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Isolation and Characterization of a Mesophilic Bacterium Capable of Chemolithotrophic Growth on Ferric Iron Using PCR Amplified Gene Coding for 16S rDNA in Iron Mine Overburden Spoil.

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ABSTRACT

Iron (trace element) plays an important role in bacterial metabolism and biogeochemical redox reactions in an ecosystem. Iron mining disrupts ecosystem stability and function due to the mine overburden spoil. Being deficient in nutrients and pyrite (FeS_2) contamination, the iron mine spoil harbors specific groups of microbial community (chemolithotrophs), which reduced Fe(III) that act as an electron sink to Fe(II) specifically by the iron reducing bacteria. Besides, microbial reduction of Fe(III) influences iron geochemistry and organic matter mineralization. Realizing the facts, the iron reducing bacterium was isolated from fresh iron mine overburden spoil, which was found to be gram-negative, rod shaped, obligately and facultatively chemolithotroph with optimal growth at pH-5.0 at 37°C. The study suggested that the bacterium was observed to be mesophilic and acidophilic in nature with specific growth rate 0.057 hr^{-1} and 0.087 hr^{-1} in chemolithotrophic, heterotrophic conditions respectively. Thermal death time was found to be 2½hr (chemolithotrophic) and 2hr (heterotrophic) at 60ºC. The culture dependent approach including molecular phylogenetic analysis based on 16S rDNA sequence homology suggested that the isolated bacterium belongs to the genus *Cupriavidus* with close affiliation with other heavy metal tolerant microbial populations that have the ability to thrive in such hostile environment.

Keywords: 16S rDNA, mine overburden spoil, PCR amplification, iron reducing bacteria.

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INTRODUCTION

The iron mining activities scars the original landscape by the formation of iron mine overburden spoil, which resulted alternations in geochemical cycles, disrupts ecosystem stability and function (Anjeneyulu *et al.,* 2011; Verma *et al.,* 2012). Although iron is required by the living organisms in trace amounts, but the discharge and deposition of iron ore in iron mine overburden spoil have become an environmental concern owing to their adverse effects on soil flora and fauna (Lin *et al.,* 2004; Anjeneyulu *et al.,* 2011). Being deficient in biologically rich top soil, devoid of nutrient and supportive microflora, the mine spoil created in the aftermath of iron mining can not able to support vegetation and represents stack hostile environment with altered geomorphic system (Felleson, 1999; Singh, 2004). Long-term mining activities not only reduce crop productivity, but also damage aquatic ecosystems, which lead to substantial changes in microclimates. From ecological standpoints, the disturbance ravaged by iron mining poses challenges warranting extensive scientific research. Different soil indicators such as soil texture, bulk density, water holding capacity, moisture content, soil pH *etc*. can serve as potential indicators of soil quality. Besides, the level of organic C, total N and extractable P defines substrate availability that bring about changes in the microbial community structure necessary to sustain soil structural attributes and functions. Moreover, the microbial characterizations are being evaluated as sensitive indicators of soil health because of the clear relationships between microbial populations, vegetation, and soil. Thus, the physico-chemical as well as microbial characterizations have been reported to be the key determinants in microbial ecology studies influencing the relative distribution, diversity and behaviour of soil microbes (Baker and Banfield, 2003; Mishra *et al.,* 2011; Kujur and Patel, 2014), which paves the way of greater understanding in the direction of soil fertility.

Iron has two main redox states *i.e.* reduced ferrous (Fe II), and oxidized ferric (Fe III) form (Straub *et al.,* 2004). Some microbes utilize iron either as electron acceptor (iron reducing) or electron donor (iron oxidizing) for their energy metabolism (Blake and Johnson, 2000; Emerson, 2000; Kostka *et al.,* 2002). Biological Fe(III) reduction is a major driver in the functioning of the biosphere, as Fe(III) is one of the most abundant electron sinks for the oxidation of organic matter (Lovley, 1991), as well as its bioavailability in extremely acidic environments due to its greater solubility. Based on their nature of accepting electrons, the iron reducing bacteria are divided into two groups: (i) aerobic iron reducers (use O_2 as terminal electron acceptor), (ii) anaerobic iron reducers (use CH_3COO^- as electron acceptor) (Lovley *et al.,* 1992). The aerobic iron reducers are difficult to culture and consequently little information about these microorganisms and their role in iron cycling is known. However, the acidophilic iron reducers are rational due to the stability of Fe(III) at high acidic pH. The mechanism of Fe(III) reduction appears to involve in reductive dissolutions by the soil microbes (Bridge and Johnson, 1998). The following chemical reactions explained the process of Fe(III) reduction, which suggested that pyrite oxidation is dependent upon the regeneration of (Fe^{3+}), which is reduced to (Fe^{2+}) on reaction with pyrite (Johnson, 2003).

$$14Fe^{2+} + 3.5O_2 + 14H^+ \rightarrow 14Fe^{3+} + 7H_2O$$
$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+$$
$$FeS_2 + 3.5O_2 + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+$$

