International Journal of Advanced Research in Biological Sciences
ISSN : 2348-8069
www.ijarbs.com

Research Article

Isolation and characterization of a chemolithotrophic bacterial strain from fresh coal mine Overburden spoil

Jitesh Kumar Maharana, Chandini Dakua and Amiya Kumar Patel*

School of Life Sciences, Sambalpur University,At/Po- Jyoti Vihar, Burla- 768019, Odisha (India)
*Corresponding author

Abstract

Coal mine overburden spoil represents a disequilibrated geomorphic system. Extremities in conditions allow the existence of chemolithotrophs as initial colonizers in such hostile environment. Microbial exploration and profiling of unique bacterial strain has been the subject of microbiological research leading to the discovery of new species. The present study was designed to isolate a chemolithotrophic bacterial strain from fresh coal mine overburden spoil using sulphate reducing medium, which was found to be gram-negative, rod shaped, obligate and facultative chemolithotroph. Optimum growth was observed at 37°C and pH 7. Growth analysis revealed that the bacteria can grow both in chemolithotrophic and heterotrophic culture condition with specific growth rate 0.138 hr^{-1} and 0.084hr^{-1} respectively. Microbial characterization suggested that the isolated bacterium is thermo-tolerant with thermal death time 3hr at 60ºC. Antibiotics sensitivity test revealed the potency of different antibiotics against the bacterium. Molecular phylogeny analysis using neighbor-joining method revealed 99% sequence similarity with *Ralstonia mannitolytica* SN82F48, and 98% sequence identity with *Ralstonia solanacearum* CMR15, *Ralstonia solanacearum* FQY_4 and *Ralstonia solanacearum* FQY_4. The study indicated that the isolated bacterium exhibited close affiliation with the genus *Ralstonia*, which includes a group of microbes from ecologically diverse niches that were classified as members of *Burkholderia*.

Keywords: Mine overburden spoil, 16S rDNA, RAPD fingerprinting, PCR amplification.

Introduction

Extensive coal mining activities resulted pit scarred landscape with huge mine overburden spoil leading to land degradation with adverse changes in soil textural, structural attributes and geochemical cycles (Mummey *et al.*, 2002; Ghose, 2004; Gairola and Soni, 2010). Being deficient in plant nutrients and lack of biologically rich top soil, the fresh coal mine overburden spoil represents a disequilibriated geomorphic system (Jha and Singh, 1991; Ghose, 1996; Kundu and Ghose, 1998; Maharana and Patel, 2013; Ramesh *et al.*, 2014), and poses problems for pedogenesis and revegetation (Jha and Singh, 1991). The soil quality status of fresh coal mine overburden spoil has been substantiated by several workers (Ghose, 1996; Kuyucak, 2002; Dutta and Agrawal, 2002; Banerjee *et al.*, 2004; Machulla *et al.*, 2005; Singh and Singh, 2006; Ekka and Behera, 2011).

The fresh coal mine overburden spoil represents unique geomorphic system with hostile environment. Being pyrite (FeS_2) as the major contaminant, it often results in auto-oxidation (Johnson, 2002; Satyanarayana *et al.*, 2005), consequently leading to burning of coal that elevates the temperature in mine spoil (Johnson, 2002). Besides, hydrogen sulphide, one of the major outcomes gets converted into sulphuric acid causing a decline in pH resulted acidic environment (Satyanarayana *et al.*, 2005). Extremities in temperature (Caruso *et al.*, 2005; Sethy and Behera, 2009), pH (Sethy and Behera, 2009; Maharana and Patel, 2013, 2014), nutrient deficiency (Ledin and

Pedersen, 1996; Johnson, 2003), and heavy metal contamination (Maiti, 2007; Cravotta, 2008; Kandeler *et al.,* 2010) thrive the microbial community in such harsh environment. However, the fresh coal mine spoil is not sterile and support specific bacterial community that has physiological adaptability to such hostile environment. The extremities allow the existence of chemolithotrophs (Mummey *et al.,* 2002; Ghose, 2004; Cismasiu, 2010; Nancucheo and Johnson, 2011; Ramesh *et al.,* 2014) and other bacterial genera involved in bio-solubilization, bioaccumulation or bio-fixation of metallic ions (Ellis *et al.,* 2003) in such thermo-acidic environment (Maiti, 2007). Chemolithotrophs have the ability to utilize inorganic compounds, and derive energy through ATP synthesis coupled with the oxidation of electron donor.

The proteobacteria, nitrospira, firmicutes and acidobacteria were reported to be the dominant microbial populations in fresh coal mine spoil (Baker and Banfield, 2003). Proteobacteria constitutes a large domain of prokaryotic microorganisms with different shapes (sphere, rod and spiral), and gram-negative with lipopolysaccharides outer membrane. Proteobacteria can be classified into five classes such as alpha, beta, gamma, delta and epsilon. Alphaproteobacteria grows at very low levels of nutrients and have unusual morphology. Betaproteobacteria consist of several groups of aerobic or facultative bacteria that are highly versatile in their degradation capacities, but also contain chemolithotrophs (Gupta, 2000) and often use nutrient substances that diffuse away from areas of anaerobic decomposition of organic matter (hydrogen gas, ammonia, methane) and includes chemoautotrophs. Gammaproteobacteria are the largest class. Deltaproteobacteria include bacteria that are predators of other bacteria and important contributors to sulphur cycle. Epsilonproteobacteria are slender gram-negative rods that are helical or curved. They are also motile by flagella and are microaerophilic (Williams *et al.,* 2010).

