© 2023, Centre for Advanced Research in Agricultural Sciences
Research Journal of Agricultural Sciences
Volume 14; Issue 02 (Mar-Apr 2023); pp 362–367

Open Access

ISSN: 0976-1675 (P)
ISSN: 2249-4538 (E)

*Full Length Research Article*

# An Optimized PEG-NaCl Protocol for Soil DNA Isolation from Wheat Rhizosphere Region for Metagenomics Applications

Deenali N. Patel*[1] and Farida P. Minocheherhomji[2]

[1-2] B. P. Baria Science Institute, Navsari - 396 445, Gujarat, India

## Abstract

In order to unravel the unexplored microbial community, it is necessary to develop an inhibitor-free DNA isolation procedure. One of the major challenges for soil metagenomics study is to develop a method to capture the diverse characteristics of soil microbial communities. During soil DNA isolation, one of the co-extractant is humic acid which will interfere with the PCR amplification. In the present work, three different PEG-NaCl methods for soil DNA isolation were compared with a commercial kit available. Here we have developed an optimized PEG-NaCl protocol for the extraction of soil genomic DNA from two different wheat crop rhizosphere. For efficient cell lysis, we have used a combination of physical (heating), chemical, and mechanical lysis method. To remove humic acid impurities calcium chloride ($CaCl_2$) and polyvinylpolypyrrolidone (PVPP) were incorporated in lysis buffer. To precipitate DNA, Polyethylene glycol (PEG), Sodium chloride (NaCl) and Sodium acetate was used. An additional purification of extracted DNA was done by using Chloroform: Isoamyl mixture and spin column and then eluted with TE buffer. The yield of the DNA isolated by using modified method was 57.33±2.3115 ug/g of soil. The purity of DNA extracted by using this method was equivalent to 1.8. As compared to the commercial kit available, the modified PEG-NaCl method gave good quality of DNA suitable for the downstream applications like sequencing and cloning. The DNA isolated by this method served as a template for PCR amplification using Prokaryotic 16s primer. This method will provide a good quality DNA for subsequent metagenomic analysis.

***Key words*:** Wheat rhizosphere, Soil DNA isolation, PEG (Polyethylene Glycol) - NaCl (Sodium Chloride), Humic acid, PVPP (Polyvinylpolypyrrolidone), Metagenomics

Soil DNA isolation is a key step towards a successful metagenomics project. The DNA extracted from the soil samples are often contaminated by humic acid substances which is an inhibitory agent to PCR enzymes. So, for successful DNA extraction procedure the DNA extracted must be free of any contaminants and should be suitable for PCR and other downstream applications like cloning. The present study provides a modified protocol for soil DNA extraction which is validated by its 16s PCR amplification. The significance of this protocol is that the researchers will be helpful in extracting DNA which is of high quality and quantity applicable for all the metagenomics procedures.

In order to study the uncultivable microbial communities, present in soil, the use of molecular biology techniques is a must. There is a huge amount of information present in the genes present in the soil community. In order to study them, Soil DNA isolation is a foremost step to be performed [1]. Major problem lies in the DNA extraction from soil is co-extraction of humic acid along with DNA. The humic acids have same charge and size characteristics that it often isolated with the DNA of interest. This will create problem in downstream processes such as PCR [2]. The presence of humic acid will influence the quality and purity of DNA as well as affects the downstream processes such as PCR, restriction digestion and molecular cloning [3]. One perquisite forms a successful metagenomic approach is isolation of high molecular weight DNA free of any impurities. There are two main steps involved in soil DNA isolation protocol viz. Cell lysis and precipitation and purification of extracted DNA. Cell lysis is done by physical, mechanical, and chemical or combination of all the three methods. For the precipitation of DNA, different chemical components such as NaCl, Sodium acetate, Potassium acetate, Polyethylene glycols, ethanol and isopropyl alcohols are often used [4]. For the purification of DNA, Phenol-chloroform-isoamyl alcohol as well as spin columns are used widely [5].

## MATERIALS AND METHODS

*Soil sample*

Received: 07 Dec 2022; Revised accepted: 12 Feb 2023; Published online: 06 Mar 2023

**Correspondence to:** Deenali N. Patel, B. P. Baria Science Institute, Navsari - 396 445, Gujarat, India, Tel: +91 9726668660; E-mail: deenalidhangar123@gmail.com

**Citation:** Patel DN, Minocheherhomji FP. 2023. An optimized PEG-NaCl protocol for soil DNA isolation from wheat rhizosphere region for metagenomics applications. *Res. Jr. Agril Sci*. 14(2): 362-367.

