Microbial Variants from Iron Ore Slimes: Mineral Specificity and pH Tolerance

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Abstract This paper describes the isolation of the native bacterial strains from the iron ore mines slime pond and its extremophilic characteristics. The two microbial isolates designated as CNIOS-1 and CNIOS-2 were grown in selective silicate broth at pH 7.0 and the organisms were tested for their selective adhesion on silicate and alumina minerals. The silicate bacteria with their exopolymers are very potent to grow over aluminosilicates. It was established that CNIOS-1 grew preferentially in the presence of silicate mineral compared to CNIOS-2 which grew in the presence of alumina. The organisms were tested for growth at various pH and trials were carried to define their efficacy for eventual applications to remove gangue minerals of silica and alumina from the raw material.

Keywords Iron ore slimes · Silica · Alumina · Bacteria · Adhesion

Introduction

In India there are many iron ore mines which continue to generate huge amount of slimes [1]. The iron ore slimes contain high level of undesirable minerals which are dumped in storage ponds, called slime ponds. It is estimated that a

typical iron ore slime contains 54 % iron and some other gangue minerals such as SiO₂, Al₂O₃, P₂O₅, TiO₂, MgO, CaO. Out of these components SiO₂ (5 % w/w) and Al₂O₃ (8 % w/w) are the major contaminants [2]. The presence of these contaminants in the huge tonnage volumes of slime poses several difficulties for effective recycling in the iron making process. Due to the close association of aluminosilicate with iron in the material, microbial weathering is poised to affect the iron, causing iron seepage into the ground water system [3–7]. Physico-chemical processes like gravity separation, magnetic separation and flotation have been attempted world over to remove these impurities from the slime, but none of these approaches turned satisfactory because of very fine size (25 µm) of the raw material. Drawback of physical and chemical methods can be overcome by bio-beneficiation which is a process in which undesirable minerals can be selectively removed with the help of microbes [8]. There are very meager reports on the isolation and applications of such microbes. Zhou et al. [8] reported screening, identification, and desilication potential of a silicate bacterium, *Bacillus mucilaginous* in bauxite or at pH 7.2 which was able to remove silica at 30 °C and pH 7.5 with maximum illite concentration 1 %. Similarly, Liu et al. [5] extracted K⁺ and SiO₂ from silicate mineral by *B. mucilaginous* in liquid culture from the crystal lattice of feldspar. This was facilitated by organic acid and polysaccharides. Regards to the role of these minerals in bacterial growth, Kompantseva et al. [6] investigated the interaction between the halo-alkaliphilic non-sulfur purple bacteria, *Rhodovulum steppense* isolated from Soda Lake that grew in presence of layered aluminosilicates: mica and clay minerals. In presence of *R. steppense*, process of saturation of the minerals with the bases was promoted indicating aluminosilicate to have a positive effect on bacterial growth with a better exchange of minerals in solution due to high pH. The

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leaching of silica from the silicate minerals may occur as a result of the participation of both exopolysaccharides [9] and organic acid as bidentate ligands forming bacteria-mineral system [10, 11]. These bacteria due to their ability to survive in extreme environments like temperature, pH variation, salinity and mineral concentration in mines can be classified under the group of extremophiles [12]. Thus, our effort was to investigate the iron ore slime environment wherein the microbial species have existed since many years and can prove useful in technological application to separate silica and alumina making slimes rich in iron values.

This paper highlights the isolation of such extremophiles from silica–alumina rich iron ore slimes and their phenotypic and molecular characterization, and elucidating its specific features for their suitability in the mineral beneficiation process. It also emphasizes the characteristics of mineral surface that influence bacterial adhesion.

