Isolation, Identification and Characterization of Bacteria of Coal Mine Soil at Sonepur Bazari of Raniganj Coalfield, West Bengal

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Abstract
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

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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; Raniganj Coalfield; 16S rRNA analysis; soil bacteria.*

**INTRODUCTION**

Each soil habitat has unique and well adapted microbial guilds whose functionalities maintain its nutritional status, degrade pollutants and control different pests, and diseases. 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 16S rRNA 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, the bacteria were isolated from the soils of Sonepur Bazari area at Raniganj Coalfield and cultured by Nutrient Agar medium.
MATERIALS AND METHODS

- Survey of study area
  Sonepur Bazari of Raniganj Coalfield Limited, West Bengal, India
  Raniganj Coalfields- an area of 1530 sq.kms spreading over Burdwan, Birbhum, Bankura, and Purulia Districts in West Bengal and Dhanbad District in Jharkhand. Heart of Raniganj Coalfield is, however, in Burdwan District bounded by Ajoy River in North and Damodar River in South.

- Sampling
  The soil samples were collected from Sonepur Bazari of Raniganj 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 S rRNA sequencing.

- 16S rRNA Sequencing
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 water bath. 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 microcentrifuge 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 the remaining 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 at 14,000 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. Care should be taken to avoid touch with the filter.

15. Incubate the tubes for 5 minutes at room temperature and Centrifuge at 6000 rpm 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 (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 deoxynucleotide substrates 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 generate an extended region of double stranded DNA.

Primer details:

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence Details</th>
<th>Number of Base</th>
</tr>
</thead>
<tbody>
<tr>
<td>27F</td>
<td>AGAGTTTGATCMTGGCTCAG</td>
<td>20</td>
</tr>
<tr>
<td>1492R</td>
<td>TACGGYTACCTTGTTACGACTT</td>
<td>22</td>
</tr>
</tbody>
</table>

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:

Denaturation
Annealing
Extension

Purification of PCR Production

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

Sequencing

Single-pass sequencing was performed on each template using below 16s rRNA 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 3730xI
The 16s r 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 Sonepur Bazari at Raniganj Coalfield Limited, West Bengal, 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 16S rRNA 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 mining environments.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Sporulation</th>
<th>Cell Shape</th>
<th>Gram staining</th>
<th>Motility</th>
<th>Colony Shape</th>
<th>Colony Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus cereus</td>
<td>Spore former</td>
<td>Rods</td>
<td>+</td>
<td>Motile</td>
<td>Circular</td>
<td>White</td>
</tr>
<tr>
<td>Bhargavaea cecembensis</td>
<td>Non-spore former</td>
<td>Rods</td>
<td>+</td>
<td>Non-motile</td>
<td>Circular</td>
<td>White</td>
</tr>
</tbody>
</table>

**Table 1:** Bacterial Characteristics

16S Ribosomal RNA Gene, Partial Sequence

AAAGGCACGATAACTACTTAGGTTAACCTTGGTTACCACAATCGTGACCC
CAGATAAAAACCTTATGTCACACACCTCTCGTGTTGACGGGCGGGGTTGAC
AAGGACCGGGAACGTATTCCACCCGCAGCTGCATGCAGGATTACTAGGC
ATTCCAGCTTCATGTAGCAGCGAGTGGCAGCTACATCCGAACTGAGAACG
GTTTTATGAGATTAGCTCCACCTCCGCGTTGAGCTCTTTGTACCGTCC
ATTGTAGCAGCTGTGAGCCAGTGATGATGATGAGCT
CATCCCCACCTTCCTCCGTTGTCACCCGCGATCAGATCCTAAATGAGTCACA
CTTAATGATGGAACACTAGCAAGGGTTGGCTGCAGTGGGGACTTAAAC
CTGTCAGGTTGGAACACTAGCAAGGGTTGGCAGCTGTCAGTGGGGACTTAAAC
CTGTCAGGTTGGAACACTAGCAAGGGTTGGCAGCTGTCAGTGGGGACTTAAAC
CTGTCAGGTTGGAACACTAGCAAGGGTTGGCAGCTGTCAGTGGGGACTTAAAC
CTGTCAGGTTGGAACACTAGCAAGGGTTGGCAGCTGTCAGTGGGGACTTAAAC
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GATTCAAGTTAGGACGCCAGGGTGTGCTCACTGTCTGTGATATAGGAGCG
AGAGTTAAGCTGGTGTGTTGTGTTTTTGTATCAGATTAGTATGATTTTGAG
GGGTAAGGCTTATAGTCTTGTGTTTTTGGTGCTGCGCCACATCTTTTTCTT
GGTGTGGAGTATAGGTTG GGG

Figure 2: Bhargavaea cecembensis

16S Ribosomal RNA Gene, Partial Sequence
ACTAAGGGTGATACGCTCATTAGGTGCTACGCTGAGGACGTCAGGTAAC
TACACTGGTGATTCCCTGCGGGACGCGGTGATAGTGTAGAATGCTGCGG
CCTCCTGACGTGACGTCGCGGACGATGGTATGAGGTATGCTGAGTCC
GCAATGGACGAAAGTCTGAGGAGGAACCCGGTAACCTACCTACCTAGC
GCGGCAGTGCTGCGGGGACGACGTCGAGGGGAGTTGAACGTTGCGCGGG
GGATGTGGTTGGATGTGGGGGCTGCGGGGCAGTGGCACACCTACCTAC
CCTCCTGACGGAGAGGTATGCTGAGTCCGCAGGACGTCGCGGACGATGGT
AGGTAGGACGTCGCGGACGATGGTACGAGGACGTCGCGGACGATGGT
GGG

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 Sonepur Bazari at Raniganj 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 Raniganj Coalfield. And microorganism in soil are critical for the maintenance of soil function in both natural
and 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.

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