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Research Article

An Improved Method for Extraction of Soil Microbial DNA for Metagenomics Study

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A B S T R A C T

Metagenomics enables the genome wide study of unculturable microorganisms in any habitat.[1] Standardization of microbial DNA isolation protocol is the first and foremost step for molecular characterization of unculturable microbes, DNA isolation methods from soil needs to be standardized. During the last 10 years, a number of protocols for DNA extraction from environmental samples have been reported.[2] A number of methods are being used for lysis of cells in soil samples. In the present study, four different methods (i) by using both CTAB and SDS,[3] (ii) only CTAB,[4] (iii) enzymatic (lysozyme) lysis,[5] and (iv) sonication[6] for different time duration were used. It was found that the first three methods yielded very less amount of DNA and the quality ratio (A260/280) was also low, which indicated impurity in DNA. Genomic DNA isolation protocol of community DNA was optimized with sonication (30 sec) and modifications made in standard CTAB method[4] and good quality and considerably higher yield was obtained in the method involving sonication followed by extraction with CTAB and SDS in the present study.

Keywords: 16S rDNA, Humic Acidmetagenomics, Soil Microbial DNA

Recommended Equipment

- Sonicator (BBRAUN LABSONIC U)
- Centrifuge (KUBOTA)
- PCR Machine (BIO METRA)
- Gel-Electrophoresis unit (Bangalore Genei)
- Gel-doc unit (Gel Doc S-Mini Bis-Bioimaging. System, USA)
- Spectrophotometer (UNICAM)
- Deep freezer (-80ºC)

Minor Instruments

Spinner, Vortex, pH meter, Water-bath, Autoclave, Weighing Balance.

Method Details

Soil Sampling

The experimental material in the present study consisted of soil samples which were collected from rhizosphere of two arid plant species viz., ker and pearl millet along with soil samples from adjacent non-rhizospheric areas of Swami Keshwanand Rajasthan Agricultural University, Bikaner. During the Kharif season (in the month of September) three plants each from three different location of pearl millet were uprooted randomly and the soil from root zone of pearl millet field were collected. The samples were kept

separately in aseptic bags and immediately transported to lab under cold condition (4ºC) for further processing. Ker is perennial woody arid tree. Three ker plants of approximately the same age were also randomly selected. Samples were collected from the root zone at 30 cm depth. All these samples were thoroughly mixed, air-dried, grounded and allowed to pass through 2.0 mm sieve separately and stored at -80ºC in deep freezer for further analysis.

Standardization of Method for DNA Isolation

Different DNA extraction methods were used initially to obtain good quality and quantity of soil microbial DNA (Table 1). Soil community nucleic acid was extracted from 15 g of each of the six soil samples by extraction procedure based on CTAB method as described by[4] with some modification which is described as follows fifteen gram soil sample was well homogenized with 15 ml 2X CTAB DNA extraction buffer (100 mMTris, 20mM EDTA, 1.4M NaCl, 2% CTAB, 1% SDS and 2 µl/ml ß-mercaptoethanol) in capped polypropylene tubes and sonicated with titanium microtip operated at a power setting of 15 W (4/10) for 50% active cycles of 30 sec duration using BBRAUN LABSONIC U sonicator and incubated for 1 hr at 60^{0}C with occasional mixing by gentle swirling (in water bath). After removing from water bath one volume of chloroform: Isoamyl alcohol (24:1) was added and mixed by inversion for 15 min to ensure emulsification of the phases followed by spinning at 15,000 rpm for 15 min (Kubota centrifuge), after that aqueous phase was taken and transferred to another tube. Two volumes of ice cold absolute alcohol were added to precipitate DNA. DNA-CTAB complex was then precipitated as a fibrous network, lifted by Pasteur pipette and was transferred to the washing solution. Twenty ml of 70 percent alcohol was, added to the pellet of DNA and kept for 20 minutes with gentle agitation. The pellet was collected by centrifugation at 5,000 rpm for 5 min at 20ºC. The tubes were inverted and drained on a paper towel. The pellet was dried over-night after covering with parafilm with tiny pores. The pellet was re-dissolved in 200 µl of TE buffer by keeping over night at 4ºC without agitation. It was purified by gel elution and column purification method.

