

A Rapid and Economical Method for Separation of Humic Substances and DNA for PCR and Cloning from Soil Samples

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A simple, cost effective and rapid method is described for the separation of total community DNA and humic substances from the soil samples for metagenomic studies. This method was based on hot detergent lyses method for isolation of less sheared high molecular DNA from any soil sample followed by gel exclusion chromatography for complete removal of coextracted humic substances. Purity of the DNA samples isolated by this method was confirmed by restriction digestion and by the amplification of 16S rRNA gene. Four highly contaminated soil samples were evaluated by this method and recovery was found to be 12-20 $\mu\text{g/g}$ of soil with average size > 25kb in 6-7 hrs. This method can be used as a substitute for expensive kits used in rapid separation of DNA and humic substances from the soil samples.

Key words: Humic Acid, Metagenomic DNA, 16SrRNA, Restriction and Digestions.

Direct isolation of DNA from environmental samples has revolutionized the field of microbial ecology. Till date only 2% of the existing microbes has been cultivated using the best of culturing techniques. Rest of 98% of the micro flora remained uncultivated (Cowan, 2000 and Amann *et al.*, 1995). Many workers have attempted to increase the yield of DNA from soil samples by using several physical treatments like, bead beating and sonication to lyse indigenous

microbial cells (Jizhong *et al.*, 1996, Liesack *et al.*, 1992 and Orgam *et al.*, 1987). Such treatments can shear high molecular weight DNA of size up to 5-10 kb or less. Such sheared DNA may not be suitable for the construction of cosmid and bacmid libraries, where the average insert recommended size is 30kb and 1Mb respectively (Rondon *et al.*, 2000). Microbial cells from soil samples pose particular challenges in obtaining high yield of unshared high molecular weight DNA as they may remain tightly bound to soil colloids, high in clay nor organic matters (Tasi *et al.*, 1992 and Jizhong *et al.*).

Higher yields of DNA from soil and sediments are usually obtained by direct lyses methods because of the ability to extract non-bacterial and extra-cellular DNA (Asa *et al.*, 1999). Direct isolation of the soil DNA also

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contains substantial amount of other soil components like humic substances, which share same physico-chemical properties with nucleic acids and interfere with transforming and detection processes (Smalla *et al.*, 1993 and Tsai *et al.*, 1992). It has been reported that coextracted humic like substances inhibits restriction endonucleases, Taq polymerase and other DNA modifying enzymes. They contribute to the decreases in the efficiencies of DNA-DNA hybridizations reactions (Steffan *et al.*, 1998 and Tebe *et al.*, 1998). To access microbial metagenome from environmental samples, an efficient method for rapid extraction and purification of soil DNA is became primary requirement of microbial ecologist.

The objective of present work was to develop and test a nonselective method for isolation of DNA from environmental samples, like soil, sediments. Purified DNA should free from any contaminant (humic substances), ready for PCR based analysis and can be cloned in a range of vectors for different purposes. Here a modified method is described that is based on the conventional hot detergent lyses. The coextracted inhibitors were removed by gel exclusion chromatography. Purity of extracted DNA samples were confirmed by amplification of 16 sRNA genes and also by restriction digestion analysis.

MATERIAL AND METHODS

Sampling

Four different soil samples were used to evaluate the efficiency of DNA extraction and purification protocol. All soil samples were different in their habitat; sulfur springs of Yumthang valley of Sikkim, India (YS) contain high content of organic contents like sulfur. In Kargil India (KI) climatic conditions are extremely cold and Yamuna River, India (YR) is one of the highly polluted rivers in the country always exposed to toxic industrial wastes. A Garbage dumping site, Delhi, India (GD) contains a variety of microbes involved in biodegrading pathways. All soil samples were collected from the mentioned places between 5-10cm depths and stored at 4°C until tested in laboratory. Prior to storage root particles were removed with help of fine forceps to avoid contamination of plant DNA.

Bacterial strains

Rhizobium meliloti (Rcd 301) was used as a control bacterial strain. Rcd 301 was grown in nutrient broth (Hi media) at 30°C for 18 hrs.

