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Development of Simple Methods of DNA Extraction from Environmental Samples for Monitoring Microbial Community Based on PCR

KAZUNARI SEI[1,2], KEN-ICHIRO ASANO[1], NAOHIRO TATEISHI[1], KAZUHIRO MORI[2], MICHIHIKO IKE[1], TETSURO KOHNO[2], and MASANORI FUJITA[1]

[1]Department of Environmental Engineering, Osaka University
/2-1 Yamadaoka, Suita, Osaka 565-0871, Japan
[2]Department of Civil and Environmental Engineering, Yamanashi University
/4-3-11 Takeda, Kofu, Yamanashi 400-8511, Japan

Abstract

Studies were made so as to establish simple and rapid DNA extraction methods for PCR-based monitoring of microbial community in the water/soil environment. Several kinds of cell-lysis enzyme, chemical agents, and mechanical treatments (proteinase K, SDS (sodium dodecyl sulfate), CTAB (cetyltrimethyl ammonium bromide), PVPP (polyvinylpolypyrrolidone), freeze-and-thaw, and ultrasonication) were comparatively investigated solely or in combinations for their DNA extracting capability against each 3 water and soil samples inoculated with the PCR-targeting bacterium, *Pseudomonas putida* BH. For water samples, cell lysis with proteinase K allowed to detect the target bacterium at a sensitivity at 10^1 cells/m*l* against backgrounds of indigenous bacteria at 10^4–10^5 cfu/m*l* with the DNA recovery of ca. 25–55 %, when coupled with the phenol-chloroform extraction and ethanol precipitation. However, the other alternatives investigated showed considerable inhibitory effects on the PCR amplification and were, therefore, less sensitive. For soil samples, ultrasonication in addition to the uses of proteinase K and SDS in the presence of a high concentration of chelating agent was the most effective, although purification of the DNA extracts with a spun column were required in addition to the phenol-chloroform extraction and ethanol precipitation. This method enabled the PCR-mediated detection of the target bacterium at 10^1–10^2 cells/g of the soil samples where 10^7–10^9 cfu/g of indigenous bacteria existed and the DNA yield was 80–95 %. The methods established here seem to be able to extract a most or considerable portion of the DNA from a variety of environmental samples with a sufficiently high purity for PCR amplification. These methods also seem routinely applicable, because the procedures are very simple and do not contain time-consuming and labor-full operations.

Key words : DNA extraction, PCR, monitoring of microbial community, proteinase K, SDS, ultrasonication

INTRODUCTION

The increasing importance of molecular biological techniques in the fields of microbial ecology and environmental microbiology have developed methods of extraction, purification, amplification, detection, quantification, and analyses of nucleic acids from environmental samples[1-16]. Extraction of microbial DNA and/or RNA from natural environments coupled with their analyses based on PCR and/or hybridization has become an especially

useful tool to detect microorganisms that cannot be cultured under the laboratory conditions[17], to monitor the selected or genetically engineered microorganisms[2,6] and the particular genes disseminated into indigenous microbes by gene transfer[18], and to reveal genotypic diversity[19] and its change in microbial ecosystems[20].

Myriads of methods for extracting DNA from environmental samples have been already developed by many researchers up to date. However, each method has been tested on a limited number of samples and/or developed for case-by-case or specialized purposes, consequently, most of the methods are not always applicable to a wide variety of the environmental samples and/or purposes. For example, the DNA extraction method developed by Sparagano[12] was suitable for river water samples but not for the samples containing sediments because PCR inhibition occurred in samples with sediment, while the method of Volossiouk *et al.*[15] was suitable for typical farm soils but not for soils containing large amounts of clay because PCR inhibition occurred. Although the method of Ogram *et al.*[1] showed a high recovery of DNA from river sediments, the DNA extracts were not suitable for PCR amplification because severe DNA fragmentation occurred[14,16]. On the other hand, the method of Zhou *et al.*[16] extracted DNA suitable for PCR from soil samples, however, the DNA recovery was considerably lower because loss of DNA was great in purification step using column and agarose gel electrophoresis. Further some of the methods need time-consuming, labor-full and/or complicated procedures, therefore, they are not available for routine and/or intensive uses.

