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IDENTIFICATION AND CHARACTERIZATION OF DOMINANT BACTERIA IN COAL FLY ASH AMENDED SOIL

Sitara Jabeen *et al.*

Fly ash

Earthworm

Aeromonas punctata

Bacillus cereus

Phylogeny

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SITARA JABEEN, SURUCHI KUMARI, PRIYANKA SAHA, NEHA YADAV, SARITA KUMARI, BHARTI S. RAIPAT[1] AND M. P. SINHA*

Deptt. of Zoology, Ranchi University, Ranchi - 834 008

[1]Deptt. of Zoology, St. Xavier's College, Ranchi - 834 001

E mail: m_psinha@yahoo.com

ABSTRACT

The experiment has been conducted to assess the ameliorating effect of native earthworm (*Drawida wilsi*) on bacterial population on coal fly ash amended soil in proportion of 5, 10 and 15%.It was found that the bacterial count has been enhanced due to presence of earthworm which bring about changes in the soil. In 5% amendment with earthworm, consecutive increase in the bacterial population has been observed within 30 days of experiment from 31 ± 0.529 X 10^9 to 39.8 ± 1.0510^9. On the other hand a decrease in the count has been observed in 10 and 15% amendment (21.7 ± 0.3610^9 to 16.56 ± 0.4010^9 and 15.3 ± 1.1010^9 to 8.46 ± 0.3210^9). Whereas in the absence of earthworm the bacterial count varied in the three concentrations as 31 ± 0.529 10^9 to 25.36 ± 1.05910^9, 21.7 ± 0.3610^9 to 16.76 ± 1.5310^9 and 15.3 ± 1.1010^9 to 4.16 ± 0.4510^9. The concentration of fly ash in the amended soil had significant impact on the bacterial population both with and without earthworm while the duration or time interval did not show significant impact in both the cases as revealed by two way ANOVA($F = 0.039$, df 2, 2; $p > 0.10$ (NS), $F = 16.82$, df 2, 2; $p < 0.01$ (with earthworm); $F = 4.5073$, df 2, 2; $p > 0.10$(NS); $F = 25.651$, df 2, 2 $p < 0.01$ (without earthworm). Genomic analysis of two dominant bacteria *Aeromanas punctata* strain JM10 and *Bacillus cereus* strain Probio 32 was done to have an insight of the bacteria. The phylogenetic study revealed the evolutionary status to be similar in comparison to 10 other similar taxa. *Aeromonas* was found to be high while *Bacillus* was comparatively less stable.

***Corresponding author**

INTRODUCTION

Fly ash is an amorphous mixture of ferro-alumino-silicate minerals generated from the combustion of ground or powdered coal at 400–1500°C and belongs to the coal combustion by-products in power plants produced from bituminous, subbituminus, and lignite combustion. Flyash production depends on the quality of the coal, which contains a relatively high proportion of ash that leads to 10-30% fly ash formation. In India 75% of electricity is generated by coal based thermal power plants. 112 million ton of this kind of waste were produced in India during 2005-06 of which 4 mt is released into the atmosphere (Jamwal, 2003). Percentage ash utilization of the total ash generated in different countries amounts to more than 85% in West Germany, 100% in Denmark, 85% in France, 50% in UK, 45% in China and 38% in India. Kalra *et al*. (1997) have reported that FA production in India will exceed 140 million ton by 2020. Nearly 50-60% of the fly ash is being stored at plant dump sites and other sites intended for this purpose. The disposal of such a huge amount of FA is one of the major problems of developing countries and is usually disposed in basins or landfills near the power plants (Kishor *et al.,* 2010). Fly ash is sometimes used in buildings, construction of roads, embankment and cement industries. Its alkaline character and a high concentration of mineral substances have resulted in attempts at using it as fertilizer or amendment to enhance the physico-chemical properties of soil. The FA contains a high concentration of toxic heavy metals such as Cu, Zn, Cd, Pb, Ni, Cr etc. (Rautaray *et al*., 2003; Lee *et al*., 2006; Tiwari *et al*., 2008) along with low nitrogen and phosphorus content and pH ranged from 4.5 to 12.0 depending on the S-content of parental coal. Fly ash is disposed of either by dry or wet methods. In dry disposal, the fly ash is dumped in landfills and fly ash basins. In the wet method, the fly ash is washed out with water into artificial lagoons and is called pond ash. Both methods ultimately lead to dumping the fly ash on open land, which degrades the soil and endangers human health and the environment. Fly ash particles are small enough to escape emission control devices and easily get suspended in the air. Repeated exposure to fly ash can cause irritation in eyes, skin, nose, throat and respiratory tract and result in arsenic poisoning (Carlson and Adriano, 1993). Therefore, disposal and utilization of fly ash needs careful assessment to prevent conversion of arable land into landfills and accumulation of toxic metals in soil (Petruzzelli, 1989) and use it as an ameliorant for problem soils. Fly ash has a vast potential for use in agriculture, forestry and wasteland reclamation due to its excellent soil ameliorating properties (Adriano *et al*., 1979, 1980; Capp, 1978; Fail and Wochok, 1977; Aitken and Bell, 1985). Earthworms play a major role in increasing the fertility of the soil. It acts on the soil structure by ingestion of soil, partial breakdown of organic matter, intimate mixing of these fragments, ejection of this material as surface or subsurface casts and the formation of excavations such as burrows. (Peres *et al.,* 1998).

