The Bioscan 8(1): 243-252, 2013 (Supplement on Taxonomy)
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.in

MOLECULAR AND PHYLOGENETIC STUDY OF BACTERIA RESISTANT TO COAL FLY ASH

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KEYWORDS
16S rDNA
Aeromonas punctata
Bacillus
Kocuria
Phylogeny

Received on :
21.11.2012

Accepted on :
14.02.2013

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ABSTRACT

The assessment of the bacteria in coal fly ash has been a critical issue with respect to its enormous production and utilization purpose. 16S rDNA based fingerprints revealed the resistant bacteria to coal fly ash and the molecular analog was studied. The bacteria observed were *Aeromonas punctata, Bacillus cereus, Bacillus probio* 32 and *Kocuria*. The consensus sequences along with the significant alignment of the bacteria were revealed in turn helping in the formulation of their unrooted phylogenetic tree.

INTRODUCTION

Fly ash, an amorphous mixture of ferro-alumino-silicate minerals, has been estimated to reach 170 million ton in 2012 (Chatterjee, 2011). Such huge amount generation of FA is proving to be a critical issue. Bacterial population can influence carbon or mineral cycles and have the ability to colonize harsh environments (Jabeen and Sinha, 2012a). Microbes are the important elements of the soil environment as they participate in the degradation of the organic matter and make the nutrient available to the other soil organisms (Jabeen *et al*., 2010). Further incorporation of earthworm ameliorates the nutrient profile of fly ash amended soil helping to increase its usability percent in agricultural prospects thereby restraining its harmfulness as elaborated by Jabeen and Sinha (2012b).

Molecular methods used in ecological studies usually involve the separation of PCR amplicons on the basis of DNA nucleotide sequence differences (Reynolds and Surridge, 2009). Consensus oligonucleotides produce DNA bands by agarose gel electrophoresis following PCR amplification. These band patterns provided unambiguous DNA fingerprints of different eubacterial species and strains. Widespread distribution of these repetitive DNA elements in the genomes of various microorganisms and BLAST enable rapid identification of bacterial species and strains and be useful for the analysis of prokaryotic genomes (Jabeen and Sinha, 2012). Sequencing of DNA is a powerful tool for gathering information about organisms and their environment. High bacterial diversity could be revealed using molecular techniques targeting directly either the diversity of the 16S rDNA (Amann *et al*., 1995) as most used genetic marker for molecular phylogenetic studies (Ueda *et al*., 1995). Fly ash can promote soil microbial activity and mixing with an organic substrate enhances its benefits, which assumes importance owing to eco-friendly disposal of fly ash. The application of wastes to soil as a recycling option can only be sustained if there are demonstrable 'ecological benefits' which is usually justified in terms of elevated organic carbon and its effect on soil conditions and stimulation of microbial activity and nutrient supply and this is sustainable only if threshold levels of pollutants does not exceed (Jabeen and Sinha, 2011).

In the present study the presence of microorganisms in fly ash was estimated with their molecular description.

MATERIALS AND METHODS

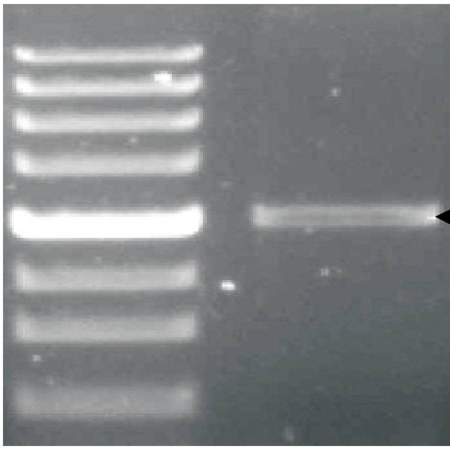
Sample of coal combustion by product, Fly ash, was collected for the study from the ash disposal site of Patratu thermal Power Plant situated in the state of Jharkhand.

Experimental setup

The laboratory experiment was performed to study the molecular analog of bacteria in fly ash amended soil. Soil for the experiment was collected from the agro-ecosystem near the Ranchi University campus. It was air dried, grinded and sieved using 1mm mess sieve. The fly ash was also air dried and mixed with the soil in a proportions 15% fly ash and stored in plastic trays in four replicates.

Enumeration of bacterial population

Bacterial population was estimated from CFA amended soil



Lane 1 - DNA marker; Lane 2 - 16 S rDNA amplicon band
Figure 1: Agarose gel image of 1500bp of 16S rDNA amplicon band

CATGCAAGTCGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTT
GCCGGCGAGCGGCGGACGGGTGAGTAATGCCTGGGAAATT
GCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATACGC
CCTACGGGGGAAAGCAGGGGACCTTCGGGCCTTGCGCGATTGGATATGCC
CAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACG
ATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACG
GTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGG
GAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTT
GTAAAGCACTTTCAGCGAGGAGGAAAGGTCAGTAGCTAATATCTGCTGG
CTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAG
CCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGC
GTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCC
CCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAG
AGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAA
ATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCT
GGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAAC
AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAG
GCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAA

Figure 2: Forward primer for *Aeromonas punctata* (814 bp)