In this study, it is aimed to establish generalized DNA extraction methods applicable for intensive monitoring of microbial ecology with PCR techniques, of which requirements are (i) a whole or major portion of DNA contained in the samples should be recovered, (ii) the DNA extracts should have a high purity and a least mechanical damage suitable for PCR amplification and analyses, (iii) the methods should be applicable to a wide variety of environmental samples of diverse natures, and (iv) the methods should be rapid, simple and possibly cost-effective enough for routine applications.

LITERATURE REVIEW AND EXPERIMENTAL DESIGN

A literature review Previously-reported typical protocols for extraction of environmental DNA are summarized in Table 1 from our literature review. In general most of the protocols are composed of the following sequential steps; (a) cell lysis, (b) DNA extraction (and partial purification of DNA), and (c) further purification of the DNA extracts.

Cell lysis (a) is performed so as to release the intracellular DNA, and is performed by the treatments with enzymes (lysozyme, proteinase K, etc.), chemical agents (SDS (sodium dodecyl sulfate), CTAB (cetyltrimethyl ammonium bromide), TritonX-100, GuSCN (guanidine thiocyanate), phenol, etc.), and/or mechanical treatments (bead beating, freeze-and-thaw, ultrasonication, etc.) in most cases. Depending on sample properties and purposes the different types of the cell lysis treatments have been applied in combination. The DNA extraction step (b) is performed to separate DNA from other cell components such as polysaccharides and proteins, and environmental impurities such as soil particles and humic substances. In this step, DNA extraction with phenol or phenol-chloroform, direct DNA precipitation with PEG (polyethylene glycol), ethanol or isopropanol precipitations, and silica or glass milk adsorption of DNA were often employed. Of them phenol-chloroform extraction could serve as both cell lysis and DNA extraction steps in the study of Sparagano[12]. Further purification of DNA (c) is occasionally performed to obtain DNA of high purity enough for PCR or other molecular analyses, especially in case that the DNA extracts obtained by the previous steps can contain much impurities. Often-employed operations for this step include hydroxyapatite column chromatography, CsCl-ethidium bromide (EtBr) density gradient ultracentrifugation, and agarose gel electrophoresis followed by DNA elution (e.g., Elutip-d elution) and spun column (resin) treatments.

Generally preparation of DNA extracts from

Table 1 Operations in cell lysis, DNA extraction, and further purification steps

References	Cell lysis	DNA extraction	Further purification	Target environment
Boom *et al.* (1990)[1]	• GuSCN and TritonX-100 [room temperature, 10 min]	• silica or diatom adsorption • GuSCN wash • 70% ethanol wash • acetone wash	–	• pure bacterial culture
Sparagano (1993)[12]	• phenol-chloroform extraction		–	• river water
Wilson (1994)[13]	• SDS and proteinase K [37℃, 1 hr]	• CTAB-NaCl extraction • phenol-chloroform extraction • isopropanol precipitation	–	• marine surface water
Ogram *et al.* (1987)[4]	• SDS [70℃, 1 hr] • bead beating	• ethanol precipitation or PEG precipitation	• KAc precipitation • CsCl-EtBr gradient centrifugation • hydroxyapatite chromatography	• marine and reservoir sediments
Tsai and Olson (1991)[5]	• lysozyme [37℃, 2 hr] • SDS and freeze-and-thaw	• Elutip-d elution • ethanol precipitation	–	• subsurface soil and settling pond sediment
Picard *et al.* (1992)[8]	• ultrasonication • freeze-and-thaw	• Elutip-d elution • ethanol precipitation	–	• subsurface soil (silt loam)
Trevors *et al.* (1992)[30]	• lysozyme and zymolyase [4℃, 1.5 hr] • SDS (or Brij 58, sarkosyl, deoxycholic acid) with/without PVPP and proteinase K [4℃, 16 hr]	• phenol-chloroform extraction • ethanol precipitation	–	• surface soil (loam)
Smalla *et al.* (1993)[11]	• lysozyme [4℃ or 37℃, 30 min] • SDS and bead beating	• phenol-chloroform extraction	• CsCl precipitation • KAc precipitation • glass milk or spermine purification	• loamy sand and silt loam soil
Mor'e *et al.* (1994)[31]	• bead beating or SDS and freeze-and-thaw	• NH_4Ac precipitation • SpinBind DNA extraction cartridge	• agarose gel electrophoresis • SpinBind DNA extraction cartridge	• groundwater sediment
Porteous *et al.* (1994)[42]	• ultrasonication • lysozyme and Novozym [37℃, 1 hr] • SDS and GuSCN [68℃, 1 hr]	• isopropanol precipitation	–	• surface agronomic, urban, and natural soil
Zhou *et al.* (1996)[16]	-pretreatment for gram positive bacteria- • grinding of frozen sample • SDS and freeze-and-thaw -general procedure- • CTAB and proteinase K [37℃, 30 min] • SDS [65℃, 2 hr]	• chloroform extraction • isopropanol precipitation	• agarose gel electrophoresis • spun column	• standard soil

