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An efficient and economical method for extraction of DNA amenable to biotechnological manipulations, from diverse soils and sediments

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Abstract

Aims: An attempt was made to optimize a new protocol for isolation of pure metagenomic DNA from soil samples.

Methods and Results: Various chemicals ($FeCl_3$, $MgCl_2$, $CaCl_2$ and activated charcoal) were tested for their efficacy in isolation of metagenomic DNA from different soil and compost samples. Among these trials, charcoal and $MgCl_2$ when used in combination yielded highly pure DNA free from humic acids and other contaminants. The DNA extracted with the optimized protocol was readily digested, amplified and cloned. Moreover, compared with a well-established commercial DNA isolation kit (UltraClean™ Soil DNA Isolation Kit), our method for DNA isolation was found to be economical. This demonstrated that the method developed can be applied to a wide variety of soil samples and allows handling of multiple samples at a given time.

Conclusions: The optimized protocol developed has successfully yielded pure metagenomic DNA amenable to biotechnological manipulations.

Significance and Impact of the Study: A user-friendly and economical protocol for isolation of DNA from soil and compost samples has been developed.

Introduction

Microbial diversity has proven to be a repository of micro-organisms, novel genes, metabolic pathways and their products. The fact that 99% of the microbial diversity is unculturable (Torsvik and Ovreas 2002) suggests that largely untapped genetic and biological pool is still unexploited. Inability to culture a vast majority of micro-organisms from a large variety of habitats limits our understanding of microbial ecology, diversity and important enzymes or other bioactive molecules. Therefore, it would be necessary to focus on investigating microbial communities *en masse* rather than simply going for culture-dependent approaches. An efficient method for isolation of nucleic acids directly from environmental samples could provide a gateway to access the entire metagenome including the unculturable microbes.

Soil being a rich source of microbes, about 4×10^7 prokaryotic cells per gram, (Richter and Markewitz 1995) has been the ecosystem of choice for many metagenomic studies and has emerged as a promising source for many novel genes. However, ineffective cell disruption, smaller size of isolated DNA, unbiased representation of all microbial genomes and above all coprecipitation of contaminants are some of the bottlenecks in successful studies of metagenomics. The major contaminants coprecipitating with the isolated environmental DNA are humic acids, phenolics and heavy metals (Hinoue *et al.* 2004). They interfere with biotechnological processing of the isolated DNA, such as PCR amplification (Tsai and Olson 1992; Smalla *et al.* 1993), restriction digestion (Porteous and Armstrong 1991) and transformation (Holben *et al.* 1988) thus hampering the downstream processing of DNA. Different methods for purification of soil metagenomic DNA have been reported, such as hydroxyapatite columns (Torsvik 1980; Purdy *et al.* 1996), potassium acetate (Hu *et al.* 2010), cetyl trimethyl ammonium (CTAB) (Cho *et al.* 1996; Zhou *et al.* 1996),

polyvinyl polypyrrolidone (PVPP) (Gray and Herwig 1996; Frostegard *et al.* 1999; Wechter *et al.* 2003; Guobin *et al.* 2008), gel filtration resins (Moran *et al.* 1993; Jackson *et al.* 1997; Miller 2001; Lakay *et al.* 2007; Sharma *et al.* 2007), CsCl density centrifugation (Holben *et al.* 1988; Walia *et al.* 1990; Leff *et al.* 1995), chemical flocculation (Braid *et al.* 2003; Dong *et al.* 2006), ion exchange and size exclusion chromatography (Erb and Wagner-Dobler 1993; Leff *et al.* 1995; Kuske *et al.* 1998; Hurt *et al.* 2001), agarose gel electrophoresis followed by excision and DNA extraction from the gel matrix (Malik *et al.* 1994; More *et al.* 1994; Zhou *et al.* 1996; LaMontagne *et al.* 2002), Silica (Ranjard *et al.* 1998; Kauffmann *et al.* 2004; Rojas-Herrera *et al.* 2008), activated charcoal (Desai and Madamwar 2006; Verma and Satyanarayana 2011) and ligninolytic enzyme complex (Sharma *et al.* 2012). However, most of these protocols suffer from one or other limitations like being time-consuming, laborious and costlier. Some of the purifying agents (PVPP and CTAB) have been reported to be unreliable (Braid *et al.* 2003). Therefore, developing an efficient and economical method for removing the full spectrum of inhibitors to get pure DNA with higher yield is a necessity. Moreover, it is imperative that the purifying agent being used should not hamper the downstream processing of the isolated metagenomic DNA. Keeping in view, an attempt has been made to develop a novel method to isolate DNA from different environmental samples, which can make it possible to get DNA with high yield and purity.

In this study, a combination of activated charcoal and $MgCl_2$ was used to obtain pure metagenomic DNA from soil samples. Activated charcoal, by the virtue of its high porosity and adsorption rate, has been used for long to remove pollutants from air or water. It has the ability to remove a number of nonbiodegradable organic substances including humic acid, lignin sulfonate, tannic acid, arabic gum along with several biodegradable substances from waste water (Seo and Ohgaki 2001). Activated charcoal has been used for obtaining pure plasmid (Kim *et al.* 2010) and plant genomic DNA (Barra *et al.* 2012). Use of activated charcoal in combination with other purifying agents, such as anion exchange resins (Desai and Madamwar 2006) and PVPP (Verma and Satyanarayana 2011), has also been reported in isolation of pure metagenomic DNA from activated sludge samples and soil, respectively. In addition, chemical flocculation using multivalent cations has also been practiced as a standard method for the removal of suspended organic solids from drinking water. Flocculation has been defined as a process wherein colloids precipitate from suspension in the form of flocs, thus clearing the solution and the chemicals that promote flocculation are termed as flocculants. Chemical flocculation has also been used to potentially remove the inhibitors coprecipitating with the soil metagenomic DNA (Braid *et al.* 2003; Dong *et al.* 2006).

