International Journal of New Technology and Research (IJNTR)
ISSN:2454-4116, Volume-2, Issue-5, May 2016 Pages 08-15

Microbial Characterization and Phylogenetic Analysis of a Chemolithotrophic Bacterial Strain Isolated from Acid Mine Drainage

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***Abstract*— Excessive coal mining activities disrupt the ecosystem stability and function. The overburden when exposed to air and water, it forms acid mine drainage. Being deficient in soil nutrients and pyrite (FeS2) as major contaminants with heavy metals, it harbors specific groups of microbes especially chemolithotrophs. Realizing the facts, the present study is based on the isolation and identification of the bacterium isolated from acid mine drainage. The isolated bacterium was found to be Gram negative and round shaped cocci under the microscope, the bacterium is obligately and facultatively chemolithotroph and requires an optimal temperature of 37°C for optimal growth and proliferation. The study suggested that the bacterium was observed to be acidophilic in nature. The thermal resistance of the isolated bacterium was found out to be 2hrs 60°C. Besides, its growth pattern revealed that the isolated bacterium has a tendency to shift its metabolism from chemolithotrophy to heterotrophy culture conditions. The isolated bacterium showed sensitivity as well as resistant against different antibiotics. In addition, the culture dependent approach based on PCR amplification the 16S rDNA sequence was used to identify the bacterium isolated from acid mine drainage. Molecular phylogenetic analysis based on 16S rDNA sequence homology using neighbor-joining method suggested that the isolated bacterium belongs to Pseudomonas sp. with close affiliation with other microbial populations that have the ability to thrive in such hostile environment. All sequences used in the study were obtained from the GenBank (http://www.ncbi.nlm.nih. gov).**

***Index Terms*—16S RDNA, Acid Mine Drainage, Acidophilic, Pseudomonas.**

I. INTRODUCTION

Acid mine drainage refers to the outflow of acidic water from the metal contaminants in coal mines. The drainage flowing from or caused by surface mining, deep underground mining or coal seams that is typically highly acidic with elevated levels of dissolved metals is known as acid mine drainage (AMD). The formation of AMD is primarily a function of the geology; hydrology and mining technology employed for the mine site [1], [7], [20], [36]. Acidic sulphur rich waste waters are the byproducts of a variety of industrial operations such as galvanic processing and scrubbing of flue gases at power plants and mining sites [29], [31], [59]. This unavoidable byproduct of the mining and mineral industry are generally characterized by high concentrations of dissolved heavy metals, salts, sulphate, pyrite contamination, acidic PH which perpetuates to be a consequential water pollution quandary in the mining industries [4], [5], [19], [32], [64], [65], [66]. When water comes in contact with the minerals in coal, refuse or overburden of mine operation, the resulting water customarily becomes acidic in nature, which can severely degrade the aquatic habitat as the quality of water has become toxic, can easily corrode infrastructure, incrustation and other effects of dissolved constituents [23], [27], [51], [57], [63], [72], [76]. Aquatic organisms accumulate cumbersomely heavy metals directly from contaminated water and indirectly via the food chain. AMD occurs by mining and processing of metal ores/coals can promote AMD generation through exposing sulphide minerals in the form of pyrite contaminant to both oxygen and water [2], [32], [71]. The major components of mine water acidity are proton acidity, associated with pH and mineral acidity cognate to dissolve metal contents [18].

The amount of AMD engenderment depends on the size of the exposed surface area of sulphide mineral whereas the concentration of dissolved heavy metal ions depends on the type and quantity of sulphide minerals present as the host rock composition in the form of pyrite contamination in coal mining sites [49] [73]. Besides, chemical effects of acid mine drainage; physical effects such as incremented turbidity from soil erosion, accumulation of coal fines and smothering of the stream substrate from precipitating metal compounds may also occur [58], [68]. Precipitation of ferric hydroxide may result in consummate layering of the stream bottom, filling in crevices in rocks and making the substrate unstable and unfit for habitation by benthic organisms [25]. Trace metals, such as zinc, cadmium and copper, which may also be present in mine drainage are prodigiously toxic.