water samples was carried out through the protocols composed of the cell lysis and DNA extraction steps, while those for soil and sediment samples additionally needed the purification step. Because soil and sediment samples contain much more amounts of various organic matters and other impurities which can inhibit the recovery and PCR amplification and/or other analyses of DNA compared with water samples. Therefore, sometimes higher concentrations of chelating agents were used and/or other chemical agents such as PVPP (polyvinylpolypyrrolidone), which can reduce the effect of impurities in the environmental samples, were added to the cell lysis buffer to improve the recovery and purity of DNA for soil or sediment samples.

Experimental design of this study In order to develop DNA extraction methods applicable for intensive monitoring of microbial community with PCR techniques, comparative studies were performed on several types of cell lysis treatments for water and soil samples in this study. The cell lysis (a) with enzyme (proteinase K), chemical agents (SDS, CTAB, and PVPP), and mechanical treatments (freeze-and-thaw and ultrasonication) were investigated solely or in combinations for their DNA extracting capability.

As for the DNA extraction (b) and purification (c) steps, phenol-chloroform extraction followed by ethanol precipitation and spun column purification was used,

respectively, throughout the experiments of the comparative studies, because only these methods seem to be able to satisfy all the requirements (i) to (iv) mentioned in the introduction part. The other DNA extraction and purification methods apparently possess disadvantages or problems compared with the selected methods (phenol-chloroform extraction/ethanol precipitation and spun column purification). For example, direct DNA precipitations with chemical agents tend to co-precipitate much impurities in the samples[1], and silica or glass milk adsorption causes significant damage of DNA molecules from shearing[4]. DNA purification with hydroxyapatite column chromatography, EtBr density gradient ultracentrifugation, and agarose gel electrophoresis with Elutip-d elution are too laborious and time-consuming to be applied routinely. Further, they require special apparatus and sometimes cause a significant loss of DNA yields[5].

In total 6 and 24 different DNA extraction protocols were comparatively investigated against water and soil samples, respectively, as summarized in Table 2. These protocols were evaluated from the viewpoints of the recovery (yield) and purity (suitability for PCR amplification) of the DNA of the target microbe seeded to the water and soil samples.

MATERIALS AND METHODS

Bacterial strain *Pseudomonas putida* BH[21] was used as the seed microbe throughout this study. The *pheB* gene encoding catechol 2, 3-dioxygenase[22] carried by this strain (1 copy per cell) was chosen as the target gene for PCR amplification. For experiments, *P. putida* BH was cultivated with LB medium[3] for about 3 hours at 30 ℃ at 100 rpm on a reciprocal shaker. The bacterial cells were seeded to water and soil samples to give densities of 1.0×10^5 cells/m*l* and 1.0×10^7 cells/g (wet weight of soil), respectively. Cell density of this strain was determined by microscopic direct counting with a counting chamber.

