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RESPONSES OF MICROBIAL POPULATIONS USING METAGENOMICS OF SELECTIVE COAL MINE GENERATED WASTELANDS IN RANIGANJ COAL FIELD

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ABSTRACT

Environmental havoc we experience today is largely the outcome of deforestation, reckless mining, rapid urbanization, industrialization and overexploitation of natural and irrational use of agrochemicals. Coal exploitation can lead to destruction of terrestrial and aquatic environments thus soil, air and water become polluted and biodiversity loss occurs. Mining activities especially open cast type for coal generates a large amount of waste, deposited at the soil surface. Because of a harsh condition, these wastelands require reclamation. Different physical, chemical, and biological remediation technologies have been developed during previous decades however microbe-based technologies are more suitable in a large mining site and efficient for improving the quality of disturbed landscapes. Considering the importance of all these perspectives studies, this work was designed to assess the microbial responses by using metagenomic study in overburden dump soil of Bankola and Nagrakunda areas of Raniganj Coalfield in West Bengal.

KEY WORDS : Coal mine soil, Soil microbes, 16S rRNA based metagenome sequencing.

INTRODUCTION

Microbes are very useful indicators for spoil quality development and serve as an assessment criteria of successful rehabilitation of ecologically disturbed areas (Ramesh *et al.,* 2014). The soil microbial community characterization is a key tool for soil overall health determination. In soil, the measurement of microbial community may certainly be used for ecological structure, processes and biodiversity determination; thus this type of microbial measurement re-create the connection between the biota and restoration of function in degraded systems. For the success of restoration processes, the one way of approach is to comprehensively determine the soil microbial community characteristics of the disturbed system. The bacteria and fungi play important role in mine drainage ecosystem by using nitrogen as a key nutrient source. The growth and activity of microbe in soils are affected by its physicochemical and biological properties of soil. Because of these microbial processes, the soil environment gets transformed in its geochemical properties.

MATERIALS AND METHODS

Survey of study sites

Bankola: Geographically the area is located with Latitude 23°39′52.9"N, Longitude 87°14′32"E, Elevation 109 and Way Point 376. The nearest town of this area is Ukhra. Opencast type Mining is found in Bankola controlled by Eastern Coalfield Limited (ECL). Total mining area of Bankola is 54000 m² and total overburden area is 540 m². One overburden is found in this area.

Nagrakunda: Geographically the area is located with Latitude 23°39′52.9"N, Longitude 87°14′32"E, Elevation 79 and Way Point 375. The nearest town of this area is Pandaveswar. Opencast type Mining is found in Nagrakunda controlled by Eastern Coalfield Limited (ECL). Total mining area of Nagrakunda is 731951 m² and total overburden area

is 283290 m². Three overburdens are found in this area.

Sampling

To analyze microbiological properties the spoil samples were collected from Bankola and Nagrakunda coal mine of Raniganj colliery. Then mine overburdens were divided into two blocks and spoils were collected randomly from each block (1-15 cm soil depth) by using digging pits. Now this soil sample is referred to as "subsample" which was thoroughly mixed to make one "composite sample" that obtained from all overburden sites.

Microbiological analysis

Bacterial populations were determined for different spoil samples of each overburden site by using standard plate count dilution methodology. In this method, nutrient agar medium was used for microbial growth on petriplate. Sterile conditions were maintained throughout the entire process. 1 gram of soil sample was collected from each spoil samples and then diluted in sterilized tap water to obtain the bacterial concentration that was countable on the petriplate. The l mL spoil sample was taken and volume was made up I 00mL with sterile water which was further serially diluted to get l0*⁴ dilution. For isolation of microbe, the diluted samples were plated onto nutrient agar media by using spread plate technique and incubated at 37 C for 24 hours to 48 hours. Sub-culturing was done onto nutrient agar medium by using streak plate technique. Then the isolated colonies of bacterial cultures were taken and again incubated at 37 U for 24-48 hours. The microbial populations were enumerated as colony-forming units (CFU) from this serial dilution of spoil suspensions. The microbial colonies were counted by using three replica plates and the average values were calculated. For each spoil sample, the microbial populations were considered from the number of microbes multiplied by the dilution factor.