AMD is composed by a series of intricate geochemical and microbial reactions that solely depends upon the physicochemical properties of the coal mine overburden spoil. Besides, the acidophilic chemolithotrophs and low pH with high temperature acts as a catalyst for the acid-forming reaction. The entire process of acid mine drainage can be expounded in the form of following equations [11], [17], [62], [78].

$$FeS_2(s) + 3.75O_2 + 3.5H_2O \rightarrow Fe(OH)_3(s) + 2SO_4^{2-} + 4H^+ + \text{heat} \quad (1)$$

$$FeS_2(s) + 3.5O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+ \quad (2)$$

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$$Fe^{2+} + 0.25O_2 + H^+ \rightarrow Fe^{3+} + 0.5H_2O \quad (3)$$

$$FeS_2(s) + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+ \quad (4)$$

$$Fe^{3+} + 3\,H_2O \rightarrow Fe(OH)_3(s) + 3\,H^+ \quad (5)$$

Out of these, equations 1.2 and 1.3 are carried out by the chemolithotrophic bacterial oxidation specifically in the acidic and aerobic environment and results in more production of acidic discharge [70], [77]. Studies have betokened that AMD generation due to the chemolithotrophic oxidation is much more expeditious than the geochemical oxidation [14], [47], [48], [74], [79], [80]. Thus, in acid-generating environments, the typical sequence is pyrite oxidation by reaction 1.4 to produce Fe2+, which is then oxidized to Fe3+ by bacteria via reaction 1.3; the Fe3+ is then available for further pyrite oxidation. Albeit, O2 is not directly consumed in the pyrite-oxidizing step, it is obligatory for the regeneration of Fe3+ to perpetuate the pyrite-oxidation cycle. Therefore, the chemolithotrophic oxidation specifically the oxidation of Fe2+ to Fe3+ is referred as the rate circumscribing step for an AMD generation [21], [28], [81]. Fe(OH)3 is commonly referred to as 'yellow boy' [21], [28] and this Fe(OH)3 is the acid mine drainage, which inhibits the vegetation growth and pollutes the environment leading to deleterious effects [67].

However, it is a chemically involute, but biologically simple cycle that is dominated by microbial communities with autotrophic (both photoautotrophic and chemoautotrophic), heterotrophic and decomposers [6], [30]. The characterization of soil microbial communities is a very useful implement in determining the overall health of the soil systems. The comprehensive determination of soil microbial community characteristics is one way of approach for the success of restoration processes [55]. The characterization is a very broad term that can cover many aspects of the soil microbes [82]. Higher levels of bacterial and fungal diversity quantification can be useful for its treatment. Several investigators substantiate the concept [22], [34], [35], [42], [43], [44], [53], [54].

In recent years, the molecular tool has emerged as a promising robust tool for studying the soil microbial communities. The 16S rDNA sequencing approach is marginally unique among methods of community analysis, which allows rapid quantitative assessment of the relative abundance and distribution of specific phylogenetic groups of microorganisms in soil [46], [50]. Quantification of the microbial community has utility as an indicator of the reestablishment of connecting links between the biota and recuperation of function in degraded system. The connection between soil microbial communities and other characteristics of acid mine drainage is an important one to demonstrate if they are to be convincingly advocated for wider utilization as an ecological designator [40]. The microbes (bacteria and fungi) may play a significant role at the base of the overall mine drainage ecosystem by providing a supply of nutrient nitrogen. Before the development and implementation of next generation remediation strategies, there is the need to identify the microbes responsible and determine how they are interrelated in the ecosystem in order to understand what conditions trigger the microbial generation of acid mine drainage [8], [9], [13], [60]. One of the major obstacles encountered in studying the ecology of these microbes is the arduousness involved in isolating, identifying, screening and enumerating individual microbial strain from an environment, which contains a plethora of strains with homogeneous metabolic requisites. The application of 16S rDNA sequence analysis has revolutionized the study of both microbial ecology and phylogeny [12], [41], [69].