Soil DNA isolation

Soil samples were taken and ground with help of mortar pastel in ice, to homogenize the soil. To one gm of each soil taken and mixed with 4 ml of TEN buffer (tris 100mM, EDTA 10mM and NaCl 100mM) and 2ml of 10% SDS. The tubes were kept the at 65°C for 30 minutes. 1 ml of 10% CTAB and 2ml of 5M NaCl were added to the tubes. Contents of the tube were mixed by gentle inversion and kept at for 65°C 30 min. Buffer equilibrated phenol (qualigen) was added to the tubes, mixed by gentle inversion and kept at 65°C for 1 h, with a loose cap (to avoid bumping). Tubes were spun at 13000 rpm for 15 min, upper aqueous layer was collected in a fresh tube by using a large bore tip and further extracted with phenol:chloroform:isoamyl alcohol as mentioned in Sambrook *et al.*, 1989. To remove traces of phenol, aqueous was layer re-extracted with equal volume of chloroform. Finally aqueous solution of DNA was precipitated with 0.7 volume of isopropanol at-20°C for 2h. Tubes were spun at 13,000 rpm for 30 min. and pellet was washed with 70% alcohol. Brown colored pellet of crude soil DNA was dried at room temperature and dissolved in 300µl of TE buffer (tris 10mM, pH 8.0 and EDTA 1mM). Same method was used for the isolation of control bacterial DNA from culture pellet of *Rhizobium meliloti*.

Removal of humic substances

Sephadex G-200 (gel beads) were swollen in TE buffer (pH 7.0) for overnight at 4°C, upper layer containing fine particles were removed with help of a glass pipettes. Granular solution of polyvinylpyrrolidone (PVPP) was prepared in TE buffer (pH 7.0) and drop wise added to the swollen gel beads to a final concentration of 30 mg/ml. Slurry was mixed well and loaded on a 5ml disposable syringe containing 0.22 µm filter on the mouth (Fig. 1). Columns were packed by spinning at 800 rpm for 10 min in a swinging bucket rotor at 4°C. When the content of column was 2/3 of the original volume of slurry, 300µl of brown colored crude DNA was loaded slowly to the top of the column. The column was placed carefully in the collector

and spun at 500 rpm for 5 min in a swinging bucket rotor at 4°C. After centrifugation light color supernatant was collected and again loaded top of the column for centrifugation. This procedure was repeated three times to get the colorless solution of DNA. Aqueous colorless purified solution of soil DNA was transferred to a micro centrifuge tube and extracted by phenol: chloroform:isoamyl alcohol and chloroform as mentioned above. Aqueous solution of DNA was again precipitated with 0.1 volume of sodium acetate (3M pH 5.2) and 3 volume of absolute ethanol as mentioned in Sambrook *et al.*, 1989. Finally pellet of DNA was dissolved in 100ul of autoclaved MQ water and checked on 0.7% agarose gel for determination of the DNA concentration.

PCR amplification of 16S rRNA gene

Amplification of full length 16S rRNA gene was performed as described by Weisburg *et al.* 1991 using universal primers fd1 (5' AGTTTGATCCTGGCTCA 3') and rP2 (5' ACGGCTACCTTGTTACGACTT 3'). In a 100µl reaction volume, 20 pmol of each primer was used for 20ng soil DNA template. Amplification was performed on an automated thermocycler (MJ Research, USA) using 3U Taq polymerase (NEB), and the recommended buffer system. Amplification profile is as follows: 95°C for 5min followed by 30 cycles of 95°C for 1min, 55°C for 1min, 72°C for 1 min and a final extension of 7 minute (Fig. 3-4).

Restriction analysis of soil and bacterial DNA samples

Restriction digestions of the purified soil DNA and control bacterial genomic DNA was carried out using enzymes EcoRI and BamHI (NEB) with respective buffers. In each reaction 5µg DNA was digested with 10U of each enzyme in the final reaction volume of 30µl and incubated at 37°C for 10h. The movement of restricted DNA on 0.7% agarose gel, confirmed digestion of DNA by respective enzyme as shown in Fig. 5.

