Acidithiobacillus ferrooxidans: A bioleaching bacteria for better iron(II) oxidation ability

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ABSTRACT

The selection of improved strains of *Acidithiobacillus ferrooxidans* with better colonization & iron(II) oxidation abilities is a crucial need for biohydrometallurgy. Here we present a method for selection of the morphological variants of *Acidithiobacillus ferrooxidans* in thiosulfate containing semi-solid growth medium. For selection of variants we used standard culture of *Acidithiobacillus ferrooxidans* NCIB 8455 strain and *Acidithiobacillus ferrooxidans* strain isolated from Indian acid-mine water. All the strains were identified by their growth in liquid 9k medium and characterized at molecular level by the presence of insertion sequences IS *T2* and IS *T445*. The conservation of unique restriction sites within the two known insertion sequences of *A. ferrooxidans*, IS *T2* and IS *T445,* were confirmed by observing no polymorphism in the restriction fragment lengths of the individual amplicons generated from wild type and variant strains by using specific IS *T2* and IS *T445* primers. The selected variants showed stable heritable characters with marked difference from those of the wild type, in their colonizing ability on reduced sulfur compounds in agar medium, colony morphology and iron oxidation capability. We demonstrated the method for selection of Hyphal colonies which had 2.6 fold and 2.08 fold higher iron oxidation capability and ability to colonize at much higher rate with altered morphology than the wild type strain of *A. ferrooxidans*. These variants might prove as efficient microbe for bioleaching.

Key words: *A. ferrooxidans*, IS *T2*, IS *T445*, bioleaching.

INTRODUCTION

The role of improved bacterial strain in biohydrometallurgy processes has yet not received due attention. The efficiency of the processes is usually evaluated by the activity of individual species of chemolithotrophic bacteria, e.g. *A. ferrooxidans*, *A. thiooxidans*, etc. Moreover, its role in metal solubilization of sulfide ore leaching processes has been well demonstrated and the process is currently being implemented commercially in many places[1]. Notably, in bioleaching operations there are also many associated microorganisms besides other sulfur oxidizing bacteria and heterotrophic organisms[2]. *Acidithiobacillus ferrooxidans* formerly called *Thiobacillus ferrooxidans* is an extreme acidophilic chemolithotrophic, gram-negative eubacterium, which is well adapted to low pH (1.6) environment. It is active in the solubilization of copper and in the processing of refractory gold ores in bioleaching operations and also a major contributor to acid mine drainage in copper and coal mines. It derives the energy required for its growth, and more particularly for nitrogen and carbon fixation, through the biological oxidation of ferrous(II) to ferric(III) state of iron or reduced sulphur compounds to sulphuric acid at pH 2 to 4 using oxygen as the ultimate electron acceptor[1,3]. For many years *T. ferrooxidans* was considered to be the most important microorganism in commercial bioleaching and biooxidation plants that operate at 40°C or less[4].

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There are several specific obstacles associated with the identification and enumeration of microorganisms in a bioleaching operation. Strain identification through the insertion elements present in genomic DNA has been used[5,6,7]. Insertion elements are dynamic in the genome, and the rate of transposition found to be typically greater than the rate of deletion[8,9]. The genome of *A. ferrooxidans* contains two distinct families (1 and 2) of repetitive DNA elements[10]. The nucleotide sequence of family 2 member (IS *T2*) is 1408 bp long and has structural features similar to

those of IS elements[6]. While IS *T445* (family 1) is 1219 bp long and is bordered by a perfect repeat of 8 bp with no mismatches and the terminal repeats can be further extended to 23 or 48 bp with 9 or 26 mismatches[7].

The present investigation was started with an aim to generate and select the variants which can grow faster on the ore particles with better iron(II) oxidation capability, in turn may have better bioleaching properties. In the present paper we described the protocol for selecting and characterizing improved strains of *A. ferrooxidans*, which had higher rate of iron(II) oxidation and better colonizing ability. Furthermore, these variants may have the potential for bioleaching of metals from natural ores. The benefit may be taken of the fact that the microorganisms adhere to specific sites on the mineral surface to get energy by oxidation process.

