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# 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

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ABSTRACT

Extraction of DNA from soil samples using standard methods often results in low yield and poor quality making them unsuitable for community analysis through polymerase chain reaction (PCR) due to the formation of chimeric products with smaller template DNAs and the presence of humic substances. The present study focused on the assessment of five different methods for metagenomic DNA isolation from soil samples on the basis of processing time, purity, DNA yield, suitability for PCR, restriction digestion and mDNA library construction. A simple and rapid alkali lysis based on indirect DNA extraction from soil was developed which could remove 90% of humic substances without shearing the DNA and permits the rapid and efficient isolation of high quality DNA without the requirement of hexadecyltrimethylammonium bromide and phenol cleanup. The size of DNA fragment in the crude extracts was >23 kb and yield 0.5–5 μg/g of soil. mDNA purification using Sephadex G-50 resin yielded high concentration of DNA from soil samples, which has been successfully used for 16S rDNA based amplification of a 1500 bp DNA fragment with 27F and 1492R universal primers followed by restriction digestion and mDNA library construction.



## 1. Introduction

DNA extraction from environmental samples has become an essential tool for constructing metagenomic DNA (mDNA) libraries to reveal the genotypic diversity which requires high quality DNA (Amann et al., 1995; Borneman and Triplett, 1997; Hugenholtz et al., 1998; Stackebrandt et al., 1993; Tiedje et al., 1997; Zhou et al., 1997). It is widely accepted that more than 99% of the microorganisms present in natural environments are not readily cultivable and therefore not accessible for biotechnology or basic research (Torsvik et al., 1990). Although laboratory enrichment culture bears only a limited biodiversity, to overcome the limitation of cultivation methods, several DNA based molecular approaches have been developed to explore the diversity and potential of the microbial communities. However, many workers have attempted to increase DNA quality and yield from soil samples by using severe chemical and physical treatments such as bead beating and sonication to lyse microbial cells. All such treatments caused shearing of DNA making it unsuitable for community analysis based on Taq DNA PCR analysis owing to the risk of forming chimeric products with smaller template DNAs (Liesack et al., 1991; Holben, 1994; Tsai and Olson, 1992; Smalla et al., 1993). Isolation of good quality DNA from contaminated environments is often complicated, as polyphenols and polysaccharides present abundantly in such samples which become difficult to eliminate using standard DNA extraction protocols (Porteous and Armstrong, 1991). These compounds co-precipitate with DNA and interfere with subsequent analytical reactions such as enzymatic modification of DNA, PCR analysis and reduction of the transformation efficiency as well as DNA hybridization specificity (Steffen and Atlas, 1988; Tebbe and Vahjen, 1993; Yeates et al., 1998). The removal of humic substances is a critical step following DNA extraction (Rajendhran and Gunasekaran, 2008; Jackson et al., 1997; Rajendhran et al., 2011). However, there are reports of proteinase K and sodium dodecyl sulfate (SDS) reducing the humic acid contamination (Singh et al., 2013). Aromatic compounds such as humic substances and polyphenols from soil samples can be eliminated using cation-exchange resins and detergents (Jacobsen and Rasmussen, 1992), polyvinylpyrrolidone (PVP) (Koonjul et al., 1999), hydroxyapatite (Roh et al., 2005) and activated charcoal (Desai and Madamwar, 2007) but the purification compromises with the yield of quality DNA.

For a successful mDNA library construction, humic substance-free cloneable DNA from environmental samples is a prerequisite (Rajendhran and Gunasekaran, 2008). The present study focused on isolation of pure and an optimized DNA yield isolated from soil samples using different methods. Based on comparative study of five different DNA extraction methods, a modified rapid method providing higher yield and quality of mDNA is presented for the extraction, purification and 16S rDNA based polymerase chain reaction and mDNA library construction.

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## 2. Materials and methods

### 2.1. Soil sample collection

Soil samples were collected from a bakery industry in Tezpur town, Assam, India (26°42′3″N 92°49′49″E). Soil and sediments were homogenized by manual mixing, frozen in liquid nitrogen, transported on dry ice and stored at −20 °C.

### 2.2. Physical and chemical characterization of soil samples

Soil samples were air dried, weighed and physical and chemical characterizations were carried out. Soil samples used for particle size analysis were pre-treated with hydrogen peroxide to remove organic materials and then dispersed using sodium hexametaphosphate and sodium carbonate. Wet sieving was carried out to separate the soil particles of >0.060 mm in diameter. The pH of soil was determined in 1:1 (wt/wt) soil–water slurry. The total organic carbon was determined after removing inorganic carbon in 10% HCl followed by boiling and washing with distilled water.

### 2.3. mDNA extraction

mDNA from the soil samples was extracted using five different methods viz. M1, M2, M3, M4 and M5. Methods M1, M2 and M3 were performed as outlined in Table 1. In M4, a commercial miniprep kit, was performed as per manufacturer's instruction (Mobio Ultraclean soil DNA isolation kit). Method M5 is a modification of the protocol described by Porteous and Armstrong (1991) as outlined in Table 2.

### 2.4. DNA isolation from gram positive and gram negative bacteria using the M5 method

Method M5 was used to isolate genomic DNA from *Bacillus subtilis* and *Escherichia coli* as representative gram positive and gram negative DNA to validate the utility of the extraction method for cultivable bacteria.