Four soil samples were collected from two different regions of Gujarat cultivating wheat viz. Two samples ($G_1$ and $L_1$) collected from South Gujarat region (21.124857 Longitude, 73.112610 Latitude) and another two samples ($N_1$ and $N_2$) were collected from North Gujarat region. Samples from each site were well mixed, air dried, grounded and stored at -20 °C until further analysis. The soil samples were then subjected to physico-chemical analysis by different methods as shown in (Table 1). Soil pH, moisture, total potassium, organic matter, and organic carbon were measured according to FCO protocol-1985. The total phosphorus present in soil was measured as per the protocol given by Sommers *et al*. [6], and available phosphorus was determined by Olsen *et al*. [7]. Total nitrogen estimation was done by methodology as per Boltz *et al*. [8].

Table 1 Physicochemical characteristics of soil samples

| SITE | ID | pH | Moisture (%) (FC O-1985) | Total phosphorus (Kg/ha) | Available phosphorus (kg/ha) | Total potassium (mg/g) | Organic matter (%) | Organic carbon (%) | Total nitrogen (mg/kg) |
|---|---|---|---|---|---|---|---|---|---|
| South Gujarat | $G_1$ | 7.38±0.05 | 18.41±0.744 | 0.2819±0.002 | 3.5908±0.117 | 0.1337±0.040 | 31.33±2.100 | 18.17±1.220 | 15.36±0.231 |
| | $L_1$ | 7.58±0.02 | 9.00±0.099 | 0.1652±0.007 | 2.0699±0.075 | 1.3332±0.040 | 28.73±3.100 | 16.663±1.801 | 2.46±0.213 |
| North Gujarat | $N_1$ | 7.62±0.03 | 11.66±0.642 | 0.1987±0.007 | 2.3131±0.071 | 1.6011±0.027 | 14.46±0.503 | 8.39±0.292 | 68.52±0.882 |
| | $N_2$ | 7.30±0.05 | 11.00±0.115 | 0.2036±0.004 | 2.2348±0.071 | 1.6653±0.048 | 15.80±1.090 | 9.16±1.104 | 15.33±0.189 |

*Methods for DNA extraction*

Four DNA extraction methods were evaluated in this study with respect to the quality and purity of extracted DNA using two different types of rhizospheric soils. A soil DNA extraction kit, PEG-NaCl method and two modified PEG-NaCl method were compared for obtaining a high recovery DNA with good yield and purity.

*Method 1: DNA extraction using Soil DNA Extraction Kit (HiPurA™ Soil DNA Purification Kit): ("HiMedia- Soil DNA extraction kit- MB-542,")* [9]

DNA was extracted from soil according to the manufacturer's instruction (HiMedia). This method involves cell lysis by using soil lysis buffer and bead beating method, followed by incubating at 65 $^0$ C for 20 mins and vortexing for 10 mins. The supernatant was collected after centrifugation at 13,000 g at room temperature for 1 minute and mixed with 250 ul IRSH (Inhibitor Removal Solution). Vortex and incubate at 40$^0$ C for 5 minutes. Centrifuge at 13,000 g for 1 minute and add 1.2 ml binding buffer solution into supernatant. Load the bound DNA onto the spin column. Centrifuge at 13,000 g for 1 minute. Wash the column twice with the wash solution and centrifuge it at 13,000 g for 2 minutes. Elute the DNA by adding 100 ul elution buffer and centrifuge at 13,000 g for 1 minute.

*Method 2: DNA Extraction Using PEG-NaCl Method* [5]

Take 250 - 500 mg of soil sample. Add 750 ul soil lysis buffer (120 Mm $Na_2HPO_4$, 10% SDS, 0.1g PVPP). Keep /incubate it at 65$^0$ C for 1 hr. Centrifuge at 7,000 g at 40$^0$ C for 5 minutes. Transfer the supernatant to a new collection tube. Add ½ volume of 50% PEG and 1 volume of 0.6 M NaCl. Mix the solution by gently vortexing/ inverting the tubes 3-4 times. Add 1 volume of chloroform: isoamyl alcohol (24:1). Mix the solution by gently inverting the tubes. Centrifuge at 13,000 g at 4$^0$ C for 1 minute. Collect the aqueous phase and add ¼ volume of 3M sodium acetate (pH 5.2) . Add 2 volumes of ice-cold ethanol (100% ethanol). Mix the solution by gently inverting the tubes 3-4 times. Incubate overnight at -20 $^0$C which results in high yield of purity of DNA. Centrifuge at 13,000g for 1 minutes at 4$^0$ C. Remove the ethanol and air dry the pellet for 15- 20 minutes. Dissolve the pellet in 100ul TE buffer.

