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Defining optimum metagenomic procedure for microbial diversity analysis in wheat rhizosphere

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ABSTRACT

Bacteria are an important part of the soil micro flora. Conventional plate or liquid culture often cannot emulate the environmental conditions to which these microorganisms are adapted. Thus, these methods do not necessarily provide comprehensive information on the diversity and biotechnological potential of a soil or water sample. Metagenomics circumvents the unculturability and genomic diversity of most microbes, which is the biggest roadblock to advances in clinical and environmental microbiology. In this study certain soil DNA extraction procedures were studied and evaluated for microbial DNA yield from different regions of North India. Soil samples under wheat crop were collected in sterile condition at a depth of 10-15 cm below the soil surface in the months of Jan-May. All samples were analyzed for their physical properties. DNA was extracted metagenomically by three methodologies after making certain modifications. Purity of isolated DNA was checked by taking O.D at 260 and 280nm. Purest form of DNA was obtained by ASM method as compared to the other two methods. This method was applicable on all soil types and even on old soil samples with fair degree of efficiency. 17.7-40.98 µg of DNA was obtained with a purity ratio of A260/A230 and A260/A280 as 1.456 and 1.680 respectively. Size of DNA obtained was found to be > 23 Kb. DNA obtained by ASM method was used directly for PCR as there were negligible amount of inhibitors left due to addition of $CaCl_2$.

Keywords: Soil DNA extraction, Soil microorganisms, Bacterial Diversity, $CaCl_2$.

INTRODUCTION

Soil is a complex ecosystem, delimited by physicochemical parameters that hold enormous number of living organisms [1].The Rhizosphere is an active zone of soil processes dependent upon plant and microbial co-operation. Since bacteria are reservoirs of biomolecules with potential use in health care and industrial applications [2,3], hence they are an important part of the soil micro flora. Bacterial diversity is the major driving force of fundamental metabolic processes in rhizosphere-dynamic environment and a number of bacterial species are associated with the plant rhizosphere [4,5], therefore a basic understanding of diversity of soil biota is required in order to preserve the integrity, function and long term sustainability of natural and managed terrestrial ecosystems. Obtaining bacteria in pure culture is typically the first step in investigating bacterial processes. However, standard culturing techniques account for 1% or less of the bacterial diversity in most environmental samples [6]. Conventional plate or liquid culture often cannot reconstitute the environmental conditions; hence these methods do not provide comprehensive information on the diversity and biotechnological potential of a soil or water sample [7].

Metagenomics, allow rapid access to microbial diversity and thus facilitate the discovery of new groups of microorganisms [8]. Over the last two decades, methods for extraction of DNA from soil samples for DNA analysis of all types have been markedly improved. Several methods for extracting microbial DNA from soil or other environmental samples have been described. Many of these techniques employ extensive purification steps to ensure that the DNA is suitable for use in the polymerase chain reaction [9-13] as development of modern techniques, especially PCR had made it possible to compare bacterial isolates more carefully and critically [14] The major disadvantage of DNA extraction directly from soil is the Co- extraction of other organinc components, such as humic and fluvic acids adequately present in soil which prevent subsequent molecular analysis. Soils are therefore

one of the most challenging environmental matrices for obtaining microbial DNA. Every type of soil sample, because of its own nature, requires optimization of the extraction and purification methods. Thus, there is a need of a standard metagenomic procedure which is most suitable and applicable to samples derived from diverse environmental habitat for molecular ecology research.

In this paper we describe an effective and versatile method for soil DNA extraction from various geographical locations of North India by drawing comparison between three different techniques primarily based on their lysis efficiency. The effectiveness of the three methods was evaluated by UV spectrophotometry, DNA yield and purity study and single gene amplification.

MATERIALS AND METHODS

2.1 Soil Sampling

Soil samples under wheat crop were collected from different geographical locations of North India rich in wheat plantation such as Bijvasan, New Delhi (28.38°N lat and 77.13°E long); Sonepat, Haryana (28.98°N lat and77.02°E long); Aligarh, U.P (27.88°N lat and 78.08°E long); Gaya, Bihar (24.78°N lat and 85.0°E long); Karnal, Hayana (29.68°N lat and76.98°E long). Wheat plant was chosen for this study as it is the major staple food in North India. Six samples from each site were collected at a depth of 10-15 cm below soil surface in the months of Jan – May. The samples were kept at 4° C immediately and were analyzed for their % moisture content, pH, and total carbon content according to standard protocol.