Keeping the above facts into consideration, the present study was designed to isolate and characterize the isolated bacterium for a better understanding of the geo-microbiology of the AMD, which may provide the scientific justification for more practical and efficacious remediation strategies [55]. Therefore, in the present study, the bacterium was isolated from acid mine drainage and cultured using AMD media. The detailed microbial characterization of the isolated bacterium may have the utility as an indicator for the re-establishment of the connecting link between the soil biota and the restoration of function in acid mine drainage.

II. Materials and Methods

A. 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). The acid mine drainage is devoid of successful vegetation. The area experiences a semi-arid climate with annual rainfall 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 performed from acid mine drainage randomly from five different sites, which were mixed thoroughly to obtain a 'composite sample'. The AMD samples were aseptically packed in sterilized polypropylene vials and brought to the laboratory. The water sample of the acid mine drainage sample was stored at 4°C until analyzed.

B. Isolation of Bacteria

The bacterium was isolated by inoculating 100µl of the water sample of acid mine drainage in 50ml of AMD medium (K2HPO4 - 4g, KH2PO4 - 1.5g, (NH4)2SO4 - 0.3g, MgSO4 - 0.5g, FeSO4.7H2O - 0.018g, Yeast Extract - 5g, Na2S2O3.5H2O - 10g, Agar - 15g per liter), pH adjusted to 4 with 1N H2SO4, which was used for isolation, cultivation and maintenance of bacteria. The flask was subjected to incubation at 37°C for 72hrs till the absorbance reaches to ~0.2. About 100µl of the bacterial culture was streaked onto the solidified AMD agar using streak plate technique and incubated at 37°C for 72hrs for development of the colonies. These steps have been repeated in order to obtain the pure culture which can be treated as the master culture for further studies.

C. Gram's Stain Response

The gram strain response of the isolated bacterium was performed by making a smear of bacterial culture with the help of an inoculation needle onto a clean, dry and sterilized glass slide, heat fixed. Care was taken so that the smear should be thin and uniform. Then 1/2 drops of crystal violet were added. After a few minutes, gram's iodine was added, washed with alcohol, and air dried followed by the addition of 1/2 drops of safranin strain. Excess staining and heating should be avoided. The slide was washed slowly, air dried and observed under 100X magnification using the phase contrast microscope.

D. Optimal pH for Growth

Optimum pH required for the growth of isolated bacterium was determined by serial dilution technique. The culture was serially diluted upto (108) folds, and 100µl of diluted culture was spread onto solidified AMD agar with different pH (2, 3, 4, 5, 6, 7, 8, 9 and 10) individually, and subjected to incubation at 37°C for 72hrs. The pH dependent growth response of the isolated bacterium was estimated predicated on the variation in CFU counts.

E. Growth Analysis

The growth response of the isolated bacterium was performed at optimum pH utilizing AMD medium in chemolithotrophic, heterotrophic (supplemented with 10g glucose/l) and mixotrophic (chemolithotrophic + heterotrophic) culture condition individually. About 100µl of bacterial culture was inoculated in 50ml of AMD 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 response of the isolated bacterium in heterotrophic as well as in mixotrophic culture conditions.

F. Thermal Death Time Determination

The microbial cell gets inferred and killed over the maximum range of temperature. Thermal death time (TDT) of the isolated bacterium was determined by inoculating 100µl culture in 5ml of AMD medium and subjected to heating at 60ºC for different time intervals. Thereafter, the culture was streaked onto the already solidified AMD agar individually and incubated at 37ºC for 72hrs for the development of the bacterial colonies.

G. Antimicrobial Activities

The antimicrobial sensitivity test was performed through disc diffusion technique following Kirby-Bauer's method [37]. About 100µl of pure bacterial culture isolated from AMD was spread onto AMD agar. The disc of different antibiotics such as amikacin, amoxycillin, azithromycin, cefixime, cefotaxime, chloramphenicol, ciprofloxacin, erythromycin, gentamycin, kanamycin, levofloxacin, norfloxacin, ofloxacin, rifampicin, roxythromycin, streptomycin and tetracycline having the concentration (0.5mg/ml) were placed (in triplicates) in each petridish and were incubated at 37ºC for 72hrs. After the incubation, the degree of sensitivity contributed by different antibiotics against the isolated bacterium was estimated by measuring the diameter of zone of inhibition, which indicated the potency of the antibiotics.

