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Methods for the Extraction of DNA from Water Samples for Polymerase Chain Reaction

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Methods for the extraction of DNA from water samples were approximated. Four different procedures of DNA extraction were carried out with pellets obtained from centrifugation of 4 liter water samples. The recovery efficiency and purity of DNA extracted by each method from different sources were compared. DNA yield varied with extraction methods. Method I, which involves enzymatic and freeze-thaw lysis steps and phenol and phenol-chloroform purification of extracted nucleic acid, showed a significantly higher yield and purity than the other methods. The use of glass beads in the DNA extraction methods improved the purity of DNA suitable for PCR. Bovine serum albumin in the PCR reaction mixture was useful in reducing inhibitory effects of contaminants. The efficacy of an extraction method was determined by the detection of the *aer* gene of *Aeromonas hydrophila* with PCR. The lower limit of detection of *A. hydrophila* from seeded tap water was 2 CFU/ml in PCR when method I was used for DNA preparation.

Key words: DNA extraction, water sample, PCR, BSA, *Aeromonas hydrophila*

Methods traditionally used for the analysis of community diversity in environmental samples were the fluorescence antibody and culture enrichment techniques. However, it is well recognized that only a minor fraction of the constituents of naturally occurring communities can be recovered in pure culture (17, 21). Techniques that involve DNA extraction, followed by either direct DNA probing or amplification by the polymerase chain reaction, have been developed as more sensitive ways to assess the microbial communities of natural environments.

PCR is a technique that possesses rapidity, sensitivity, and specificity and can be employed to facilitate rapid analysis of microbial communities. In particular, PCR amplification has the potential for monitoring pathogens and indicator bacteria in the environment. This method has been used to detect pathogenic microorganisms in foods (6, 12), in clinical samples (15), in air samples (1) and in soil and sediment (3, 4, 8, 18, 19, 20). To apply molecular techniques to the study of microbial communities, DNA should be isolated directly from environmental samples.

Direct isolation of nucleic acid from the environment may be useful in several respects, including the estimation of total biomass, detection of specific organisms and genes, estimation of species diversity, and cloning application (14). Molecular analysis of microbial communities requires the extraction of high-quality nucleic acid. However, the purification of nucleic acid from environmental samples for PCR is not without its problems. The humic acid and phenolic compounds present in samples are known to reduce the efficiency of restriction enzymes (10), PCR (19) and even the specificity of hybridization (16). The development of methods to optimize each step of the protocol, including lysis of bacteria and DNA purification, are needed to detect specific microorganism by PCR amplification.

In this work, we modified several methods developed for DNA extraction from soil and sediment samples and applied these protocols to water samples. We also compared the yield and purity of extracted DNAs. To investigate the usefulness of the DNA extraction methods, DNA was extracted from water samples which were artificially contaminated by an appropriate bacterial dilution and used in PCR.

Materials and Methods

Sample collection

To evaluate the efficiency of the DNA extraction and purification procedures, water samples were collected from five sites, including creek, lake, hos-

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pital sewage, influent of sewage plant and sea. The numbers of culturable bacteria were counted by spreading on plate counting agar for fresh water or Zobell agar for sea water.

DNA extraction methods

Four liters of each water sample were centrifuged at 16,000×*g* for 30 min. The pellets were collected and divided into four subsamples. DNA was extracted from the subsamples by each of the four methods. The extraction procedures are as follows.

Method I. The pellet was suspended in 300 µl of lysis solution (150 mM NaCl, 100 mM sodium EDTA, pH 8.0) containing 15 mg/ml lysozyme, and incubated at 37°C for 2 hr with agitation at 20-min intervals, subseguently 300 µl of 10% sodium dodecyl sulfate were added. Three cycles of freezing in a -70°C ethanol bath and thawing in a 65°C water bath were conducted to release DNA. After the freeze-thaw cycle, an egual volume of buffer saturated phenol was added. The top aqueous layer was collected and then mixed with 200 ul of phenol and 200 µl of chloroform mixture (chloroform-isoamylalcohol, 24:1). After the phenol-chloroform extraction, DNA was precipitated with ethanol (18).

