IJNRD.ORG ISSN : 2456-4184
INTERNATIONAL JOURNAL OF NOVEL RESEARCH AND DEVELOPMENT (IJNRD) | IJNRD.ORG
An International Open Access, Peer-reviewed, Refereed Journal

# Isolation, Identification and Characterization of Bacteria of Coal Mine Soil at Pandwa of Rajhara Coalfield, Jharkhand

**Manoj Kumar Singh**

Research Scholar, Department of Chemistry, R C I T, University of R C U Bishrampur, Palamu, Jharkhand.

**Dr. K.K Mishra**

H. O. D, Department of Chemistry, R C I T, Bishrampur, Palamu, Jharkhand.

## Abstract

Mining is the process of extracting useful materials from the earth. The dust and noxious fumes inhaled by miners make them vulnerable to pulmonary diseases. The water sources in the region get contaminated due to mining. Dumping of waste and slurry leads to degradation of land, soil, and increase in stream and river pollution. Mining means any naturally occurring substance from earth for useful purposes. During this mining process, several changes take place in the physical, chemical, and microbiological characteristics of soil as a result of storage. Soil is a system, in which constant interface between soil minerals and microorganisms control the physicochemical and biological properties of global ecosystem. Microbial properties have been reported to be useful indicators of soil quality and could possibly serve as assessment criteria of successful rehabilitation of ecologically disturbed areas. The present study was conducted to determine dominant bacterial species in the soils of Sonepur Bazari at Raniganj coalfield. During this study, the soil samples were collected in different sites and the bacteria was isolated, identified biochemically and characterized by phylogenetic analysis using 16SrRNA sequencing technique. Two bacterial isolates, i.e. S-1 and S-2 were obtained by using Nutrient agar medium. The isolates were identified morphologically, biochemically and also by phylogenetic analysis using 16SrRNA sequencing technique. The molecular identification of 16SrRNA gene sequences showed that the isolates had 95% similarity to the genus. The results showed that between the two different strains Bacillus cereus is a common soil bacteria whereas Bhargavaea cecembensis is a newly found bacteria in soil.

**Keywords:** Bacillus cereus, Bhargavaea, cecembensis, Rajhara Coalfield, 16S rRNA, Analysis, soil bacteria

## INTRODUCTION

### Isolation of Bacterial isolate

The soil microorganisms were isolated by serial dilution technique on nutrient agar medium (NAM). One gram of soil from sample were separately suspended in 10 ml of distilled water and mixed well for 15 minutes and vortexes. Each suspension was serially diluted from 10-1 to 10-6. Each soil habitat has unique and well adapted microbial guilds whose functionalities maintain its nutritional status, degrade pollutants and control different pests, anddiseases. Bacteria are found everywhere that researchers have been clever enough to sample. They are found in the deepest ocean sediments, the highest atmospheric altitudes, at extremes of temperatures and ice, associated with the most heavily polluted sites. Soils contain phylogenetic groups of bacteria that are globally distributed and abundant in terms of the contributions of individuals of those groups to total soil bacterial communities. However, only a few bacteria have been reported to live in the soil of coal mines (20). Coal mine spoil overburden represents a physically disturbed habitat for the existence of soil organism (5, 8, and 10) due to internal high temperature (6, 18) and low pH (2, 9). Currently, most soil bacteria belong to phylogenetic groups that have few or no known representatives (7). The characterization of the microbial community within a soil sample is a very useful tool in determining the overall health of the soil. Measurement of the soil microbial community may certainly be used to determine biodiversity, ecological processes and structures. A comprehensive determination of soil microbial community characteristics is one way of approach for the success of restoration processes. Characterization is a very broad term that can cover many aspects of the soil microbes
(19) .The diversity of the bacterial communities in soil is extraordinary. In recent years, real-time PCR has emerged as a promising tool for studying soil microbial communities. This technique allows a relatively rapid yet quantitative assessment of the abundances of specific phylogenetic groups of microorganisms in soil (9). Measurement of the microbial community has utility as an indicator of the reestablishment of connections between the biota and restoration of function in degraded system. The application of 16SrRNA sequence analysis has, however, revolutionized the study of both microbial ecology and phylogeny. Hence, simply trying to raise the number of responsible microorganisms, a better understanding of the geo-microbiology of that area may provide the scientific foundation for more practical and effective remediation strategies. Therefore in the present work, thebacteria were isolated from the soils of Pandwa area at Rajhara Coalfield and cultured by Nutrient Agar medium.

