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MICROBIAL DNA EXTRACTION FROM SOIL BY DIFFERENT METHODS AND ITS PCR AMPLIFICATION

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ABSTRACT – To understand the occurrence of particular microbes *in situ*, using nucleic acid technology, the direct isolation of DNA from soil comes as an emerging technology in comparison to cell cultivation. The development of techniques in molecular biology has led to their application to microbial ecology. The extraction of DNA from soil, followed by the application of Polymerase Chain Reaction (PCR) to amplify a gene common to all organisms can provide information about microbial community structure, microbial diversity, evolution and taxonomy. Thus different methods were developed and compared for DNA extraction from the soil and evaluated on the basis of PCR-based 16S ribosomal DNA analysis.

Key words : Mannitol-CTAB, Mannitol-PCI, Mannitol PEG-NaCl, Microbial DNA extraction, PCR, 16S ribosomal DNA analysis.

INTRODUCTION

Soil is a complex environment, which is a major reservoir of microbial genetic diversity (Robe et al, 2003). Soil is dominated by the solid phase (Rolf, 2004) and the soil Microorganisms are localized in close association with soil particles, e.g. with complexes of clay–organic matter (Foster, 1988).The diversity of microorganisms in soil is critical to the maintenance of good soil health, because microorganisms are involved in many important functions such as soil formation, toxin removal, and elemental cycles of carbon, nitrogen, phosphorus, and others. Previously, studies on the development of microbial communities required their isolation from soil sample, followed by a series of morphological and biochemical tests to identify them. However, the majorities of microbes in the environment are not readily cultivable (Moyer *et a*l, 1994; Marchesi *et al,* 1998). Furthermore, culture dependent community structure analysis produce spatial and heavily biased results. Later the use of 16S ribosomal nucleic acid (rRNA) techniques has facilitated the molecular identification of a wide variety of yet uncultivated microorganisms (Head and Edwards, 1999) and novel microbial groups in various environments (Torsvik *et al*, 1990). There are two approaches for the extraction of DNA from soil: (i) the bacterial fractionation approach, which involves the separation of bacterial cells from the bulk of the soil prior to cell lysis and recovery of bacterial community DNA, and (ii) the direct lysis approach in which bacterial cells are lysed directly in the presence of the soil matrix. Direct lysis methods are more often used because these are less time-consuming, and give better recovery, resulting in an extracted DNA more representative of the whole microbial community present in that particular sample. However, the direct lysis has major disadvantage as other organic soil components, such as co-extraction of humic and fluvic acids with DNA. Since these contaminants can prevent subsequent molecular analysis, such as restriction digestion, Polymerase Chain Reaction (PCR), and cloning (Olson *et al*, 1991), extensive purification steps were required to successfully amplify a PCR product, including cesium chloride-ethidium bromide (CsCl-EtBr) density gradient centrifugation or the use of commercial reagents (Borneman *et al,* 1996; Silva M C *et al*, 1994). These steps increase both the complexity and the cost of the technique.

Various methods for extracting microbial DNA from soil have been reported. These techniques employ extensive purification steps to ensure that the DNA is suitable for use in PCR (Tsai and Olson 1991; Holben *et al*, 1994; Zohu *et al,* 1996; Miller *et al*, 1999; Roose-Amsaleg, *et al*, 2001). Thus, selection of an appropriate DNA extraction and purification procedure from among the procedures that have been described to date remains a major problem in the application of molecular techniques for studies of soil and sediment microbial communities It is therefore important to develop a suitable soil DNA extraction procedure and its PCR amplification. The present study describes in detail six methods for extracting DNA from soil that involve minimal purification prior to PCR amplification. Furthermore, among them three are mannitol-based extraction methods which yield better quality of DNA for PCR analysis. An additional advantage is that these methods require only 1 gm of soil sample

and result in analysis of soil sample in a short span of time. Thus a PCR product is obtained rapidly and inexpensively from small amount of soil.

