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Microbial Characterization of A Chemolithotrophic Bacterial Strain Isolated from Iron Mine Overburden Spoil

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ABSTRACT

The undesirable legacies of iron mining activities lead to unintended consequences in soil subsystem with altered geomorphic system influencing ecosystem processes. The textural and elemental composition of iron mine spoil with nutrient deficiency determines the characteristic soil microbial communities by providing habitat and nutrient resources. Extremities of iron mine overburden spoil allow the existence of specific group of microbial populations especially chemolithotrophs, which reduce Fe^{3+} to Fe^{2+} thereby influencing the biogeochemistry and mineralization of organic matter. In addition, microbes influence soil functioning due to their involvement in nutrient cycling, biotransformation, formation of stable microaggregates and structural stability. Being the initial colonizers, their presence and activity is crucial for subsequent colonization of other microbial groups in iron mine spoil over time. Therefore, emphasis has been given to isolate a chemolithotrophic bacterial strain and subsequently characterized and identified by 16S rDNA sequencing. The isolated bacterium was found to be gram-negative, obligatory and facultative chemolithotroph, which exhibited maximal growth in culture medium with pH 9 at 45°C. The bacterium was observed to be alkalophilic and moderately thermophilic exhibiting higher growth rate in heterotrophic condition as compared to chemolithotrophic culture condition. Molecular phylogenetic analysis based on 16S rDNA sequence homology of the isolated bacterium revealed its close resemblance with Methylobacterium organophilum DSM 18172T (99% sequence identity), which possess heavy metal resistance genes, regulators and metal binding proteins having the ability to survive even in such adverse environmental conditions.

Keywords*: Iron mine spoil, Methylobacterium, 16S rDNA, PCR amplification.*

INTRODUCTION

Mining activities involve the excavation of surface layer pilling it over unmined land and thus form different age series mine spoil dumps referred as overburdens[1]. Open cast iron mining often leads to land degradation with adverse changes in soil textural and structural attributes[2].

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The fresh iron mine overburden spoil contains several sulfide minerals such as pyrite (FeS_2), chalcopyrite ($CuFeS_2$), pyrrhotite (FeS), arsenopyrite (FeAsS), galena (PbS), sphalerite (ZnS), pentlantite (FeNiS) and cobaltite (CoS)[3], which does not support vegetation and therefore the post-mining consequences create negative impact on ecosystem processes. Due to the extremities in conditions, it allows the existence of specific groups of microbes including chemolithotrophs[5], acidophiles[5,6] and thermophiles[7] in fresh iron mine overburden spoil. Being the initial colonizers, their presence and activity is important for subsequent colonization of other microbial groups in fresh iron mine spoil.

Microbiologically expedited oxidation of sulphidic minerals in fresh iron mine spoil is consequential in microbial ecology studies for its prominent and multifarious uses. The chemolithotrophs utilize inorganic salts for their metabolic activities in association with the oxidation reduction reactions to derive energy for their growth and proliferation[8]. Sulfide oxidation depends on several factors including water accessibility, oxygen concentration, Fe^{+3} concentration, acidity, microbial population and temperature[9]. The dissociation of iron and sulphur causes acidification and formation of acid mine drainage[10] (equation 1). Pyrite is the major waste product generated from the mining operations, which is being oxidized in presence of dissolve oxygen either to produce ferric hydroxide with sulfate and 4 protons (equation 2) or ferrous iron with sulfate and 2 protons (equation 3). The ferric hydroxide is an idealized phase and the ferrous iron oxidizes slowly in acidic condition. Further, the oxidation of pyrite by the ferric iron is a complex process, which can be carried out both in the presence or absence of dissolved oxygen[11] through different biochemical and microbial mediated reactions[12] (equation 4). Further, the Fe^{2+} is rapidly oxidized to Fe^{3+} at circum neutral pH, which suggested oxygen as the key regulatory factor influencing pyrite oxidation in such adverse environment. Therefore, the ferric iron (Fe^{+3}) acts as the strong oxidant for all types of reduced sulfur species, which do not allow the accumulation of sulfoxy anions[13].