GTGGTAACGCCCTCCCGAAGGTTAAGCTATCTACTTCTGGTGCAACCC
ACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGT
ATTCACCGCAACATTCTGATTTGCGATTACTAGCGATTCCGACTTCAT
GGAGTCGAGTTGCAGACTCCAATCCGGACTACGACGCGCTTTTTGG
GATTCGCTCACTATCGCTAGCTTGCAGCCCTCTGTACGCGCCATTG
TAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGACGT
CATCCCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCCTTGAGTT
CCCACCATTACGTGCTGGCAACAAAGGACAGGGGTTGCGCTCGT
TGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCC
ATGCAGCACCTGTGTTCTGATTCCCGAAGGCACTCCCGTATCTCTA
CAGGATTCCAGACATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCA
TCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCA
TTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGATTTAACGC
GTTAGCTCCGGAAGCCACGTCTCAAGGACACAGCCTCCAAATCGACAT
CGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCC
CCACGCTTTCGCACCTGAGCGTCAGTCTTTGTCCAGGGGGCC
GCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCATTTCACCG
CTACACCTGGAATTCTACCCCCCTCTACAAGACTCTAGCTGGAC
AGTTTTAAATGCAATTCCCAGGTTGAGCCCGGGGCTTTCACAT
CTAACTTATCCAACCGCCTGCGTGCGCTTTACGCCCAGTAATTCC

Figure 3: Reverse primer for *Aeromonas punctata* (913 bp)

CATGCAAGTCGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCG
GCGAGCGGCGGACGGGTGAGTAATGCCTGGGAAATTGCCCAG
TCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATACGCC
CTACGGGGGAAAGCAGGGGACCTTCGGGCCTTGCGCGATTGGATA
TGCCCAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAG
GCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGA
ACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAA
TATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTG
TGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAA
AGGTCAGTAGCTAATATCTGCTGGCTGTGACGTTACTCGCAGAAGA
AGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGT
GCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGG
TTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGC
ATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCA
GGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGC
GAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGT
GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT
GTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAACG
CGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCA
AATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTA
ATTCGATGCAACGCGAAGAACCTTACCTGGCCTTGACATGTCTGGA
ATCCTGTAGAGATACGGGAGTGCCTTCGGGAATCAGAACACAGGTG
CTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC
GCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTAATGGTGGG
AACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGAT
GACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTA
CAATGGCGCGTACAGAGGGCTGCAAGCTAGCGATAGTGAGC
GAATCCCAAAAAGCGCGTCGTAGTCCGGATTGGAGTCTGCAAC
TCGACTCCATGAAGTCGGAATCGCTAGTAATCGCAAATCAGAATGTTGC
GGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGG
AGTGGGTTGCACCAGAAGTAGATAGCTTAACCTTCGGGAGGGCGTTACCAC

Figure 4: Consensus sequence of 16S rDNA gene of *Aeromonas punctata* strain JM10 (1418 bp)

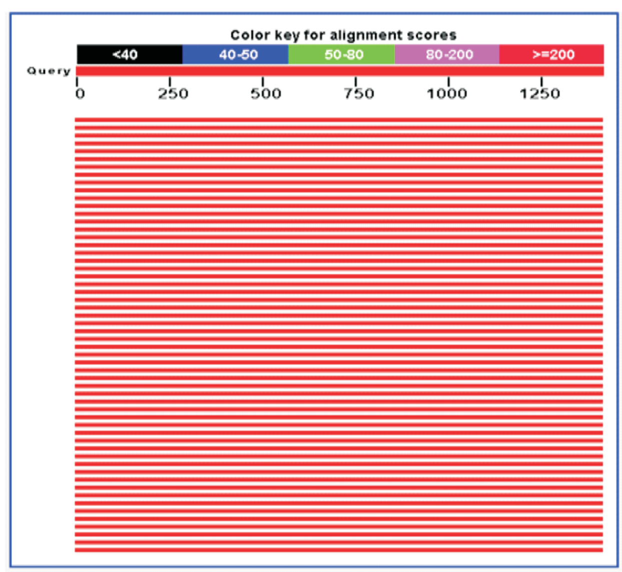


Figure 5: BLAST hits for *Aeromaonas punctata*

by dilution plate count 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 9mL of sterilized deionized water by shaking for 30 mins. 1mL inoculant was taken from the aliquots of 1: 10^7 dilutions of the primary suspension (1g soil in 10mL distilled water). Each dilution was plated in Petri plates (100 mm dia) containing Czapak Dox Agar (Thom and Raper, 1945) media for the bacterial culture. The media was prepared using peptone - 10 g/L, NaCl- 5g/L, Beef extract- 10g/L, Agar- 15g/L and the pH was maintained at 7. After 24h incubation

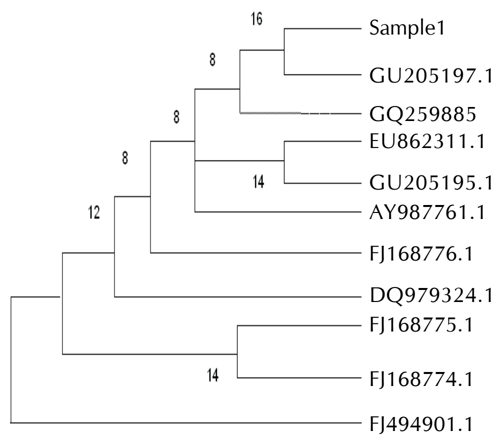


Figure 6: Phylogenetic tree depicting the evolutionary status of *Aeromonas punctata* strain JM10

GCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGG
ACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATA
ACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTC
GAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGT
CGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGAT
GCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGA
AAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTA
AAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCA
CCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCA
GCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTG
GGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAA
GCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGA
CTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGG
TGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGC
GACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTG
GGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAA
GTTAACGCATTAAGCACTC

Figure 7: Forward primer (833bp) for *Bacillus cereus* strain *Probio 32*

CACCTTAGGCGGCTGGCTCCAAAAAGGTTACCCCACCGACTTCGGGTG
TTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGA
ACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTT
CATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAACGGTTTTATG
AGATTAGCTCCACCTCGCGGTCTTGCAGCTCTTTGTACCGTCCATTGTAG
CACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCC
CCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTTA
ATGATGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACC
CAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCAC
TCTGCTCCCGAAGGAGAAGCCCTATCTCTAGGGTTTTCAGAGGATGT
CAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCT
CCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGCCTTGCG
GCCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAACTTCAGCACTAA
AGGGCGGAAACCCTCTAACACTTAGCACTCATCGTTTACGGCG
TGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCG
CGCCTCAGTGTCAGTTACAGACCAGAAAGTCGCCTTCGCCACT
GGTGTTCCTCCATATCTCTACGCATTTCACCGCTACACATGGAAT
TCCACTTTCCTCTTCTGCACTCAAGTCTCCCAGTTTCCAATGACCCTCC

Figure 8: Reverse primer (848bp) for *Bacillus cereus* strain *Probio 32*

of the Petri plates at an ambient temperature of 38 ± 2°C for 48h, the bacterial colonies were counted.