Microbiological constraints of mine spoil have been the subject of microbiological research and therefore involved consummate exploration and physiological profiling of unique bacterial strains having bio-prospecting potential. Besides, impact of anthropogenic disturbances on soil microbes and ecological properties altering ecosystem function may lead to the discovery of new species (Rawlings *et al.,* 1999). The nucleic acid based cultural dependent approach targeting small subunit 16S rDNA sequences by PCR amplification coupled with sequence analyses is considered as the most accurate measures for the assessment of microbial community structure (McDonald *et al.,* 1997; Ellis *et al.,* 2003; Okabayashi *et al.,* 2005). Because, the rDNA gene is the most conserved (least variable) in all organisms, which are remarkably similar even in distantly related microbes. The rDNA molecules that code for ribosomal RNA have long been recognized for their utility as molecular chronometers (Woese, 1987), and possess a higher degree of structural and functional conservation. Because of the similarity exist in rDNA sequence even in distantly related microorganisms; the sequences can be precisely aligned helpful in making differences among the microbes. The rRNA (rDNA) has been extensively used to determine evolutionary relatedness, phylogeny and to estimate rate of species divergence among bacteria. However, the larger rDNA molecules contain many domains with independent rates of sequence change relating to their functional and structural attributes (Kent and Triplett, 2002).

The present study was designed with an aim to isolate bacterial strain from fresh coal mine overburden spoil and to identify through cultivation dependent approach based on PCR amplified 16S rDNA sequence analysis. Further, phylogenetic analysis was performed using 16S rDNA sequence of the isolated bacterial strain with the corresponding sequences derived from the microbial databases based on sequence homology for accurate detection of closer resemblance bacterial strain as well as the total amount of sequence change.

Materials and Methods

Study site and sampling

The present study was carried out in the Basundhara (west) open cast colliery, Ib valley coalfields area of Mahanadi Coalfields Limited (MCL), Sundargarh, Odisha (Geographical location: 22º 03' 58" - 20º 04' 11" north latitude and 83º 42' 46" - 83º 44' 45" east longitude). Topologically, the area is hilly sloppy to plateau. The thickness of top soil in the site varies from (0.15-0.30) mtr (average: 0.22 mtr). Because of extensive mining activities and limited top soil, the fresh coal mine overburden spoil is devoid of successful vegetation. The area experiences semi-arid climate with annual rain fall 1514 mm yr^{-1}, annual

average temperature 26°C and relative humidity 15% with three distinct seasons *i.e.* summer (March to mid June), rainy (mid June to mid of October) and winter (October to February). Tropical dry deciduous forest is considered to be the natural vegetation of the site.

Sampling was done from fresh coal mine overburden spoil randomly from soil depth (0-15) cm by digging pits (15x15x15) cm^3 size, aseptically packed in sterilized polypropylene vials and brought to the laboratory. The mine overburden spoil sample was homogenized, sieved (0.2 mm mesh) and stored at 4°C until analyzed.

Isolation of bacteria

Bacterial pellet obtained from culture was inoculated into sulphate reducing medium (SRM, Himedia-M803) (Starkey, 1937). The bacterium was isolated by inoculating 100µl culture in 50ml of SRM medium [(Part A: K_2PO_4 - 0.5g; peptic digest- 2g; beef extract-1g; $MgSO_4$- 2g; Na_2SO_4- 1.5g; $CaCl_2$- 0.1g; Part B: $Fe(NH_4)_2SO_4$- 0.392g; $C_6H_7NaO_6$- 0.1g; and Part C: $C_3H_5NaO_3$- 3.5g per liter, pH 7.5], which was used for isolation, cultivation and maintenance of bacteria (Eaton *et al.*, 2005). The flask was subjected to incubation at 37°C for 24hr till the absorbance reaches to ~0.2. About 100µl culture was streaked onto the solidified agar using streak plate technique and incubated at 37°C for 24hr for development of colonies. These steps have been repeated in order to obtain the pure culture.

Gram's stain response

Gram strain response of the isolated bacterium was performed by making a smear of bacterial culture on a sterilized glass slide, heat fixed followed by addition of 1/2 drops of crystal violet. After few minutes, gram's iodine was added, washed with alcohol, and air dried followed by addition of 1/2 drops of safranin strain. The slide was washed, dried and observed under the microscope.

Optimal pH for growth

Optimum pH required for growth of the isolated bacterium was determined by serial dilution technique. The culture was serially diluted upto (10^{-8}) folds, and 100µl of diluted culture was spread onto solidified SRM agar with different pH (2, 3, 4, 5, 6, 7, 8, 9, and 10) individually, and subjected to incubation at 37°C for 24hr. The pH dependent growth response of the isolated bacterium was estimated based on the variation in CFU counts.

Growth analysis

The growth response of the isolated bacterium was performed at optimum pH using SRM medium in chemolithotrophic as well as heterotrophic culture condition (supplemented with 10g glucose/l) individually. About 100µl culture was inoculated in 50ml of SRM medium without glucose and incubated at 37ºC for different time intervals. Absorbance was measured at 640nm against control. Similar strategies were performed to determine the growth analysis of the isolated bacterium in heterotrophic culture condition.

Thermal death time determination

Thermal death time (TDT) of the isolated bacterium was determined by inoculating 100µl culture in 5ml of SRM medium, and subjected to heating at 60ºC for different time intervals. Thereafter, the culture was streaked onto already solidified SRM agar individually, and incubated at 37ºC for 24hr for the development of colonies.

Antimicrobial activities

Antimicrobial sensitivity test was performed by disc diffusion technique following Kirby-Bauer's method (Madigan and Martinko, 2006). About 100µl culture was spread onto SRM agar. The disc of different antibiotics such as amikacin, amoxycillin, cefotaxime, chloramphenicol, ciprofloxacin, erythromycin, gentamycin, kanamycin, levofloxacin, ofloxacin, rifampicin, roxythromycin, streptomycin and tetracycline having concentration (0.5mg/ml) were placed (in triplicates) in each petridish, and were incubated at 37ºC for 24hr. Degree of sensitivity contributed by different antibiotics against the bacterium was estimated by measuring the diameter of zone of inhibition, which indicated the potency of antibiotics.