The effect of soil fertilization with fly ash has been quite explored although the effect of this on the soil microbes is not well covered in the literature. The microbes are the important elements of the soil environment as they participate in the degradation of the organic matter and make the nutrients available to other soil organisms. This favors the formation of soil aggregates and immobilizes the heavy metals and stimulate the activity of soil enzymes viz., dehydrogenase, urease and phosphatases etc., (Pati and Sahu, 2004). A great amount of elements (C, K, Ca, Mg, Cu, Zn and Mn) get into the soil as a result of ash used at different doses and may probably change the chemical as well as physicochemical soil properties which intern may determine the biological properties irrespective of the crop (Yelledhalli *et al.,* 2007). Therefore, the study was aimed at determining the effect of graded levels of fly ash on the bacterial activity in the amended soil with and without earthworm to watch out the ameliorating effect of the earthworm. Finally the identification and characterization of the dominating count of the microbes (bacteria) was done.

MATERIALS AND METHODS

The laboratory experiment was carried out using fly ash (Table 1) collected from Patratu Thermal Power Plant and soil (Table 2) from the nearby area of the Ranchi University. The soil texture was somewhat clay

loamy which was amended with 5, 10 and 15% of ash. This was kept as a control with the test samples inoculated with native earthworms (*Drawida Wilsi*) from cropland near Morabadi Campus, Ranchi. The six combinations of the fly ash amended soil with and without earthworms were kept in quadruplicate plastic trays and sample soil for microbial study was collected from it.

Bacterial population was estimated in with and without earthworm inoculated amended soil by dilution plate method (Waksman, 1922). The isolation of bacteria from soil samples was initiated by taking 1g of soil from each composite and transferring it to sterilized test tube for suspension in 9 mL of sterilized deionized water by shaking for 30 mins. 1 mL inoculant was taken from the aliquots of 1: 10^7 dilutions of the primary suspension (1 g soil in 10 ml distilled water). Each dilution was plated in Petri dishes containing Czapak Dox Agar (Thom and Raper, 1945) media for the bacterial culture. For each amendment three replicates of Petri dishes were prepared. After 24 hr incubation of the Petri dishes at ambient temperature of 38 ± 2°C, the bacterial colonies were counted. The dominant colonies were pointed out and gram staining was done. From the bacterial culture there were two dominating colonies which were further pure cultured using the solid agar slant streak plating method for the genomic analysis.

Genomic analysis

DNA extraction and purification

DNA was isolated from the culture. Its quality was evaluated on 1.2 Agarose Gel (Fig. 1). A single band of high molecular weight DNA has been observed.

PCR amplification and sequencing

Fragments of 16 Sr DNA gene was amplified by PCR from the isolated DNA. A single discrete PCR amplicon band of 1500bp was observed when resolved on Agarose Gel. The PCR amplicon was purified to remove contaminants.