MATERIAL AND METHODS

The soil sample was collected from Kukrail Forest, Lucknow, UP, India. Soil samples were immediately placed on dry ice, mixed, and then stored at -20°C prior to DNA extraction. Subsample (approximately 1 g) of soil was set aside for DNA extraction.

DNA Extraction Methods Investigated

Six DNA extraction methods were evaluated in this study using single type of soil. Three methods involved mannitol-based DNA extraction and grinding with liquid nitrogen while others were based on polyethylene glycol-sodium chloride (PEG-NaCl) method without liquid nitrogen, bead beating without liquid nitrogen and sodium phosphate-sodium dodecyl sulfate (Na_2HPO_4-SDS) based method.

DNA extraction using mannitol method with liquid nitrogen

DNA was extracted from 1 g of various soil samples using direct lysis with liquid nitrogen followed by methods involving mannitol with cetyl trimethyl ammonium bromide (CTAB), mannitol with PEG precipitation and mannitol with phenol/chloroform (PCI). In each method 1 g of soil samples were mixed with 1 ml of DNA extraction buffer comprising of 0.2 M Tris-HCl (pH 8.0), 0.02 M Na_2EDTA (pH 8.0), 5 M NaCl, 10% SDS 10% CTAB and 1 M mannitol in centrifuge tubes and incubated at 65°C in water bath for 1 hour with occasional stirring. This was followed by centrifugation at 12,000 rpm for 15 min at 4°C. Further, it was followed by three types of treatments which are described below.

Protocol I- Polyethylene glycol-NaCl (PEG-NaCl) with mannitol

After centrifugation, half volume PEG and 1 volume of NaCl were added to the supernatant and incubated at 4°C for overnight. The pellet was recovered by centrifugation at 12,000 rpm and dissolved in 50 µl TE buffer (Tris-HCl 10 mM, Na_2EDTA 1 mM; pH 8.0). The DNA was then extracted with PCI followed by the addition of 1/10th volume of 3 M sodium acetate (pH 5.2) and 2 volumes ethanol. The pellet was redissolved in 20 µl TE buffer.

Protocol II- Phenol-chloroform-isoamyl alcohol (PCI) with mannitol

In this method, after centrifugation, the supernatant was extracted with an equal volume of PCI followed by centrifugation at 12,000 rpm at 4°C. Aqueous layer of PCI was precipitated with 1/10th volume of 3 M sodium acetate (pH 5.2) and 2 volumes ethanol and the pellet was recovered by centrifugation at 12,000 rpm and dissolved in 20 µl TE buffer.

Protocol III- Cetyl trimethyl ammonium bromide (CTAB) with mannitol

In this protocol, 0.5 M NaCl and 0.5 M CTAB were added to the supernatant after centrifugation and incubated at room temperature for 10 min. This was followed by addition of equal volume of PCI and centrifugation at 12,000 rpm at 4°C. Aqueous layer was precipitated as above and the pellet was dissolved in 20 µl TE buffer.

Protocol IV- DNA extraction using PEG/NaCl method without liquid nitrogen

DNA was extracted from soil samples by directly lysing the cells with liquid N_2. One gram soil sample was mixed with 10 ml of DNA extraction buffer [120 mM Na_2HPO_4 (pH 6.8), 5% SDS (w/v) and 0.02 g polyvinyl polypyrrolidone (PVPP)] in centrifuge tubes and incubated for 1 h at 65°C with occasional stirring. The supernatant were collected after centrifugation at 1,000 rpm for 15 min at room temperature and mixed with half volume PEG and 1 volume of NaCl and incubated at 4°C for overnight. The pellet was recovered by centrifugation at 12,000 rpm and dissolved in 1 ml of TE buffer. The DNA was purified using sodium acetate and ethanol precipitation and the pellet was redissolved in 50 ìl TE buffer.