Water and soil samples Water samples were collected from the Yodo river, Osaka, Japan (River sample; SS 19.4 mg/*l*, pH 6.9), the Lake Biwa, Shiga, Japan (Lake sample; SS 4.6 mg/*l*, pH 7.1), and the Ise Bay, Mie, Japan (Sea sample; SS 33.2 mg/*l*, pH 7.8). Soil samples were collected from 3 sites of the

Table 2 Outline of the comparative study on DNA extraction methods

Methods	Cell lysis			DNA extraction[a]	DNA purification
	Buffer	Enzymatic/chemical treatment	Mechanical treatment		
			Water samples		
W1	SB	(none)	(none)	phenol-chloroform	(none)
W2	SB	proteinase K	(none)	phenol-chloroform	(none)
W3	SB	SDS	(none)	phenol-chloroform	(none)
W4	SB	CTAB	(none)	phenol-chloroform	(none)
W5	SB	(none)	freeze-and-thaw	phenol-chloroform	(none)
W6	SB	(none)	ultrasonication	phenol-chloroform	(none)
			Soil samples		
S1-L/H	LB/HB	proteinase K	(none)	phenol-chloroform	spun column
S2-L/H	LB/HB	proteinase K + PVPP	(none)	phenol-chloroform	spun column
S3-L/H	LB/HB	proteinase K + SDS	(none)	phenol-chloroform	spun column
S4-L/H	LB/HB	proteinase K + CTAB	(none)	phenol-chloroform	spun column
S5-L/H	LB/HB	proteinase K	freeze-and-thaw	phenol-chloroform	spun column
S6-L/H	LB/HB	proteinase K + PVPP	freeze-and-thaw	phenol-chloroform	spun column
S7-L/H	LB/HB	proteinase K + SDS	freeze-and-thaw	phenol-chloroform	spun column
S8-L/H	LB/HB	proteinase K + CTAB	freeze-and-thaw	phenol-chloroform	spun column
S9-L/H	LB/HB	proteinase K	ultrasonication	phenol-chloroform	spun column
S10-L/H	LB/HB	proteinase K + PVPP	ultrasonication	phenol-chloroform	spun column
S11-L/H	LB/HB	proteinase K + SDS	ultrasonication	phenol-chloroform	spun column
S12-L/H	LB/HB	proteinase K + CTAB	ultrasonication	phenol-chloroform	spun column

[a] Coupled with ethanol precipitation for DNA recovery.
Abbreviations: SB, Standard Buffer; LB, Low Buffer; HB, High Buffer

bacterial cell[24].

Determination of DNA purity by PCR The purity of DNA extracts prepared by each method was compared on the detection limit of PCR amplification that was targeted *pheB* gene, as previously described[16]. The PCR amplification was conducted in 30 cycles with denaturation at 95 ℃ for 60 s, annealing at 5 0 ℃ for 30 s, and extension at 72 ℃ for 60 s using Program Temp Control System PC-800 (ASTEC, Fukuoka, Japan). The sequences of the primer set to amplify 921 bp DNA fragment from *pheB* gene of *P. putida* BH were 5'-ATGAAAAAAGGCGGAATTCGCC CCG-3': PHE-1 and 5'-TCAGGTGAGCA CGCTCGAGAAACGT-3': PHE-2. Aliquots (10 μl) of the PCR products were analyzed by electrophoresis on a 1.0 % agarose gel stained with 0.5 $\mu g/ml$ ethidium bromide.

RESULTS AND DISCUSSION

Comparative study on DNA extraction methods for water samples Results of the comparative study on the DNA extraction methods for water samples are summarized in Tables 4 and 5. Five frequently-employed enzymatic, chemical, or mechanical cell lysis operations (proteinase K, SDS, CTAB, freeze-and-thaw, and ultrasonication treatments) were tested coupled with phenol-chloroform extraction.