2.2 Soil DNA extraction

Three methods for community DNA isolation were studied after making certain modifications in pre-existing methods and designated as ASM; ASN and ASA methods. These are discussed below:

2.2.1 ASM method **:- (**modified Zhou's method**)** [11] 5g of soil sample and 13.5 ml of DNA extraction buffer (100mM TRIS –HCl, pH- 8.0; 1.5 M NaCl,) were mixed. 100 µl of Proteinase K (10mg per ml) was added and the samples were incubated at 37º C for 30 min.1.5 % $Cacl_2$ was added to it and sample was vortexed for 30 seconds. 1.5 ml of 20% SDS was added and Samples were incubated at 70ºC for 15-20 min and centrifuged at 10,000 g for 10 min. The supernatant thus obtained was subjected to isopropanol precipitation.

2.2.2 ASN method:- (Modified Kuse's method) [13] 10 ml of TENS buffer (50mM TRIS, pH- 8.0; 20mM disodium EDTA; 10mM NaCl; 1% SDS) was added to 5 g of soil samples .The soil samples were then mixed thoroughly by vortexing and the solution was then incubated in boiling water for 10 min.The samples were then centrifuged at 10,000g for 10 min. The soil pellet was resuspended in 7.5 ml of TEN buffer and exposed to three sets of thermal shocks by immersion of the tubes at -20°C for 10 min followed by rapid thawing in 65ºC water bath. After centrifugation at 10,000g, the supernatant were collected in a separate tube.

2.2.3 ASA method: - (Modified Tsai method) [9]. 5g soil sample was added to 10 ml of 120 mM sodium phosphate buffer, pH-8.0 by horizontal shaking at 150 rpm for 15 min. The slurry was pelletized by centrifugation at 10,000g for 10 min.The pellet was washed again with phosphate buffer, resuspended in 10 ml of lysis solution I, containing 15 mg of lysozyme per ml and Incubated in a 37 º C water bath for an hour with agitation at 20 to 30 min interval and then 10 ml of lysis solution II (0.1 M NaCl; 0.5 Tris HCl, pH-8.0; 10 % SDS) was added and the solution was incubated at 70ºC for 15 min followed by centrifugation at 10,000g for 10 min to get the supernatant.

Common Steps: Each supernatant which we got from the different methods was mixed with an equal volume of phenol,chloroform, isoamyl alcohol (25:24:1 v/v). The aqueous phase was recovered by centrifugation; 10µl of 3M sodium acetate was added and was further precipitated with 0.6 volume of isopropanol at room temperature for 1 hour. The pellet of crude nucleic acid was obtained by centrifugation at 10000 g for 5 min at room temperature, washed with cold alcohol and resuspended in sterile deionized water to give a final volume of 50 µl and stored at -20 ºC. For PCR reactions the DNA extract was diluted to 1:10 volume with double distilled water.

The purity and quality of DNA was assessed spectrophotometrically by calculating A260/A280 and A260/230 ratios and electrophoresis on 0.7% agarose gel respectively with 1 μg^{-1} ml ethidium bromide. 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 the calculation was based on the value of 1.0 A260 unit = 50 µg/ml of DNA, taking into account the dilution factor of the sample [15] .

2.3 PCR amplification

DNA isolated using different methods were further evaluated by PCR. Two pairs of universal primers, anchoring different regions of conserved bacterial 16S rRNA gene were used. They were 27f/1525r (universal bacterial fact sheet, 2004) and 968f/1406r [16,17]. PCR was conducted in 50µl reaction solution containing1U taq polymerase (Banglore genei) 1XPCR buffer (Banglore genei). Primer and dNTP- concentration were 10 pmole and 100µmole, respectively. The DNA extract was diluted with water (1:10) and 10 µl was used for PCR. Each set of primers were first screened with different PCR-temp-cycling parameters. PCR condition for primer set 1: The amplification of primer set 1 (27f/1525r) was performed in a thermal block (Bioneer thermal block) by using 30 reaction cycles, each consisting of the following steps involving initial denaturation at 95˚ C for 3 min, 94˚C for 30 sec, 50˚C for 30 sec, 72˚ C for 1 min,and final extension at 72˚ for 7 min. The amplification of primer set 2 (968f/1406r) was performed in a thermal block by using 35 reaction cycles, each consisting of the following steps involving initial denaturation at 94˚ C for 5 min, 94˚C for 1 min, 55˚C for 1 min, 72˚ C for 2 min and final extension at 72˚C for 10 min. The PCR product was analyzed on 1.5% and 2% agarose gel respectively with ethidium bromide staining.