Genomic DNA isolation

Genomic DNA was isolated using bacterial genomic DNA isolation kit (Chromous bacterial genomic DNA

Spin-50). About 750µl of 1X suspension buffer was mixed with 100mg of bacterial pellet followed by addition of 5µl of RNaseA with intermittent mixing for 5-6 times, and kept at 65ºC for 10min. Then, 1ml of lysis buffer was added with intermittent mixing for 5-6 times, and kept at 65ºC for 15min. The mixture was centrifuged at 13000g at room temperature and supernatant was collected in a 2ml vial. Supernatant was loaded onto the spin column (600µl each time), and centrifuged at 13000g for 1 min at room temperature. The content of the collection tube was discarded. Then, 500µl of 1X wash buffer was added to the column and centrifuged at 13000g for 3 min at room temperature. Then, the spin column was placed in a fresh 1.5ml vial followed by addition of 50µl of warm elution buffer (already kept at 65ºC), and centrifuged at 13000g for 1min at room temperature. The eluted DNA sample was collected, and resolved by 1.5% agarose gel electrophoresis to estimate the quality and quantity of the template used subsequently for PCR amplification.

RAPD fingerprinting

Eight isolates were selected and cultured individually in SRM medium at 37ºC for 24hr. DNA was isolated from individual isolates and subjected to RAPD screening using 8 primers (OPA09, OPC02, OPD03, OPJ07, OPN12, OPS04, OPU05 and OPZ04) (Genei^{TM}, Bangalore) in order to obtain a specific fingerprinting pattern. PCR mix of 25µl was prepared (MilliQ water- 14.67µl; 10X assay buffer- 2.5µl; dNTPs mix- 1µl; $MgCl_2$- 0.5µl; Taq polymerase- 0.33µl; primer- 1µl; template DNA- 5µl) for RAPD amplification. Amplification was carried out in thermo cycler programmed for 5 min of initial denaturation at 94ºC followed by 45 cycles of 94 ºC for 1 min denaturation, 37ºC for 1 min Annealing, 72ºC for 2 min extension, and final extension at 72ºC for 10 min. The amplified PCR products were electrophoresed in 1.5% agarose gel containing ethidium bromide @ 0.5µg ml^{-1} in 1X TAE buffer for 1h at 50 volts. Further, the gel was visualized under UV transilluminator and documented in Gel-Doc XR (Bio-Rad, USA).

PCR amplification

PCR amplification of 16S rDNA was performed using universal primers to confirm the size and approximate quantity of generated amplicons (Chan *et al.*, 2007). The reaction mixture (100µl) was prepared (template DNA- 1µl; forward primer- 400ng; reverse primer- 400ng; dNTPs (2.5mM each)- 4µl; 10X Taq DNA polymerase assay buffer- 10µl; Taq DNA polymerase (3U/µl)- 1µl; make up the volume to 100µl with Milli Q water). The universal primers were used for PCR amplification (forward: 5'-AGHGTBTGHTCMTGNCTCAS-3' and reverse: 5'-TRCGGYTMCCTTGTWHCGACTH-3').

Amplification was performed using thermal cycler (ABI2720, Applied Biosystems, USA) with an initial denaturation at 94°C for 5 min; 35 cycles denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and elongation at 72°C for 1 min and 30 sec; and final extension step at 72°C for 15 min. PCR products were stored at 4°C for further analysis. Amplification products were electrophoresed in 1.5% agarose gel containing ethidium bromide @ 0.5µg ml^{-1} in TAE buffer for 2hr at 50 volts. A total of 2.5µl loading buffer (1X TAE, 50% glycerol, 0.25% xylene cyanol) was added to each reaction. After electrophoresis, the gel was observed under UV-transilluminator, and documented in Gel-Doc XR (Bio-Rad, USA). The size of the amplicon was determined using 500bp DNA ladder (Bangalore Genei Pvt. Ltd., Bangalore, India) and Quantity One software. To test the reproducibility, the reactions were repeated twice.

Gel extraction

The amplified DNA was cut from agarose gel and kept in a 2ml microcentrifuge tube and weighted. To 1 volume of gel, 3 volumes of gel extraction buffer was added, and incubated at 55ºC for 5-10 min with intermittent mixing by inverting tubes for complete solubilization of agarose. Then, 1 volume of isopropanol was added and loaded onto the spin column (600µl each time), and centrifuged at 13000g for 1 min at room temperature. Then, 500µl of wash buffer was added to the column and centrifuged at 13000g for 3 min at room temperature. The content of the collection tube was discarded. Then, 15µl of elution buffer was added, centrifuged at 13000g for 1 min at room temperature, and the purified DNA was collected for further analysis.

16S rDNA sequencing and analysis

The 16S rDNA fragment was subjected to sequencing based on the chain termination reaction (Imhoff *et al.*,

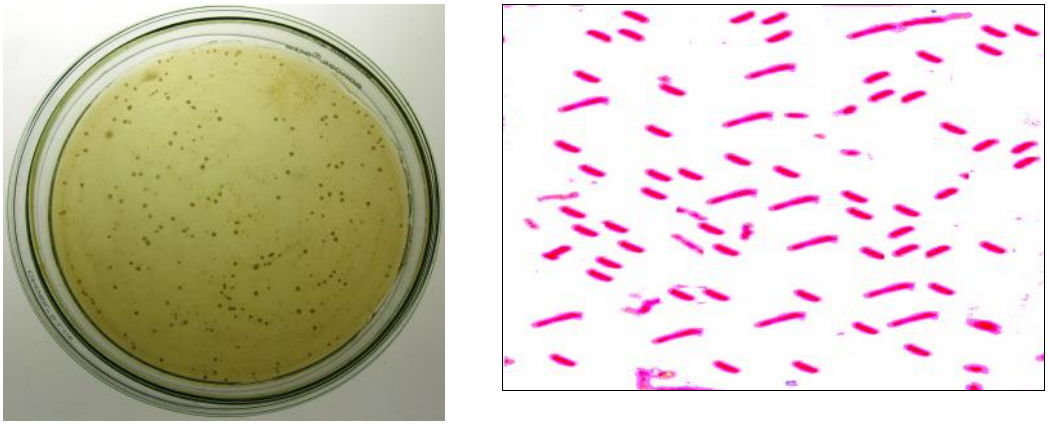
2003) using 'BigDye terminator (version 3.1) sequencing Ready Reaction kit' in automated ABI 3130 genetic analyzer (PE Applied Biosystems) using PCR amplification primers such as forward (5'-AGHGTBTGHTCMTGNCTCAS-3') and reverse (5'-TRCGGYTMCCTTGTWHCGACTH-3'). Sequencing mixture (10µl) included BigDye terminator Ready Reaction mix- 4µl; template (100ng/ul)- 1µl; primer (10pmol/λ)- 2µl; MilliQ water- 3µl). The PCR conditions includes 25 cycles with initial denaturation at 96°C for 5 min, denaturation at 96°C for 30 sec, hybridization at 50°C for 30 sec followed by the final elongation at 60°C for 1hr 30 min.