MATERIALS AND METHODS

Materials

Taq DNA Polymerase, dNTP mix and assay buffer were procured from Banglogenei. Primers were synthesized from Life Technologies. Restriction enzymes used for the digestion reactions were procured from Banglogenei, and Life Technologies. For electrophoresis all Bio-Rad reagents are used. Other biochemical reagents were of AR grade and purchased from Merck.

Culture details

IB 1 and IB 2 cultures were the clonally purified culture from *Acidithiobacillus ferrooxidans* NCIB 8455 (National collection of Industrial Bacteria). AS 1 and AS 2 cultures were enriched from acid mine water obtained from RRL Bhubneshwar in 9k liquid medium. Hyphal and PDC (pin dot colonies) cultures are the morphological variants of clonally purified cultures selected on thiosulfate agarose plates.

Culture media details

All strains (IB 1, IB 2, AS 1 and AS 2) of *A. ferrooxidans* referred as mother culture, were grown in liquid 9k medium, purified clonally by serial dilution technique on 9k agarose plates[11], and subcultured in liquid medium containing inorganic sulfur[12].

Morphological variants selection and characterization

After sub-culturing of mother cultures twice in the liquid inorganic sulfur medium, variants were selected on thiosulfate containing agarose plates[13]. These variants were further grown and maintained in liquid medium containing inorganic sulfur.

The morphological variants of culture IB 1, IB 2, AS 1, and AS 2 were selected in the following medium at 30^oC: 200ml of solution A containing $(NH_4)_2SO_4$, 3.0g; $MgSO_4.7H_2O$, 0.5g; $CaCl_2.2H_2O$, 0.25g; $Na_2S_2O_3.5H_2O$, 7.0g; adjusted to pH 4.0 with 1(N) H_2SO_4 and autoclaved at 5lb/inch^2 for 10min, was added to 800ml of solution B containing 4.0g agarose separately autoclaved at 15lb/inch^2 for 15min.

The genomic DNA was prepared for all the mother cultures and variants strains by the modified Marmur's method[10]. The PCR reactions were performed using Taq DNA Polymerase with 50ng genomic DNA in 50µl containing 200µM each of deoxynucleoside triphosphates, 500nM each of the primer (F1 5'-GGCTCTTCTGCGGATTGA-3', R1 5'-GGCTCTTCG TCATTTTCA-3', from the ends of the IS *T445* sequence) and (F1 5'-GAGCTATAGTCAAATCTG-3', R1-GAGCTATGCTCGAAAGTG-3', from the ends of the IS *T2* sequence); and the buffer with 1.5mM $MgSO_4$. The reactions were programmed as follows: 94^oC for 5min and then subjected to 30 cycles each consisting of 30s at 94^oC, 30s at 55^oC and 1min at 72^oC, and finally at 72^oC for 7min to complete extension.

Iron(II) oxidation studies

For iron(II) oxidation studies, these variants and wild type cultures were grown in liquid-9k medium twice, and iron grown cultures taken out from the shaker at regular intervals to determine Fe^{2+} concentration by titration with $KMnO_4$ solution[14]. To 5ml of iron grown culture, 2ml of 10N H_2SO_4 was added and $KMnO_4$ solution was run from the burette till the end point was obtained. $KMnO_4$ solution acted as self indicator.

RESULTS

Selection of morphological variants

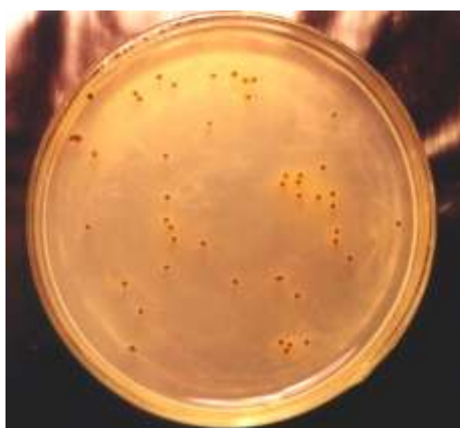

Figure 1: Regular colonies of *A. ferrooxidans* wild type culture IB 2 on 9k solid plates.