$$2FeS_2 + 7.5O_2 + H_2O \rightarrow Fe_2(SO_4)_3 + H_2SO_4 \quad (1)$$

$$FeS_2 + 3.75O_2 + 3.5H_2O \rightarrow Fe(OH)_3 + 2H_2SO_4 \quad (2)$$

$$FeS_2 + 3.5O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+ \quad (3)$$

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

Microbes are an integral part of ecosystem involved in various functions such as nutrient cycling, biogeochemical processes, biotransformation and decomposition of organic matter *etc*. However, the anthropogenic activities including mining operations inflict dramatic shift in functional attributes influencing the geochemistry of their surrounding environment. Thus, the microbial characterization is prerequisite in determining their relative distribution indicating the overall soil health. In addition, microbes active in pyrite oxidizing environment obtain energy from the intermediate sulphur species preferably at low pH with moderately higher temperature[14,15,16]. The flexibility with respect to their genomic organization allowed them to adapt in wide spectrum of extreme environments[17]. The iron oxidizing proteobacteria play an important role in ferric iron reduction, which appears to be widespread among the acidophilic proteobacteria[17]. Besides, many acidophilic bacteria oxidize ferrous iron into soluble ferric iron form, which is more readily reduced than the amorphous or crystalline forms[18]. Several studies substantiated the concept and suggested that these microbes can be used for bioremediation of metal contaminated soil subsystem[19,20, 21].

Microbial characterization using culture dependent approach provides an insight into the microbial community structure and supplements a glimpse of their ecophysiological potential influencing ecosystem function. Bacterial isolates were taxonomically characterized through amplification, sequencing and subsequent analysis for identification[22,23,24]. Besides, molecular characterization through cultivable and non-cultivable based approaches is used for microbial diversity assessment. The potential use of 16S rDNA sequence informatics is to allow the accurate identification of the novel bacteria[25,26]. The absolute rate of change in 16S rDNA sequence is not known, which suggested that it does mark evolutionary distance and relatedness between microbes[27,28]. Being remarkably conserved and not influenced by the

shift in environmental changes[29], the 16S rDNA sequences from the distantly related microbes can be precisely aligned in order to illustrate genetic variability. The 16S rDNA sequence has been extensively used to determine evolutionary relationships[30,31,32] and is less likely to be influenced by the lateral gene transfer[33,34]. Thus, the 16S rDNA sequence provides an excellent, robust and reproducible tool, which has been extensively used for the identification of unknown microorganism with no prior knowledge.

Keeping in view, the present study was designed for microbial characterization of the bacterium isolated from fresh iron mine overburden spoil. Besides, the 16S rDNA sequence analysis can better identify the poorly described, rarely isolated or phenotypically aberrant microbial strains, because the 16S rDNA sequence analysis can discriminate the bacterial strains more accurately than is possible with phenotypic traits.

MATERIALS AND METHODS

Study Site and sampling

The study was carried out in Thakurani iron ore mines in Noamundi (85° 28' 02.61" east longitude and 22° 8' 33.93" north latitude), maintained by M/s. Sri Padam Kumar Jain sponge mines Private Ltd., which is located in the revenue district of West Singhbhum, Jharkhand, India. The study site is situated away from the mean sea level *i.e.* about 581m altitude. Mean annual temperature and humidity is around 19.67°C and 20% respectively.

Spoil sampling was done in accordance with the general soil microbiological method. The fresh iron mine overburden spoil was collected randomly from (0-15) cm soil depth by digging pits (15 x 15 x 15) cm^3 size. The composite samples were sieved (0.2 mm) and stored at 4°C until analyzed.

Isolation of the bacterium

The bacterium was isolated using standard agar plate method[35,36]. Sulphate reducing bacteria medium (SRB) has the following composition: Part-A (Peptic digest of animal tissue: 2g/l, Beef extract: 1g/l, $MgSO_4$: 2g/l, Na_2SO_4: 1.5g/l, K_2PO_4: 0.5g/l, $CaCl_2$: 0.1g/l); Part-B ($Fe(NH_4)_2SO_4$: 0.392g/l, $C_6H_7NaO_6$: 0.1g/l); Part-C ($C_3H_5NaO_3$: 3.5g/l), which was used for isolation, cultivation and maintenance of the bacterium. About 100µl culture was streaked on sulphate reducing bacteria agar and was incubated at 45°C for 48 hrs. Isolated colonies were randomly taken and these steps were repeated in order to obtain pure culture.

Microbial characterization

Gram stain response of the isolated bacterium was performed through differential straining of the heat fixed smear of bacterial culture with crystal violet followed by the addition of 1/2 drops of saffranin stain and observed under the microscope.

