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Study of Soil Microbiological Properties

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Abstract: Two strategies have been developed to improve DNA recovery in terms of yield, purity and unbiased representation of the microbial diversity. However, amplification of DNA from soil is often inhibited by co-purified contaminants. Furthermore, DNA is also suitable for PCR amplification using various DNA targets. This review presents an overview of the available methods to achieve this challenging objective. DNA was extracted from 100 g of soil using direct lysis with glass beads and SDS followed by potassium acetate precipitation, polyethylene glycol precipitation, phenol extraction and isopropanol precipitation.

Key words: Purification, soil, DNA, RNA.

1. Introduction

Genetically modified bacteria were the first organisms to be modified in the laboratory, due to their simple genetics [1]. These organisms are now used for several purposes, and are particularly important in producing large amounts of pure human proteins for use in medicine. The two important requirements for metagenomic DNA extraction are efficient cell lysis and purification of DNA from the complex milieu of an environmental sample. The impossibility to culture most microorganisms from environmental samples is a fundamental obstacle to understanding microbial ecology and diversity [2]. The use of DNA-based techniques can overcome this limitation by allowing the fate of particular genes or organisms to be monitored directly in environmental samples. Techniques to extract DNA from soil and sediment initially used large samples of 100 g. These extracts were usually contaminated with humic acids which interfered with subsequent molecular biological manipulations. Extensive purification steps were then required to successfully amplify a PCR product, including CsCl-ethidium bromide density gradient centrifugation [10], or the use of commercial reagents. These steps increase both the complexity and the cost of the technique. This paper describes in detail a method for extracting DNA from soil which involves minimal purification prior to PCR amplification. The method is compared to other commonly used DNA extraction methods. A PCR product was obtained rapidly and inexpensively from large amounts of soil, even when contaminated with heavy metals. A rapid, inexpensive, large-scale DNA extraction method involving minimal purification has been developed that is applicable to various soil types [10].

Upon considering the limitations of previous methods (variable efficiency, time consuming and high cost), the current study focused on developing a rapid inexpensive method for extraction of metagenomic DNA with sufficient quantity and purity to be broadly suitable for metagenomic applications. Since, cell lysis and purification are the key steps in metagenomic DNA extraction; this study includes a particular focus on these two factors. Cell lysis is accomplished by homogenizing with glass powder that is obtained from laboratory waste glassware [9]. Silica, the major component of ground glass powder, has been widely used for DNA extraction from various sources including soils and sediments, tissues and blood of transgenic animals and plasmid from *E. coli.* Autoclaved silica-based sand has been reported for extraction of fungal DNA, and glass powder along with skim milk was used for detection of *Phytophthora infestans* [8].

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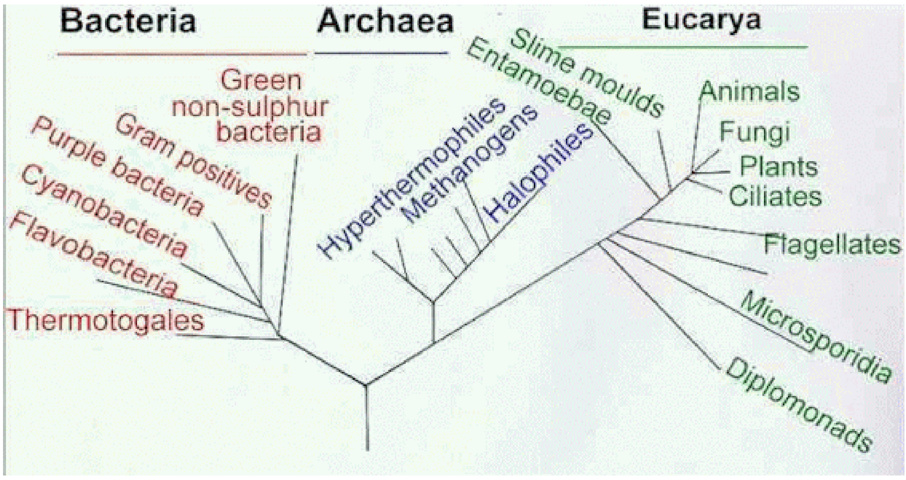

Fig. 1 Classification placement of microorganisms.