Method II. The pellet was resuspended in 300 µl of GuSCN lysis binding buffer (5.3 M guanidine thiocyanate, 10 mM dithiothreitol, 1% Tween 20, 300 mM sodium acetate, 50 mM sodium citrate, pH 7.0), and then 3.8 µl of 0.5 M NaCl and 26.5 µl of CTAB/NaCl (10% hexadececylcetyl-trimethylammonium bromide in 0.7 M NaCl) were added. The mixture was incubated at 65°C for 10 minutes. Fifteen microliters of resuspended glass matrix (Geneclean; Bio101) were then added to the solution, and the mixture was incubated at room temperature for 15 min with mixing. The impurities were removed according to the directions of manufacturer (15).

Method III. The pellet was suspended in 1 ml TE and 0.25 ml of 5 M NaCl was added. The mixture was centrifuged at 14,000×*g* for 15 min. The pellet was resuspended in 550 µl of TE and 50 µl of freshly prepared lysozyme (10 mg/ml). After incubation at 37°C for 60 min, 50 µl of proteinase K (10 mg/ml) was added and the mixture was incubated at 37°C for 30 min. Samples were then heated at 65°C for 10 min and 160 µl of 10% SDS was added. After 10 min of incubation, samples were centrifuged at 14,000×g at 4°C for 1 h and the supernatant was collected for DNA purification using the Geneclean kit (4).

Method IV. The pellet was suspended in 450 µl DNA extraction buffer (100 mM Tris-HCl, pH 8.0, 100 mM sodium EDTA, 100 mM sodium phosphate, 1.5 M NaCl, 1% CTAB) and 30 µl of proteinase K (10 mg/ml) was added. After shaking for 30 min at 37°C, 50 µl of 20% SDS was added. Samples were incubated at 65°C for 2 h and then centrifuged at 14,000×g for 10 min. The supernatant was collected for DNA purification and the pellet was resuspended in 450 µl of DNA extraction buffer and 50 µl of 20% SDS for further extraction. Supernatants were mixed in an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol). The aqueous phase was recovered by centrifugation and precipitated with ethanol. The pellet of crude nucleic acids was suspended in 100 µl TE (22).

Following DNA extraction, all DNA samples extracted the by four methods were finally purified with the Geneclean kit as described above. Bound DNA was eluted by incubation in 50 µl of TE (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) at 50°C for 5 min with periodic mixing. Following centrifugation at 14,000×g for 2 min, the elute was transferred to a new tube.

DNA measurement

The concentration of DNA was measured by fluorometry with a TKO100 fluorometer (Hoefer Scientific Instruments) by the assay protocol provided by the manufacturer.

PCR amplification of 16S rDNA

The suitability of the isolated DNA for PCR was estimated using primers for eubacterial 16S rRNA genes. The 16S rDNA was selectively amplified from purified DNA by using PCR with oligonucleotide primers designed to anneal to conserved positions in the 3' and 5' regions of bacterial 16S rRNA genes. The forward primer (5'-TNA NAC ATG CAA GTC GAI CG) corresponded to positions 49 to 68 of *Escherichia coli* 16S rRNA gene (2), and the reverse primer (5'-GGY TAC CTT GTT ACG AC TT) corresponded to the complement of positions 1510 to 1492. This primer set has been used to amplify eubacterial small-subunit (16S) rRNA genes from total-community genomic DNA (7). The reaction mixture consisted of 10 ng of template DNA, 2.5 U of Taq DNA polymerase (Boehringer Mannheim), 5 µl of 10x PCR amplification buffer (100 mM Tris-HCl, 25 mM $MgCl_2$, 500 mM KCl, pH 8.3), 1 M of each primer, 200 µM of deoxyribonucleotide triphosphates, and distilled water up to a final volume of 50 µl. A total of 30 PCR cycles were run with the GeneAmp PCR system (Perkin-Elmer Cetus) under the following conditions: DNA denaturation at 94°C for 30 sec, primer annealing at 55°C for 30 sec, and DNA extension at 72°C for 30 sec. After the final cycle, reactions were terminated by keeping them at 72°C for 7 minutes.

Table 1. DNA yield of each method and heterotrophic bacterial numbers of five different water samples

Water sample	DNA yield (μg/L)				CFU/ml[a]
	Method I	Method II	Method III	Method IV	
Creek water	1.20	0.36	0.60	0.72	4.1×10^4
Lake water	1.98	0.42	0.78	0.84	1.2×10^2
Hospital sewage	3.00	0.78	0.90	0.96	1.5×10^6
Sewage influent	2.04	0.36	0.60	0.90	3.5×10^5
Sea water	0.84	0.24	0.30	0.36	1.3×10^3

[a]Heterotrophic bacterial numbers were counted on plate counting agar or zobell agar (sea water).

