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BIOREMEDIATION OF ACID MINE DRAINAGE : A PROTEOMIC AND GENOMIC APPROACH

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ABSTRACT

The most significant environmental issue that has arisen in the mining industries over the last two decades is that of Acid Mine Drainage or Acid Rock Drainage (AMD/ARD) from tailing dumps and waste mined rock piles. *Bacillus subtilis* and its metabolic products have been used in our studies. In this work, selective separation of pyrite from oxides such as quartz and calcite as well as galena (sulphide mineral) was achieved. Through microbially induced flotation, pyrite could be separated with 90% efficiency from quartz and calcite leading to desulphurisation and depyritization making the process environmentally benign. Mineral specific proteins were found in extracellular proteins isolated from the cell free extract when *B. subtilis* was grown in presence of minerals through SDS-PAGE. The suppression of some genes was observed in the RAPD profile of the genomic DNA in presence of minerals. The cells of *B. subtilis* proved to be 45-60% efficient in the biosorption of lead metal ions at 10 and 50ppm of lead metal ion. The exopolymers extracted from *B. subtilis* proved to be 50-60% efficient in biosorption of lead metal ions. This study shows possibilities for development of eco friendly technologies for bioremediation.

Key Words : Bioremediation, Acid Mine Drainage, Lead, Sulphide, Proteins

INTRODUCTION

Pyrite is usually found with valuable metal sulphides such as galena. Economical extraction of the valuable metal demands selective depression of pyrite from the associated metallic sulfides during flotation.[1] Conventional methods make use of toxic chemicals like xanthates and cyanides leading to potentially disastrous environ mental consequences. The application of micro organisms in bioflotation and bioflocculation has been critically reviewed.[2] Sulfide minerals such as pyrite (FeS_2) are oxidized to sulfate when water containing oxygen, infiltrates the tailings.[3] Separation of pyrite from galena, quartz and calcite has also been studied with relevance to mitigation of acid mine drainage. *Bacillus subtilis* is a Gram-positive neutrophilic persiflagellated aerobic, catalase positive capsulated bacterium commonly found in soil indigenously associated with many mineral deposits.[4] Heavy metals are released into the environment through industrialization which poses a great danger for humans. Biosorption and bioaccumulation processes have been reviewed for different metals with different microorga nism.[5] Selective separation of pyrite from galena, quartz and calcite was achieved through microbially induced flotation and flocculation. The extracellular protein profile of bacterial cells grown in absence and presence of the minerals has been characterized through SDS-PAGE and suppression of some genes was observed in RAPD profile of genomic DNA in presence of minerals.

The cells and exopolymers of *B. subtilis* proved to be efficient in the biosorption of lead metal ions this study shows possibilities for development of eco friendly technologies for bioremediation.

MATERIAL AND METHODS

Minerals

Handpicked mineral samples of pyrite, galena, quartz and calcite were procured from Alminrock Indscer Fabriks, Bangalore, India. The purity of all mineral samples was determined through chemical, X-ray and mineralogical analyses and it was ascertained to be around 99%. The above

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samples were ground in a porcelain ball mill, sieved and fractioned to obtain different size fractions. The − 105+75 μm size fraction was used in flotation - 37 μm for adsorption and 8-10 μm for flocculation studies.

Bacterial culture

Strain of *Bacillus subtilis* (NCIM-2655) used in this study was obtained from National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India subcultured in luria broth[6] incubated at 30°C on orbitek rotary shaker maintained at 200 rpm and the cell concentration was determined with haemocytometer.

Bacterial growth in the presence of minerals

Bacillus subtilis was grown in presence of minerals in LB medium at 5% pulp density, before using for bacterial growth the minerals were steam sterilized to kill endogenous organisms. Sub culturing was done weekly and adaptation to minerals was considered achieved when the growth rate of adapted strain was identical to that of the control cells grown in absence of minerals.