RESULT AND DISCUSSION

Crude DNA extracted from different soil samples using different methods results in a brown colored pellet indicating co-extraction of other components like humic substances. Soil

DNA extracted using modified C-TAB method is compared with conventional cell lyses methods (Fig. 2). The present procedure yields a better DNA in terms of quantity. The amount of DNA extracted using the present protocol in soil samples 1 and 3 were comparable to that of bacterial control. Samples 1 and 3 were collected

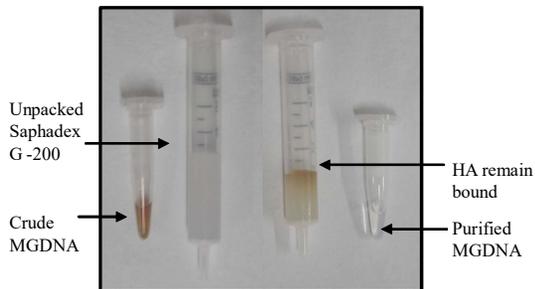


Fig.1. Syringe column with sephadex gel beads and polyvinylpyrrolidone

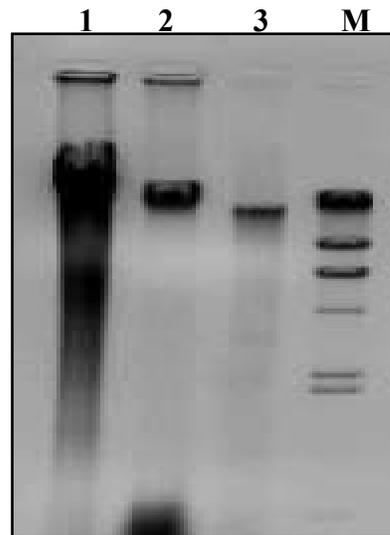


Fig. 2. Gel electrophoresis (0.7% agarose) of soil DNA isolated using different lyses methods, where Lane1. Genomic DNA isolated from bacterial culture pellet (*Rhizobium meliloti*) using standardized cell lyses method, Lane 2. Soil crude DNA isolated from soil sample KI, Lane 3. Soil crude DNA isolated from sample KI using conventional cell lyses methods, Lane 4. RE Hind III digested lambda phage DNA (size marker).

from a hot spring and a polluted river respectively. Soils from these two regions are thought to have a high microbial load and, a higher amount of DNA. DNA from soil sample 2 has been isolated from an uninhabitable site containing no vegetation. Soil pH was also alkaline, thus explaining a lower microbial load (Fig. 3).

DNA obtained using this procedure was used for further purification using different methods and tested for its quality using restriction enzyme digestion and PCR amplification of 16S rRNA gene. The PCR product obtained from the DNA isolated by this method developed in the present study is better in comparison to other conventional methods. Since universal 16S rRNA gene primers were used for this study, the result indicates that modified C-TAB method developed for DNA isolation yields better DNA better quantity wise and quality wise (Fig. 4).

To further check for the quality of DNA obtained through this method developed in this study, RE digestion of DNA obtained from

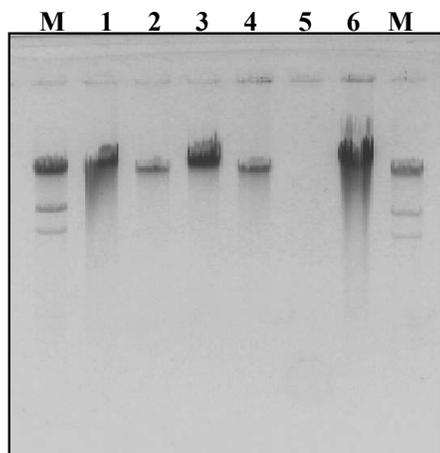


Fig.3. Gel electrophoresis (0.7% agarose) of total DNA extracted from soil, where RE Hind III digested lambda phage DNA (size marker) (Lane M) and crude soil DNA extracts from different soil samples, Lane 1. Yumthang valley of Sikkim, India (YS), Lane 2. Kargil India (KI), Lane 3. Yamuna River, India (YR), Lane 4. Garbage dumping site, Delhi, India (GD) Lane 5 Negative control. Lane 6. Genomic DNA isolated from same amount of bacterial culture pellet (*Rhizobium meliloti*).

different soil samples were carried out using Eco RI and Bam HI. A smear in both the cases indicates and confirms that DNA obtained through modified C-TAB method yields better quantity of DNA as compared to conventional methods (Fig. 5). In this method the column used for purification of soil DNA is also very effective in removing humic acid and other co-extracted products. To further confirm the effectiveness of the combination of these two procedures, DNA was extracted and purified from different soil samples using the techniques discussed above. PCR amplification of the 16S rRNA gene in these soil samples were carried out that yielded a good amount of amplicon (Fig. 6).

