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ISOLATION AND CHARACTERIZATION OF BACTERIA FROM RUSTED IRON MATERIALS

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Abstract

Seventy bacterial isolates were recovered from different rusted iron materials using modified NP glucose, 9K and sulfur oxidizing media. Thirty four isolates were selected after primary screening on the basis of their growth in modified NP glucose medium. Out of 34 isolates 5 belonged to the genus *Kurthia*, 6 *Thiobacillus*, 16 *Bacillus* and remaining 7 were *Acidiphilium, Oscillospira, Sulfobacillus, Alicyclobacillus, Neisseria, Pseudomonas* and *Rahnella.* One of the isolated organisms, *Bacillus megaterium* (F16/2), was found to be thermophilic. The organisms showed better growth in presence of iron salts like ferrous sulfate, ferric sulfate and ammonium ferric citrate. Five isolates were found to transform ferrous (Fe^{2+}) to ferric (Fe^{3+}) of which *Bacillus subtilis* (F8) was most efficient. A good number of bacteria were associated with oxidation of iron.

Introduction

Iron is one of the most abundant elements on earth and widely known for its multifarious uses. It is impossible now to imagine life without mineral resources, for they are inseparably connected with the economic and cultural development of man (Kourimsky 1992).

Activities of microbes are very important in the turnover of organic and inorganic matter on earth (Agate 1995). Neutrophilic bacteria associated with ferric (Fe^{3+}) oxide precipitation have been known for a long time. Some species were shown to precipitate ferric (Fe^{3+}) oxides during heterotrophic metabolism. Emerson and Moyer (1997) suggested that bacterial Fe^{3+} iron oxidation might promote coupling between iron oxidation and reduction by producing amorphous or poorly crystalline ferric (Fe^{3+}) oxides, which are readily available for ferric (Fe^{3+}) reducing bacteria such as, *Thiobacillus ferrooxidans, Sulfolobus* sp. and *Bacillus megaterium* (Sobolev and Roden 2001).

It has now been realized that organisms which are capable of transforming metals and inorganic compounds such as, iron, copper, uranium, sulfur, manganese etc. can be utilized not only for extracting minerals from poor ores but at the same time with careful planning they can also be utilized for environmental management, such as by removing excess iron from the water bodies and similar environments (Agate 1995).

At present geomicrobiology may not look like a promising proposition in Bangladesh, a mineral-poor country, but the great potentiality of the microorganisms involved in bioleaching may be applied for different biotechnological processes. Conceiving this concept the present work was undertaken to isolate iron associated bacteria from rusty materials and to test their potentiality in various mineralogical processes.

Materials and Methods

Rusted materials including iron bars, rods, plates and scrappings were collected from Mirpur, Dhaka and Chittagong Shipyard for the isolation of iron bacteria. Small portions of samples were inoculated in 9K medium (Paknikar and Agate 1995) [Solution A: $(NH_4)_2SO_4$ 2.0 g, $MgSO_4$ 0.5 g,

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dipotassium hydrogen orthophosphate 0.025 g, distilled water 400 ml, pH 2.5 (adjusted with 5N H_2SO_4). Sterilized at 15 psi for 20 minutes. Solution B: $FeSO_4.7H_2O$ 40.0 g, distilled water 300 ml, after pH adjustment of distilled water to 2.5 $FeSO_4.7H_2O$ was dissolved in it and the solution was filter sterilized. Finally solutions A and B were mixed aseptically]. The inoculated flasks were incubated at 30ºC on a mechanical shaker (MRK Recipro Box Shaker, Japan) and were shaken regularly (2 h/day) at 62 rpm for a week.

Bacteria were isolated using modified NP glucose medium (Paknikar and Agate 1995), sulfur oxidizing bacterial medium (SAB 1957), nutrient agar and nutrient broth media. The pH of the media was adjusted to 6.5 and inoculated plates were incubated at 37ºC for 48 h. Various types of bacterial colonies mostly circular, entire and non-spreading in nature appeared. The selected colonies were maintained in pure culture (modified NP glucose medium). A total of 70 isolates from 9 samples were obtained. After primary screening 34 isolates were selected for morphological, cultural and biochemical studies following standard laboratory manuals (Berkeley and Ali 1994, Staley *et al.* 1989, Sneath *et al.* 1986, Krieg and Holt 1984).