When phenol-chloroform extraction was applied without any other enzymatic, chemical, or mechanical treatment (control ; W1), the DNA yields were only 7.2 to 11.2 % (Table 4). When the samples were treated with proteinase K (W2) or SDS (W3) prior to phenol-chloroform extraction, the DNA yield was considerably increased independent of the water samples, though the use of CTAB (W4) was not effective at all. Application of the mechanical cell lysis operations, freeze-and-thaw (W5) and ultrasonication (W6), also gave very high yields of DNA for the fresh water (river and lake) samples; it seems that almost all DNA was recovered (76. 0 to 109 %). On the other hand, these mechanical treatments were not so effective for enhancing the DNA yield from the sea water sample.

In order to determine whether the DNA extracts were sufficiently pure suitable for PCR or not, amplification of the *P. putida* BH-derived sequence was tried using the primer set PHE-1 and PHE-2 (Table 5). No PCR signal was observed from non-inoculated samples, indicating absence of the DNA sequence of the target microbe in the environmental samples (data not shown). The positive signal of the PCR amplification was observed from 1 to 2 of triplicated DNA extracts prepared by the phenol-chloroform extraction without additional treatment (W1), suggesting that PCR-dependent detection of a target microbe at approximately 10^5 cells in 1 ml of water samples is possible by this simplest DNA extraction procedure. The DNA extracts prepared by the proteinase K

Table 4 Comparison of various DNA extraction methods for water samples: DNA yields (%)

Methods	Samples used for DNA extraction		
	River water	Lake water	Sea water
W1	9.3±1.1	11.2±2.7	7.2±1.3
W2	53.8±8.8	55.9±4.5	23.8±7.3
W3	88.3±2.1	70.3±5.1	63.9±1.5
W4	0.2±0.2	0.9±0.3	0.06±0.04
W5	104±20.0	105±10.0	21.0±9.1
W6	109±12.0	76.0±8.0	8.9±1.5

DNA yields (%) are expressed by mean value ± standard deviation in triplicated experiments. Details of each methods are given in Table 2.

Table 5 Comparison of various DNA extraction methods for water samples: PCR amplification (DNA purity)

Methods	Samples used for DNA extraction		
	River water	Lake water	Sea water
W1	+ + −	+ + −	+ − −
W2	+ + +	+ + +	+ + −
W3	− − −	− − −	− − −
W4	− − −	+ − −	− − −
W5	− − −	+ − −	− − −
W6	− − −	− − −	+ − −

Results of the triplicated experiments are shown as positive (+) and negative (−) signals. e.g. + + + indicates that the anticipated DNA fragment was amplified (observed) in all the triplicated trials. Details of each method are given in Table 2.

treatment showed more reproducible amplification; all the extracts except for one from the sea water showed positive signals, indicating that more sensitive detection of the target microbe was possible by this method (W2). However, the other chemical or mechanical treatments resulted in inferior PCR amplification, nevertheless some of them gave very high DNA yields. Especially no positive signal was observed from any samples when the SDS treatment was employed for extracting DNA (W3). The reason can be attributed to that SDS remaining in the DNA extracts inhibited *Taq* DNA polymerase activity in the PCR[25]. On the other hand, the reasons for poor PCR amplification of the mechanically-treated samples (W5 and W6) may be that the shearing force injured or fragmentated the extracted DNA to generate damaged or smaller-size fragments which are unsuitable for PCR analyses[8,26]

Considering both DNA yield and suitability for PCR, the proteinase K treatment followed by phenol-chloroform extraction (W2) was selected as the best method from water samples among the tested methods. In order to determine the detection limit of the target microbe by PCR, water samples were inoculated with *P. putida* BH at different densities (10^0 to 10^5 cells/m*l*) and the selected method was applied to extract DNA from these samples. The DNA extracts from water samples which were inoculated with BH at more than 10^1 cells/m*l* reproducibly gave positive signals by the PCR against the background of 10^4 to 10^5 cells/m*l* of indigenous microbes in the samples (data not shown).

