0273-2289/1559-0291 (Online)/06/134/0097/$30.00

Comparative Study of Methods for Extraction and Purification of Environmental DNA From Soil and Sludge Samples

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Received August 16, 2005; Revised December 2, 2005; Accepted December 6, 2006

Abstract

An important prerequisite for successful construction of a metagenome library is an efficient procedure for extracting DNA from environmental samples. We compared three indirect and four direct extraction methods, including a commercial kit, in terms of DNA yield, purity, and time requirement. A special focus was on methods that are appropriate for the extraction of environmental DNA (eDNA) from very limited sample sizes (0.1 g) to enable a highly parallel approach. Direct extraction procedures yielded on average 100-fold higher DNA amounts than indirect ones. A drawback of direct extraction was the small fragment size of approx 12 kb. The quality of the extracted DNA was evaluated by the ability of different restriction enzymes to digest the eDNA. Only the commercial kit and a direct extraction method using freeze-thaw cell lysis in combination with an in-gel patch electrophoresis with hydroxyapatite to remove humic acid substances yielded DNA, which was completely digested by all restriction enzymes. Moreover, only DNA extracted by these two procedures could be used as template for the amplification of fragments of several 16S rDNA, 18S rDNA groups under standard polymerase chain reaction conditions.

Index Entries: Environmental DNA; metagenome; purification; direct extraction; indirect extraction; activated sludge.

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Introduction

Cultured microbes have revealed tremendous chemical resources and utility; however, the vast majority of as yet unknown microorganisms from environmental samples could well be a far larger source of new molecular structures. Current estimates indicate that less than 1% of the total microbial community observed under the microscope can be cultivated with known cultivation techniques *(1–9)*. There is likely to be further diversity within species *(10–12)*, which current phylogenetic analysis cannot resolve. The methodological protocols for understanding aspects of these resources have been dramatically developed in recent years. Until now, numerous researchers have improved methods for extracting pure environmental DNA (eDNA). In particular, the problem of coextracting humic substances that severely inhibit polymerase chain reaction (PCR) amplification reactions and cloning procedures were addressed in several publications *(13–28)*. These different methods for the extraction of eDNA from environmental microbes vary in the degree of shearing, purity, and yield of the isolated eDNA. Although many of these protocols have been employed for years, only recent studies have begun to compare their efficiency and reliability *(15,16)*. There are basically two different approaches to extract eDNA: either the cells are separated from the environmental samples prior to cell lysis (indirect cell extraction), or the microbes are lysed within the environmental samples (direct cell extraction).

In the present study, three indirect and four direct cell extraction methods, including one commercially available procedure, were compared regarding DNA yield and purity after extraction and purification from environmental samples. The quality of the obtained eDNA was verified by several PCR amplification reactions and restriction enzyme digestions.

Materials and Methods

Sampling

Soil was collected in Stuttgart at the Institute of Technical Biochemistry (University of Stuttgart, Germany). Sediment was collected from a settling pond in the lake Bärensee in Stuttgart (Germany). Activated sludge was collected from a sewage plant at the Institute for Sanitary Engineering, Water Quality and Solid Waste Management of the University of Suttgart (Germany). All methods were carried out with 0.1 g of sample, stored at –20°C before use. All samples were expressed as wet weights.

Indirect Extraction Methods

Method 1 *(17)*

Environmental samples (0.1 g) were collected and suspended in 1 mL of buffer (20 m*M* Tris-HCl, 10 m*M* EDTA, 10 m*M* ε-aminocaproic acid, pH 8.0) and incubated at 4°C for 10 h with shaking. The particles were

collected by low-speed centrifugation (2900*g*) for 5 min. The microbial cell fraction was centrifuged and embedded into an agarose plug. The plug was incubated at 37°C for 1 h in extraction buffer (100 m*M* EDTA, 10 m*M* Tris-HCl, 50 m*M* NaCl, 0.2% [w/v] deoxycholate, 1% [w/v] *N*-lauroyl sarcosine, 1 mg/mL of lysozyme; pH 8.0), then transferred into a buffer, containing 2 mg/mL of proteinase K, 1% (w/v) lauroyl sarcosine, and 500 m*M* EDTA and incubated overnight at 50°C with gentle shaking. Agarose plugs were placed in a 0.5% agarose gel containing 2% polyvinylpyrrolidone (PVP) in the first half and no PVP in the other half. Gel electrophoresis was performed overnight at 4°C, 5 V/cm. The eDNA was extracted from gel with a QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany), and the resulting DNA was dialyzed (20 m*M* Tris-HCl, pH 7.8; 10 m*M* NaCl; 0.2 *M* EDTA) and concentrated in an evaporator. For analysis of the fragment size, 10 g of environmental sample was suspended in 10 mL of buffer (Tris-HCl, 10 m*M* EDTA, 10 m*M* ε-aminocaproic acid, pH 8.0). The following procedures for eDNA extraction were the same as described previously.

