International Journal of Microbial Science, ISSN (online), 2582-967X, Volume 6, Issue 2, October 2025, pp. 53-57
Available at https://internationaljournalofmicrobialscience.com/

Original Article

Contaminated soil of the Malshiras, Bhuleshwarnagar, Purandar Taluka, Pune District reports the presence of uncultured bacteria

[1]Beg IR, [2]Dhanure RG,[3] Phulaware MS, [4]Dhakane RD
[1,4]Department of Microbiology, Jayawantrao Sawant Commerce and Science College, Hadapsar, Pune, Maharashtra, India.

Article Info

Article history:
Received: September 04, 2025
Accepted: October 16, 2025
Published: October 30, 2025

Corresponding Author:Dhakane RD
Email: rajeshdhakhane001@gmail.com

Keywords: uncultured bacteria, 16S rRNA sequencing, BLASTn, Neighbor joining method, Kimura 2 -Parameter technique.

Abstract

This study investigates the presence of residing microbial communities in the contaminated soil. Soil health in Purandar region is diverse due to agricultural and geological landscape. While previous studies have documented various soil properties and nutrient indices across Pune, the microbial and ecological impact of localized soil contamination and local waste accumulation in areas like Malshiras, Buleshwar Nagar remains under reported. The primary objective of this study is to assess the microbial diversity or non-conventional bacterial species of contaminated soil. Soil sample was collected from specific impacted area with reference to control and was analysed through DNA extraction and PCR products were amplified through universal (forward and reverse) primers through sequencing of 16S rRNA gene for molecular identification to bypass the traditional culture methods to identify the microbial communities present in contaminated soil. Sequence analysis using the BLASTn against NCBI of Genbank database reveals the presence of uncultured bacterium. The presence of this unique microbial diversity shaped by contamination in environmental conditions highlights the hidden presence of bacteria and recommends on further biochemical research and metagenomics studies to evaluate metabolic studies in the Malshiras Village.

1. Introduction:

Microorganisms play a vital role in degrading the contaminants in polluted soils. As indispensable members of soil ecosystem, microorganisms drive the biochemical breakdown and synthesis of these microbial communities are central to sustaining nutrient cycling and enhancing soil fertility through successive

Biochemical breakdown and synthesis of these microbial communities are central to sustaining nutrient cycling and enhancing soil fertility through successive stages [1]. The efficacy of this process stems from the evolutionary adaptability of microorganisms to shifting environments and generally known as the primary drivers of pollution [1].

Contaminants and xenobiotic are discharged into the environment by commercial, agricultural activities or inappropriate storage. Hence, contaminants in soil are one of the major concerns in the environment. These Xenobiotic or recalcitrant compounds did not get degraded by bacteria present in soil. If these

compounds did not get degrade, they cause soil pollution lead to imbalance of ecosystem [2].

Soil associated microorganisms also exhibit in high heterogeneity making it difficult for cultivating them in laboratory. Some bacterial taxa arising from the soil environment may exhibit in relatively slow growth rates or require complex source and develop bacterial taxa originating insights by the specific nutrient supply, such as when bacteria exhibit interdependent nutrients relationship in their natural soil environment [3]. Over 33% of major bacterial lineages are solely identified via rRNA sequence data & represented exclusively by environmental molecular signatures because they are highly unknown for uncharacterized taxa and microbial dark matter constituents of significant source of biochemical and metabolic profiles [4].

For sequence alignment and taxonomic classification the full length 16S rRNA gene (1,500bp) is sufficiently large [5]. However, nearly full length of 16S rRNA gene from numerous bacterial genera can be amplified using just one pair of primer through partial sequencing (500bp), the other primers are effective for abundance of distinct sequences [6]. For instance, using this gene sequencing method has revealed notably view of broad range of microorganisms and their groups in soil or ecological conditions [7].

