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Enhanced method for microbial community DNA extraction and purification from agricultural yellow loess soil

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In this study, novel DNA extraction and purification methods were developed to obtain high-quantity and reliable quality DNA from the microbial community of agricultural yellow loess soil samples. The efficiencies of five different soil DNA-extraction protocols were evaluated on the basis of DNA yield, quality and DNA shearing. Our suggested extraction method, which used CTAB, EDTA and cell membrane lytic enzymes in the extraction followed by DNA precipitation using isopropanol, yielded a maximum DNA content of 42.28 ± 5.59 μg/g soil. In addition, among the five different purification protocols, the acid-treated polyvinyl polypyrrolidone (PVPP) spin column purification method yielded high-quality DNA and recovered 91% of DNA from the crude DNA. Spectrophotometry revealed that the ultraviolet A_{260}/A_{230} and A_{260}/A_{280} absorbance ratios of the purified DNA were 1.82 ± 0.03 and 1.94 ± 0.05, respectively. PCR-based 16S rRNA amplification showed clear bands at ~1.5 kb with acid-treated PVPP–purified DNA templates. In conclusion, our suggested extraction and purification protocols can be used to recover high concentration, high purity, and high-molecular-weight DNA from clay and silica-rich agricultural soil samples.

Keywords: microbial community DNA, metagenomics, soil texture, DNA extraction purification

Introduction

The microbial diversity of environmental samples is enormous; however, only 1–10% of the microbial population can be cultured through the traditional isolation techniques. Numerous works in the literature have indicated that most bacteria in environmental samples cannot be isolated via the recognized methods. In recent years, researchers have paid great attention to the culture-independent metagenomic approach, using either a sequence- or a functional-based application. Metagenomics is a reliable alternative approach for providing insights into microbial diversity. This approach has also been considered as a promising molecular method for the isolation and identification of novel and unusual proteins, enzymes, and secondary metabolites from soil samples (Forsberg *et al.*, 2012; McGarvey *et al.*, 2012; Selvin *et al.*, 2012; Yeh *et al.*, 2013). In addition, this approach has become one of the powerful research tools to overcome the limitation of the traditional culture-based methods. Construction of a metagenomic library requires a sufficiently great quantity of high-quality DNA, which makes the extraction and purification of DNA from the environmental samples a critical step (Wilkinson *et al.*, 2002). The molecular techniques used in metagenomic studies, including DNA extraction from the environmental samples, followed by purification, restriction digestion, cloning, and sequencing, are affected mainly by humic acid contaminants. Humic substances are known to inhibit the activities of imperative enzymes that are used in molecular studies, such as *Taq* DNA polymerase and restriction enzymes. Even at extremely low concentrations, humic acid substances significantly influence the binding efficiency and annealing of double-stranded DNA in polymerase chain reaction (PCR) amplification. Hence, to obtain high-quality microbial community DNA, researchers have developed different types of protocols and applied modified methods in each step. The efficiency of soil microbial community DNA extraction depends on the soil quality and chemical composition of the soil, including sand, clay, and silt. Several previous studies have indicated that the extraction process is also influenced by the tight interaction of microorganisms on soil colloids, the formation of clay-organic matter aggregates, and the interaction of DNA with the soil matrix (Harry *et al.*, 1999). The soil samples contain microbial populations including bacteria, actinomycetes, fungi, protozoa, mycelia, spores, and different types of unicellular and multicellular organisms (Krsek and Wellington, 1999). However, the data on high quality DNA extractions from soil communities are significantly limited. This may be due to different trace elements, pH, and clay contents of the soil and sediments. A variety of extraction protocols have been applied to obtain high-quality microbial community DNA from diverse environmental samples.

Over the past two decades, different protocols of physical, chemical, and enzymatic lysis have been developed for direct DNA extraction. Generally, common physical disruption methods have been employed, such as sonication (Yeates *et al.*, 1997), bead beating (Kozdroj and Van Elsas, 2000), freezing-

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thawing (Tsai and Olson, 1990), liquid nitrogen freezing-thawing (Erb and Wagner-Dobler, 1993), and microwaving (Orsini and Romano-Spica, 2001). Although these techniques yield high DNA concentrations, a major drawback of direct lysis is the coextraction of extra humic acid, cellulose-derived compounds, and other phenolic compounds. The lysis buffer commonly contains detergents such as sodium dodecyl sulfate (SDS) (Miller *et al*., 1999) and sarkosyl (Smith and Tiedje, 1992), however in recent years a variety of chemical lysis approaches have been used to obtain higher purity DNA, including high-temperature boiling with polyvinyl polypyrrolidone (PVPP), phenol, chloroform, ethylenediamine tetraacetic acid (EDTA), cetyl trimethylammonium bromide (CTAB), and Triton X-100, respectively. Arbeli and Fuentes (2007) used polyethylene glycol (PEG) as a precipitate instead of isopropanol, which resulted in a higher reduction of PCR inhibitors without loss of DNA concentration. The final method of DNA extraction is an enzymatic digestion step that frequently employs lysozyme and proteinase K to quicken the process and increase the DNA yield.

The extracted DNA product is light brown to dark brown in color owing to the presence of phenolic compounds. As mentioned above, because these contaminants inhibit the activities of DNA polymerase and restriction-digestion enzymes, further purification is required to obtain greater purity. So far, several purification methods-including Sephadex spin columns, ion exchange chromatography, gel filtration chromatography, agarose gel electrophoresis, PVPP, bovine serum albumin (BSA), gelatin, and skim milk-have been developed to remove PCR inhibitors (Romanowski *et al*., 1993; More *et al*., 1994; Harry *et al*., 1999; Kauffmann *et al*., 2004). Amsaleg *et al.* (2001) reported that each step of the purification procedure suffers from shortcomings of inappropriate removal of humic acids and high cost, and every additional step inevitably results in DNA loss. The choice of extraction and purification protocol should consider the desired concentration and quality of the recovered DNA.

Because yellow loess is an aeolian sediment formed by the accumulation of wind-blown silt, typically in the 20–50 μm size range, twenty percent or less clay and balance equal parts sand and silt that are loosely cemented by calcium carbonate, its moisture content is relatively low as 10–15%. And main chemical composition is as follows: 50–60% silica (SiO_2), 8–12% alumina (Al_2O_3), 2–4% ferric ion (Fe_2O_3), 0.8–1.1% ferrous ion (FeO), 0.5% titanium oxide (TiO_2), and Manganese oxide (MnO), 4–16% calcium oxide (CaO), and 2–6% Magnesium oxide (MgO). These indicate that yellow loess is a unique environmental habitat for growth of microorganisms, from which DNA is difficult to extract and purify.