2. Materials and Methods

2.1 Soil DNA Purification Protocol

2.1.1 Preparation of the Sample

(1) Spin Columns: Add 550 µL of inhibitor to remove resin to each empty spin column to be used. Centrifuge for 1 min at 2,000×*g* to pack the column. (2) Decant flow-through and place the column in the same collection tube. (3) Add another 550 µL of inhibitor to remove resin to each packed column. Centrifuge for 2 min at 2,000×*g*. (4) Move the column to a clean 1.5-mL collection tube [3]. Pellet Wash Solution 1: For 50 Extractions Kit: Add 45 mL of ethanol to the pellet wash solution before first use. For 5 Extractions Kit: Add 4.5 mL of ethanol to the pellet wash solution before first use.

2.1.2 Cell Lysis

Weigh out 100 mg of the soil sample into a 1.5 mL tube. (2) Add 250 µL of soil DNA extraction buffer and 2 µL of proteinase K; vortex briefly. (3) (Optional) To increase the yield of DNA, shake the tube at 37 °C for 10 min or vortex for 2 min. Add 50 µL of soil lysis buffer and vortex briefly. (5) Incubate at 65 °C for 10 min. (6) Centrifuge for 2 min at 1,000×*g*. (7) Transfer 180 µL of the supernatant to a new tube. (8) Add 60 µL of protein precipitation reagent, mix thoroughly by inverting the tube. (9) Incubate on ice for 8 min. Centrifuge the tube for 8 min at maximum speed. (10) Carefully transfer 100-150 µL of the supernatant directly onto the prepared spin column. (11) Centrifuge for 2 min at 2,000×*g* into the 1.5 mL tube. Discard the column. (12) Add 6 µL of DNA precipitation solution, vortex briefly. Incubate the tube at room temperature for 5 min. (13) Centrifuge for 5 min at maximum speed. Carefully decant the supernatant. (14) Wash the pellet with 500 µL of pellet wash solution. Invert to mix then spin for 3 min at maximum speed. Carefully decant the supernatant. (15) Repeat the wash and spin. (16) Resuspend the pellet in 300 µL of TE Buffer [6].

2.2 Troubleshooting DNA Extractions

DNA is not amplified by PCR. (1) Optimize cycling conditions. Decrease the annealing temperature of the cycling profile by 2 °C or more. Some primer pairs require a lower annealing temperature (less stringent conditions) when amplifying soil DNA. (2) Use less starting material. Some environmental samples contain significantly larger amounts of enzymatic inhibitors. When using these samples, begin the extraction with less starting material (50 mg). (3) Load less extract onto the column.

(4) Rewash the pellet with the pellet wash solution. This step is important in removing residual inhibitors of DNA amplification. Eliminate the vortex mixing step. Eliminate the 2 min vortex mixing step when extracting the DNA. Shake at 37 °C instead or simply skip this step entirely.

Table 1 Physicochemical analysis of soil samples.

pH	3.90
Organic matter %	5
Field capacity 0.33 bar	7.05
CEC (cmol)	1.1
As (mg/kg)	< 3
Hg (mg/kg)	< 0.7
Zn (mg/kg)	5
Cr (mg/kg)	3.3
Cd (mg/kg)	< 0.4
Ni (mg/kg)	1.7
Pb (mg/kg)	15
Cu (mg/kg)	9.5
Mn (mg/kg)	13

Soil (loamy sand) was collected on campus at Semnan University in Iran. The Sokan Semnan National Park Station samples represent the extremes of pristine vs. polluted soils and were compared by further soil testing.