Adsorption studies

For adsorption tests, one gram each of individual mineral powder (−37 μm) was suspended in 100ml of 10^{-3} M KCL solution at the desired pH in the presence of a known concentration of bacterial cells in 250ml erlenmeyer flasks. The suspension was agitated for 1hour at pH 6.0–7.5 on an orbital shaking incubator at 200rpm at 30°C.[7] Adsorption of proteins from the bacterial cell free extract EP before and after mineral interaction was also similarly established.

Ruthenium red adsorption

A known concentration of cells was interacted with 20ml of a known concentration of ruthenium red at desired pH value. The mixed solution was agitated in an orbital shaking incubator (200rpm and 25-28°C) for 1hour. The suspension was then centrifuged at 10,000×g at 10°C. The supernatant was analyzed using a Shimadzu model UV-260 spectrometer for determination of residual concentration of ruthenium red at a wavelength of 535nm.[8]

Protein assay and extraction of extracellular /intracellular proteins

The proteins present in the cell free extract of *B. subtilis* before and after interaction with minerals and when grown in presence of minerals were quantified using bradford reagent.[9] For extracellular protein extraction one liter of two-day old batch culture was taken and centrifuged. Protein was precipitated by ammonium sulphate method and dialysed against 0.1M, Tris-hydrochloride buffer of pH7. The concentration of extracellular proteins isolated was determined by bradford method.[10]

Microflotation tests

1 gram of desired mineral was pulped in 100 ml of bacterial cell suspension, cell free extract and EP at neutral pH incubated on a rotary shaker for 1 hour. After interaction with the bacterial cells, cell free extract and EP mineral particles were separated through decantation. The conditioned minerals were transferred to a modified hallimond tube and floated using nitrogen at a flow rate of 20 ml/min for 3 min. The settled and floated fractions were separated dried and weighed.[11] In some tests the minerals were also conditioned with specific collectors such as quartz : SOKEM-524c, calcite : Sodium oleate, pyrite and galena : Sodium/potassium isopropyl xanthate.

Gel Electrophoresis for characterization of extracellular proteins

12%SDS-PAGE gel was used for this purpose[12] and the protein was then run on gel and analyzed.

Genomic DNA isolation

Genomic DNA was isolated from 5-15 μg of DNA found in 1ml-1.5ml of overnight grown culture the procedure was followed by as given in. Genomic DNA was then amplified through PCR using fluorescent labeled RAPD primer and PCR products were loaded on two percent agarose gel.

Scanning Electron Microscopic analysis

SEM studies were carried out using a FEI Sirion, high resolution electron microscope. The mineral particles were interacted with bacterial cells collected by centrifugation and subjected to chemical fixation using 2.5% glutaraldehyde and dehydrated in grade series of ethanol.[13]

Extraction and partial purification of bioflocculant

Fully grown culture was centrifuged at 10000 xg for 10 minutes to separate cell pellet. Supernatant was poured into two volume of cold ethanol and

kept for overnight at 4°C to precipitate the bioflocculant and resulting precipitate was collected by centrifugation at 10000x g for ten minutes and washed by re dissolving in distilled water.[14]

Characterization of partially purified bioflocculant

Protein content of polymer was determined according to bradford method. Total sugars were determined by phenol-sulphuric acid method.[15] Infra-Red spectra (IR) of biopolymer -rs were measured on a KBr disk to determine the functional groups of bioflocculants.

Characterization of biopolymers by Thin Layer Chromatography (TLC)

TLC analysis was performed on silica G-50 plates with butanol, acetic acid and water (3:1:1) as the developing phase and spot was detected by spraying agent with 20% H_2SO_4 in methanol followed by drying at 120°C for 5 to 10 minutes. Authentic monosaccharide standards (Glucose, Fructose and Galactose) were run along with test samples[16,17] and retardation factor is calculated by R_f=distance travelled by solute/distance travelled by solvent.