The purpose of this study was to develop novel methods for efficient extraction and purification of microbial community DNA from agricultural yellow loess soil (AYLS) samples. To obtain high quantity of DNA from six soil samples, five different extraction methods were investigated. In addition, five different purification methods were developed to gain high-quality DNA from crude extracted DNA. The purity of DNA was assessed both by analysis of the A_{260}/A_{230} and A_{260}/A_{280} spectrophotometry absorbance ratios and by analysis of 16S rRNA gene amplification.

Materials and Methods

Soil sample collection and characterization

AYLS samples were collected in sterile plastic bags from three different sites around Jeollanamdo, the southwestern province in South Korea: Muan-Hyeongyeong (34°59′25.63″N, 126°28′54.07″E), Yeongam-Sinbuk (34°53′25.04″N, 126°41′33.35″E), and Yeongam-Miam (34°41′57.20″N, 126°34′18.94″E). The collected soil samples were labeled AYLS01, AYLS02, AYLS03, AYLS04, AYLS05, and AYLS06. All soil samples were immediately transferred to the laboratory and stored at -20°C until further use. To identify the soil characteristics, its textures were analyzed by the method described by Kathiravan *et al.* (2011) with a slight modification. The required amount of sterilized soil sample was placed in a glass jar and mixed with an equal volume of distilled water. The resulting soil-water mixture was vigorously stirred for 1 h and then left without further agitation for 1 day. Next, the volume of each particle size was visually measured and the percentage of sand, silt, and clay was calculated with reference to a soil texture analysis chart. The AYLS trace element composition was analyzed as follows. Briefly, 1 g of homogenized soil was placed into a Teflon vessel containing a solution of 20 ml concentrated HNO_3:HCl (3:2), after which the total volume was increased by a further 50% by the addition of ultrapure water. The sample vessels were sealed and stored at 120°C overnight. After incubation, the vessels were allowed to cool, and 10 ml of ultrapure water was added when the acid-extracted digestate reached room temperature. This digestate was further diluted into a 100- to 1000-fold series, and injected into inductive-coupled mass spectroscopy (ICP-MS) (Elan DRC II, PerkinElmer). The pH values of all soil samples were determined by using a glass electrode in a soil:water ratio of 1:1.25. The moisture content of the samples was determined by drying 10 g of soil samples at 100°C for 2 days. The concentration of humic acid was determined using a UV-visible spectrophotometer at a wavelength of 230 nm.

Soil DNA extraction methods

Five different methods were used for DNA extraction, as described below:

Method 1: The PowerSoil® DNA Extraction Kit (MO BIO Laboratories, Inc.): AYLS microbial community DNA extraction was performed according to the manufacturer's protocol using 250 mg of the soil sample. For further purification, the resultant DNA was washed three times with 70% ethanol.

Method 2: Modified Porteous method (Porteous *et al*., 1994): Briefly, 100 mg of soil sample and 350 μl of homogenization solution A [250 mM NaCl, 100 mM Na_2·EDTA, and 0.2% CTAB (w/v), pH 8.0] were mixed by vortexing for 30 sec. The samples were then sonicated (Branson 5200 sonicator-bath) at room temperature for 3 min. The resultant products were treated with 10 μl proteinase K (10 mg/ml), 10 μl lysozyme (10 mg/ml), and 2 μl glusulase (1,000 U/ml). The tubes were vortexed for 10 sec and incubated at 37°C for 1 h. Each sample was treated with 350 μl of lysis solution B [250 mM NaCl, 100 mM Na_2·EDTA, and 4% SDS (w/v), pH 8.0] and 50 μl of 5 M guanidine isothiocyanate. The samples

were incubated at 68°C for 1 h and then centrifuged for 15 min at 12,000 × *g* at 4°C. The resultant supernatant was mixed with 0.6× volume of isopropanol and incubated at -20°C for 30 min, followed by centrifugation for 15 min at 12,000 × *g*. The pellet was washed three times with 70% ethanol, centrifuged, air dried, and then resuspended in 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0).

Method 3: Modified Yeates method (Yeates *et al*., 1997): Yeates's protocol with a minor modification was applied for DNA extraction. Briefly, 1 g of soil was mixed with 2 ml of extraction buffer (100 mM Tris-HCl, 100 mM Na_2·EDTA, 1.5 M NaCl, pH 8.0) and 1 g of sterilized glass beads, before being blended in a mini-bead beater (Biospec instruments) for 2 min at 50 shaking speed/min. The sample was incubated at 65°C for 10 min then centrifuged for 15 min at 12,000 × *g*. The supernatant was transferred to centrifuge tubes containing a half volume of PEG (30%, w/v)/NaCl (1.5 M) and incubated at room temperature for 2 h. Then, 0.5 M potassium acetate (7.5 M) was added to the samples, which were transferred to ice for 5 min and then centrifuged at 12,000 × *g* for 30 min at 4°C to precipitate proteins and polysaccharides. The aqueous phase was extracted with the addition of equal volumes of phenol/chloroform and chloroform/isoamyl alcohol (Sambrook *et al*., 1987), and DNA was precipitated by adding 0.6× volume of isopropanol. After 2 h at room temperature, the samples were centrifuged at 12,000 × *g* for 30 min and the DNA was resuspended in 250 µl of TE buffer.

Method 4: Modified Bürgmann method (Bürgmann *et al*., 2001): The Bürgmann protocol was used with a slight modification. Briefly, 0.5 g soil sample and 0.5 g glass beads were suspended in 1 ml of extraction buffer (0.2 M Na_3PO_4, 0.1 M NaCl, 50 mM EDTA, pH 8.0) containing 2 µl glusulase (1,000 U/ml) and incubated with agitation at 200 rpm for 30 min at 37°C. DNA extraction was performed by using a mini-bead beater for 1 min of 10 cycles at 50–60 Hz, and thereafter DNA was purified by adding 2 ml chloroform/isoamyl alcohol (24/1, v/v). DNA precipitation was performed by the addition of 3 ml of a precipitation solution (20% PEG 6000, 2.5 M NaCl), followed by incubation at 37°C for 1 h and then centrifugation at 12,000 × *g* for 5 min. The resultant pellet was washed three times with 70% ice cold ethanol, air dried, and resuspended in 1 ml of TE buffer.