Elution of DNA

Genomic DNA was eluted containing agarose gel slice and weighed carefully. Three volumes of gel solubilizer (i.e. for 100 mg use 300 µl) was added and incubated at 50^{o}C for 10 minutes or until the agarose gel slice has completely dissolved for better dissolution of gel, mixed by vortexing every 2-3 min. The colour of the mixture was checked if pink/red appears then 10 µl of 3M sodium acetate was added with pH 5.0 and mixed to bring it back to yellow. To about 100 µl of isopropanol was added to every 100 mg of agarose gel weighted. The spin column was kept in a 2ml collection tube and sample was added to the column. It was centrifuged for 1 min and flow through was discarded and the column was placed back in the same collection tube. Then, 700 µl of diluted wash buffer was added and spun for 1miniute. Flow through was discarded and the column was placed back in the same collection tube. Empty column was centrifuged for 2 min to ensure complete removal of wash buffer. Spin column was placed in a new 1.5 ml tube and 50 µl of elution buffer was added to the centre of the membrane set the column stand at room temperature for 5 min and then centrifuged for 1 min to elute the DNA. Elution is repeated one more time as described in the previous step. For the second elution 1.5 ml tube was used to prevent dilution of the first elute.

DNA purification by Fast DNA Spin Kit

The Fast DNA Spin Kit is designed to extract PCR- ready genomic DNA in less than 30 min. The rapid DNA extraction method precludes the use of harmful organic solvents such as phenol and choloroform. The kit is recommended for isolation of DNA from small amount of soil (1g) directly. However, we used it to purify DNA isolated by the CTAB procedure described above. This will enhance the DNA yield. To 200 µl of extracted DNA from CTAB method using sonication taken in lysed matrix E tube 978 µl sodium phosphate buffer and 122 µl MT buffer were added. Lysing matrix E tubes was centrifuged at 14,000 rpm for 30 sec and the supernatant was then transferred to a clean tube. About 250µl of PPS reagents was added and mixed by shaking the tube by hand 10 times. It was then centrifuged at 14,000 rpm for 5 min to pellet the precipitate followed by transfer of supernatant to a clean tube. Then it was placed on a rotator for 2 min to allow binding of DNA to matrix. The tube was placed in a rack for 3 min to allow settling of silica matrix. From the tube 500 µl of supernatant was removed carefully to avoid settled binding matrix. Supernatant was discarded and resuspended in binding matrix in the remaining amount of supernatant. The mixture (600 µl) was transferred to a spin filter and centrifuged at 14,000 rpm for 1 min. To the spun filter 500 µl salt ethanol wash solution was added and centrifuged at 14,000 rpm for 1 min. Flow-through was decanted and spin filter was replaced in catch tube than it was centrifuged at 14,000 rpm for 2 min to dry the matrix of residual wash solution. SPINTM Filter was removed and placed in fresh kit-supplied Catch Tube. Air drying the SPINTM filter for 5 minutes at room temperature, 50 µl DES (DNase/ pyrogen free water) was added and gently stirred matrix on filter membrane with a pipette tip or vortex/finger flips to resuspend the silica for efficient elution of the DNA.It was then centrifuged at 14,000 rpm for 1 min transfer eluted DNA to catch tube. DNA was ready for processing.

Gel Analysis

The integrity of DNA was assessed by agarose gel analysis

by casting 150 ml agorose gel (0.8%) in 0.5X TBE (Tris Borate EDTA) buffer containing 0.5 µg / ml of ethidium bromide. About 2 µl of DNA per sample was loaded in each well and known amount of uncut λ phage DNA was also loaded as control. Electrophoresis was conducted at 50 V for 1 h and then the gel was visualized under UV light using transilluminator. Presence of single compact band at the corresponding position to lambda phage DNA indicated high molecular weight genomic DNA.