In this study, we identified that the soil present in Malshiras Village contains uncultured bacterium by cultured based technique through isolating uncountable colonies and using 16sRNA sequencing gene marker. Many less research has been done on the role of uncultured bacteria in contaminated soil, hence, this research has been undertaken. This work provides insights into microbial diversity of uncultured bacteria in contaminated soil and establishes a foundation for future bioremediation research.

2. Materials and Methods:

2.1 Sample collection and processing

Soil sample was collected from slightly contaminated site of Malshiras Village, Purandar taluka, Pune district, Maharashtra, India. Then, it was aseptically transferred to laboratory of Jayawantrao Sawant Commerce and Science college, Pune, Maharashtra, India and stored at 4^0C in refrigerator. For serial dilution, an initial amount of soil suspension is diluted to reduce the number of microbes present in soil samples [8]. A measured volume of the diluted sample was spread onto solidified nutrient agar medium and incubated plates for 24 hours at room temperature to allow the growth of isolated colonies of microbes present in sample (figure1).

The isolated colonies are picked and streaked onto fresh nutrient agar slants until a pure culture was obtained [9]. The pure culture was submitted to Pro Genome Life Science Pvt. Limited, Chhatrapati Sambhajinagar, Maharashtra, India for further molecular identification through 16S rRNA Gene marker by partial sequencing (figure 2).

2.2 Molecular identification

2.2.1 DNA Extraction and PCR amplifications

The genomic DNA of the bacterial isolate was extracted by ProGenome Life Science by DNA Extraction kit (In House Microbial Kit) following manufactures manual instructions. The quality of isolate was verified by 1% of concentration agarose gel electrophoresis [10]. The resulting DNA was observed on gel and noted using the Vilber documenting system [11].

The fragments of isolate‘s 16S rRNA gene amplified by the universal primer such as 27 forward primer (AGAGTTTGATCCTGGCTCAG) and 1492 reverse primer (GGTTACCTTGTTACGACTT) [12]. After resolved on 1.2% agarose gel, the single band of distinct PCR amplicons was obtained at 52^0C (figure 3) [11]. The PCR reaction composition consists of HiPCR® Master Mix 12.5 µl, the forward 1.5 µl and DNA template of 3.0 µl and nuclease free water 6.5 µl (table.2) for 16S rRNA gene amplification.

2.2.2 Cycle sequencing and purification

The Big Dye Terminator V.3.1 Cycle Sequencing Kit (Applied Bio systems, Inclusions) was implemented for the cycle sequencing reaction by subsequent purification of exonuclease-I and Shrimp Alkaline Phosphatase enzymes to eliminate unincorporated DNA strands and leftover primers [13]. The conditions for thermal cycle were 35 cycles of 30 seconds at 96^0C, 15 seconds at 55^0C, and 4 minutes at 60^0C after an initial denaturation period of 2 minutes at 96^0C were maintained. Cycle sequencing was followed by ethanol precipitation for sequencing cleanup, template disintegration in HiDi formamide, and bidirectional sequenced in ABI 3730 Genetic analyzer [14].

2.2.3 Sequence alignment and assembly phylogenetic analysis

The PRISM 3730×I Genetic analyser (Applied Bio systems, USA) was used to process the PCR products for direct bi-directional sequencing. CLUSTAL W in MEGA 11 was used to align the resultant DNA sequence, which was then manually edited and trimmed to provide full

sequence [15], [20]. The sequence similarity was then used to confirm the species. The BLASTn tool was used to perform homology searches against NCBI GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). All places with gaps and locking data information were included for examination when the phylogenetic tree was constructed with neighbour joining method [17]. 1000 bootstrap re-sampling were used to compute clade supports [16],[18].

3. Results:

The present study conducted on identification of uncultured bacterium present in the contaminated soil by using culture base technique and molecular identification 16S rRNA gene sequencing.