*Method 3: DNA Extraction Using PEG-NaCl Column Based:* [10]

Take 250-500 mg f soil sample. Add 900 ul soil lysis buffer (120 mM $Na_2HPO_4$, 10% SDS, 0.1 g PVPP). Mix by inverting or gently vortexing. Vortex for 10 minutes. Keep / incubate it at 65$^0$C for 20 minutes. Vortex for 10 minutes. Centrifuge at 13,000 g at 4$^0$ C for 5 minutes. Transfer the supernatant to a new collection tube. Add 1/10th volume sodium acetate (3 M), ½ volume PEG (50%) and 1 volume NaCl (0.6 M). Allow to precipitate at -20$^0$ C for 45 minutes. Thaw the tube and centrifuge at 13,000 g for 5 minutes at 4 °C. Discard the supernatant. Dissolve the pellet in TE buffer (500 ul). Add 500 ul binding buffer solutions (equal volume - 30% binding buffer (5M Guanidine Thiocyanate, 70% alcohol) into supernatant. Load onto the spin column. Centrifuge at 13,000 g for 1 minute. Wash the column twice followed by 1$^{st}$ wash - Add 500ul wash solution (50% ethanol) into the column and centrifuge it at 12,000 rpm for 1 minute and 2$^{nd}$ wash - Add 500 ul wash solution (70% ethanol) and centrifuge at 13,000 g for 1 minutes. Add 100 ul of elution buffer. Centrifuge at 13,000 g for 1 minute.

*Method 4: DNA Extraction Using PEG-NaCl Modified Method:* [10]

Take 250 - 500 mg of soil sample. Add glass beads. Add 900 ul Extraction buffer (120 mM $Na_2HPO_4$, 10% SDS, 0.1 g PVPP). Add 200 ul $CaCl_2$ (200 mM). Mix by inverting or gently vortexing. Vortex for 10 minutes. Keep/ incubate it at 65$^0$ C for 1 hr. Vortex for 10 minutes. Centrifuge at 13,000 g at 4$^0$ C for 5 minutes. Transfer the supernatant to a new collection tube. Repeat the lysis step (500 ul extraction buffer+ 100 ul $CaCl_2$). Add 1/10$^{th}$ volume sodium acetate (3M), ½ volume PEG (50%) and 1 volume NaCl (0.6 M). Allow to precipitate at -20$^0$ C for 45 minutes. Thaw the tube and centrifuge at 13,000 g for 2 minutes at 4$^0$ C. Discard the supernatant. Dissolve the pellet in TE buffer (300 ul). Add 1 volume chloroform: isoamyl alcohol (24:1). Centrifuge at 13,000 g for 1 minute. Take the aqueous phase in new Eppendorf tube. Add 1 volume binding buffer (30% binding buffer, 70% ethanol) . Load onto the spin column. Centrifuge at 13,000 g for 1 minute. Wash the column twice followed by 1$^{st}$ wash- Add 500ul wash solution (50% ethanol) into the column and centrifuge it at 12,000 rpm for 1 minute and 2$^{nd}$ wash- Add 500 ul wash solution (70% ethanol). Centrifuge at 13,000 g for 1 minute. Add 100 ul of elution buffer (TE) and centrifuge at 13,000 g for 1 minute.

*Analysis of extracted DNA*

*Qualitative and quantitative analysis*

The purity of the extracted DNA was determined by taking absorbance at 230, 260, and 280 nm. A pure DNA has the $A_{260}/A_{280}$ ratio as 1.8 and the $A_{260}/A_{230}$ ratio as 2.0, whereas DNA contaminated with protein will have an $A_{260}/A_{280}$ ratio lower than 1.8 [11]. The quality of the extracted DNA was estimated by measuring $A_{260}/_{280}$ and $A_{260}/_{230}$ ratios using Nanodrop (Thermo Fischer Scientific USA) and running 5 ul of extracted DNA on 1% agarose gel electrophoresis. The quantity of DNA was estimated using 1x dsDNA High sensitivity assay kit (Invitrogen by Thermo Fischer Scientific) and measured by using Qubit 4 Fluorometer (Thermo Fischer Scientific USA).