Mass spectrometry

Molecular weight of bioreagents was measured with Kompact (SEQ) KRATOS analytical time of flight mass spectrometry and with a MALDI (Matrix Asserted Laser Desoprtion/Ionisation) technique.[18]

RESULTS AND DISCUSSION

Bacterial growth

The growth curve of *Bacillus subtilis* was plotted with cell count as a function of time as depicted in **Fig. 1(a)** and **Fig. 1(b)** To illustrate their morphological characteristics. Maximum growth attained was $1.5x10^9$cells/ml and pH was also measured and was found to remain constant at about 7.0-7.6. Similar growth kinetic studies of *Bacillus subtilis* have been conducted.[19]

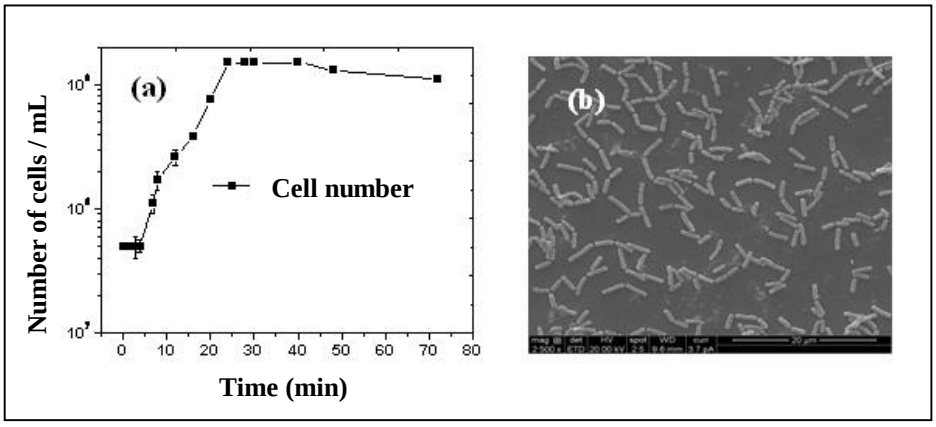

Fig. 1 : (a) Bacterial cell number as a function of time during growth of *Bacillus subtilis* (b) SEM micrograph of *Bacillus subtilis*

Adsorption of bacterial cells and extracellular proteins

Typical scanning electron micrographs depicting adhesion of bacterial cells on to pyrite, galena, quartz and calcite are illustrated in **Fig. 2**. As could be readily seen, profuse and significant adhesion of bacterial cells could be seen on pyrite, compared to calcite and quartz minerals. The adsorption behavior of bacterial cells on to pyrite, galena, calcite and quartz as a function of time is illustrated in **Fig. 3** at pH 6.0-7.5. Adsorption density of bacterial cells on to pyrite was 9×10^8 cells/g in less than 5 min, 1.2×10^8 cells/g in almost 5 min onto calcite as well as galena and 1×10^8 cells/g in 5 min onto quartz. Pyrite exhibited the highest affinity for the cells compared to galena, calcite and quartz. Similar studies were conducted by Usha and Natarajan[20] with respect to the separation of quartz and calcite from yeast cells. Yeast cells shows profuse attachment on to calcite and was least on quartz. Generation of cell surface polysaccharides when grown in the presence of minerals was also estimated through ruthenium

red adsorption method and amount of protein present in cell free extract before and after interaction with the minerals was estimated by Bradford method as illustrated in **Table 1**.