H. 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 and the 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 the addition of 50µl of warm elution buffer (already kept at 65ºC), and centrifuged at 13000g for 1 min at room temperature. The eluted DNA sample was collected and resolved by 1% agarose gel electrophoresis to estimate the quality and quantity of the template used subsequently for PCR amplification.

I. PCR Amplification

PCR amplification of 16S rDNA gene sequence was performed using universal primers to confirm the size and approximate quantity of the generated amplicons [10]. 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 used in the study for the PCR amplification were as follows (forward: 5'- AGHGTBTGHTCMTGNCTCAS -3' and reverse: 5'- TRCGGYTMCCTTGTWHCGACTH -3'). Amplification was performed using a thermal cycler (ABI2720, Applied Biosystems, USA) with an initial denaturation at 95°C for 5 min; 35 cycles denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, and elongation at 72°C for 1.30 min; and final extension step at 72°C for 7 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 a 500bp DNA ladder (Bangalore Genei Pvt. Ltd., Bangalore, India) and Quantity One software. To test the reproducibility, the reactions were repeated twice.

J. 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 55°C 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.

K. 16S rDNA Sequencing and Analysis

The 16S rDNA fragment was subjected to sequencing based on the chain termination reaction [26] using 'BigDye terminator (version 3.1) sequencing Ready Reaction kit' in automated ABI 3500 genetic analyzer (PE Applied Biosystems) using PCR amplification primers such as forward primer (5'- AGHGTBTGHTCMTGNCTCAS -3') and reverse primer (5'- TRCGGYTMCCTTGTWHCGACTH -3'). The sequencing mixture (10µl) included BigDye terminator Ready Reaction mix- 4µl; template (100ng/ul)- 1µl; primer (10pmol/λ)- 2µl; MilliQ water- 3µl). The PCR conditions implemented in the study include 25 cycles with an 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 were 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/) [3]. 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 by using MEGA (version-7.0) packages [83] with p-distance using neighbor-joining method [58]. Bootstrap values were calculated from 1000 replications to represent the evolutionary history of the taxa [16] using the MEGA program.

III. Results and Discussion

A. Isolation of the Bacterium

AMD broth was used for the isolation, cultivation and maintenance of the bacteria. The bacteria derives energy for growth and cell maintenance from the reduction of oxidized sulphate compounds under acidic conditions, the AMD medium was supplied with K2HPO4, KH2PO4, (NH4)2SO4, MgSO4, FeSO4.7H2O, Yeast Extract and Na2S2O3.5H2O. The isolated bacteria derive its metabolic energy by utilizing the organic compounds from the supplied medium for better growth, which is the distinct feature of the bacterial isolation procedure [24]. Bacterial colonies appeared on the AMD agar plates incubated at 37ºC for 72hrs (Fig 1a). Usually the colonies were 1-2 mm in, diameter, and smooth, circular, aerobic in nature, low convex, reddish pink in colour and greater opacity of their size (Fig 1b).

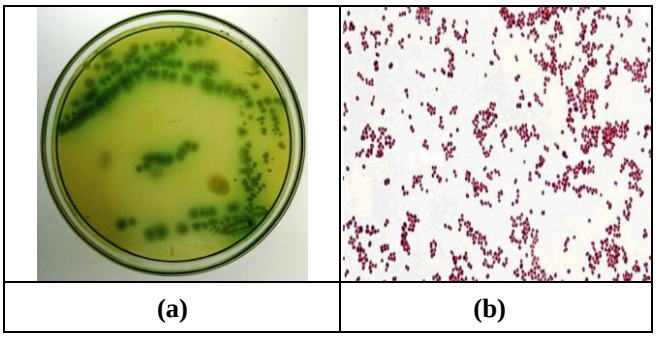

Fig 1. (a) Petridish Showing Isolated Colonies of Isolated Bacterium from Acid Mine Drainage; (b) Gram's Stain Response of the Isolated Bacterium.

Besides, the isolated bacterium was subjected to grow on AMD 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 Log CFU count (10.589) (pH 4). The existence of some bacteria, even in acidic environments survives by creating circum neutral pH substantiated the concept [38], [39], [61], [77].