Optimum pH required for growth of the isolated bacterium was determined by the serial dilution technique (10^8 folds). About 100µl diluted culture was spread individually onto the solidified SRB medium maintained at different pH and subjected to incubation at 45ºC for 24 hrs. The optimal pH range was estimated through the enumeration of bacterial CFU.

Thermal death time of the bacterium was determined by inoculating 100µl bacterial culture in 5ml of SRB medium and subjected to incubation at 60ºC for different time interval. After incubation, the bacterial culture was streaked onto the solidified SRB agar individually and incubated at 45ºC for 24 hrs for development of colonies. The reduction in CFU counts revealed the time dependence on the temperature induced effect on the bacterium.

Antimicrobial sensitivity test of the bacterium was performed through disc diffusion technique following Kirby-Bauer's method[37] using different antibiotics such as amikacin, moxycillin, azithromycin, cefotaxime, ciprofloxacin, erythromycin, gentamycin, kanamycin, levofloxacin, norfloxacin, ofloxacin, rifampicin, roxythromycin, streptomycin and tetracycline with the concentration (0.5 mg/ml) in triplicates and incubated at 45ºC for 72 hrs. The diameter of the zone of inhibition is measured.

Growth analysis of the isolated bacterium was performed using SRB medium both in chemolithotrophic (without glucose) as well as heterotrophic culture condition (with 10g/l glucose) individually and

incubated at 45ºC for different time intervals. The growth response of the bacterium was determined by taking absorbance at 640 nm against control.

Genomic DNA isolation

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

Gel extraction of PCR products

Amplified PCR product was cut from the gel and kept in 2ml microcentrifuge tube. About 3 volumes of gel extraction buffer was added to 1 volume of gel and incubated at 55°C for 10 min with intermittent mixing. Then, 1 volume of isopropanol was mixed with the gel extracted solution, loaded to spin column (600µl each time) and centrifugation at 13000g for 1 min at room temperature. Then, 500µl of wash buffer was added and centrifuged at 13000g for 3 min at room temperature. The content in the collection tube was discarded. The spin column was placed in a fresh 1.5ml microcentrifuge tube with 15µl of elution buffer and centrifuged at 13000g for 1 min. 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[38] using 'Big-Dye terminator sequencing ready reaction kit' (version 3.1) (Applied Biosystems) through automated ABI 3500 XL genetic analyzer using primers (Forward: 5'-AGHGTBTGHTCMTGNCTCAS-3' and Reverse: 5'-TRCGGYTMCCTTGTWHCGACTH-3'). The sequencing mixture (10µl) includes Big-Dye terminator ready reaction mix: 4µl; template (100ng/ul): 1µl; primer (10pmol/λ): 2µl; Milli Q water: 3µl. The PCR conditions includes 25 cycles with initial denaturation at 96°C for 1 min, denaturation at 96°C for 10 sec, hybridization at 50°C for 5 sec followed by the final elongation at 60°C for 4 min.

The 16S rDNA sequence was aligned using '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' at NCBI[39]. The representative sequences were retrieved and aligned using CLUSTAL W to generate multiple sequence alignment. The computed alignment was manually checked and corrected. The resulting sequences were analyzed for chimera using QIIME (Version 1.5) (http://www.qiime.org). The final sequence of 16S rDNA was deposited in the GenBank using BankIt submission tool. Further, the evolutionary distances were computed by using MEGA (Version 6.0)[40] with *p*-distance using neighbor-joining method[41]. Bootstrap values were calculated from 1000 replications to represent the evolutionary history of the taxa[42] using MEGA (Version 6.0).