From the bacterial culture the specific dominating colonies were further pure cultured using the solid agar slant streak plating method for genomic analysis.

GCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTA
GCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAG
ACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACAT
TTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCA
CTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAA
CGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTG
ATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG
AGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACG
GAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAAC
TCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCAC
CTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATT
GGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAA
AGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGAC
TTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGA
AATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTT
TCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGC
AAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGT
GCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACG
CATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAA
CTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCAT
GTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTG
ACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTCGGGAG
CAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTG
AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAG
TTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACA
AACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTT
ATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAG
CTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTC
AGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAAT
CGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCC
CGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAA
CACCCGAAGTCGGTGGGGTAACCTTTTTGGAGCCAGCCGCCTAAGGTG

Figure 9: Consensus Sequence of 1427bp revealed for *Bacillus cereus* strain *Probio* 32

Genomic analysis

DNA extraction and purification

DNA was isolated from the pure culture of the bacterial colony. Its quality was evaluated on 1.2 agarose gel. A single band of high molecular weight DNA has been observed.

PCR amplification and sequencing

Fragments of 16S rDNA gene were amplified by PCR from the above 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}1 genetic analyzer. Consensus sequence of 1418bp rDNA gene was generated from forward and reverse sequence data using aligner software. The sequences obtained were compared with the nrdatabase of NCBI genbank data base using BLAST search program (http://www.ncbi.nlm.nih.gov) (Marchler-Bauer *et al*., 2002; Pruitt *et al*., 2005)

Phylogenetic data analysis

Ten maximum identical score were aligned using multiple

Figure 10: 161 BLAST hits for *Bacillus cereus* Probio 32

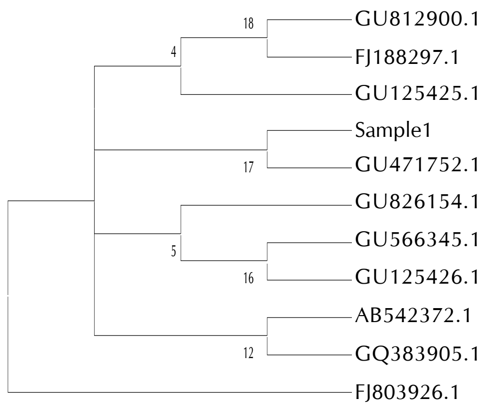


Figure 11: Phylogenetic tree showing the evolutionary history of different strains of *Bacillus cereus*

TGCAAGTCGAACGATGATCTCCCGCTTGCGGGGGTGATTAGTGGC
GAACGGGTGAGTAATACGTGAGTAACCTGCCCCTGACTCTGGGATAA
GCCTGGGAAACCGGGTCTAATACTGGATACGACTCCTCATCGCATGGT
GGGGGGTGGAAAGGGTTTGACTGGTTTTGGATGGGCTCACGGCCTAT
CAGCTTGTTGGTGGGGTAATGGCTCACCAAGGCGACGACGGGTAGC
CGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCA
GACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGGA
AGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTC
GGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCCACAAGTGAC
GGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAG
CCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTG
GGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCTGTGAA
AGCCCGGGGCTCAACCCC

Figure 12: Forward primer for *Kocuria* of 550bp

alignment software program Clustal W (Thompson, 1994). 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). The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed

CCTTCGACGGCTCCCTCCCACAAGGGGTTAGGCCACCGGC
TTCGGGTGTTACCAACTTTCGTGACTTGACGGGCGGTGTGTACA
AGGCCCGGGAACGTATTCACCGCAGCGTTGCTGATCTGCGAT
TACTAGCGACTCCGACTTCATGAGGTCGAGTTGCAGACCTCAAT
CCGAACTGAGACCGGCTTTTTGGGATTAGCTCCACCTCACAGTA
TCGCAACCCTTTGTACCGGCCATTGTAGCATGCGTGAAGCCCAA
GACATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCC
GAGTTGACCCCGGCAGTCTCCTATGAGTCCCCACCATCACGTG
CTGGCAACATAGAACGAGGGTTGCGCTCGTTGCGGGACTTAAC
CCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTG
TCCACCGACCCCGAAGGGAAACCCCATCTCTGGGGTAGTCCGG
TGAATGTCAAGCCTTGGTAAGGTTCTTCGCGTTGCATCGAATTAA
TCCGCATGCTCCGCCGCTTGTGCGGGCCCCCGTCAATTCCTTTG
AGTTTTAGCCTTGCGGCCGTACTCCCCAGGCGGGGCACTTAAT
GCGTTAGCTACGGCGCGGAGAACGTGGAATGTCCCCCACACC
TAGTGCCCAACGTTTACGGCATGGACTACCAGGGTATCTAATC
CTGTTCGCTCCCCATGCTTTCGCTCCTCAGCGTCAGTAACAG
CCCAGAGACCTGCCTTCGCCATCGGTGTTCCTCCTGATATCTGCG
CATTTCACCGCTACACCAGGAATTCCAGTCTCCCCTACTG
CACTCTAGTCTGCCCGTACCCACTGCAGACCCGGGGTTG
AGCCCCGGGCTTTCACAGCAGACGCGACAAACCGCCTA
CGAGCTCTTTACGCCCAATAATTCCGGACAACGCTTGCG
CCCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCC
GGCGCTTCTTCT

Figure 13: Reverse primer for Kocuria (985 bp)

(Felsenstein, 1985).