*PCR amplification*

Soil DNA was amplified by PCR using a PCR Prima-96™ Thermal Cycler (HiMedia). Each 25 $\mu$L PCR mixture contained 2 ul (1: 10 dilution) Soil DNA (approx. 10 ng/ul), 12.5 $\mu$L PCR Master Mix (Bioline), 1 $\mu$L of forward and reverse primers (0.5 $\mu$M). The 16S rRNA regions were amplified by using 16S rRNA primers, namely, (FD1) 5′-CAGAGTTTGATCCTGGCTCAG -3′ and (RD1) 5′-AAGGAGGTGATCCAGCC- -3′ [12]. The amplification cycle consisted of an initial denaturation step of 3 minutes at 94°C, followed by 30 cycles of 1 minute at 94°C (denaturation), 1 minute at 59°C (annealing), and 1 minute at 72°C (extension), with a final extension step for 3 min at 72°C. For visualizing PCR products, 5 $\mu$L of the amplified product was electrophoresed on 1% agarose gel in 1X TAE buffer, stained with ethidium bromide (EtBr 0.5 $\mu$g/mL) and analyzed by gel documentation system (HiMedia).

## RESULTS AND DISCUSSION

For studying cultivation independent microorganisms, it is essential to isolate good quality of DNA free of PCR inhibitors. Soil DNA isolation is influenced by many factors such as cell lysis, DNA bound to soil particles, co-extraction of humic acid, degradation of DNA and many more. For effective cell lysis, both mechanical and chemical lysis should be employed [13). In the present study, physical, mechanical, and chemical methods were employed for efficient cell lysis. In the case of Soil DNA isolation by HiMedia (Fig 1) and PEG-NaCl method (Fig 3-4), it resulted in poor quality DNA and it was not suitable for PCR amplification [5], [14]. In case of PEG-NaCl Column based (method 1), DNA yield and purity was increased but still not suitable for PCR for all the soil samples (Fig 5-6). On the counterpart in the PEG-NaCl Chloroform-isoamylalcohol method DNA purity and yield was very good and was suitable for PCR amplification for all the four samples (Fig 7-8). The comparison of yield and purity of the all four methods are shown in the (Table 2).

Table 2 Comparison of yield and purity of DNA Extraction methods

| Method | DNA yield (ug/g) | A260/280 | A230/260 |
|---|---|---|---|
| HiPurA™ Soil DNA Purification Kit | 3.276667±1.9007 | 0.93±0.0435 | 0.24±0.0929 |
| PEG-NaCl [5] | 3.17±1.9600 | 0.91±0.0650 | 0.49±0.0602 |
| PEG-NaCl Modified method 1 | 7.85±1.0500 | 1.45±0.0550 | 0.68±0.0602 |
| PEG-NaCl Modified method-2 | 54±7.14 | 1.81±0.1159 | 1.97±2.1947 |

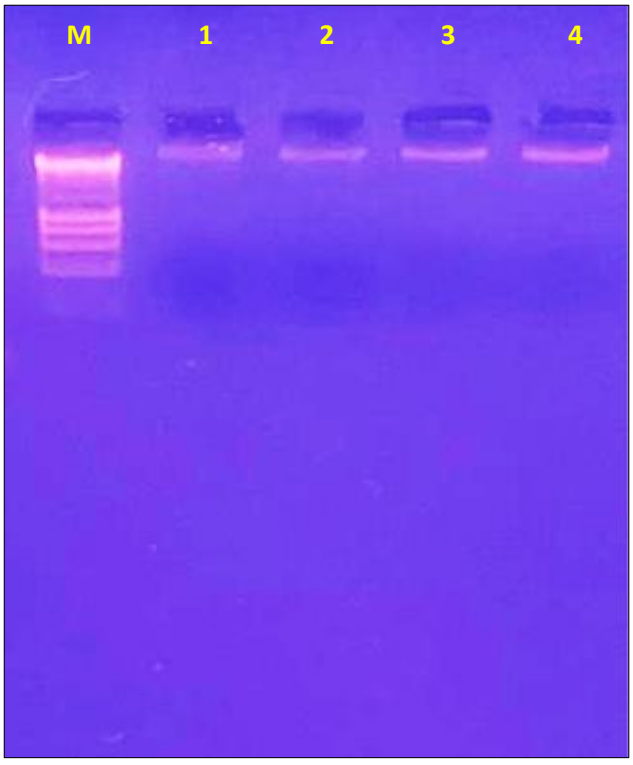


Fig 1 Soil DNA extraction by Hi Media Kit: Lane M- DNA ladder (1Kbp), Lane 1- G1, Lane 2- L1, Lane 3- N1, Lane 4- N2


Fig 2 Visualization of PCR amplification products of soil DNA isolation using 16S rRNA by HiMedia protocol: Lane M- 1Kbp Ladder, Lane1- Control, Lane 2- G1, Lane 3- L1, Lane 4- N1 and Lane- N2