The selected isolates were tested for their growth responses in different iron salt containing media by the disc diffusion method (Atlas *et al.* 1995). Isolates were seeded by pour plate method and then the paper discs impregnated with selected salt solutions [*viz.* $FeSO_4.7H_2O$; $Fe_2(SO_4)_3$ and $C_6H_8FeO_7.NH_3$] were placed on the surface of the agar plates. Each disc was 6 mm in diameter and the concentration of salt was 50 µg/disc. After 24 h of incubation, the plates were observed and the zones of inhibition and stimulation were recorded.

An experiment was designed to study the ability of transformation of ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron salts in solution. *Bacillus subtilis* (F8) and *Thiobacillus thioparus* (F33, F34, F36 and F37) isolates were used and the method suggested by Jackson (1973) was followed for the determination of ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron. Modified NP glucose medium (without $FeSO_4.7H_2O$) was four times diluted and to it buffer (pH 6.5) was mixed in 4 : 1 ratio and then ammonium ferric citrate was added as a source of iron.

Sequencing of 16S rRNA gene of strain F8: Molecular characterization of *Bacillus subtilis* (F8) was done based on alignment of partial sequence of 16S rRNA gene. For the partial amplification of 16S rRNA gene and for automated sequencing following primer pairs were used:

5'-16S rRNA: CCAGACTCCTACGGGAGGCAGC

3'-16S rRNA: CTTGTGCGGGCCCCCGTCAATTC

PCR condition applied to amplify the 16S rRNA gene:

Initial denaturation	:	95° C for 5 min	
Denaturation	:	94° C for 1 min	for 30 cycles
Primer annealing	:	60° C for 30 sec	
Polymerization	:	72° C for 30 sec	
Final extension	:	72° C for 5 min	

PCR amplified DNA was gel purified using Qiagen kit and sent for automated sequencing. The sequence generated from automated sequencing of PCR amplified DNA was analyzed through BLAST (http://blast.ncbi.nlm.nih.gov/) program to find out possible similar organism through alignment of homologous sequences.

Results and Discussion

A total of 34 isolates were considered of which five were members of the genus *Kurthia*, 6 of *Thiobacillus*, 16 of *Bacillus* and remaining 7 were *Acidiphilium*, *Oscillospira*, *Sulfobacillus*,

Alicyclobacillus, *Neisseria*, *Pseudomonas*, *Rahnella.* Isolate F8 was identified as *Bacillus subtilis* through sequence comparison of 16S rRNA gene in a standard alignment program, BLAST (http://blast.ncbi.nlm.nih.gov/) (Fig. 1). Vegetative cells and Gram staining reactions of 3 isolates, *Alicyclobacillus*, *Bacillus megaterium* and *Thiobacillus thioparus*, were shown in Fig. 2.

(a) F8: 16sRNA gene (partial sequence)

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GACGAACTCCCACGGGGAATGCTTACTGGGTTGACTACAGCACTAAGAGGCGGAACCCTGTTGCACTGGAAGATCATCCTTT
ACGGCGTGGACTACCANGGTATCTAATCCTGTTCGCTCCCCACGCTTTCACTCCTCAGCGTCAGTTACAGACCAGAGAGTCG
CCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCCACTCTCCTCTTCTGCACTCAAGT
TCCCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTTTCACATCAAACTTAAGAAACCGCCTGCGAGCCCTTTACGC
CCAATAATTCCGGACAACGCTNGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTA
CCGTCAAGGTACCGCCCTATTCGAACGGTACTTGTTCTTCCCTAACAACAGAGCTTTACGATCCAAAAACCTTCATCACTCA
CGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCAAAAGATTCCCTACTGCTGCCTCCCGTA
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(b) gb|FJ573170.1| *Bacillus subtilis* strain E1-2 16S ribosomal RNA gene, partial sequence Length=1488

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 Score = 870 bits (471), Expect = 0.0
 Identities = 526/553 (95%), Gaps = 6/553 (1%)
 Strand=Plus/Minus

Query  42   ACT-CCCACGGGGAATGCTTACTGGGTTGA-CTACAGCACTAAGAGGCGG-AACCCTGTT  98
            ||| |||| ||||| |||||| || ||| | || |||||||||| ||||| |||||  |
Sbjct  911  ACTCCCCAGGGGGAGTGCTTAATGCGTT-AGCTGCAGCACTAAGGGGCGGAAACCCCCTA  853

Query  99   GCAC-TGGAAGATCATCCTTTACGGCGTGGACTACCANGGTATCTAATCCTGTTCGCTCC  157
             ||| | | |  ||||| |||||||||||||||||| ||||||||||||||||||||||
Sbjct  852  ACACTTAGCA-CTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCC  794