Dissimilatory Fe(III) reducing bacteria obtain energy by coupling the oxidation of hydrogen or organic compounds to the reduction of ferric iron oxides catalyzed by the membrane associated enzyme ferric reductase (Lonergan *et al.,* 1996; Lovley, 1997; DiChristina *et al.,* 2002; Richter *et al.,* 2012). The iron reducing bacteria may utilize H_2, short and long-fatty acids (Coates *et al.,* 1995), amino acids, sugars and aromatic compounds as electron donors as well as 0_2, nitrate, S°, sulfate, humic substances, contaminant metals, metalloids and chlorinated solvents as alternative electron acceptors for Fe(III) reduction (Erbs and Spain, 2002), which quickly reduces to its own iron oxides and hydroxides at higher pH. Soil microbes involved in Fe(III) reduction at neutral pH (pH ≥ 5) are enigmatic for both geochemical and microbiological reasons, because Fe(III) will rapidly reduce to Fe(II) (Stumm and Morgan, 1981; Ehrlich, 1997).

Several investigations suggested the existence of chemolithotrophic iron reducing bacteria as dominant microflora involved in biotransformation and biomineralization (Bazylinski and Moskowitz, 1997; Fredrickson *et al.,* 2001; Roh *et al.,* 2003, 2006; Gadd, 2010). Iron reducing acidophiles have been subdivided into three groups on the basis of their optimum growth temperature (Norris, 1990), such as mesophiles (gram-negative bacteria; *Pseudomonas ferrireductans, Shewanella oneidensis, Alteromonas putrefaciens*, *Shewanella putrefaciens*, *Thiobacillus ferrooxidans*, *Leptospirillum ferrooxidans*, and *Leptospirillum ferriphilum*), moderate

thermophiles (mostly gram-positive, and spore-forming bacteria; *Rhodobacter capsulatus*, *Thermincola potens*, *Geobacter metallireducens*), and extreme thermophiles (archaea; *Acidianus* and *Sulfolobus* sp.). Ferric iron reductions by mesophilic chemolithotrophic and heterotrophic acidophiles have also been reported (Balashova and Zavarzin, 1980; Ghauri and Johnson, 1991; Johnson and McGinness, 1991; Pronk and Johnson, 1992) that thrive in such extreme conditions can bring about metal mobilization either by reduction or oxidation of iron. Besides, many neutrophilic are also able to reduce Fe(III), the ability to conserve energy to support growth by coupling organic matter oxidation exclusively to Fe(III) reduction appears to be more restricted among neutrophilic bacteria (Lovley, 1995).

The analysis of soil microbial communities has relied on the culture based techniques using selective media designed to maximize the recovery of diverse microbial populations. Mining activities lead to species extinction in fresh mine spoil, which may be due to the accelerated oxidation/reduction of pyrite (FeS_2) and other sulphidic minerals (Johnson and Hallberg, 2005), heavy metal contamination (Guo *et al.,* 2011; Burkhardt *et al.,* 2011), acid mine drainage (Gaikwad and Gupta, 2008; Nancucheo and Johnson, 2011), which prompted extensive research on the discovery of new species that might bring changes in ecosystem functioning in such hostile environment. In particular, the development of 16S rDNA-based method enables cultivation dependent analysis of bacterial community involving polymerase chain reaction (PCR) amplification, cloning and sequence analysis of 16S rDNA genes used for the detection and characterization of nucleic acid sequences within the samples (Embley and Stackebrandt, 1996). Besides, comparative study of small subunit (SSU) 16S rDNA molecules is particularly suited due to the fact that (i) they are found universally in all the three domains (Bacteria, Archaea, and Eucarya) (Woese *et al.,* 1990), (ii) these molecules are composed of highly conserved regions as well as the regions with considerable sequence variation, and hence because of these differential rates of sequence evolution, phylogenetic relationships at different hierarchical levels can be measured from comparative sequence analyses, (iii) due to its larger size and the presence of several secondary structural domains in SSU 16S rDNA molecule, the phylogenetic information held in it can be further enhanced. Consequently, evolutionary changes in one domain do not affect the rate of change in other domains. Finally, the SSU 16S rDNA sequence can be amplified using polymerase chain reaction (PCR) and rapidly sequenced for the detection of microbial 16S rDNA genes, which can be compared with other known microorganisms in order to provide a better understanding about the microorganism.

However, the microbial reduction of Fe(III) in fresh iron mine overburden spoil are poorly understood. Besides, the rapid auto reduction of Fe(III) makes the study of iron reducing bacteria under oxic environments challenging. Therefore, the potential of the iron bacteria for iron cycling between the aerobic oxidation and anaerobic reduction should be studied. Keeping the above facts into consideration, the present investigation was designed to characterize the isolated bacterium from fresh iron mine overburden spoil through different microbiological study. Besides, the cultivation-dependent approach based on PCR-amplified 16S rDNA sequence followed by phylogenetic analysis was performed with the corresponding sequences retrieved from the microbial databases with closer resemblance bacterial strain based on 16S rDNA sequence homology for accurate detection. Such information holds potential as the complementary criteria for restoration ecology studies in order to assess the role of different soil microorganism in facilitating mine spoil genesis, activity, biodiversity and ecosystem function.