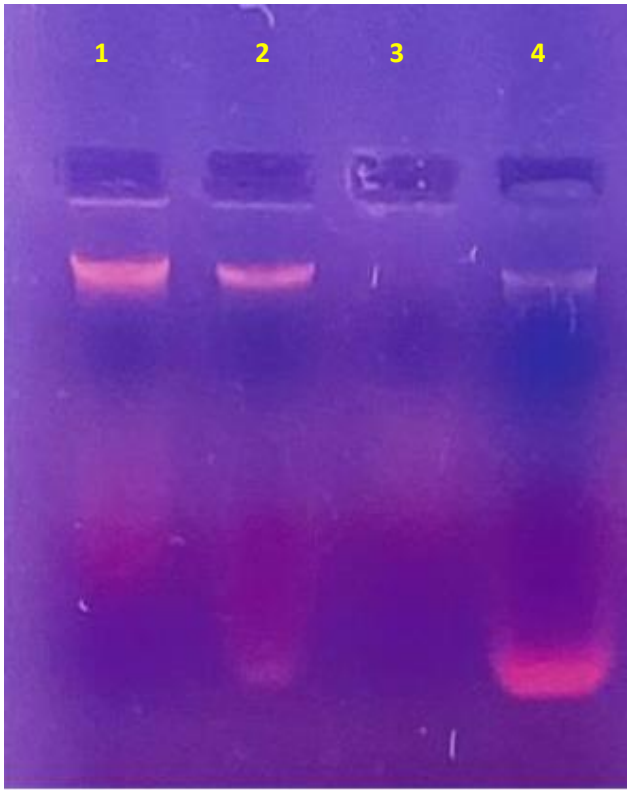


Fig 3 Soil DNA extraction by PEG-NaCl method: Lane M-DNA Ladder (1Kbp), Lane 1- G1, Lane 2- L1, Lane 3- N1, Lane 4- N2

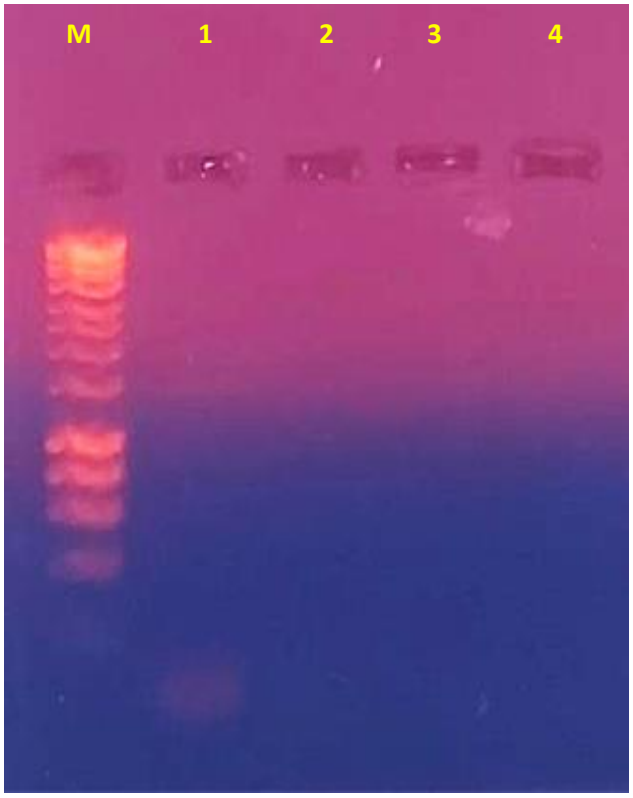


Fig 4 Visualization of PCR amplification products of soil DNA isolation using 16S rRNA by PEG-NaCl method: Lane M- 1Kbp Ladder, Lane1- G1, Lane 2- L1, Lane 3- N1, Lane 4- N2