Method 2 *(27)*

Environmental samples (0.1 g) were collected and suspended in 1 mL of buffer (100 m*M* Tris-HCl, 100 m*M* sodium EDTA, 0.1% [w/v] sodium dodecyl sulfate [SDS], 1% [w/v] cetyltrimethylammonium bromide [CTAB], pH 8.0) and homogenized in a blender (Ultra-Turrax T25) for three 1-min intervals. The particles were collected by low-speed centrifugation (2900*g*) for 10 min. The supernatant was centrifuged at high speed (20,800*g*) for 20 min. After the cells were collected, they were washed in 1 mL of Chrombach buffer (300 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0), and the pellet was resuspended in 100 mL of lysis buffer, lysozyme (50 mg/mL), and proteinase K (10 mg/mL) solution and incubated at 37°C for 30 min. Lysis was carried out chemically by adding 100 mL of 20% (w/v) SDS solution and incubating for 1 h at 65°C. After phenol:chloroform:isoamyl alcohol (25:24:1) extraction, DNA was precipitated with isopropanol, and then the pellet was subsequently precipitated with 70% ethanol. Crude DNA pellets were dissolved in 100 mL of TE buffer (10 m*M* Tris-HCl, 1 m*M* sodium EDTA, pH 8.0). Crude DNA extracts were purified by gel electrophoresis using 1% (w/v) agarose gels. eDNA was extracted from gel with a QIAEX II Gel Extraction Kit (Qiagen). For analysis of the fragment size, 10 g of environmental sample was suspended in 10 mL of buffer (100 m*M* Tris-HCl, 100 m*M* sodium EDTA, 0.1% [w/v] SDS, 1% [w/v] CTAB, pH 8.0). The following procedures for eDNA extraction are the same as described previously.

Method 3 *(25)*

Environmental samples (0.1 g) were collected and 0.1 g of Chelex 100 (Bio-Rad, Richmond, CA) was added to 1 mL of 0.1% (w/v) Na-deoxycholate and 2.5% (w/v) polyethylene glycol (PEG) 6000 solution. After shaking at 4°C for 1 h, environmental samples and Chelex 100 were removed by

low-speed centrifugation (2900*g*). The microbial cell fraction was collected by centrifugation and washed in 500 mL of TE buffer (33 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0). Lysis was performed chemically with 100 mL of 20% (w/v) SDS and 500 mL of 5 *M* NaCl with lysozyme (10 mg/mL) and incubated for 1 h at 65°C. The supernatant was collected by centrifugation and suspended in 500 mL of TE buffer (pH 8.0). DNA was purified by ion-exchange chromatography with a Q-Sepharose column (25-mL volume; Pharmacia Biotech, Erlangen, Germany) with a linear gradient (Eluent A: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0; Eluent B: 10 m*M* Tris-HCl, 1 m*M* EDTA, 1 *M* NaCl, pH 8.0). For analysis of the fragment size, 10 g of environmental sample and 10 g of Chelex 100 were added to 10 mL of a 0.1% (w/v) Na-deoxycholate solution containing 2.5% (w/v) PEG 6000. The following procedures for eDNA extraction are the same as described previously.

Direct Extraction Methods

Method 4 *(18)*

Environmental samples (0.1 g) were collected and suspended in 1 mL of extraction buffer (50 m*M* Tris-HCl, 25 m*M* EDTA, 0.1% [w/v] SDS, 0.1% [w/v] PVP, pH 8.0). Samples were centrifuged at 8100*g* for 1 min and resuspended in 500 mL of a lysis buffer (50 m*M* Tris-HCl, 25 m*M* EDTA, 3% [w/v] SDS, 1% [w/v] PVP, pH 8.0). Tubes were heated in a microwave oven at 600–700 W for 1 min. A 500-mL volume of prewarmed (65°C) extraction solution (10 m*M* Tris-HCl, 1 m*M* EDTA, 300 m*M* sodium acetate, 1% [w/v] PVP) was added to the sample. Phenol:chloroform:isoamyl alcohol (25:24:1) was added and mixed by inversion. DNA precipitation was performed with isopropanol, and the DNA pellet was washed with 70% ethanol and resuspended in 100 mL of TE buffer (pH 8.0).

Method 5 *(22)*

Environmental samples (0.1 g) were collected and suspended in 1 mL of extraction buffer (100 m*M* Tris-HCl, 100 m*M* sodium EDTA, 1.5 *M* NaCl, pH 8.0). Glass beads (0.1 g, 0.5 mm; BioSpec Products, Bartlesville, OK) were added, and the sample was blended in a Bead-Beater (Retsch) for 20 min. One hundred microliters of 20% (w/v) SDS was added and incubated at 65°C for 1 h and centrifuged at 8100*g* for 10 min. The supernatants were transferred to tubes containing 500 mL of PEG solution (30% [w/v] PEG, 1.5 *M* NaCl) and incubated at room temperature for 2 h. Samples were centrifuged at 14,000 rpm for 20 min. The aqueous phase was extracted with phenol:chloroform:isoamyl alcohol (25:24:1), and DNA was precipitated by adding 0.6 vol of isopropanol. DNA was collected by centrifuging at 20,800*g* for 30 min. Conventional electrophoresis was used to remove humic substances. DNA was purified using a QIAEX II Gel Extraction Kit (Qiagen) and resuspended in TE buffer (pH 8.0).