3.1 Isolation, sample preparation by cultured based technique

Isolated colonies were observed on nutrient agar plate after incubation at 37^0C for 24 hours (figure 1). Distinct colonies were picked and streaked onto nutrient agar slants. Pure culture was obtained with uncountable colonies of bacteria after incubation at 37^0C for 24 to 48 hours (Figure 2).

Figure 1: Isolation of microbes on solidified nutrient agar.

(a) (b)

Figure 2: Obtained pure culture of bacteria.

2.2 Molecular Identification through 16SrRNA Gene Marker

The amplification of the 16S rRNA region of the bacterial consortia by using (forward and reverse primers; table 1)

Table 1: Preparation of polymerase chain reaction composition.

Component	Component Volume
HI-PCR® REDy Master Mix	12.5 µl
Forward primer	1.5 µl
Reverse primer	1.5 µl
Template DNA	3.0 µl
Nuclease free water	6.5 µl
Total reaction volume	22 µl

The PCR amplification of 16S rRNA gene was successfully done for identification of uncultured bacterium (RSGO1 Sample). A single identifiable band at about 1500 base pair found by gel electrophoresis was in line with targeted region's anticipated size. There was no primer dimer or general amplification observed.

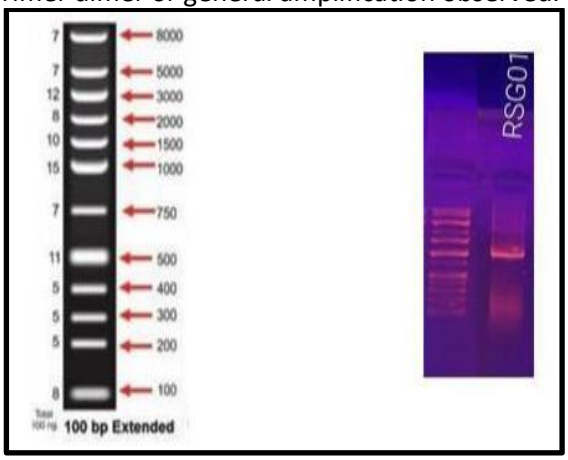

Figure 3: PCR product was obtained on 1.2% agarose gel by gel electrophoresis. The PCR products shows a single band confirming successful amplification at targeted region of 16S rRNA gene at 1500bp using 27 forward, 1492 reverse primer.
Lane 1: DNA Ladder at 100bp extended
Lane 2: PCR product of uncultured bacterium species (RSGO1 sample)

By using Bio edit software, the consensus sequence was generated from forward and reverse sequence data in fasta file format as follows:

GACTNCCTTATGGGTAGAAAANTGCATCTGGTTNNGACTA
AACCGCNCTACGGGTAGTGANNNGAATGTACCTGAAGAA
TATGGATCGGGCAGCTCCGTGNCTGCAGCCGCGGTAATAC
GNAGTATGCNAGCGTTATCCTGATTTATTGGGTTTAAATCG
TGCGTATGCGTCACTATAAGTCAGGGGTGAACGACGGCAG
TTCAACTGTCGCAGTGCCCTTGATACTGAAGTGATTGAATG
CGGTTGAAGACGGCGGCATGAGACAAGTAGCGGTGAAAT
GCATAGATACGTCTCAGAACACCGATCGCGAAGGCACCTG
TATAAGCCGTTATTGACGCTGATGCACGAAAGCGTAGGGA
TTGACCAGGATTAGATACCCTGGTAGTCCACGCCCTAAACG
ATGATGACTTGAGGTTTGCGATATACCGTAAGCGTCCAAA