The purifying agents used in the current protocol are relatively inexpensive as compared to purifying agents such as anion resins and hydroxyapatite columns, thus making this protocol economical. Moreover, the purifying agent was added to the extraction buffer, avoiding a separate purification step and thereby saving time. The protocol developed in this study has been tested on diverse soil types, proving its applicability for metagenomic studies of various ecosystems. The isolated DNA was also found to be pure and amenable for biotechnological manipulations.

Material and methods

Soil sample collection and analysis

Soil samples were collected from wood and litter decaying areas from University of Delhi South Campus, New Delhi; Mangrove forest area, Orissa; and compost samples from Okhla sewage treatment plant, New Delhi; soil from termite mounts from the forest canopy in University of Delhi South Campus, New Delhi, India. The soil samples were sieved using 2 mm mesh size, and the samples were stored at −20°C for further use.

Optimization of protocol for isolation of purified soil metagenomic DNA

Cell lysis and addition of purifying agents

The soil samples (10 g each) were homogenized by crushing gently with liquid nitrogen in a prechilled mortar and pestle and were transferred to a sterilized tube containing 20 ml lysis buffer (pH 8·0; Tris HCl, EDTA, NaCl, Lysozyme and CTAB). Chemical flocculants ($CaCl_2$, $MgCl_2$ and $FeCl_3$) at varying concentrations (10, 20, 30, 40, 50 and 60 mmol l^{-1} each) and activated charcoal (0·5, 1·0, 1·5 and 2·0% w/v) were tested for purification of metagenomic DNA by adding them to the lysis buffer. The samples were mixed thoroughly with the lysis buffer by vortexing briefly and incubated at 37°C for 1 h with intermittent mechanical shaking.

Precipitation of DNA

After incubation, the samples were centrifuged at 10 000 ***g*** for 15 min and the soil pellets were discarded. The supernatants were treated with phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and chloroform : isoamyl alcohol (24 : 1) successively for the removal of proteins and other impurities. Thereafter, the DNA was precipitated with equal volume of polyethylene glycol (PEG) 8000 (5·0% w/v) and then pelleted by centrifugation at 7000 ***g***, 4°C for 15 min

followed by washing with 80% (v/v) ethanol. Thereafter, the DNA samples were dissolved in 50 μl TE buffer (0·5×) and stored at −20°C. Metagenomic DNA isolated without the addition of any of the multivalent cations or activated charcoal served as control.

Determination of yield and purity of isolated metagenomic DNA

The purity and yield of metagenomic DNA isolated from all the four soil samples were determined spectrophotometrically (Specord; Analytik Jena, Germany) analysis. Absorbance at different wavelengths, 260, 280 and 340 nm, was recorded for all the DNA samples. $A_{260/280}$ ratio was calculated for quantifying protein contamination, whereas A_{340} was used for the level of humic acid content. Yield of DNA isolated was calculated using the A_{260} absorbance values. The size of the isolated metagenomic DNA fragments was determined by agarose gel electrophoresis using λ *Hin*dIII marker.

Evaluation of minimal inhibitory concentration of the purifying agents added

To confirm that the purifying agent *per se* does not inhibit the downstream processing of the isolated metagenomic DNA, the minimum concentration of each purifying agent which could inhibit PCR was determined. This concentration was taken as minimal inhibitory concentration (MIC) of the purifying agent being used. To determine the MIC of the purifying agents, pure bacterial genomic DNA was extracted using a commercial kit (GenElute™ Bacterial Genomic DNA kit; Sigma-Aldrich, St. Louis, MO) following the manufacturer's protocol. Purifying agents were added at varying concentrations (Table S1) to the isolated bacterial genomic DNA and mixed well, and the samples were amplified using fungal ITS primers. The minimum concentration of purifying agent responsible for PCR inhibition was observed and recorded.

Selection of purifying agent and its optimal concentration

The most suitable purifying agent and its concentration for the optimized isolation and purification protocol were selected on the basis of purity and yield of metagenomic DNA isolated.

Validation of purity of DNA isolated under optimized conditions

Metagenomic DNA was isolated from four different soil samples following the optimized conditions, and its purity was validated by PCR, restriction digestion and cloning.

PCR and restriction digestion assay

The isolated metagenomic DNA was amplified using the bacterial-specific 16S rRNA (F 5′ AGAGTTTGATCCTGG CTCAG 3′ and R 5′ ACGGCTACCTTGTTACGAC 3′) as well as fungal-specific ITS region (ITS 1 5′ TCCGTAGGT GAACCTGCGC 3′ and ITS 4 5′ TCCTCCGCTTATTGA TATGC 3′) primers. These primers are universal primers for bacteria and fungi, respectively, and therefore, were chosen for DNA amplification. The reaction mix (total volume 25 μl) consisted of isolated metagenomic DNA (0·5 μg), 0·51 μM of each forward and reverse primers, 0·2 mmol l^{-1}M dNTP′s, 1·5 U of Taq polymerase, 2·5 μl 10× buffer and water. The optimized PCR conditions used for bacterial 16S rRNA; initial denaturation of 5 min at 95°C, followed by 30 cycles of 30 s at 90°C, 45 s at 56°C at 90 s at 72°C, final extension at 72°C for 5 min, whereas for fungal-specific ITS regions, the conditions were, initial denaturation initial of 5 min at 95°C, followed by 30 cycles of 60 s at 90°C, 45 s at 56°C and 45 s at 72°C, final extension at 72°C for 5 min. The amplified products were visualized on 0·8% agarose gels.