Query  158  CCACGCTTTCACTCCTCAGCGTCAGTTACAGACCAGAGAGTCGCCTTCGCCACTGGTGTT  217
            |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  793  CCACGCTTTCGCTCCTCAGCGTCAGTTACAGACCAGAGAGTCGCCTTCGCCACTGGTGTT  734

Query  218  CCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCCACTCTCCTCTTCTGCACT  277
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  733  CCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCCACTCTCCTCTTCTGCACT  674

Query  278  CAAGTTCCCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTTTCACATCAAACTT  337
            |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
Sbjct  673  CAAGTTCCCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTTTCACATCAGACTT  614

Query  338  AAGAAACCGCCTGCGAGCCCTTTACGCCCAATAATTCCGGACAACGCTNGCCACCTACGT  397
            |||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  613  AAGAAACCGCCTGCGAGCCCTTTACGCCCAATAATTCCGGACAACGCTTGCCACCTACGT  554

Query  398  ATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTA  457
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  553  ATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTA  494

Query  458  CCGCCCTATTCGAACGGTACTTGTTCTTCCCTAACAACAGAGCTTTACGATCCAAAAACC  517
            |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
Sbjct  493  CCGCCCTATTCGAACGGTACTTGTTCTTCCCTAACAACAGAGCTTTACGATCCGAAAACC  434

Query  518  TTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCAAAAGATTCCCTACT  577
            |||||||||||||||||||||||||||||||||||||||||||||  ||||||||||||||
Sbjct  433  TTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACT  374