Detection limit of bacteria

The lower limit of detection of bacterial cells by PCR was examined for *Aeromonas hydrophila* Ah 65. A suspension of bacterial cells was serially diluted and seeded into 100 ml water. Total DNA from each dilution was extracted by method I. The minimum number of cells detectable by PCR was assayed using *Aeromonas* spp. specific primers. Primers used in the PCR targeted a 209 bp fragment of the *aer* gene coding for β-hemolysin (9). The nucleotide sequences of the two primers aero-1 and aero-2 were 5'-CCA AGG GGT CTG TGG CGA CA and 5'-TTT CAC CGG TAA CAG GAT TG, which corresponded to positions 645 to 664 and 834 to 853 of the aerolysin gene sequence, respectively. PCR conditions were the same as above.

Results and Discussion

Methods of DNA extraction from environmental samples can be divided into two categories: (i) direct lysis and (ii) cell extraction. Direct lysis involves treatment of sample with one or more of the following; lysozyme, SDS, proteinase K, guanidine thiocyanate, and freeze-thawing. Cell extraction involves separation of bacterial cells from particle by shaking in a washing buffer followed by differential centrifugation. The isolation of nucleic acids from aquatic samples first requires the harvesting of bacterial cells from the environmental samples. An efficient means of recovering bacteria from aquatic environments is ultrafiltration (14). However, environmental water samples, which contain excessive amounts of colloidal debris, can not be filtered. Filtration is also limited by the capacity of the filtration apparatus. In cases where very large samples are to be processed, alternative extraction methods that can accomidate increased sample sizes are needed. We used the centrifugation method, which can concentrate relatively large volumes of water, to precipitate particles and bacterial cells in the aquatic environmental samples. Since water type and microbial community characteristics will influence DNA recovery, we selected five different environmental water samples for choosing the appropriate extraction and purification method.

DNA yields varied with extraction methods and source of samples, from 240 ng to 3 μg of DNA per 1000 ml of water sample (Table 1). Total DNA yields were the greatest with method I and lowest with method II regardless of sample sources. The extraction methods presented here all yielded positive, linear correlations between cell number and recovered DNA.

Fig. 1 shows an ethidium bromide-stained agarose gel used to visualize the DNA extracted from lake water. All four methods yielded DNA with low fragmentation. Although the DNA yield of method IV was greater than that of method II (Table 1), the band intensity of method IV in the agarose gel was fainter than that of method II (Fig. 1). These

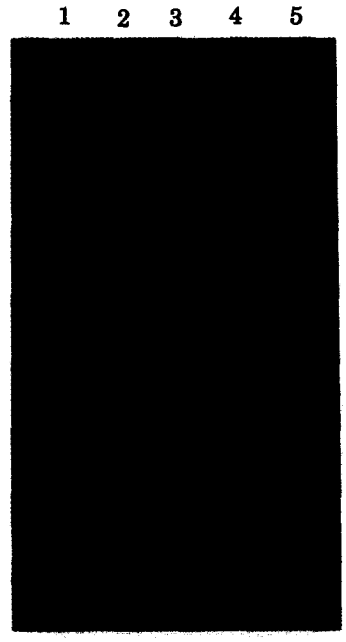

Fig. 1. Agarose gel electrophoresis of total DNA extracted from lake water samples by four different methods. Ten microliters of DNA were loaded on 1.2% agarose gel. Lanes: 1, lambda DNA digested with *Eco*RI-*Hin*dIII; 2, method I; 3, method II; 4, method III; 5, method IV.

results indicate that the DNA extracted by method IV was highly fragmented during the extraction procedure. The high degree of DNA fragmentation should be avoided because small template DNAs may produce chimeric products during PCR (21). The extent of the fragmentation did not differ among sample sources (data not shown).