2.3 DNA Extraction from Soil Using Bead Beating

Extraction buffer (100 mL of 100 mM Tris-HCl (pH 8.0), 100 mM sodium EDTA (pH 8.0), 1.5 M NaCl)) was mixed with 100 g (wet weight) of soil. Glass beads (100 g, Bio-Spec Products, Bartesville, U.S.) were added and the sample blended in a Bead-Beater (Bio-Spec Products) for 2 min. Sodium dodecyl sulphate (SDS) was added (10 mL; 20 %) and blending continued for further 5 s. The sample was incubated at 65 °C for 1 h, transferred to centrifuge bottles (250 mL) and centrifuged at 6,000*g* for 10 min. The supernatant was collected, and the soil pellet re-extracted with further extraction buffer (100 mL), incubation at 65 °C for 10 min and centrifugation as described above. Supernatants were transferred to centrifuge tubes (50 mL) containing a half-volume of polyethylene glycol (30%)/sodium chloride (1.6 M), and incubated at room temperature for 2 h. Samples were centrifuged (10,000*g* for 20 min) and the partially purified nucleic acid pellet was resuspended in 20 mL of TE (10 mM Tris-HCl, 1 mM sodium EDTA, pH 8.0). Potassium acetate (7.5 M) was added to a final concentration of 0.5 M. Samples were transferred to ice for 5 min then centrifuged (16,000*g*, 30 min) at 4 °C to precipitate proteins and polysaccharides. The aqueous phase was extracted with phenol/chloroform and chloroform/isoamyl alcohol and DNA was precipitated by adding 0.6 volume isopropanol. After 2 h at room temperature, DNA was pelleted by centrifugation (16,000*g* for 30 min) and resuspended in TE (1 mL).

2.4 DNA Extraction Using Sonication

Extraction buffer (100 mL) was mixed with soil (50 g) on ice. The mixture was sonicated using a High Intensity Ultrasonic Processor (Vibra Cell) with a standard 13 mm horn solid probe for 150 s. The sample was cooled in ice and the sonication repeated. SDS was added (10 mL; 20%) and the sample incubated at 65 °C for 1 h. The sample was transferred to centrifuge bottles (250 mL) and centrifuged at 6,000*g* for 10 min. The supernatant was collected, and the soil pellet re-extracted with further extraction buffer (50 mL), incubated at 65 °C for 10 min and centrifuged were adopted as above. Extraction was then continued as per bead beating method [4].

2.5 DNA Extraction Using Enzymatic Lysis

Extraction buffer (100 mL) containing proteinase K (5 mg) was mixed with soil (50 g) in 250 mL centrifuge tubes. The sample was incubated at 37 °C for 30 min with shaking at 180 rpm. SDS was added (10 mL; 20%) and the sample incubated at 65 °C for 90 min. The supernatant was collected after centrifugation at 6,000*g* for 10 min at room temperature. Extraction was continued as per bead beating method.

2.6 DNA Extraction from Bacterial Cells Isolated from Soil

The bacterial fraction of soil was separated from the inorganic or humic layer by a differential centrifugation technique. Bacterial cells were lysed using lysozyme and the DNA purified using ammonium acetate precipitation and ethanol precipitation. DNA was resuspended in TE.

3. Test for Co-extraction of Contaminants

Co-extracted humic acids are the major contaminant when DNA is extracted from soil. These compounds absorb at 230 nm whereas DNA absorbs at 260 nm and protein at 280 nm. To evaluate the purity of the extracted DNA, absorbance ratios at 260 nm/230 nm (DNA/humic acids) and 260 nm/280 nm (DNA/protein) were determined.

4. Polymerase Chain Reaction (PCR)

DNA (1 mL of 1:50 dilution) was mixed with 9 mL of Genereleaser™ (Bioventures Inc., Murfreesboro, Tennessee, USA) in a 0.5 mL tube and overlaid with 2 drops of sterile mineral oil. Genereleaser™ is a proprietary agent that sequesters inhibitors of PCR. Negative controls containing water only, and Genereleaser™ only, were included in each set of reactions. Reaction tubes were heated on the high setting of a 650 W microwave oven for 7 min (4,550 W/min) in a microwave transparent rack (Bioventures Inc.). An Erlenmeyer flask containing 100 mL of water was included as a microwave sink. Tubes were incubated for at least 10 min at 80 °C in an Omn-E PCR machine (Hybaid). PCR master mix (40 μL) was then added to each tube. Final concentrations of reagents were as follows: 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl (pH 9.0), 0.01% (*w*/*v*) Tween 20, 2 mM $MgCl_2$, 0.5 mM of each primer, 0.2 mM of each deoxyribonucleotide triphosphate, and 1 U Red Hot DNA Polymerase (Advanced Biotechnologies, Surrey, UK). The following thermal cycle was performed: 94 °C 3 min (1 cycle), 94 °C 1 min, 55 °C 1 min, 72 °C 2 min (35 cycles), 72 °C 5 min (1 cycle) [1].