## MATERIALS AND METHODS

- Survey of study area

Pandwa of Rajhara Coalfield Limited, Jharkhand, India

Rajhara Coalfields- spreading over Palamu, Garhwa, in Jharkhand and Aurangabad in Bihar. Rajhara Coalfield is, however, in Palamu, District bank of Koel River.

- Sampling

The soil samples were collected from Sonepur Pandwa of Rajhara Coalfield region. Top soil was scrapped off and about 100 gm soil samples were collected and mixed thoroughly, put in a polythene bag and sealed with rubber bands then carried into the laboratory for analysis.

- Microbiological analysis

By using nutrient agar medium and serial dilution method microbiological study were performed. Microbial colonies were enumerated as colony forming unit (CFU). Viable cells will be assessed from the plates after incubation at 37°C in a BOD incubator.

- Subculture

It was done by streak plate method taking the isolated colonies of bacterial cultures which were obtained from spread plate method and again incubated at 37°C for 24-48 hrs.

- Identification of Bacteria

Identification of the selected isolates was carried out morphologically, Biochemically and also using 16 SrRNA sequencing.

- 16SrRNA Sequencing

16s rRNA sequencing is a culture-free method to identify and compare bacterial diversity from complex microbiomes or environments that are difficult to study. It is commonly used to identify bacteria present within a given sample down to the genus and/or species level. In research, 16S rDNA PCR will continue to be used to identify novel bacterial species, characterise species-specific pathogenicity and as a gold-standard assay to compare against when evaluating new assays. It is also used in combination with cutting-edge techniques, such as next-generation sequencing.

Broad-range 16S ribosomal RNA (rRNA) gene polymerase chain reaction (PCR) is used for detection and identification of bacterial pathogens in clinical specimens from patients with a high suspicion for infection.

- **Nucleic acid extraction and purification**

- **DNA Extraction**

1. Lysis/homogenization: Cells grown in monolayer should be lysed by suspend 1-3 colonies aseptically and mixed with 450 µl of "B Cube" lysis buffer in a 2 ml microcentrifuge tube and lyse the cells by repeated pipetting.
2. Add 4 µl of RNAse A and 250 µl of "B Cube" neutralization buffer.
3. Vortex the content and incubate the tubes for 30 minutes at 65˚C in waterbath. To minimize shearing the DNA molecules, mix DNA solutions by inversion.
4. Centrifuge the tubes for 15 minutes at 14,000 rpm at 10 ˚C.
5. Following centrifugation, transfer the resulting viscous supernatant into a fresh 2 ml micro centrifuge tube without disturbing the pellet.
6. Add 600 µl of "B Cube" binding buffer to the content and mix thoroughly by pipetting and incubate the content at room temperature for 5 minutes.
7. Transfer 600 µl of the contents to a spin column placed in 2 ml collection tube.
8. Centrifuge for 2 minutes at 14,000 rpm and discard flow-through.
9. Reassemble the spin column and the collection tube then transfer theremaining 600 µl of the lysate.
10. Centrifuge for 2 minutes at 14,000 rpm and discard flow-through.
11. Add 500 µl "B Cube" washing buffer I to the spin column. Centrifuge at14,000 rpm for 2 mins and discard flow-through.
12. Reassemble the spin column and add 500 µl "B Cube" washing buffer II and Centrifuge at 14,000 rpm for 2 mins and discard flow-through.
13. Transfer the spin column to a sterile 1.5 ml microcentrifuge tube.
14. Add 100 µl of "B Cube" Elution buffer at the middle of spin column. Careshould be taken to avoid touch with the filter.

15. Incubate the tubes for 5 minutes at room temperature and Centrifuge at 6000rpm for 1min.
16. Repeat the above mentioned step 14 and 15 for complete elution. The buffer in the microcentrifuge tube contains the DNA.
17. DNA concentrations were measured by running aliquots on 1% agarose gel.
18. The DNA samples were stored at -20°C until further use.

- **PCR**

Polymerase chain reaction (abbreviated PCR) is a laboratory technique for rapidly producing (amplifying) millions to billions of copies of a specific segment of DNA, which can then be studied in greater detail. PCR involves using short synthetic DNA fragments called primers to select a segment of the genome to be amplified, and then multiple rounds of DNA synthesis to amplify that segment.