The sequences of 16S rDNA gene of the isolated bacterium was aligned using the 'JustBio online bioinformatics tool' (http://www.justbio.com) and assembled into a contiguous chain. Besides, the 16S rDNA sequence was subjected to homology search using 'BLAST' search at NCBI (http://www.ncbi.nlm.nih.gov/) (Altschul *et al.,* 1997). The representative sequences were retrieved and aligned using CLUSTAL W to generate multiple sequence alignments. The computed alignment was then manually checked and corrected, and the resulting sequences were analyzed for chimera using QIIME (version 1.5) software (http://www.qiime.org). The final sequence of 16S rDNA was deposited in GenBank using BankIt submission tool. Further, the evolutionary distances were computed using MEGA (Version-6.0) packages (Tamura *et al.,* 2013) with *p*-distance using neighbor-joining method (Saitou and Nei, 1987). Bootstrap values were calculated from 1000 replications to represent the evolutionary history of the taxa (Felsenstein, 1985).

Results and Discussion

Isolation of the bacterium

SRM medium was used for isolation, and cultivation of chemolithotrophic bacterial strain that derive energy through redox reaction using sulphate, which is the key feature employed in isolation procedure (Crundwell, 2003). Distinct colonies were appeared on SRM agar incubated at 37ºC for 24hr, which were observed to be smooth, circular, and greater opacity of size (Figure 1a). The colony diameter were estimated to be approx. (1-2) mm. The bacteria derives energy for growth and maintenance using hydrogen as electron donor from $Fe(NH_4)_2SO_4$, $C_6H_7NaO_6$ and $C_3H_5NaO_3$, and sulphate compounds ($MgSO_4$, Na_2SO_4) as electron acceptor, that are being supplemented in SRM medium (Rawlings *et al.,* 1999; Kelly and Wood, 2000). The isolated bacterium is found to be obligately and facultatively chemolithotrophic strain (Rawlings *et al.,* 1999; Baker and Banfield, 2003; Deb *et al.,* 2004). Further, the isolated bacterium was found to be gram negative, which appears pink in color and rod shaped structure (Figure 1b).



(a) **(b)**

Figure 1. (a) Petridish showing isolated colonies of isolated bacterium from fresh coal mine overburden spoil; (b) Gram's stain response of the isolated bacterium.

Besides, the isolated bacterium was subjected to grow on SRM agar with different pH (2, 3, 4, 5, 6, 7, 8, 9, and 10) individually in order to determine the optimal pH required for their growth. It is evident from the data that the isolated bacterium exhibited higher growth in terms of CFU count (31 x 10^{-8}) closer to neutral (pH 7). The existence of some bacteria even in acidic environments survive by creating circum neutral pH substantiated the concept (Sethy and Behera, 2009; Maharana and Patel, 2013, 2014).

Growth analysis

Growth response of the isolated bacterium was determined using SRM medium in chemolithotrophic and heterotrophic culture conditions at 37°C by plotting a graph taking time of incubation (hr) along X-axis and absorbance at 640nm along Y-axis (Figure 2). Under chemolithotrophic culture condition (without glucose), the lag phase was continued upto 5hr of incubation and then log phase continued till 18hr of incubation with concomitant increase in medium pH from 7.0 to 8.0. However, in case of heterotrophic culture condition with glucose in SRM medium showed that the lag phase continued upto 10^{th} hr of incubation followed by log phase upto 48 hr of incubation (Figure 2).

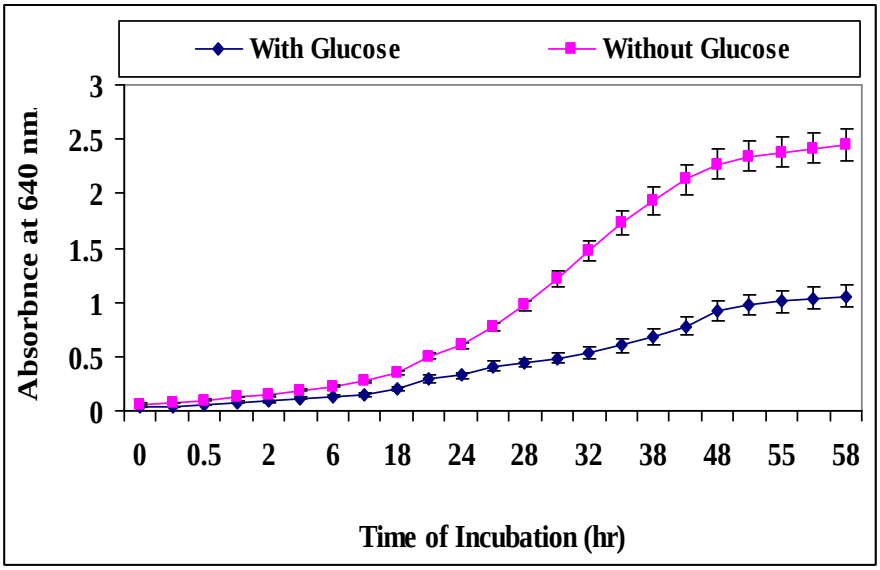


Figure 2. Growth response of isolated bacterium in SRM medium in chemolithotrophic as well as heterotrophic culture condition at 37°C.

The growth analysis revealed reatively slower growth rate and sustained for a longer period in heterotrophic condition, which may be due to low energy yielding states. Therefore, sodium lactate was taken as growth factor, which accelerates bacterial growth. The specific growth rate of isolated bacterium exhibited lower categorical magnification in heterotrophic (0.084 hr^{-1}) as compared to chemolithotrophic (0.138 hr^{-1}) culture condition, which might be due to the versatile physiology of isolated bacteria by switching over from chemolithotrophic to heterotrophic condition (Jorgensen and Nelson, 2004). However, it suggests that the isolated bacterium was mostly chemolithotrophic in nature but simultaneously it has the proclivity to shift towards heterotropy when organic carbon in the form of glucose was available as nutrient. The study indicated that the colony forming units of chemolithotroph in fresh coal mine overburden spoil in spite of hostile environment was observed to be relatively higher as compared to heterotrophic culture condition.