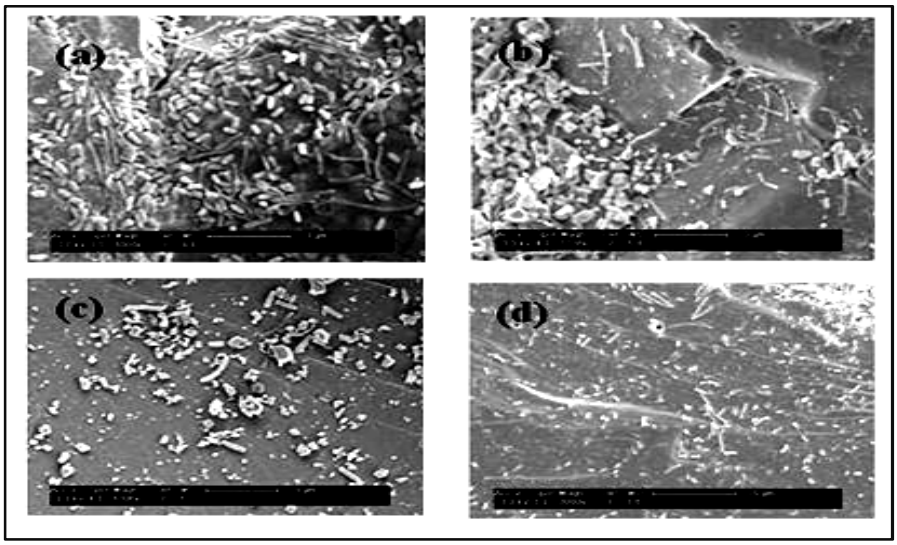

Fig. 2 : Scanning electron micrographs of *B.subtilis* attached on (a) pyrite (b) galena (c) quartz and (d) calcite

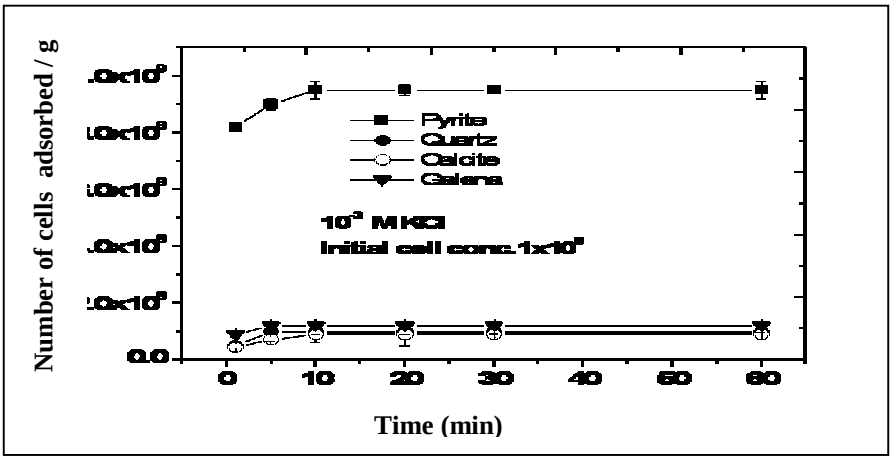

Fig. 3 : Adsorption density of *B. subtilis* cells onto minerals as function of time

Table 1: Percent adsorption of ruthenium red onto bacterial cells and amount of protein present in cell free extract before and after interaction with the minerals

Bacterial cell growth conditions	Amount of protein (µg/g)	Percent adsorptin
	Bradford reagent	Ruthenium red
Control	70 ± 0.76	70.4 ± 0.86
Pyrite	50 ± 1.8	93.8 ± 2.0
Galena	85 ± 1.6	43 ± 2.0
Calcite	81 ± 0.99	81.5 ± 0.7
Quartz	91 ± 1.34	60.2 ± 0.7

Significant value $p<0.05$ for $n = 5$

Percent ruthenium red adsorption on cells will indicate increased presence of polysaccharides while the amount of protein present after interaction is estimated by bradford method. Bacterial growth in presence of galena and quartz promoted enhanced protein secretion followed by growth in presence of calcite. On the other hand in presence of pyrite less protein was generated and more of ruthenium dye was absorbed due to more production of polysaccharides. In presence of galena, quartz less polysaccharides was formed compared to that of pyrite but in case of calcite equal amount of proteins and polysaccharides was generated. From surface chemical view point increased surface affinity towards proteins (as in the case of galena, calcite and quartz) promotes hydrophobicity. Enhanced surface presence of polysaccharides on the other hand (as in the case of pyrite) would render the mineral more hydrophilic.

Surface affinity of extracellular proteins on to minerals was then established through adsorption density measurements as illustrated in **Fig. 4**. Quartz exhibited the highest adsorption density for proteins when compared to galena, calcite and pyrite. Bacterial growth in the presence of pyrite resulted in a decrease in protein generation. Research work conducted by Poorni and Natarajan[21] shows that on interaction with hematite with *B. subtilis* more amount of polysaccharide was generated and on interaction with kaolinite protein secretion was found to be more.