Method 5: Our suggested method: An alternative and efficient method was developed to obtain high-quantity, -quality, and -molecular-weight microbial community DNA. In this protocol, 250 mg of soil sample in 1.5 ml Eppendorf tubes was mixed with 270 µl of DNA extraction buffer [100 mM Tris-HCl, 100 mM Na_2·EDTA, 100 mM NaH_2PO_4, 1.5 M NaCl, and 1% (w/v) CTAB pH 8.0], 2 µl proteinase K (10 mg/ml), and 2 µl glusulase (1,000 U/ml), followed by agitation at 200 rpm for 30 min at 37°C. After shaking, 30 µl of 20% (w/v) SDS was added and incubated in a 65°C water bath for 2 h with gentle mixing every 20 min. The supernatant was collected after centrifugation at 6,000 × *g* for 10 min at room temperature and transferred into 1.5 ml Eppendorf tubes. About 10 µl of RNase A (10 mg/ml) was added to 1.5 ml tubes, which were incubated at room temperature for

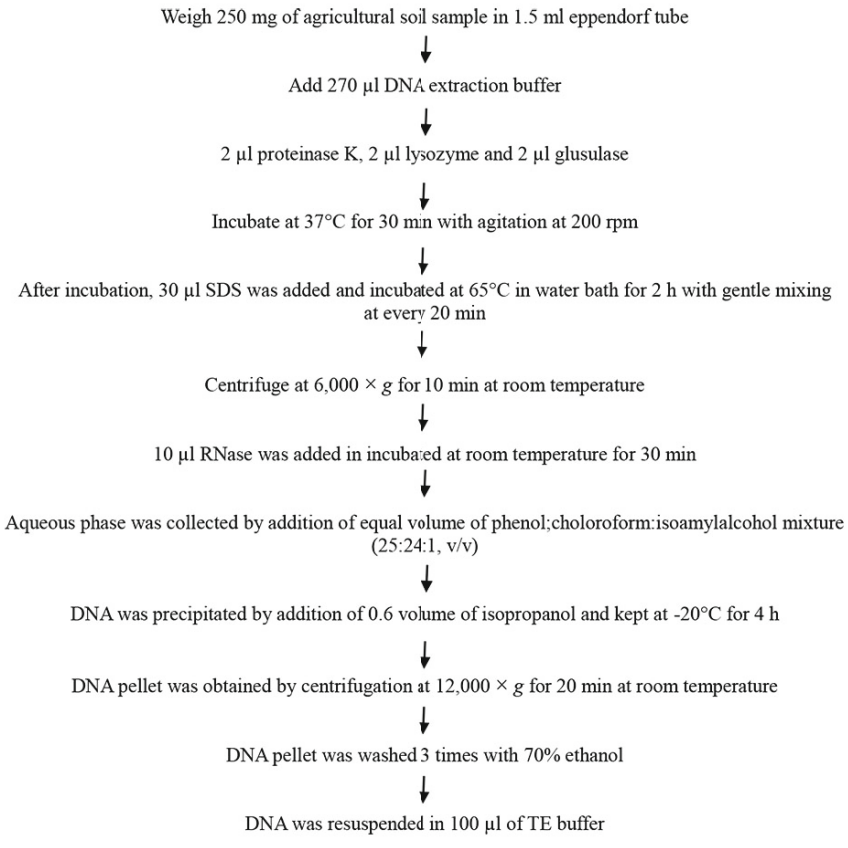

Fig. 1. Schematic diagram of microbial community DNA extraction from AYLS samples as suggested by this study.

30 min, and then mixed with an equal volume of phenol: chloroform:isoamyl alcohol (25:24:1, v/v). Next, the aqueous phase was obtained by centrifugation at 6,000 × *g* for 10 min at room temperature and precipitated with 0.6× volume of isopropanol at -20°C for 4 h. The samples were centrifuged at 12,000 × *g* (20 min, room temperature) and the resulting pellet was washed three times with ice cold 70% ethanol and resuspended in 100 µl of TE buffer (Fig. 1).

DNA purification methods

Removal of humic acids and phenolic compounds from a soil sample is very difficult and therefore DNA purification is a critical step. The presence of humic acid is revealed by the development of a brownish color in the recovered DNA extract. This crude DNA is not suitable for PCR amplification or restriction digestion. Generally, during purification, humic acid and contaminated proteins can be removed; however, a significant amount of DNA will inevitably be lost. In this study, five different DNA purification methods were applied and compared as follows:

Method 1: Genomic DNA purification kit: The extracted soil DNA was further purified using a genomic DNA purification kit (Nucleogen Biotechnology) according to the manufacturer's instructions, using 100 µl of sample with slight modifications. Briefly, 20 mg polyvinyl pyrrolidone (PVP) was added to the spin columns, which were then washed three times with ice cold 70% ethanol to remove the excess contaminants present in the extracted sample.

Method 2: Sephadex G-100 spin column: The Sephadex G-100 spin column was constructed by adding 25 mg Sephadex G-100 into the commercial spin column, followed by washing with sterilized DNase-free water. The excess water was removed by centrifugation. Approximately 100 µl of the extracted DNA was added into the spin column. The contaminants were washed with ice cold 70% ethanol, and then 100 µl of TE buffer was added to the spin column and left for 2 min. The purified DNA was collected in a collection tube by centrifuging the spin column at maximum speed for 2 min.

Method 3: PVP-low melting point (LMP) agarose gel: The PVP-LMP agarose gel was prepared by the addition of 1.5% (w/v) PVP and 1% (w/v) LMP agarose in 100 µl of 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA; pH 7.0). This mixture was dissolved by heating in a microwave oven for 3 min and then cooled to 50°C. After cooling, 10 µl Top Red nucleic acid gel stain (Genomicbase) was added and the mixture was poured into a gel casting tray. After setting the gel, about 40 µl of crude DNA sample was added into each well. Electrophoresis was performed for 30 min at 100 V. The separated DNA was visualized using UV light. After electrophoresis, the separated bands were extracted using a commercial kit.

Method 4: Formamide–agarose gel purification (our suggested method): A 2% agarose solution in 1× TAE was prepared in a sterile glass beaker, heated in a microwave oven, and left to cool to 45°C. It was mixed briefly to ensure that the agarose solution was homogeneous. Then, 100 µl of 2% agarose was mixed with 100 µl of DNA extract. A solution of 1 ml 80% formamide prepared in a 1.3 M NaCl solution was added into the agarose-DNA mixture before being inverted slowly then incubated at 4°C for 1 h. After incubation, the formamide was removed by centrifugation at 6,000 × *g* for 10 min. Purified DNA was obtained using a commercial gel extraction kit.