Materials and Methods

Samples

Samples of iron ore slimes for this work were collected from the old slimes dam of Joda Mines, Tata Steel, India. Samples were collected in sterilized plastic buckets from a depth of about 1.5 m of two different sites, namely fresh slurry dumps and dried vegetation soil. The aforesaid samples were transported to the laboratory for storage at room/cold temperature depending on usage. Chemical analysis indicated the presence of 43.68 % Fe, 9.888 %

Fig. 2 Cell morphology of mixed colonies in silicate media (*halo zone* around bacilli indicates thick EPS layer)

SiO_2 and 13.48 % Al_2O_3 as the major constituents. The sample was subjected to various characterization studies such as XRD, EPMA and QEMSCAN. The XRD study was carried out by Bruker X-ray diffractometer using $\text{Cu-K}\alpha$ radiation operated at 40 kV and 30 mA. Micro-morphological, mineralogical characterization, and elemental distribution in different phases present in the slime sample were investigated using point and bulk analysis in EPMA.

Bacterial Isolation, Growth Conditions and Characterization

Silica rich minimal (SRM) media composed of peptone (1 g/L), yeast extract (1 g/L), glucose (20 g/L), ammonium

Fig. 1 QEMSCAN (*left*) and EPMA (*right*) analysis of iron ore slimes used for bacterial isolation

sulfate (0.05 g/L) and magnesium trisilicate (5 g/L), was used for isolation of bacterial species from the collected samples. The pH was adjusted to 6.8 ± 0.2 using 2 N NaOH and 10 N H₂SO₄. The solid media were prepared by adding agarose and the streak plate method was preferred for differential growth of microbes. The plates were incubated at 37 °C for 24 h and the colonies were observed by acid-fast staining using carbol fuchsin. The cellular morphology was observed in the LEICA DM6000-BM microscope. These mixed isolates were purified and

separated into two different strains. The organisms grown in plates were transferred into broth after three rounds of purification. Biochemical tests which included gram staining, motility test, endospore staining, capsule staining, IMViC reaction, catalase test, starch hydrolysis test, oxidase test, gelatin hydrolysis test, triple sugar iron (TSI) test, and carbohydrate fermentation test, were performed.

The bacterial cells harvested were further taken for molecular characterization by isolation of DNA, RNA and for 1200 bp sequencing of 16S rRNA as per the standard protocol [13, 14]. The bacterial sample(s) based on 16S rRNA were processed and the identification report was generated using RDP Database and the confidence in identification is limited by both the availability and the extent of homology shown by the ~1200 bp sequence of each sample with its closest neighbor in the database. For this reason, each isolate is reported with the first five hits observed in the said database with 1195961 sequences included in the search. The screening was based on 7-base

Table 1 Biochemical characteristics of both isolates from iron ore slimes in SRM media

S. no.	Characteristics	CN-10S-1	CN-10S-2
1.	Staining		
	Gram	Gram positive	Gram positive
	Endospore	Positive	Negative
	Capsule/acid fast	Positive	Positive
	Negative	Positive	Positive
2.	Motility	Highly motile	
3.	Morphological characteristics		
	Plate	Scaliriform	Punctuate
	Microscope	Thick long rods	Thin rods
4.	Indole	Negative	Negative
5.	Voges Proskauer	Negative	Negative
6.	Citrate utilisation	Positive	Negative
7.	Nitrate reduction	Negative	Negative
8.	Gelatin hydrolysis	Negative	Negative
9.	Caesin hydrolysis	Positive	Negative
10.	Starch hydrolysis	Positive	Positive
11.	Anaerobic growth	Negative	Negative
12.	Growth at pH		
	2.0	No growth	No growth
	4.0	No growth	No growth
	6.0	Meager growth	Meager growth
	8.0	Prolific growth	Prolific growth
	10.0	Found growing	Found growing
13.	Growth at temperature (°C)		
	35	Prolific growth	Prolific growth
	45	Found growing	Found growing
	55	No growth	No growth
14.	Acid from sugars		
	Lactose	Negative	Negative
	Xylose	Positive	Positive
	Maltose	Positive	Positive
	Fructose	Negative	Negative
	Dextrose	Positive	Positive
	Galactose	Positive	Positive
	Raffinose	Negative	Negative
	Trehalose	Negative	Negative

Fig. 3 Cell morphology on CN-10S-1 (a) and CN-10S-2 (b)

oligomers and further phylogenetic analysis was carried out.