Comparative study on DNA extraction methods for soil samples In a preliminary study, the DNA extraction method established for water samples was applied to soil samples, resulted in no amplification of the target DNA fragment from any samples, while the DNA yield ranged from approximately 10 to 75 %. When the DNA extracts were further purified by the spun column, loss of the DNA was trivial (less than 5 %), however, positive signals were rarely obtained by PCR (data not shown). This suggested that much impurities which inhibit PCR was contained in the soil samples and that they could not be sufficiently removed by the combination of proteinase K treatment, phenol-chloroform extraction, and spun column purification.

To overcome this problem, several modifications were added for establishing the DNA extraction method suitable for soil samples. Comparative studies were performed on the concentration of chelating agent in the extraction buffer, and on the uses of chemical agents (SDS, CTAB, and PVPP) and mechanical treatments (freeze-and-thaw and ultrasonication) in addition to the proteinase K treatment (Table 2). The results are summarized in Tables 6 and 7.

As shown in tables 6 and 7, mechanical treatments were much effective for increasing DNA yield and purity (suitability for PCR), especially when used with High Buffer which contains chelating agents and sodium chloride at high concentration. According to the previous report, chelating agents protect the

Table 6 Comparison of various DNA extraction methods for soil samples: DNA yield (%)

Methods	Samples used for DNA extraction		
	A	B	C
S1-L	52.1±8.3	71.1±5.9	9.0±3.5
H	23.1±6.3	2.0±1.8	16.4±6.7
S2-L	65.8±19.7	17.6±1.5	10.6±8.9
H	8.6±3.5	18.0±3.3	34.1±2.8
S3-L	65.4±3.6	74.4±7.9	4.6±3.7
H	21.8±5.3	16.6±2.0	8.7±1.5
S4-L	22.4±3.2	48.4±7.9	0.3±0.2
H	26.1±4.0	17.1±2.5	7.6±2.4
S5-L	39.7±5.4	34.4±10.0	14.8±8.2
H	26.2±4.0	34.2±5.4	28.8±13.9
S6-L	16.5±1.9	29.5±0.6	5.3±1.6
H	91.0±3.6	78.5±2.6	64.5±3.3
S7-L	76.6±1.3	60.5±5.6	< 0.1
H	43.8±6.6	38.3±12.2	68.8±3.3
S8-L	62.1±14.4	77.8±16.3	< 0.1
H	44.8±7.5	41.6±3.9	43.3±6.8
S9-L	70.1±5.5	62.7±7.1	4.8±5.0
H	44.6±7.2	54.3±5.7	37.2±5.9
S10-L	41.1±4.2	14.0±2.1	26.5±5.9
H	89.1±3.2	80.8±7.8	57.5±4.4
S11-L	76.5±23.2	68.6±1.5	8.5±2.1
H	87.9±2.5	80.1±10.4	95.8±17.3
S12-L	15.0±2.0	10.6±3.0	< 0.1
H	51.0±5.3	80.7±7.6	80.7±9.4

DNA yields (%) are expressed by mean value ± standard deviation in triplicated experiments. Details of each methods are given in Table 2.

Table 7 Comparison of various DNA extraction methods for soil samples: PCR amplification (DNA purity)