Method 6 *(29)*

Environmental samples (0.1 g) were collected and suspended in 1 mL of TENC buffer (100 m*M* Tris-HCl, 10 m*M* EDTA, 100 m*M* NaCl, 1% [w/v] CTAB, 1 mL of 50 mg/mL proteinase K, pH 8.0). The samples were frozen in liquid nitrogen for 5 min and subsequently thawed at 65°C for 10 min. Then, 100 mL of 20% (w/v) SDS was added, and the sample was incubated at 65°C for 10 min. After centrifuging at 20,800*g* for 10 min, the supernatant was transferred into a sterile tube. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the combined supernatant and mixed by inversion. The supernatant was collected, and 1 mL of isopropanol and 70% ethanol was added before centrifuging at 20,800*g* for 5 min. Crude DNA was resuspended in 50 mL of TE buffer (pH 8.0). Crude DNA was loaded onto 0.5% agarose gel. One part of the agarose gel contained hydroxyapatite, to remove humic substances *(29)*. A 40-mL volume of melted 0.5% low melting point (LMP) agarose was gently mixed with 2 g of hydroxyapatite resin (Fluka, Buchs, Switzerland). The mixture was poured into sterile rectangular molds and rotated with a ROTAMIX RM1 (ELMI) to ensure a uniform distribution of the resin in the agarose gel prior to solidification. Then the gel patch was extracted from the mold. After fixing the gel patch in an electrophoresis rack, melted 0.5% LMP agarose was poured into the rack and electrophoresis was carried out at 120 V for 20 min. Subsequent DNA purification was carried out with a QIAEX II Gel Extraction Kit (Qiagen).

Method 7 (*SoilMaster*™ DNA Extraction Kit;
Epicentre, Madison, WI, www.epibio.com)

The eDNA of environmental samples was extracted by the *SoilMaster* DNA Extraction Kit according to the manufacturer's recommendations. Environmental samples (0.1 g) were collected and suspended in 250 mL of DNA extraction buffer. Two microliters of proteinase K and 50 mL of lysis buffer were added. The sample was incubated at 65°C for 10 min. After centrifuging at 2900*g* for 2 min, the supernatant was transferred to a sterile tube. For protein precipitation, 60 mL of protein precipitation reagent was added and mixed thoroughly by inverting. After centrifugation, the supernatant was placed directly on a spin column with the inhibitor removal resin. After repeating wash and spin, the precipitated eDNA was resuspended in 300 mL of TE buffer.

Restriction Endonuclease Treatment and PCR

Restriction enzyme digestions were carried out to examine the digestibility of eDNA obtained by four different direct extraction methods. The digestions (*Bam*HI, *Eco*RI, *Hin*dIII, *Sma*I, *Xho*I; Fermentas) were done under conditions provided by the manufacturer. After incubation for 30 min, the DNA fragments were resolved in a 1% agarose gel. Purified eDNA extracted from environmental samples was used as template in the PCR. PCR amplification was conducted using *Taq* DNA polymerase

(Eppendorf, Hamburg, Germany) under conditions provided by the manufacturer. PCR was conducted with an Eppendorf thermal cycler as follows: 2 min of denaturation at 94°C, followed by 35 amplification cycles (1 min at 94°C, 1 min at the optimized annealing temperature [Table 4], 1 min at 72°C [extension]), with a final 10-min 72°C extension step after cycling was complete).

Determination of Purity and Yield of eDNA

To evaluate the purity of the extracted DNA, absorbance ratios at 260 nm/230 nm and 260 nm/280 nm were determined *(27)*. A high A_{260}/A_{230} ratio of greater than 2 indicates pure DNA. A low ratio indicates contamination with humic substances. An A_{260}/A_{280} ratio of less than 1.7 indicates protein contamination. Quantification of the extracted eDNA was also carried out using a spectrophotometric method *(27)*.

DNA Sequencing

The DNA sequencing reaction was carried out on both strands of double-stranded templates using a BigDye Terminator Cycle Sequencing Kit RR-100 (Applied Biosystems, Weiterstadt, Germany). Sequencing was performed with an ABI Prism™ 377 DNA Sequencer (Perkin Elmer, Shelton, CT).

Results

Three indirect and four direct DNA extraction methods from environmental samples were compared in terms of DNA yield, purity, and time requirement. For the direct extraction methods, cell lysis was performed in the presence of the sample matrix, whereas the indirect approach involved the extraction of cells prior to cell lysis. The examined methods mainly differ in the type of cell lysis and DNA purification (Table 1). The direct extraction methods included microwave thermal shock (method 4), mechanical disruption by bead beating (method 5), and freeze-thaw cycles combined with in-gel patch electrophoresis (method 6). In the commercial kit, a hot detergent lysis process was combined with spin column purification (method 7). In the indirect extraction methods, cells were lysed enzymatically with lysozyme. In all cases, a sample volume of 0.1 g was used in order to compare the results with those of the tested commercial kit. The DNA yields of the three examined indirect extraction methods were between 0.02 and 0.06 mg/g of soil (wet wt) (Table 2), with the highest DNA concentration obtained with method 2 from Holben *(27)*. Direct extraction methods yielded about 100-fold higher DNA concentrations in the range between 1.1 and 11.4 mg/g of soil. The highest DNA concentrations, 9.7 mg/g on average, from environmental samples were obtained with method 6, whereas the commercial kit yielded the lowest DNA concentrations (1.5 mg/g on average) of the four examined direct extraction methods. No significant differences were observed among the DNA yields