RESULTS AND DISCUSSION

The genomic analysis of the bacterial colonies were done to know the resistant bacteria to fly ash content in the soil.

The 75% dominating bacteria in the Petri plate, the most resistant to fly ash, were the punctiform whitish colonies, assigned as sample 1. It was identified to be *A. punctata* strain JM10 (Gen Bank Accession Number: GU205197.1) on the basis of the nucleotide homology and phylogenetic analysis. Fragment of 16S rDNA gene was amplified by PCR from the isolated bacterial DNA, revealed a single discrete PCR amplicon band of 1500 bp when resolved on agarose gel (Fig. 1). The forward and reverse primers used for the bacterial DNA sequencing were 8F and 1492R primers revealing two different regions of the 16S rDNA with 814 bp and 913 bp respectively (Fig. 2, 3). A consensus sequence of 1418 bp rDNA gene (Fig. 4) was obtained.

BLAST reports the sequence similarity by 100 blast hits to identify the homolog to the query sequence and infer the unknown bacterium (Fig. 5). 10 homologous sequences were inferred from the BLAST with similarity of about 200 amino acid sequences. The significant alignment table (Table 1) revealed the homologous bacteria to the identified bacterium *i.e. Aeromonas punctata* strain JM10 with the respective gene bank accession number. Additionally, it showed the maximum score of 2614 which was equivalent to the total score except for the uncultured bacterium clone Niu10 which had 2617 maximum score. The query coverage was almost 100% with expected value to be zero showing the sequences to be highly homogenous. The maximum identification for the homologies was 99%.

The unrooted phylogenetic tree was constructed on the basis of the distance matrix which exhibits the dissimilarity between the nucleotide sequences of the respective homolog strains of bacterium, *Aeromonas punctata*. The distance matrix table

TGCAAGTCGAACGATGATCTCCCGCTTGCGGGGGTGATTAGTG
GCGAACGGGTGAGTAATACGTGAGTAACCTGCCCCTGACTCT
GGGATAAGCCTGGGAAACCGGGTCTAATACTGGATACGACTC
CTCATCGCATGGTGGGGGGTGGAAAGGGTTTGACTGGTTTTG
GATGGGCTCACGGCCTATCAGCTTGTTGGTGGGGTAATGGCT
CACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCG
GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG
CAGCAGTGGGGAATATTGCACAATGGGCGGAAGCCTGATGCA
GCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCT
CTTTCAGCAGGGAAGAAGCCACAAGTGACGGTACCTGCAGAA
GAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA
GGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCG
TAGGCGGTTTGTCGCGTCTGCTGTGAAAGCCCGGGGCTCAACC
CCGGGTCTGCAGTGGGTACGGGCAGACTAGAGTGCAGTAGGG
GAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCA
GGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCTGTTACT
GACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGAT
ACCCTGGTAGTCCATGCCGTAAACGTTGGGCACTAGGTGTGGG
GGACATTCCACGTTCTCCGCGCCGTAGCTAACGCATTAAGTGCC
CCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATT
GACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCG
ATGCAACGCGAAGAACCTTACCAAGGCTTGACATTCACCGGACTA
CCCCAGAGATGGGGTTTCCCTTCGGGGTCGGTGGACAGGTGGT
GCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC
CGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTGATGGT
GGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTG
GGGATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACG
CATGCTACAATGGCCGGTACAAAGGGTTGCGATACTGTGAGGTG
GAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATTGAGGTCTGCA
ACTCGACCTCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCA
ACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTC
AAGTCACGAAAGTTGGTAACACCCGAAGCCGGTGGCCTAACCCCT
TGTGGGAGGGAGCCGTCGAAGG

Figure 14: Consensus Sequence of 1401bp revealed for *Kocuria* sp HO-9042

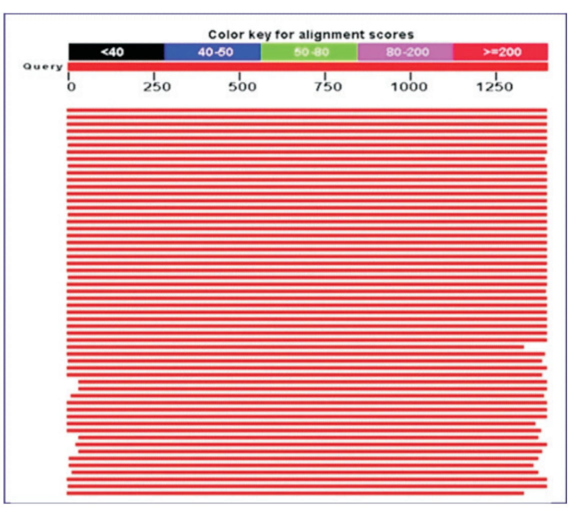


Figure 15: Blast hits for *Kocuria*

(Table 2) showed similarity in the sequences except for bacterial strain *Aeromonas* sp. B27. Here a dissimilarity of 0.0007 has been observed among the selected strains of bacteria.

The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1988) 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) was shown next to the branches or at the nodes. The evolutionary distances were computed in the units of the number of base substitutions per site. Codon positions included were1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated from dataset. There were a total of 1417 positions in the final set.