Methods	Samples used for DNA extraction														
	A					B					C				
	10^{5a}	10^{4a}	10^{3a}	10^{2a}	10^{1a}	10^{5a}	10^{4a}	10^{3a}	10^{2a}	10^{1a}	10^{5a}	10^{4a}	10^{3a}	10^{2a}	10^{1a}
S1-L	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
H	+	−	−	−	−	+	−	−	−	−	+	−	−	−	−
S2-L	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−
H	+	−	−	−	−	+	−	−	−	−	+	−	−	−	−
S3-L	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
H	+	−	−	−	−	+	−	−	−	−	+	−	−	−	−
S4-L	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
H	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−
S5-L	+	+	−	−	−	+	+	−	−	−	+	−	−	−	−
H	+	+	+	−	−	+	+	+	−	−	+	−	−	−	−
S6-L	+	+	−	−	−	+	+	−	−	−	−	−	−	−	−
H	+	+	+	+	−	+	+	+	+	−	+	−	−	−	−
S7-L	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−
H	+	+	+	−	−	+	−	−	−	−	+	−	−	−	−
S8-L	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−
H	+	+	+	−	−	+	+	+	−	−	+	−	−	−	−
S9-L	+	+	−	−	−	+	+	−	−	−	+	−	−	−	−
H	+	+	+	−	−	+	+	+	−	−	+	−	−	−	−
S10-L	+	+	−	−	−	+	+	−	−	−	−	−	−	−	−
H	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S11-L	+	−	−	−	−	+	−	−	−	−	+	−	−	−	−
H	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S12-L	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−
H	+	−	−	−	−	+	−	−	−	−	+	−	−	−	−

[a] DNA was extracted from the soil samples inoculated with *P. putida* BH to be corresponding numbers of the cells/g-soil (wet weight). +, amplification of the fragment; −, no amplification. Details of each method are given in Table 2.

extracted DNA from nuclease activity and sodium chloride provides dispersing effect to the solution[8], therefore it can be considered that High Buffer could protect extracted DNA from digestion or re-absorption onto soil-derived impurities. Though chelating agents at high concentration are known to inhibit the activity of the *Taq* DNA polymerase[25], no PCR inhibition was observed in this study. When comparing 2 mechanical treatments used here, the ultrasonication led to more sensitive PCR amplification than the freeze-and-thaw as a whole, while DNA yield was not so different between both treatments. As for the addition of chemical agents, SDS and PVPP generally gave higher DNA yield and purity, while CTAB was much less effective. As previously described, both SDS and mechanical treatments for water samples caused inhibitory effects on the PCR amplification in spite of high DNA yields, however, such inhibitory effects were not clearly observed for soil samples. It seems that, when applied to soil samples, SDS formed a complex with impurities and it might be removed to be lower concentrations during the DNA extraction and purification steps, resultantly it gave little or no inhibition for *Taq* DNA polymerase activity. On the other hand the reason for no PCR inhibition by the mechanical treatments for soil samples may be that the co-presence of soil particles and impurities reduced the effects of shearing force onto released DNA, caused less damage or fragmentation.

Considering both DNA yield and suitability for PCR, the best method for extracting DNA from soil samples among the tested methods seemed to be the combination of proteinase K, SDS, and ultrasonication treatments in the High Buffer followed by the phenol-chloroform extraction, ethanol precipitation, and spun

column purification (S11-H). By using this extraction method, very high DNA yields (> 80 %) and sensitive PCR amplification results were obtained from all soil samples regardless of their soil structure and carbon contents. The combination of the proteinase K, PVPP, and ultrasonication treatments (S10-H) showed good results almost comparative to the best one, however, the DNA yield from the soil sample C was considerably lower (about 60 %).

When the selected method (S11-H) was applied, the detection limit of the inoculated microbe by PCR from 3 soil samples where 10^7 to 10^9 cells/g of indigenous microbes existed was 10^1 to 10^2 inoculated cells/g (data not shown). Previous studies reported similar or a little lower sensitivity of PCR-mediated detection of microbes in soil samples, though some of the employed DNA extraction methods in these studies were more complicated and/or time-consuming than our method; e.g. the detection limits reported were 100 *Nitrobacter* cells/g-soil[27], 70 *Escherichia coli* cells or 500 copies of target DNA/g-sediment[9], 100 plasmids of *Corynebacterium glutamicus*/g-soil[28] and 800 *Desulfitobacterium frappieri* PCP-1 cells/g-soil[29].

CONCLUSIONS

Based on the results of comparative studies performed here, the direct DNA extraction methods for water and soil samples were developed as shown in Figs. 1 and 2, respectively.