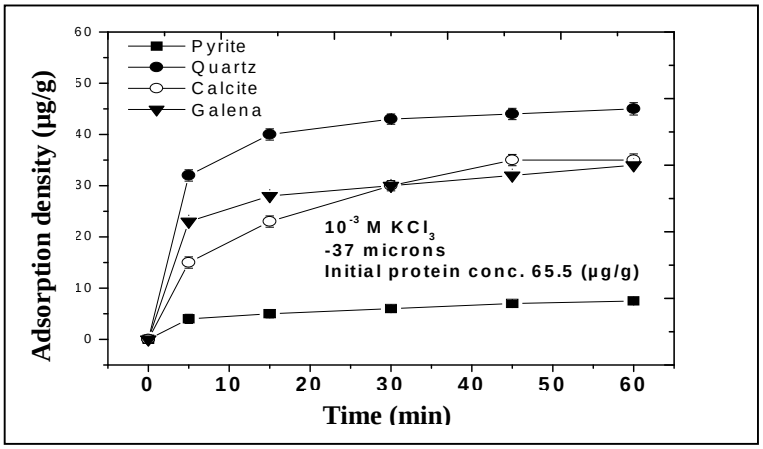

Fig. 4 : Adsorption behavior of EP secreted by *Bacillus subtilis* in presence of quartz, calcite, pyrite and galena

Microbially - induced flotation

Flotation of galena, quartz, calcite and pyrite was studied before and after interaction with bacterial cells, cell free extract and EP at pH 6.0-7.5. From **Table 2**, it can be observed that percent flotation recovery of galena was about 92% and 73% on interaction with bacterial cells and cell free extract and on interaction with EP percent floatation was 75%. Percent flotation recovery of quartz was about 92.5% and 91.1% on interaction with bacterial cells and cell free extract and on interaction with EP percent floatation was 73.5%. Such an enhancement in hydrophobicity of quartz is due to mineral-induced proteins secreted by bacteria into the cell free extract during growth in the presence of quartz. In case of calcite the recovery was 74.6%, 55.6% and 66.4% on interaction with bacterial cells, cell free extract and EP respectively. In case of pyrite significant depression of flotation was observed after interaction with bacterial interaction.

Selective flotation tests also were carried out using 1:1 mixtures of pyrite-calcite and pyrite-quartz and pyrite-galena under the above conditions and similar results could be obtained. Under all conditions of bacterial pretreatment whether interacted with cells, cell-free extract or exopolymer pyrite flotation was seriously impaired. The above results clearly indicate that interaction with *B. subtilis* confers surface hydrophobicity on galena, quartz and calcite while similar biotreatment renders pyrite more and more hydrophilic.

As reported by Yang et al.[22] Separation of hematite from quartz was achieved successfully with 65-70% flotation recovery using *Rhodococcus erythropolis*. Usha and Natarajan have found that the yeast cells adapted to quartz showed 90% separation of quartz from calcite.

Table 2 : Weight percent flotation of pyrite, galena, calcite and quartz after interaction with *B. subtilis* cells, cell free extract and EP

Mineral	No bacterial interaction	*In presence of mineral specific collectors (10^{-4} M)	After interaction with cells (1h)	After interaction with Cell free extract (1h)	In presence of EP
Quartz	13.6 ± 1.1	98.8 ± 1.0	92.5 ± 0.4	91.1 ± 0.8	73.5 ± 0.7
Pyrite	09 ± 0.9	83.5 ± 0.6	7.0 ± 0.9	3.1 ± 0.7	5.5 ± 1.0
Calcite	11.8 ± 0.5	94.2 ± 1.4	74 ± 0.8	55.6 ± 0.6	66.4 ± 0.5
Galena	25 ± 2.0	96 ± 0.7	92 ± 0.7	73 ± 1.0	75 ± 2.0

Significant value $p<0.05$ for $n = 5$

Protein, Genomic DNA and RAPD-PCR fingerprinting profiles of *Bacillus subtilis*

SDS-PAGE profile of extracellular proteins extracted from *B. subtilis* was compared with that of proteins extracted from mineral adapted cell free extract **(Fig. 5)**. Different groups of proteins were secreted by mineral grown cells compared to a regular strain grown in the absence of minerals and molecular weight of proteins secreted in presence of minerals. The molecular weights were obtained from MALDI-TOF analysis and the results of control, quartz, calcite, galena and pyrite were found to be 10, 15, 20, 53 and 14 kDa respectively.