Oligonucleotide Primers

Forward and reverse DNA sequencing reaction of PCR amplicon was carried with 8F and 1492R primers using BDTv 3.1 Cycle Sequencing Kit on ABI 3730^{x}l Genetic Analyzer. Consensus sequence of 1418bp rDNA gene was generated from forward and reverse sequence data using aligner software.

Phylogenetic data analysis

Ten maximum identical score were aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database. The evolutionary tree was constructed by the Neighbor-joining method (Saitou and Nei, 1987) with the MEGA4 program (Tamura *et al.,* 2007). The evolutionary distances were computed using Kimura 2-parameter method (Kimura, 1980).

Statistical Analysis

The data were subjected to the analysis of variance (ANOVA).

RESULTS AND DISSCUSSION

Bacterial estimation

The bacterial count in different proportion of fly ash amendment with and without earthworm was done. It was found that the bacterial population on zero day was consecutively more in 5% amendment than the 10 and 15% and the population grew in the same pattern up till 30 days of experimentation. It was observed that the population was higher in the amended soil incubated with earthworm in comparison to one without earthworm. The population raised from $31 \pm 0.529 \times 10^9$ to $39.8 \pm 1.05 \times 10^9$ in 5% amendment with earthworm whereas on the other hand the count fell down from to $31 \pm 0.529 \times 10^9$ to $25.36 \pm 1.059 \times 10^9$ in amendment without earthworm. In 10% amendment the population decreased in both with and without earthworm from $21.7 \pm 0.36 \times 10^9$ to $16.56 \pm 0.40 \times 10^9$ and $21.7 \pm 0.36 \times 10^9$ to $16.76 \pm 1.53 \times 10^9$. There was a drastic decrease in 15% amendment from $15.3 \pm 1.10 \times 10^9$ to $8.46 \pm 0.32 \times 10^9$ and $15.3 \pm 1.10 \times 10^9$ to $4.16 \pm 0.45 \times 10^9$ in both with

Table 1: Physical properties of fly ash

Parameters	Values
Colour	Gray
Shape	Sperical
Bulk Density(g cm^{-3})	1-1.8
Specific Gravity(g $^{cm-3}$)	1.90-2.55
Moisture content(%)	18-38
Cohesion(kg m^{-2})	Negligible
Clay (%)	1-10
Silt(%)	8-85
Sand(%)	7-90
Gravel(%)	0-10

Table 2: Characteristics of the soil sample used for the bacterial estimation

Chracteristics	Value (M±SD; n=3)
pH	5.81±0.07
Organic carbon (mg C g^{-1} soil)	6.52±0.11
Nitrogen(mg N g^{-1} soil)	0.78±0.01
Phosphorous(kg P hec^{-1} soil)	27.9±0.62
Potassium (kg K hec^{-1} soil)	148.0±0.49

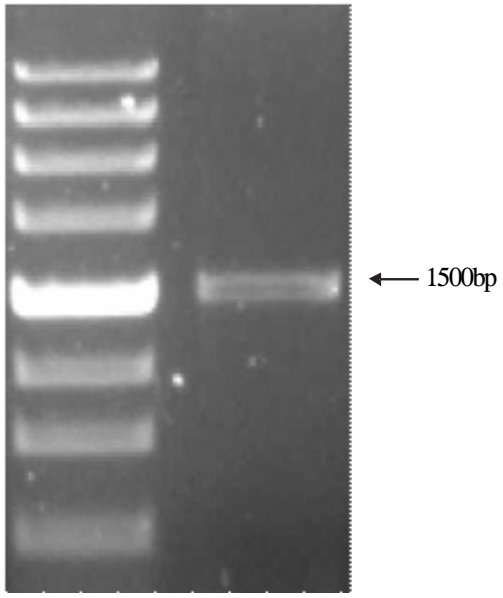

Figure 1: Gel Image
Lane 1 - DNA marker; Lane 2 - 16 Sr DNA amplicon band

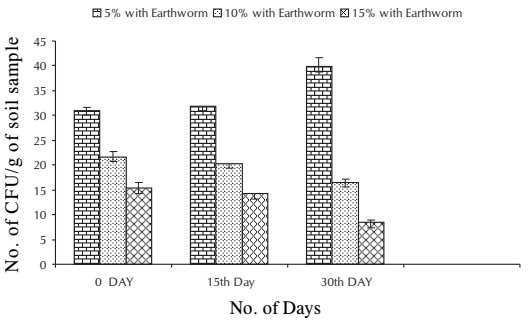

Figure 2: Number of CFU/g of soil samples with earthworms in time interval of 15 days