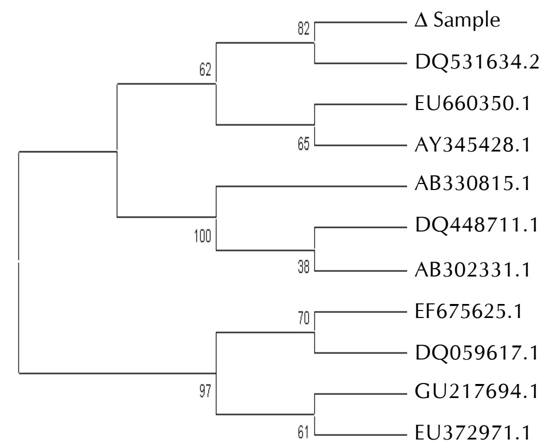


Figure 16: Phylogenetic tree for *Kocuria* sp HO-9042

The phylogenetic tree (Fig. 6) showed high level of 16S rDNA similarity between the 10 strains of the bacterium. In particular, *Aeromonas punctata* strain JM10 showed high similarity 16S rDNA value with highest bootstrap value shown in the dendrogram *i.e.* 16 at the node with the sample, thereby, inferring it to be the same. This monophyletic group of sample and *Aeromonas punctata* strain JM10 (GU205197.1) showed close relatedness with *Aeromonas punctata* strain 159 (GQ259885.2) with bootstrap value of 8. Uncultured bacterium clone Niu10 and *Aeromonas punctata* strain JW04 share identical value of 16S rDNA with a bootstrap value of 14. The above group of strain was relatively similar to strain RK 65541 with bootstrap value 8. The strain 219c is identical with slight change in any of the ambiguous nucleotide to the above set of strains with the value 8. Further the group is in relation with strain MPT4 with bootstrap value 12. Strain 360c and 176c share identical 16S rDNA with bootstrap value 14. This is in turn related to *Aeromonas* sp. B27 with certain changes in the nucleotide sequences.

The evolutionary tree shows that JM10 is closely related to the strain 159 and is distantly related to *Aeromonas* sp B27. These 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 distantly related with low bootstrap value showing considerable divergence between *Aeromonas punctata* strains. Phylogenetic divergence was also observed between *Aeromonas salmonicida* and *A. bestiarum* (Soler, 2004). Divergence among the strains might be in accordance to biovars, serovars or the morphovars which is the variation characterized by the biochemical, physiological, morphological or by the antigenic properties.

Table 1: Significant alignment revealing homologues of bacterium *Aeromonas punctata*

Accession	Description	Max score	Total Score	Query coverage	E value	Max ident
EU862311.1	*Uncultured bacterium clone* Niu10	2617	2617	99%	0.0	100%
GQ259885.2	*Aeromonas punctata* strain 159	2614	2614	100%	0.0	99%
GU205197.1	*Aeromonas punctata* strain JM10	2614	2614	100%	0.0	99%
GU205195.1	*Aeromonas punctata* strain JW04	2614	2614	100%	0.0	99%
FJ494901.1	*Aeromonas* sp. B27	2614	2614	100%	0.0	99%
FJ168776.1	*Aeromonas punctata* strain 219c	2614	2614	100%	0.0	99%
DQ979324.1	*Aeromonas punctata* strain MPT4	2614	2614	100%	0.0	99%
FJ168775.1	*Aeromonas punctata* strain 176c	2614	2614	100%	0.0	99%
FJ168774.1	*Aeromonas punctata* strain 360c	2614	2614	100%	0.0	99%
AY987761.1	*Aeromonas punctata* strain RK 65541	2614	2614	100%	0.0	99%

Table 2: 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	

Table 3: Homologues of *Bacillus cereus* strain *Probio* 32

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
GU812900.1	Bacillus cereus strain JBS10	2636	2636	100%	0.0	100%
GU826154.1	Bacillus cereus strain Q34	2636	2636	100%	0.0	100%
GU566345.1	Bacillus sp. R5(2010)	2636	2636	100%	0.0	100%
GU471752.1	Bacillus cereus strain Probio-32	2636	2636	100%	0.0	100%
AB542372.1	Bacillus sp. TSA4w	2636	2636	100%	0.0	100%
GU125426.1	Bacillus cereus strain IMAU80004	2636	2636	100%	0.0	100%
GU125425.1	Bacillus cereus strain IMAU80003	2636	2636	100%	0.0	100%
GQ383905.1	Bacillus sp. 4CCS8	2636	2636	100%	0.0	100%
FJ188297.1	Bacillus cereus strain BU040901-022	2636	2636	100%	0.0	100%
FJ803926.1	Bacillus cereus strain 0-9	2636	2636	100%	0.0	100%

Table 4: Distance matrix table For the *Bacillus cereus* strain *Probio* 32

Sample 2	1		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GU812900.1	2	0.0000		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GU826154.1	3	0.0000	0.0000		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GU566345.1	4	0.0000	0.0000	0.0000		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GU471752.1	5	0.0000	0.0000	0.0000	0.0000		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
AB542372.1	6	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000	0.0000	0.0000	0.0000	0.0000
GU125426.1	7	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000	0.0000	0.0000	0.0000
GU125425.1	8	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000	0.0000	0.0000
GQ383905.1	9	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000	0.0000
FJ188297.1	10	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000
FJ803926.1	11	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	

