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Isolation, Identification And Characterization of Bacteria In Godavarikhani Open Cast – III Coal Mine Soil of the Singareni Collieries In Andhra Pradesh.

Ramesh.M[1*], Anbusaravanan.N[2] and Loganathan.A[3]
[1]*Assistant Professor in Zoology, Government Arts College (Autonomous), Karur District,Tamilnadu.*
[2]*Associate Professor in Zoology, Periyar EVR College (Autonomous), Tiruchirappalli District,Tamilnadu.*
[3]*Deputy Superintendent (Analytical), the Singareni Collieries Company Limited, Bhupalpalli, Warangal District, Andhra Pradesh.*

Abstract: *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 purpose of this research is to characterize the microbial community in the soils of Godavarikhani open cast – III coal mine of the Singareni collieries to determine the dominant bacterial species. During this study, the soil samples were collected in different seams and the bacteria was isolated, identified biochemically and characterized by phylogenetic analysis using 16SrRNA sequencing technique. Three bacterial isolates, i.e. BDRC1, BDRC2 & BDRC3 were obtained by using LB agar medium culture. 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 99% similarity to genus Bacillus. The results showed that the strains were closely related to each other. These bacteria are common soil bacteria that are well characterized.The characterization of microbial activity holds potential as complementary criteria for evaluating rehabilitation progress on mine discard sites.*

Keywords: *coal mine soil; soil bacteria; 16s rRNA analysis.*

I. Introduction

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. [17].

Coal mine soil also has various types of Thiobacillus sp. and Methanogene sp. Thiobacillus ferrooxidans was the first organism isolated from acidic bioleaching environments.It shares its environmental niche with other acidophilic bacteria which have a similar physiology and which can directly or indirectly compete for available inorganic substrates. It is therefore impossible as yet to define the precise role and importance of each organism in these dynamic populations, as the intimate link between microbial physiology and sulfide bio hydrometallurgy is incompletely understood [17] [11].

Coal mine spoil overburden represents a physically disturbed habitat for the existence of soil organism [3],[4],[5], due to internal high temperature profile [6],[7],and low pH [8],[9]. In spite of such extremities, the coal mine spoil is not a microbiologically sterile habitat and often harbours specific group of thermoacid tolerant, chemolithotrophic and heterotrophic bacteria [10],[11]. The earlier microbiological studies [12]on coal mine spoil overburdens of Basundhara coal field area of Mahanadi coal field limited, Orissa revealed the isolation of thermo and pH resistant Gram negative bacilli and cocci. This study revealed Gram negative bacteria (both bacilli and cocci) to be in a major proportion of total colony forming units of bacterial population in the fresh coal mine spoil. There have been also reports about the prevalence of Gram negative bacteria from coal mine spoils of different geographical regions [13],[14].

Currently, most soil bacteria belong to phylogenetic groups that have few or no known representatives [15]. It was noted that an increase in iron and sulfate reducing bacteria were more pronounced closer to the landfill. Overall, most studies conclude that the iron and sulfate-reducing species exist beneath the landfill. It is also noted that the landfill leachate does alter the chemistry of the groundwater nearby the landfill thus allowing favorable anoxic conditions for the iron and sulfate reducing bacteria [16].

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. That microbial measurement has utility as an indicator of the re-establishment of connection between the biota and restoration of function in degraded

systems. 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 [2].

The diversity of the bacterial and fungal communities in soil is extraordinary. High levels of bacterial and fungal diversity make quantifying and characterizing soil microbial communities a daunting task. In recent years, quantitative PCR (qPCR, also referred to as real-time PCR) has emerged as a promising tool for studying soil microbial communities. qPCR is based on the real-time detection of a reporter molecule whose fluorescence increases as PCR product accumulates during each amplification cycle. The qPCR approach is somewhat unique among methods of community analysis in that it allows for a relatively rapid yet quantitative assessment of the abundances of specific phylogenetic groups of microorganisms in soil [19].

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 link between soil microbial measurements and other characteristics of a mine is an important one to demonstrate if they are to be convincingly advocated for wider use as ecological indicators [20].

The microbes (bacteria and fungi) may play a significant role at the base of the overall mine drainage ecosystem by providing a supply of nutrient nitrogen. There is a need for a better understanding of mine reclamation ecosystem and their microbial origins [1]. Before we can develop and implement the next generation of remediation strategies, we need to identify the microorganisms responsible and determine how they are interrelated in this ecosystem in order to understand what conditions trigger the microbial generation of acid-mine drainage[18].

One of the major obstacles encountered in studying the ecology of these organisms is the difficulty involved in isolating, identifying, and enumerating individual species and strains from an environment which contains a plethora of strains with similar metabolic requirements. The application of 16S rRNA sequence analysis has, however, revolutionized the study of both microbial ecology and phylogeny [17].