Table 1
Comparison of Examined Indirect and Direct DNA Extraction Methods[a]

	Indirect extraction method			Direct extraction method			
	Quaiser et al. *(17)* (method 1)	Holben *(27)* (method 2)	Jacobsen and Rasmussen *(25)* (method 3)	Orsini and Romano-Spica *(18)* (method 4)	Yeates et al. *(22)* (method 5)	Roh et al. *(29)* (method 6)	Commercial kit (method 7)
Soil removal	Gently shaking in buffer (4°C, 10 h); low-speed centrifugation	Homogenization in buffer (SDS, CTAB); low-speed centrifugation	Shaking in buffer (4°C, 1 h) + cation-exchange resin; low-speed centrifugation	—	—	—	—
Cell lysis	Agarose plug, enzymatic lysis with lysozyme and proteinase K	Lysozyme and proteinase K + chemical lysis (SDS, 65°C, 1 h)	Lysozyme and chemical lysis (SDS + NaCl) at 65°C, 1 h	Microwave thermal shock	Bead beating	Freeze-thaw + proteinase K	Hot detergent lysis + proteinase K
Protein removal	—	P:C:I (25:24:1)	—	P:C:I (25:24:1)	P:C:I (25:24:1)	P:C:I (25:24:1)	Precipitation reagent
DNA purification	Two-phase electrophoresis (PVP) + gel extraction	Gel electrophoresis + gel extraction	Ion-exchange chromatography (Q-Sepharose)	—	Conventional gel electrophoresis + gel extraction	In-gel patch electrophoresis (HA) + gel extraction	Spin column with inhibitor removal resin
Spent time	48 h	4 h	7 h	30 min	5.5 h	2.5 h	1 h

[a]HA, hydroxyapatite; P:C:I, phenol:chloroform:isoamyl alcohol.

Table 2
Amount and Purity of eDNA Extracted From Environmental Samples

Type of purification	Amount of DNA (μg/g [wet wt])[a]			Purity[b]					
				A_{260}/A_{280}			A_{260}/A_{230}		
	Soil	Sediment	Activated sludge	Soil	Sediment	Activated sludge	Soil	Sediment	Activated sludge
Indirect									
Method 1	3.0×10^{-2}	3.0×10^{-2}	1.5×10^{-2}	1.48 ± 0.03	1.51 ± 0.02	1.65 ± 0.02	1.73 ± 0.05	1.75 ± 0.04	1.71 ± 0.03
Method 2	6.0×10^{-2}	4.2×10^{-2}	2.4×10^{-2}	1.49 ± 0.03	1.59 ± 0.03	1.51 ± 0.03	1.62 ± 0.03	1.63 ± 0.04	1.75 ± 0.02
Method 3	4.5×10^{-2}	3.5×10^{-2}	1.7×10^{-2}	1.65 ± 0.02	1.67 ± 0.03	1.59 ± 0.02	1.67 ± 0.02	1.73 ± 0.05	1.74 ± 0.03
Direct									
Method 4	4.5 ± 1.1	5.1 ± 0.8	4.5 ± 0.7	1.68 ± 0.05	1.71± 0.05	1.77 ± 0.05	1.61 ± 0.07	1.68 ± 0.07	1.73 ± 0.05
Method 5	6.5 ± 0.8	6.7 ± 0.7	5.9 ± 0.8	1.72 ± 0.05	1.71 ± 0.07	1.81 ± 0.04	1.63 ± 0.03	1.71 ± 0.03	1.76 ± 0.04
Method 6	8.3 ± 1.2	9.5 ± 0.9	11.4 ± 0.6	1.89 ± 0.03	1.85 ± 0.02	1.89 ± 0.03	1.98 ± 0.03	2.03 ± 0.02	1.97 ± 0.03
Method 7	1.9 ± 0.3	1.5 ± 1.1	1.1 ± 0.5	1.75 ± 0.02	1.76 ± 0.02	1.85 ± 0.03	1.89 ± 0.03	1.87 ± 0.03	1.82 ± 0.02

[a]Values are means of four independently purified samples with standard errors.
[b]The ratios were calculated from spectrophotometric measurements.

received from the different types of environmental samples: soil, sediment, or activated sludge.

The cost of the extraction efficiency of direct extraction methods is the resulting eDNA fragments of about only 12 kb in size, owing to shearing processes (Fig. 1). Starting with 0.1 g of environmental sample, no bands were visible on the agarose gel in the case of indirect extraction methods. However, when the indirect extraction procedures were applied to a 10-g soil sample, very large DNA fragments could be obtained (Fig. 2). The DNA obtained by indirect extraction methods 1–3 had very little size distribution. There was a clear band of approx 40 kb by methods 1 and 2 and even larger DNA fragments of more than 50 kb by method 3 with nearly no smear of smaller DNA fragments.

The A_{260}/A_{280} ratio corresponding to direct extraction methods was in all cases at least 1.7, indicating a sufficient removal of protein contaminations. In the case of indirect extraction methods, only method 3 applied to soil and sediment and method 1 applied to activated sludge produced a value of 1.7. Concerning the removal of humic acid contaminations, indicated by an A_{260}/A_{230} ratio of more than 2, only method 6 gave satisfactory results.