Microbial identification

16S rRNA Sequencing based metagenome sequencing

Extraction of DNA

1. Lysis (homogenization): Cells should be lysed by suspending 1 or 2 colonies aseptically and mixed with 450 ìl of "B Cube" lysis buffer in a 2 ml microcentrifuge tube and the cells were lysed by repeated pipetting.
2. Then add 4 µl of RNAse A and 250 ìl of "B Cube" neutralization buffer.
3. Vortex all content and the tubes were incubated for 30 minutes at 65 °C to 68 °C in a water bath that minimize the shearing of DNA molecules and mix the DNA solutions by inversion.
4. Then centrifuge the tubes at 14,000 rpm at 10 °C for 15 minutes.
5. After centrifugation, transfer all the resulting viscous supernatant into a fresh microcentrifuge tube (2 ml) without disturbing the pellet.
6. After that 600 µl of "B Cube" of binding buffer were added to the content and mix thoroughly by pipetting then the content was incubated at room temperature for 5 minutes.
7. Then transfer 600 µl of the contents to a spin column placed in 2 ml of collection tube.
8. Then centrifuge at 14,000 rpm for 2 minutes and discard the flow-through.
9. Reassemble the spin column and the collection tube and transfer the remaining 600 µl of the lysate.
10. Then centrifuge at 14,000 rpm for 2 minutes and discard flow-through.
11. Then add 500 µl "B Cube" washing buffer I to the spin column and centrifuge at 14,000 rpm for 2 mins, discard flow-through.
12. After that reassemble the spin column and add 500 µl "B Cube" washing buffer II and repeat Centrifugation at 14,000 rpm for 2 mins and discard flow-through.
13. Then transfer the spin column into a sterile microcentrifuge tube of 1.5 ml.
14. Add 100 µl of "B Cube" Elution buffer at the middle of the spin column. To avoid touch with the filter, care should be taken.
15. The tubes incubate for 5 minutes at room temperature and Centrifuge at 6000 rpm for 1min.
16. Repeat the above mentioned 14 and 15 number step for complete elution. The buffer contains the DNA in the microcentrifuge tube.
17. DNA concentrations were measured by running aliquots on 1% agarose gel.
18. The DNA samples were stored at -20°C for further use.

PCR Protocol (Polymerase Chain Reaction)

It is a process that uses primers to amplify specific cloned or genomic DNA sequences with the help of

unique enzyme. PCR uses the DNA polymerase enzyme that directs the DNA synthesis from deoxynucleotide substrates of a single-stranded DNA template. When DNA polymerase is annealed to a longer template DNA, it adds nucleotides to the 3′ end of a custom-designed oligonucleotide. If a synthetic oligonucleotide is annealed to a single-stranded template that contains a region complementary to the oligonucleotide then DNA polymerase can use the oligonucleotide as a primer and elongates 3′ end to form an extended region of double-stranded DNA.

Composition of the Taq Master Mix

- Taq DNA polymerase is given in 2X Taq buffer
- 0.4mM dNTPs,
- 3.2mM $MgCl_2$ and
- 0.02% bromophenol blue

PRIMER DETAILS

Primer Name	Sequence Details
16F	AGAGTTTGATCMTGGCTCAG
27F	AGAGTTTGATCMTGGCTCAG
16R	TACGGYTACCTTGTTACGACTT
1492R	TACGGYTACCTTGTTACGACTT

5 ìl of isolated DNA were added in 20 ìl of PCR reaction solution (1.5 ìl of Forwarding Primer and Reverse Primer, 5 ìl of deionized water, and 12 ìl of Taq Master Mix). Perform PCR using the following thermal cycling conditions.

Denaturation

The DNA template is heated at 94°C for 3 minutes. As a result, the weak hydrogen bonds (hold DNA strands together in a helix) were broken down and allowing the strands to separate creating single-stranded DNA.

Annealing

The mixture is cooled to anywhere from 94! for 30 sec, 50! for 60 sec, and 72! for 60 sec. It allows the primers to bind (anneal) to their complementary sequence in the template DNA.

Extension

The reaction is then heated to 72° C for 10 mins, the optimal temperature for the DNA polymerase to act. At this step, DNA polymerase was adding nucleotides onto the primer in a sequential manner, using the target DNA as a template and extends the primers.