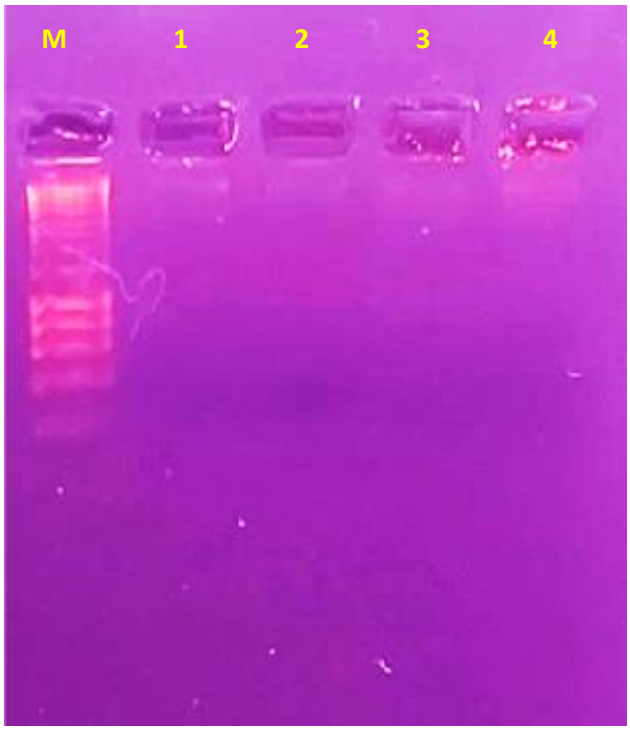


Fig 5 Soil DNA extraction by PEG-NaCl (Column based-Method 1): Lane M-DNA Ladder (1Kbp), Lane 1- G1, Lane 2- L1, Lane 3- N1, Lane 4- N2.

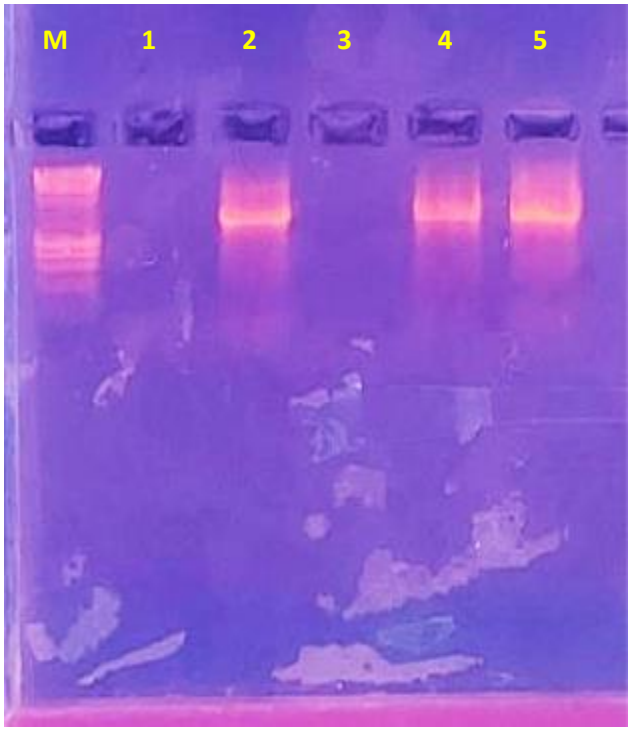


Fig 6 Visualization of PCR amplification products of soil DNA isolation using 16S rRNA by PEG-NaCl (Column based-method 1) : Lane M- 1Kbp Ladder, Lane1- Control, Lane 2- G1, Lane 3- L1, Lane 3- N1 and Lane-N2

For efficient removal of humic acid, PVPP was incorporated in the lysis buffer in the PEG-NaCl method. But alone PVPP was not sufficient enough to remove humic acid impurities; therefore PEG- NaCl method was modified by the addition of spin column step. Still purity of DNA (Deoxyribonucleic acid) was not good enough. To increase DNA purity, furthermore, Chloroform: isoamyl alcohol was added to remove impurities like organic compounds and other phenolic impurities [15]. This modification gave good results in terms of DNA yield and purity. The DNA yield and Purity obtained through PEG-NaCl modified method has been shown in (Table 3).

Table 3 The DNA yield and Purity by PEG-NaCl modified method

| PEG-NACL (Modified) | DNA yield (ug/g) | A260/280 | A230/260 |
|---|---|---|---|
| $G_1$ | 8.69±0.7217 | 1.61±0.0602 | 1.91±0.8444 |
| $L_1$ | 8.88±0.6671 | 1.77±0.0208 | 2.46±0.1106 |
| $N_1$ | 57.33±2.3115 | 1.70±0.1159 | 1.97±2.194 |
| $N_2$ | 12.08±1.2204 | 1.69±0.0862 | 1.75±0.2977 |