As can be seen in Table 1, indirect extraction methods were more time-intensive than direct extraction methods, because of the additional matrix removal step. Methods 1, 2, and 3 required 48, 4, and 7 h, respectively. By contrast, methods 4 and 7 were the fastest procedures, requiring 30 min and 1 h, respectively. Methods 5 and 6 required 5.5 and 2.5 h, respectively.

The purity of the extracted eDNA was investigated by the ability of several restriction enzymes to digest it. Table 3 shows the results of the restriction enzyme treatment of eDNA obtained by direct extraction methods. None of the crude extracts could be digested by the five restriction enzymes *Bam*HI, *Eco*RI, *Hin*dIII, *Sma*I, and *Xho*I. Furthermore, although a reduction in the dark brownish color of the crude eDNA was observed after microwave thermal shock (method 4) and mechanical cell lysis in combination with conventional gel electrophoresis (method 5), eDNA extracted by method 4 still could not be digested by *Bam*HI, *Eco*RI, or *Hin*dIII. *Sma*I and *Xho*I caused a partial digestion of the extracted DNA. eDNA extracted by method 5 could not be digested by *Bam*HI or *Eco*RI and only partially digested by *Hin*dIII, *Sma*I, and *Xho*I. However, when purified by methods 6 and 7, extracted eDNA was completely digested by all five restriction enzymes (Table 3). Restriction digestion could not be performed with eDNA purified by indirect extraction (methods 1, 2, and 3), owing to low DNA concentrations received by 0.1 g of sample.

The quality of the eDNA purified by the four direct extraction methods was also investigated by the ability to amplify a region of the rRNA of several phylogenetic groups representing different components of the microbial communities. These groups include members with a broad range of microbial cell sizes and growth habits. Six different sets of primers were used (Table 4). 16S rDNA and 18S rDNA amplification analysis by

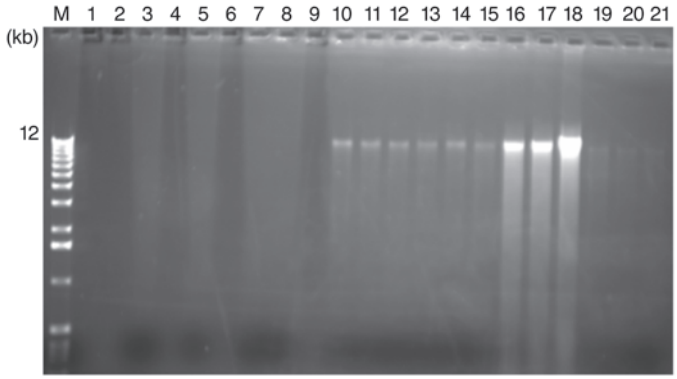

Fig. 1. Comparison of different methods for extraction of eDNA from environmental samples. M, DNA marker; eDNA extracted by method 1 from soil (lane 1), sediment (lane 2), and activated sludge (lane 3); eDNA extracted by method 2 from soil (lane 4), sediment (lane 5), and activated sludge (lane 6); eDNA extracted by method 3 from soil (lane 7), sediment (lane 8), and activated sludge (lane 9); eDNA extracted by method 4 from soil (lane 10), sediment (lane 11), and activated sludge (lane 12); eDNA extracted by method 5 from soil (lane 13), sediment (lane 14), and activated sludge (lane 15); eDNA extracted by method 6 from soil (lane 16), sediment (lane 17), and activated sludge (lane 18); eDNA extracted by method 7 from soil (lane 19), sediment (lane 20), and activated sludge (lane 21).

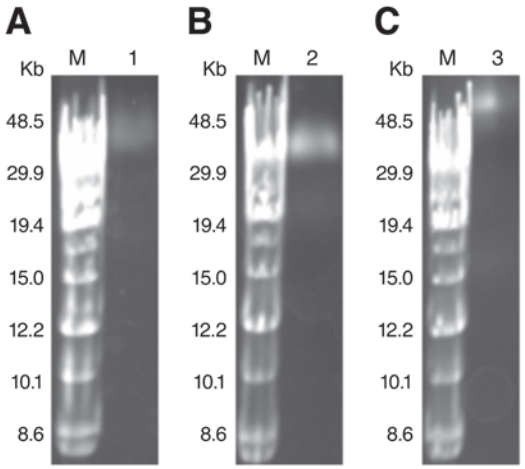

Fig. 2. eDNA extracted by indirect methods of 10-g soil sample by **(A)** method 1, **(B)** method 2, and **(C)** method 3. M, lambda mix marker.

methods 6 and 7 resulted in successful PCR amplifications of gene fractions of the expected size (Fig. 3). All targeted microbial groups were detected under standard PCR conditions using eDNA as template. The PCR result of Gram-positive bacteria with high G+C content proved the lysis of bacteria. Detection of the prokaryotic small subunit rRNA, the internal transcribed spacer (ITS) region for lichen fungi, which represents the fungal

Table 3
Restriction Enzyme Digestion of eDNA Purified from Environmental Samples

	Digested by[a]														
	*Bam*HI			*Eco*RI			*Hind*III			*Sma*I			*Xho*I		
Type of purification	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Direct cell extraction															
Method 4	–	–	–	–	–	–	–	–	–	±	±	±	±	±	±
Method 5	–	–	–	–	–	–	±	±	±	±	±	±	±	±	±
Method 6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Method 7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[a]A, soil; B, sediment; C, activated sludge; +, complete digestion; ±, partial digestion; –, no digestion.