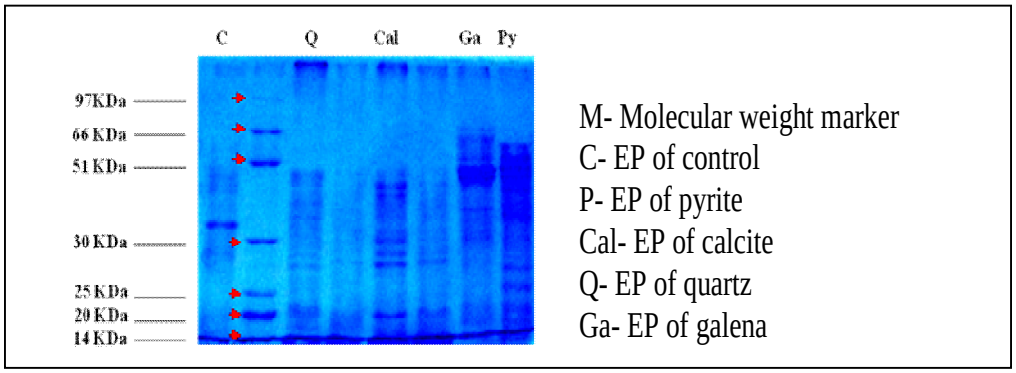

Fig. 5 : SDS-PAGE of EP secreted by *Bacillus subtilis* in presence of quartz, calcite, pyrite and galena

The concentrations of EP measured trough UV-visible spectroscopy was found to be in accordance with that obtained from MALDI-TOF. Concentration (µg/µl) of EP in control, quartz, calcite, galena and pyrite was found to be 0.25, 1.55, 1.4, 1.7 and 1.2 respectively.

Genomic DNA of *B. subtilis* in presence and absence of minerals was studied **(Fig. 6(a))**. In all the isolates the genomic DNA was found to be of 23,130 bp. RAPD-PCR analysis was carried out to look into the genomic alterations of bacterial genomic DNA grown in presence of minerals. RAPD-PCR fingerprinting was carried out for gaining better understanding of the different base pair bands present in the bacteria in absence and presence of minerals **(Fig. 6(b))**. Prominent band of 500bp was seen in both control and bacteria grown in presence of minerals. 1kb band was prominently seen only in control and calcite adapted DNA where as the 1kb band was very light in bacteria grown in presence of quartz. 1kb band was absent in bacteria grown in presence of pyrite and galena.

