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Genomic Analysis of Bacterium Isolated from Amended Coal Fly Ash by 16S rDNA

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Abstract : Fly ash being the problematic solid waste must be managed in eco-friendly way. For the same the present study was conducted to analyse the FA resistant bacteria by16S rDNA PCR. The identified bacterium was *Kocuria* sp HO-9042 with Gen bank Accession No: DQ531634.2. PCR amplification of the 16S rDNA gene from *Kocuria* sp revealed 8F and 1492R sequences with 550bp and 985bp respectively and a consensus sequence of 1401bp. The distribution of 100 BLAST hits on the 1401 query sequence showed the 10 homologs with about 200 nucleotide similarities with the homologues showing close relatedness on the basis of the sequence similarity in the significant alignments. The present investigation suggests that *Kocuria sp* HO-9042 in coal fly ash amended soil will also help in improving soil fertility.

Key words : Fly ash, 16S rDNA, *Kocuria*, PCR, BLAST.

Introduction

Fly ash (FA), a coal combustion residue of thermal power plants has been regarded as a problematic solid waste all over the world (Pandey and Singh, 2010). It is an amorphous ferro-alumino silicate with a matrix very similar to soil. Addition of FA to soil may improve the physico-chemical properties as well as nutritional quality of the soil and the extent of change depends on soil and FA properties. In view of the high cost of disposal and environmental management, utilization of FA in agricultural sector could be a viable option. Its use in agriculture was initially due to its liming potential and the presence of essential nutrients, which promoted plant growth and also alleviated the nutrient deficiency in soils (Mittra *et al*., 2005).

In soil, microorganisms by virtue of the exo-enzymatic activities are considered as primary decomposers playing key role in mineralization and demineralization process facilitating cycling of minerals in biosphere (Rodriquez *et al*., 2011) resulting in the fertility of the soil. Bacterial population can influence carbon or mineral cycles and have the ability to colonize harsh environments. However, little efforts have been made in studying the microbial ecology of such soils. In such soils or sites affected with fly ash, introduction of beneficial soil microorganisms and their establishment, colonization and survival along with their role in improving soil fertility and interaction with plant roots will reveal more information on developing strategies for faster remediation of such sites. Amplification of 16S rDNA and analysis of amplicon diversity using techniques such as TGGE and DGGE are valuable tools for exploring microbial diversity in the natural environments (Macrae, 2000). 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. As there is paucity of knowledge about the bacterial identification which are resistive to fly ash, the present work was conducted to identify the resistive bacterium in the fly ash amended soil using 16S rDNA.

Materials and Methods

Fly ash for the laboratory experiment was collected from Patratu Thermal Power Plant, Ranchi and was amended with soil from agro-ecosystem of Ranchi University campus. Table 1 depicts the edaphic profile of soil and fly ash. The amendment was done in the proportion of 5% FA. The bacterial culture was prepared using sample from the FA and soil mixture 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 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 plates (100 mm dia) containing Czapak Dox Agar media for the bacterial culture. The white circular colonies with entire margin and raised elevation were isolated from the bacterial culture by streak method and pure cultured using solid agar media for slant preparation which was used for the genomic analysis.

Table 1: Edaphic profile of soil and fly ash (mean±SD, n=4)

Parameters	Soil	Fly ash
pH	5.81±0.1	6.85±0.31
O. C (g %)	0.31±0.21	0.12±0.11
O.M (g %)	0.54±1.1	0.21±0.3
Nitrogen (mg N/g soil)	0.078±0.41	0.175±0.22
Phosphorus (mg P/g soil)	0.0279±0.17	0.13±0.23
Potassium (mg K/g soil)	1.48±0.41	1.9±0.14
EC(m.Mhos/cm)	0.23±0.04	0.56±0.21

Genomic analysis

DNA extraction and purification

DNA was isolated from the pure culture of the bacterial colony. Tris – EDTA (10mM Tris-HCl, 1mM EDTA; pH 8) buffer and lysozyme (10 mg/mL) were added in the pelleted cells of the dominant isolate and incubated for 30 min at room temp. SDS and proteinase K(10U/µL) were added and incubated at 55°C for 2h. DNA was extracted with phenol, chloroform and iso-amyl alcohol and was precipitated with ethanol and dissolved in TE buffer (Wawer and Muyzer 1995). Its quality was evaluated on 1.2 Agarose Gel which revealed a single band of high molecular weight DNA.