Determination of thermal death time

Microorganisms can grow only over a restricted range of temperature defined by three cardinal temperatures (optimum, lower and maximum). When this temperature is increased over the maximum for growth, cells are inferred and killed as the key cellular components get destroyed. Thermal death rate is a first order process at a given lethal temperature. Thermal death time of isolated bacterium in chemolithotrophic and heterotrophic culture condition was found to be 3hr and 2hr at 60ºC respectively. The estimated CFU

count revealed that there is an increasing trend of death with respect to the increase in exposure time at 60ºC. Comparative analysis suggested that the isolated bacterium under chemolithotrophic condition was found to be thermo-tolerant as compared to heterotrophic condition (Evans and Rose, 1995; Rawlings *et al.*,, 1999).

Antimicrobial activities

Antibiotic sensitivity test revealed clear circular zone of inhibition, and the degree of sensitivity against the isolated bacterium was determined with respect to the different antibiotics (Table 1).

Table 1. Degree of sensitivity contributed by different antibiotics (0.5 mg/ml) against the isolated bacterium. Diameter of zone of inhibition expressed in (mm ± SD).

Antibiotics	Diameter in zone of inhibition (in mm)	Antibiotics	Diameter in zone of inhibition (in mm)
Amikacin	8.6 ± 1.2	Kanamycin	12.6 ± 1.1
Amoxycillin	7.8 ± 1.3	Levofloxacin	12.1 ± 1.3
Cefotaxime	12.6 ± 1.1	Ofloxacin	11.3 ± 1.5
Chloramphenicol	13.1 ± 1.4	Rifampicin	19.8 ± 2.4
Ciprofloxacin	14.8 ± 1.5	Roxythromycin	45.5 ± 3.5
Erythromycin	17.5 ± 1.2	Streptomycin	36.8 ± 2.8
Gentamycin	8.1 ± 1.1	Tetracycline	25.8 ± 3.1

RAPD fingerprinting

RAPD fingerprinting was performed using 8 different decamer primers (OPA09, OPC02, OPD03, OPJ07, OPN12, OPS04, OPU05 and OPZ04) in order to estimate the genetic variability among eight different bacterial isolates from fresh coal mine overburden spoil using SRM medium in chemolithotrophic culture condition. For the purpose, the amplified DNA products were resolved through 1.5% agarose gel electrophoresis. The DNA marker analysis showed that the primers OPA09 and OPD03 (lane 1 and 3) provide similar banding pattern, while the remaining amplicons failed to generate any bands. The analysis depicted similar banding patterns indicating similar bacterial strain (Figure 3).

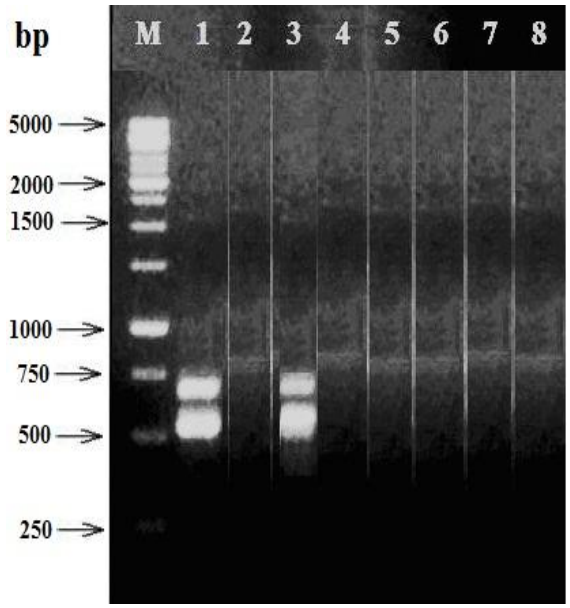


Figure 3. RAPD fingerprinting of eight different bacterial isolates from fresh coal mine overburden spoil using 8 decamer primers for estimation of genetic variability.

PCR amplification

The identification of the isolated bacterium becomes a challenging mission to provide insight into microbial community function and microbial diversity among bacterial isolates. PCR amplification of 16S rDNA gene of the bacterium using two primers (Forward: 5'-AGHGTBTGHTCMTGNCTCAS-3' and Reverse: 5'-TRCGGYTMCCTTGTWHCGACTH-3') generated a single band with amplicon size of ~1.5 Kb on 1.5% agarose gel (Figure 4).

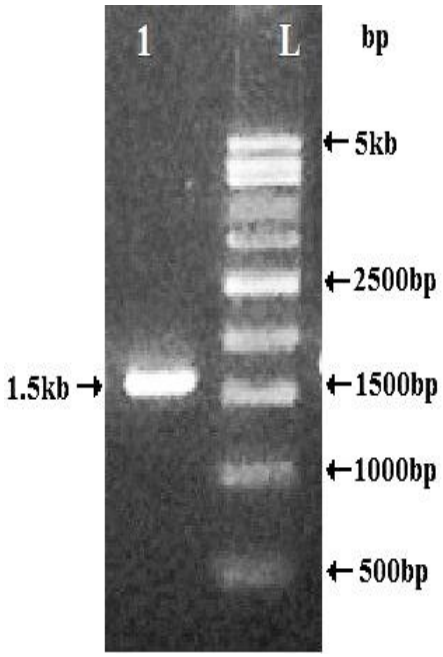


Figure 4. PCR amplification of 16S rDNA gene of the isolated bacterium from fresh coal mine overburden spoil. (Lane L: DNA Ladder; Lane 1: the amplicon size ¼1.5Kb of the bacterial isolates)

BLAST analysis and sequence homology

The amplified 16S rDNA gene products was excised from agarose gel and subjected to sequencing. The 16S rDNA nucleotide sequence information of the bacterial isolate was subjected to homology search using BLAST. Highest degree of homology exhibited by the 16S rDNA nucleotide sequence indicated by the BLAST analysis were represented (Table 2).