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 at different strata of coalmine of Godavarikhani Open Cast Project – III in Ramagundam Area and cultured by means of solid and liquid media.

II. Materials And Methods

2.1. Study area

Godavarikhani Open Cast Project – III coal mine (Ramagundam Area) of Singareni Collieries Company Limited is situated in Karim Nagar District of Andhra Pradesh. Geographically Godavarikhani is located at 18.8000° N 79.4500° E. It has an average elevation of 179 meters (590 feet) and is situated in the Godavari Valley coalfields.

2.2. Collection of Samples and Isolation of Bacteria

The soil samples were collected in sterile vials from the top to bottom in various seams. The diluted samples were plated onto isolation media (LB agar) by pour plate method and incubated at 37^{o} C for 24 hours. Sub-culturing was done by streak plate method taking the isolated colonies of bacterial cultures which were obtained from pour plate method and again incubated at 37^{o} C for 24-48 hrs.

2.3. Identification of Bacteria

Identification of the selected isolates was carried out morphologically, Bio-Chemically and also using 16s rDNA ribotyping.

2.4. Nucleic acid extraction and purification

10ml of overnight grown bacterial culture was transferred into 5 eppendorf tubes and centrifuged for 5 min at 5000 rpm. Supernatant was discarded. Pellet was resuspended in 1ml of extraction buffer by pipetting up-and-down repeatedly. Suspension was transferred to a sterile 2-ml microcentrifuge tube and centrifuged for 10 min at 10000 rpm. 300 µl of both phenol and chloroform/isoamyl alcohol was added to the pellet and centrifuged for 3min at 10,000 rpm or until phases were well separated. With a sterile pipette tip, aqueous phase was transferred to a new 2 ml tube. 500 µl of chloroform was added to supernatant.

2.5. Characterization of bacteria using 16s rDNA typing

2.5.1. PCR Amplification of the 16s rDNA gene

49 µl of the "PCR mix" was pipetted in ice bucket into the 0.2 mL microcentrifuge tube. The PCR mix contains the forward and reverse primers, dNTPs, Taq polymerase, $MgCl_2$ and PCR reaction buffer. 1µL of PCR mix was added to the cell solution.

We used the following universal bacterial primers: 16s Forward (5-AGAGTTTGATCATGGCTCAG-3) and 16s Reverse (5-GGTTACCTTGTTACGACTT-3) were used to characterize the unknown bacterial species. 49 µl of the above mix was added to 1µl of prepared template DNA per PCR reaction.

2.5.2. DNA Sequencing

In our sequencing reactions, we used dideoxynucleotides labeled with different colored fluorescent tags. Also, in DNA sequencing only one primer was used, so only one of the two strands was used as a template in the sequencing reaction. Once the results of the DNA sequencing are known, we were able to search the database of known sequences for a match to this sequence. The resulted sequence was compared to the Gen Bank database at the National Centre of Biotechnological Information (NCBI) by using BLAST (Basic Local Alignment Search Tool) for sequences similarity.

III. Results

During this study, three bacterial isolates, i.e. BDRC1, BDRC2 & BDRC3 were isolated from soil from the Godavarikhani Open Cast Project – III coal mine (Ramagundam Area) of Singareni Collieries Company Limited is situated in Karim Nagar District of Andhra Pradesh, India, by using LB agar medium culture. The molecular identification of 16SrRNA gene sequences showed that the isolates had 99% similarity to genus Bacillus sp. Sequence analysis of the 16S rRNA genes of 3 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. The identified sequences were deposited in GenBank (Accession Number: KJ643909 – KJ643911).

Table1. Colony and cell characteristics of isolates

Isolate	Colony			Cell		
	Shape	Colour	Elevation	Edge	Shape	Gram reaction
BDRC1	circular	Yellowish white	convex	entire	Bacillus(Rod)	positive
BDRC2	circular	Yellowish white	convex	entire	Bacillus(Rod)	positive
BDRC3	circular	Milky white	convex	entire	Bacillus(Rod)	positive

Three bacterial isolates were obtained based on different in characteristics of colony and cell. Colony and Cell characteristics showed that they had similar shape, but all of them had distinct diameters at the same age; it indicated that all of them were of same types.