Table 5: Significant alignment table revealing 10 homologs of *Kucoria*

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
DQ531634.2	Kocuria sp. HO-9042	2588	2588	100%	0.0	100%
EU660350.1	Kocuria rosea strain CT22	2555	2555	100%	0.0	99%
AY345428.1	Bacterium K2-25	2553	2553	100%	0.0	99%
DQ448711.1	Kocuria sp. CNJ770 PL04	2510	2510	100%	0.0	99%
EF675625.1	Kocuria sp. RM1	2497	2497	100%	0.0	98%
AB302331.1	Actinobacterium C18 gene	2481	2481	99%	0.0	98%
GU217694.1	Kocuria sp. ljh-7	2475	2475	100%	0.0	98%
AB330815.1	Actinobacterium C20	2471	2471	99%	0.0	98%
DQ059617.1	Kocuria aegyptia strain YIM 70003	2459	2459	99%	0.0	98%
EU372971.1	Kocuria sp. E7	2453	2453	100%	0.0	98%

Table 6: Distance matrix table

Sample –A	*1*		*0.000*	*0.001*	*0.001*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*	*0.002*
DQ531634.2	2	0.000		0.001	0.001	0.002	0.002	0.002	0.002	0.002	0.002	0.002
EU660350.1	3	0.001	0.001		0.000	0.002	0.002	0.002	0.002	0.002	0.002	0.002
AY345428.1	4	0.001	0.001	0.000		0.002	0.002	0.002	0.002	0.002	0.002	0.002
DQ448711.1	5	0.005	0.005	0.005	0.005		0.002	0.002	0.002	0.002	0.000	0.002
EF675625.1	6	0.006	0.006	0.006	0.006	0.008		0.002	0.001	0.002	0.002	0.001
AB302331.1	7	0.005	0.005	0.005	0.005	0.000	0.008		0.002	0.002	0.000	0.002
GU217694.1	8	0.006	0.006	0.005	0.005	0.008	0.003	0.008		0.002	0.002	0.001
DQ059617.1	9	0.007	0.007	0.007	0.007	0.010	0.004	0.010	0.004		0.002	0.002
AB330815.1	10	0.005	0.005	0.005	0.005	0.000	0.008	0.000	0.008	0.010		0.002
EU372971.1	11	0.006	0.006	0.006	0.006	0.008	0.003	0.008	0.001	0.004	0.008	

Table 7: Significant alignment table revealing 10 homologs of *Bacilllus cereus* strain MBL13

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
GQ148914.1	Bacillus cereus strain MBL13	2623	2623	100%	0.0	100%
GU190368.1	Bacillus sp. Ts-116	2614	2614	99%	0.0	99%
FJ932761.1	Bacillus thuringiensis strain 61436	2612	2612	100%	0.0	99%
DQ289058.1	Bacillus cereus isolate HKS 2-1	2610	2610	99%	0.0	99%
HM047298.1	Bacillus thuringiensis strain ZJU03	2606	2606	100%	0.0	99%
GU269268.1	Bacillus cereus strain P-12	2606	2606	100%	0.0	99%
GU250444.1	Bacillus cereus strain BFE 5384	2606	2606	100%	0.0	99%
GU250443.1	Bacillus cereus strain BFE 5392	2606	2606	100%	0.0	99%
GU120652.1	Bacillus thuringiensis strain IWF24	2606	2606	100%	0.0	99%
EU622630.1	Bacillus sp. NS-4	2606	2606	100%	0.0	99%

Table 8: Distance matrix table

PH-2	*1*		*0.000*	*0.001*	*0.001*	*0.001*	*0.001*	*0.001*	*0.001*	*0.001*	*0.001*	*0.001*
GQ148914.1	2	0.000		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
GU190368.1	3	0.001	0.001		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
FJ932761.1	4	0.001	0.001	0.001		0.001	0.001	0.001	0.001	0.001	0.001	0.001
DQ289058.1	5	0.002	0.002	0.001	0.001		0.001	0.001	0.001	0.001	0.001	0.001
HM047298.1	6	0.002	0.002	0.003	0.001	0.001		0.000	0.001	0.000	0.000	0.000
GU269268.1	7	0.002	0.002	0.003	0.001	0.001	0.000		0.001	0.000	0.000	0.000
GU250444.1	8	0.001	0.001	0.002	0.001	0.001	0.001	0.001		0.001	0.001	0.001
GU250443.1	9	0.002	0.002	0.003	0.001	0.001	0.000	0.000	0.001		0.000	0.000
GU120652.1	10	0.002	0.002	0.003	0.001	0.001	0.000	0.000	0.001	0.000		0.000
EU622630.1	11	0.002	0.002	0.003	0.001	0.001	0.000	0.000	0.001	0.000	0.000	

The second resistant bacterium identified was *Bacillus cereus* strain Probio 32 (Gen Bank Accession Number: GU471752.1). PCR of fragment of 16S rDNA from the isolated bacterial DNA, showed the amplicon band of 1500 bp when resolved on agarose gel (Fig. 1).The forward and reverse primers used for the bacterial DNA sequencing were also 8F and 1492R revealing two different regions of the 16S rDNA with 833 bp and 848bp respectively (Fig. 7, 8) . A consensus sequence of 1427 bp rDNA gene (Fig. 9) was obtained for the bacterium *Bacillus cereus*.

The 161 BLAST HITS (Fig. 10) on the query sequence revealed the homologs with more than 200 amino acids sequence similarity. The significant alignment table (Table 3) showed the 10 homologous taxa with maximum score 2636 equivalent to total score. The query coverage was 100% with maximum identification of 100%. The minimum expected value of zero showed the maximum similarity among the homologues. The distance matrix value (Table 4) depicted the no dissimilarity among the 10 homolog of the sampled bacterium.

The unrooted phylogenetic tree showed the convergent evolution between the homologues. The dendrogam showed the 11 strains of bacteria of which 8 strains are grouped (Fig. 11). The tree depicted the sample to be identical to *Bacillus cereus* strain Probio-32 with the bootstrap value 17. *Bacillus cereus* strain JBS10 and strain BU040901-022 showed identical nucleotide sequences with bootstrap value 18.