GAAAGCGTTAAGTGGTCCACGTGGGGAGTACGCCCACTAA
AGTGAAATCAAAGGAATTGCGCGGGGCCCGCACAAGCGG
GGGAACTTGTGGTTTAATTGGATGATACGACCGGAACCTT
ACCCGGGCATGAAAGTTAATGAAGGGAGCAGAGCCGTCTC
GATCTTTAGGACCGGGAAACTAGGGGCGTCATGGGCTTGG
TTAGTTAGATCCTGGGGTTGTTGGGTTAAGTCCCGCAACGA
GCGTAACCCGTTTGTTTAATGTACAGCAGGTAAATGTGGG
GAGTCTAAACGGACCACATGCGCAGGAAGAGGGGAAGGG
GGGAGGACGTCAAGTCATCATGGCCATTACGTCCGGGGTT
ACACACTTGTTACAATGGATGGTACAGCGGGCAGGTA
CATAGCACTGTGATGCCAATCTCGAAAAGCTATTCACAGTT
CGGATCGGGGTCTGCAACTCGACCCCGTGAAGTTGGATTC
GGTAGTAATCGCATATCAGCAATGACACGGTGA

According to NCBI gene bank database, the matrix showed 1096 maximum score, 100% query coverage, 0.0 E-value (table 2).

Table 2: RSGO1 sample showing similarity searches in sequence alignment

Sr. no	Sample ID	Description	Max. Score	Total Score	Query cover age	E-value	Percent Similarity
1	RSGO 1	*Uncultured bacterium*	1096	1096	100%	0.0	87.07%

The neighbor joining approach was used to deduce evolutionary history. The evolutionary history of bacterial taxa under study was assumed to be represented by the bootstraps consensus tree which was derived from 1000 replicates. Partitions that were replicated in less than 50% of bootstrap replicates were collapsed.

The Kimura 2 parameter technique was used to calculate the evolutionary distances which were expressed in terms of number of base pair substitution per site. In this investigation, 12 nucleotides sequence was involved. For all base pair sequences, ambiguous positions were eliminated (pairwise deletion options). In the final dataset, there were 1483 positions. MEGA 11 was used to carry out evolutionary studies.

4. Discussion:

Microbial examination of polluted soil samples isolated from Malshiras village, Bhuleshwar Nagar, Purandar Taluka, Pune District resulted in the isolation and culture of bacterial strains in the laboratory by cultured based technique and identification through partial 16S rRNA gene sequencing.

Molecular evaluation revealed that this isolate showed substantial sequence similarity to bacterial taxa that have previously been reported as uncultured or poorly documented in public databases. The presence of these bacterial taxa in polluted soils may be connected with local environmental variables, including farming practices and anthropogenic factors [21] [22]. Environmental stressors can influence microbial community composition by favoring species capable of adapting to specific ecological niches [23].

The fact that these isolates are most common in slightly polluted soils suggests that they may be important for the environment. These organisms might have metabolic pathways engaged in breakdown processes that were previously unknown as these soil microbes may have key roles in organic matter breakdown, nitrogen cycling, and maintenance [24]. Enzymes produced from uncultured soil bacteria often exhibit structural traits linked with adaptability to certain environmental circumstances, suggesting their role in ecosystem functioning and pollutant transformation [25].

The successful cultivation and identification through partial 16S rRNA of isolates related to previously uncultured species constitutes a crucial step toward understanding their ecological functions and physiological properties [26]. However, this finding recommends further metagenomic studies and biochemical research is required to demonstrate their unique metabolic abilities management.

5.Conclusion:

This study investigated that the contaminated soil of Malshiras Village, Purandar region consists of uncultured bacterium identified by 16S rRNA gene sequencing method.

Author contributions: GD: collected sample, SP: Performed experiment for preparation of sample, RB: wrote the manuscript, RD: Developed an idea and verified the manuscript.

Competing interest: Authors declare that no conflict of interest exists.

Ethical statement: This work does not violate ethical guidelines

Grant support details: The authors did not get funding from any agency to complete this work.

Acknowledgement: The authors are thankful to the Principal, Vinod Kulkarni, Jaywantrao Sawant Commerce and Science College, Hadapsar, Pune, Maharashtra, India. They are also thankful to Mrs. Archana Ghadge, head of department of Microbiology, Dr. Sudipta Banerjee, Mrs. Pradnya Joshi, Mr.