MATERIALS AND METHODS

STUDY SITE

The present investigations were carried out in iron mining area at Noamundi (geographical location: 85° 30' 33.61" east longitude and 22° 9' 49.96" north latitude) maintained by Tata Iron Steel Corporation Limited (TISCO) located in the revenue district of West Singhbhum, Jharkhand, India (Figure 1). Tropical dry deciduous forest is considered to be the natural vegetation of the area. However, rapid industrialization not only led to the decline of forest cover, but also generated a number of new, old and abandoned iron mine overburden due to increased mining activities in the area. The annual mean temperature and humidity was estimated to be 19.67°C and 20% respectively. The area experiences three distinct seasonal tropical climate with intense summer (April to June), monsoon (July to September) and winter (December to February).

SPOIL SAMPLING

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

ISOLATION OF THE BACTERIUM

Isolation of the bacteria was performed using standard agar plate method (Hallberg and Lindstrom, 1994; Johnson, 1995). Citrate broth was used for isolation, cultivation and maintenance of the iron reducing bacteria from the fresh iron mine overburden spoil (Clark *et al.,* 1967; Greenberg *et al.,* 1998), with the following medium composition [$(NH_4)_2SO_4$- 0.5g; $NaNO_3$- 0.5g; $MgSO_4$- 0.5g; K_2HPO_4- 0.5g; $CaCl_2$- 0.2g; $Fe(NH_4)_3(C_6H_5O_7)_2$- 10g; Agar-1.5% per liter, and the pH was adjusted to 5.0 with 1N HCl]. About 100 µl soil suspension was inoculated in 50ml of citrate broth, and was incubated at 37°C for 48hr (absorbance ~0.2) without shaking. About 100µl culture was spread onto citrate agar, and incubated at 37°C for 72hr to get bacterial colonies. Isolated colonies were randomly taken, and these steps were repeated (4 - 5) times to obtain pure culture.

CHARACTERIZATION OF THE ISOLATED BACTERIUM

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 one or two drops of crystal violet. After few minutes, gram's iodine was added, washed with alcohol, air dried followed by the addition of 1/2 drops of safranin strain. The slide was washed with distilled water, air dried and observed under the microscope.

OPTIMAL pH FOR GROWTH

Optimum pH requirement of the isolated bacterium for growth was determined by serially diluting the pure culture upto (10^{-8}) folds, and about 100µl diluted culture was spread individually onto the solidified citrate agar maintained at different pH (1, 2, 3, 4, 5, 6 and 7). The petriplates were subjected to incubation at 37°C for 72hr. The effect of pH on bacterial growth was estimated based on microbial enumeration in terms of variation in CFU count.

GROWTH ANALYSIS

The growth analysis of the isolated iron reducing bacteria was performed using citrate broth (with optimal pH) in chemolithotrophic culture condition (without glucose) as well as in heterotrophic culture condition with glucose (10g/l) individually. About 100µl bacterial culture was inoculated in each 50ml citrate broth (without glucose) and incubated at 37ºC for different time intervals (0hr, 1hr, 2hr, 3hr, 4hr, 6hr, 12hr, 18hr, 24hr, 26hr, 28hr, 30hr, 32hr 36hr, 38hr, 42hr, 48hr, 60hr, 65hr and 72 hr), and the absorbance was measured at 640nm against control. Similar strategies were followed for growth analysis of the isolated bacterial strain in heterotrophic culture condition using citrate broth with glucose.

THERMAL DEATH TIME

Thermal death time (TDT) of the isolated iron bacteria from fresh iron mine overburden spoil was determined by inoculating 100µl culture in each 5ml of citrate broth, and subjected to incubation at 60ºC for different time interval (control, 15min, 30min, 45min, 1hr, 1½hr, 2hr, 2½hr, and 3hr). After incubation, the culture was streaked onto the solidified citrate agar individually, and incubated at 37ºC for 72hr for the development of colonies.

ANTIMICROBIAL ACTIVITIES

Antimicrobial sensitivity test was performed by the disc diffusion technique following Kirby-Bauer's method (Madigan and Martinko, 2006). About 100µl culture was spread on the solidified citrate agar (with optimal pH). Thereafter, 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 same concentration (0.5mg/ml) were placed (in triplicates) individually in each petridish, and were incubated at 37ºC for 72hr. The degrees of sensitivity contributed by different antibiotics against the isolated bacterium were determined by measuring the diameter of zone of inhibition, which indicated the potency of the 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 culture followed by addition of 5µl of RNaseA with intermittent mixing for 5-6 times, and kept at 65ºC for 10min. Besides, 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 RT, and the supernatant was collected in a 2ml vial. The supernatant was loaded on the spin column (600µl each time) and centrifuged at 13000g for 1 min at RT. The content of the collection tube was discarded. About 500µl of 1X wash buffer was fed into the column and centrifuged at 13000g for 3 min at RT. 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 1min at RT. The eluted DNA sample was resolved by 1% agarose gel electrophoresis to estimate quality and quantity of template for PCR amplification.