B. Growth Analysis

The growth response of the isolated bacterium was determined using the AMD medium in chemolithotrophic, heterotrophic and mixotrophic culture conditions at 37°C by plotting a graph taking time of incubation (hr) along X-axis and absorbance at 640nm along Y-axis (Fig 2). Under the chemolithotrophic culture condition (without glucose), the lag phase was continued upto 2hrs of incubation and then log phase continued till 72hrs of incubation. However, in case of heterotrophic culture condition with glucose in AMD medium showed that the lag phase continued upto 1hr of incubation followed by log phase upto 78 hr of incubation. Similarly, in mixotrophic (chemolithotrophic + heterotrophic) culture condition, the lag phase continued upto 1hr and thereafter the log phase continued till 78hrs with concomitant increase in medium pH from 4.0 to 5.5 (Fig 2).

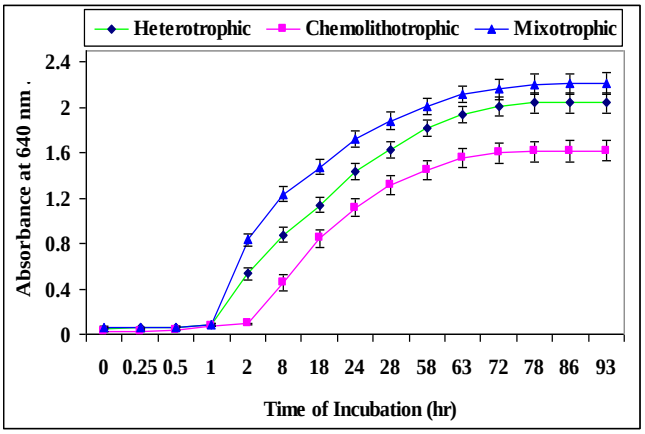

Fig 2. Growth Response of the Isolated Bacterium in Chemolithotrophic (Without Glucose), Heterotrophic (With

Glucose) and Mixotrophic (Chemolithotrophic & Heterotrophic) Culture Condition at 37°C.

The growth analysis revealed reatively slower growth rate and sustained for a longer period in chemolithotrophic culture condition, which may be due to low energy yielding states. Therefore, yeast extract was taken as growth factor, which accelerates bacterial growth. The specific growth rate of isolated bacterium exhibited lower categorical magnification in chemolithotrotrophic (0.085 hr-1) as compared to the heterotrophic (0.113 hr-1) and mixotrophic (0.135 hr-1) culture conditions, which might be due to the versatile physiology of isolated bacteria by switching over from chemolithotrophic to heterotrophic culture condition [33]. The study suggested that the isolated bacterium was mostly chemolithotrophic in nature but simultaneously it has the proclivity to shift its metabolism towards heterotrophy when organic carbon in the form of glucose was available as the source of nutrient and energy. The study indicated the chemolithotrophic nature of the bacterium isolated from the acid mine drainage based on the occurrence of bacterial CFU in AMD agar in spite of the hostile environment.

C. *Antimicrobial Activities*

Antibiotic sensitivity test revealed clear circular zone of inhibition in the petriplates. The degree of their sensitivity against the isolated bacterium was determined with respect to the different antibiotics (Fig 3).

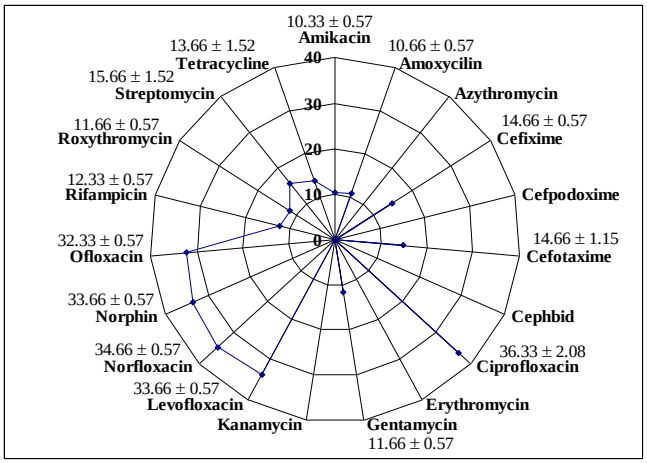

Fig 3. Effect of antibiotics having concentration (0.5 mg/ml) against the isolated bacterium. Diameter of zone of inhibition expressed in (mm ± SD); n = 5.