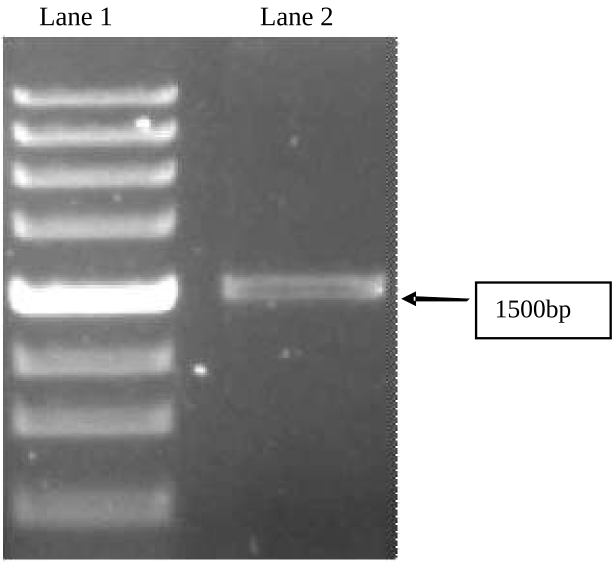
PCR amplification and sequencing 16S rDNA gene

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. 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 1401bp rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the nrdatabase of NCBI genbank database (Pruitt *et al*., 2005). Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. The nucleotide database was searched with the sequences obtained with NCBI BLAST tool (http:// www.ncbi.nlm.nih.gov/BLAST) (Altuschul et *al*., 1997)

Results

The bacterial culture of the soil fly ash mixture expressed morphologically different colonies. The circular colonies from the culture on genomic analysis

Figure 1: Agarose gel image showing the 1500bp of 16S rDNA amplicon band



Lane 1 - DNA marker
Lane 2 - 16 S rDNA amplicon band

by 16S rDNA polymerase chain reaction was found to be *Kocuria sp.* HO-9042 (Gen Bank Accession Number: DQ531634.2) on the basis of nucleotide homology. 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 of the bacterium *Kocuria sp.* HO-9042 with 550bp and 985 bp respectively (Fig: 2 & 3). The forward and reverse sequence data revealed a consensus sequence of 1401bp (Fig. 4) referring to the most common nucleotide or amino acid at a particular position after multiple sequences are aligned. The consensus sequence showed which residues were most abundant in the alignment at each position.

Further, the BLAST reports the sequence similarity by 100 blast hits to identify the homologs to the query sequence and infer the unknown bacterium (Fig. 5). 16S rDNA gene of the bacterium was used as the input sequence to determine the bacterial homologs using 1401bp of the query sequence and assessing the similarity. 10 homologous sequences were inferred from the BLAST with similarity of about 200 amino acid or nucleotide sequences with slight variations. The nucleotide database were searched with the sequences obtained using NCBI BLAST tool and showed 99% similarity with 16S rDNA gene of the sampled bacterium in the database sequences. Based on these characteristics and sequence analysis, the isolate was identified as *Kocuria sp* HO-9042.

The significant alignment table (Table 2) revealed the homologous bacteria to the identified bacterium in accordance to the Gen Bank Database. The table depicted the variation in the maximum score of the 10 homologous taxa which were equivalent to the total score with *Kocuria sp* HO-9042 showing 2588 score. The minimum expected value of zero showed the maximum similarity among the homologues revealing them to be the homogenous strains of *Kocuria*. 99% similarity was observed for *Kocuria rosea* strain CT22, Bacterium K2-25 and *Kocuria* sp. CNJ770 PL04 followed by 98% sequence similarity for *Kocuria* sp. RM1, *Actinobacterium* C18 gene, *Kocuria* sp. ljh-7, *Actinobacterium* C20, *Kocuria aegyptia* strain YIM 70003 and *Kocuria* sp. E7 to the identified bacterium *Kocuria* sp. HO-9042.

Figure 2: Forward primer (8F) for *Kocuria sp* HO-9042 of 550bp

TGCAAGTCGAACGATGATCTCCCGCTTGCGGGGGTGATTAGTGGCGAACGGGTGAGTAATACGTGAGTAAC
CTGCCCCTGACTCTGGGATAAGCCTGGGAAACCGGGTCTAATACTGGATACGACTCCTCATCGCATGGTGGG
GGGTGGAAAGGGTTTGACTGGTTTTGGATGGGCTCACGGCCTATCAGCTTGTTGGTGGGGTAATGGCTCACC
AAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCC
TACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGGAAGCCTGATGCAGCGACGCCGCGTGAGGGAT
GACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCCACAAGTGACGGTACCTGCAGAAGAAGC
GCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAA
AGAGCTCGTAGGCGGTTTGTCGCGTCTGCTGTGAAAGCCCGGGGCTCAACCCC