Purification of PCR Production

From PCR products, removed unincorporated PCR primers and dNTPs by using Montage PCR Clean-up kit (Millipore). The PCR product was sequenced using the 27F/1492R primers. An ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems) was used for sequencing reactions.

Sequencing protocol

Using below 16s rRNA universal primers, single-pass sequencing was performed on each template. Then the fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. After that, the samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Bioinformatics protocol

1. The 16s r RNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of query sequence was performed with the closely related sequence of blast result followed by multiple sequence alignment.
2. For multiple alignments of sequences, the program MUSCLE 3.7 was used (Edgar, 2004). The program G-blocks 0.91b was used to cure the resulting aligned sequences. This G-blocks removes poorly aligned positions and divergent regions (Talavera and Castresana 2007). The program PhyML 3.0 aLRT was suited for phylogeny analysis and HKY85 as Substitution model.
3. PhyML was shown more accurate than other existing phylogeny programs using simulated data while being one order of magnitude faster. For tree rendering, the program Tree Dyn 198.3 was used (Dereeper *et al.*, 2008).

Quality Check

DNA QC

Extracted DNA from the samples was subjected to Nano Drop and GEL Check before being taken for PCR amplification.

The Nano Drop readings of 260/280 at an ~ value of 1.8 to 2 is used to determine the DNA's quality.

PCR Ampliqon QC

The amplified 16s PCR Product is purified and subjected to GEL Check and NanoDrop QC.

The NanoDrop readings of 260/280 at an ~ value of 1.8 to 2 is used to determine the DNA's quality.

RESULTS

16S rRNA based metagenome sequencing analysis [Figure 1 (B1) and Figure 2 (N1)] showed that isolates to be encompassing extremely low taxonomic diversity and the spoil were found to have comparable microbial community structures in Bankola and Nagrakunda area. At the phylum level, both were co-dominated by Acidobacteria and Firmicutes each of which accounted for ~4% of the total notable reads of either metagenome. Other