PCR AMPLIFICATION

PCR amplification of 16S rDNA was performed using a set of universal primers (F: 5'-AGAGTTTGATCMTGGCTCAG-3' and R: 5'-TACGGYTACCTTGTTACGACTT-3') to conform the size and approximate quantity of the generated amplicon (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 enzyme (3U/µl): 1µl; volume adjusted to 100µl with Milli Q water). Amplification was performed in thermal cycler (My Cycler, Bio-Rad, USA) with initial denaturation of 5min at 94°C; 35 cycles of 30 sec denaturation at 94°C, 30 sec annealing at 55°C, and 1 min elongation at 72°C; and final elongation at 72°C for 5 min. The PCR products were stored at 4°C. The amplified products were electrophoresed in 1.5% agarose gel containing ethidium bromide @ 0.5µg ml^{-1} in TAE buffer for 2h at 50V. A total of 2.5µl loading buffer (1X TAE, 50% glycerol, 0.25% xylene cyanol) was added to each reaction. Thereafter, the gel was observed under UV-transilluminator, documented in Gel-Doc XR (Bio-Rad). The size of the amplicon was determined using 500bp DNA ladder, and Quantity One software. The reactions were repeated twice in order to test the reproducibility.

GEL EXTRACTION OF PCR PRODUCTS

The amplified product was cut from agarose gel and kept in a 2ml microcentrifuge tube and weighted. About 3 volumes of gel extraction buffer was added to 1 volume of gel, and incubated at 55°C for 5-10 min with intermittent mixing till the solubilization of agarose. Then, 1 volume of isopropanol was mixed with the extracted solution and loaded on to the spin column (600µl each time), centrifuged at 13000g for 1 min at RT. Then, 500µl of wash buffer was added to the column and centrifuged again at 13000g for 3 min at RT. The content of the collection tube was discarded. The spin column was placed in a fresh 1.5ml microcentrifuge tube and 1.5µl of elution buffer was added followed by centrifugation at 13000g for 1 min at RT. The purified DNA was collected for further analysis.

16S RDNA SEQUENCING AND ANALYSIS

The purified amplified 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' (PE Applied Biosystems) in an automated ABI 3500 XL genetic analyzer (PE Applied Biosystems) using both

forward and reverse PCR amplification primers (forward primer: 5'-AGAGTTTGATCMTGGCTCAG-3' and the reverse primer: 5'-TACGGYTACCTTGTTACGACTT-3'). The sequencing mixture (10μl) included BigDye 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 gene sequences of iron reducing bacteria 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, manually checked and corrected, and 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 GenBank using BankIt submission tool. The evolutionary distances were computed by using MEGA (version-4.0) (Tamura *et al.,* 2007) with *p*-distance using neighbor-joining method (Saitou and Nei, 1987). The bootstrap values were calculated from 1000 replications to represent the evolutionary history of the taxa (Felsenstein, 1985).

RESULTS AND DISCUSSION

PHYSICO-CHEMICAL CHARACTERIZATION

The textural analysis revealed (87.8 ± 2.1)% sand, (7.8 ± 0.5)% slit and clay (4.4 ± 0.6)% in fresh iron mine overburden spoil. The bulk density was estimated to be (1.852 ± 0.019) g/cm^3, which regulates space, air and water availability to the soil microorganisms (Foissner, 1992). The moisture content and water holding capacity was found to be (6.643 ± 0.103)% and (22.501 ± 1.015)% respectively. Soil pH exhibited by the fresh iron mine overburden spoil was in the acidic range (pH 6.14), which may be due to the pyrite (FeS_2) and other sulphidic minerals contaminant (Johnson and Hallberg, 2005; Guo *et al.,* 2011; Burkhardt *et al.,* 2011). However, minimal organic carbon (0.142 ± 0.009)%, total nitrogen (0.004 ± 0.001)% and available phosphorous (70.445 ± 0.043) μgP/g spoil were exhibited by the fresh mine spoil. It is evident from the data that the fresh mine spoil represents nutrient deficient situation with minimal clay percentage (Kujur and Patel, 2012), which acts as an absorption sink for organic matter (Marshman and Marshall, 1981) influencing soil aggregate formation, structural stability and nutrient retention.