To evaluate DNA purity, DNA samples extracted by the four methods were used as templates for PCR. Eubacterial 16S rRNA universal primers were used in PCR as positive controls to check the quality of the DNA and to show that eubacterial DNA was present. Substances that inhibit *Taq* polymerase activity are present in many DNA samples and can limit the use of PCR. Humic substances, which are mixtures of complex polyphenolics produced during the decomposition of organic matter and ubiquitous in natural soil and water, are known inhibitors of PCR. In many cases, however, the source of inhibition is not known. To remove contaminants contained in DNA samples, extensive purification methods are required for amplifying target DNA by PCR. For this purpose, commercially available matrices have been used to purify total nucleic acid obtained by classical procedure (15). In this work, we used a glass matrix to purify DNA samples extracted by the four methods described above. Fig. 2 shows amplification of 1.5-kb DNA fragments by PCR with template DNA extracted from water samples. Amplification of the 16S rRNA genes was successful regardless of sample source when the DNA purified by methods I or IV was used as template (Fig. 2, lane 1 and 4). These are equivalent in size to the DNA amplified from *Pseudomonas syringae* DNA (lane 5) with the same primers. However, no amplification was observed when the DNA extracted by method II from hospital sewage, influent of sewage plant and marine sample (Fig. 2C, D and E, lane 2) and the DNA extracted by method III from lake and marine samples (Fig. 2B, E, lane 3) were used for PCR amplification. These results suggest that inhibitory substances were present in DNA samples prepared by method II and III.

To relieve interference, various additives have been included in PCR mixtures (11, 12). Among these, bovine serum albumin (BSA) has been widely used to overcome the inhibitory effect (5). Indeed the addition of BSA to PCR enhanced the reaction with DNA of method III (Fig. 3). Since the method I showed the greatest total DNA yield of the four