The established method for water samples (Fig. 1) could recover more than 60 % from fresh water samples, though the yield was a little lower from a sea water sample. The PCR-mediated detection limit of the target microbe was between 10^0 and 10^1 added cells/m*l*, indicating no or very little inhibition of PCR reaction. The procedure was very simple and could be completed within 3 to 4 hours in a small scale (1.5-m*l* microcentrifuge tube).

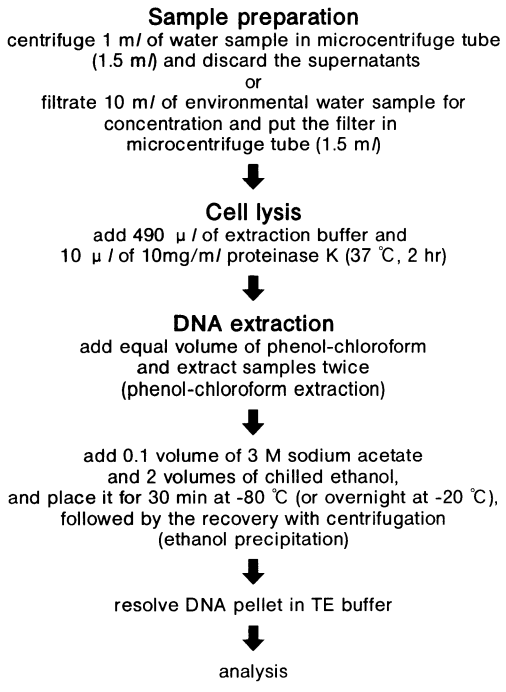

Fig. 1 Established DNA extraction method for water environmental samples

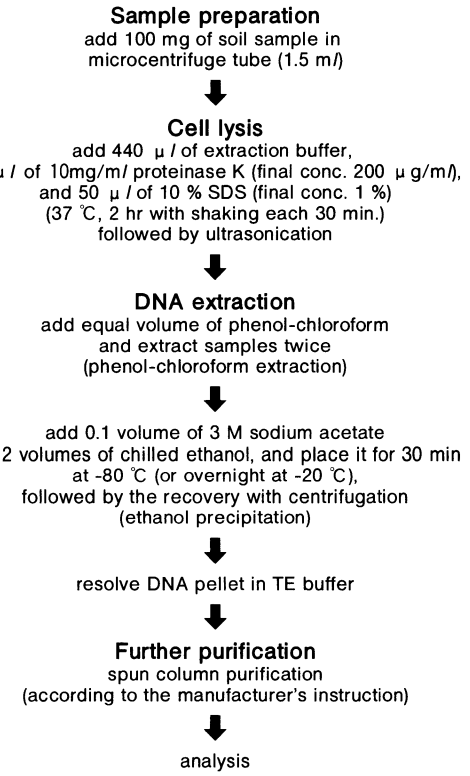

Fig. 2 Established DNA extraction method for soil environmental samples

On the other hand, the established method for soil samples (Fig. 2) gave the DNA yield more than 80 % and enabled sensitive detection of the inoculated microbe by PCR without apparent inhibition (detection limit, 10^1 to 10^2 added cells/g soil, i.e. 10^0 to 10^1 cells/microcentrifuge tube) regardless of the soil characteristics. The procedure could be completed within 4 to 5 hours without complicated operations and required no expensive and/or specialized equipments, therefore, was routinely-applicable.

Although detailed data are not shown in this article, these methods with or without minor modifications could be successfully applied to analyze microbial communities in several other water, activated sludge, sediment, and soil samples, providing over 80 % of DNA recovery and sufficient suitability for PCR (data not shown). This implies that the established methods are applicable regardless of the bacterial types.

Thus, the established DNA extraction methods are considered to satisfy the requirements (i) to (iv) for general protocols applicable for intensive monitoring of microbial ecology with PCR techniques. They can be very helpful for rapid and routine monitoring of specific functional microbes and microbial flora in bioremediation and waste/wastewater treatment processes.

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