BACTERIAL ISOLATION AND CHARACTERIZATION

Citrate broth was used for isolation, cultivation and maintenance of iron bacteria from fresh iron mine overburden spoil. The medium composition has specific functions such as K_2HPO_4 provides buffering to the medium, $MgSO_4$, $(NH_4)_2SO_4$ and $CaCl_2$ are the ion sources that stimulate bacterial metabolism, $NaNO_3$ acts as nitrogen source, and $Fe(NH_4)_3(C_6H_5O_7)_2$ is used as carbon source as well as acidity regulator (Greenberg *et al.,* 1998). The bacterium derive energy through oxidation reduction reaction using inorganic iron triggered by the chemical reduction of $[Fe(NH_4)_3(C_6H_5O_7)_2]$ in the medium, and growth rate was found to be optimum under anaerobic (not strictly anoxic) and acidic pH without shaking, which is the distinct features of the bacterial isolation procedure (Bridge and Johnson, 1998; Johnson and McGinness, 1991).

Distinct colonies of the iron bacteria appeared on citrate agar after incubation at 37ºC for 72hr, which suggested that the isolated bacterium is a mesophile. Bacterial growth was not observed on solid media containing Fe(III) iron when they were incubated aerobically in shake flasks, which may be due to inhibition of Fe(III) reduction in aerobic condition. In contrast, when the cultures were incubated anaerobically without shaking, the growth and development of bacterial colonies were apparent on the petridish containing ferric iron. However, the reduction of Fe(III) by some strains of mesophilic heterotrophic acidophiles has been found to be more rapid and extensive when the bacteria were grown under (micro)aerobic conditions than when they were grown under anaerobic conditions (Johnson and McGinness, 1991). Further, the bacterial colonies were observed to be 0.5-1 mm in diameter, round, smooth, flat in size, and convex colonies that were transparent (Figure 2a).

The initially orange/dark brown color of the medium became increasingly bleached to pale brownish yellow/bleached brown color around the developing bacterial colonies indicating ferric iron reduction (Bridge

and Johnson, 1998), and the bacterium was observed to be acidophilic, obligately and facultatively chemolithotrophic strain (Bacelar-Nicolau and Johnson, 1999; Bridge and Johnson, 1998; Beller *et al.,* 2006). The change in color may be due to the fact that brown ferric ammonium citrate contains (16.5-18.5)% Fe, 9% NH_3, and 65% hydrated citric acid, that leads to the formation of Fe(II) ion readily supplying electrons to exchange and react with sulfide, together sulfide and iron form pyrite, an iron bearing soil mineral with a favorable reductive capacity (Schroder *et al.,* 2003; Coupland and Johnson, 2008). In addition, some soil microbes secrete metabolic intermediates such as citrate or specially synthesized siderophores that chelate Fe(III), and make it accessible for reduction and cellular uptake (Guerinot, 1994; Ehrlich, 1997). Further, the microscopic studies revealed that the isolated bacterium was found to be motile, non-spore forming and gram-negative, which appears pink in color and rod shaped structure (Figure 2b).

Further, the isolated bacterium was subjected to grow on citrate agar with different pH (pH- 1, 2, 3, 4, 5, 6, and 7) culture conditions individually in order to determine the optimal pH required for growth based on microbial enumeration in terms of CFU. The result showed that highest CFU count (3.5×10^{-8}) was exhibited in culture medium with pH-5, which suggested that the bacteria isolated from the fresh iron mine overburden spoil was acid-tolerant (acidophilic) (Wichlacz and Unz, 1981; Pronk and Johnson, 1992; Bridge and Johnson, 1998; Garcia-Balboa *et al.,* 2009).

GROWTH ANALYSIS

Growth analysis of the isolated bacterium was performed with citrate medium containing glucose and without glucose culture condition at 37°C (Figure 3). Under chemolithotrophic culture condition (without glucose), the lag phase of the isolated bacterium was continued upto 4hr of incubation. Thereafter, the log phase was continued till 48hr of incubation at 37°C, where the growth of the iron bacteria took place exponentially. Growth of the iron bacteria in citrate medium (without glucose) produced its own iron oxides and hydroxides, while pH of the culture showed an increasing trend from 5.0 to 6.3. However, in case of the heterotrophic culture condition (with glucose) in citrate medium showed that the lag phase of the isolated bacterium continued upto 2hr of incubation followed by log phase upto 65hr of incubation (Figure 3). The stationary phase was initiated after 65hr of incubation followed by change in pH from 5.0 to 6.5.

The growth analysis suggested slow growth rate and sustained for a longer period in chemolithotrophic condition due to low energy yielding states. The specific growth rate (μ) in case of chemolithotrophic culture condition (0.057 hr^{-1}) was calculated to be comparatively less as compared to heterotrophic (0.087 hr^{-1}) culture condition, which might be due to the versatile physiology of the iron bacteria by switching over from chemolithotrophic to heterotrophic condition (Hallberg and Johnson, 2003) suggesting that the bacterium is “heterotrophically inclined” that requires exogenous organic carbon for better growth (Ghauri and Johnson, 1991).