D. *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 culture condition was found to be 2hrs at 60ºC. The estimated CFU count revealed that there is an increasing trend of death with respect to the increase in exposure time at 60ºC. The comparative analysis suggested that the isolated bacterium was found to be thermo-tolerant [15], 56].

E. *PCR Amplification*

The identification of isolated bacterium becomes a challenging mission to provide insight into the microbial community function and associated microbial diversity among the bacterial isolates. The PCR amplification of 16S rDNA gene of the isolated 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% agarose gel (Fig 4).

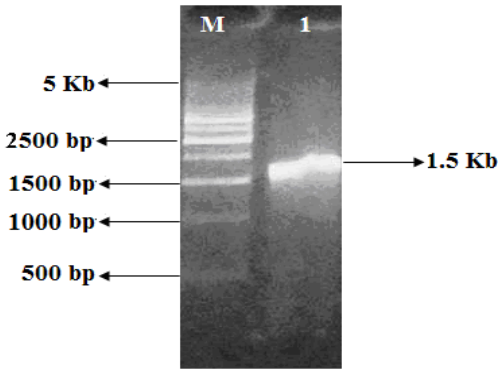

Fig 4. PCR amplification of 16S rDNA gene of isolated bacterium isolated from the acid mine drainage. (Lane M: marker DNA; Lane 1: represents the amplicon size ~1.5Kb of the bacterial isolate).

F. *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 1).

The analysis suggested that the 16S rDNA gene sequence of the isolated bacterium isolated from the water sample of acid mine drainage shared 99% sequence identity with the 16S rDNA of Pseudomonas aeruginosa PAO1 (NC_002516.2), Pseudomonas resinovorans NBRC 106553 (NC_021499.1) and Pseudomonas aeruginosa I-M-1-1-1 (KU570306.1); 97% sequence identity with Pseudomonas stutzeri A1501 (NC_009434.1) and Pseudomonas balearica DSM 6083 (NZ_CP007511.1). Besides, it exhibited 96% sequence identity with 16S rDNA of Pseudomonas putida KT2440 (NC_002947.3) and Pseudomonas entomophila L48 (NC_008027.1). In addition, it shared 95% sequence identity with Pseudomonas alkylphenolia KL28 (NZ_CP009048.1) and Pseudomonas rhizosphaerae DSM 16299 (NZ_CP009533.1) respectively (Table 1). Further, it showed 91% sequence identity with Cellvibrio japonicus Ueda 107 (NC_010995.1) respectively (Table 1).

Table 1. 16S rDNA sequence homology of the query sequence (KX034405) with respect to closely related 10 subject sequences in microbial databases using BLAST analysis.

Microbial strain	NCBI Accession No.	Query coverage	E-value	Identity (%)
Pseudomonas aeruginosa PAO1	NC_002516.2	99%	0.0	99%
Pseudomonas resinovorans NBRC 106553	NC_021499.1	96%	0.0	99%
Pseudomonas aeruginosa I-M-1-1-1	KU570306.1	99%	0.0	99%
Pseudomonas stutzeri A1501	NC_009434.1	99%	0.0	97%
Pseudomonas balearica DSM 6083	NZ_CP007511.1	96%	0.0	97%
Pseudomonas putida KT2440	NC_002947.3	99%	0.0	96%
Pseudomonas entomophila L48	NC_008027.1	99%	0.0	96%
Pseudomonas alkylphenolia KL28	NZ_CP009048.1	99%	0.0	95%
Pseudomonas rhizosphaerae DSM 16299	NZ_CP009533.1	99%	0.0	95%
Cellvibrio japonicus Ueda 107	NC_010995.1	95%	0.0	91%