Additionally, the growth kinetics for each strain was evaluated by culturing the isolate in SRM media and hourly measurement of turbidity using a spectrophotometer and determination of the cell count using a Petroff Hauser chamber.

Bacterial Adaptation and Tolerance Studies

The microbial species were then adapted in pure silicon and aluminium oxide powder in the presence of media (subtracting Si/Al fraction of the media). The culture was subjected to routine sub-culturing and adaptability of both isolates on the individual components was established. The microbial species were then used for extraction of metabolites which were estimated using pyridine–acetic anhydride method [15]. An enriched bacterial culture was also tested for its ability to secrete exopolysaccharides by centrifuging the suspension at 20,000 rpm yielding an organic acid rich fraction. The remaining cell harvest was added with lysis buffer and vortexed vigorously in the presence of chloroform while incubating at room temperature. This mixture was centrifuged and the supernatant was added to purification buffer in fresh tube, and re-centrifuged in programmable mode of 10 min. The pellet obtained was washed with ethanol and Tris–HCl, and sonicated yielding cellular EPS [16]. Solution chemistry, including pH, dissolved organic carbon (DOC), and nutrients, is known to affect microbial attachment behavior focusing our studies to examine the pH tolerance of isolates. The pH tolerance of the strains was monitored in broth by changes in pH/ E_{SHE} , organic acid production and microscopic observations, as pre isolates and also during adaptation.

Results and Discussion

Characterization of Iron Ore Slimes

QEMSCAN analysis (Fig. 1a) was performed to evaluate the association of SiO_2 and Al_2O_3 in the slimes. QEMSCAN analyses that the regions with higher concentrations of iron have relatively very less presence of alumina and silica. Similar inference can be gained from the EPMA–EDAX (Fig. 1b) which clearly shows the inevitable presence of higher Si–Al ratio causing the decrease in Fe content in the sample. XRD phase analysis reveals hematite and goethite as the major iron bearing mineral phases. Kaolin, gibbsite and, quartz are other minerals present as gangue phase in the slime. Goethite phase is around 50 % which contains Al and Si distributed inside the matrix.

Isolation of Native Strain, Biochemical Characterization with Evaluation of Growth Kinetics

The vegetation soil sample of iron ore slime used for microbial isolation revealed mixed colonies (Fig. 2), which indicated a remarkable EPS layer (Fuschia colored) around the cells indicating the presence of high exopolysaccharides. These mixed isolates were purified and separated into two different strains which were further biochemically characterized (Table 1). The isolate denoted as CNIOS-1 was slimy in nature; whereas, another pure culture denoted as CNIOS-2 was rough in appearance and texture. Both species were determined to be gram positive rods. CNIOS-1 was characterized with thick rods, (Fig. 3a) whereas CNIOS-2 formed relatively thin rods, though in chains (Fig. 3b). The fresh slurry dumps didn't harbor any microbe due to much harsh physical environment, as the samples belonged to the ditch near outlet.

The growth pattern of the two strains was investigated by culturing the isolate CNIOS-1 and CNIOS-2 in SRM

Fig. 4 Change in cell count with respect to optical density at 660 nm against time. **a** CNIOS-1 and **b** CNIOS-2

Fig. 5 Phylogenetic tree of the bacteria isolated (YH64 and YH221) and their related strains based on 16S rRNA gene sequences. Distances were calculated from nucleic acid sequences by using the ClustalW program. The *scale bar* indicates the number of substitutions per site

media. Measurement of turbidity at 660 nm in spectrophotometer and determination of the cell count using a Petroff Hauser chamber was carried out at an interval of 1 h to study their growth kinetics. The change in cell count of CNIOS-1 and CNIOS-2 with respect to OD against time is shown in Fig. 4a, b. A plot between $\ln$ (cell count/initial cell count) vs time showed a straight line to obtain the specific growth rate (μ). R^2 value was found to be 0.904 which indicated a reasonable fit to the growth equation. The generation time of CNIOS-1 at pH 7 and 35 °C was estimated to be 1.9 h. Similarly for strain CNIOS-2, the generation time was calculated to be 1.7 h.