Method 5: Acid-treated PVPP (AT-PVPP) spin column (our suggested method): Acid treatment of PVPP was performed by the addition of 10 g of insoluble PVPP to 1 L of 1 M HCl. This reaction mixture was incubated at room temperature for 24 h. After incubation, the solution was filtered through Whatman filter paper No. 1. The filtered PVPP was added to 1 L of 20 mM phosphate buffer (pH 8.0) and mixed by stirring for 2 h. This washing process was repeated with the same buffer until the pH of filtered PVPP suspension reached 7.0. After washing, the AT-PVPP residue was air dried over-

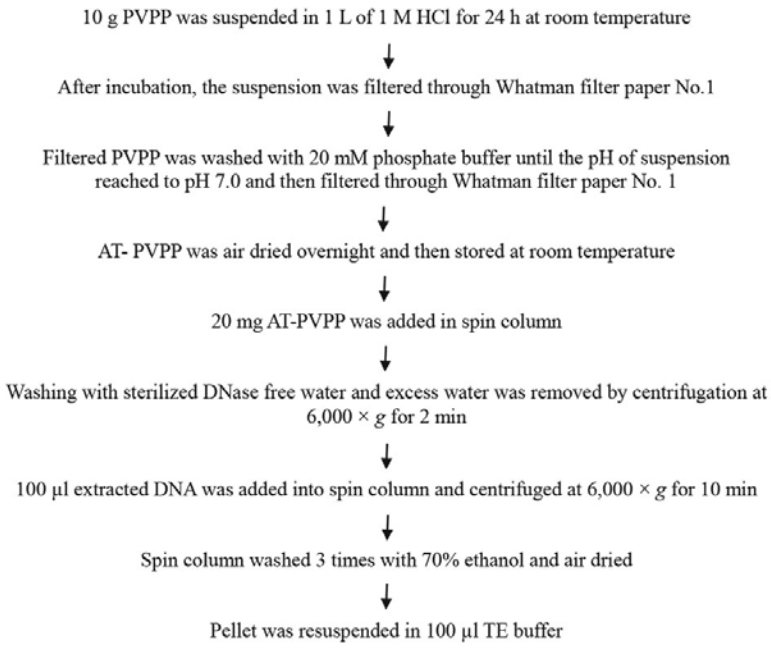

Fig. 2. Schematic purification steps of crude DNA by AT-PVPP.

Table 1. Soil properties of agricultural yellow loess

Property		Sample Nos.					
		AYLS01	AYLS02	AYLS03	AYLS04	AYLS05	AYLS06
pH		6.5 ± 0.04	7.2 ± 0.03	6.8 ± 0.07	6.2 ± 0.05	6.8 ± 0.04	7.1 ± 0.06
Soil type		Loam	Loam	Sandy loam	Sandy clay loam	Sandy loam	Clay loam
Soil texture[a]	Sand (%) (2-0.05 mm)	41.4 ± 1.2	50.7 ± 2.4	59.6 ± 1.9	52.6 ± 2.1	43.6 ± 1.6	38.7 ± 2.3
	Silt (%) (0.05-0.002 mm)	43.0 ± 3.2	39.8 ± 1.8	30.0 ± 1.3	26.9 ± 1.8	39.0 ± 1.7	41.7 ± 1.9
	Clay (%) (<0.002 mm)	15.6 ± 1.7	9.5 ± 0.5	10.4 ± 0.6	20.4 ± 1.1	17.4 ± 0.9	19.6 ± 1.4

[a] The soil type was determined with soil texture analysis chart.

night at room temperature. About 20 mg AT-PVPP was added to the DNA purifying spin column and washed with sterilized DNase-free water. The excess water was removed by centrifugation. Approximately 100 µl of the extracted DNA was added into the spin column and washed three times with 70% ethanol to remove the excess contaminants present in the extracted sample. In this purification method, the eluant was collected, and DNA was washed with 70% ethanol, air dried, and then resuspended in 100 µl of TE buffer (Fig. 2).

PCR amplification

16S rRNA genes were amplified by PCR from the soil-extracted community DNA templates using universal bacterial primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1494R (5′-TGACTGACTGAGGYTACCTTGTTAC-3′). PCR amplification of 16S rRNA genes was performed using two templates; crude DNA obtained using our suggested protocol, and DNA that had been purified using the AT-PVPP approach. A total volume of 20 µl PCR mixture was used, containing 1 µl undiluted DNA template, 2.5 µl 10× PCR buffer (TaKaRa Bio Inc.), 2 µl 20 mM $MgCl_2$, 1 µl 1% (w/v) BSA, 1 µl 1.5% (w/v) PVP, 0.5 µl dNTPs, 1 µl each of forward and reverse primers, 0.2 U of *Taq* DNA polymerase (TaKaRa Bio Inc.), and 9.5 µl DNase-free water. The PCR conditions were as follows: 1 cycle of 5 min at 94°C, then 30 cycles of denaturation at 94°C for 1 min; annealing at 60°C for 1.3 min; and extension at 72°C for 1.3 min, followed by a final extension at 72°C for 20 min. About 2 µl of PCR-amplified products were analyzed by 0.8% agarose gel electrophoresis in 1× Tris/borate/EDTA buffer.

Results and Discussion

Soil properties

The texture of AYLS samples was analyzed to define the percentage of clay, silt, and sand present in soil. Generally, the sand particles were settled at the bottom, the silt was layered above the sand, and the clay lay on top in the measuring jar. The general properties of the AYLS samples are listed in Table 1. In this experiment, all six samples were found to be loam, sandy loam, and sandy clay loam with pHs of 6.2–7.2. The soil type plays an important role in DNA extraction; for example, higher clay content is an especially problematic factor (Lakay *et al*., 2007). Furthermore, the size of soil pores among sand, silt, and clay determine the interaction of soil particles with microorganisms. As mentioned earlier, the efficiency of DNA extraction depends on clay content and organic matter content. Moreover, microorganisms strongly bind with clay through a variety of binding forces (Bakken and Lindahl, 1995). The high clay content leads to an explanation of lowered DNA yields because of the adsorption of free DNA onto the clay particles. Young *et al.* (2014) reported that soil samples containing high levels of clay and organic compounds also influence DNA extraction. Furthermore, the AYLS04 DNA was found to be more dark brown in color than other samples, due to coextraction of high humic acid contaminants.