Protocol V- DNA extraction using bead beating without liquid N_2

Extraction buffer [120 mM K_2HPO_4 (pH 8.0), 5% CTAB in 120 mM K_2HPO_4 (pH 8.0), 0.5 ml PCI) was mixed with 1 g (wet weight) of soil. Glass beads 0.5 g, were added and the sample blended in a bead-beater for 2 min with intermittent cooling on ice. The sample was incubated at 65°C for 1 h, transferred to centrifuge tubes and centrifuged at 8,000 rpm for 15 min. The supernatant was collected and re-extracted. Thereafter, supernatants were transferred to centrifuge tubes containing 1 volume of (24:1) chloroform:isoamyl alcohol, vortexed and centrifuged for 10 min at 12,000 rpm. The supernatant was collected and DNA was precipitated by addition of 0.1 volumes of 3 M sodium acetate and 2 volumes ethanol. The pellet was redissolved in 20 ìl TE buffer.

Protocol VI- DNA extraction using sodium phosphate buffer and SDS method with bead beating and liquid nitrogen

In this method, 10 ml sodium phosphate buffer (120 mM, pH 8.0) and 0.1% Tween 80, pH 7.0 were added in 1 g of soil sample. The mixture was incubated under

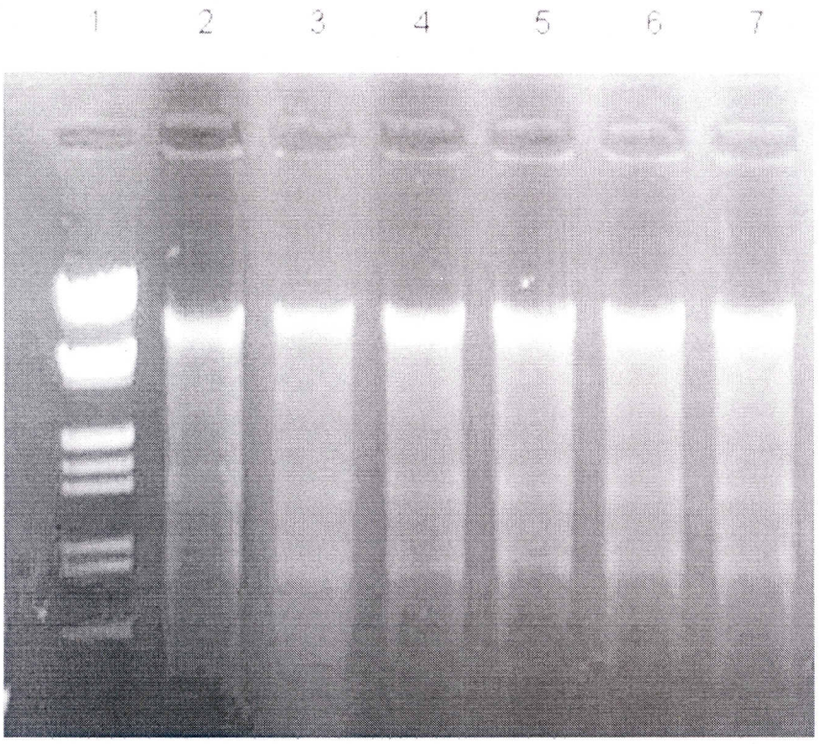

Fig. 1: Soil DNA extracted by different methods showing smear on agarose gel.

[Lane 1- Marker; Lane 2- Mannitol-PEG/NaCl method; Lane 3- Mannitol PCI method; Lane 4- Mannitol–CTAB method; Lane 5-- PEG /NaCl method without liquid nitrogen; Lane 6-Sodium phosphate buffer & SDS method with bead beating & liquid nitrogen; and Lane 7- Bead beating without liquid nitrogen].