THERMAL DEATH TIME

Thermal death time (TDT) of the isolated bacterium in chemolithotrophic (without glucose) and heterotrophic (with glucose) culture conditions was found to be 2½hr and 2hr respectively at 60ºC. There is an increasing trend of death with respect to the increase in exposure time at 60ºC. Comparative analysis of thermal death time suggested that the iron bacterium under chemolithotrophic culture was found to be relatively more thermo-tolerant as compared to heterotrophic culture condition (Johnson *et al.,* 1992; Bridge and Johnson, 1998; Bacelar-Nicolau and Johnson, 1999; Coupland and Johnson, 2008).

ANTIMICROBIAL ACTIVITIES

Antibiotic sensitivity test revealed clear circular zones of inhibition, and the degree of sensitivity against the isolated iron bacterium was determined. Clear differential zone of inhibition were observed in case of Azithromycin, Cefotaxime, Ciprofloxacin, Erythromycin, Levofloxacin Norfloxacin, Ofloxacin, Rifampicin, Streptomycin and Tetracycline. However, the bacterium did not show any sensitivity against Amikacin, Amoxycillin, Cefixime, Chloramphenicol, Gentamycin, Kanamycin and Roxythromycin (Table 1).

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Table 1: Degree of sensitivity contributed by different antibiotics against iron reducing bacteria. Diameter of zone of inhibition (3 replicates) is expressed in (mm ± SD).

Antibiotics	Diameter in zone of inhibition (mm)	*Antibiotics*	Diameter in zone of inhibition (mm)
Amikacin	Nil	Kanamycin	Nil
Amoxycillin	Nil	Levofloxacin	34 ± 1.3
Azithromycin	28.6 ± 0.5	Norfloxacin	23.3 ± 0.6
Cefixime	Nil	Ofloxacin	31.6 ± 0.8
Cefotaxime	27 ± 1.1	Rifampicin	12.6 ± 1.1
Chloramphenicol	Nil	Roxythromycin	Nil
Ciprofloxacin	10 ± 1.2	Streptomycin	9.3 ± 0.9
Erythromycin	12 ± 0.8	Tetracycline	12 ± 0.8
Gentamycin	Nil		

PCR AMPLIFICATION

The isolation and identification of the iron reducing bacteria from fresh iron mine overburden spoil becomes a challenging mission for microbial ecologists to unravel the diversity. Such investigations provide insight into the microbial community structure and function, and the importance of microbial diversity among the bacterial isolates in the fresh iron mine overburden spoil. PCR amplification of 16S rDNA gene of the isolated bacterium from fresh iron mine spoil with two primers (Forward: 5'-AGAGTTTGATCMTGGCTCAG-3' and Reverse: 5'-TACGGYTACCTTGTTACGACTT-3') generated a single band amplicon size of ~1.5 Kb on 1% agarose gel (Figure 4).

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 isolated iron reducing bacterium was subjected to homology search using BLAST analysis. Highest degree of homology exhibited by 16S rDNA nucleotide sequence was represented (Table 2).

Table 2: 16S rDNA sequence homology of the query sequence (KJ801564) 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 pickettii 12J 1323	NC 010678.1	100%	0.0	99%
Ralstonia solanacearum GMI1000	NC 003296.1	100%	0.0	99%
Cupriavidus necator N-1	NC 015726.1	100%	0.0	96%
Cupriavidus metallidurans CH34	NC 007974.2	100%	0.0	95%
Cupriavidus taiwanensis LMG 19424	NC 010530.1	100%	0.0	95%
Herbaspirillum seropedicae SmR1	NC 014323.1	99%	0.0	94%
Ralstonia eutropha JMP134	NC 007347.1	100%	0.0	94%
Burkholderia pseudomallei K96243	NC 006350.1	100%	0.0	94%
Burkholderia mallei SAVP1	NC 008785.1	100%	0.0	93%
Pandoraea pnomenusa 3kgm	NC 022904.1	100%	0.0	93%
Burkholderia thailandensis E264	NC 007650.1	100%	0.0	93%
Thiobacillus denitrificans ATCC 25259	NC 007404.1	100%	0.0	91%
Thiomonas intermedia K12	NC 014153.1	100%	0.0	90%
Comamonas testosteroni CNB-2	NC 013446.2	99%	0.0	89%

The analysis suggested that the 16S rDNA gene sequence of the isolated iron reducing bacterium shared 99% sequence identity with the 16S rDNA of *Ralstonia pickettii* 12J 1323 (NC 010678.1) and *Ralstonia solanacearum* GMI1000 (NC 003296.1). Besides, it exhibited 96% sequence identity with 16S rDNA of *Cupriavidus necator* N-1 (NC 015726.1); 95% sequence identity with *Cupriavidus metallidurans* CH34 (NC 007974.2) and *Cupriavidus taiwanensis* LMG 19424 (NC 010530.1) respectively (Table 2). In addition, it shared 94% sequence identity with *Herbaspirillum seropedicae* SmR1 (NC 014323.1), *Ralstonia eutropha* JMP134 (NC