The purity of metagenomic DNA was further validated by partial restriction digestion using the restriction enzymes *Sau*3AI, *Bam*HI and *Hin*dIII. One microgram of the isolated DNA was treated with 1 U of restriction enzyme in a total reaction mixture of 20 μl and incubated at 37°C for 10 min. The enzymes were heat inactivated at 80°C, and the digested DNA was fractionated on 1·0% agarose gel.

Metagenomic DNA isolated using activated charcoal and $MgCl_2$ (as per the optimized method), $MgCl_2$ and activated charcoal separately as purifying agents and DNA without any purifying treatment (control) were analysed with quantitative PCR. PCR (Total volume 20 μl) consisted of isolated metagenomic DNA (1 μl), 1 μl of each forward and reverse fungal ITS primers, 9 μl of Master mix (Light cycler Master SYBR Green; Roche, Mannheim, Germany) and 8 μl of distilled water. Thermocycling was performed with a denaturation step of 10 min at 94°C, followed by 45 cycles, each cycle consisting of the following steps: 10 s at 94°C, 30 s at 56°C and 45 s at 72°C. The accumulation of amplification products was visualized by fluorescence measurement of nonspecific binding of SYBR green to the new degenerated DNA amplicons. Quantitative results for both treated and untreated samples were analysed by LIGHT CYCLER software 3.0 (Roche) assisted calculations of cross-pointing (CP) values. The CP values stand for the number of PCR cycles that result in an increase in fluorescence of sample background threshold value.

The soil metagenomic DNA was also extracted with various existing protocols (Tables 3 and 4) as well as commercially available kit (Ultra Clean™ Soil DNA Isolation Kit; Mo Bio Laboratories, Inc., Carlsbad, CA) following

