2 U for Dynazyme (Finnzymes Co.) in a 1× working buffer (Finnzymes Co.) containing 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100. The PCR was then carried out for 27 cycles at each of the following temperatures; 94℃ (1.5 min for denaturation), 62℃ (1.5 min for annealing), and 72℃ (2.0 min for extension) with a 20 min extension at 72℃ in the last cycle. All amplifications were performed on a GeneAmp PCR system 2400 (Perkin Elmer).

Gel electrophoresis

Electrophoresis was carried out in 1% agarose at 10 V/cm for 30 min (2% agarose for PCR products). The buffer system contained 90mM Tris-borate, 2 mM EDTA (pH 8.0). *Eco*RI-*Hind*III-cut bacteriophage lambda DNA and PCR marker (Promega) were used for molecular size markers.

Results

The method for the preparation of DNA sample to be employed for the PCR-amplification of 16S rRNA gene sequences was developed in this study for molecular ecological study on soil bacterial community. To optimize extraction, purification, and PCR procedures, two types of soil samples (KA and OF) were collected and examined. DNA was isolated directly from the soil samples by SDS-based extraction method. Fig. 1 shows ethidium bromide-stained agarose gel used to visualize the DNA extracts after RNase treatment. The average DNA fragment size was 23Kb. There was not a severe shearing in DNA even when DNA extracts were purified twice with Elutip-d column. 38.4~49.5 and 399.5~449.9 μg of DNA per g dry soil KA and OF, respectively, were extracted (Table 1). These were considered reasonable yields assuming that average bacteria contain 9.0×10^{-3} μg of DNA (*Escherichia coli*) (7) and less than 0.1~1% of total bacteria is culturable on laboratory media (4). The density of heterotrophic bacteria from soil KA and OF represented 0.063 and 6.21 g of DNA per g soil, which would correspond 0.1~0.2 and 1.4~1.6% of total extracted DNA, respectively. These results support the observations that only a very small portion of the natural bacteria is culturable. However, the purity of DNA extracts was considerably low. A_{260}/A_{280} and A_{260}/A_{230} ratios of soil KA and OF were 1.07 and 0.56~0.75. In particular, the level of humic acid-like impurities, represented by A_{260}/A_{230} ratio, was very low. We attributed the lower A_{260}/A_{230} ratio of soil OF than that of soil KA to the fact that DNA extract from soil OF still contained more humic

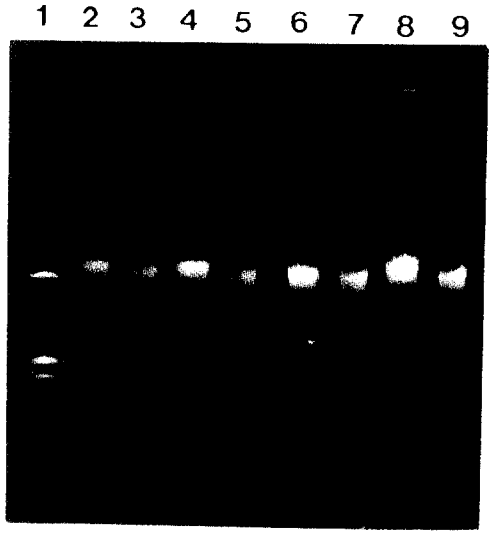

Fig. 1. Agarose gel electrophoresis of DNA directly extracted from soil KA (lane 2 to 5) and OF (lane 6 to 9). Lane 1, *Eco*RI-*Hind*III-cut bacteriophage lambda DNA molecular size marker; 2 and 6, crude DNA extract; 3 and 7, Elutip-d column purified DNA extract; 4 and 8, crude DNA extract treated with CTAB; 5 and 9, Elutip-d column purified DNA extract treated with CTAB.