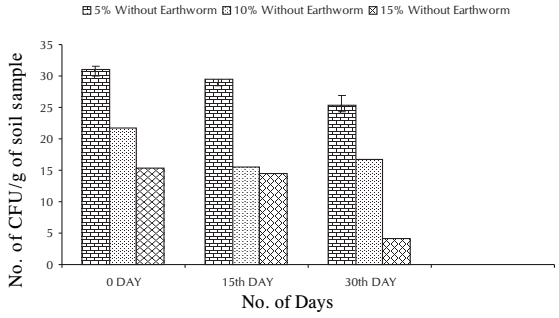

Figure 3: Number of CFU/g of soil samples without earthworms in time interval of 15 days

and without earthworm respectively, showing the concentration of fly ash to be in high proportion (Fig. 2 and 3).The above result revealed that the amended soil with earthworm showed considerably higher bacterial count in comparison to the one without earthworm. The enhancement in the population with earthworm was due to the ameliorating effect of the earthworms. Earthworm activity may raise N_2O emissions from agro-ecosystems. Rather than emitting N_2O themselves, earthworms are thought to enhance soil microbial activity (nitrification, denitrification and nitrifier denitrification) by changing physico-chemical properties, excreting mucus, and increasing available carbon (Lubbers *et al.*, 2010). The count and activity of soil bacteria depend on a number of factors. The climate, type and physico-chemical properties of the soil, the composition of species and toxic substances including heavy metals (Yeledhalli *et al.*, 2007). In this study, the coal fly ash modified the soil bacteria count and its effect depends on the level of fly ash application. Amendment of Class F, bituminous fly ash to soil at a rate of 505 Mg ha^{-1} did not cause any negative effect on soil microbial communities and improved the populations of fungi, including arbuscular mycorrhizal fungi and gram-negative bacteria as revealed from analysis of community fatty acids (Schutter and Fuhrmann, 2001). This showed that low level of fly ash had positive effect on microbial activity and also helped in the enhancement of gram negative bacteria as *Aeromonas punctata* in the present study. Fly ash on addition in soil is reported to decrease bulk density, improve soil porosity, increase water-holding capacity, decrease

Table 3: Two way analysis of variance (without earthworm)

Source of Variation	SS	df	MS	F	P-value	F crit
Between days	79.9538	2	39.9769	4.50736	0.094461	6.944272
Between concentration	455.0234	2	227.5117	25.65174	0.005231	6.944272
Error	35.477	4	8.86925			

Table 4: Two way analysis of variance (with earthworm)

Source of Variation	SS	df	MS	F	P-value	F crit
Between days	1.720564	2	0.860282	0.039785	0.961371	6.944272
Between concentration	728.5472	2	364.2736	16.84651	0.011262	6.944272
Error	86.49238	4	21.62309			

surface encrustation change soil pH and increase the electrical conductivity of soil (Chang *et al.*, 1977; Page *et al.*, 1979; Elseewi *et al.*, 1980). Fly ash may either have a positive and negative effect on plant growth and yielding if not used in optimum doses. The effect is determined primarily by chemical composition and the ash dose applied. In a study by Kalara *et al* ., 2003, application of 5 to 12 tones ha^{-1} yr^{-1} has modified the soil physico-chemical properties viz., reduced the bulk density, increase the water holding capacity, improvement in the exchangeable calcium and magnesium status of the soil which enhanced the wheat yield. The greater application of fly ash doses decreased the yield of crop due to pozzolonic effect of fly ash in soil which induced the poor aeration and compaction. The beneficiary role of low level of fly ash is further suggested as application of fly ash in low doses in the agricultural fields is suitable for better crop management (Fail and Wochok, 1977). The differences in treatments with and without worms were found significant between amendments and not significant between days. (Table 3 and 4).