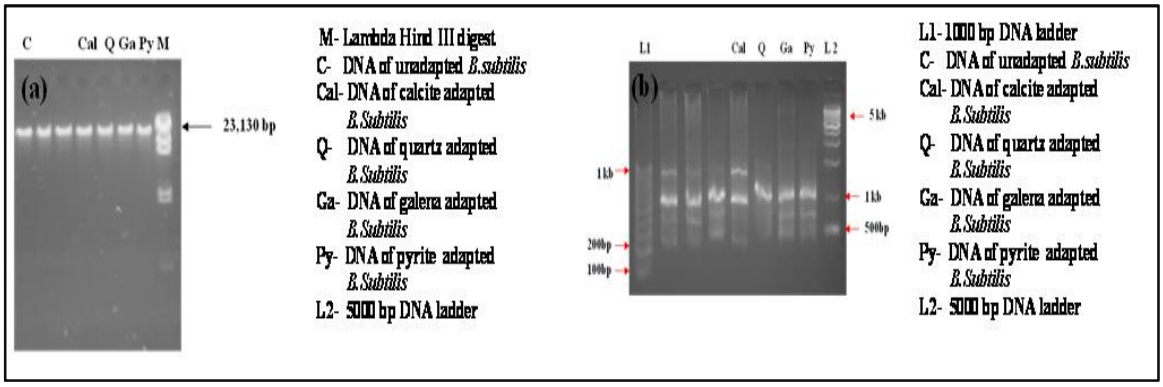

Fig. 6 : (a) Genomic DNA of *B.subtilis* (b) RAPD amplification patterns of PCR products of *B.subtilis*

This shows the possibility of some genomic alterations in the bacterial genomic DNA in presence of galena and pyrite. Based on the RAPD results, the RAPD-PCR method can be used as an investigational tool for mineral induced genomic alterations. Furthermore the present results suggest that RAPD-PCR fingerprinting in conjugation with gene sequencing can be a powerful screening tool for searching for mineral induced specific genomic variations which are caused when bacteria is grown in presence of the minerals. Research work carried out by Sabari and Natarajan[23] have shown the presence of mineral specific proteins extracted from the cell free extract of sulphate reducing bacteria grown in presence of quartz and hematite. As reported by Vasanthakumar et al.[24] it is very much evident that DNA played a vital role in the selective flotation of sphalerite from galena with respect to *B. megaterium*.

Bioremediation of lead through *B. subtilis* cells and exopolymers

The cells of *B. subtilis* were used in the bioremediation of lead. The SEM micrograph of bacteria in absence and presence of lead (**Fig. 7(a)**). In presence of lead the bacterial cells are slightly elongated due to the toxic effects of lead. Bacteria were grown in presence of 10, 50 and 100 ppm of lead concentration. *B. subtilis* was found to be tolerant to Pb metal ion up to 50 ppm and later on the growth was hindered. Growth of bacteria in presence of Pb **(Fig. 7(b))**. It was naturally tolerant up to 50 ppm with good growth of 10^9cells/mL in 24 hours. Bioremediation of arsenic heavy metal ion upto 2500 ppm using *Acidithio bacillus ferrooxidans* has been reported by Chandra Prabha and Natarajan.[25]

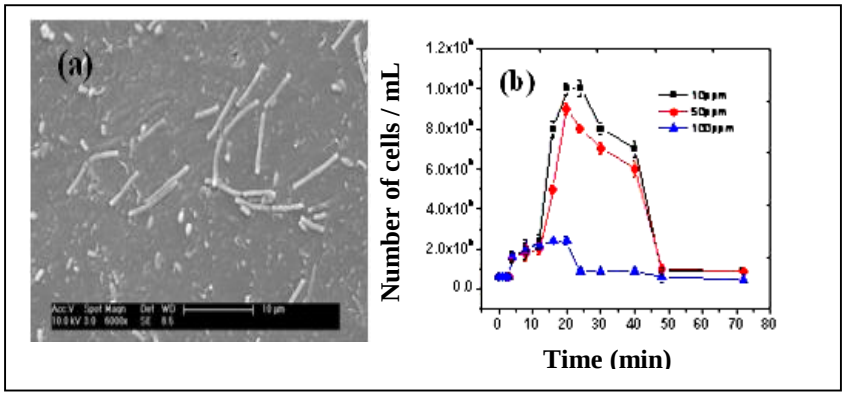

Fig. 7 : (a) SEM micrograph of *B. subtilis* grown in presence of lead metal ions (b) growth of *B. subtilis* in presence of lead metal ion

In presence of cells the percent of lead ion removed was found to be 60% for 10ppm and 45% for 50ppm. Exopolymer was extracted from the cell free extract of the bacteria, **(Fig. 8(a)** and **Fig. 8(b))** and later the biosorption tests were performed, the

amount of protein in the exopolymer was found to be 5µg/g and polysaccharide was found to be 95µg/g. Similar studies have been carried out by Gomaa[26] using *Pseudo-monas aeruginosa* for the bioreme-diation of heavy metal ions like arsenic, lead, mercury by using the exopolymers secreted by the bacteria.[27-31]