Table 1. The yield and purity of DNA extracts

Treatments	Soil sample					
	KA			OF		
	Yield[a]	A_{260}/A_{280}	A_{260}/A_{230}	Yield	A_{260}/A_{280}	A_{260}/A_{230}
Crude DNA extract						
-CTAB	49.5	1.07	0.75	399.5	1.07	0.56
+CTAB	38.4	1.10	0.80	449.9	1.14	0.79
Single column[d]						
-CTAB	11.3	1.11	1.02	216.0	1.08	0.70
+CTAB	10.8	1.10	1.06	196.6	1.17	0.79
Double column[e]						
-CTAB	8.0	1.11	1.38	116.0	1.08	0.70
+CTAB	7.8	1.10	1.67	80.6	1.15	0.79

[a] yield (μg DNA/g dry soil)
[b] extraction buffer containing no CTAB
[c] extraction buffer containing 1% CTAB
[d] DNA extracts purified once with Elutip-d column
[e] DNA extracts purified twice with Elutip-d column

contaminants than that of soil KA even after extraction.

In the extraction step in this study, two approaches-addition of CTAB in extraction buffer and purification with commercial DNA purifying column (Elutip-d column)-were utilized to remove humic contaminants from DNA extracts. However, the ability of CTAB to precipitate and remove humic contaminants was poor. The A_{260}/A_{230} ratio was not significantly changed whether CTAB was used (Table 1). DNA extract taken from the extraction buffer, to which CTAB was added during the extraction step, showed slightly increased A_{260}/A_{280} and A_{260}/A_{230} ratios of DNA extracts from both soil KA and OF. CTAB did not completely remove humic compounds. So, each DNA extract was further purified once or twice with commercial DNA purifying column (Elutip-d). DNA extracts from soil KA could be purified only a little once or twice with Elutip-d column. However, it was no use purifying the DNA extracts from soil OF. Moreover, rather than impurities were eliminated when DNA extracts were purified with Elutip-d column, the yield decreased (46~72% and 28~59% loss of DNA at first and second purification steps, respectively). Because the pure DNA solution exhibits more than 1.8 of A_{260}/A_{280} and A_{260}/A_{230} ratio (16), the purification step using Elutip-d column was not effective in removing humic contaminants, though crude extracts were purified even twice with the column, and not recommended as a purification procedure for DNA extract from soil.

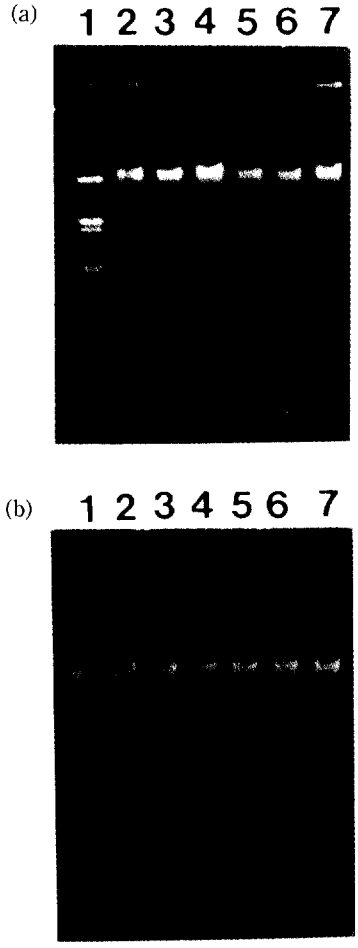

Fig. 2. Agarose gel electrophoresis of restriction endonuclease-digested DNA extracted from soil KA (a) and OF (b) purified with (lane 2 to 4) or without (lane 5 to 7) CTAB and Elutip-d column. Lane 1. *EcoR*I-*Hind*III-cut bacteriophage lambda DNA molecular size marker. Lane 2 and 5, *EcoR*I-digested DNA extract; 3 and 6, *Hind*III-digested DNA extract; 4 and 7, *EcoR*I-*Hind*III-digestion of the DNA plus uncut bacteriophage lambda DNA.

The susceptibility of DNA extracts to restriction endonuclease was tested to evaluate the maleficence of humic impurities which interfere with the enzyme reaction and because the application of restriction enzyme analysis to directly extracted DNA may allow more precise studies of *in situ* gene rearrangement or amplification. Most of the crude extracts and Elutip-d column purified extracts from the two soil samples were not digested completely by *EcoR*I and *Hind*III (Fig. 2). So as to verify the existence of inhibitors in the DNA extracts, undigested lambda DNA was added to DNA extracts and then extract mixtures (DNA extract plus undigested lambda DNA) were treated with restriction endonuclease, *EcoR*I and *Hind*III. In this case, only the DNA extract mixture from soil KA which was treated with CTAB and purified twice with Elutip-d column was partially digested by restriction enzymes. Fig. 2a lane 4 shows weak DNA bands at the position below the chromosomal band. Judging from the results obtained above, it seemed that DNA extracts still contained considerably high amounts of humic impurities, enough to inhibit enzyme reactions even after the purification steps.