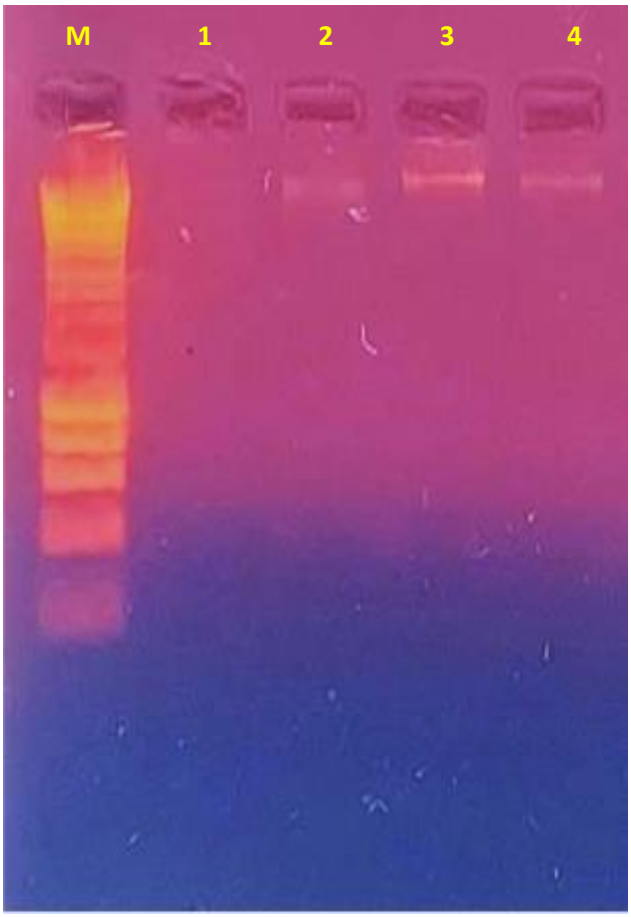


Fig 7 Soil DNA extraction by PEG-NaCl (Modified- Method 2): Lane M-DNA Ladder (1Kbp), Lane 1- G1, Lane 2- L1, Lane 3- N1, Lane 4- N2

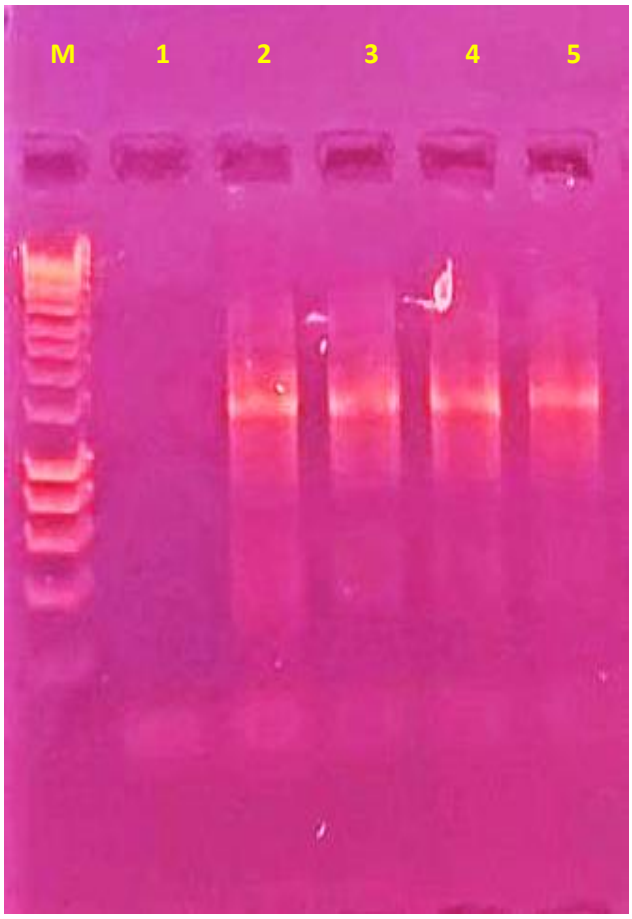


Fig 8 Visualization of PCR amplification products of soil DNA isolation using 16S rRNA by PEG- NaCl (Modified-method 2) : Lane M- 1Kbp Ladder, Lane1- Control, Lane 2- G1, Lane 3- L1, Lane 4- N1 and Lane-N2

16s rRNA PCR amplification was carried out to check the quality of the isolated DNA of all the four methods. In the case of method 1-3, PCR amplification was not profound for all the samples as compared to method 4. High quality PCR amplicons was observed in both the modified PEG-NaCl method. In each case, an amplified product corresponds to the expected size (~1500 bp). For PCR amplification, of soil DNA it requires humic acid free DNA which might otherwise interfere with the DNA polymerase enzyme. Extensive purification steps are required to remove the humic acid impurity which is a cost-effective procedure. The DNA isolated by using PEG-NaCl modified method was suitable for PCR amplification by using prokaryotic 16S Universal primers. The study suggested that addition of purification of chloroform: isoamyl alcohol in the PEG-NaCl column-based method gave very good yield of DNA which was suitable for amplification for both types of rhizospheric soil as compared to other three methods. It also proves that addition of PVPP in the lysis buffer precipitated out humic acid impurities which might otherwise interfere with the PCR amplification.