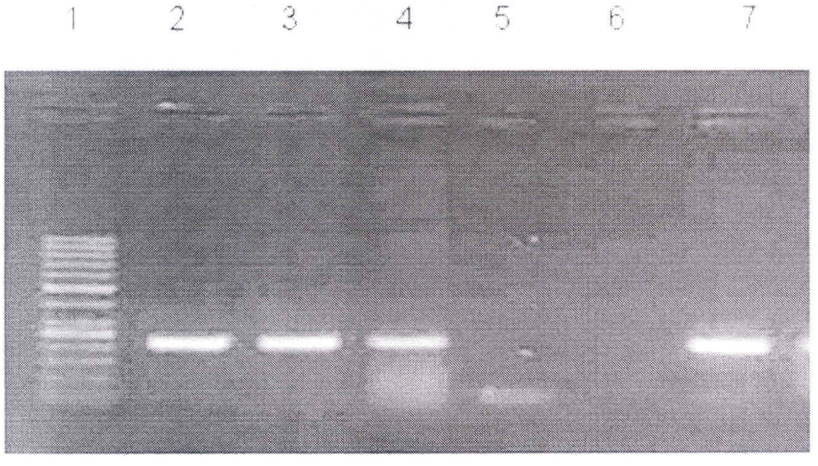

Fig. 2 : PCR amplification products using 16S rRNA specific primers showing bands on agarose gel.

[Lane1- Marker; Lane 2- Mannitol-PEG/NaCl method; Lane 3- Mannitol-PCI method; Lane 4- Mannitol–CTAB method; Lane 5- PEG/NaCl method without liquid nitrogen; Lane 6- Bead beating without liquid nitrogen; Lane 7- Sodium phosphate buffer & SDS method with bead beating & liquid nitrogen].

constant agitation overnight at room temperature. The supernatant from this suspension was centrifuged at 5,000 rpm for 10 min. The pellet was washed four times with TE buffer and the cells were lysed mechanically by maceration of the liquid nitrogen-frozen pellet. The macerate was transferred to a centrifuge tube to which 2 ml TE buffer and an equivalent volume of phenol-chloroform-isoamyl alcohol (25:24:1) were added, and the mixture was centrifuged at 5,000 rpm for 10 min. DNA from the collected supernatant was precipitated by adding 0.7 volume ice-cold isopropanol and 1/10th volume of 3 M sodium acetate and incubating this mixture for 2 h at -20°C. The pellet was redissolved in 20 ìl TE buffer.

Determination of Purity and Yield of DNA

The concentration of the DNA in the sample was measured by monitoring the absorbance of a dilute solution of the sample at 260 nm, and calculation was based on the value of 1.0 A^{260} unit = 50 ìg/ml of DNA, taking into account the dilution factor of the sample (Sambrook *et al,* 1989). Purity of the DNA was determined by taking absorbance at 230, 260 and 280 nm. A_{260} /$_{A280}$ and A_{260} /A_{230} ratios were calculated to evaluate levels of protein and humic acid impurities respectively in both types of extraction methods.

PCR amplification of Soil Extracted DNA:

DNA samples were subjected to PCR amplification using 16S rRNA-specific primers. Each 25 ml PCR mixture contained 1 ml template DNA, 2.5 ml 1X PCR buffer, 1 ml of each deoxyribonucleoside triphosphate (100 mM dNTP), 1 ml of forward

Table 1 : Prokaryotic 16S rRNA forward and reverse primers used for PCR amplification.

Primer	Sequence
Forward	5' AGAGTTTGATCCTGGCTCAG 3'
Reverse	5' GGTTACCTTGTTACGACTT 3'

Table 2 : Amount of DNA extracted by various isolation protocols

DNA Extraction Protocol	Amount (µg/ml)
Protocol I	2.25
Protocol II	2.50
Protocol III	2.75
Protocol IV	0.75
Protocol V	0.80
Protocol VI	1.25

Table 3 : Comparison of DNA extraction methods for humic acid contamination.

DNA Extraction Protocol	A 260/230	A 260/280
Protocol I	1.89	1.80
Protocol II	1.94	1.85
Protocol III	2.05	1.85
Protocol IV	1.10	1.25
Protocol V	1.20	1.42
Protocol VI	1.24	1.69

and reverse primers (Table 1) and 0.5 ml Taq DNA polymerase. The amplification cycle consisted of an initial denaturation step of 5 min at 94°C, followed by 35 PCR cycles each involving 1 min at 94°C (denaturation), 1 min at 59°C (annealing) and 2 min at 72°C (extension), and a final extension step for 10 min at 72°C.