Quantification of DNA

The quantitation of DNA was done by observing it at 260 nm and 280 nm wavelengths by using a UV-VIS spectrophotometer (UNICAM) for this 1500 µl T.E. buffer was taken in a cuvette and spectrophotometer was calibrated at 260 nm as well as at 280 nm wavelengths then 5-15 µl of DNA was added, mixed properly and absorbance (A) was recorded at both 260 and 280 nm.

DNA concentration was estimated by employing the following formula:

Amount of DNA (µg / µl) = A260 x 50 x dilution factor/1000.

Quality of DNA was judged from the ratio of A values recorded at 260 and 280 nm.

Result

Genomic DNA isolation from two soil samples was standardized by modifying the standard protocol of CTAB method.[4] Initially, standard CTAB method was followed for genomic DNA extraction which resulted in lower yield (0.53 µg/g and 1.26 µg/g) and inferior quality DNA (A260/280 1.00 and 1.36). The quantity and quality of DNA yielded in all these modifications is given in Table 1. To improve the quality and extraction efficiency, addition of SDS and lysozyme along with standard protocol was attempted. But the enzymatic lysis involving lysozyme also yielded low quantity (0.73 µg/g and 0.86 µg/g) and quality DNA (A260/280 1.20 and 1.30) for two samples. Addition of anionic detergent, SDS along with CTAB, yielded maximum DNA (1.53 µg/g and 1.4 µg/g) in both the samples but failed in enhancing its quality (A260/280 1.35 and 1.40) as shown in Figure 1. In order to achieve acceptable quality and quantity of DNA, a sonication step was incorporated prior to extraction methods using CTAB and SDS. The sonication treatment increased the yield however, the quality remained inferior which was evident from the ratio of A260/280 values ranging from 1.06 to 1.22 (Table 2) Therefore, an additional purification step using Fast DNA Soil Kit (Qbiogene Fast DNA spin kit) was attempted. After this purification, the DNA yield declined and ranged from 3.45 µg/g to 4.05 µg/g of soil, but the quality improved (A260/280 ranged from 1.56 to 1.72 is shown in Figure 2). These results collectively revealed that sonication, extraction with CTAB along with SDS and purification steps essentially resulted in superior quality and significant yield of soil DNA as shown in Table 3. Genomic DNA of good quality is the prime requisite for molecular studies. DNA isolation in soil community is cumbersome because of the humic acid present in the soil, which co-precipitate with DNA and interfere with PCR reactions. Hence, the standardization of protocol for DNA isolation is the most critical step and this experiment was carried out to fulfill this objective.

Table 1.DNA quantitation and quality analysis in different treatments of DNA isolation of metagenome

Method	Absorbance		Ratio of A_{260}/A_{280}	Conc. of DNA (µg/ µl)	Yield in µg/g of soil
	A_{260}	A_{280}			
CTAB	0.008	0.008	1.00	0.040	0.53
	0.019	0.014	1.36	0.095	1.26
CTAB and SDS	0.023	0.017	1.35	0.115	1.53
	0.021	0.015	1.40	0.05	1.40
Enzymatic lysis	0.013	0.010	1.30	0.065	0.86
	0.011	0.009	1.20	0.055	0.73

Table 2.Yield and quality of DNA with sonication treatment before purification

Samples	A_{260}	A_{280}	Ratio of A_{260}/A_{280}	Conc. of DNA (µg/ µl)	Yield in µg/g of soil
PMR-1	0.038	0.034	1.12	0.57	7.6
PMR-2	0.059	0.054	1.09	0.88	11.8
PMR-3	0.071	0.063	1.13	1.06	14.2
KR- 1	0.060	0.049	1.22	0.90	12.0
KR-2	0.054	0.050	1.08	0.81	10.8
KR-3	0.053	0.050	1.06	0.79	10.6