Pertaining to the goal of this study, it was investigated whether each final DNA extract prepared by each extraction and purification method was suitable for PCR as a template DNA. When the DNA extracts were employed as the template without adding BSA to reaction buffer, all PCRs failed to amplify 16S rRNA gene sequences (land 2 to 5 of Fig 3 (a) and (b)). Similar to restriction endonuclease, PCR seemed to be interfered by the humic impurities in the DNA extracts. On the other hand, in DNA extracts from sample KA, where BSA was added to PCR buffer, PCR took place successfully whether the DNA extracts were purified with Elutip-d column or not. Fig. 3 lane 6 to 9 shows the PCR products whose size is 150~300 bp as a result of the amplification of the conserved position of eubacterial 16S rDNAs (9). Among the DNA extracts from soil OF, only the CTAB treated and double Elutip-d column purified DNA extract was

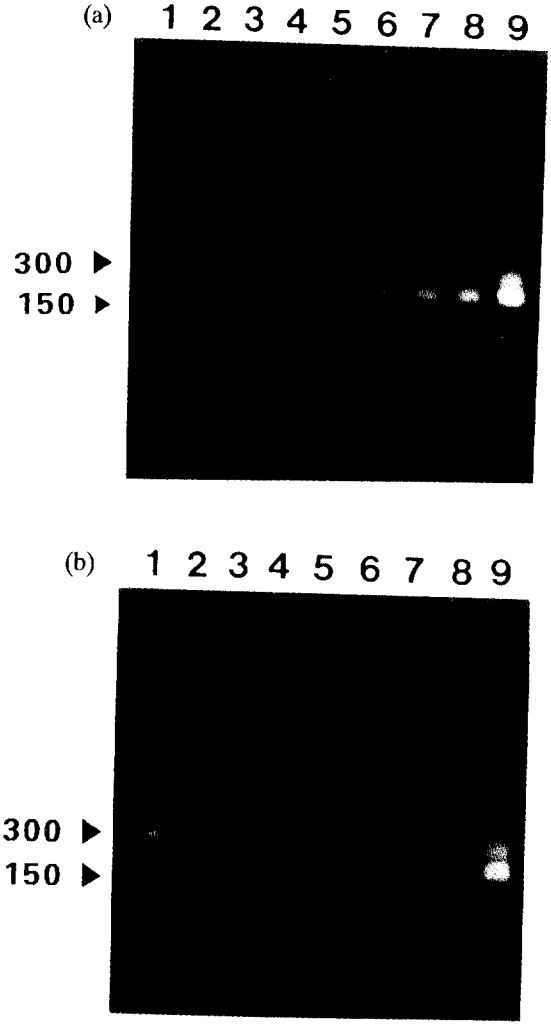

Fig. 3. Agarose gel electrophoresis of PCR products employing DNA extracts isolated from soil KA (a) and OF (b) as template DNA when BSA was added to reaction buffer (lane 6 to 9) or not (lane 2 to 5). Lane 1, PCR marker; 2 and 6, crude DNA extract; 3 and 7, Elutip-d column purified crude DNA extract; 4 and 8, CTAB-treated DNA extract; 5 and 9, CTAB-treated and Elutip-d column purified DNA extract.

compatible with PCR only when BSA was added to the PCR reaction buffer (Fig. 3 lane 9). However, resolving PCR products more than that shown in Fig. 3 was impossible on agarose gel. This requires more minute techniques such as SSCP/heteroduplex pattern analysis (9) to figure out the profile of the amplified sequences.