Query  578  GCTGCCTCCCGTA  590
            |||||||||||||
Sbjct  373  GCTGCCTCCCGTA  36
```

Fig. 1. Sequencing of 16S rRNA gene of strain F8. (a) partial sequence of 16S rRNA gene and (b) alignment shows the isolate F8 has 95% identities with *Bacillus subtilis* strain E1-2.

Growth response of the selected isolates at different pH levels are shown in Fig. 3. *Thiobacillus intermedius* (F9), *Oscillospira* (F12), *Sulfobacillus* (F13), *Rahnella aquatilis* (F32) and *Thiobacillus thioparus* (F33) showed better growth at pH 4.5.

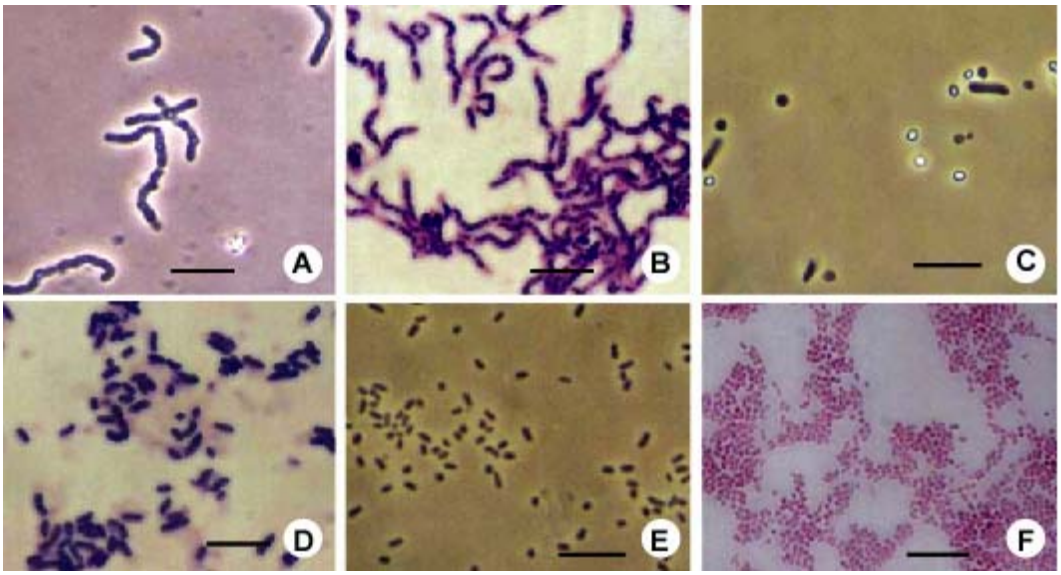

Fig. 2. Photomicrograph showing vegetative cells and Gram staining reaction of of *Alicyclobacillus* (A & B), *Bacillus megaterium* (C & D) and *Thiobacillus thioparus* (E & F). Bars = 10 µm.

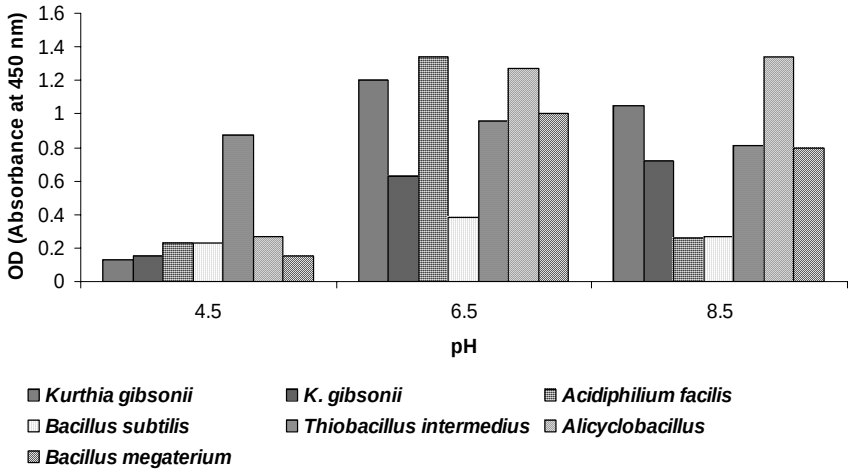

Fig. 3. Growth response of seven isolates at three different pH.

Ehrlich and Brierley (1990) mentioned that the optimum temperature range of iron oxidizing bacteria is generally from 45 to 50ºC but they are active over a wide range of temperature. In present study the optimum temperature for growth was found in between 30 and 40ºC (data not shown).

Out of 34 selected isolates 17 were tested for their tolerance to NaCl at 0, 2, 5, 7, and 10% (data not shown). The maximum growth of the organisms was found in between 0 and 2% NaCl. *Sulfobacillus* (F13), *Bacillus acidocaldarius* (F15) and 3 isolates of *B. megaterium viz.* F16/1, F16/2 and F18 showed better growth at 10% NaCl.

All the 34 selected isolates were tested for their oligodynamic reaction to different iron salt concentration (Atlas *et al.* 1995, Müller 1985). Some of the observations of oligodynamic observation test are shown in the Fig. 4. The growth of *Kurthia gibsonii* (F2), *Bacillus acidocaldarius* (F15), *B. megaterium* (F19), *Neisseria elongata* (F24), *Pseudomonas pseudoalkaligenes* (F28), *B. megaterium* (F30), *Thiobacillus thioparus* (F33, F36 and F37) were inhibited with $FeSO_4.7H_2O$. The growth of 4 isolates of *Kurthia gibsonii* (F1, F3, F5 and F6), *Acidiphilium facilis* (F7), *Bacillus subtilis* (F8), *Oscillospira* (F12), S*ulfobacillus* (F13) [Fig. 4-B1], *B. megaterium* (F25 and F25/1), *B. firmus* (F26 and F27), and *T. thioparus* (F35) were inhibited just around the salt impregnated disc of high concentrations on the other hand; these isolates showed growth stimulation at lower concentration, which was called oligodynamic reaction.

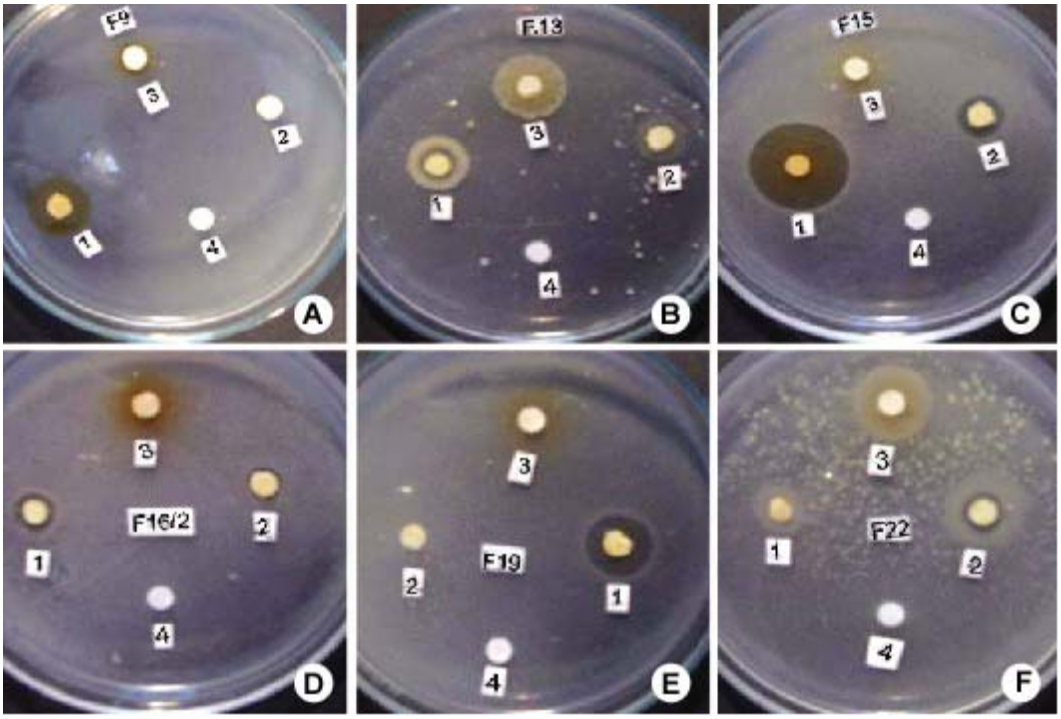