PMR-pearl millet rhizosphere; KR-kerrhizosphere

Table 3.Yield and quality of DNA obtained after spin column purification

Samples	A_{260}	A_{280}	Ratio of A_{260}/A_{280}	Conc. of DNA (µg/ µl)	Yield in µg/g of soil
PMR-1	0.079	0.049	1.61	1.18	3.95
PMR-2	0.074	0.043	1.72	1.11	3.70
PMR-3	0.081	0.052	1.56	1.21	4.05
KR-1	0.072	0.043	1.67	1.08	3.60
KR-2	0.069	0.041	1.68	1.03	3.45
KR-3	0.070	0.041	1.70	1.05	3.50

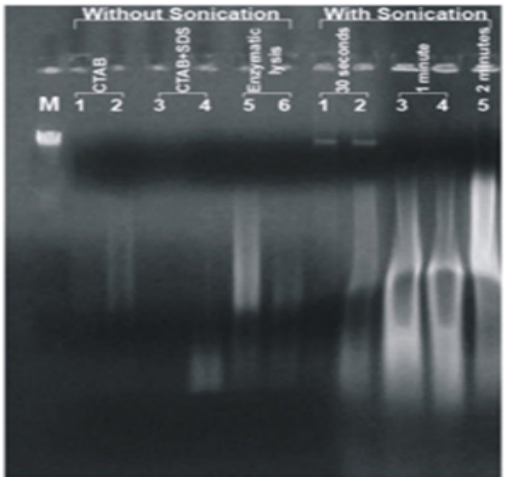

Figure 1.Agarose Gel Profile of Genomic DNA Isolated from Soil with and without Sonication

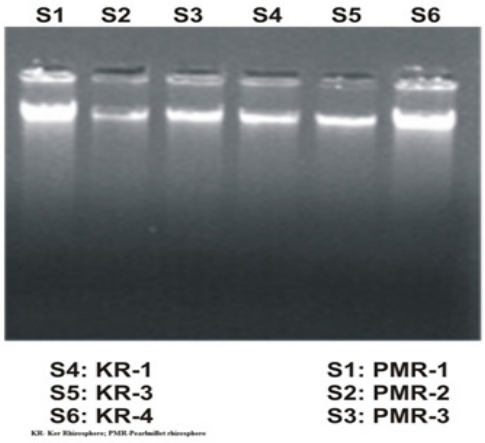

Figure 2.Whole Community DNA (metagenome) of Micro organisms from Rhizosphere of Ker and Pearl Millet Isolated by Sonication Treatment for 30 seconds

Validation

In order to check the quality of the DNA, bacterial diversity studies using ribotyping of soil samples of rhizosphere and non-rhizosphere of ker and pearl millet was employed for conclusive results. The 16S rDNA gene were amplified with bacterial specific universal primers P1 (5' AGAGTTTGATCCTGATCCTGGCTCAG 3') and P2 (5' TACCTTGTTACGACTT 3'). PCR reaction was performed in final volume of 25 µl containing 2.5 µl 10X Assay Buffer with $MgCl_2$ (Bangalore Genei), 0.19 µl of *Taq* polymerase (3U/µl), 2.0 µl dNTPs, 1 µl primer-1 (10pM/ µl) (OPERON TECHNOLOGIES), 16.3 µl deionised water and 2.5µl template DNA (25ng/µl). PCR was performed for 35 cycles in 'Thermocycler' (Bio metra) at 94 °C for 1 minute, 48.0 °C for 1 minute and 72 °C for 1 minute for 16S rDNA gene amplification. The PCR products, were analyzed on 1% Agarose gel (Himedia, molecular grade), prepared in 1X TBE buffer containing 0.5 µg/ml of ethidium bromide. The amplified products were electrophoresed for 3-3.5 hours at 100 V with cooling. The gel was viewed under UV trans-illuminator and photographed by digital camera (Gel Doc S- Mini Bis Bioimaging. System, USA). The PCR amplification of 16S rDNA gene was performed using bacterial specific primers (27F and 1492R) which resulted in an amplicon size of 1500 bp.

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