## CONCLUSION

In the present study, efficient soil DNA extraction procedures have been reported, which are simple and efficient and do not require elaborate instrumentation and yield good quality DNA suitable for the metagenomics applications. The present study showed that an additional step of using PEG-NaCl- Sodium acetate with inclusion of chloroform: isoamyl alcohol was useful to achieve desirable purity of isolated DNA. The PCR amplification procedures involve several enzymatic reactions where the enzyme DNA polymerase requires sites, which should be contamination-free. It is suggested that the initial treatment with PVPP Polyvinylpolypyrrolidone) and $CaCl_2$ will precipitate will remove polyphenolic compounds such as humic acid present in the soil. PEG-NaCl-Na acetate will enhance DNA concentration. Thus, these modified PEG-NaCl method help not only in providing good yield of DNA but also provides good quality of extracted soil DNA applicable for downstream processing such as PCR amplification and cloning procedures. These modified protocols may offer an easy

method for metagenomics applications such as microbial population dynamics and studies such as gene expression.

***Acknowledgement***

*The authors of this study acknowledge the Wheat Research Station, Bardoli for providing soil samples for conducting the present study*.

## LITERATURE CITED

1. Stursa P. 2009. Approaches for diversity analysis of cultivable and non-cultivable bacteria in real soil. *Plant Soil Environ.* 389-396.
2. Zhou J, Bruns MA, Tiedje JM. 1996. DNA recovery from soils of diverse composition. *Appl. Environ. Microbiology* 62(2): 316-322. Doi: 10.1128/aem.62.2.316-322.1996.
3. Wnuk E, Waśko A, Walkiewicz A, Bartmiński P, Bejger R, Mielnik L, Bieganowski A. 2020. The effects of humic substances on DNA isolation from soils. *Peer Journal* 8: e9378.
4. Narayan A, Jain K, Shah AR, Madamwar D. 2016. An efficient and cost- effective method for DNA extraction from athalassohaline soil using a newly formulated cell extraction buffer. *3 Biotech* 6(1): 62. https://doi.org/10.1007/s13205-016-0383-0
5. Kamble SS, Gunasekaran A, Gawankar SA. 2020. Achieving sustainable performance in a data-driven agriculture supply chain: A review for research and applications. *International Journal of Production Economics* 219: 179-194.
6. Sommers LE, Nelson DW. 1972. Determination of total phosphorus in soils: a rapid Perchloric acid digestion procedure. *Soil Science Society of America Journal* 36(6): 902-904.
7. Olsen SR. 1954. *Estimation of available phosphorus in soils by extraction with sodium bicarbonate* (No. 939). US Department of Agriculture.
8. Boltz DF. 1958. Colorimetric determination of nonmetals. *Colorimetric determination of non-metals.* Chemical Analysis, Vol. VIII. Interscience Publishers, New York-London 1958. 1. Aufl., XII, 372.
9. HiPurA Soil DNA purification kit- MB542 Kamble, Asmita & Singh, Harinder. (2020). Different Methods of Soil DNA Extraction. BIO-PROTOCOL. 10. 10.21769/BioProtoc.3521.
10. Fatima F, Pathak N, Rastogi Verma S. 2014. An improved method for soil DNA extraction to study the microbial assortment within rhizospheric region. *Mol. Biol. Int*. 2014: 518960. Doi: 10.1155/2014/518960. Epub 2014 Sep 15. PMID: 25302120; PMCID: PMC4181777.
11. Russell JS. 2001. *Molecular Cloning—A Laboratory Manual.* Cold Spring Harbor Laboratory Press.: Cold Spring Harbor, NY, USA.
12. Velazquez-Sepulveda I, Orozco-Mosqueda, Ma. Del Carmen, Prieto-Barajas C, Santoyo, Gustavo. 2012. Bacterial diversity associated with the rhizosphere of wheat plants (*Triticum aestivum*): Toward a metagenomic analysis. *Phyton* 81: 81-87. 10.32604/phyton.2012.81.081.
13. Yeates C, Gillings, Michael, Davison AD, Nanda A, Duncan V. 1998. Methods of microbial DNA extraction from soil by PCR amplification. *Biological Procedures Online* 1: 40-47. 10.1251/bpo6.
14. Devi SG, Fathima AA, Radha S, Arunraj R, Curtis WR, Ramya M. 2015. A rapid and economical method for efficient DNA extraction from diverse soils suitable for metagenomic applications. *PLoS One* 10(7):e0132441. Doi: 10.1371/journal.pone.0132441. PMID: 26167854; PMCID: PMC4500551.
15. Verma SK, Singh H, Sharma PC. 2017. An improved method suitable for isolation of high-quality metagenomic DNA from diverse soils. *3 Biotech* 7(3): 171. Doi: 10.1007/s13205-017-0847-x.