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007347.1), *Burkholderia pseudomallei* K96243 (NC 006350.1), and 93% sequence identity with *Burkholderia mallei* SAVP1 (NC 008785.1), *Pandoraea pnomenusa* 3kgm (NC 022904.1), *Burkholderia thailandensis* E264 (NC 007650.1). Further, it showed 91% sequence identity with *Thiobacillus denitrificans* ATCC 25259 (NC 007404.1), 90% with *Thiomonas intermedia* K12 (NC 014153.1) and 89% with *Comamonas testosteroni* CNB-2 (NC 013446.2) (Table 2).

MOLECUAR PHYLOGENETIC ANALYSIS

The 16S rDNA gene sequence obtained from the iron reducing bacteria after sequencing was subjected to phylogenetic analysis conducted in MEGA 4.0 (Tamura *et al.,* 2007). The evolutionary distances were computed using the p-distance using neighbor-joining method (Saitau and Nei, 1987) and were in the units of the number of base substitutions per site (Figure 5). The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). The phylogenetic tree was linearized assuming equal evolutionary rates in all lineages (Takezaki *et al.,* 2004). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 701 positions in the final dataset. Since all the clusters showing bootstrap values above 50%, the tree likeness of the original (unrandomized) tree was statistically well resolved (Figure 5).

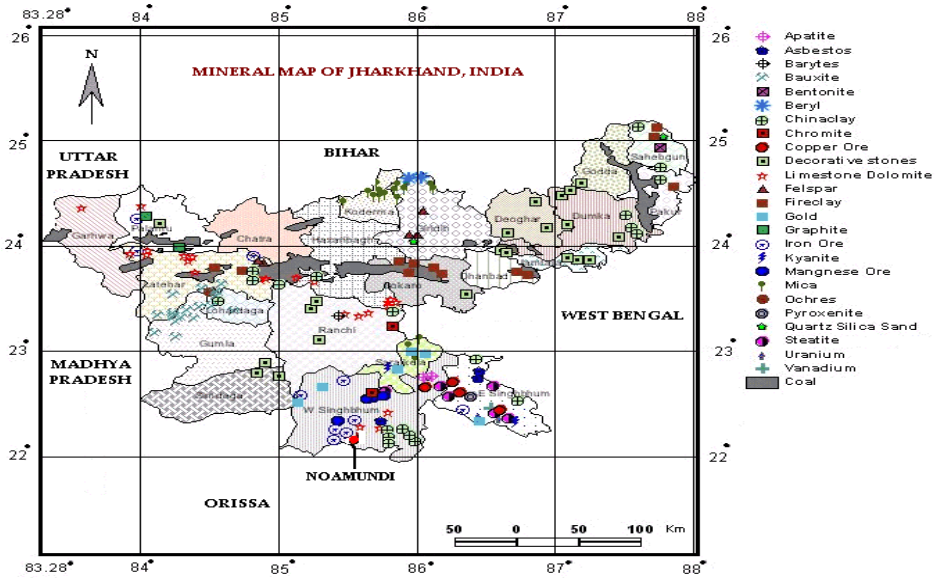
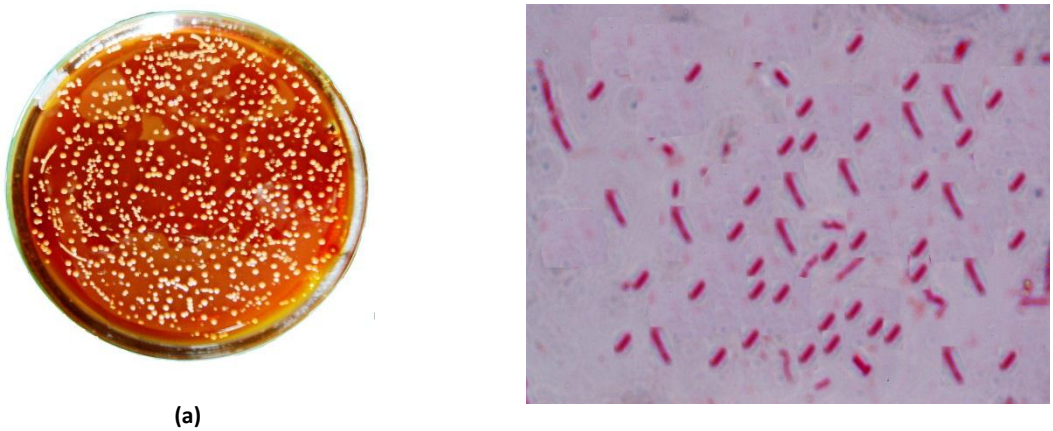


Figure 1. Geographical location and the mineral map of study site, Jharkhand, India.