Table 4
Primer Pairs Used in PCR to Detect Phylogenetic Groups of Native Microorganisms in Environmental Samples

Organism group	Primer pair region and reference	DNA sequence	Product (bp)	Optimized annealing temperature (°C)
Bacteria	Prokaryotic small subunit rRNA *(39)*	5'AGAGTTTGATCCTGGCTCAG 5'AGAAAGGAGGTGATCCAGCC	1536	50
	Bacillus species and relatives *(40)*	5'AGGGTCATTGGAAACTGGG 5'CGTGTTGTAGCCCAGGTCATA	600	55
	High G+C Gram-positive bacteria *(40)*	5'GAGTTTGATCCTGGCTCAG 5'GCCATTGTAGCACGTGTGCA	542	63
	Streptomyces species and related taxa *(41)*	5'GGCCTTCGGGTTGTAAACC 5'CTTTGAGTTTTAGCCTTGCGGC	1243	60
Fungi	ITS for lichen *(42)*	5'GCGGAAGGATCATTACTGA 5'GGGTATCCCTACCTGATCCG	565	56
Eukaryote	rRNA ITS *(43)*	5'TCCGTAGGTGAACCTGCGG 5'TCCTCCGCTTATTGATATGC	584	58

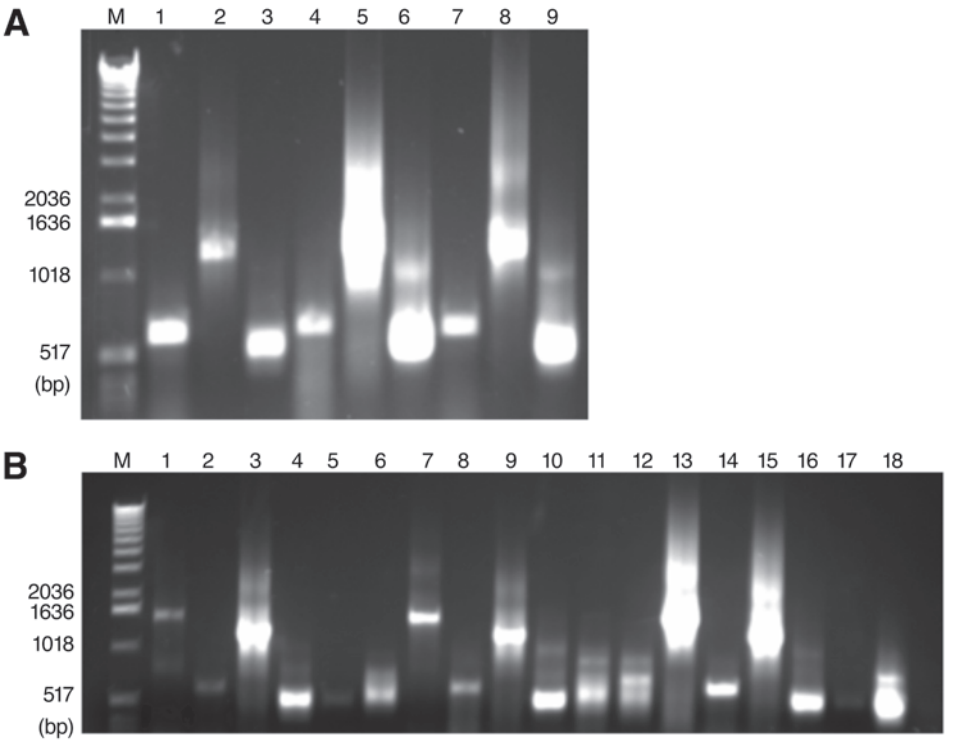

Fig. 3. PCR amplification analysis of eDNA extracted from environmental samples by **(A)** method 6 and **(B)** method 7. (A) M, DNA marker; bacillus species and relatives region from soil (lane 1), sediment (lane 4), and activated sludge (lane 7); streptomyces species and related taxa region from soil (lane 2), sediment (lane 5), and activated sludge (lane 8); high G+C Gram-positive bacteria region from soil (lane 3), sediment (lane 6), and activated sludge (lane 9). (B) M, DNA marker; prokaryotic small subunit rRNA region of soil (lane 1), sediment (lane 7), and activated sludge (lane 13); bacillus species and relatives region of soil (lane 2), sediment (lane 8), and activated sludge (lane 14); streptomyces species and related taxa region of soil (lane 3), sediment (lane 9), and activated sludge (lane 15); high G+C Gram-positive bacteria region of soil (lane 4), sediment (lane 10), and activated sludge (lane 16); ITS region for lichen fungi of soil (lane 5), sediment (lane 11), and activated sludge (lane 17); eukaryotic rRNA ITS region of soil (lane 6), sediment (lane 12), and activated sludge (lane 18).

part of the symbioses of a fungus and a cyanobacteria in lichens, and the eukaryotic rRNA ITS region were very clearly in the three types of environmental samples extracted by method 7. The corresponding results obtained by method 6 were already described in a previous publication *(29)*. By contrast, when using the eDNA extracted by methods 4 and 5 as template for the PCR, no amplification product was observed. Negative controls without eDNA as template also resulted in no PCR product (data not shown).