Gel Electrophoresis

For visualizing extracted DNA and PCR products, 5 ml of the suspension was electrophoresed on 0.8% agarose gel in 1X TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA). Gels were stained with EtBr and photographed under transmitted UV light using BIORAD Gel DocTM XR. A 100 bp DNA size marker was included on the gel for size analysis.

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; representative lysis of microorganisms. It is important to select an extraction and purification method, which yields DNA of suitable quantity and purity. The extraction methods are strongly influenced by several parameters, such as incomplete cell lysis, DNA adsorption to soil surfaces, extraction of humic acid contaminants, and DNA degradation. In this present study, six methods of DNA extraction were tested for their ability to fulfill these requirements. Various DNA isolation protocols are compared on the basis of DNA yield (Figure 1 and Table 2). A good amount of DNA was obtained in all tested protocols. DNA yield was increased by using liquid nitrogen and bead beating. It was further improved by adding mannitol in the extraction buffer. Trevors *et al*, (1992) found that the SDS-based cell lysis protocol gave the highest DNA yields in comparison with freezing-thawing lysis protocols. The mechanical treatment was more effective and less selective than chemical lysis and attributed to increased cell lysis. According to Frostegard *et al*, (1999) grinding homogenizes the soil and increases the release of bacteria from inner compartment, making them available for subsequent lysis. The inclusion of mannitol in extraction buffer which was not earlier reported has been proved to increase the efficiency of extraction. On the basis of this finding it has been proposed that mannitol may also play an important role in chemical lysis of the cells along with other reagents like CTAB, SDS, EDTA and Tris-HCl which leads to high yield of DNA. Mannitol appears to be essential for the protection of soil DNA against cell damage under the stress conditions. These conditions may be the effect of inclusion of above reagents such as CTAB, SDS, EDTA and Tris-HCl) in the extraction buffer. It may be possible that effect of these reagents may cause some extent of degradation or shearing of DNA due to which the yield of DNA decreases. Moreover, mannitol preserves the soil DNA. Among the three mannitol-based protocols analyzed, CTAB method gave very good results as compared to PCI and PEG/NaCl methods. When mannitol was used in soil DNA extraction, yield of DNA was increased, hence it is proposed that mannitol plays the role of a preservative in soil DNA extraction. The size of extracted DNA ranged from less than 20 kb to greater than 500 bp in size. 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.

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 interfere in enzymatic manipulations of DNA. The humic materials in soil have similar size and charge characteristics to DNA resulting in their co-purification, evident by the extractions being brown in colour (Holben, 1994). Humic contaminants also interfere in DNA quantitation since this exhibit absorbance at both 230 nm and at 260 nm, the later used to quantitate DNA (Liesack, 1991; Olson, 1992). 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. Similarly a high 260/280 ratio (>1.8) 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 and PEG/NaCl method with liquid nitrogen consistently extracted DNA with higher 260/230 and 260/280 ratios. This indicated that the DNA was contaminated with fewer humic acid-like compounds. Furthermore, mannitol based methods removed humic acid material very effectively in contrast to other methods of extraction.

Amplification of DNA by PCR was used to determine its quality. The size of amplicons was estimated as 225 bp. Among the applied methods, DNA extracted from all three mannitol-based and Na_2PO_4-SDS with liquid nitrogen methods provides efficient PCR amplification. However, other methods were not suitable for PCR amplification even though giving an acceptable level of DNA. PCR involves successive enzymatic reactions and since DNA polymerase have found to be inhibited by as little amount of humic acid like extracts (Tsai and Olson, 1992) requires contamination-free sites, the described methods allow the use of large-scale preparations providing greater probability of detecting genes present in low abundance in the soil environment. Because this method is applicable to even the more challenging heavily contaminated soils, molecular microbial biodiversity assessment can now be more readily applied. Moreover, greater DNA recovery reflected a more representative (diverse) sample of DNA from the microbial community. New tools to rapidly compare the DNA diversities of extracts are needed to better estimate the effectiveness of DNA extraction protocols. Furthermore, development of procedures for extracting DNA directly from soil and determining DNA sequences will eventually lead to test-kits that could be used to identify and quantify such microbes *in situ*.

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