RESULTS AND DISCUSSION

Table 1-Physical analysis of soil

Sample Site	pH	% Moisture Content	% CO_3^{2-} content
Bijwasan, Delhi	7.12	4.98	8.71
Sonepat, Haryana	7.68	2.78	10.04
Aligarh, UP	7.10	3.66	9.68
Gaya, Bihar	6.73	2.59	9.90
Kernal, Haryana	7.15	3.81	10.02

The physical and chemical properties of the soils from different sample site, used in DNA extraction were quite different (Table 1). The pH of soils as measured by pH meter ranged between 6.73 to7.68. Similarly % moisture content was found to be maximum in Bijvasan, New Delhi Soil and minimum in Gaya, Bihar soils indicating their texture to be different. Sonepat soil was characterized by highest Carbonate content of 10.04% per g dry soil and the reason may be high pH content and presence of free CO_3^{2-} ions

Extraction of DNA from soil is always problematic and any particular method will have its own predicaments. The extraction methods are strongly influenced by several parameters such as incomplete lysis, DNA degradation and contaminants such as aromatic proteins and humic acids. The co-extracts of soil DNA act as a strong inhibitor of enzyme based downstream processes such as PCR and RFLP which requires contamination free sites [18,19]. Molecular analysis of soil DNA require a DNA extraction method that produce DNA of high molecular weight, free from inhibitors such as humic acids and fulvic acids and that, representative of all microbes within the soil sample. Typically there are two steps to extract DNA from prokaryotes and eukaryotes: disruption of cells to release the contents, and extraction of DNA from lysate using a phenol/chloroform/alcohol mixture and ethanol/isopropanol precipitation [20]. Though numerous methodologies are available for soil DNA extraction which yield good results they are time consuming and laborious, and most often the DNA extract is not completely purified. The different methodology differs on the account of chemical and mechanical force used for cell disruption and DNA precipitation. In order to obtain purified DNA in lesser time three methods of Soil DNA extraction were studied in parallel. As shown in table 2 and figure 1 the purity and quality of DNA extracted from test samples with different methods have notable difference. Although high molecular weight DNA (23 kb) is obtained in all the three methods, different methods gave rise to yield and purity discrepancies.

Table2. DNA yield and purity

	R1	R2	Conc.	R1	R2	Conc.	R1	R2	Conc.
Bijvasan, Delhi	1.456±0.01	1.680±0.03	40.98±1.34	1.082±0.002	1.248±0.15	33.6±2.31	0.886±0.081	1.147±0.008	41.13±2.50
Sonepat, Haryana	1.238±0.008	1.524±0.11	35.85±1.60	0.987±0.008	1.138±0.006	21.15±1.45	0.832±0.007	1.011±0.014	35.05±1.65
Aligarh, U.P	1.235±0.098	1.328±0.006	40.74±1.95	0.883±0.010	1.107±0.091	31.35±2.88	0.710±0.003	0.98±0.0071	37.6±2.03
Gaya, Bihar	1.246±0.041	1.410±o.065	36.15±1.08	0.988±0.006	1.198±0.063	24.6±0.75	0.83±0.068	1.117±0.011	21.6±1.98
Karnal, Haryana	1.436±0.005	1.634±0.12	39.75±1.17	0.996±0.004	1.10±0.003	29.88 ±1.89	0.844±0.001	1.198±0.63	39.81±1.17

Shown is the comparative detail of methods ASM, ASN and ASA respectively in terms of R1 (A260/A230), R2(A260/A280) and Concentration of DNA in µg/g dry soil. Where R1 and R2 indicate humic acid and aromatic proteins contamination respectively

The results show that, ASA method, got the highest total DNA yield (41.1µg/g dry soil), but was associated with the lowest A260/A230 and A260/A280 ratios indicating high humic acid and protein contamination respectively. ASM method yielded the best DNA with lighter broad spectra and good yield (40.98±1.34 µg/g dry soil) and purity ratio of (A260/230 and A260/280) 1.456±0.01 and 1.680±0.03 respectively. Therefore ASM method proved to be an efficient and reproducible method for DNA extraction as it yields yields good quality DNA with minimum shearing and impurity whereas with ASN and ASA methods, DNA is sheared and may not be a good starting material for further analysis. The use of $Cacl_2$ in method 1 (ASM method) increases the efficiency of the protocol by eliminating impurities like humic acids and fulvic acids. Humic acids were precipitated by $CaCl_2$ at a concentration higher than 1% whereas a concentration higher than 4% results in DNA precipitation [21].