Fig. 4A-F. Photographs showing growth responses toward different iron salts *viz.* $FeSO_4.7H_2O$ (1); $Fe_2(SO_4)_3$ (2); $C_6H_8FeO_7NH_3$ (3) and blank (4). The paper discs soaked in different iron salt solutions were used for this bioassay. A. *T. intermedius* (F9), B. *Sulfobacillus* (F13), C. *B. acidocaldarius* (F15), D. *B. megaterium* (F16/2), E. *B. megaterium* (F19), F. *B. megaterium* (F22).

Seven isolates of *B. megaterium viz.* F16/1, F17, F18, F20, F21, F22 and F31 showed growth stimulation around the disc, which indicated their ability to use $FeSO_4.7H_2O$ at a higher concentration. Bopp *et al.* (1983) mentioned that there are several bacterial strains that contain genetic determinants of resistance to heavy metal.

Growth of *Kurthia gibsonii* (F1 and F2), *Alicyclobacillus* (F14), *B. acidocalderius* (F15), *B. megaterium* (F16/1, F16/2, F19, F20, F21, F25, F25/1 and F31), *Neisseria elongata* (F24), *B. firmus* (F26 and F27) and *T. thioparus* (F36) were inhibited in the presence of $Fe_2(SO_4)_3$. On the other hand, *Sulfobacillus* (F13) and *T. thioparus* (F33) showed growth stimulation, indicating possible utilization of the tested salts. *Bacillus megaterium* (F22) showed oligodynamic reaction (Fig. 4-F2). Except *Kurthia gibsonii* (F2 and F5) and *T. thioparus* (F37), all the strains showed growth stimulation in the presence of $C_6H_8FeO_7NH_3$.

From the transformation experiment it was observed that the 5 isolates transformed Fe^{2+} and Fe^{3+} iron to varying degrees (Table 1). *Bacillus subtilis* (F8) showed 17.5 µg/ml ferrous iron against 35 µg/ml of the control, and 297 µg/ml of ferric iron against 222.5 µg/ml of the control after ten days of incubation. Similarly isolate *Thiobacillus thioparus* (F33) showed 5.0 µg/ml of Fe^{2+} and 145 µg/ml of Fe^{3+} iron. This organisms could be useful for metal extraction from low grade ores.

Table 1. Transformation of Fe^{2+} and Fe^{3+} iron by five isolated bacteria.

Isolates	Concentration of iron (µg/ml)	
	Fe^{2+}	Fe^{3+}
Control	35	222.5
Bacillus subtilis (F8)	17.5	297.5
Thiobacillus thioparus (F33)	5.0	145
T. thioparus (F34)	7.5	27.5
T. thioparus (F36)	12.5	192.5
T. thioparus (F37)	17.5	142.5

Microorganisms transform the iron to get energy during active growth. In anaerobic respiration Fe^{3+} serve as an electron acceptor (Myers and Myers 1994). Results of the experiment were indicative of transformation of iron in culture medium which needs confirmation. Other serious workers interested in the transformation of metals reported their findings from time to time (Grishin *et el.* 1985, Dispirito and Tuovinen 1982).

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