In addition, metal contaminants are often coextracted with DNA from soil because of their similar physicochemical properties. The analysis of the mineral contents in the AYLS samples facilitates the selection of the DNA extraction method (Table 2). The concentrations of trace elements were determined using ICP-MS after soil extraction with acid extraction (conc. HNO_3/conc. HCl). It is known that Fe and Si concentrations in soil samples have a major effect on soil DNA extraction. The Fe content in AYLS03 was found to be higher than that in other soil samples tested (Table 2). Fe may be present in the form of iron oxides and iron hydroxides in agricultural soils (Kozdroj and Van Elsas, 2000). DNA extraction increases with an increase in Fe concentration in the soil sample and *vice versa*. On the other hand, the presence of high Si in the soil may retard the release of DNA from clay particles, which subsequently affects the efficiency of DNA extraction from soil. The concentrations of soluble ele-

Table 2. Trace elements composition of AYLS samples analyzed by ICP-MS

Samples	Concentration of trace elements (mg/g of soil)[a]					
	Fe	K	Ca	Mg	Mn	Si
AYLS01	20660 ± 32	2657 ± 13	1312 ± 5	2252 ± 10	222 ± 5	38.4 ± 2.1
AYLS02	18770 ± 25	1980 ± 10	1600 ± 5	2040 ± 15	319 ± 6	41.1 ± 3.0
AYLS03	23100 ± 39	1693 ± 11	514 ± 6	1417 ± 7	270 ± 5	34.7 ± 1.4
AYLS04	22330 ± 23	2786 ± 12	2804 ± 7	2567 ± 14	185 ± 4	40.2 ± 2.0
AYLS05	15030 ± 18	1215 ± 10	795 ± 5	1147 ± 6	162 ± 7	41.0 ± 1.9
AYLS06	14060 ± 12	496 ± 5	274 ± 6	1604 ± 7	321 ± 8	34.7 ± 2.0

[a] Soil samples were prepared by acid treatment method.

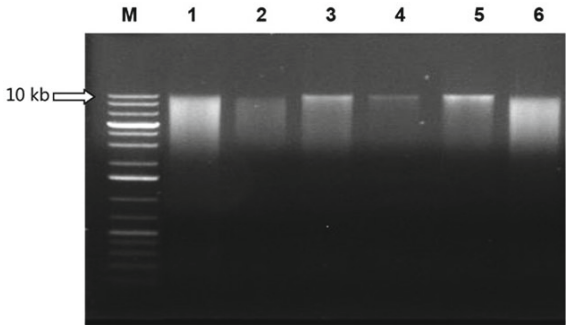

Fig. 3. Agarose gel electrophoresis of soil microbial community DNA extracted as per the Powersoil® DNA extraction protocol. Lanes: M, molecular marker; 1, AYLS01; 2, AYLS02; 3, AYLS03; 4, AYLS04; 5, AYLS05; and 6, AYLS06.

ments varied significantly, but can be ordered by abundance with the following: K>Mg>Ca>Mn>Si (Table 2). High metal concentrations in the soil can negatively affect microbial activities, provoking a low mineralization of organic materials during plant growth.

Soil DNA extraction

For functional- and sequence-based DNA analysis, it is essential to develop novel protocols that yield high quantity and quality DNA. However, DNA extraction from soil is extremely complicated due to coextraction of humic acid and other contaminants. The successful extraction depends on the type of soil, because the soil's composition requires optimization of the extraction protocol used. It is important to obtain DNA samples that are free of or contain an extremely low concentration of humic contaminants. In order to evaluate the best protocol for DNA extraction from agricultural soil samples, we used a commercial kit and four different methods with slight modifications. DNA yields from six AYLS samples varied considerably from sample to sample depending on the extraction method used.

The microbial community DNA extractions from six samples using the commercial PowerSoil® DNA extraction kit showed different sizes of fragments on agarose gel (Fig. 3), and yielded very low DNA concentrations (2.47–6.96 ± 1.56 μg/g soil) (Fig. 4). It is known that highly fragmented DNA may allow generation of chimeric amplicons during PCR amplification (Liesack *et al.*, 1991). The purity of extracted DNA was determined based on the spectrophotometry absorption ratios at A_{230}, A_{260}, and A_{280}, where the absorption peaks of coextracted humic acids and phenolic compounds with DNA are visible at 230 nm, whereas those of DNA and protein are observed at 260 and 280 nm, respectively (Yeates *et al.*, 1997). An A_{260}/A_{230} ratio greater than 2 and an A_{260}/A_{280} ratio greater than 1.7 indicate high-purity DNA, whereas the lower absorbance ratios indicate the contamination of DNA with humic acid and protein, respectively. The A_{260}/A_{230} and A_{260}/A_{280} ratios of DNA extracted from six AYLS samples by the PowerSoil® DNA extraction method were 1.28–1.58 and 1.13–1.64, respectively. Our results show that the commercial DNA extraction kit was not suitable for problematic soil samples that contain high levels of organic compounds, clay or heavy metals. Moreover, because commercial kits are typically optimized for a small volume of soil sample, their uses for obtaining high-quantity and -quality DNA are often limited. Therefore, we tested four alternative DNA extraction methods.

A modified Porteous DNA extraction protocol yielded a high DNA concentration of 15.89 ± 1.34 μg/g soil from AYLS01 and a low concentration of 9.31 ± 0.77 μg/g soil from AYLS05 (Fig. 4). This method adopts sonication to disturb the microbial cell wall and release DNA. Although a long period of sonication was required to obtain high DNA concentration, excessive sonication makes DNA highly fragmented (data not shown). The absorption ratios of DNA extracted by this method were assessed as 0.80 ± 0.01 for A_{260}/A_{230} and 1.04 ± 0.02 for A_{260}/A_{280}. These ratios indicate that the extracted DNA samples were highly contaminated with humic acid and proteins. As shown in Fig. 4, a modified Yeates DNA extraction method yielded a DNA concentration of 23.62 ± 4.65 μg/g soil from AYLS03, however, the amounts of DNA extracted from other soil samples were relatively low and the absorption ratios of A_{260}/A_{230} and A_{260}/A_{280} ratios were found

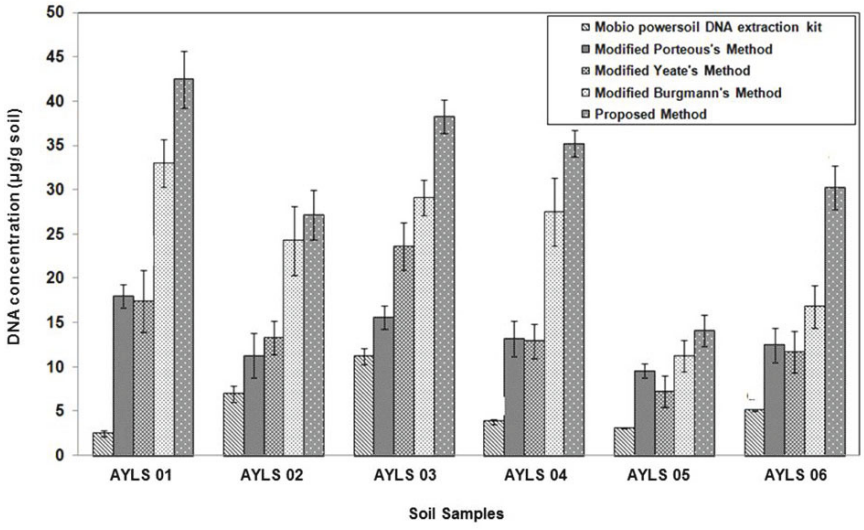

Fig. 4. Microbial community DNA concentrations (μg/g soil [dry wt]) obtained from various extraction protocols. The results are presented as mean ± SD of triplicate experiments.