This group was related to strain IMAU80003 with the value 4. Identical nucleotide sequence were also seen in *Bacillus* sp. R5 (2010) and strain IMAU80004 with bootstrap value 16. Further, it's closely related to *Bacillus cereus* strain Q34 with a value 5. *Bacillus* sp. TSA4w shared similar nucleotide sequence with *Bacillus* sp. 4CCS8 having bootstrap value 12. Further, the above groups were distantly related to *Bacillus cereus* strain BU040901-022.

Xu and Cote (2003) stated that *Bacillus anthracis*, *Bacillus cereus*, *Bacillus mycoides* and *Bacillus thuringiensis* belongs to the same group from 40 Bacillaceae studied species. The phenotypic and genotypic similarities between all four species have been well documented (Ash, 1991). The genomes of these three species show high levels of similarity; for example, they share almost identical 16S rDNA sequences (CDC, 2001)

B. cereus often considered at most, a soil-dwelling opportunistic pathogen (Jensen *et al.*, 2011). The bacterium *Bacillus cereus* produces a non-hemolytic enterotoxin, known as Nhe which is the major food poisoning toxin. The three

TGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGA
CGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAA
CCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGG
CTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTA
ACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACA
CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAA
TCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGAT
GAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTT
GAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACG
TGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATT
ATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACG
GCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGG
AAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAAC
ACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGC
GCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGA
AGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAA
GGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTG
GAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGG
TCTTGACATCCTTTGACAACCCTAGAGATAGGGCTTCTCCTTC

Figure 17: Forward primer of 986 bp for *Bacillus cereus* strain MBL 13

GGCGGCTGGCTCCAAAAGGTTACCCCACCGACTTCGGGTGTT
ACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGG
GAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATT
CCAGCTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGA
ACGGTTTTATGAGATTAGCTCCACCTCGCGGTCTTGCAGCTCTTT
GTACCGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGC
ATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGG
CAGTCACCTTAAAGTGCCCAACTAAATGATGGCAACTAAAATCAA
GGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACG
AGCTGACGACAACCATGCACCACCTGTCACTCTGCTCCCGAAGG
AGAAGCCCTATCTCTAGGGTTGTCAAAGGATGTCAAGACCTGGT
AAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTT
GTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGCCTTGCGGCCGT
ACTCCCCAGGCGGAGTGCTTAATGCGTTAACTTCAGCACTAAAGG
GCGGAAACCCTCTAACACTTAGCACTCATCGTTTACGGCGTGGAC
TACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCA
GTGTCAGTTACAGACCAGAAAGTCGCCTTCGCCACTGGTGTTCCTC
CATATCTCTACGCATTTCACCGCTACACATGGAATTCCACTTTCCTC
TTCTGCACTCAAGTCTCCCAGTTTCCAATGACCCTCCACGGTTGAG
CCGTGGGCTTTCACATCAGACTTAAGAAACCACCTGCGCGCGCTT
TACGCCCAATAATTCCGGATA

Figure 18: Reverse primer of 916 bp for *Bacillus cereus* strain MBL 13 (915 bp)

proteins in the Nhe toxin are called NheA, NheB and NheC (Phung, 2013).

The next bacterium identified was *Kocuria* sp. HO-9042 (GenBank Accession Number: DQ531634.2). The 1500 bp amplicon band obtained by PCR of 16S rDNA from isolated DNA of the bacteria was sequenced with forward and reverse primers 8F and 1492R (Fig. 12, 13) of 550bp and 1401 bp obtaining a consensus sequence of 1401bp (Fig. 14).

The 100 BLAST HITS on the query sequence here revealed the homologues with more than 200 amino acids sequence similarity (Fig. 15). The significant alignment table (Table 5) depicted the variation in the maximum score of the 10 homologous taxa which were equivalent to total score. The query coverage was 100% with maximum identification of 99%. The minimum expected value of zero showed the maximum similarity among the homologues. The distance

TGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGG
CGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGAT
AACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCAT
GGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCC
GCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACG
ATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGA
CACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCA
ATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCT
TTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAA
TAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACT
ACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGG
AATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGT
GAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGAC
TTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATG
CGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCT
GTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAG
ATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAG
GGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTG
GGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCC
CGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGA
AGAACCTTACCAGGTCTTGACATCCTTTGACAACCCTAGAGATAGGGCT
TCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGC
TCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTT
GATTTTAGTTGCCATCATTTAGTTGGGCACTTTAAGGTGACTGCCGG
TGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCC
TTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAG
AGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCG
TTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAG
CTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAAT
ACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAG
TTTGTAACACCCGAAGTCGGTGGGGTAACCTTTTGGAGCCAGCCGCC

Figure 19: Consensus sequence of 1420 bp for *Bacillus cereus* strain MBL 13

matrix value (Table 6) depicted the dissimilarity among the 10 homolog of the sampled bacterium. The *Kocuria* sp. HO-9042 showed a dissimilarity of 0.001 in the nucleotide sequences with *Kocuria rosea* strain CT22, 0.005 with *Kocuria* sp. CNJ770 PL04.0.001 with Bacterium K2-25, 0.006 with *Kocuria* sp. RM1, 0.005 with *Actinobacterium* C18 gene, 0.006 with *Kocuria* sp. ljh-7, 0.007 with *Actinobacterium* C20, 0.005 with *Kocuria aegyptia* strain YIM 70003 and 0.006 with *Kocuria* sp. E7.