Sunil Hadke, Mrs. Dipali Banakar for their support in this work.

References:

1. Mbachu AE, Chukwura EI, Amalachukwu M. Role of microorganisms in the degradation of organic pollutants: a review. Energy Environ Eng. 2020;7(1):1-11.[Google Scholar]
2. Bodor A, Bounedjoum N, Vincze GE, Erdei J, Palagyi A, Kovacs K, et al. Challenges of unculturable bacteria: environmental perspectives. Rev Environ SciBiotechnol. 2020;19(1):1–22.[ResearchGate]
3. Kazarina A, Wiechman H, Sarkar S, et al. Recovery of 679 metagenome-assembled genomes from different soil depths along a precipitation gradient. Sci Data. 2025; 12:521.[PubMed]
4. Harris JK, Kelley ST, Pace NR. New perspective on uncultured bacterial phylogenetic division OP11. Appl Environ Microbiol. 2004;70(2):845-849.[PubMed].
5. Janda JM, Abbott SL. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. J ClinMicrobiol. 2007;45(9):2761-2764. .[PubMed] [ResearchGate]
6. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol. 1991 Jan;173(2):697-703..[PubMed]
7. Smets W, Leff JW, Bradford MA, McCulley RL, Lebeer S, Fierer N. A method for simultaneous measurement of soil bacterial abundances and community composition via 16S rRNA gene sequencing. Soil BiolBiochem. 2016; 96:145-151.[Google Scholar]
8. Sanders ER. Aseptic laboratory techniques: plating methods. J Vis Exp. 2012;(63): e3064.[PubMed].
9. Janssen PH, Yates PS, Grinton BE, Taylor PM, Sait M. Improved culturability of soil bacteria and isolation in pure culture using liquid serial dilution. Appl Environ Microbial. 2002;68(5):2391-2396..[PubMed]
10. Lee PY, Costumbrado J, Hsu CY, Kim YH. Agarose gel electrophoresis for the separation of DNA fragments. J Vis Exp. 2012;(62):3923. .[PubMed]
11. Kharel Sitaula R,Janani MK, Madhavan HN , Biswas J. Outcome of polymerase chain reaction (PCR) analysis in 100 suspected cases of infectious uveities. J Ophthalmic Infect. 2018;8(1):2.[PubMed]
12. Frank JA, Reich CI, Sharma S, Weisbaum JS, Wilson BA, Olsen GJ. Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. Appl Environ Microbiol. 2008;74(8):2461-2470.[Google Scholar] [PubMed]
13. Hanke M, Wink M. Direct sequencing of PCR products using exonuclease I and shrimp alkaline phosphatase. BioTechniques. 1994;17(5):858-860.
14. Swerdlow H, Gesteland R. Capillary gel electrophoresis for rapid, high-resolution DNA sequencing. Nucleic Acids Res. 1990;18(6):1415-1419.[PubMed]
15. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994;22 b(22):4673-4680..[PubMed]
16. Altschul SF, Gish W, Miller W, Myers E.W, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215(3):403–410.[PubMed]
17. Saitou N, Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. MolBiolEvol. 1987;4(4):406-425.[PubMed]
18. Felsenstein J. Confidence limits on phylogenies: An approach using the bootstrap. Evolution. 1985;39(4):783-791.[PubMed]
19. Kimura M. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J MolEvol. 1980;16(2):111-120.[PubMed]
20. Tamura K, Stecher G, Kumar S. MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. MolBiolEvol. 2021; 38(7):3022-3027.[PubMed]
21. Delbari AS, Kulkarni DK, Saptarshi PG. Heavy metal contamination in soils along highways near Pune, India. Int J PollutAbat Technol. 2012;1(1):35-39. [Research Gate]
22. Patturajan V, Jaybhaye RG, Shouche Y. Effect of shifting cultivation on soil health and microbial diversity in BhorTaluka of Pune District. Indian J Nat Sci. 2021 Aug;12(67):33102-33110.[Research Gate]
23. Allison SD, Martiny JB. Resistance, resilience, and redundancy in microbial communities. ProcNatlAcadSci U S A. 2008 Aug 12;105 Suppl 1:11512-9. [PubMed]
24. Murphy CL, Sheremet A, Dunfield PF, Spear JR, Stepanauskas R, Woyke T, et al. Genomic analysis of the yet-uncultured Binatota reveals broad methylotrophic, alkane-degradation, and pigment production capacities. mBio. 2021;12 (3):e00985-21. [PubMed]
25. Bhattacharya S, Panda AK. In silico analysis of phylogeny, structure, and function of enzymes from uncultured soil bacteria. BMC Microbiol. 2021;21(1):89.[PubMed]
26. Joseph SJ, Hugenholtz P, Sangwan P, Osborne CA, Janssen PH. Laboratory cultivation of widespread and previously uncultured soil bacteria. Appl Environ Microbiol. 2003;69(12):7210-5.[PubMed]