Polymerase Chain Reaction (PCR) is a process that uses primers to amplify specific cloned or genomic DNA sequences with the help of a very unique enzyme. PCR uses the enzyme DNA polymerase that directs the synthesis of DNA from deoxynucleotidesubstrates on a single-stranded DNA template. DNA polymerase adds nucleotides to the 3` end of a custom-designed oligonucleotide when it is annealed to a longer template DNA. Thus, if a synthetic oligonucleotide is annealed to a single-stranded template that contains a region complementary to the oligonucleotide, DNA polymerase can use the oligonucleotide as a primer and elongate its 3` end to generatean extended region of double stranded DNA.

- **Primer details:**

| Primer Name | Sequence Details | Number of Base |
|---|---|---|
| 27F | AGTGTTTGATCMTGGCTCAG | 20 |
| 1492R | TAGGGYTACCTTGTTACGACTT | 22 |

Add 5 µl of isolated DNA in 20 µl of PCR reaction solution (1.5 µl of Forward Prime and Reverse Primer, 5 µl of deionized water, and 12 µl of Taq Master Mix). Perform PCR using the following thermal cycling conditions:

1.Denaturation 2. Annealing 3.

Extension

- **Purification of PCR Production**

PCR purification produces pure DNA strands for use in a multitude of applications including ligation, enzyme digestion, sequencing, labeling experiments, microarray analysis, and in vitro transcription, and it can be done quickly, efficiently, and in a cost effective manner.

Removed unincorporated PCR primers and dNTPs from PCR products by usingMontage PCR Clean up kit .The PCR product was sequenced using the 27F/1492R primers.

- **Sequencing**

Single pass sequencing means one directional sequencing either of any of 5 prime or 3 prime site of either forward or reverse primer of gene of interest or plasmid.

Single-pass sequencing was performed on each template using below 16SrRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to

electrophoresis in an ABI 3730xl Sequencer. The 16Sr RNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of query sequence with the closely related sequence of blast result was performed followed by multiple sequence alignment.

## RESULTS AND DISCUSSION

During this study, two bacterial isolates, i.e. S-1 & S- 2 were isolated from soil of Pandwa at Rajhara Coalfield Limited, Palamu, India, by using Nutrient agar medium culture. The molecular identification of 16SrRNA gene sequences showed that the isolates had 95% similarity to genus *Bacillus* sp and *Bhargavaea* sp. Sequence analysis of the 16SrRNA genes of 2 representative strains revealed that all of the strains were closely related to strains which have been sequenced previously and also confirmed the phylogenetic diversity of bacteria present in coal miningenvironments.

**Table 1:** Bacterial Characteristics

| Bacteria | Sporulation | Cell Shape | Gram staining | Motility | Colony Shape | Colony Color |
|---|---|---|---|---|---|---|
| *Bacillus cereus* | Spore former | Rods | + | Motile | Circular | White |
| *Bhargavaea cecembensis* | Non-spore former | Rods | + | Non-motile | Circular | White |

***Figure 1: Bacillus cereus***

- **16S Ribosomal RNA Gene, Partial Sequence**

AAAGGCACGATAACTACTTAGGTTAACCTTGGTTACCACAATCGTAACCC
CAGATAAAACTCTATGGTCAAACTCTCGTGGTGTGACGGGCGGGGAGTAC
AAGGACCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACAAGCG
ATTCCAGCTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAAAACG
GTTTTATGAGATTAGCTCCACCTCGCGGTCTTGCAGCTCTTTGTACAGTCC
ATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTAACGT
CATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAAAGTGACCAA
CTTAATGATGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACATAAC
CCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTAACTC
TGCTCCCGAAGGAGAAGCCCTATCTCTAGGGTTTTCATAAGATGTAAAGA
CCTGGTAAAGTTCTTCACGTTGCTCCAATTAAACCACATGCTGCACAGCTT
GTGCGGGCCCCCGTCTATTCCTATGAGATTTAGCCTTCCGGCAGTAATCCT
CCTGGGGAGTAGTTAAAGCTGTAAAATCTGCACTTAAGAATGGAAACAGT
CTCTCTATGAGTAGTCTAGAATCTCGGGGAGGGGTGACAAAGGGGASTAT
GATTCAAGTTAGGACGCCAGGGTGTGCTCACTGTCTGTGATATAGAAGCG
AGAGTGTTACTGGGTTGTGGTTTTTGTGATTCAGATTAGTATGATTAGGAG
GGGTAAGGCTTATAGTTTAGTTGGTTTGGTGTGCGCGCACATCTTTATCTT
GGTTGTGGAGTTAGGTGTTTGG GGG