Table 3. Comparison of the quantity of crude DNA obtained by different extraction methods from diverse types of soil samples

No	Soil type	Extraction buffer	Detergent (% SDS)	Precipitating agent	Maximum DNA concentration (μg/g soil)	Reference
1	Paddy soil	NaCl, EDTA, and lysozyme	10	Isopropanol	20.17	Islam *et al.* (2012)
2	Paddy soil	NaCl	1	Isopropanol	11.36	Islam *et al.* (2012)
3	Paddy soil	NaCl, CTAB and proteinase K	20	Isopropanol	18.65	Islam *et al.* (2012)
4	Pristine polluted soil	NaCl, EDTA and glass beads	20	PEG/NaCl	23.50	Yeates *et al.* (1997)
5	Bakery industry soil	EDTA and NaCl	4	PEG	3.8	Sagar *et al.* (2014)
6	Compost and organic rich soil	NaCl and EDTA	1	PEG	20.0	LaMontagne *et al.* (2002)
7	Arable soil	NaCl, EDTA, glass beads and skim milk	2	Potassium acetate	3.76	Ikeda *et al.* (2004)
8	AYLS	PowerSoil® DNA extraction kit	-	-	6.96	This study
9	AYLS	NaCl, EDTA, glusulase and glass beads	-	PEG/NaCl	33.71	This study
10	AYLS	NaCl, EDTA and glusulase	4	Isopropanol	16.86	This study
11	AYLS	Tris, NaCl, EDTA, glusulase and glass beads	20	PEG/NaCl	18.35	This study
12	AYLS	Tris, EDTA, NaCl, CTAB, proteinase K, lysozyme and glusulase	20	Isopropanol	42.48	This study (proposed method)

to be 0.92 ± 0.04 and 1.23 ± 0.06, respectively. The extracted DNA was also highly contaminated with proteins and humic acids. When we used a modified Bürgmann DNA extraction protocol, 33.8 ± 2.71 μg/g soil of DNA was extracted from AYLS01 (Fig. 4). It seems that a relatively higher DNA yield was due to glass bead-beating cell disruption, and the PEG precipitated DNA extraction. A combination of PEG and NaCl could provide an alternative to isopropanol precipitation, although the purity was low, as assessed by the absorbance ratios of 0.86 ± 0.02 at A_{260}/A_{230} and 1.27 ± 0.03 at A_{260}/A_{280}. The extracted DNA was still contaminated with high contents of humic acid and proteins. Based on these results, it is concluded that the above-mentioned DNA extraction protocols are not suitable for obtaining high quantity and quality of DNA.

Therefore, we suggested an alternative soil DNA extraction protocol, which yielded the highest DNA concentration among the methods used in this study. The maximum DNA yield was 42.48 ± 5.59 μg/g soil from AYLS01, which had loam soil and a pH of 6.5. We exploited the combined action of chelating agents (EDTA and CTAB), which were added into the DNA extraction buffer. These long-tailed surfactants turn random coil DNA structures into the compact globular structure and subsequently increase the efficiency of DNA precipitations. Also, these chemical agents help to detach microbes from soil matrix. Additionally, the collective actions of hydrolytic enzymes such as lysozyme, proteinase K, and glusulase break microbial cells and release more DNA. Agarose gel electrophoresis of soil microbial community DNA extracted by our suggested protocol showed that the size of extracted crude DNA was found to be > 10 kb, and no fragmented DNA was noted (data not shown). The range of absorption ratios of A_{260}/A_{230} of six soil samples was from 0.52 to 0.96. Similarly, that of A_{260}/A_{280} of extracted crude DNA was 1.24–1.43. The quantity of DNA was high enough to study metagenomics, and was much higher than the maximum DNA concentrations obtained from other methods (Table 3). Soil type, DNA extraction buffer composition, SDS concentration, and precipitating agents influence successful DNA extraction.

DNA purification

Metagenomic studies requires highly purified and plentiful high-molecular-weight DNA, because humic acid and protein contaminants have severe negative effects on DNA polymerase, restriction enzymes, DNA ligase, and DNA-DNA hybridization. To remove the contaminants from crude DNA, several purification methods have been adopted. The conventional purification methods have many limitations, including low quality products, significant DNA loss, and comigration of phenolic compounds. Therefore, in this study, we applied five different purification methods that enhance purity and high-molecular-weight DNA and incur low DNA loss.

A commercial DNA purification kit consists of a silica-coated spin column and a DNA collection tube. The binding buffer in the column neutralizes the silica's surface negative charge due to its high ionic strength, so that it helps binding of DNA to the silica surface. The unbound humic acid contaminants were eluted during centrifugation. By increasing the elution buffer temperature, the purified DNA can be obtained. The resultant DNA showed the absorbance ratios of A_{260}/A_{230} and A_{260}/A_{280} as 1.62 and 1.75, respectively, and was therefore a good quality. However, the major drawback of this method is a notable loss (41.7%) of DNA caused by inappropriate binding of DNA with the silica surface in the column.

When the crude DNA was purified by PVP–agarose gel electrophoresis and subsequent gel extraction, the A_{260}/A_{230} and A_{260}/A_{280} ratios of purified DNA were 1.68 and 1.81, respectively. These ratios indicate that the purified DNA was also of good quality. The high purification is obtained by the strong hydrogen interaction of PVP with humic acid and phenolic compounds. This complex retards comigration during electrophoresis. However, because a remarkable loss of DNA (56.8%) was incurred by this method, PVP–agarose gel purification failed to yield the required quantity of purified DNA. This confirms the findings of Young *et al.* (1993), who mentioned that although PVP reduces electrophoretic mobility of DNA, it strongly binds to humic acid.

The Sephadex G-100 mini-column purification method

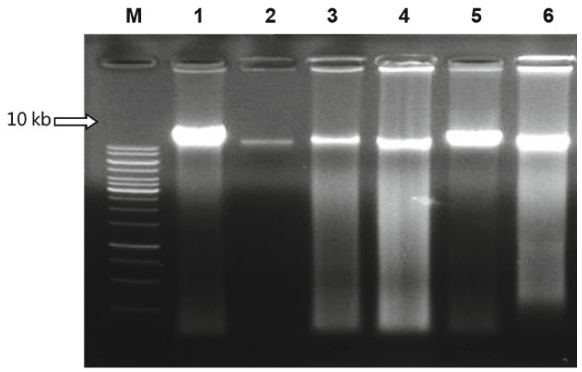

Fig. 5. Agarose gel electrophoresis of DNA after formamide-LMP agarose gel plug purification. Lanes: M, molecular marker; 1, AYLS01; 2, AYLS02; 3, AYLS03; 4, AYLS04; 5, AYLS05; and 6, AYLS06.