Cite this article as: Beg IR, Dhanure RG, Phulaware MS, Dhakane RD. Contaminated soil of the Malshiras, Bhuleshwar Nagar, Puranda rtaluka, Pune district reports the presence of uncultured bacterium. Int.J.Micro 2025;6(2):53-58.

APPENDICES

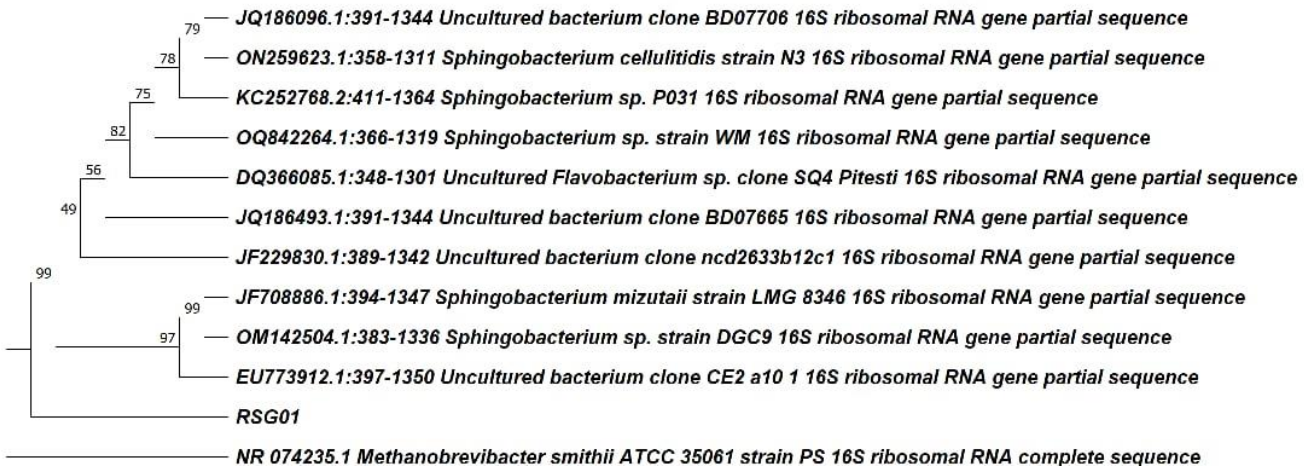

Figure 4: Phylogenetic analysis of the bacterial culture with sample RSGO1 ID under study.

Abbreviations:
rRNA: Ribosomal Ribonucleic Acid
°C: Degree Celsius
PCR: Polymerase Chain Reaction
DNA: Deoxyribonucleic acid
µL: Microliter
Clustal W: Cluster Alignment Weighted
MEGA 11: Molecular Evolutionary Genetics Analysis Version 11
NCBI: National Center Biotechnology Information