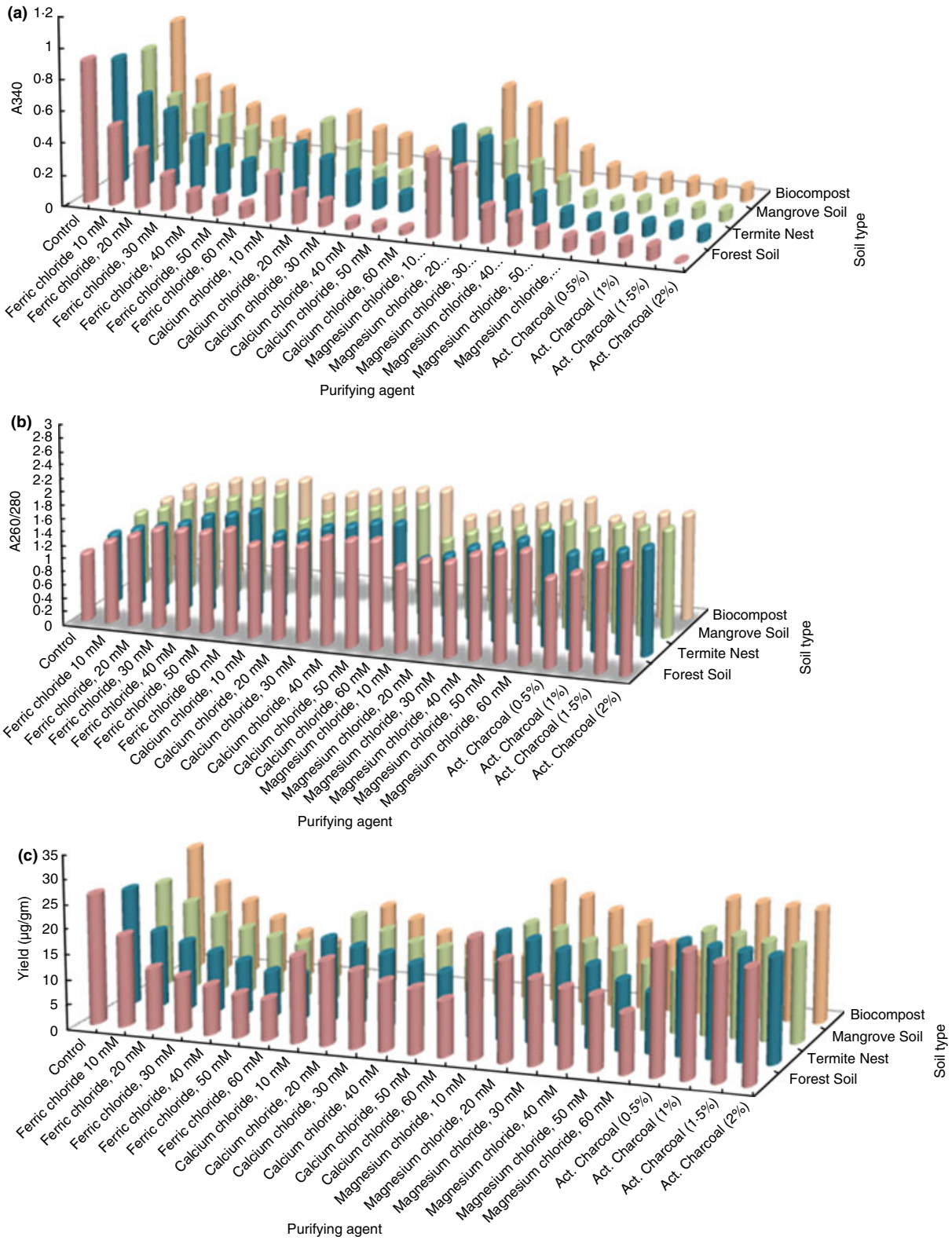

Figure 1 Effect of the purifying agents on the purity and yield of metagenomic DNA extracted from various soil samples. (a) Effect of the purifying agents on $A_{260/280}$ values of the extracted DNA samples. (b) Effect of the purifying agents on humic acid content of the extracted DNA samples. (c) Effect of the purifying agents on yield of the extracted DNA samples.

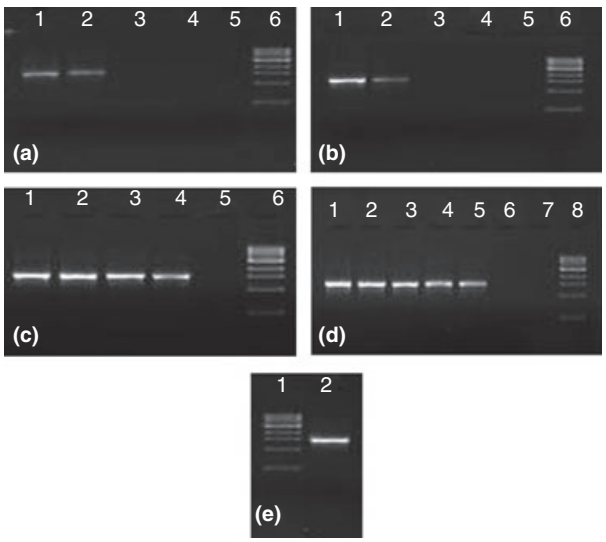

Figure 2 Determination of minimal inhibitory concentration of various purifying agents tested in current study using PCR amplification of fungal-specific ITS region. (a) $FeCl_3$, Lane 1 – 0.02 mmol l^{-1} $FeCl_3$, Lane 2 – 0.04 mmol l^{-1} $FeCl_3$, Lane 3 – 0.06 mmol l^{-1} $FeCl_3$, Lane 4 – 0.08 mmol l^{-1} $FeCl_3$, Lane 5 – 0.1 mmol l^{-1} $FeCl_3$, Lane 6 – NEB 1 kb marker. (b) $CaCl_2$, Lane 1 – 0.2 mmol l^{-1}, Lane 2 – 0.4 mmol l^{-1}, Lane 3 – 0.6 mmol l^{-1}, Lane 4 – 0.8 mmol l^{-1}, Lane 5 – 1.0 mmol l^{-1}, Lane 6 – NEB 1 kb marker. (c) $MgCl_2$, Lane 1 – 7 mmol l^{-1} $MgCl_2$, Lane 2 – 8 mmol l^{-1} $MgCl_2$, Lane 3 – 9 mmol l^{-1} $MgCl_2$, Lane 4 – 10 mmol l^{-1} $MgCl_2$, Lane 5 – 11 mmol l^{-1} $MgCl_2$, Lane 6 – NEB 1 kb marker. (d) Activated charcoal, Lane 1 – 0.6% activated charcoal, Lane 2 – 0.8% activated charcoal, Lane 3 – 1% activated charcoal, Lane 4 – 1.5% activated charcoal, Lane 5 – 2% activated charcoal, Lane 6 – 2.5% activated charcoal, Lane 7 – 3% activated charcoal, Lane 8 – NEB 1 kb marker. (e) $MgCl_2$ + activated charcoal – Lane 1 – NEB 1 kb marker, Lane 2 – $MgCl_2$ (10 mmol l^{-1}) + activated charcoal (1% w/v).

manufacturer's protocol. All these methods were compared in terms of yield and purity of the DNA with the metagenomic DNA obtained using the protocol developed in this study (Tables 3 and 4).

The cost for processing 1 g of soil sample and the amounts of all the chemicals used in the currently optimized protocol were taken into account, and total cost of the protocol was estimated. The estimated cost of the method developed was compared with UltraClean™ Soil DNA Isolation Kit (Mo Bio Laboratories, Inc.; Table 5).

Construction of metagenomic library

The metagenomic DNA (1 μg) obtained following the optimized protocol was partially digested with *Sau*3AI, and the digested metagenomic DNA was size fractionated on 1·0% (w/v) agarose gel. The fragments ranging from 1 to 9 kb were purified from the gel and ligated to *Bam*HI-digested pUC19 vector. The ligated product was transformed into *Escherichia coli* DH5α competent cells.

Table 1 Comparison of crossing over point (C.P.) values of the purifying agents in different soil samples as determined by quantitative PCR (qPCR)

C.P. values	Termite nest soil	Aravali forest soil	Mangrove forest	Compost
$MgCl_2$ only	45	–	–	–
Activated charcoal only	39	42	–	–
$MgCl_2$ + activated charcoal	25	24	27	29
Control	–	–	–	–

–: means that crossing over point was not achieved even after the 45th cycle. Control refers to the DNA sample extracted without $MgCl_2$ and activated charcoal treatment.