The analysis suggested that the 16S rDNA gene sequence of the isolated bacterium isolated from fresh coal mine overburden spoil shared 99% sequence identity with the 16S rDNA of *Ralstonia mannitolilytica* SN82F48 (NZ_CP010799.1) and 98% with *Ralstonia solanacearum* CMR15 (NC_017559.1), *Ralstonia solanacearum* FQY_4 (NC_021745.1), *Ralstonia pickettii* 12D (NC_012856.1) respectively (Table 2). Besides, it exhibited 96% sequence identity with 16S rDNA of *Cupriavidus taiwanensis* LMG19424 (NC_010528.1), *Cupriavidus metallidurans* CH34 (NC_007974.2), *Cupriavidus necator* N-1 (NC_015726.1) and 95% sequence identity with *Ralstonia pickettii* DTP0602 (NC_022515.1), *Cupriavidus basilensis* 4G11 (NZ_CP010537.1), *Ralstonia eutropha* JMP134 (NC_007348.1) respectively. In addition, it shared 92% sequence identity with *Pandoraea pnomenusa* 3kgm (NC_022904.2), *Herbaspirillum seropedicae* SmR1 (NC_014323.1), *Burkholderia mallei* KC_1092 (NZ_CP009943.1), *Burkholderia pseudomallei* PB08298010 (NZ_CP009550.1) respectively (Table 2).

Table 2. 16S rDNA sequence homology of the query sequence with respect to closely related 14 subject sequences in microbial databases using BLAST analysis.

Microbial strain	NCBI accession number	Query coverage	E-value	Identity (%)
Ralstonia mannitolilytica SN82F48	NZ_CP010799.1	99%	0.0	99%
Ralstonia solanacearum CMR15	NC_017559.1	99%	0.0	98%
Ralstonia solanacearum FQY_4	NC_021745.1	99%	0.0	98%
Ralstonia pickettii 12D	NC_012856.1	99%	0.0	98%
Cupriavidus taiwanensis LMG19424	NC_010528.1	99%	0.0	96%
Cupriavidus metallidurans CH34	NC_007974.2	99%	0.0	96%
Cupriavidus necator N-1	NC_015726.1	99%	0.0	96%
Ralstonia pickettii DTP0602	NC_022515.1	99%	0.0	95%
Cupriavidus basilensis 4G11	NZ_CP010537.1	99%	0.0	95%
Ralstonia eutropha JMP134	NC_007348.1	99%	0.0	95%
Pandoraea pnomenusa 3kgm	NC_022904.2	99%	0.0	92%
Herbaspirillum seropedicae SmR1	NC_014323.1	99%	0.0	92%
Burkholderia mallei KC_1092	NZ_CP009943.1	99%	0.0	92%
Burkholderia pseudomallei PB08298010	NZ_CP009550.1	99%	0.0	92%

Molecuar phylogenetic analysis

The 16S rDNA gene sequence obtained from the isolated bacteria (CMS 05) was subjected to phylogenetic analysis conducted in MEGA 6.0 (Tamura *et al.*, 2013). The evolutionary history was inferred using the Neighbor-Joining method (Saitau and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches (Felsenstein, 1985). The tree was drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the *p*-distance method (Nei and Kumar, 2000) and were in the units of the number of base differences per site. The analysis involved 15 nucleotide sequences from the dataset (complete deletion option). All positions containing gaps and missing data were eliminated. There were a total of 903 positions in the final dataset. Since all the clusters showing bootstrap value values above 50%, the tree likeness of the original (unrandomized) tree is statistically well resolved (Figure 5).