Bacillus sp. BDRC1 16S ribosomal RNA gene, partial sequence

GenBank: KJ643909.1

1 tctgttgtta gggaagaaca agtgctagtt gaataagctg gcaccttgac ggtacctaac
61 cagaaagcca cggctaacta cgtgccagca gccgcggtaa tacgtaggtg gcaagcgtta
121 tccggaatta ttgggcgtaa agcgcgcgca ggtggtttct taagtctgat gtgaaagccc
181 acggctcaac cgtggagggt cattggaaac tgggagactt gagtgcagaa gaggaaagtg
241 gaattccatg tgtagcggtg aaatgcgtag agatatggag gaacaccagt ggcgaaggcg
301 actttctggt ctgtaactga cactgaggcg cgaaagcgtg gggagcaaac aggattagat
361 accctggtag tccacgccgt aaacgatgag tgctaagtgt tagagggttt ccgcccttta
421 gtgctgaagt taacgcatta agcactccgc ctggggagta cggccgcaag gctgaaactc
481 aaaggaattg acgggggccc gcacaagcgg tggagcatgt ggtttaattc gaagcaacgc
541 gaagaacctt accaggtctt gacatcctct gaaaacccta gagatagggc ttctccttcg
601 ggagcagagt gacaggtggt gcatggttgt cgtcagctcg tgtcgtgaga tgttgggtta
661 agtcccgcaa cgagcgcaac ccttgatctt agttgccatc attaagttgg gcactctaag
721 gtgactgccg gtgacaaccg gaggaaggtg gggatgacgt caaatcatca tgccccttat
781 gacctgggct acacacgtgc tacaatggac ggtacaaaga gctgcaagac cgcgaggtgg
841 agctaatctc ataaaaccgt tctcagttcg gattgtaggc tgcaactcgc ctacatgaag
901 ctggaatcgc tagtaatcgc ggatcagcat gccgcggtga atacgttccc gggccttgta
961 cacaccgccc gtcacaccac gagagtttgt aacacccgaa gtcggtgggg taacctttt
1021 ggagccagcc gcctaaggtg ggacagatga ttggggtgaa gtcgtaacaa ggtagccgta

Bacillus sp. BDRC2 16S ribosomal RNA gene, partial sequence
GenBank: KJ643910.1

1 gtggggcgtg gcttattaca tgcagtcgag cgaatggatt aagagcttgc tcttatgaag
61 ttagcggcgg acgggtgagt aacacgtggg taacctgccc ataagactgg gataactccg
121 ggaaaccggg gctaataccg gataacattt tgaaccgcat ggttcgaaat tgaaaggcgg
181 cttcggctgt cacttatgga tggacccgcg tcgcattagc tagttggtga ggtaacggct
241 caccaaggca acgatgcgta gccgacctga gagggtgatc ggccacactg ggactgagac
301 acggcccaga ctcctacggg aggcagcagt agggaatctt ccgcaatgga cgaaagtctg
361 acggagcaac gccgcgtgag tgatgaaggc tttcgggtcg taaaactctg ttgttaggga
421 agaacaagtg ctagttgaat aagctggcac cttgacggta cctaaccaga aagccacggc
481 taactacgtg ccagcagccg cggtaatacg taggtggcaa gcgttatccg gaattattgg
541 gcgtaaagcg cgcgcaggtg gtttcttaag tctgatgtga aagcccacgg ctcaaccgtg
601 gagggtcatt ggaaactggg agacttgagt gcagaagagg aaagtggaat tccatgtgta
661 gcggtgaaat gcgtagagat atggaggaac accagtggcg aaggcgactt tctggtctgt
721 aactgacact gaggcgcgaa agcgtgggga gcaaacagga ttagataccc tggtagtcca
781 cgccgtaaac gatgagtgct aagtgttaga gggtttccgc cctttagtgc tgaagttaac
841 gcattaagca ctccgcctgg ggagtacggc cgcaaggctg aaactcaaag gaattgacgg
901 gggcccgcac aagcggtgga gcatgtggtt taattcgaag caacgcgaag aaccttacca
961 ggtcttgaca tcctctgaaa accctagaga tagggcttct ccttcgggag cagagtgaca
1021 ggtggtgcat ggttgtcgtc agctcgtgtc gtgagatgtt gggttaagtc ccgcaacgag
1081 cgcaaccctt gatcttagtt gccatcatta agttgggcac tctaaggtga ctgccggtga
1141 caaaccggag gaaggtgggg atgacg