5. Gel Electrophoresis

An aliquot (7 μL) of each amplification reaction was analyzed on 2% *w*/*v* agarose gels cast and run in TBE buffer (pH 8.3). Gels were stained with ethidium bromide and photographed using transmitted U.V. light and Polaroid film. A 100 base pair marker (Pharmacia, LKB) was included on every gel.

6. Results and Discussion

DNA extraction from soil has three requirements: extraction of high molecular weight DNA; extraction of DNA free from inhibitors for subsequent molecular biological manipulations to be performed; and representative lysis of microorganisms within the sample. In this paper, we tested a number of DNA extraction methods for their ability to fulfill these requirements [10].

Table 2 Comparison of DNA extraction methods using a single soil.

Method*	Number of samples	A260230	A260280
Bacterial cells	4	0.83=0.03	1.10=0.003
Chemical lysis	10	1.06=0.03	1.31=0.03
Sonication	4	1.20=0.10	1.41=0.07
Bead beating	6	1.82=0.05	1.69=0.02

* DNA diluted 1:100.

Table 3 Crude DNA ratios for different soil samples extracted using bead beating.

Sample*	Soil type	A260230	A260280
Western	Clay loam	1.22	1.42
University	Clay loam	1.83	1.71
Sokan Ku-Ring Gai	Loamy sand	1.03	1.3
Balmain power station	Loamy sand	1.33	1.53

* DNA diluted 1:100.

DNA extracted using sonication was more degraded than the one obtained with the other tested methods. The size of extracted DNA ranged from less than 500 bp to greater than 20 kb. Methods that shear DNA, such as sonication, generally result in DNA of 100-500 bp. Higher molecular weight DNA is desirable for PCR since the greater the size of the DNA, the less likely is the formation of chimeras during PCR. The bead beating method used here performed better than those previously reported which usually extract DNA of less than 10 kb in size. The DNA extraction methods that did not use sonication all produced DNA of greater than 20 kb.

Organic matter is the major source of inhibitors that may be co-extracted from soil with the microbial DNA. In particular, humic acids pose a considerable problem and will interfere in enzymatic manipulations of DNA [1]. DNA polymerases have been found to be inhibited by as little as 1 μL of undiluted humic-acid-like extract, regardless of the amount of DNA present [7].

The humic materials in soil have similar size and charge characteristics to DNA resulting in their co-purification, evidenced by the extractions being brown in colour. Humic contaminants also interfere in DNA quantitation since they exhibit absorbance at both 230 nm and at 260 nm, the later used to quantitate DNA. This characteristic can be used to determine the level of contamination of humic material by examining absorbance ratios. A high 260/230 ratio (> 2) is indicative of pure DNA, while a low ratio is indicative of humic acid contamination and a high 260/280 ratio (> 1.7) is indicative of pure DNA, while a low ratio is indicative of protein contamination. When the DNA extraction methods were compared (Table 2), the bead beating method consistently extracted DNA with higher 260/230 and 260/280 ratios. This indicated that the DNA was contaminated with fewer humic acid-like compounds. Although the extracts were still brown in colour, dilution of the DNA to 1:50 from all methods was suitable to produce a PCR product. Heavy metal ions, such as those present in the Balmain soil (Table 1), also contribute to inhibitory effects. Here we have demonstrated that a PCR product from soil DNA contaminated with humic acids and heavy metals can be obtained without the use of expensive purification products.

To determine the diversity of microorganisms from which DNA had been extracted, different primer sets were tested, including both multi- and single-copy genes. The multi-copy targets included the prokaryotic small subunit rRNA, prokaryotic rRNA intergenic spacer region, the eukaryotic rRNA internal transcribed spacer (ITS) region, the ITS region for lichen fungi, and the HSP70 family of proteins while the low abundance targets included fungal β-tubulin, and *nif*H genes. With dilution of DNA from each extraction technique, successful PCR amplification was achieved with all primers tested (Fig. 2).