RESULTS AND DISCUSSION

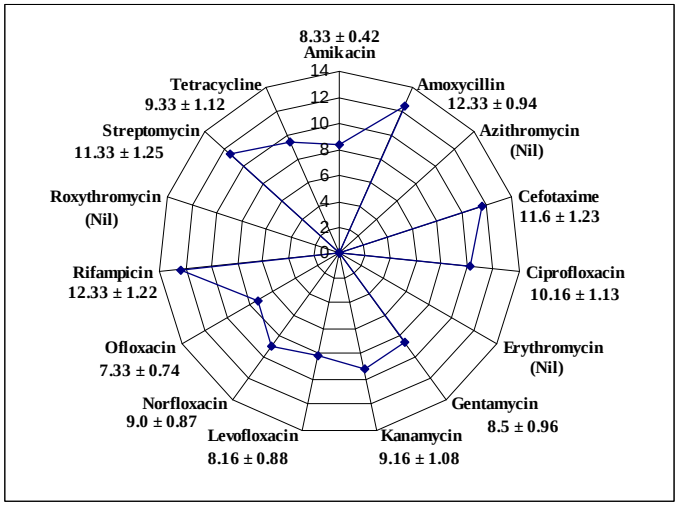
Microbial characterization

Sulphate reducing broth (SRB) was used for isolation, cultivation and maintenance of the bacterium. The bacterium isolated from the fresh iron mine overburden spoil derives its energy for growth and proliferation from the reduction of the oxidized sulphate compounds under alkaline conditions. The bacterial colonies appeared on the SRB agar were observed to be (1-2) mm in diameter, smooth, circular, low convex and greater opacity in their sizes. Besides, the gram stain response revealed that the isolated bacterium was rod-shaped gram negative and reddish pink pigmented bacteria. The CFU count in medium with different pH revealed that the bacterium exhibited relatively higher grow rate at pH 9. The bacterium exhibited a decline trend with respect to their CFU counts upto 2½ hrs, but no colony was observed at 3

hrs of incubation at 60ºC. Thus, the thermal death time of the bacterium was found to be 3 hrs at 60ºC and suggested to be moderately thermo-tolerant[43,44].

Antibiotics sensitivity test revealed clear circular zone of inhibition, which indicated the potency of antibiotics against the isolated bacterium (Figure 1). The study suggested that the isolated bacterium was found to be resistant against azithromycin, erythromycin and roxythromycin, while sensitive towards amikacin, amoxicillin, cefotaxime, ciprofloxacin, gentamicin, kanamycin, levofloxacin, ofloxacin, rifampicin, streptomycin and tetracycline.

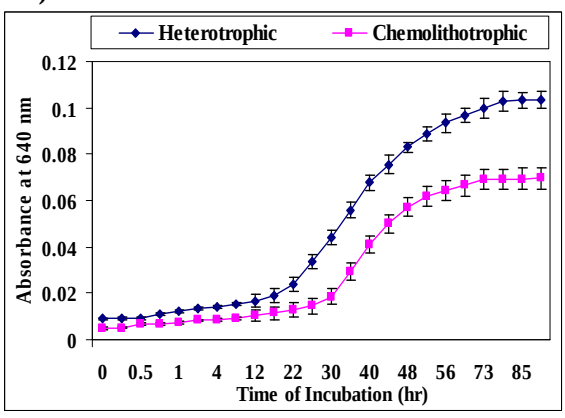
Fig. 1: Antibiotics sensitivity against the isolated bacterium revealed the diameter of zone of inhibition expressed in (mm ± SD); n= 3



Growth analysis

Growth analysis of the bacterium was done using SRB medium in chemolithotrophic (without glucose) and heterotrophic culture condition (with 10g/l glucose) at 45ºC (Figure 2). The lag phase of the bacterium continued upto 30th hr of incubation in chemolithotrophic culture condition, where as it continued upto 22nd hr of incubation in heterotrophic culture condition. The exponential phase continued till 73rd hr of incubation in chemolithotrophic culture condition. However, the log phase started after 22nd hr and continued till 78th hr of incubation in heterotrophic culture condition. The specific growth rate (μ) of the bacterium in heterotrophic condition (0.068 hr^{-1}) was comparatively higher than chemolithotrophic culture condition (0.019 hr^{-1}). It is evident from the study that the bacterial growth rate was found to be relatively slower in chemolithotrophic culture condition, which may be due to the slower rate of energy yielding activities. Further, the shift in growth rate can be explained due to the tendency of bacterium to switch over from the chemolithotrophic to heterotrophic condition supplemented with extraneous organic carbon[45].