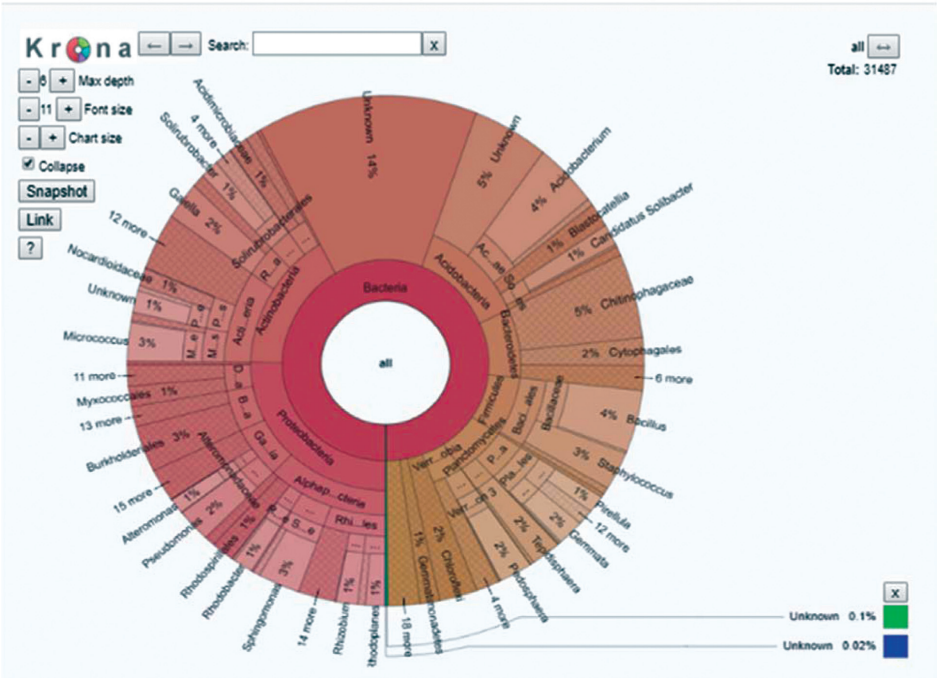

Fig. 1. Metagenomic study of soil in Nagrakunda

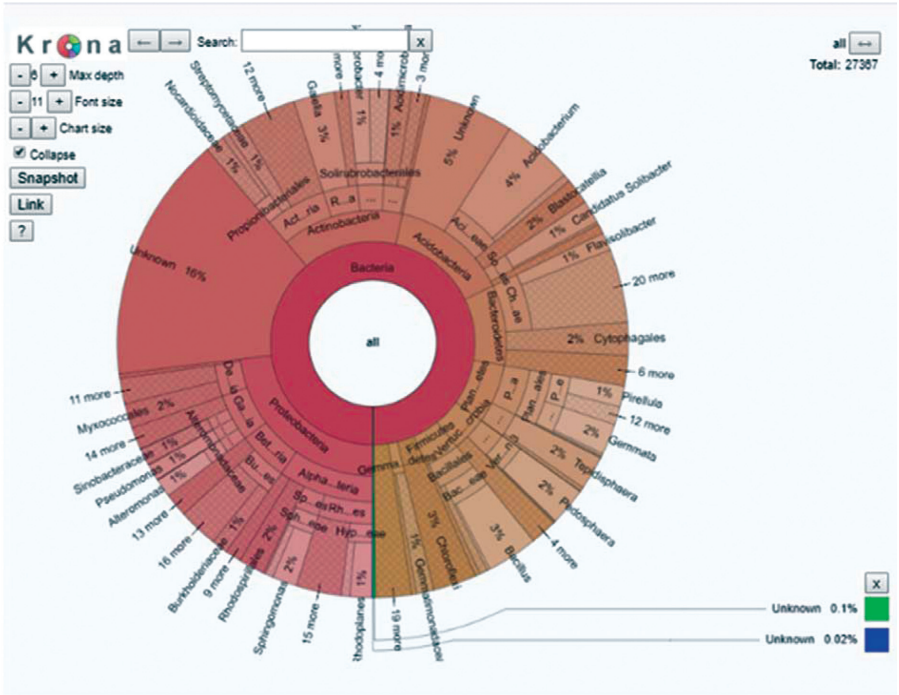

Fig. 2. Metagenomic study of soil in Bankola

bacterial phyla having ~2% attendance in the metagenomes of Bankola and Nagrakunda were *Proteobacteria, Gaiellaceae, Planctomycetes, and Verrucomicrobia*. Top ten most abundant genera (Table 1) *Vicinamibacter* sp., *Bacillus* sp., *Acidobacterium* sp., *Sphingomonas* sp., *Micrococcus* sp., *Staphylococcus* sp., *Gariella* sp., *Pseudomonas* sp., *Pedosphaera* sp., *Tepidisphaera* sp. were found in Bankola area and *Gariella* sp., *Pedosphaera* sp., *Tepidisphaera* sp., *Gemmata* sp., *Sphingomonas* sp., *Candidatus solibacter, Rhodoplanes* sp. were present in Nagrakunda data point. As many as 7 genera within 10 genera were common to the Bankola and Nagrakunda lists, while only 3 were unique to each of them. This underscored the similar structure of the microbial community in those overburden dump areas.

At the phylum level (Figure 3), both were co-dominated by *Acidobacteria* and *Firmicutes* each of which accounted for ~4% of the total notable reads of either metagenome. Other bacterial phyla having ~2% attendance in the metagenomes of Bankola and Nagrakunda were *Proteobacteria, Gaiellaceae, Planctomycetes, and Verrucomicrobia.*

Top ten most abundant genera (Figure:4) *Vicinamibacter* sp., *Bacillus* sp., *Acidobacterium* sp., *Sphingomonas* sp., *Micrococcus* sp., *Staphylococcus* sp., *Gariella* sp., *Pseudomonas* sp., *Pedosphaera* sp., *Tepidisphaera* sp. were found in Bankola area and *Gariella* sp., *Pedosphaera* sp., *Tepidisphaera* sp.,