The transformants were plated on Luria-Bertani (LB) medium containing ampicillin.

Results

Optimization of protocol for isolation of purified metagenomic DNA

The chemical flocculants (divalent/multivalent cations; $CaCl_2$, $FeCl_3$ and $MgCl_2$) and activated charcoal were tested for their capability to remove humic acids and other contaminants from soil metagenomic DNA. Irrespective of the flocculating agents tested, the purity of the extracted metagenomic DNA increased with the increase in concentration of the purifying agent (Fig. 1), while the DNA yield was observed to be reduced with the increase in concentration of the cations (Fig. 1). Among the chemical flocculants, $MgCl_2$ caused a minimum loss in DNA yields. $CaCl_2$ even at the minimum tested concentration of 10 mmol l^{-1} led to one-third decreases in the DNA yield as compared to the control samples, while $FeCl_3$ lead to more than 30% loss in the DNA yield at same concentration. However, activated charcoal caused a very low loss in DNA yield and also improved purity of the metagenomic DNA extracted. But, neither $MgCl_2$ nor activated charcoal alone purified the DNA to the requisite level as DNA samples with $A_{260/280}$ ratio > 1·7 are not fit for biotechnological manipulations (Verma and Satyanarayana 2011; Fig. 1). The MIC of all the purifying agents was found to vary from as low as 0·06 mmol l^{-1} in case of $FeCl_3$ to 11 mmol l^{-1} in $MgCl_2$ (Fig. 2). In case of activated charcoal, the MIC was 2% (Table S1).

Therefore, a combination of 10 mmol l^{-1} $MgCl_2$ and 1% activated charcoal was found to be optimal for the removal of inhibitors from the extracted DNA samples. Quantitative PCR also demonstrated that the combination of activated charcoal and $MgCl_2$ brought about higher purification of DNA than when tried individually

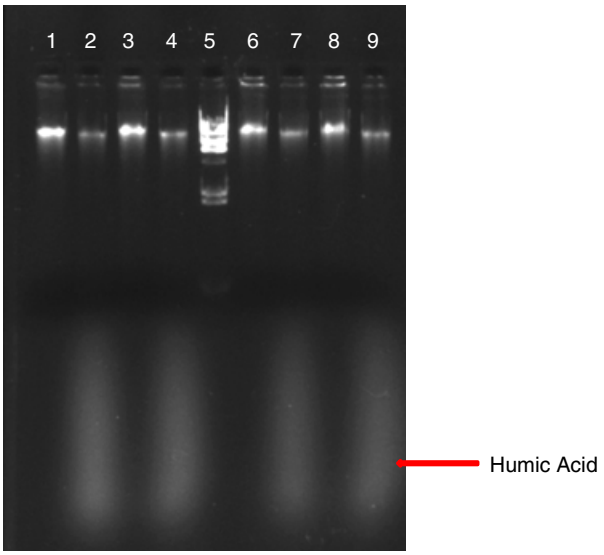

Figure 3 Agarose gel electrophoresis (0.8% agarose gel) of the metagenomic DNA extracted using the optimized protocol. Lane 1 – Metagenomic DNA isolated from Aravali Forest Soil Sample by the optimized protocol, Lane 2 – Control DNA isolated from Aravali Forest Area, Lane 3 – Metagenomic DNA isolated from Biocompost Sample by the optimized protocol, Lane 4 – Control DNA isolated from Biocompost Sample, Lane 5 – Phage Lambda DNA *Hin*dIII marker, Lane 6 – Metagenomic DNA isolated from Termite Nest Sample by the optimized protocol, Lane 7 – Control DNA isolated from Termite Nest Sample, Lane 8 – Metagenomic DNA isolated from Mangrove Forest Sample by the optimized protocol, Lane 9 – Control DNA isolated from Mangrove Forest Sample.

(Table 1). Therefore, activated charcoal (1·0%) in combination with $MgCl_2$ (10 mmol l^{-1}) was taken as the optimized purifying agents in this protocol.

Recovery of soil metagenomic DNA using the optimized protocol

The metagenomic DNA isolated with the optimized method from all the four soil samples had a high molecular weight (Fig. 3) and was pure as indicated by $A_{260/280}$ (for protein) and A_{340} (for humic acid) values (Table 2). The A_{340} values decreased considerably after the purification process, depicting almost complete removal of the humic acids from the extracted metagenomic DNA samples. The metagenomic DNA yield ranged between 24·4 μg g^{-1} of soil in case of Aravali forest soil sample and 19·07 μg g^{-1} of soil in Mangrove forest soil sample (Table 2). Higher yield and purity of metagenomic DNA was obtained using the optimized protocol compared with various manual protocols and a commercial kit tested (Tables 3 and 4).

When comparative cost of the optimized method was analysed, it was found that it approx. costed US 0·36 for processing 1 g of soil sample (Table 5). On the other hand, the procedure for isolation of DNA from 1 g soil sample following protocol as per UltraClean™ Soil DNA Isolation Kit (Mo Bio Laboratories, Inc.) costed 8·40. It is evident that in comparison with commercially available kit, the price of the good quality DNA isolation from soil was found to be more than 20-fold lower than UltraClean™ Soil DNA Isolation Kit (Mo Bio Laboratories, Inc.). This however does not include the costs related to storage, packaging and distribution.