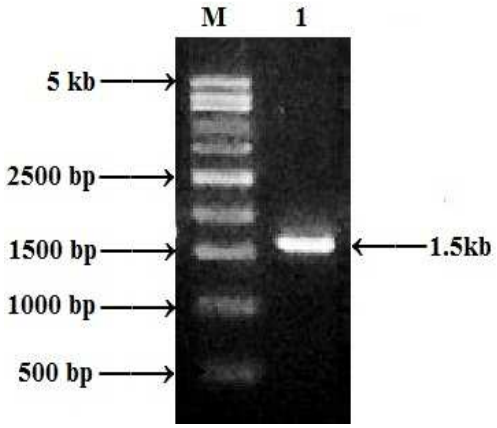
Fig. 2: Growth response of the bacterium in chemolithotrophic (without glucose) and heterotrophic (with glucose) culture condition at different time of incubation at 45°C



PCR amplification

The identification of bacterium becomes a challenging mission to unravel diversity. PCR amplification of 16S rDNA with forward: 5'-AGHGTBTGHTCMTGNCTCAS-3' and reverse: 5'-TRCGGYTMCCTTGTWHCGACTH-3' primers generated a single band with the amplicon size of ~1.5 Kb on 1.2% agarose gel (Figure 3).

Fig. 3: PCR amplification of 16S rDNA of the bacterial isolate revealed the amplicon size ~1.5 Kb (Lane M: marker DNA; Lane 1: amplicon size of ~1.5Kb)



BLAST analysis and sequence homology

The 16S rDNA amplified products was excised from the agarose gel and subjected to sequencing. The 16S rDNA nucleotide sequence information of the isolated bacterium from fresh iron mine overburden spoil was subjected to homology search using BLAST. The highest degree of sequence homology exhibited by the 16S rDNA sequence was presented (Table 1). The analysis suggested that the 16S rDNA sequence of the bacterium isolated from fresh iron mine overburden spoil shared 99% sequence identity with the 16S rDNA of *Methylobacterium organophilum* DSM 18172T (LN681562.1), *Methylobacterium organophilum* L-21 (KF831025.1), *Methylobacterium organophilum* S-18 (KF831000.1), *Methylobacterium organophilum* ATCC 27886 (NR_041027.1), *Methylobacterium organophilum* JCM 2833 (NR_115551.1), *Rhizobiales* bacterium OS2BR22 (JN233013.1), *Methylobacterium organophilum* NBCS08 (GQ281072.1), *Methylobacterium organophilum* NS6 (AB298391.1) and *Methylobacterium organophilum* TBA-R2-005 (DQ872157.1). Besides, it exhibited 97% sequence identity with the 16S rDNA of *Methylobacterium sp.* S47 (GU731244.1) and *Methylobacterium sp.* MBIC4305 (AB024616.1). In addition, the 16S rDNA of the isolated bacterium shared 91% sequence identity with *Bradyrhizobium japonicum* USDA 110 (NC_004463.1); 89% sequence identity with the 16S rDNA of *Sinorhizobium fredii* NGR234; 88% sequence identity with the 16S rDNA of *Sinorhizobium meliloti* 1021 (NC_003047.1) and *Sinorhizobium medicae* WSM419 (NC_009636.1). Further, it showed 87% sequence identity with the 16S rDNA of *Mesorhizobium ciceri biovar biserrulae* WSM1271 (NC_014923.1) and 85% sequence identity with the 16S rDNA of *Rhodospirillum rubrum* ATCC 11170 (NC_007643.1) respectively (Table 1).

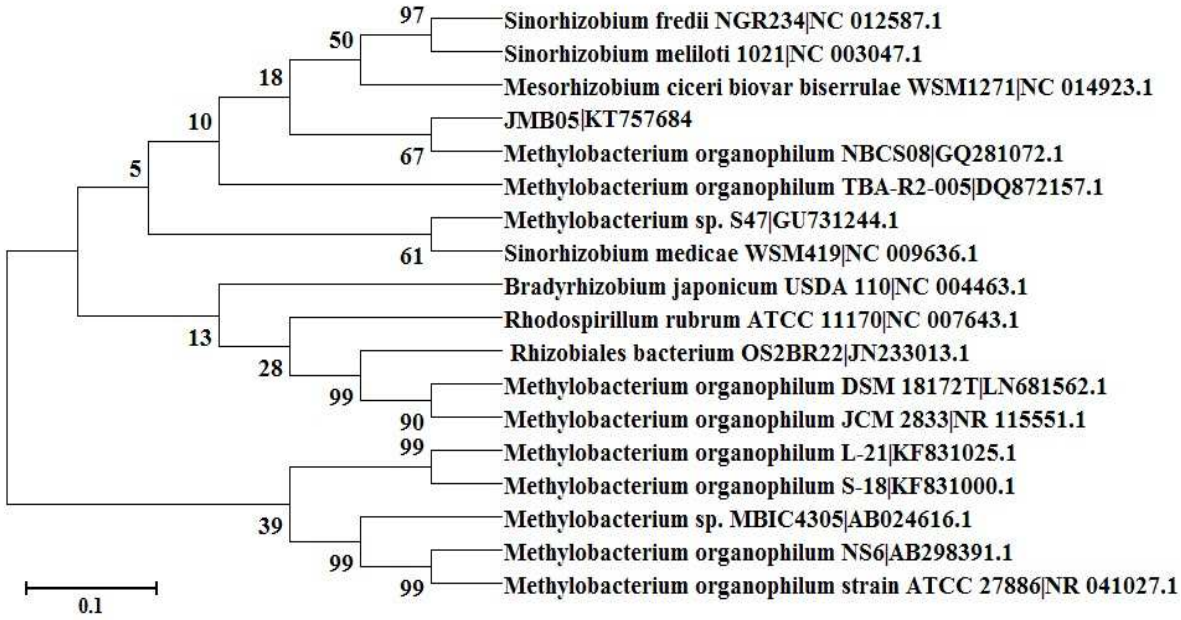
Table 1. 16S rDNA sequence homology of the query sequence (accession number: KT757684) with the closely related sequences from databases using BLAST analysis