Discussion

The applicability of different soil extraction and DNA purification methods to obtain suitable eDNA for molecular biology procedures from very small sample sizes was examined. The sample size was limited to 0.1 g, because this amount enables a highly parallel sample processing. The results showed that this small sample size is insufficient for indirect extraction methods. This correlates with former descriptions of indirect extraction procedures in which usually 10–100 g of soil was used to obtain enough DNA for further cloning steps *(30–32)*. Gabor et al. *(15)* reported 10- to 100-fold lower DNA yields obtained by indirect extraction methods compared with direct DNA extraction. In addition, Tien et al. *(20)* obtained on average a 10-fold higher amount of DNA with direct lysis methods compared to indirect extraction. The overall highest DNA yield in the present study was obtained by method 6, which was recently developed by our group *(29)*. Freeze-thaw cell lysis in combination with gel electrophoresis with a hydroxyapatite patch inside the agarose gel resulted in higher amounts of DNA than mechanical cell lysis by bead beating combined with conventional gel electrophoresis. Miller et al. *(33)* compared the efficiency of bead-beating cell lysis with that of freeze-thaw lysis and found that bead beating is superior to the freeze-thaw technique. Thus, the increased amount of DNA obtained by method 6 compared with method 5 might be related to the different purification procedures. Moreover, in terms of purity and humic acid removal, method 6 gave the best results, followed by the examined commercial kit. The hydroxyapatite resin present in a part of the agarose gel causes an effective binding of the humic substances. Alternatively, CsCl–ethidium bromide equilibrium density centrifugation or purification procedures using a chromatographic column might be used to obtain pure enough DNA, but these methods are time-consuming and the former method requires an ultracentrifuge *(25,34)*.

A disadvantage of direct extraction methods is the rather small DNA fragment size. Similar to the results of the present work, Lloyd-Jones and Hunter *(16)* obtained DNA fragments of about 12 kb by direct extraction methods using chemical lysis and bead-beating cell lysis. However, there are several examples of metagenomic libraries derived from eDNA from direct extraction procedures that resulted in the discovery and heterologous expression of new enzymes *(32,35–38)*. The fragment sizes obtained by indirect extraction methods are substantially higher *(15)*. Fragment sizes extracted from soil can reach up to 400 kb *(30)*.

The big advantage of direct extraction methods is that they are much faster than indirect extraction methods. The SoilMaster commercial kit provided eDNA that was sufficiently pure to be used as template for PCR after 1 h, whereas the purity of the extracted DNA could be further enhanced by in-gel patch electrophoresis (method 6). This method requires about 2.5 h, which is still suitable for high-throughput screening.

Acknowledgments

We thank Holger Schulze for valuable discussions and critical review of the manuscript. We are also grateful to Karl-Heinrich Engesser and Manfred Roth from the Institute for Sanitary Engineering, Water Quality and Solid Waste Management of the University of Stuttgart (Germany) for their generous cooperation. This work was supported by grants from Korea-Germany Exchange Program of the Korea Science and Engineering Foundation (KOSEF).