Validation of amenability of the extracted metagenomic DNA for downstream processing

The purity of the metagenomic DNA isolated from soil samples and its amenability for downstream processing were validated by restriction digestion and amplification of conserved domains of the genomes of the microorganisms (16S rRNA in case of bacteria and ITS in case of fungi). Further, metagenomic libraries were also constructed from the isolated metagenomic DNA. The isolated metagenomic DNA samples were readily digested by all the three restriction enzymes tested: *Sau*3AI, *Bam*HI and *Hin*dIII (Fig. 4). These metagenomic DNA samples could be successfully amplified using bacterial 16S rRNA and fungal-specific ITS primers, giving amplification products of 1600 and approx. 650 bp, respectively (Fig.

Table 2 Properties of metagenomic DNA isolated from various soil samples using the optimized protocol

	DNA purity				Yield (μg g^{-1})		
	Control		DNA isolated with optimized protocol				
Soil type	$A_{260/280}$	A_{340}	$A_{260/280}$	A_{340}	Control	DNA isolated with optimized protocol	Size (kb)
Termite nest soil	1·048 ± 0·065	0·790 ± 0·059	1·76 ± 0·067	0·056 ± 0·007	23·46 ± 0·67	20·83 ± 0·87	~20
Aravali forest soil	1·021 ± 0·097	0·89 ± 0·069	1·74 ± 0·054	0·064 ± 0·009	26·12 ± 0·93	24·42 ± 0·52	~20
Mangrove forest soil	1·108 ± 0·068	0·740 ± 0·083	1·72 ± 0·076	0·058 ± 0·006	21·20 ± 0·58	19·07 ± 0·49	~20
Compost	1·06 ± 0·072	0·815 ± 0·051	1·73 ± 0·062	0·062 ± 0·008	24·99 ± 0·63	22·78 ± 0·43	~20

Table 3 Comparison of purity of metagenomic DNA samples isolated using earlier reported methods and currently optimized protocol

References	$A_{260/280}$				A_{340}			
	Termite nest soil	Aravali forest soil	Mangrove forest soil	Compost	Termite nest soil	Aravali forest soil	Mangrove forest soil	Compost
Braid *et al.* (2003)	1·344 ± 0·021	1·261 ± 0·030	1·115 ± 0·026	1·17 ± 0·033	0·061 ± 0·017	0·097 ± 0·029	0·083 ± 0·016	0·094 ± 0·020
Desai and Madamwar (2006)	1·469 ± 0·018	1·371 ± 0·023	1·431 ± 0·037	1·41 ± 0·017	0·083 ± 0·015	0·091 ± 0·021	0·079 ± 0·011	0·089 ± 0·018
Verma and Satyanarayana (2011)	1·315 ± 0·024	1·301 ± 0·021	1·24 ± 0·025	1·27 ± 0·021	0·087 ± 0·022	0·092 ± 0·039	0·073 ± 0·027	0·093 ± 0·025
Sagova-Mareckova *et al.* (2008)	1·359 ± 0·029	1·312 ± 0·036	1·392 ± 0·027	1·322 ± 0·013	0·081 ± 0·015	0·096 ± 0·024	0·070 ± 0·029	0·085 ± 0·028
Dong *et al.* (2006)	1·257 ± 0·034	1·176 ± 0·019	1·244 ± 0·022	1·206 ± 0·014	0·076 ± 0·026	0·093 ± 0·037	0·072 ± 0·015	0·087 ± 0·023
Ultra Clean™ Mo Bio Soil DNA isolation kit	1·478 ± 0·019	1·397 ± 0·020	1·421 ± 0·017	1·38 ± 0·023	0·086 ± 0·012	0·091 ± 0·027	0·081 ± 0·018	0·097 ± 0·014
Current study	1·761 ± 0·067	1·742 ± 0·054	1·721 ± 0·076	1·738 ± 0·062	0·056 ± 0·007	0·064 ± 0·009	0·058 ± 0·006	0·062 ± 0·008

S1). However, no amplification was observed in the control (untreated) samples.

The amplified ITS and 16S rRNA genes were cloned in pGEMT easy vector, and libraries were constructed for all the four soil samples. Sequencing of these libraries revealed the presence of diverse groups of bacteria (proteobacteria, firmicutes, actinobacteria) and fungi (ascomycetes, basidiomycetes, zygomycetes and deuteromycetes (data not shown). This demonstrated that the method developed for extraction of DNA can be used to extract DNA from a majority of microbial lineages with equal efficiency. Further, the extracted metagenomic DNA was cloned successfully to construct a metagenomic library containing 5×10^6 CFU ml^{-1}, which confirmed the purity of the extracted metagenomic DNA. The metagenomic DNA samples extracted from all the four soil samples could be preserved at −20°C without any loss in yield or change in purity for six months.

Discussion

In the current study, an attempt has been made to optimize procedure for isolation of high-purity metagenomic DNA from soil samples. The protocol uses enzymatic (lysozyme), chemical (CTAB) as well as mechanical (crushing under liquid nitrogen) strategies for cell lysis, thus ensuring efficient cell disruption and recovering metagenome representing the diverse soil microbial community. Gentle crushing with excess of liquid nitrogen lead to proper homogenization of the soil, lysis of micro-organisms with resistant cell walls and avoided shearing of the metagenomic DNA. The use of CTAB and lysozyme to the lysis buffer for facilitation of cell lysis has already been reported (Gray and Herwig 1996; Sharma *et al.* 2012). Combined use of mechanical, chemical and enzymatic lysis strategies lead to efficient cell lysis allowing the isolation of DNA from various groups of bacteria and fungi as proved by the sequencing of 16S and ITS libraries.