Thiosulfate - agarose did not allow wild type cells to form colonies (Figure 1), but is selectively effective in developing the two kinds of specific colony morphology variants within *A. ferrooxidans* population at a frequency of 10^{-7} to 10^{-8} $cell^{-1}$ after about 7-8 days of incubation. Two types of colonies appeared on thiosulfate – agarose semi-solid media, when IB 2 culture of *A. ferrooxidans* was plated in 10^{-8} dilution. First white dots, which further spreaded as branched hyphaes of the fungus dissemination and grew very fast to cover the entire surface (Figure 2), named as Hyphal colonies and second, as pin-dot type colonies. The Hyphal colonies on second day of their appearance were 4-5 cm in diameter. These variants grew like wild type in ferrous sulfate containing broth but with altered morphology on ferrous-iron-agarose plates (Figure 2). Whereas, pin dot colonies were like pin head of 0.5-1mm in diameter which maintained its colony feature even after several generations in sulfur media. While IB 1, AS 1 and AS 2 culture of *A. ferrooxidans* gave only Hyphal colonies. These variants were found to maintain their phenotypic character during further generations and showed 100% colony forming efficiency on thiosufate agarose plates (Figure 2).

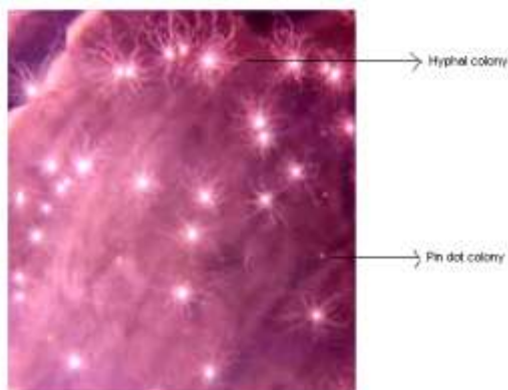

Figure 2: Variant (Hyphal and pin dot) colonies of *A. ferrooxidans* IB 2 wild type mother culture on thiosulfate agarose plates.

Characterization of *Acidithiobacillus ferrooxidans* strains:

The genome of *A. ferrooxidans* contains at least two different repetitive DNA elements. These two distinct elements, termed IS *T2* and IS *T445* (or IST *fe1* family 1 repetitive element) were shown to exhibit the characteristics of typical

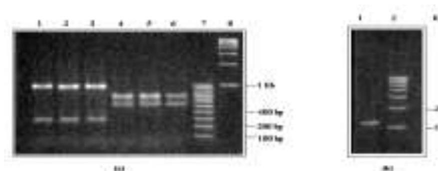

Figure 3: Agarose gel electrophoresis of (a) restriction digestion product of IS *T445* PCR amplicon, lane 1, 2, & 3, Pst I digested product; lane 4, 5, & 6, Nco I digested product for IB 2 culture of *A. ferrooxidans* wild type and its two variants, Hyp I and Hyp III respectively; lane 7, 100 bp marker; lane 8, 1kb marker; (b) lane 1, 1.3 kb IS *T445* amplicon from IB 2 culture of *A. ferrooxidans* wild type; lane 2, 1 kb marker.

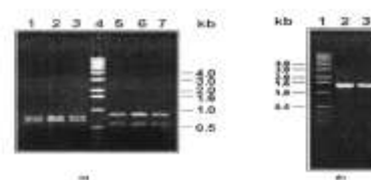

Figure 4: Agarose gel electrophoresis of (a) restriction digestion product of IS *T2* PCR amplicon, lane 1, 2, & 3, Bam HI digested product for IB 1 wild type and variants Hyp I and Hyp III respectively; lane 4, 1kb marker; lane 5, 6 & 7 Nco I digested product for IB 2 culture of *A. ferrooxidans* wild type and its two variants, Hyp I and Hyp III respectively; (b) lane 1, 1 kb marker; lane 2 & 3, 1.4 kb IS *T2* amplicon from IB 2 culture of *A. ferrooxidans* wild type and Hyp I variant.