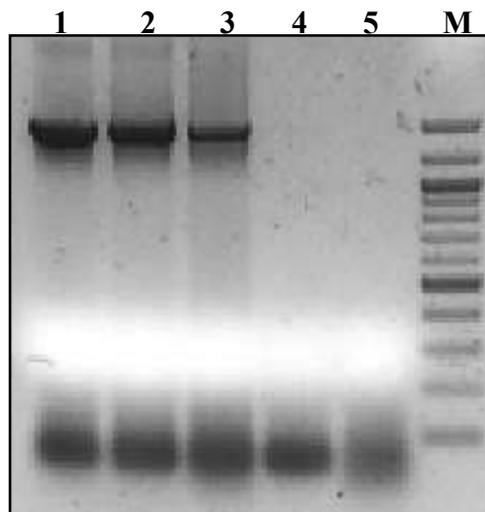


Fig. 4. Gel electrophoresis (1.5% agarose) where, PCR amplification of full-length 16S rRNA gene with template DNA purified by different methods of humic acid removal. Lane 1. Product of PCR amplified 16S rRNA gene using bacterial genomic DNA (*Rhizobium meliloti*) as a template, Lane 2. Product of PCR amplified 16S rRNA gene using T1 (column purified + PEG purified soil DNA from sample KI), Lane 3. Product of PCR amplified 16S rRNA gene using T2 (column purified soil DNA from sample KI), lane 4. Product of PCR amplified 16S rRNA gene using T3 (crude soil DNA of sample KI dialyzed against TE buffer for overnight) Lane 5. Product of PCR amplified 16S rRNA gene using T4 (crude soil DNA isolated from sample KI), Lane M. 100 base pair standard DNA marker from NEB

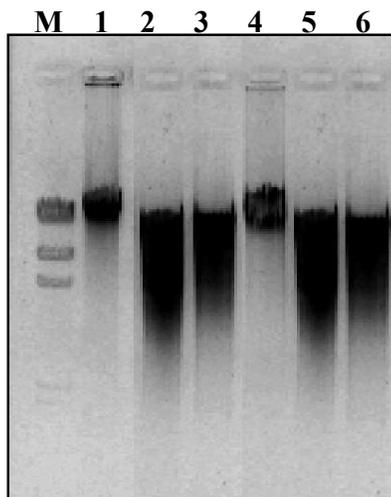


Fig. 5. Gel electrophoresis (0.7% agarose) shown, Restriction digestion analysis of soil sample YR and bacterial genomic DNA (*Rhizobium meliloti*). Lane 1. Purified soil DNA isolated from sample YR, lane 2-3. YR DNA digested with restriction endonucleases EcoRI and BamHI respectively, Lane 4. genomic DNA isolated from bacterial culture of *Rhizobium meliloti*, Lane 5-6. Genomic DNA of *Rhizobium meliloti* digested with restriction endonucleases EcoRI and BamHI respectively.

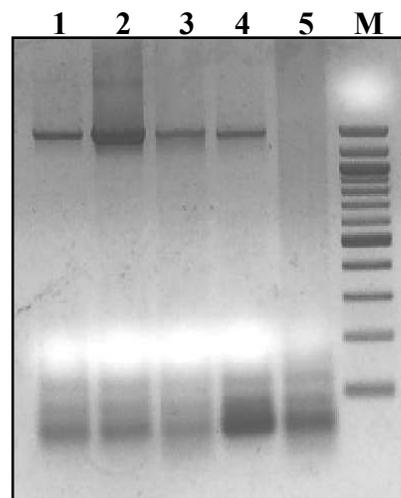


Fig. 6. Gel electrophoresis (1.5% agarose) shown, PCR amplification of 16s rRNA gene from different purified soil DNA. Lane 1-4. Product of PCR amplified 16s rRNA gene using humic acid free purified soil DNA preparations as a source of template (YS, KI, YR and GD respectively), Lane 5. Negative control doesn't contain any template DNA, Lane M. 100 base pair standard DNA marker from NEB

CONCLUSION

In summary, the present method for the DNA extraction from the soil samples is rapid, simple and inexpensive compared to commercially available kits. Humic acid free DNA could be extracted from the four highly contaminated soil samples. Because of gentle nature of the procedure the amount of total DNA recovered from soil sample was 12-20ug/gm of soil with average size > 25kb in 6-7 hrs. Thus this modified method is not only a convenient and cost-effective (7-8 times cheaper than kits) procedure for the isolation of pure DNA from different soil samples, but also has the potential for use in the construction of metagenomic libraries.

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