Among various purifying agents tested, 10 mmol l^{-1} magnesium chloride and 1% w/v activated charcoal were selected on the basis of their contribution in purification and improving yield of metagenomic DNA. Both of these purifying agents resulted in purity ratios higher than the control, suggesting their significant role in removal of contaminants coprecipitating with the soil metagenomic DNA (Fig. 1). $CaCl_2$ and $FeCl_3$ were to cause substantial loss in the DNA yield. And both divalent cations inhibited the PCR even at very low concentrations and therefore were found unsuitable for purifying the metagenomic DNA (Table S1). Bickley *et al.* (1996) reported that divalent calcium ions interfere with the interaction between the polymerase enzyme and magnesium ions, which acts as a cofactor for the enzyme. Similarly, $FeCl_3$

Table 4 Comparison of yield of metagenomic DNA samples isolated by various methods and currently optimized protocol

References	Yield (μg g^{-1})			
	Termite nest soil	Aravali forest soil	Mangrove forest soil	Compost
Braid *et al.* (2003)	6·96 ± 0·31	9·28 ± 0·57	5·78 ± 0·37	11·09 ± 0·45
Desai and Madamwar (2006)	17·65 ± 0·22	19·33 ± 0·47	18·25 ± 0·19	18·16 ± 0·74
Verma and Satyanarayana (2011)	15·26 ± 0·76	17·73 ± 0·92	15·81 ± 0·25	16·26 ± 0·64
Sagova-Mareckova *et al.* (2008)	13·49 ± 0·27	15·51 ± 0·56	11·93 ± 0·74	14·21 ± 0·63
Dong *et al.* (2006)	11·98 ± 0·62	13·84 ± 0·20	10·94 ± 0·85	12·36 ± 0·51
Ultra Clean™ Mo Bio Soil DNA isolation kit	15·37 ± 0·34	16·09 ± 0·05	14·51 ± 0·17	17·19 ± 0·24
Current study	20·83 ± 0·87	24·42 ± 0·52	19·07 ± 0·49	22·78 ± 0·43

Table 5 Tentative cost analysis of the currently optimized protocol

Material used	Rate of the material (US $)	Amount of material used in the protocol	Approximate cost of the material (US $)
Phenol (Tris equilibrated)	$ 17·74 for 60 ml	10 ml	$ 2·96
Chloroform	$ 4·93 for 500 ml	29·4 ml	$ 0·29
Liquid nitrogen	$ 0·32 per litre	200 ml	$ 0·06
EDTA	$ 3·2 per 100 g	0·744 g	$ 0·02
Tris	$ 13·22 per 100 g	0·2428 g	$ 0·03
NaCl	$ 12·09 per 500 g	1·75 g	$ 0·04
Lysozyme	$ 14·03 per g	2 mg	$ 0·03
CTAB	$ 3·39 per 50 g	1 g	$ 0·07
$MgCl_2$	$ 19·48 per 500 g	0·402 g	$ 0·0015
Activated charcoal	$ 4·03 per 500 g	0·2 g	$ 0·0016
Isoamyl alcohol	$ 12·09 per 500 ml	0·6 ml	$ 0·0144
PEG (8000)	$ 38·01 per kg	1·25 g	$ 0·05
Total cost for processing 10 g soil/sediment sample using currently optimized protocol			$ 3·57
Total cost for processing 1 g soil/sediment sample using currently optimized protocol			**$ 0·357**
Total cost for processing 1 g soil/sediment sample using Ultra Clean™ Mo Bio Soil DNA isolation kit			$ 8·39

CTAB, cetyl trimethyl ammonium·

The cost analysis of the optimized protocol is based on the cost of materials used in isolation of DNA from soil/sediments.

The bold value is the cost of processing 1 g soil/sediment using our protocol.