Discussion

In the initial stage of this study, various methods for extracting DNA from soil had been considered after referring to sundry literatures. Among them, the methods of Tsai and Olson (20) and Erb and Wagner-Döbler (3) had been chosen and reproduced by us. Finally we used the our own method which was modified from the procedures of the above-mentioned to increase purity and convenience. They used approximately 1 g of soil for DNA extraction. In this case, however, the yield of DNA varied and could be severely decreased when the bacterial number was low or the extraction efficiency decreased because of minor technical problems in sample handling. Therefore we used 5 g of soil for extraction and conducted four freeze-thaw cycles for releasing DNA from cells, and shortened other things, such as incubation time required for lysozyme and RNase treatments. However, the DNA extracts obtained by direct extraction method contained such high amounts of humic acid-like impurities that they could not be compatible to PCR.

Humic substances, which are able to inhibit PCR (21), are mixtures of complex polyphenolics produced during the decomposition of organic matter and are abundant in natural soil. Humic acid-like impurities were usually found in crude DNA extracts isolated by using the SDS-based and freeze-and-thaw method (20) although some of them were lost during the phenol-chloroform extraction step (21). Phenolic compounds are known to bind to proteins by forming hydrogen bonds with peptide bond oxygens, and to inactivate proteins like enzymes (10). Therefore, extensive purification is required to generate PCR-compatible DNA extract. However, because the purification procedure is time-consuming and incommodious, and decreases the yield of extraction, a more satisfying approach should be used to solve the problem of PCR inhibition.

In this study, by adding BSA in the reaction buffer, the inhibition of several impurities has been relieved during PCR (8). BSA has been widely used during isolation of organelles and enzymes from plants to scavenge endogenous phenolic contaminants and is also known to bind lipids via hydrophobic forces and anions by virtue of its high lysin content (10). Therefore, BSA may be able to scavenge a variety of contaminants which can bind to and inactivate proteins or enyzmes of interest, and so it can prevent their binding and inactivation of DNA polymerase. BSA has been added to PCR buffer to relieve inhibition from samples containing endogenous protease activity (14), so BSA may provide an alternative substrate and thereby protect the DNA polymerase (8). We suggest the use of BSA in PCR buffer to relieve inhibition by humic contaminants in DNA extract. However, in the case of the soil containing high amounts of humic materials such as soil OF in this study, other purification steps should be conducted to remove humic contaminants. If not, PCR cannot be performed successfully. But humic contaminants might be removed by

freezing too. We stored the DNA extracts in the freezer (-20°C), and after the samples were frozen, the humic fraction which showed a dark brown colour was separated from the bulk DNA extract and stayed at the top of the solution. Then the humic fraction could be removed by pipetting and PCR could be performed by employing the resulting extracts as template. The DNA extracts even from soil OF could be purified by this procedure. We, however, could not elucidate the reason why humic impurities, PCR inhibitors, were removed by this procedure. It was considered that the differences in the freezing point and the density between humic fraction and pure DNA fraction generated during freezing might separate them. Nevertheles this procedure was not free from the blemish.

Contrarily, cell extraction methods, described in the introduction section, produce high purity of DNA extract. But this needs a large amount of soil, and is very complex and time-consuming. More importantly, most of the total bacteria cannot be extracted by cell extraction methods. Holben *et al* (6) reported the recovery of about 33% of the bacterial cells from soil. So, it cannot reflect the whole bacterial community in an objective environment. On the other hand, the direct extraction (SDS-based extraction) method results in 92~99% recovery of the DNA from bacteria (22). Crude DNA yield from the direct extraction method agreed reasonably well with expected yield based on microbial counts (22, in this study). We extracted 38.4~49.5 and 399.5~449.9 μg of DNA per g dry soil KA and OF, respectively. These were considered reasonable yields assuming that average bacteria contain 9.0×10^{-9} μg of DNA (*Escherichia coli*) (7) and less than 0.1~1% of total bacteria is culturable on laboratory media (4). The density of heterotrophic bacteria from soil KA and OF represented 0.063 and 6.21 μg of DNA per g soil, which would correspond 0.1~0.2 and 1.4~1.6% of total extracted DNA, respectively. These results support the observations that only a very small portion of the natural bacteria is culturable. In this study, we developed the preparation method of DNA to be employed as template DNA for PCR. The proposed method for the preparation of DNA from soil and for PCR is as follows: SDS-based extraction using extraction buffer containing lysozyme and CTAB, freeze and thaw cycles, phenol-chloroform extraction, PCR with reaction buffer containing BSA. This procedure will be a rapid and simple method for molecular microbiological study on soil environments.

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