Microbial strain	NCBI accession number	Query coverage	E-value	Identity (%)
Methylobacterium organophilum DSM 18172T	LN681562.1	100%	0.0	99%
Methylobacterium organophilum L-21	KF831025.1	100%	0.0	99%
Methylobacterium organophilum S-18	KF831000.1	100%	0.0	99%
Methylobacterium organophilum ATCC 27886	NR_041027.1	100%	0.0	99%
Methylobacterium organophilum JCM 2833	NR_115551.1	100%	0.0	99%
Rhizobiales bacterium OS2BR22	JN233013.1	100%	0.0	99%
Methylobacterium organophilum NBCS08	GQ281072.1	100%	0.0	99%
Methylobacterium organophilum NS6	AB298391.1	100%	0.0	99%
Methylobacterium organophilum TBA-R2-005	DQ872157.1	100%	0.0	99%
Methylobacterium sp. S47	GU731244.1	100%	0.0	97%
Methylobacterium sp. MBIC4305	AB024616.1	100%	0.0	97%
Bradyrhizobium japonicum USDA 110	NC_004463.1	100%	0.0	91%
Sinorhizobium fredii NGR234	NC_012587.1	100%	0.0	89%
Sinorhizobium meliloti 1021	NC_003047.1	100%	0.0	88%
Sinorhizobium medicae WSM419	NC_009636.1	100%	0.0	88%
Mesorhizobium ciceri biovar biserrulae WSM1271	NC_014923.1	100%	0.0	87%
Rhodospirillum rubrum ATCC 11170	NC_007643.1	100%	0.0	85%

Molecular phylogenetic analysis

The 16S rDNA sequence obtained from the bacterium was subjected to phylogenetic analysis using MEGA 6.0[40]. Evolutionary distance was computed using the *p*-distance using NJ method[46] reflecting the units of the number of base substitutions per site. The evolutionary history was inferred using the Neighbor Joining method[41]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches[42]. The dendrogram is drawn to scale with branch length in the same unit as the evolutionary distance used to infer phylogeny (Figure 4). All positions containing gaps and missing data were eliminated. There were a total of 1117 positions in the final datasets.

Fig. 4: Dendrogram based on NJ method showing the relatedness between 16S rDNA sequence of the bacterium and its closest relative sequences retrieved from databases



CONCLUSION

Microbial characterization in such adverse environment emphasizes the understanding of their ecophysiological role and metabolic processes influencing the ecosystem functions. The present study was aimed to isolate chemolithotrophic bacterium, which have potential use in bioremediation of the sulphur contaminated iron mine overburden spoil. Molecular phylogenetic analysis based on 16S rDNA sequence homology suggested that the bacterium isolated from fresh iron mine spoil exhibited closer resemblance with *Methylobacterium organophilum* DSM 18172T (99% sequence identity), which possess heavy metal resistance genes, regulators and metal binding proteins having the ability to resist the adverse environment with heavy metal contaminations. The 16S rDNA sequence of the isolated bacterium was submitted to NCBI GenBank (Accession no. KT757684).

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