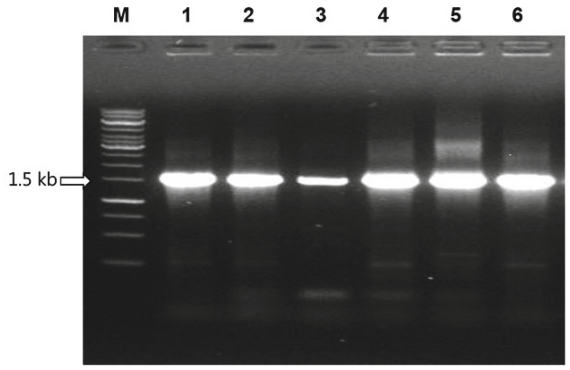

Fig. 7. Agarose gel electrophoresis of PCR-amplified products of purified DNA after AT PVPP spin column purification. Lanes: M, molecular marker; 1, AYLS01; 2, AYLS02; 3, AYLS03; 4, AYLS04; 5, AYLS05; and 6, AYLS06.

allowed effective removal of humic contaminants from crude DNA. The A_{260}/A_{230} and A_{260}/A_{280} ratios were found to be 1.66 and 1.78, respectively. The high-molecular-weight humic acid substances passed through the larger pore size of the Sephadex G-100 matrix. Although this method was better than DNA purification kit, a loss of about 19% of DNA was still observed. Sephadex G-50, Sephadex G-200, and Sephadex 4B were ineffective in purifying DNA from soil samples.

The formamide–LMP agarose gel plug purification method showed effective removal of humic acids. After the contaminated DNA was mixed with formamide, LMP-agarose, and NaCl, the humic acid and phenolic compounds tightly interacted with formamide, while the DNA strongly binds with the agarose gel. To prevent denaturation of DNA by formamide, NaCl was used as a stabilizer. The absorption ratios of A_{260}/A_{230} and A_{260}/A_{280} were 1.78 and 1.86, respectively, which indicates the high purity of the DNA. The purified DNA from each of the six soil samples was visualized by band analysis after electrophoresis (Fig. 5). All of the DNA fragment sizes were larger than 10 kb without shearing fragments, however this protocol also lost a meaningful amount (20%) of DNA.

Therefore, we suggested a novel AT-PVPP spin column purification protocol to minimize DNA loss with high purity.

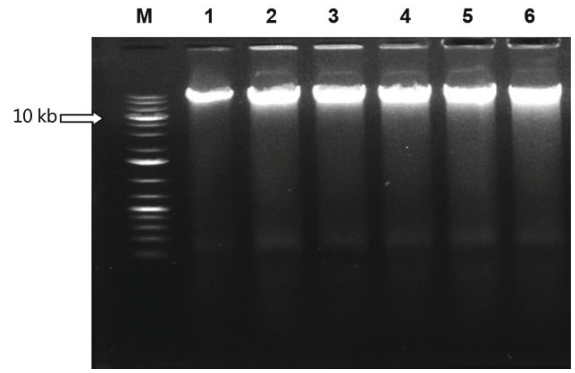

Fig. 6. Agarose gel electrophoresis of DNA after AT-PVPP spin column purification. Lanes: M, molecular marker; 1, AYLS01; 2, AYLS02; 3, AYLS03; 4, AYLS04; 5, AYLS05; and 6, AYLS06.

The absorbance ratios of A_{260}/A_{230} of six DNA samples ranged from 1.82 to 2.03 and those of A_{260}/A_{280} ranged from 1.89 to 2.05. It suggests that this method was found to yield the highest quality DNA and to be the most simple, most convenient, and least time-consuming protocol. DNA purity was further evaluated by agarose gel electrophoresis (Fig. 6). The band profile indicated that DNA sizes were larger than 10 kb in all six soil samples. Moreover, DNA loss in AT-PVPP spin column purification was significantly lower than the other methods (9%).

Furthermore, the quality of purified DNA by the AT-PVPP plug method was confirmed through 16S rRNA gene amplification by PCR. There were no appropriate sizes of 16S rRNA amplicon bands when the crude DNA was used as a template but clear primer dimer bands appeared in gel electrophoresis (data not shown). This is due to the fact that humic acid binds more strongly to *Taq* polymerase than to DNA strands. The purified DNA with AT-PVPP spin column showed high-quality clear bands with a PCR amplification product of ~1.5 kb in all soil samples (Fig. 7). Consequently, it was suggested that our proposed DNA extraction and purification methods were found to be the most reliable, simple, and cost-effective for obtaining microbial community DNA from all kinds of soil samples. In addition, it was found that each DNA color purified by each method showed "clear to white".

Conclusion

The main objective of a DNA extraction protocol is to obtain high DNA yields of high purity by a method that is convenient, less time-consuming, and cost-effective. Our assessment of DNA extraction methods depends mainly on the soil type, pH, and clay content of the soil samples. Our proposed DNA extraction method permits a wide range of DNA extraction from bacteria, fungi, and soil-associated organisms. The results demonstrated 3- to 7-fold increased DNA extraction from all six AYLS samples compared to previously described methods. Among the five purification methods assessed, the AT-PVPP spin column purification protocol was found to be the best choice to obtain high

concentrations of high-purity and high-molecular-weight DNA. The purity absorbance ratios of A_{260}/A_{230} and A_{260}/A_{280} were found to be 1.82 ± 0.03 and 1.94 ± 0.05, respectively. Both novel DNA extraction and purification methods were suitable for use in a large-scale study involving the comparative analysis of microbial diversity depending on soil types. However, further research is required to evaluate the efficiency of the purified DNA with restriction enzymes and DNA ligase for successful larger-sized DNA cloning.

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References

Amsaleg, C.L.R., Garnier-Sillam, E., and Harry, M. 2001. Extraction and purification of microbial DNA from soil and sediment samples. *Appl. Soil Ecol.* **18**, 47–60.

Arbeli, Z. and Fuentes, C.L. 2007. Improved purification and PCR amplification of DNA from environmental samples. *FEMS Microbiol. Lett.* **272**, 269–275.

Bakken, L.R. and Lindahl, V. 1995. Recovery of bacterial cells from soil. Nucleic Acids in the Environ, pp. 9–27. Springer-Verlag, Berlin, Germany.