G. Molecuar phylogenetic analysis

The 16S rDNA gene sequence obtained from the isolated bacterium after sequencing was subjected to phylogenetic analysis conducted in MEGA software Version 7.0 [83]. The evolutionary history was inferred using the Neighbor-Joining method [58]. The optimal tree with the sum of branch length = 1.02066136 is shown. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed [16]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [16]. The evolutionary distances were computed using the p-distance method [45] and are in the units of the number of base differences per site. The analysis involved 11 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1272 positions in the final dataset. The dendrogram analysis based on the neighbor-joining phylogenetic tree showed the relationship between the 16S rDNA gene sequence of the isolated bacterium from acid mine drainage and their closest relative sequences retrieved from microbial databases (Fig 5).

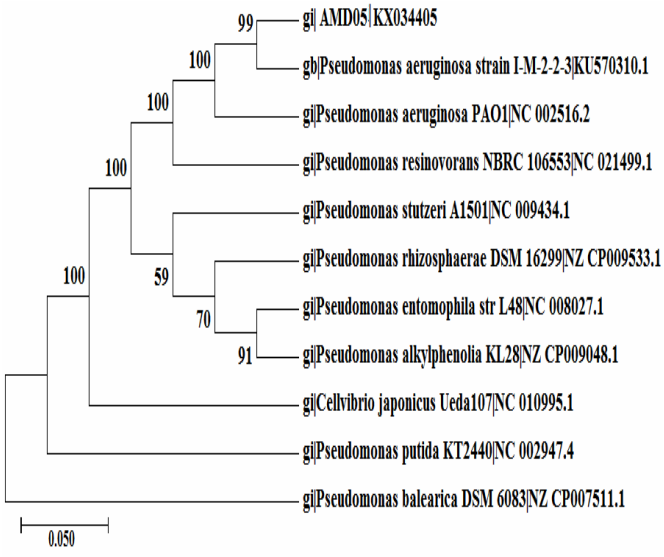

Fig 5. Neighbor-joining phylogenetic trees showing the relationship between the 16S rDNA gene sequence of the isolated bacterium from acid mine drainage and their closest relative sequences retrieved from microbial databases. The numbers in parentheses correspond to the accession number.

Further, the estimates of evolutionary divergence between the 11 sequences are shown in the form of a matrix with an overall average of 0.237. The standard error estimates are shown above the diagonal (Table 2). The nucleotide sequence of the isolated Pseudomonas sp. has been submitted to NCBI GenBank (Accession No. KX034405).

Table 2. Estimates of Evolutionary Divergence Between the 10 Subject Sequences and One Query Sequence.

	1	2	3	4	5	6	7	8	9	10	11
1	****	0.000	0.003	0.004	0.013	0.013	0.006	0.006	0.006	0.009	0.000
2	0.000	****	0.003	0.004	0.013	0.013	0.006	0.006	0.006	0.009	0.000
3	0.010	0.010	****	0.004	0.013	0.013	0.005	0.005	0.006	0.008	0.003
4	0.025	0.025	0.021	****	0.013	0.013	0.004	0.004	0.005	0.008	0.004
5	0.643	0.643	0.641	0.641	****	0.013	0.013	0.013	0.013	0.013	0.013
6	0.573	0.573	0.572	0.572	0.640	****	0.013	0.013	0.013	0.013	0.013
7	0.039	0.039	0.035	0.020	0.634	0.575	****	0.013	0.004	0.008	0.006
8	0.044	0.044	0.040	0.022	0.637	0.572	0.012	****	0.004	0.008	0.006
9	0.051	0.051	0.047	0.029	0.635	0.575	0.020	0.018	****	0.008	0.006
10	0.092	0.092	0.090	0.081	0.641	0.583	0.079	0.083	0.087	****	0.009
11	0.000	0.000	0.010	0.025	0.643	0.573	0.039	0.044	0.051	0.092	****

ACKNOWLEDGEMENTS

The authors were thankful to the Coordinator, Biotechnology, and Head, School of Life Sciences, Sambalpur University, Odisha for providing the necessary laboratory facilities during the study. Further, the help rendered by several persons during sampling, data analysis and interpretation of data in several counts were duly acknowledged.

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