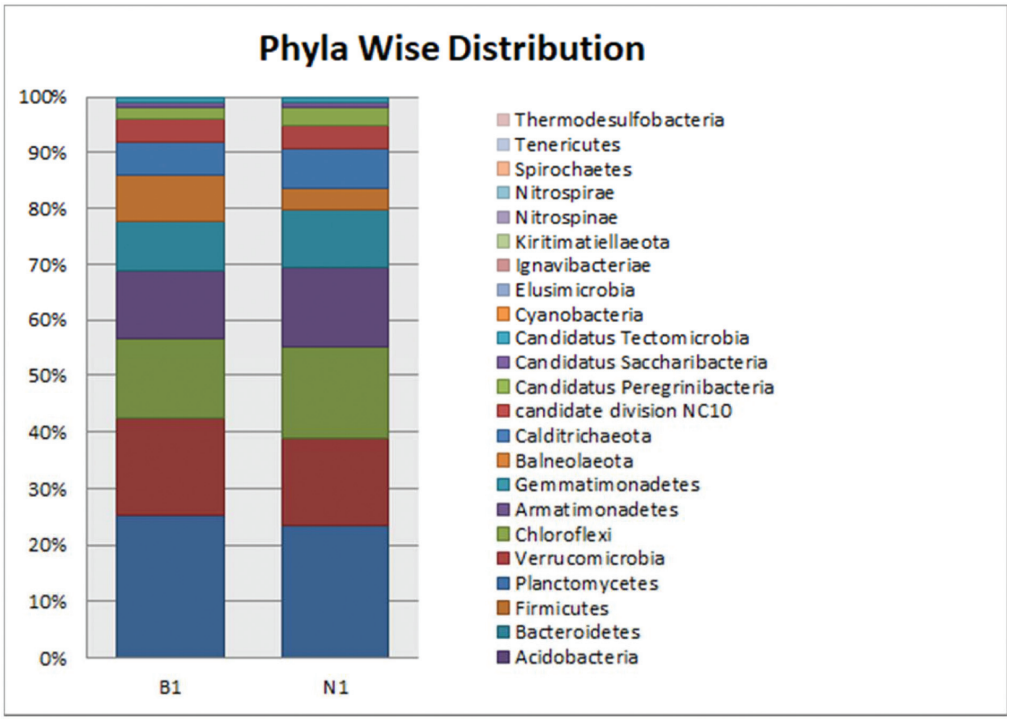

Fig. 3. Taxonomy Plot Analysis (Phyla Level)

Table: 1 Top ten Bacteria of Nagrakunda and Bankola wastelands

Top ten genera of identified bacteria at Nagrakunda	Top ten genera of identified bacteria at Bankola
Vicinamibacter sp.	*Vicinamibacter sp.*
Acidobacterium sp.	*Bacillus sp.*
Bacillus sp.	*Acidobacterium sp*
Gariella sp.	*Sphingomonas sp.*
Pedosphaera sp.	*Micrococcus sp.*
Tepidisphaera sp.	*Staphylococcus sp.*
Gemmata sp.	*Gariella sp.*
Sphingomonas sp.	*Pseudomonas sp.*
Candidatus solibacter	*Pedosphaera sp.*
Rhodoplanes sp.	*Tepidisphaera sp.*

Gemmata sp., *Sphingomonas* sp., *Candidatus solibacter, Rhodoplanes* sp. were present in Nagrakunda data point.

At the species level(Figure:5), both areas were co-dominated by *Vicinamibacter silvestris, Acidobacterium sp., Gaiella occulta, Pedosphaera parvula, Tepidisphaera mucosa, Bacillus megaterium, Rhizobium phaseoli, Micrococcus endophyticus, Micrococcus luteus, Rhodobacter blasticus, Sphingomonas insulae, Candidatus Solibacter usitatus, Gemmata sp., Alteromonas sp., Pirellula staleyi, Aciditerrimonas ferrireducens, Solirubrobacter sp., Chryseolinea serpens* that showed a similar microbial diversity at species level of these two overburden dump of Raniganj coalfield.