Identification

The two dominant bacterial colonies were identified to be *Aeromonas punctata* JM10 (GenBank Accession Number: GU205197.1) and *Bacillus cereus* strain Probio 32 (GenBank Accession Number: GU471752.1) based on nucleotide homology and phylogenetic analysis. The punctiform Aeromonas constituted about 60% of the bacterial colony and circular bacillus about 30%. Along with these, certain whitish round colonies, yellowish colonies and very few filamentous forms were observed.

Characterization

Aeromonas punctata shows punctiform appearance dominating the bacterial culture. The bacterium colony on naked eye observation was found to be of punctiform with flat elevation and entire margin. On Gram staining it was found to be of gram negative bacilli (Fig. 4). The genus *Aeromonas* are water borne Gram - negative bacteria that are ubiquitous in water, including groundwater and chlorinated drinking water (LeChevallier *et al.,* 1982; Kuhn *et al.,* 1997; Gavriel *et al.,* 1998). *Aeromonas* strains isolated from water have been shown to possess virulence traits, such as adhesions, hemolysins and cytotonic enterotoxins presumably involved with human pathogenicity (Handfield *et al.,* 1996 ; Albert *et al.,* 2000; Schubert,

Table 5: Distance matrix depicting the pairwise distance between the DNA sequences of the strains of bacteria *Aeromonas punctata*

Sample 1	1		0000.0	0000.0	0000.0	0000.0	0000.7	0000.0	0000.0	0000.0	0000.0	0000.0
EU862311.1	2	0000.0		0000.0	0000.0	0000.0	0000.7	0000.0	0000.0	0000.0	0000.0	0000.0
GQ259885.2	3	0000.0	0000.0		0000.0	0000.0	0000.7	0000.0	0000.0	0000.0	0000.0	0000.0
GU205197.1	4	0000.0	0000.0	0000.0		0000.0	0000.7	0000.0	0000.0	0000.0	0000.0	0000.0
GU205195.1	5	0000.0	0000.0	0000.0	0000.0		0000.7	0000.0	0000.0	0000.0	0000.0	0000.0
FJ494901.1	6	0000.7	0000.7	0000.7	0000.7	0000.7		0000.7	0000.7	0000.7	0000.7	0000.7
FJ168776.1	7	0000.0	0000.0	0000.0	0000.0	0000.0	0000.7		0000.0	0000.0	0000.0	0000.0
FJ168775.1	8	0000.0	0000.0	0000.0	0000.0	0000.0	0000.7	0000.0		0000.0	0000.0	0000.0
FJ168774.1	9	0000.0	0000.0	0000.0	0000.0	0000.0	0000.7	0000.0	0000.0		0000.0	0000.0
DQ979324.1	10	0000.0	0000.0	0000.0	0000.0	0000.0	0000.7	0000.0	0000.0	0000.0		0000.0
AY987761.1	11	0000.0	0000.0	0000.0	0000.0	0000.0	0000.7	0000.0	0000.0	0000.0	0000.0	

2000). The genus comprises a collection of oxidase and catalase-positive, glucose-fermenting, facultatively anaerobic, Gram-negative, rod-shaped bacteria that are resistant to the vibriostatic agent O/129 and are generally motile by means of polar flagella (Popoff, 1984). Aeromonads are autochthonous to aquatic environments worldwide and have been implicated in the aetiology of a variety of fish and human diseases, frequently including diarrhoea and occasionally systemic infections (Janda, 1991).