Molecular Characterization

The pure slants were submitted to Microbial Culture Collection of National Center for Cell Sciences, Pune, INDIA for molecular characterization and classification. The blast sequence (figure not shown) identification, which is further elevated for construction of phylogenetic tree as depicted in Fig. 5 was evaluated and confirmed with 100 % homology of CNIOS-1 and CNIOS-2 with *Bacillus cereus* ATCC C1220 and *Bacillus thuringiensis* Fh-6, respectively. These strains were renamed by the culture collection center as MCC2114 and MCC2117,

Fig. 6 CN-IOS-1 in silicate media containing SiO₂ in 20 days (10⁷–10⁸ cells/mL in 5–15 days)

Fig. 7 CN-IOS-1 in SRM media containing SiO₂ in 15 days: **a** SiO₂ surface without bacteria and **b** nanoSEM images of microbes inhabiting in and around silica vents

respectively. These sequence data have been submitted to the GenBank databases under accession numbers KM203113 and KM203114.

Growth in Presence of Alumina and Silica

The isolates CNIOS-1 and CNIOS-2 with initial cell counts in the range of 10⁶–10⁷ cells/mL were grown at a fixed amount of 5 % (w/w) of Al₂O₃ and SiO₂, respectively in the presence of media (SRM) at 35–37 °C and pH 7. During the growth, it was observed that pH decreased

substantially to 4–5 attributed by the generation of organic acids by these species [17–19]. To quantify the organic acid produced by bacteria, fermentation vessel with a 2-L capacity (BIOSTAT-B™) were used with air flow rate set at 0.5 L/min, and the pH was controlled between 7 and 8. To measure the concentrations of organic acids, HPLC equipped with a UV–visible light monitor was employed. The estimation of organic acids was done by titration with NaOH. It was calculated that CNIOS-1 and CNIOS-2 generated 2.83 N and 2.17 N of citric acid per litre of broth grown in glucose medium devoid of Si/Al sources.

Fig. 8 CN-IOS-2 in SRM media containing Al_2O_3 in 15 days (10^8 – 10^9 cells/mL in 5–15 days)

Fig. 9 CN-IOS-2 in SRM media containing Al_2O_3 in 15 days: **a** Al_2O_3 surface without bacteria and **b** nanoSEM image of microbial mat on alumina surface eliciting specificity

It was observed even after repeated sub-culturing that CNIOS-1 (Fig. 6) grew favorably in the presence of silica when fed in their respective media, as a replacement to the existing magnesium silicate salt (added during isolation). Figure 6 clearly demonstrates the growth of bacteria over a period of 20 days in presence of silica. It was observed that intercellular inclusions of silica granules were seen in the cells in 15–20 days with the rise in cell count and size [20]. This specificity was also confirmed by SEM (Fig. 7) where against the non-reacted silica surface (Fig. 7a), a microbial mat was formed on the silicate surface in presence of media (Fig. 7b). On evaluating the microbial specificity to

alumina surface, CNIOS-2 (Fig. 8) grew specifically well in the presence of Al_2O_3 . The bacteria very well colonies around alumina particles in 20 days, which is also evident from its selective adhesion properties as seen in Fig. 9. As a result of adhesion, there was a significant change in surface properties of alumina, wherein the microbial mat solubilises the particle via the organic acid metabolites and increased cell count in 20 days [21] as seen in Fig. 10.