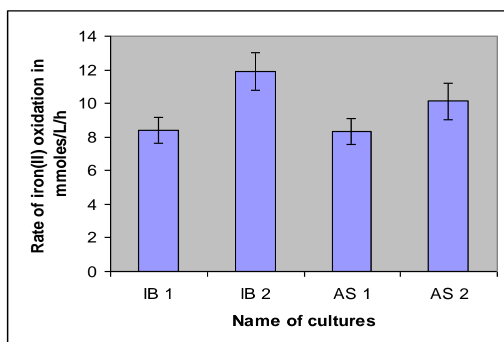

Figure 5: Comparison of rate of iron(II) oxidation by all wild type cultures of *A. ferrooxidans*.

prokaryotic insertion sequence. We had used inward primers designed from the ends of IS *T2* and IS *T445* in PCR to generate amplicons of ≈1.3 or 1.4kb corresponding to family 1 and family 2 repetitive DNA elements, respectively for all the wild type and variant strains (Fig. 3 and 4)[13]. When we digested the amplicons of IS *T445* with Pst I and Nco I and IS *T2* with Bam HI and Nco I, evidently no detectable differences in the restriction analyses among the PCR amplicons (generated from the wild type strains or variants) were noted (Figure 3 and 4). The unique site restriction (Pst I, Nco I for IS *T445* and Bam H I, Nco I in IS *T2*) analysis of IS *T445* and IS *T2* for one wild type and variants has been shown in Figure 3 and 4 respectively. By comparing the banding pattern of restricted products of IS *T445* and IS *T2* we obtained no variation in the size of restricted

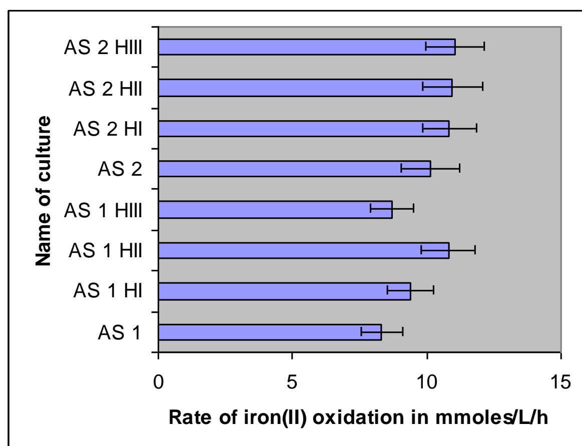

Figure 7: Comparison of rate of iron (II) oxidation by AS 1 and AS 2 wild type mother cultures and its Hyphal variants (H).

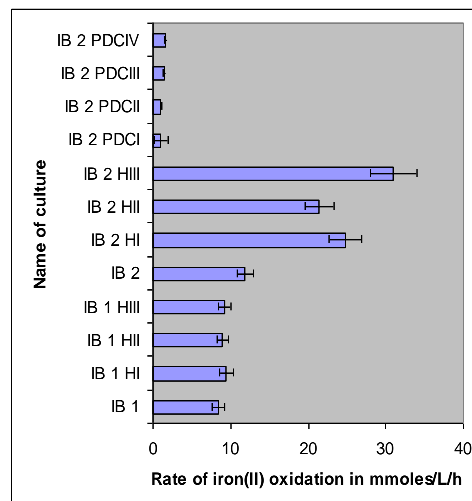

Figure 6: Comparison of rate of iron(II) oxidation by IB 1 and IB 2 wild type mother culture and its variants, Hyphal (H) and pin dot cultures (PDC).

products of wild type and variants. This finding proved that no polymorphism is detected in IS *T445* and IS *T2* with respect to enzymes Pst I and Nco I in IS *T445* and Bam H I and Nco I in IS *T2* (Figure 3 and 4) and therefore, proved the genetic consistency of the microorganism within insertion elements, a characteristic feature of *A. ferrooxidans*.

Evaluation of iron(II) oxidation ability

The efficiency of iron(II) oxidation by all the clonally purified cultures of *A. ferrooxidans* and its variants had been investigated (Figure 5 6 and 7). Based on the data obtained using wild type cultures of *A. ferrooxidans*, IB 2 showed highest rate of iron(II) oxidation i.e. 11.9 ± 1.1mmoles/L/h followed by AS 2 i.e. 10.13 ± 1.1mmole/L/h. Whereas all other wild type cultures had lower iron(II) oxidizing ability than the IB 2 and AS 2 (Figure 5).