Figure 3: Reverse primer (1492F) for *Kocuria sp* HO-9042 of 985bp

CCTTCGACGGCTCCCTCCCACAAGGGGTTAGGCCACCGGCTTCGGGTGTTACCAACTTTCGTGACTTGACGG
GCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCAGCGTTGCTGATCTGCGATTACTAGCGACTCCGACTT
CATGAGGTCGAGTTGCAGACCTCAATCCGAACTGAGACCGGCTTTTTGGGATTAGCTCCACCTCACAGTATC
GCAACCCTTTGTACCGGCCATTGTAGCATGCGTGAAGCCCAAGACATAAGGGGCATGATGATTTGACGTCAT
CCCCACCTTCCTCCGAGTTGACCCCGGCAGTCTCCTATGAGTCCCCACCATCACGTGCTGGCAACATAGAAC
GAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCT
GTCCACCGACCCCGAAGGGAAACCCCATCTCTGGGGTAGTCCGGTGAATGTCAAGCCTTGGTAAGGTTCTT
CGCGTTGCATCGAATTAATCCGCATGCTCCGCCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTAGCCT
TGCGGCCGTACTCCCCAGGCGGGGCACTTAATGCGTTAGCTACGGCGCGGAGAACGTGGAATGTCCCCCAC
ACCTAGTGCCCAACGTTTACGGCATGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCATGCTTTCGCTCCT
CAGCGTCAGTAACAGCCCAGAGACCTGCCTTCGCCATCGGTGTTCCTCCTGATATCTGCGCATTTCACCGCTA
CACCAGGAATTCCAGTCTCCCCTACTGCACTCTAGTCTGCCCGTACCCACTGCAGACCCGGGGTTGAGCCCC
GGGCTTTCACAGCAGACGCGACAAACCGCCTACGAGCTCTTTACGCCCAATAATTCCGGACAACGCTTGCG
CCCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGCGCTTCTTCT

Figure 4: Consensus Sequence of 16s rDNA gene of *Kocuria sp* HO-9042 (1401 bp)

TGCAAGTCGAACGATGATCTCCCGCTTGCGGGGGTGATTAGTGGCGAACGGGTGAGTAATACGTGAGTAAC
CTGCCCCTGACTCTGGGATAAGCCTGGGAAACCGGGTCTAATACTGGATACGACTCCTCATCGCATGGTGGG
GGGTGGAAAGGGTTTGACTGGTTTTGGATGGGCTCACGGCCTATCAGCTTGTTGGTGGGGTAATGGCTCACC
AAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCC
TACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGGAAGCCTGATGCAGCGACGCCGCGTGAGGGAT
GACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCCACAAGTGACGGTACCTGCAGAAGAAGC
GCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAA
AGAGCTCGTAGGCGGTTTGTCGCGTCTGCTGTGAAAGCCCGGGGCTCAACCCCGGGTCTGCAGTGGGTACG
GGCAGACTAGAGTGCAGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
ACACCGATGGCGAAGGCAGGTCTCTGGGCTGTTACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAG
GATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCACTAGGTGTGGGGGACATTCCACGTTCTCCGCG
CCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGG
GGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACAT
TCACCGGACTACCCCAGAGATGGGGTTTCCCTTCGGGGTCGGTGGACAGGTGGTGCATGGTTGTCGTCAGC
TCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTGATGG
TGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCT
TATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAAAGGGTTGCGATACTGTGAGGTGGAGCTAATCC
CAAAAAGCCGGTCTCAGTTCGGATTGAGGTCTGCAACTCGACCTCATGAAGTCGGAGTCGCTAGTAATCGC
AGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGTCACGAAAGTTGGT
AACACCCGAAGCCGGTGGCCTAACCCCTTGTGGGAGGGAGCCGTCGAAGG