The phylogenetic tree showed the evolutionary relationship of the related taxa (Fig. 16). The sample shared an identical nucleotide sequence with *Kocuria* sp. HO-9042 with a high bootstrap value of 82 expressing themselves to form a clad group. The other identical nucleotide sharing was seen among *Kocuria rosea* strain CT22 and Bacterium K2-25 with a bootstrap value 65. The two monophyletic groups were related to each other by the value 62. *Kocuria* sp. CNJ770 PL04 and *Actinobacterium* C18 gene also formed a monophyletic group with bootstrap value 38 and were related to *Actinobacterium* C20 by 100.

Further, two monophyletic groups were observed among *Kocuria* sp. RM1, *Kocuria aegyptia* strain YIM 70003 and *Kocuria* sp. LJH-7, *Kocuria* sp. E7 with a bootstrap value of 70 and 61.

The two groups were further related to each other by bootstrap value 97. These were very closely related showing a convergent evolution. The above phenons showed at least 70% relatedness under optimal hybridization condition.

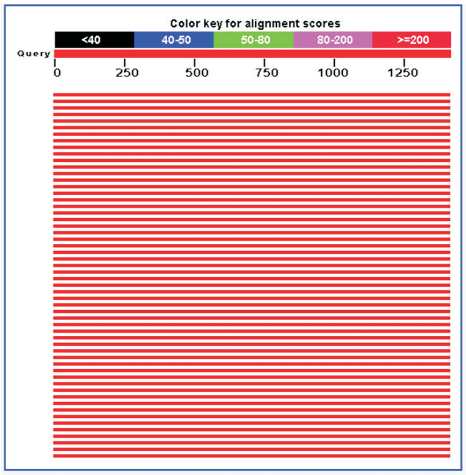


Figure 20: BLAST hits for *Bacillus cereus* MBL13

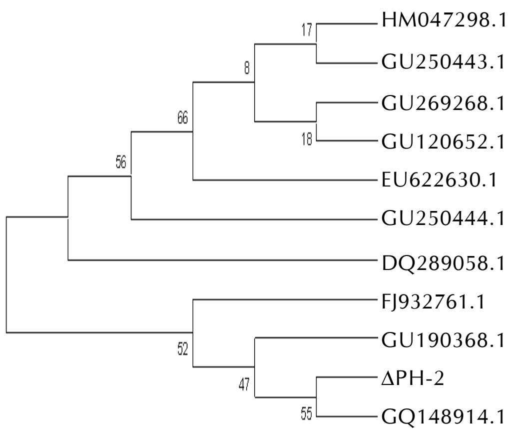


Figure 21: Phylogenetic tree depicting the evolutionary status of *Bacillus cereus* strain MBL13 (PH-2)

The phenotypic features and complete sequence of 16S rDNA revealed that *Kocuria* sp. HO-9042 strain showed 99% sequence similarity with *Kocuria rosea* strain CT22 (Stackebrandt, 1995) and 98% sequence similarity with *Kocuria* sp. RM1 and *Kocuria aegyptia* strain 71M70003 (Altuntas *et al*., 2004). Sequence similarity among the strains were ranged between 98 to 100 % which was also observed in case of *Kocuria rosea* DSM 20447(T) and *Kocuria polaris* MTCC 3702(T) with 98.1 and 97.8 % sequence similarity, respectively (Zhou, 2008).

The fourth identified bacterium was *Bacillus cereus* strain MBL13 (Gen Bank Accession Number: GQ148914.1).

Here the 1500 bp amplicon band of 16S rDNA of the isolated DNA of the bacterium by PCR was sequenced with forward and reverse primers 8F (986 bp) and 1492R (916bp) resulting in a consensus sequence of 1420bp (Fig. 17-19).

The 100 blast hits on the query sequence inferred the 10 nucleotide homologues with more than 200 amino acid sequence similarity (Fig. 20). The maximum score was high for the bacterium *Bacillus cereus* MBL13 (2623). With the minimum expected value maximum similarity among the homologs can be observed (Table 7). The distance matrix (Table 8) exhibited no dissimilarity between the bacterium and GQ148914.1 confirming its identity. Further, a dissimilarity of 0.001is shown with *Bacillus* sp. Ts-116, *Bacillus thuringiensis* strain 61436, *Bacillus cereus* strain BFE 5384 and 0.002 with *Bacillus cereus* isolate HKS 2-1, *Bacillus thuringiensis* strain ZJU03, *Bacillus cereus* strain P-12, *Bacillus cereus* strain BFE 5392, *Bacillus thuringiensis* strain IWF24 and *Bacillus* sp. NS-4.

On the account of the distance matrix the unrooted phylogenetic tree had been constructed depicting the evolutionary status of the bacterium among the other 10 homologues as shown in the Fig. 21. The sample bacterium shared identical nucleotide sequence *to Bacillus cereus* strain MBL13 with a bootstrap value of 55 forming a monophyletic group.

The clad were related to *Bacillus* sp Ts-19 with a value of 47 and thereby related to *Bacillus thuringiensis* strain 61436 with 52 bootstrap values. High up in the evolutionary tree, with more stability two monophyletic group were observed between *Bacillus thurigiensis* strain IWF24 and *Bacillus cereus* strain P-12 and between *Bacillus cereus* strain BFE 5392 and *Bacillus thuringiensis* strain ZJU03 with bootstrap value 18 and 17 respectively among the two. The two groups were related to each other with bootstrap value 8 showing 100% maximum identification. This in turn shared identical nucleotide value with a bootstrap 66. Then it's further showed relatedness with next homolog *Bacillus cereus* strain BFE 5384 with value 56 and then to *Bacillus cereus* isolates HKS 2-1. From the dendrogram its clear that two distinct groups were formed which emerged from a distinct node of the unrooted tree. The sampled bacterium was not the highly evolved among the homologs but showed convergent evolution within the group

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