References

1. Schleper, C., Jurgens, G., and Jonuscheit, M. (2005), *Nat. Rev. Microbiol.* **3,** 479–488.
2. Streit, W. R., Daniel, R., and Jaeger, K. E. (2004), *Curr. Opin. Biotechnol.* **15,** 1–6.
3. Daniel, R. (2004), *Curr. Opin. Biotechnol.* **15,** 199–204.
4. Galbraith, E. A., Antonopoulos, D. A., and White, B. A. (2004), *Environ. Microbiol.* **6(9),** 928–937.
5. Gabor, E. M., Alkema, W. B. L., and Janssen, D. B. (2004), *Environ. Microbiol.* **6(9),** 879–886.
6. Voget, S., Leggewie, C., Uesbeck, A., Raasch, C., Jaeger, K.-E., and Streit, W. R. (2003), *Appl. Environ. Microbiol.* **69,** 6235–6242.
7. Torsvik, V. and Ovreas, L. (2002), *Curr. Opin. Biotechnol.* **5,** 240–245.
8. Rondon, M. R., Goodman, R. M., and Handelsman, J. (1999), *Science* **17,** 403–409.
9. Amann, R. I., Ludwig, W., and Schleifer, K. H. (1995), *Microbiol. Rev.* **59,** 143–169.
10. Zengler, K., Toledo, G.., Rappé, M., Elkins, J., Mathur, E. J., Short, J. M., and Keller, M. (2002), *Proc. Natl. Acad. Sci. USA* **99,** 15,681–15,686.
11. Kaeberlein, T., Lewis, K., and Epstein, S. S. (2002), *Science* **296,** 1127–1129.
12. Kozdrój, J. and van Elsas, J. D. (2000), *Biol. Fertil. Soils* **31,** 372–378.
13. Kauffmann, I. M., Schmitt, J., and Schmid, R. D. (2004), *Appl. Microbiol. Biotechnol.* **64,** 665–670.
14. Robe, P., Nalin, R., Capellano, C., Vogel, T. M., and Simonet, P. (2003), *Eur. J. Soil Biol.* **39,** 183–190.
15. Gabor, E. M., de Vries, E. J., and Janssen, D. B. (2003), *FEMS Microbiol. Ecol.* **44,** 153–163.
16. Lloyd-Jones, G. and Hunter, D. W. F. (2001), *Soil Biol. Biochem.* **33,** 2053–2059.
17. Quaiser, A., Ochsenreiter, T., Klenk, H.-P., et al. (2002), *Environ. Microbiol.* **4(10),** 603–611.
18. Orsini, M. and Romano-Spica, V. (2001), *Lett. Appl. Microbiol.* **33,** 17–20.
19. Courtois, S., Frostegård, Å., Göransson, P., Depret, G., Jeannin, P., and Simonet, P. (2001), *Environ. Microbiol.* **3(7),** 431–439.
20. Tien, C. C., Chao, C. C., and Chao, W. L. (1999), *J. Appl. Microbiol.* **86,** 937–943.
21. Moreira, D. (1998), *Nucleic Acids Res.* **26,** 3309, 3310.
22. Yeates, C., Gillings, M. R., Davison, A. D., Altavilla, N., and Veal, D. A. (1997), *Lett. Appl. Microbiol.* **25,** 303–307.
23. Zhou, J., Bruns, M. A., and Tiedje, J. M. (1996), *Appl. Environ. Microbiol.* **62,** 316–322.
24. Young, C. C., Burghoff, R. L., Keim, L. G., Minak-Bernero, V., Lute, J. R., and Hinton, S. M. (1993), *Appl. Environ. Microbiol.* **59,** 1972–1974.
25. Jacobsen, C. S. and Rasmussen, O. F. (1992), *Appl. Environ. Microbiol.* **58,** 2458–2462.
26. Tsai, Y.-L. and Olson, B. H. (1991), *Appl. Environ. Microbiol.* **57,** 1070–1074.
27. Holben, W. E. (1994), in *Methods of Soil Analysis, Part 2. Microbiological and Biochemical Properties: Isolation and Purification of Bacterial DNA from Soil,* SSSA Book Series no. 5, Weaver, R. W., Angle, S., and Bottomley, P. D., eds., Soil Science Society of America, Madison, WI, pp. 727–752.
28. Santosa, D. A. (2001), *Mol. Biotechnol.* **17,** 59–64.

29. Roh, C. H., Villatte, F., Kim, B. G., and Schmid, R. D. (2005), *Electrophoresis* **26,** 3055–3061.
30. Bertrand, H., Poly, F., Van, V. T., Lombard, N., Nalin, R., Vogel, T. M., and Simonet, P. (2005), *J. Microbiol. Methods* **62,** 1–11.
31. Rhee, J. K., Ahn, D. G., Kim, Y. G., and Oh, J. W. (2005), *Appl. Environ. Microbiol.* **71(2),** 817–825.
32. Ginolhac A., Jarrin C., Gillet B., et al. (2004), *Appl. Environ. Microbiol.* **70,** 5522–5527.
33. Miller, D. N., Bryant, J. E., Madsen, E. L., and Ghiorse, W. C. (1999), *Appl. Environ. Microbiol.* **65,** 4715–4724.
34. Miller, D. N. (2001), *J. Microbiol. Methods* **44,** 49–58.
35. Yun, J., Kang, S., Park, S., Yoon, H., Kim, M. J., Heu, S., and Ryu, S. (2004), *Appl. Environ. Microbiol.* **70,** 7229–7235.
36. Knietsch, A., Bowien, S., Whited, G., Gottschalk, G., and Daniel, R. (2003), *Appl. Environ. Microbiol.* **69,** 3048–3060.
37. Henne, A., Schmitz, R. A., Bomeke, M., Gottschalk, G., and Daniel, R. (2000), *Appl. Environ. Microbiol.* **66,** 3113–3116.
38. Henne, A., Daniel, R., Schmitz, R. A., and Gottschalk, G. (1999), *Appl. Environ. Microbiol.* **65,** 3901–3907.
39. Edwards, U., Rogall, T. L., Blocker, H., Emde, M., and Bottger, E. C. (1989), *Nucleic Acids Res.* **17,** 7843–7853.
40. Kuske, C. R., Banton, K. L., Adorada, D. L., Stark, P. C., Hill, K. K., and Jackson P. J. (1998), *Appl. Environ. Microbiol.* **64,** 2463–2472.
41. Stackebrandt, E., Liesack, W., and Goebel, B. M. (1993), *FASEB J.* **7,** 232–236.
42. Yeates, C., Gillings, M. R., Davison, A. D., Altavilla, N., and Veal, D. A. (1998), *Biol. Procedures* **14,** 40–47.
43. White, T. J., Bruns, T., Lee, S., and Taylor, J. (1990), in *PCR Protocols: A Guide to Methods and Applications: Amplification and Direct Sequencing of Ribosomal RNA Genes for Phylogenetics,* Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., eds., Academic, New York, pp. 315–322.