Bürgmann, H., Pesaro, M., Widmer, F., and Zeyer, J. 2001. A strategy for optimizing quality and quantity of DNA extracted from soil. *J. Microbiol. Methods* **45**, 7–20.

Erb, R.W. and Wagner-Dobler, I. 1993. Detection of polychlorinated biphenyl genes in polluted sediments by direct DNA extraction and polymerase chain reaction. *Appl. Environ. Microbiol.* **59**, 4065–4073.

Forsberg, K.J., Reyes, A., Wang, B., Selleck, E.M., Sommer, M.O.A., and Dantas, G. 2012. The shared antibiotic resistome of soil bacteria and human pathogens. *Science* **337**, 1107–1111.

Harry, M., Gambier, B., Bourezgui, Y., and Garnier-Sillam, E. 1999. Evaluation of purification procedures for DNA extracted from organic rich samples: interference with humic substances. *Analysis* **27**, 439–442.

Ikeda, S., Watanabe, K.N., Minamisawa, K., and Ytow, N. 2004. Evaluation of soil DNA from arable land in Japan using a modified direct-extraction method. *Microbes Environ.* **19**, 301–309.

Islam, M.R., Sultana, T., Joe, M.M., Cho, J.C., and Sa, T. 2012. Comparisons of direct extraction methods of microbial DNA from different paddy soils. *Saudi J. Biol. Sci.* **19**, 337–342.

Kathiravan, M.N., Karthick, R., and Muthukumar, K. 2011. *Ex situ* bioremediation of Cr (VI) contaminated soil by *Bacillus* sp.: Batch and continuous studies. *Chem. Eng. J.* **169**, 107–115.

Kauffmann, I.M., Schmitt, J., and Schmid, R.D. 2004. DNA isolation from soil samples for cloning in different hosts. *Appl. Microbiol. Biotechnol.* **64**, 665–670.

Kozdroj, J. and Van Elsas, J.D. 2000. Bacterial community DNA extracted from soils polluted with heavy metals. *Polish J. Environ. Studies* **9**, 403–407.

Krsek, M. and Wellington, E.M.H. 1999. Comparison of different methods for the isolation and purification of total community DNA from soil. *J. Microbiol. Methods* **39**, 1–16.

Lakay, F.M., Botha, A., and Prior, B.A. 2007. Comparative analysis of environmental DNA extraction and purification methods from different humic acid-rich soils. *Appl. Microbiol.* **102**, 265–273.

LaMontagne, M.G., Michel, F.C.Jr., Holden, P.A., and Reddy, C.A. 2002. Evaluation of extraction and purification methods for obtaining PCR-amplifiable DNA from compost for microbial community analysis. *J. Microbiol. Methods* **49**, 255–264.

Liesack, W., Weyland, H., and Stackebrandt, E. 1991. Potential risks of gene amplification by PCR as determined by 16S rDNA analysis of a mixed-culture of strict barophilic bacteria. *Microb. Ecol.* **21**, 191–198.

McGarvey, K.M., Queitsch, K., and Fields, S. 2012. Wide variation in antibiotic resistance proteins identified by functional metagenomic screening of a soil DNA library. *Appl. Environ. Microbiol.* **78**, 1708–1714.

Miller, D.N., Bryant, J.E., Madsen, E.L., and Ghiorse, W.C. 1999. Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. *Appl. Environ. Microbiol.* **65**, 4715–4724.

More, M.I., Herrick, J.B., Silva, M.C., Ghiorse, W.C., and Madsen, E.L. 1994. Quantitative cell lysis of indigenous microorganisms and rapid extraction of microbial DNA from sediment. *Appl. Environ. Microbiol.* **60**, 1572–1580.

Orsini, M. and Romano-Spica, V. 2001. A microwave-based method for nucleic acid isolation from environmental samples. *Lett. Appl. Microbiol.* **33**, 17–20.

Porteous, L.A., Armstrong, J.L., Seidler, R.J., and Watrud, L.S. 1994. An effective method to extract DNA from environmental samples for polymerase chain reaction amplification and DNA finger print analysis. *Curr. Microbiol.* **29**, 301–307.

Romanowski, G., Lorenz, M.G., and Wackernagel, W. 1993. Use of polymerase chain reaction and electroporation of *Escherichia coli* to monitor the persistence of extracellular plasmid DNA introduced into natural soils. *Appl. Environ. Microbiol.* **59**, 3438–3446.

Sagar, K., Singh, S.P., Goutam, K.K., and Konwar, B.K. 2014. Assessment of five soil DNA extraction methods and a rapid laboratory-developed method for quality soil DNA extraction for 16S rDNA-based amplification and library construction. *J. Microbiol. Methods* **97**, 68–73.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1987. Molecular cloning-a laboratory manual. Cold Spring Harbor, USA, Cold Spring Harbor Laboratory Press, London.

Selvin, J., Kennedy, J., Lejon, D.P.H., Kiran, G.S., and Dobson, D.W. 2012. Isolation identification and biochemical characterization of a novel halo-tolerant lipase from the metagenome of the marine sponge *Haliclona simulans*. *Microb. Cell Fact.* **11**, 72.

Smith, G.B. and Tiedje, J.M. 1992. Isolation and characterization of a nitrite reductase gene and its use as a probe for denitrifying bacteria. *Appl. Environ. Microbiol.* **58**, 376–384.

Tsai, Y.L. and Olson, B.H. 1990. Effects of Hg^{2+}, CH_3-Hg^{+}, and temperature on the expression of mercury resistance genes in environmental bacteria. *Appl. Environ. Microbiol.* **56**, 3266–3272.

Wilkinson, D.E., Jeanicke, T., and Cowan, D.A. 2002. Efficient molecular cloning of environmental DNA from geothermal sediments. *Biotechnol. Lett.* **24**, 155–161.

Yeates, C., Gillings, M.R., Davison, A.D., Altavilla, N., and Veal, D.A. 1997. PCR amplification of crude microbial DNA extracted from soil. *Lett. Appl. Microbiol.* **25**, 303–307.

Yeh, Y.F., Chang, S.C., Kuo, H.W., Tong, C.G., Yu, S.M., and Ho, T.H. 2013. A metagenomic approach for the identification and cloning of an endoglucanase from rice straw compost. *Gene* **519**, 360–366.

Young, C.C., Burghoff, R.L., Keim, L.G., Minak-Bernero, V., Lute, J.R., and Hinton, S.M. 1993. Polyvinylpyrrolidone-agarose gel electrophoresis purification of polymerase chain reaction-amplifiable DNA from soils. *Appl. Environ. Microbiol.* **59**, 1972–1974.