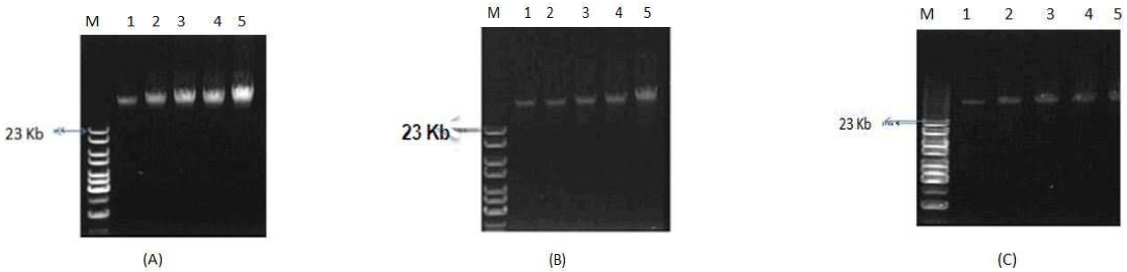

Figure 1 DNA extracted with different methods. (A) Five soil samples from different regions was extracted by method 1 as described above. M: marker digested by λ-*Hind* III: Lane 1-5 test samples. (B) soil samples from different regions was extracted by method 2 as described above. M: marker digested by λ-*Hind* III: Lane 1-5 test samples. (c) soil samples from different regions was extracted by method 3 as described above. M: marker digested by λ-*Hind* III: Lane 1-5 test samples.

Figure-2: Agarose gel electrophoresis of the V6-V9 fragments amplified by different DNA. M: DL100 bp; Lane-1 and 4: DNA extracted by method-1 with and without dilution.Lane-2 and 5: DNA extracted Lane-3 and 6: DNA extracted by method-3

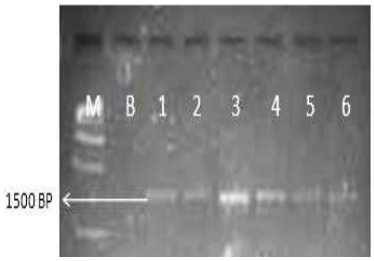

Figure-3: Agarose gel electrophoresis of the complete 16S r RNA gene fragment amplified by different DNA. M: DL1000 bp. Lane B: Blank Lane-1-by method-2; 6: DNA extracted by ASM method

To further evaluate the authenticity of most effective method for downstream processes PCR analysis was done. PCR amplification of soil extracted DNA is a good indicator of purity of the sample as humic acid contaminants can inhibit subsequent molecular reactions such as Restriction enzyme digestion, PCR [22,23] and DNA-DNA hybridization in dot plot assays [24]. DNA isolated using different methods were amplified with universal bacterial primers in PCR reactions. The results obtained in the present study show that DNA extracted by the ASM protocol was sufficiently purified for PCR amplification as shown in fig. (2 & 3).

PCR amplification of bacterial 16S rRNA gene and V6-V9 fragment was successful by ASM method only. Rest of the two methods did not show any amplicon. This result indicates that ASM method yield good quality DNA and is therefore more suitable for microbial molecular biology research. It is also observed that PCR product showed poor spectra band when undiluted DNA extracts obtained by ASM method, were used. This might be due to persistence of even a negligible amount of substances that interfere with Taq DNA polymerase. Routine dilution of the extract prior to PCR at a ratio of 1:10, yielded good results (Fig 2).

The DNA extraction procedure namely ASM method should allow the extraction of good quality DNA from wide geographical reign. The procedure is simple, consumes less time and can be routinely applied for DNA extraction

from multiple soil samples at the same time. It is better than the previous protocol as it require simple purification step and the entire procedure takes less than two hour for extraction of good quality DNA.

CONCLUSION

In conclusion the effectiveness of three soil DNA extraction methods, which based on different lytic principles for isolation of the total farmland microbial DNA were compared after making some important modification in pre existing methods. The results show that, method ASA, got the highest total DNA yields, but with the highest contamination which strongly restrained the PCR analysis. Method ASM yielded the best DNA with the highest molecular weight and purity and was more propitious to molecular ecology research. Although, DNA size analyzed by the reference λ *Hind* III marker was found to be >23kbps in community DNA by all the three methods, Method ASM yielded purest form of DNA as compared to the other two methods. The above said method was applicable on all soil types. 17.7-40.98(µg/g pellet), of DNA was obtained with a purity ratio (A260/A230 and A260/A280) of 1.456 and 1.680 respectively. The DNA was used directly for PCR by making 10 fold dilutions and showed good results.

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