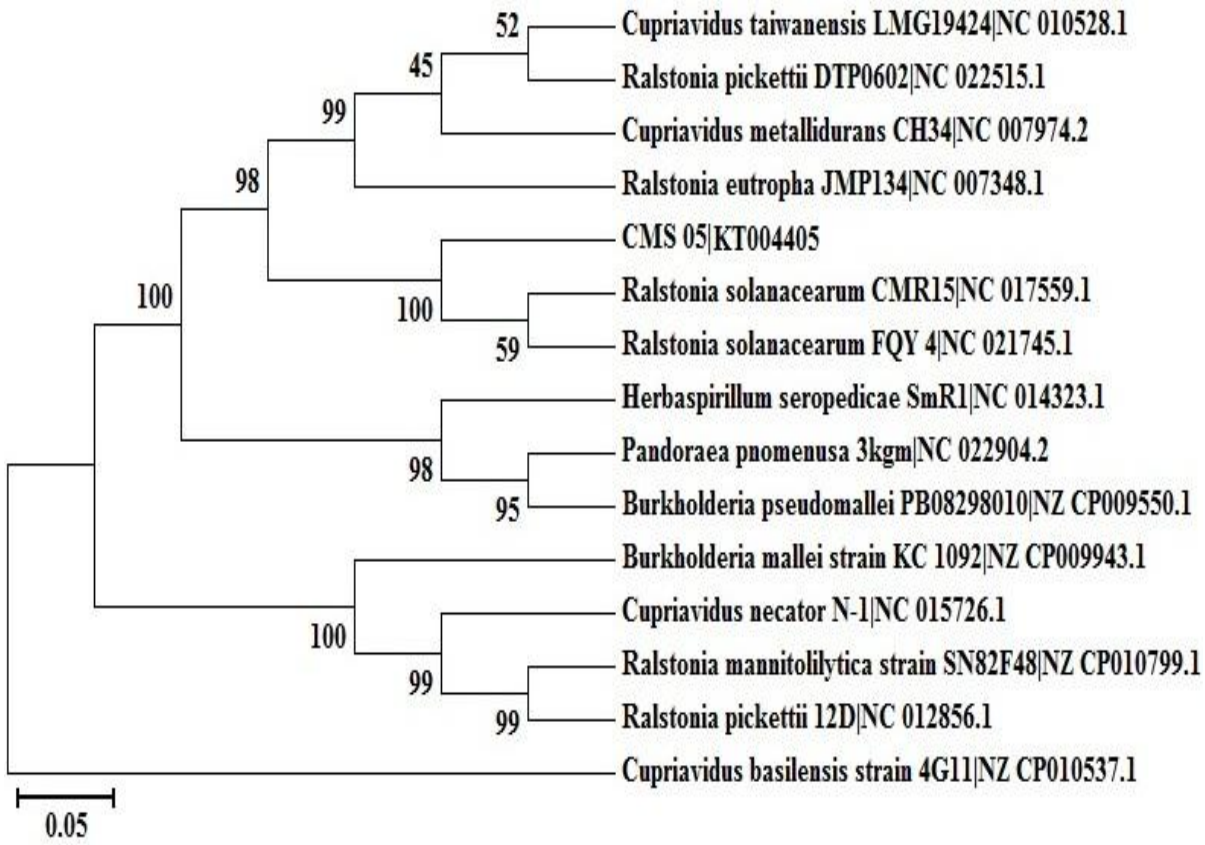


Figure 5. Neighbor-joining tree showing relationship between 16S rDNA gene of isolated bacterium with their closest relatives retrieved from microbial databases. The numbers in parentheses correspond to the accession number.

Further, the diligent and accurate identification of the isolated bacteria from fresh coal mine overburden spoil was performed by molecular phylogeny approach using 16S rDNA gene by specific PCR amplification due to its excellent reproducibility, good discriminatory power, excellent ease of interpretation and performance. The nucleotide sequence analysis suggested that the isolated bacterium (CMS 05) belongs to genus *Ralstonia*, which has been submitted to NCBI GenBank (Accession No. KT004405). The scientific classification of the isolated bacterium belongs to the phylum: *Proteobacteria*; class: *Betaproteobacteria*; order: *Burkholderiales*; family: *Ralstoniaceae*; genus: *Ralstonia*.

Acknowledgments

The authors are highly grateful to the Science and Engineering Research Board (SERB), Govt. of India for providing financial assistance and support (Grant registration No.: SERC/LS-0623/2010). We are indebted to many, who helped in field, laboratory and statistical analysis during the course of the study. The facilities provided by the Head, School of Life Sciences, Sambalpur University are also gratefully acknowledged.

References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25: 3389-3402.

Baker, B.J., and Banfield, J.F. 2003. Microbial communities in acid mine drainage. FEMS Microbiol. Ecol. 44: 139-152.

Banerjee, S.K., Mishra, T.K., Singh, A.K., and Jain, A. 2004. Impact of plantation on ecosystem development in disturbed coal mine overburden spoils. J. Trop. For. Sci. 16(3): 294-307.

Caruso, P., Palomo, J.L., Bertolini, E., Alvarez, B., Lopez, M.M., and Biosca, E.G. 2005. Seasonal variation of *Ralstonia solanacearum* biovar 2 populations in a Spanish river: recovery of stressed cells at low temperatures. Appl. Environ. Microbiol. 71:140-148.

Chan, K.G., Tiew, S.Z., and Ng, C.C. 2007. Rapid isolation method of soil *Bacilli* and screening of their quorum quenching activity. Asia Pacific J. Mol. Biol. Biotechnol. 15(3): 153-156.

Cismasiu, C.M. 2010. The acidophilic chemolithotrophic bacteria involved in the desulphurization process of lignite and pit coal from Halanga, Mintia and Petrila mines. Rom. Biotechnol. Lett. 15(5): 5602-5610.

Cravotta, C.A. 2008. Dissolved metals and associated constituents in abandoned coal-mine discharges, Pennsylvania, USA, Constituent concentrations and correlations. Appl. Geochem. 23: 166-202.

Crundwell, F.K. 2003. How do bacteria interact with minerals? Hydrometal. 71: 75-81.

Deb, C., Stackebrandt, E., Pradella, S., Saha, A., and Roy, P. 2004. Phylogenetically diverse new sulphur chemolithotrophs of α-proteobacteria isolated from Indian soils. Curr. Microbiol. 48: 452-458.

Dutta, R.K., and Agrawal, M. 2002. Effect of tree plantations on the soil characteristics and microbial activity of coal mine spoil land. Trop. Ecol. 43(2): 315-324.

Eaton, A.D., Clesceri, L.S. Rice, E.W., Greenberg, A.E. and Franson, M.A.H. 2005. Standard methods for the examination of water and wastewater: Centennial edition. Washington DC: American Public Health Association. pp. 1368.

Ekka, J.N., and Behera, N. 2011. Species composition and diversity of vegetation developing on an age series of coal mine spoil in an open cast coal field in Orissa, India. Trop. Ecol. 52(3): 337-343.

Ellis, R.J., Morgan, P., Weightman, A.J., and Fry, J.C. 2003. Cultivation dependent approaches for determining bacterial diversity in heavy metal contaminated soil. Appl. Environ. Microbiol. 69: 3223-3230.

Evans, D.R., and Rose, A.W. 1995. Experiments on alkaline addition to coal mine spoil. In: Proceedings of Sudbury. Min. Environ. 95: 49-58.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evol. 39: 783-791.

Gairola, S.K., and Soni, P. 2010. Role of soil physical properties in ecological succession of restored mine land – A case study. Int. J. Environ. Sci. 1(4): 475-480.

Ghose, M.K. 2004. Effect of opencast mining on soil fertility. J. Environ. Indus. Res. 63: 1006-1009.

Ghose, M.K. 1996. Damage of land due to coal mining and conservation of topsoil for land reclamation. Environ. Ecol. 14 (2): 466-468.

Gupta, R.S. 2000. The phylogeny of proteobacteria: relationships to other eubacterial phyla and eukaryotes. FEMS Microbiol. Rev. 24: 367-402.

Imhoff, J.F., Sahling, H., Suling, J. and Kath, T. 2003. 16S rDNA based phylogeny of sulphur oxidizing bacterial endosymbionts in marine bivalves from cold seep habitats. Mar. Ecol. Prog. Ser. 249: 39-51.