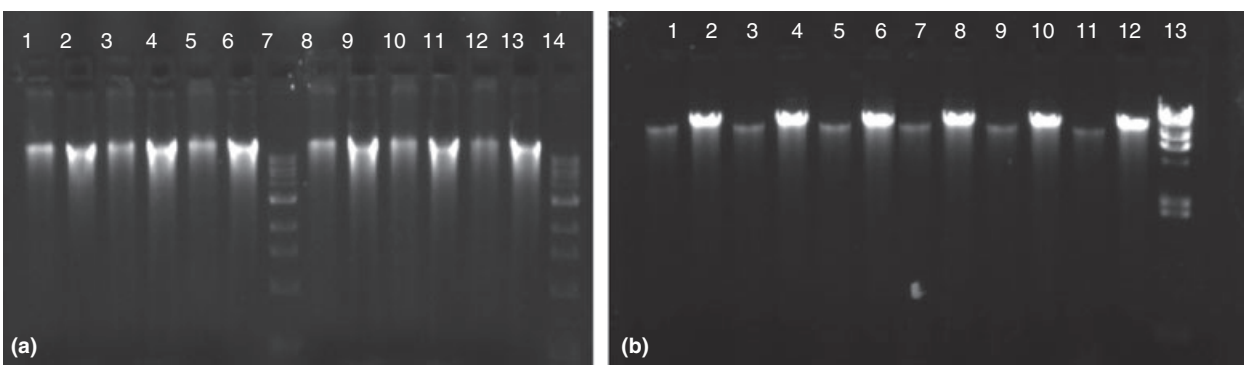

Figure 4 Agarose gel electrophoresis of partially digested metagenomic DNA isolated using the optimized protocol. (a) Lane 1, 3 and 5 – Control forest soil DNA samples digested by *Sau*3AI, *Bam*HI and *Hind*III, respectively; Lane 2, 4 and 6 – Forest soil DNA samples isolated using the optimized protocols digested by *Sau*3AI, *Bam*HI and *Hind*III, respectively; Lane 7 – NEB 1 kb Ladder; Lane 8, 10 and 12 – Control termite nest soil DNA samples digested by *Sau*3AI, *Bam*HI and *Hind*III, respectively; Lane 9, 11 and 13 – Termite nest soil DNA samples isolated using the optimized protocols digested by *Sau*3AI, *Bam*HI and *Hind*III, respectively; Lane 14 – NEB 1 kb Ladder (b) Lane 1, 3 and 5 – Control mangrove soil DNA samples digested by *Sau*3AI, *Bam*HI and *Hind*III, respectively; Lane 2, 4 and 6 – Mangrove soil DNA samples isolated using the optimized protocols digested by *Sau*3AI, *Bam*HI and *Hind*III, respectively; Lane 7, 9 and 11 – Control biocompost DNA samples digested by *Sau*3AI, *Bam*HI and *Hind*III, respectively; Lane 8, 10 and 12 – Biocompost DNA samples isolated using the optimized protocols digested by *Sau*3AI, *Bam*HI and *Hind*III, respectively, Lane 13 – λ *Hind*III marker.

has also been observed to inhibit PCR at very low concentrations (Kreader 1996).

$MgCl_2$ (10 mmol l^{-1}) and activated charcoal (1·0% w/v) were chosen as the purifying agents in the current protocol. Quantification of the PCR amplified product of the fungal ITS gene lead to the observation that in case of all the four soil samples tested, the metagenomic DNA purified by the combination of activated charcoal and magnesium chloride crossed the background thresh hold levels of fluorescence earlier than the samples treated either with activated charcoal or magnesium chloride alone (Table 1). Magnesium chloride removed the suspended organic solids and other impurities which otherwise coprecipitate with the metagenomic DNA by chemical flocculation. On the other hand, activated charcoal, being extremely porous allowed the adsorption of humic acids, phenolics and various other unknown impurities present in the DNA samples. Desai and Madamwar (2006) have reported the use of activated charcoal in isolating metagenomic DNA samples from polluted soil sediments, but they used an additional treatment with anion resins to remove all the inhibitors from the DNA. The use of anion exchange resins considerably increased the cost of the purification protocol. Similarly, Verma and Satyanarayana (2011) used PVPP along with activated charcoal for extraction of metagenomic DNA from alkaline soils. But use of PVPP was found to be unreliable by earlier researchers (Braid *et al.* 2003).

The purification step was followed by deproteinization with organic solvents. Precipitation of metagenomic DNA was achieved by 5·0% PEG. Unlike ethanol and isopropanol, PEG reduced the coprecipitation of humic substances along with the DNA, without decreasing the DNA yields. Similarly, a fourfold reduction in humic substances was reported by LaMontagne *et al.* (2002) when PEG was replaced with ethanol/isopropanol for DNA precipitation.

The protocol optimized under present investigation resulted in metagenomic DNA with higher yield and purity which is evident from $A_{260/230}$ and $A_{260/280}$ ratios. The metagenomic DNA yield varied with the soil samples (Table 2) but was comparable to that of the control samples, indicating that purification step resulted in only negligible loss of DNA. The metagenomes isolated were susceptible to restriction digestion with all three restriction enzymes: *Bam*HI, *Hin*dII and *Sau*3AI, tested here. The purity of the metagenomic DNA extracted from various soil samples were further confirmed by amplification of conserved regions of bacterial (16S rRNA) as well as fungal (ITS) communities. Successful amplification of the conserved regions indicated that the isolated DNA was an unbiased representation of both bacterial and fungal communities. Metagenomic libraries were also constructed from the extracted metagenomic DNA samples to establish the purity of the DNA samples. Thus, it can be concluded that treatment with activated charcoal and magnesium chloride removed almost all the inhibitors and contaminants of the soil metagenomic DNA.

The optimized method resulted in high yields of metagenomic DNA (Table 2) from different types of soils tested here. Our protocol when compared with various earlier protocols and commercial kit gave better DNA yield and purity (Table 3 and 4). Moreover, the protocol proved to be economical in comparison with UltraClean™ Soil DNA Isolation Kit (Mo Bio Laboratories, Inc.; Table 5). The optimized purification and extraction protocol resulted in isolation of pure metagenomic DNA fit for further biotechnological manipulations such as construction of metagenomic libraries.

A cost-effective and user-friendly protocol for extraction of soil metagenomic DNA was optimized. This method would be applicable for isolating pure metagenomic DNA to reveal unculturable and unknown microbial strains and wider communities.

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Conflict of interest

All the authors agree to the submission of this manuscript and have no conflict of interest to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Agarose gel electrophoresis of (a) Bacterial-specific 16S rDNA and (b) Fungal-specific ITS amplicons from metagenomic DNA isolated from various soil samples.

Table S1 Minimal Inhibitory Concentrations of various purifying agents tested in the current study.