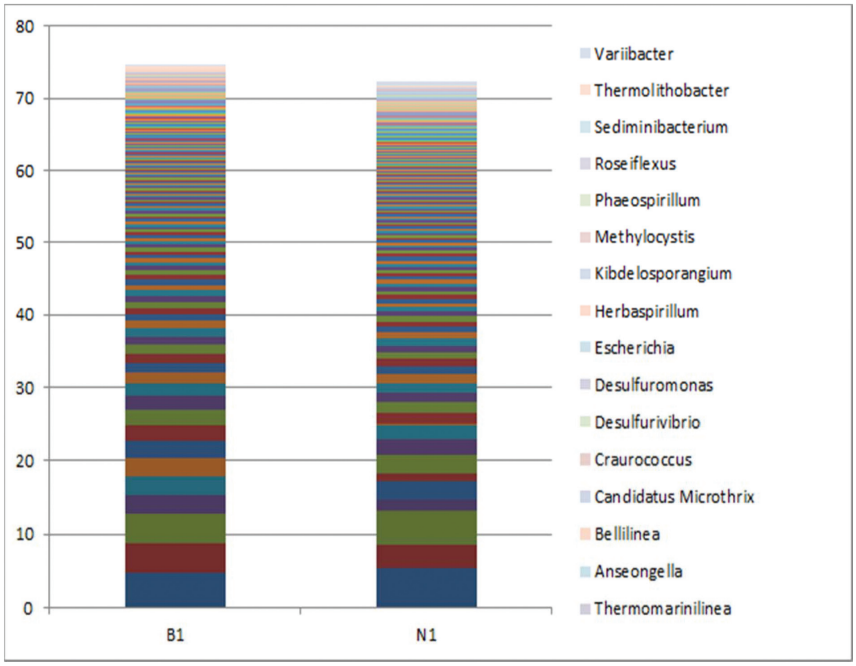

Fig. 4. Taxonomy Plot Analysis (Genus Level)

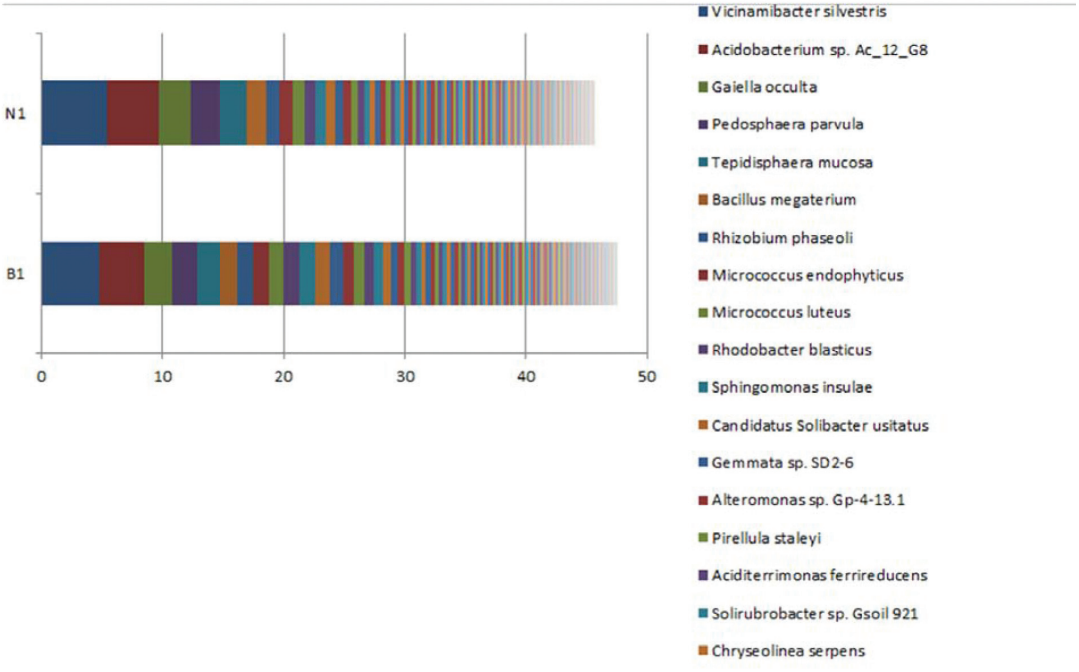

Fig. 5. Taxonomy Plot Analysis (Species Level)

DISCUSSION

This study presented emphasize on responses of microbial populations using metagenomics of Bankola and Nagrakunda coal mine generated wastelands of Raniganj coal field. Basically the dump soil always showed poor nutrient content and very low microbial communities thus it make the soil less fertile. Therefore it is very difficult to revegetate those overburden dumps as well as biological reclamation do not occur within the self-life of those soils and the soil eventually becomes unproductive. In this study, ten dominant genera were recorded that having the characteristics of plant growth promotion, developing effects on bioremediation approach and also involving in soil fertilization as well as in soil reclamation. Therefore it is evidenced that the microbial community is an effective factor of soil reclamation and remediation as well as these influences the soil fertility of coal mine dump soils at Raniganj Coalfield, West Bengal.

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