Due to ease of the method, the reduced co-extraction of inhibitors (Tables 2 and 3) and the greater confidence that bead beating would lyse all microbial cells in the soil, this was the method of choice and concentrated on for further analysis. Bead beating has been found to have a lysis efficiency of greater than 90%. The PCR results reported here provide further evidence to support this with products from both bacterial and fungal elements of the soil microbiota being obtained. The bead beating direct lysis method described here extracts between 1.5 and 2.35 mg/mL of DNA from 100 g of soil or 15-23.5 μg DNA/g soil. Extraction methods using small soil samples ranging from 5 g to 100 mg of soil have extracted 9-25 μg DNA/g soil, 12 μg/g, 1-100 μg/g, and 2.5-26.9 μg/g. The method described here is therefore at least as efficient as the above methods. Various methods are available for metagenomic DNA extraction based on chemical or mechanical lysis of microbial cells present in the soil. Among these methods, glass bead beating is considered to be an effective technique for metagenomic DNA extraction.

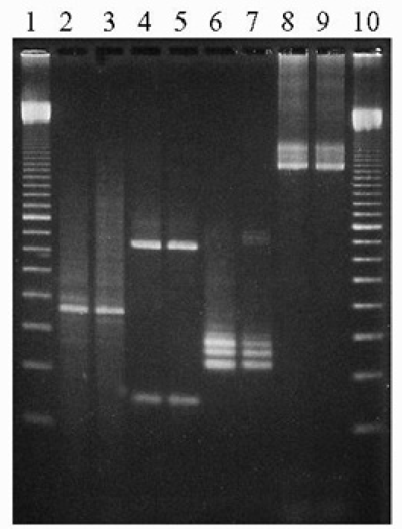

Fig. 2 Example of PCR amplification products using various DNA targets with soil extracted by enzymatic lysis or bead beating. Lane 1: 100 bp marker; lane 2: enzymatic lysis DNA with 16S rRNA primers; lane 3: bead beating DNA with 16S rRNA primers.

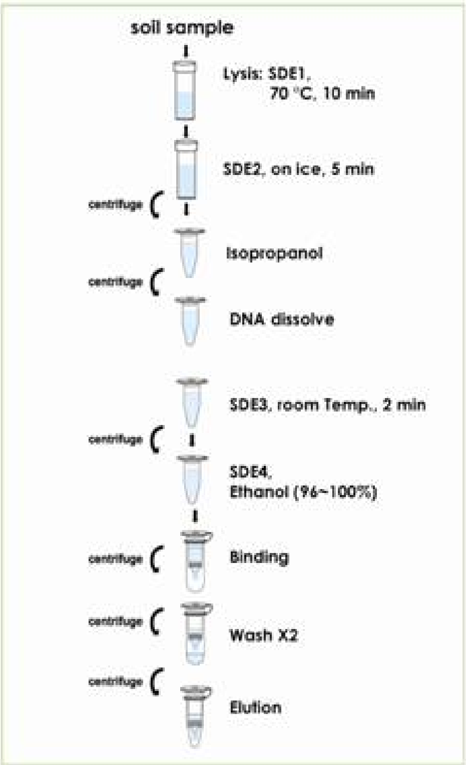

Fig. 3 In this simple and rapid process, the soil samples are homogenized and lysed by the buffer containing glass beads, Proteinase K and detergents. Provided special buffer will remove debris, proteins, and polysaccharides by precipitation and other contaminants are washed away by alcohol containing wash buffer. Finally, the purified DNA is eluted by low-salt elution buffer or water.

This method has also been modified in previous reports to be suitable for different soil types. Commercial kits such as Fast DNA SPIN kit for soil, (MP Biomedicals, Santa Ana, CA) and Ultra Clean Mo Bio Soil DNA isolation kit are also based on the method of bead beating [9]. The study of soil microbiology is summarized in Fig 3.

The focus of DNA extraction methods has moved to rapid performance of molecular techniques, avoiding extensive purification steps. Using the bead beating DNA extraction method described here, crude microbial DNA could be extracted from a variety of soil types and dilution of this DNA was sufficient for successful PCR from both high- and low-copy number genes.

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