On genomic analysis 1500bp of the 16S rDNA amplicon was used. Further the Blast report revealed that the query sequence of the bacterium showed that about more than 200 nucleotide sequences were similar to the 100 blast hits done. The unrooted phylogenetic tree (Fig. 5) has been constructed which dipicts the evolutionary history of the related strains on the basis of the distance matrix (Table 5) which exhibit the dissilimilarity between the nucleotide sequences of the respective strains of bacterium, *Aeromonas punctata.* The bootstrap consensus tree inferred from 500 replicates (*Felsenstein,* 1985) was taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) were shown next to the branches. The evolutionary distances were computed in the units of the number of base substitutions per site. Codon positions included were $1^{st}+2^{nd}+3^{rd}+$Noncoding. All positions containing gaps and missing data were eliminated from dataset. There were a total of 1417 positions in the final set. High level of 16S rDNA similarity was found between the 11 strains of the bacterium (Table 6). In particular, Aeromonas punctata strain JM10 and strain 159 shared relatively high similarity 16S rDNA value. Uncultured bacterium clone Niu10 and Aeromonas punctata strain JW04 share identical value of 16S rDNA and relatively similar to strain RK 65541. The strain 219c is identical with slight change in any of the ambiguous nucleotide. Further the strain 360c and MPT4 share identical 16S rDNA with certain changes in the nucleotide sequences of *Aeromonas* sp. B27.

Table 6 : Accession number of the 11 strains of bacterium *Aeromonas punctata*

Accession number with description	
GU205197.1	: *Aeromonas punctata strain JM10*
GQ259885.2	: *Aeromonas punctata strain 159*
EU862311.1	: *Uncultured bacterium clone Niu10*
GU205195.1	: *Aeromonas punctata strain JW04*
AY987761.1	: *Aeromonas punctata strain RK 65541*
FJ168776.1	: *Aeromonas punctata strain 219c*
DQ979324.1	: *Aeromonas punctata strain MPT4*
FJ168775.1	: *Aeromonas punctata strain 176c*
FJ168774.1	: *Aeromonas punctata strain 360c*
FJ494901.1	: *Aeromonas sp. B27*

Table 7: Accession number of the 11 strains of the bacteria *Bacillus cereus*

Accession number with description	
GU812900.1	: *Bacillus cereus strain JBS10*
GU826154.1	: *Bacillus cereus strain Q34*
GU566345.1	: *Bacillus sp. R5(2010)*
GU471752.1	: *Bacillus cereus strain Probio-32*
AB542372.1	: *Bacillus sp. TSA4w*
GU125426.1	: *Bacillus cereus strain IMAU80004*
GU125425.1	: *Bacillus cereus strain IMAU80003*
GQ383905.1	: *Bacillus sp. 4CCS8*
FJ188297.1	: *Bacillus cereus strain BU040901-022*
FJ803926.1	: *Bacillus cereus strain 0-9*

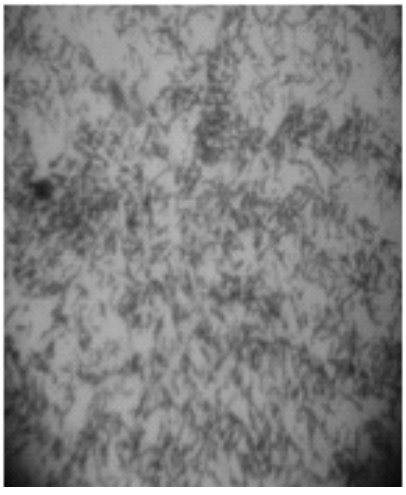

Figure 4: Gram staining of *Aeromonas punctata*

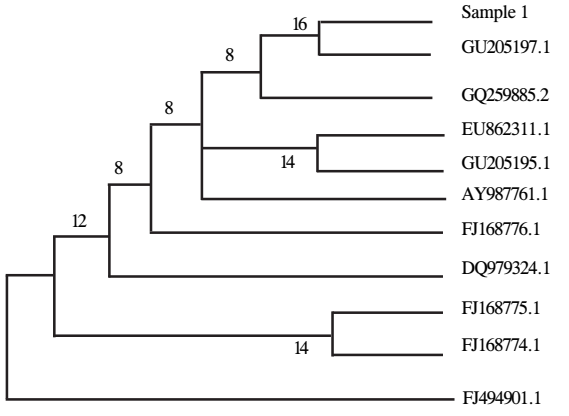

Figure 5: Phylogenetic tree showing evolutionary relationship of 11 taxas of *Aeromonas punctata* strain

The evolutionary tree shows that JM10 is closely related to the strain 159 and is distantly related to *Aeromonas* sp B27. This further demonstrated that although there is slight divergence or variation among the strains but are very much similar proving to be the homolog to the sampled bacteria. Thus, significant similarities were found between strains of the same species. This further states that the strain JM10 bears stable position in the tree. Through evolution it has reached to stability. The nucleotide sequences of the bacterial strains were found to be very much identical showing considerable convergence between *Aeromonas punctata* strains.