When we performed the evaluation of rate of iron(II) oxidation by all the wild type cultures and its variants, many of the Hyphal variants had shown increased iron(II) oxidation ability than its wild type (Figure 6 and 7). However, a marked enhancement in the rate of iron(II) oxidation by IB 2 Hyphal I and IB 2 Hyphal III had been observed in comparison to its wild type IB 2 mother culture of *A. ferrooxidans*. The Hyphal I showed 2.08 fold while the Hyphal III exhibited 2.6 fold higher

oxidation rates in comparison to the IB 2 wild type mother culture (Figure 6). Whereas, the PDC cultures originated from IB 2 wild type culture of *A. ferrooxidans* resulted in decreased iron(II) oxidation ability than the wild type. However, AS 1 and AS 2 wild type cultures of *A. ferrooxidands* resulted only in hyphal variants selection with increased iron(II) oxidation rate in comparison to wild type (Figure 7). These results clearly indicated that by growing the cells of *A. ferrooxidans* in specific media composition, specific variants selection of *A. ferrooxidans* could be achieved. These variants may be accompanied with the efficient colonizing and iron(II) oxidation ability, as shown in the present investigation.

DISCUSSION

More than 5% of total genome of *A. ferrooxidans* consists of 10 to 20 copies of both the two families of repetitive DNA[10,7]. These insertion sequences can promote spontaneous mutation under selective environments. The DNA sequence rearrangements caused IS elements to often alter the pattern of expression of nearby genes, thereby affecting various aspects of physiology (the rate of thiosulfate oxidation) and growth of the microorganisms[13]. Infact, a wide variety of DNA rearrangements may lead to genetic and phenotypic diversity and could play an important role in evolution of strains in a bacterial population[9]. For example in *Mycobacterium tuberculosis*, reduced oxygen atmosphere was shown to induce transposition of indigenous IS *6110* resulting in selection of a colony morphology mutant phenotype[15]. Since IS elements are inherited, strains which have an immediate[7] common ancestor also tend to share some or all the sites at which insertion sequences are located in the genome. Therefore, these repetitive repeats in the genome may be important in identifying the place of origin or genetic ancestry of bacterial isolates. It has been reported previously that insertion sequence transposition occurs at a high frequency in *Alcaligenes eutrophus* when nutritional conditions are altered[16]. In case of *T. ferrooxidans* ATCC 19859, it has been proved that IS *T2* insertion sequence pattern changed in response to whether it was grown on iron or sulfur medium[17]. The phenotypic switching of *T. ferrooxidans* ATCC 19859 has been reported[18] which involved the reversible change between a wild type phenotype in which both Fe(II) and sulfur (and various forms of reduced sulfur) can be oxidized and a mutant form that can only oxidize sulfur and reduced sulfur. It was also reported that the wild type state was also associated with the formation of relatively compact colonies on solid media whereas, in mutant state, the microorganisms exhibited swarming (S and H). However, the present findings did not substantiate the observations in which it was claimed that spreading phenotype was accompanied with the loss of iron(II) oxidation.

In the present investigation, we have described the protocol for selection and characterization of *A. ferrooxidans* variants and reported that variants exhibited altered status in oxidative activities towards reduced iron compounds as compared to the wild type mother strain of *A. ferrooxidans* for the first time. This result presented here may prove to have various industrial importances in bioleaching and bioremediation, and it is a real breakthrough in the isolation of high rate iron-oxidizing variants as well as their purification and selection as a single colony isolate.

CONCLUSIONS

For selection of variants of the *Acidithiobacillus ferrooxidans* which have increased iron(II) oxidation capability, two cultures originated from different places (from NCIB and Indian acid mine water) have been employed in this investigation. Specific composition of thiosulfate containing semi-solid media was capable of allowing the variant strains to grow. These variants of *Acidithiobacillus ferrooxidans* strains were characterized at molecular level by the presence of IS *T2* and IS *T445* with no polymorphism in their internal restriction sites. Two types of variants selected, and all Hyphal variants had shown increased iron(II) oxidation rates in comparison to Pin dot variants. Hyphal variant (IB 2 HIII) had shown maximum 2.6 fold enhancement in iron(II) oxidation rate in comparison to their mother culture. Therefore, it may be concluded that for industrial bioleaching, improved strains may be selected and utilized which have better performance of natural ores oxidation.

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