### 2.5. Methods of purification of crude DNA extract

The mDNA extracted from soil samples using M3 and M5 was purified following five different methods: i) MP1: Sephadex column purification (Sephadex G-50 slurry was swollen overnight and packed in to spin columns to settle down. Each of the DNA sample (100 μl) to be purified was loaded into the column and kept at room temperature for 5 min and centrifuged at 3000 rpm for 5 min); ii) MP2: silica membrane based spin column purification (commercial kit) (The DNA sample was purified using silica membrane based commercial spin column. Each DNA sample (50 μl) was loaded into the column (Ultraclean soil DNA isolation kit, Mobio, USA). As per manufacturer's instructions the column was kept at room temperature for 5 min and the purified DNA sample was eluted in 100 μl of TE Buffer); iii) MP3: electroelution (Each DNA sample (50 μl) was loaded and resolved in 0.8% agarose. High molecular weight band of mDNA was cut and transferred into a dialysis bag containing 3 volumes of electrophoresis buffer. The DNA was eluted in to the dialysis bag by electrophoresis for 1.5 h. Then the DNA sample was precipitated with isopropanol and washed with 70% ethanol followed by air drying. The sample was suspended in 100 μl TE buffer); iv) MP4 and MP5: agarose gel electrophoresis (electroelution) (MP4) and agarose gel with PVP electrophoresis (electroelution) (MP5) (Humic acid co-migrates with nucleic acid under standard electrophoretic conditions. Addition of PVP to agarose gel halts the co-migration of humic compounds by retarding its electrophoretic mobility. Each DNA sample was loaded on 0.8 % agarose gel containing 2% of PVP).

**Table 1**
Methods used for the isolation of mDNA from soil samples.

| Method | Extraction buffer | Cell lysis | Humic acid removal chemical |
|---|---|---|---|
| M1 | EDTA, CTAB, Tris–HCl, NaCl, $NaPO_4$ [Zhou et al., 1996] | SDS | CTAB |
| M2 | NaCl, Tris–HCl, EDTA [Gray and Herwig, 1996] | SDS, vortex | PVPP |
| M3 | EDTA, NaCl, Tris–HCl [Yeates et al., 1998] | Bead beating, SDS | PEG |
| M4 | As per MO-Bio kit [Ultraclean soil DNA kit, MO-Bio, USA] | As per MO-Bio kit | As per MO-Bio kit |
| M5 | EDTA, SDS, NaCl [present study] | Vortex, heating | PEG |

**Table 2**
New M5 method for quality soil DNA extraction modified from the protocol described by Porteous and Armstrong (1991).

| Step | Procedure |
|---|---|
| 1 | Weigh 750 mg of soil sample in 2 ml microfuge tube. |
| 2 | Add 1 ml of PBS buffer (pH 8.0) to the soil sample. |
| 3 | Vortex for 5 min and centrifuge at 3000 × *g* for 10 min. |
| 4 | Supernatant was transferred to 2 ml microfuge tube and 70 μl of lysis buffer (1.5 m NaCl, 0.1 M $Na_2EDTA$, 4%SDS) was added followed by incubation at 72 °C for 45 min. |
| 5 | Microfuge the sample at 13,000 × *g* for 5 min at 4 °C and the supernatant was transferred to a fresh 2 ml centrifuge tube. |
| 6 | An aliquot of 100 μl of 6 M potassium acetate and 400 μl of 50% PEG were added to the supernatant and the mixture was allowed to precipitate for 20 min at −20 °C and centrifuged at 4 °C for 5 min. |
| 7 | The supernatant was removed and the pellet was air dried. |
| 8 | The pellet was dissolved in 500 μl TE buffer (pH:8.0) and then 500 μl of chloroform was added followed by centrifugation at 13,000 × *g* at 4 °C for 5 min. |
| 9 | The chloroform extraction was repeated twice and 500 μl of isopropanol was added to the supernatant and then allowed to precipitate the aqueous DNA for 5 min at 4 °C and again centrifuged at 13,000 × *g* for 5 min. |
| 10 | The DNA pellet was suspended in 100 μl of 1× TE (10 mM Tris–HCl, 1 mM EDTA). Each experiment was performed thrice. |

### 2.6. Quantification of mDNA and humic acid

DNA quantification ($A_{260}/A_{280}$) is commonly performed to determine the average DNA concentration and its purity in a solution. Quant iT Picogreen dsDNA kit (Molecular Probes, USA) was used for the quantification of mDNA as per manufacturer's standard protocol. Fluorescence was measured using Spectra Max fluorescence microplate reader (Molecular devices, USA) at an excitation of 480 nm and emission of 520 nm. Serially diluted λ Phage DNA (1.0–100 ng/ml) was used to prepare the standard curve. The quantification of humic acid was done by absorbance of DNA sample at 340 nm using a spectrophotometer (Thermo Scientific, UV-10, Japan). The concentration of humic acid was calculated based on the standard curve prepared with serial dilution (0.1–100 μg/ml) of commercial humic acid (Merck, India). Humic compounds absorb illumination at 230 nm, protein at 280 nm, and DNA at 260 nm. Therefore, the absorbance ratios at 260/230 nm (DNA/humic acid) and 260/280 nm (DNA/protein) were used to evaluate the purity of the soil mDNA.

### 2.7. Polymerase chain reaction (PCR)

16S rRNA gene in the mDNA was amplified using the universal primers to confirm the suitability of mDNA for PCR, restriction digestion and cloning experiments. The forward primer B 27F (5′ AGA GTT TGA TCC TGG CTC AG 3′) and the reverse primer U 1492R (5′ GGT TAC CTT GTT ACG ACT T 3′) were used for PCR amplification. PCR mixture contained 1× PCR buffer, 200 μM of each dNTP, 3.0 μM $MgCl_2$, 0.2 μM of each forward and reverse primer and 2.5 U of Taq DNA polymerase (Sigma, USA) in 50 μl reaction volume. The positive control was taken