Bacillus sp. BDRC3 16S ribosomal RNA gene, partial sequence
GenBank: KJ643911.1

1 cgaacgttgg cgacatgagt ataatgcagt cgagcggaca gatgggagct tgctccctga
61 tgttagcggc ggacgggtga gtaacacgtg ggtaacctgc ctgtaagact gggataactc
121 cgggaaaccg gggctaatac cggatggttg tttgaaccgc atggttcaga cataaaaggt
181 ggcttcggct accacttaca gatggacccg cggcgcatta gctagttggt gaggtaacgg
241 ctcaccaagg cgacgatgcg tagccgacct gagagggtga tcggccacac tgggactgag
301 acacggccca gactcctacg ggaggcagca gtagggaatc ttccgcaatg gacgaaagtc
361 tgacggagca acgccgcgtg agtgatgaag gttttcggat cgtaaagctc tgttgttagg
421 gaagaacaag tgccgttcaa atagggcggc accttgacgg tacctaacca gaaagccacg
481 gctaactacg tgccagcagc cgcggtaata cgtaggtggc aagcgttgtc cggaattatt
541 gggcgtaaag ggctcgcagg cggtttctta agtctgatgt gaaagccccc ggctcaaccg
601 gggagggtca ttggaaactg gggaacttga gtgcagaaga ggagagtgga attccacgtg
661 tagcggtgaa atgcgtagag atgtggagga acaccagtgg cgaaggcgac tctctggtct
721 gtaactgacg ctgaggagcg aaagcgtggg gagcgaacag gattagatac cctggtagtc
781 cacgccgtaa acgatgagtg ctaagtgtta gggggtttcc gcccccttagt gctgcagcta
841 acgcattaag cactccgcct ggggagtacg gtcgcaagac tgaaactcaa aggaattgac
901 gggggcccgc acaagcggtg gagcatgtgg tttaattcga agcaacgcga agaaccttac
961 caggtcttga catcctctga caatcctaga gataggacgt ccccttcggg ggcagagtga
1021 caggtggtgc atggttgtcg tcagctcgtg tcgtgagatg ttgggttaag tcccgcaacg
1081 agcgcaaccc ttgatcttag ttgccagcat tcagttgggc actctaaggt gactgccggt
1141 gacaaaccgg aggaaggtgg ggatgacgtc aaatcatcat gccccttatg acctgggcta
1201 cacacgtgct acaatggaca gaacaaaggg cag

IV. Discussion

From the soil samples, three aerobic strains of Bacillus sp. were positively identified. These three Bacillus bacteria are well documented soil bacteria and are common in the depth zone where the samples were taken. These bacteria were positively involved in the production of enzymes which are essential for catalyzing reactions for organic matter decomposition and their activities are strongly influenced by organic matter content of the soil[28].Bacterial metabolism is utilized an environmentally friendly technology to reduce organic sulfur in coal by biodesulfurization [29].

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 [17].

It has been established that the genetic diversity of soil bacteria is high and that soils contain many bacterial species of lineages for which no known cultivated isolates are available. Many soil bacteria are referred to as uncultured or even nonculturable. A range of methods have been developed to study these organisms directly in their habitats. These methods are extremely useful for studying the ecology of microorganisms as parts of communities. We believe that many of these bacteria are infact culturable using relatively simple technologies [23]. The abundance, composition, and diversity of microbial communities within soils are strongly depth dependent [24]. So, in this study bacteria found in the soil samples collected from different strata of coal mine were analyzed.

Bacteria BDRC1 and BDRC2 were found in the soil collected from the top and the soil of first seam respectively. No bacteria were found in the soil of second seam. This may be attributed to the rocky substratum which does not favour the growth of bacteria. But BDRC3was found in the soil of the third seam. Seam wise bacterial diversity data are not available in the literature and so this may be the first of its kind.

Investigations of microbial composition and diversity in natural and anthropogenically impacted or created habitats is important in the characterization of such habitats, since microbes are key players in many environmental processes. Over the last few years, cultivation-independent methodologies, particularly the sequence analysis of cloned 16S ribosomal RNA genes (16S rDNA), have proven to be powerful tools for investigating the microbial diversity of environmental samples. At least as important is the specific identification of the metabolically active microorganisms, since these are responsible for the microbially driven environmental processes. Bacillus BDRC1,BDRC2 and BDRC3 can be used for knowledge of the active microorganisms in coal mines is important for the development of a better/easy strategy of mining coal, recovering metals and the development of optimal in situ bioremediation strategies, as reported by Machulla, G., Bruns, M. A. and Scow, K. M. [5].

The characterization of the small fraction of microbes that has been cultivated provides only a glimpse of their potential physiological capacity and influence on soil ecosystems. The absence of pure cultures or genome sequences makes it difficult to ascertain the roles of specific microbes in soil environments: this is particularly true for bacteria in the phylum Acidobacteria, which are broadly distributed in soils but poorly represented in culture.[26].Further work may be designed to compare the microbial communities present in different strata of coalmine soil and to find out whether these microbes are effective in the reclamation of degraded sites and it can be used in the bioleaching processes.

V. Conclusion

In this current study there are three bacterial isolates i.e. BDRC1, BDRC2 & BDRC3which were obtained from soil samples were to explore the presence of bacteria in soil sample of coalmines of Godavarikhani OCP – III (Ramagundam Area). They were characterized and their identification was confirmed by 16S rRNA sequencing. All the three bacteria were of bacillus.sp. Colony and cell characteristics showed that they had similar shape, but all of them had distinct diameters at the same age; it indicated that all of them were of same types.

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