The preferential growth patterns of the two isolates may be an indication of their potential for beneficiation of silica and alumina contaminated mineral dumps under a set of pH and other conditions. In the pH range 6–10, both the

Fig. 10 Microscopic visualization of change in properties of CNIOS-1 (a, b) and CNIOS-2 (c, d) in 10 days on SiO_2 and Al_2O_3 , respectively with SRM media (a, c—initial; b, d—10th day)

microbes individually showed good growth till pH 10.0 in the presence of silica and alumina respectively. Keeping the objective of selective removal of silica and alumina from iron ore slimes in view, both microbes were assessed microscopically for their growth in consortia at pH ranging from 5 to 10 (Fig. 11). It was surprisingly seen that the bacteria grew well at alkaline pH and especially at pH 10. The cells died at pH above 11. Growth at pH 6–10 (Fig. 11) depicts the extremophilic nature of these alkalophilic isolates [21]. They are ascertained extremophilic as the bacterial strains were isolated at neutral pH and were able to be grown in the pH range from sub-neutral/mild acid to alkaline range, which primarily indicates its tolerance of H^+/OH^- variations [22]. It was, therefore, considered to culture the alkalophiles for such applications which may prove to be effective as the slurry was alkaline in nature (around 8–9). The reason for use of both microbes in consortia with both minerals in solution was to ensure that both gangue minerals are collectively removed. Previous studies have shown that various microorganisms can enhance the dissolution of aluminosilicate minerals at low (<5) or high (>9) pH [23]. However, it was not known if they can have an effect at such pH in presence of other ions like Fe, P, etc. It is thus confirmed that these isolates preferentially attach to surfaces that are nutritionally

advantageous and have similar electrostatic characteristics resulting irreversible attachment of bacteria to aluminosilicate surfaces [24]. Whereas with SRM media, colonization occurs predominantly on the aluminosilicate surfaces. The dissolution of aluminosilicates is accompanied by a release of alkali cations (such as K^+ , Na^+ , and Mg^{2+}) and by consumption of protons, thereby increasing the acidity. More importantly, these minerals are appealing buffering agents and act as long-term sources of alkalinity [24].

Trial Studies

To test the efficacy of these isolates, experiments were carried with 20 % (w/v) iron ore slimes (Feed grade: 43.68 % Fe, 9.88 % SiO_2 and 13.48 % Al_2O_3) in two respective sets—with extracted metabolites and with bacteria for 10 days. With only metabolites, a very less improvement in the grade of Fe (46.33 %) in the residue has been observed and considered in-appropriate due to higher dissolution of iron. The metabolites were found to be more specifically able to dissolve alumina fractions, thus lowering its value to 7.56 %. Employing 20 mL broth (10 mL each of both strains), the beneficiated product was analyzed to contain 49 % Fe, with SiO_2 and Al_2O_3 being

Fig. 11 Growth of microbial consortia in SiO_2 and Al_2O_3 with SRM media at various pH

reduced to 8 and 11 %, respectively (figure not shown). There was a very unusual fall in pH to 4.5 on 10th day with the redox potential observed as <4 mV, indicating the role of metabolites. It was thus concluded that a continuous process flowsheet can be developed employing cells and metabolites, which can enrich the iron ore slimes, to remove the gangue for comprehensive utilization of such discarded fractions.

Conclusions

Two extremophilic microbes were isolated from iron ore slime samples which grew in silicate rich media at pH 7.0 and 35°C . The native microbes isolated from iron ore slimes namely CNIOS-1 and CNIOS-2 have the potential to preferentially grow in the presence of SiO_2 and Al_2O_3 . The two species characterized as *B. cereus* and *B. thuringiensis* proliferated in the pH range 6–10, and had exceptional ability to form intracellular inclusions. Better understanding of the mechanism shall have potential applications in beneficiation of poor quality/unused raw materials such as iron ore slime and low grade iron ores.

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