(a) **(b)**

Figure 2. (a) Petridish showing isolated colonies of iron reducing bacteria from fresh iron mine overburden spoil; (b) Gram's stain response of the isolated bacterium.

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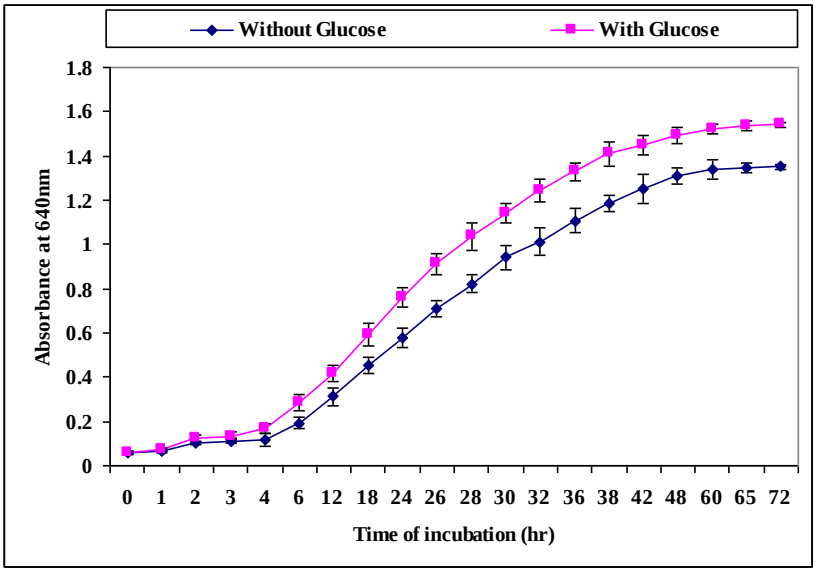

Figure 3. Growth curve of the isolated iron reducing bacterium in chemolithotrophic (without glucose) and heterotrophic (with glucose) culture condition.

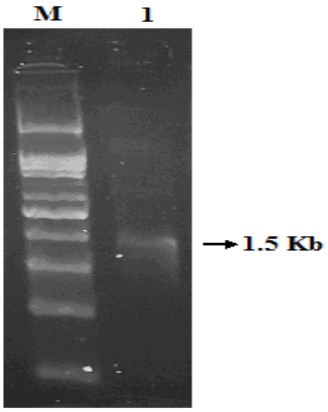

Figure 4. PCR amplification of 16S rDNA gene of iron reducing bacteria isolated from fresh iron mine overburden spoil. (Lane M: marker DNA; Lane 1: represents the amplicon size ~1.5Kb of the bacterial isolate)

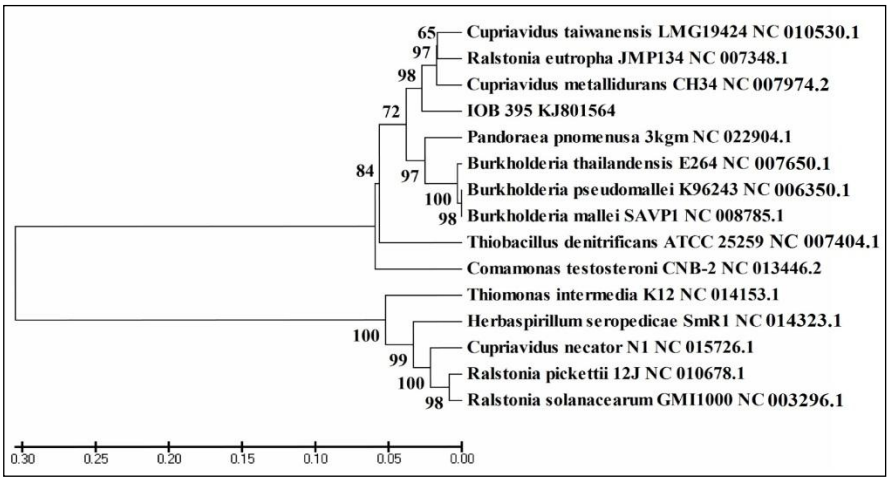

Figure 5. Neighbor-joining phylogenetic tree showing the relationship between the 16S rDNA gene sequence of the iron reducing bacteria from fresh iron mine overburden spoil and their closest relative sequences retrieved from microbial databases. The numbers in parentheses correspond to the accession number.

CONCLUSION

The dendrogram revealed that the isolated iron reducing bacterium exhibited close resemblance with *Cupriavidus metallidurans* CH34 (95% sequence identity), which possess high number of heavy metal resistance genes, regulators and metal binding proteins and also have the ability to thrive in toxic environments (Reith *et al.,* 2006). The diligent and accurate identification of iron reducing bacteria 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, which indicated that the isolated bacterium belongs to the genus *Cupriavidus*. The nucleotide sequence of the isolated *Cupriavidus* sp. has been submitted to NCBI GenBank (Accession No. KJ801564).

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