***Figure 2: Bhargavaea cecembensis***

- **16S Ribosomal RNA Gene, Partial Sequence**

ACTAAGGGTACAGGAGCTACGCTCAAGGTTTGATCCATGGCTCGGAGTCA
TACACGGTATCCTTAAATTTTCGGCGGACGGGTGAGTATCACATGAGCAA
CCTGCCTGCAAGATCGGGATAACTCCGGGAAACCGGGGCTAATACAGGAT
GGTTCCTTCCTCCGCATGGAGGAAGGCGGAAAGACGGTTTCTGCTATCAC
TTGCAGATGGGCCCGCGGCGCACTATCTGGTTGGTGGGGTAATGGACCAC
CAAGGCGACGATGCGTAGCCCACCTGAGAGGGTGATCGGCCACAATGGG
ACTGAGACACGGCCCACACTCCTACGGGAGGCAGCAGTAGGGAATATTCC
GCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGCGAAGAAGCC
TTCTGGTCGTAAAGCTCTGTTGTAAGGGAAGAACAAGTGCCGGTTAACTG
CCGGTGCCCTGACGGTACCTTACCAAAAAGCCACGGCTAACTACCAGCCA
GCAACCGCGGTAATACATACGTGGCAAGCGTTCTCCAGAATCATTATAGT
GTAAAGCGCGCGCAGCGGGTTCTTAAGTCTGATCTGAGAGCCCACAGCTC
AGCCGTCGAGGGACAGTTGAAACTGGGGAACTTGAGTGCAGTAGAGGAG
AGGGGGAGTTGAACGTTGCGCGGGGGAGATGTGTCGGGGATGTGAGTGG
GAGGACGTCGGGGGGCGGGGGACTGTGGTCGGGGACGGGATGCCACAGC
GTCAGTTACAGACCAGAAAGCCGCCTTCCCCACTGTTGTTCCCTCCACATC
TCTACGCATTTCACCGCTACACGTGGAACTCCGCTTTCCTCTTCTGAACTC
AAGTTCTCCCATTTCCAATGACCCCCAACGGTTGAGCCGTGGGGCTTTCAC
AATCAAACTTAAGAAACCGCCTGCGCGCGCTTTCCCCCCAATTATTTCTG
AAAATCTCTGCCACCTACTATTACCGCAGTTGTTGATCATACTTACATT

From the soil samples two aerobic strains were positively identified where gram positive, motile, spore-forming rod *Bacillus cereus* (Table 1, Figure 1) is a well-documented soil bacteria but gram positive, non-motile, non-spore forming rod *Bhargavaea cecembensis* (Table 1, Figure 2) is an uncommon bacteria found in coal mine soil of Pandwa at Rajhara Coalfield. Mining of coal causes massive damage of biological community. But the presence of this bacterial species explores the presence of bacteria in soil samples of coal mines of Rajhara Coalfield and microorganism in soil is critical for the maintenance of soil function in both naturalAnd managed agricultural soils because of their involvement in such key processes as soil structure formation, decomposition of organic matter, toxin removal, and the cycling of carbon, nitrogen, phosphorus, and sulphur. The isolation of microorganisms from extreme conditions or contaminated sites offers microorganisms with unusual properties and activities. Studies undertaken to examine the identification and characteristics of environmental samples revealed the true diversity of microorganisms and their unique functionality which arise from their biological system that produce enzymes to make them tolerate or adapt to their environments. The use of molecular techniques adds more precision and accuracy to the phylogenetic identification and also to the true reflection of microbial diversity [20].

*Bacillus* can be used for knowledge of the active microorganisms in the coal mines is important for the development for the better/easy strategy of mining coal , recovering metals and the development of optimal in situ bioremediation strategies [10]. Whereas *Bhargavaea* usually grown mainly on waste material, deep sea ocean sediments and newly found bacteria in soil of coalmine generated wasteland.

## REFERENCES


1. Atlas, R.M., Horowitz, A., Krichevsky, M. and Bej, A.K., (1991). Responses of microbial populations to environmental disturbance. *Microb Ecol*. 22:249- 256.
2. Bushra, J., Hasan, F., Hameed, A., Ahmed, S., (2007). Isolation of *Bacillus subtilis* MH-4 from soil and its potential of polypeptidic antibiotic production. *Pak. J. Pharm. Sci*. 20(1):26-31.