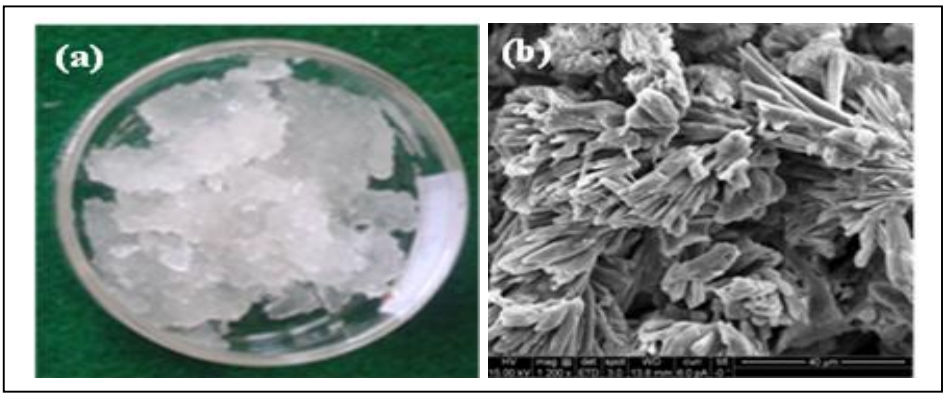

Fig. 8 : (a) Biopolymer extract (b) SEM of biopolymer

Characterization of exopolymer was done using tollens test and TLC **(Fig. 9(a)** and **Fig. 9(b)).** Presence of monosaccharides was seen which consists of glucose, fructose and galactose.[32-34] The presence of sugar was observed as black spots with R_f values of 0.45 cm and 0.4 cm (sample and standards) giving similar pattern of monosaccharides.[35-42]

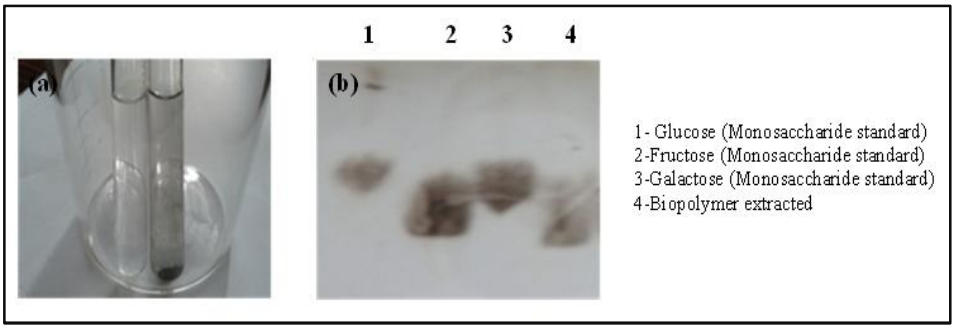

Fig. 9 : (a) Tollens test (b) TLC analysis for detection of sugars of biopolymer extracted

CONCLUSION

Cells of *B. subtilis* exhibited higher affinity towards pyrite compared to galena, quartz and calcite at neutral pH range. Pyrite was preferentially flocculated in the pH range 6.0-7.5. Through microbially induced flotation pyrite could be separated from galena, quartz and calcite, leading to desulphurisation and depyritization making the process environment - tally benign. Mineral specific proteins were found in extracellular proteins isolated from the cell free extract when *B. subtilis* was grown in presence of minerals. The cells of *B. subtilis* proved to be 45-60% efficient in the biosorption of lead metal ions at 10 and 50ppm of lead metal ion. Energy dispersive X-ray analysis further confirms biosorption of lead metal ion onto bacterial cells. In this regards bioremediation process provides an effective innovative measures for treatment of a wide variety of contaminants. The exopolymers extracted from *B. subtilis* proved to be 50-60% efficient in biosorption of lead metal ions this study shows possibilities for development of eco friendly technologies for bioremediation of acid mine drainage.

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