Figure 5: 100 BLAST hits of *Kocuria sp* HO-9042

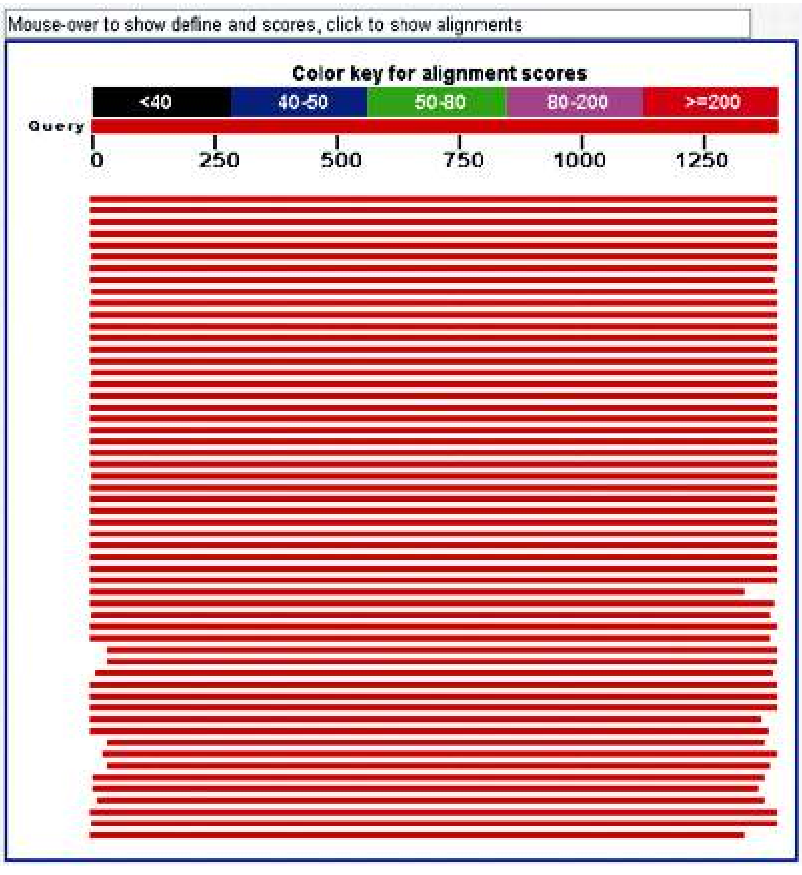


Table 2: Significant alignment table revealing 10 homologs of *Kucoria sp* HO-9042

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%

Discussion

Genomic DNA extraction, PCR mediated amplification of the 16S rDNA and sequencing of the PCR products to analyse the sampled bacterium is a highly accepted technique (Kovacs *et al*., 1999). PCR amplification of 16S rDNA using consensus bacterial primers and separation of the resultant PCR amplicons constitute the most popular molecular ecology techniques used to describe soil bacterial ecology (Macrae, 2000). Similar assessment may be done using 16S rRNA genes as in case of *Arthrobacter luteolus* (Emmanuel *et al.*, 2012)

The genus *Kocuria* (Stackebrandt *et al.*, 1995) contains four species, i.e. the type species *Kocuria rosea, Kocuria varians, Kocuria kristinae* and *Kocuria erythromyxa.* All were originally placed in the genus *Micrococcus.* Following 16S rDNA analysis the *Micrococcus,* species were shown to form an individual cluster within the *Arthrobacter-Micrococcus* line of descent (Stackebrandt *et al.*, 1995; Koch *et al.*, 1994); a cluster later described as the family *Micrococcaceae* (Stackebrandt *et al.,* 1997). Members of the genus *Kocuria* were isolated from a wide variety of natural sources including mammalian skin, soil, the rhizosphere, fermented foods, clinical specimens, fresh water and marine sediments. The *Kocuria* strains are circular, non motile gram positive, aerobic, non-encapsulated, non-halophilic, non-endospore forming, with the presence of the fatty acid anteiso $C_{15:0}$ and MK-7 (H_2) and MK-8 (H_2) as the major menaquinones (Zhou *et al.*, 2008).

The sequence similarity values determined for the type strains of members of the genus *Kocuria* ranged between 95.8 and 98.6% (Kovacs *et al*., 1999). The present work result was also in accordance to it showing similar similarity index. The sequence similarity of *K rosea* to other strain was also found to be about 98.1% (Mayilraj *et al.,* 2006). 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 as reported by Stackebrandt *et al*. (1995) and 98% sequence similarity with *Kocuria sp.* RM1 and *Kocuria aegyptia* strain 71M 70003 as found by Li *et al*. (2006).

The isolates contained all the signature nucleotides that define the family *Micrococcuceae* to which the genus *Kocuria* belongs phylogenetically (Stackebrandt *et al.,* 1997). A total of 1350 nucleotides present in all strains between positions 41 and 1458 (*E. coli* positions) were used for the analysis were also in configuration to the present study where 1401bp between 8F and 1492R sequences were used.

K. rosea was highly similar to the bacterium *Kocuria sp* HO- 9042 with 99% DNA DNA similarity index. *K. erythromyxa* was not included because, on the basis of the high 16S rDNA similarity, it can be considered a close relative of *K. rosea* (Kovacs *et al.*, 1999). The type strain *Kocuria rosea* has been reported to cause catheter related bacterium (Altuntas et al., 2004) and the majority of strains are non-pathogenic. Micro-organisms in the soil have the capability to degrade hydrocarbons and act as major agents for remediation of contaminated soil (Widada *et al*., 2002). Presence of *Kocuria sp* HO-9042 in coal fly ash amended soil will also help in the remedial activity as bacterial population have the ability to colonize harsh environments and play their role in improving soil fertility. Therefore its application to the amended FA will be beneficial on the context to the fertility of soil. Being non- pathogenic, the incorporation in the FA amended soil will be harmless. This will help in the utilization of fly ash in agricultural purpose thereby lessening the disposal problems of this emerging solid waste.

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