3. Chatterjee, S. N., Chattoraj, K., Banerjee, P., De, M.K., and Majumdar, A., (2012). Population dynamics and diversity of soil microbes in some areas of Jalpaiguri district of West Bengal, India. *Asian Journal of Microbiol. Biotech.Env. Sc.* 15 (2): 2013: 287-290.
4. Dutta, R.K., & Agrawal, M., (2002). Effect of tree plantations on the soil characteristics and microbial activity of coal mine spoil land. *Tropical Ecology*. 43 (2): 315-324.
5. Gogoi, J., Pathak, N., Dowrah, J., Deka, Boruah, H.P., (2007). In situ selection of tree species in environmental restoration of open cast coal mine wasteland. *Pro. of Int. Sem. on MPT, allied publ*. 678 -681.
6. Johnson, D.B., (2003). Chemical and microbiological characteristics of mineral spoils and drainage waters at abandoned coal and metal mines. *Water,Air, Soil poll*. 3:47-66.
7. Joseph, S.J., Hugenholtz, P., Sangwan, P., Osborne, C.a., Janssen, P.H., (2003). Laboratory Cultivation of Widespread and Previously Uncultured Soil Bacteria. *Applied and Environmental Microbiology*. 69 (12): 7210-7215.
8. Juwarkar, A.A., Jambulkar, H.P., Singh, S.K., (2004). Appropriate strategies for reclamation and revegetation of coal mine spoil dumps. In: proceedings of the national seminar on environmental engineering with special emphasis on mining environment. Sinha, I.N., Ghose, M.K., Singh, G (eds)., *Institute of Public Health Engineers Publ*. 1-9.
9. Ledin, M., and Pedersen, K., (1996). The environmental impact of mine wastes-roles of microorganisms and their significance in treatment of mine waste. *Earth-Sci. Rev*. 41: 67-108.
10. Machulla, G., Bruns, M. A., and Scow, K. M., (2005). Microbial properties of mine spoil materials in the initial stages of soil development. *Soil Sci. Soc. Am. Jr*. 69: 1069-1077.
11. Majumder, P., (2016). Microbiology of Soil Collected from Bankola and Sankerpur Kenda of Raniganj Coalfield. *International Journal of Emerging Research in Management and Technology*. 5 (6): 113-116.
12. Majumder, P., and Palit, D., (2016). Microbial diversity of Soil in Some Coal Mine Generated Wasteland of Raniganj Coalfield, West Bengal, India. *International Journal of Current Microbiology and Applied Sciences*. 5(2): 637-641.
13. Ovreas, L., (2000). Population and community level approaches for analysing microbial diversity in natural environments. *Ecol. Lett*. 3, 236–251.
14. Pace, N.R., (1999). *Microbial ecology and diversity*. ASM News 65, 328–333.
15. Ramesh, M., Anbusaravanan, N., and Loganathan, A., (2014). Isolation, Identification And Characterization of Bacteria In Godavarikhani Open Cast –III Coal Mine Soil of the Singareni Collieries In Andhra Pradesh. *IOSR Journal of Pharmacy and Biological Sciences.* 9 (6): 38-43.
16. Sadhu, K., Adhikari, K., Gangopadhyay, A., (2012). *International Journal of Environmental Sciences.*2 (3):1675-1685.
17. Urbanova, M., Kopecky, J., Valaskova, V., Sagova-Mareckova, M.,Elhottova, D., Kyselkova, M., Moenne-Loccoz, Y. and Baldrian, P., (2011). Development of bacterial community during spontaneous succession on spoil heaps after brown coal mining. *FEMS Microbiol Ecol* 78:59-69.
18. Ward, J., Cockson, A., (1972). Studies on a thermophilic bacillus: Its isolation, properties, and temperature coefficient of growth. *J. Bacteriol*. 112:1040-1042.
19. Williams, Mitchell, D., (2007). "Characterization of Microbial Activity in Soils nearby Landfills in Northwest Florida." Electronic Theses, Treatises and Dissertations. Paper 975.
20. Williamson, J.C., Johnson, D.B., (1990). Determination of the activity of soil microbial populations in stored and restored soil at opencast coal sites. Soil Biology & Biochemistry. 22(5): 671-675.