Bacillus cereus, the second dominant colony, was found to be of circular form with raised elevation and undulated margin. On Gram staining it was found to be Gram positive rod shaped bacteria (Fig. 6). *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus anthracis* all belong to the

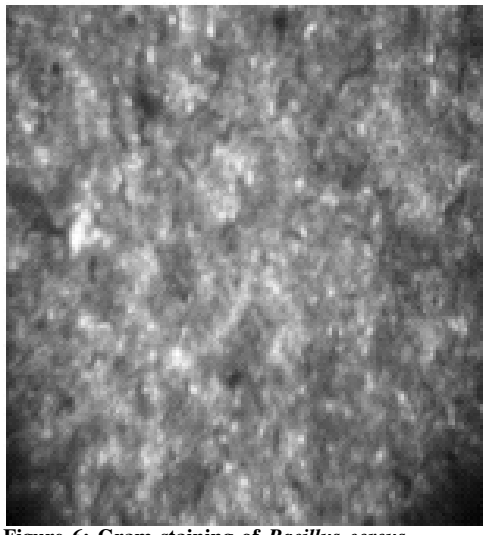

Figure 6: Gram staining of *Bacillus cereus*

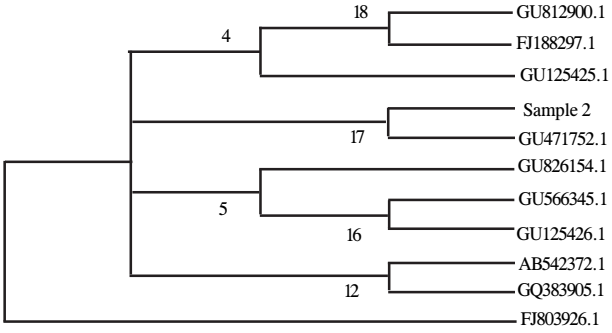

Figure 7: Phylogenetic tree showing the evolutionary history of different strains of *Bacillus cereus*

B.cereus sensu lato group of rod-shaped, Gram-positive, spore-forming bacteria (Jensen *et al.*, 2003). *Bacillus cereus* is an opportunistic pathogen causing food poisoning manifested by diarrhoeal or emetic syndromes. It is closely related to the animal and human pathogen *Bacillus anthracis* and the insect pathogen *Bacillus thuringiensis* (Ivanova *et al.*, 2003). *Bacillus anthracis* shares a very close evolutionary relationship with two other common but much less pathogenic bacterial species: *B.thuringiensis*, a well known biological insecticide (Aronson and Shai, 2001), and *B.cereus*, often considered at most, a soil-dwelling opportunistic pathogen (Jensen *et al.*, 2003). Similar assessment of the bacterium, Bacillus cereus, has been shown by the genomic analysis as done for *Aeromonas*. The phylogenetic study showed that it wasn't the most stabilized strain among all. All the strains were studied using DNA DNA hybridization and showed about 70% relatedness of the 16 S rDNA under optimal hybridization condition. Thereby, the evolutionary tree depicted that the *Bacillus cereus* strain *probio32* was homologous to the other strains (Table 7). Among all, the much evolved or the one reached to a stable position through evolution was *Bacillus cereus strain JBS10* (Fig. 7).

CONCLUSION

The study revealed that the earthworm has some ameliorating effect on the coal fly ash amended soil thereby enhancing the population in comparison to the absence of earthworm in the same concentration. Along with it was concluded that fly ash has positive effect in low doses which prove to be optimum for bacterial population. Further, on identification the bacteria *Aeromonas punctata* strain JM10 and *Bacillus cereus* strain Probio 32 were much resistant form to grow in the amended soil. Among the two *Aeromonas* was found to be highly stable in comparison to the different strains studied.

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