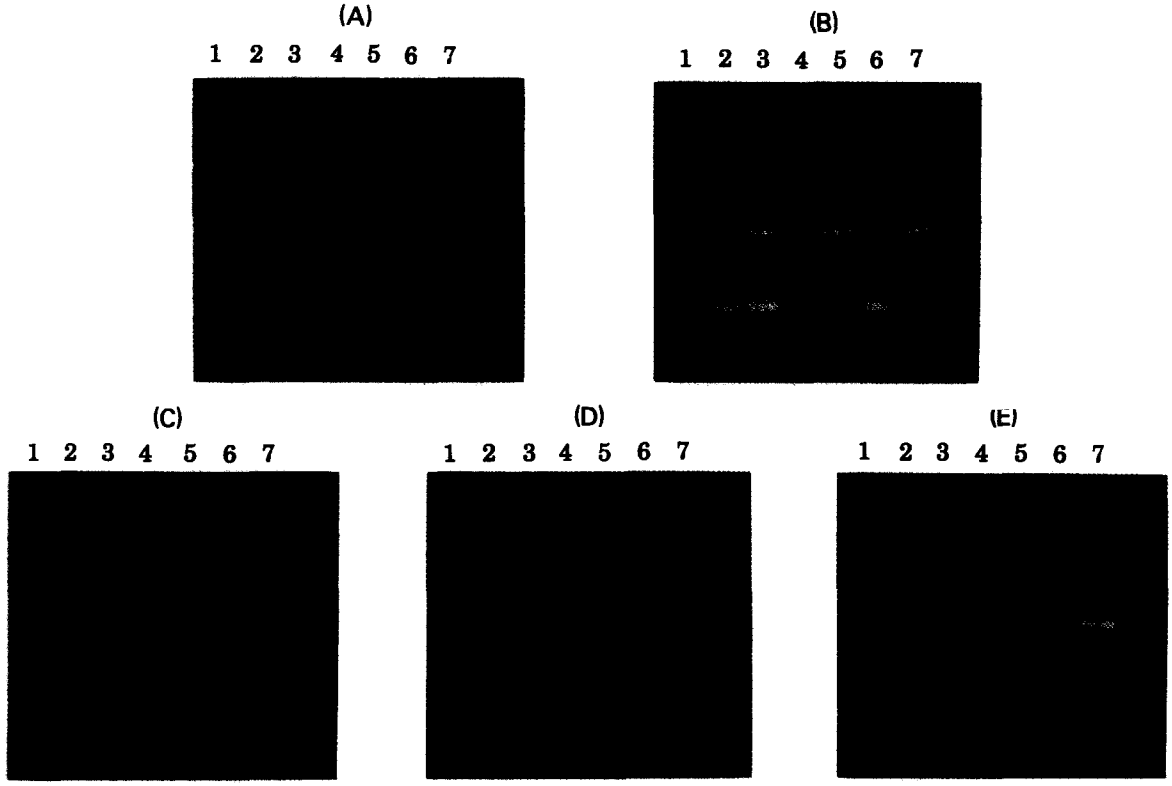

Fig. 2. Agarose gel electrophoresis of 16S rDNA amplification products from DNA of four extraction methods. Ten ng of DNA samples extracted from creek water (A), lake water (B), hospital sewage (C), influent of sewage plant (D), and sea water (E) were used for PCR amplification. Lanes: 1, lambda DNA digested with *Eco*RI-*Hin*dIII; 2, method I; 3, method II; 4, method III; 5, method IV; 6, negative control; 7, positive control with *Pseudomonas syringae* DNA.

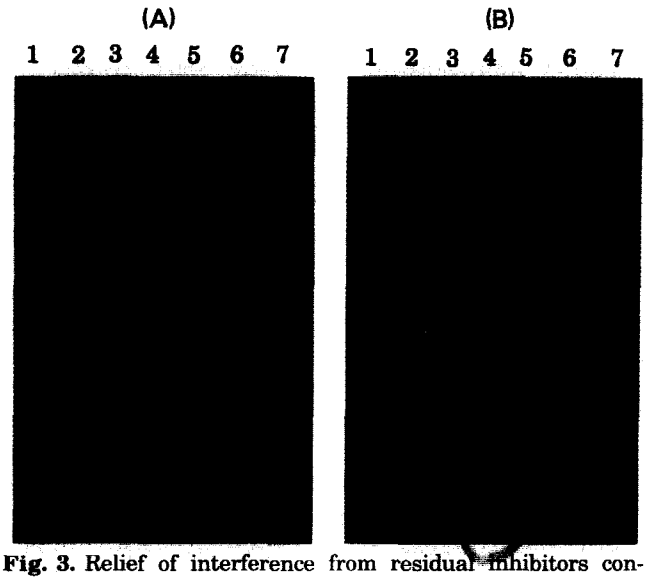

Fig. 3. Relief of interference from residual inhibitors contained in DNA, DNA extracted from lake sample by four methods was used as a template for amplification of 16S rDNA. (A) Standard PCR conditions without BSA; (B) PCR with 2 ug of BSA per reaction. Lanes: 1, lambda DNA digested with *Eco*RI-*Hind*III; 2, positive control with *Pseudomonas syringae* DNA; 3, method I; 4, method II; 5, method III; 6, method IV; 7, negative control.

methods and gave high purity, this method was used for the following experiments.

To determine the efficiency of our extraction procedure for PCR detection of bacteria, the culture of *A. hydrophila* A65 was serially diluted and seeded into 100 ml of tap water. The seeded samples were subjected to DNA extraction by method I. Extracted DNA was used as template for PCR amplification with aero-1a and aero-1b primers. These primers targeted a 209-bp fragment of the *aer* gene, encoding the beta-hemolysin, and detected template DNA only in the PCR using nucleic acid from hemolytic strains of *A. hydrophila* (9). The data show that after 30 cycles, 2×10^3 CFU was easily visualized (Fig. 4, lane 5), whereas 2×10^2 CFU was just discernible (Fig. 4, lane 6). The detection by limit by ethidium bromide staining was 2 CFU/ml of water when the method I was used for DNA extraction.

In summary, methods of extracting DNA from water samples which can be followed by PCR amplification to analyze community structure have been evaluated. Method I, which involves enzymatic and freeze-thaw lysis steps and subsequent phenol and phenol-chloroform purification of extracted nucleic acid, reproducibly extracted DNA not only in far greater quantities but also in much pure form than that produced by the other methods.

Fig. 4. PCR detection of *Aeromonas hydropila* Ah65 in water with target DNA prepared by method I, A 209-bp amplification fragment in the PCR was detected with aerolysin specific primers. Liquid culture of *A. hydrophila* was diluted and seeded into 100 ml of tap water with following CFU. Lanes: 1, *Hae* III digested ϕX174 DNA; 2, 2×10^7; 3, 2×10^6; 4, 2×10^5; 5, 2×10^4; 6, 2×10^3; 6, 2×10^2; 8, 2×10; 9, negative control; 10, positive control.

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