Jha, A.K., and Singh, J.S. 1991. Spoil characteristics and vegetation development of an age series of mine spoils in a dry tropical environment. Veg. 97: 63-76.

Johnson, D.B. 2002. Chemical and microbiological characteristics of mineral spoils and drainage water at abandoned coal and metal mines. Water Air Soil Poll. 3: 47-66.

Johnson, D.B. 2003. Chemical and microbiological characteristics of mineral spoils and drainage waters at abandoned coal and metal mines. Water Air Soil Poll. 3: 47-66.

Jorgensen, B.B. and Nelson, D. 2004. Sulphide oxidation in marine sediments: Geochemistry meets microbiology. Geol. Soc. Ame. Special Paper 379.

Kandeler, E., Tscherko, D., Bruce, K.D., Stemmer, M., Hobbs, P.J., Bardgett, R.D., and Amelung, W. 2010. Structure and function of the soil microbial community in microhabitats of a heavy metal polluted soil. Biol. Fertil. Soils. 32: 390-400.

Kelly, D.P., and Wood, A.P. 2000. Reclassification of some species of *Thiobacillus* to the newly designated genera *Acidithiobacillus* gen. nov., *Halothiobacillus* gen. nov. and *Thermithiobacillus* gen. nov. Int. J. Sys. Evol. Microbiol. 50: 511-516.

Kent, A.D., and Triplett, E.W. 2002. Microbial communities and their interactions in soil and Rhizosphere Ecosystems. Ann. Rev. Microbiol. 56: 211-236.

Kundu, N.K., and Ghose, M.K. 1998. Studies on the existing plant communities in Eastern coalfield areas with a view to reclamation of mined out land. J. Environ. Biol. 19(1): 83-89.

Kuyucak, N. 2002. Role of microorganisms in mining: generation of acid rock drainage and its mitigation and treatment. Eur. J. Min. Proc. Environ. Protec. 2(3): 179-196.

Ledin, M., and Pedersen, K. 1996. The environmental impact of mine wastes- roles of microorganisms and their significance in treatment of mine waste. Earth Sci. Rev. 41: 67-108.

Machulla, G., Burns M.A., and Scow, K.M. 2005. Microbial properties of mine spoil materials in the initial stages of soil development. Ame. J. Soil Sci. Soc. 69: 1069-1077.

Madigan, M.T., and Martinko, J.M. 2006. In: Brock biology of microorganisms, 11th (eds.) New Jersey: Pearson Education, Upper Saddle River.

Maharana, J.K., and Patel, A.K. 2013. Physico-Chemical characterization and mine soil genesis in age series coal mine overburden spoil in chronosequence in a dry tropical environment. J. Phyl. Evol. Biol. 1(1): 101-107.

Maharana, J.K., Naik, B., and Patel, A.K. 2014. Isolation and characterization of a chemolithotrophic, thiosulphate oxidizing *Bacillus* sp. from coal mine overburden spoil. J. Chem. Biol. Phy. Sci. 4(4): 3385-3397.

Maiti, S.K. 2007. Bioreclamation of coalmine overburden dumps with special emphasis on micronutrients and heavy metals accumulation in tree species. Environ. Monit. Assess. 125: 111-122.

McDonald, I.R., Kelly, D.P., Murrell, J.C., and Wood, A.P. 1997. Taxonomic relationships of *Thiobacillus halophilus*, *T. aquaesulis*, and other species of *Thiobacillus*, as determined using 16S rDNA sequencing. Arch. Microbiol. 166: 394-398.

Mummey, D., Stahl, P.D., and Buyer, J. 2002. Microbial markers as an indicator of ecosystem recovery following mine reclamation. Appl. Soil Ecol. J. 21: 251-259.

Nancucheo, I., and Johnson, D.B. 2005. Significance of microbial communities and interactions in safeguarding reactive mine tailings by ecological engineering. Appl. Environ. Microbiol. 77(23): 8201–8208.

Nei, M., and Kumar, S. 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York.

Okabayashi, A., Wakai, S., Kanao, T., Sugio, T., and Kamimura, K. 2005. Diversity of 16S Ribosomal DNA defined bacterial population in acid rock drainage form Japanese pyrite mine. J. Biosci. Bioeng. 100(6): 644-652.

Ramesh, M., Anbusaravanan, N., and Loganathan, A. 2014. Isolation, identification and characterization of bacteria in Godavarikhani open cast-III coal mine soil of the Singareni collieries in Andhra Pradesh. J. Phar. Biol. Sci. 9(6): 38-43.

Rawlings, D.E., Tributsch, H., and Hanford, G.S. 1999. Reasons why *Leptospirillum* like species rather than *Thiobacillus ferrooxidans* are the dominant iron-oxidizing bacteria in many commercial processes for the biooxidation of pyrite and related ores. Microbiol. 145: 5-13.

Saitou, N., and Nei, M. 1987. The neighbor joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425.

Satyanarayana, T., Raghukumar, C. and Shivaji, S. 2005. Extremophilic microbes: Diversity and perspectives. Curr. Sci. 89(1): 78-84.

Sethy, K. and Behera, N. 2009. Isolation of bacteria from coal mine spoil and study of their sensitivity to temperature and pH. Ecoscan. 3(3&4): 339-342.

Singh, A.N., and Singh, J.S. 2006. Experiments on ecological restoration of coal mine spoil using native trees in a dry tropical environment, India: a synthesis. New Forest. 31: 25-39.

Starkey R.L. 1937. Formation of sulphide by some sulphur Bacteria. J. Bacteriol. 33(5): 545-571.

Tamura K., Stecher, G., Peterson, D., Filipski, A., and Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30: 2725-2729.

Williams, K.P., Gillespie, J.J., Sobral, B.W.S., Nordberg, E.K., Snyder, E.E., Shallom, J.M., and Dickerman, A.W. 2010. Phylogeny of Gammaproteobacteria. J. Bacteriol. 192(9): 2305-2314.

Woese, C. 1987. Bacterial Evolution. Microbiol. Rev. 51: 221-71.

Zhilina, T.N., and Zavarzin, G.A. 1994. Alkaliphilic anaerobic community at pH 10. Curr. Microbiol. 29: 109-112.