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A PRELIMINARY STUDY ON RECOVERY OF LANTHANIDES FROM PANAMBUR BEACH (MANGALORE) WASHINGS USING *BACILLUS AERIUS*

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ABSTRACT

Bacillus aerius colonies were isolated and enumerated in the soil samples obtained from Panambur beach washings, Mangalore. The accumulation of Lanthanides in *Bacillus aerius* is characterised by using ICP OES and FTIR analysis. ICP MS is carried out to analyse the amount of lanthanides in soil. Cerium and Samarium were present in high proportion in the samples taken. The bioaccumulation studies using *Bacillus aerius* showed as samarium(sm) of 20.14 mg/l and Cerium (ce) of 8.101 mg/l. Further FTIR analysis is carried out to detect the significant changes on soil brought by bacterium.

Bacillus aerius is a gram positive bacterium, with the capacity to accumulate lanthanide elements in its external surface and that is not transported into cytoplasm. The cell wall of the Bacillus species and many other gram positive bacteria provides the bacterium with rigid and protective sacculus interposed between the cell and its environment. In the accumulation of RRE, the amount of RRE accumulated by gram positive bacteria were much higher than those of gram negative bacteria, fungi and yeast. Generally, the teichoic acid polymers in gram positive bacteria definitely confer a strong negative charge on the surface of the cell wall because of their high content of ionized phosphate groups, although a little amount of teichoic acid is found in gram negative bacteria. The binding of metal and cell wall strongly depends on teichoic acid in *Bacillus*. Therefore, it is tentatively considered that the chelate formation between the cell surface and gram positive between and metal ion becomes stronger.

Keywords: *Bacillus aerius,* Lanthanides From Panambur Beach (Mangalore)

INTRODUCTION:

Rare-earth elements (REE) are a group of seventeen chemical elements in the periodic table, in particular the fifteen lanthanides as well as yttrium and scandium as defined by the International Union of Pure and Applied Chemistry (IUPAC).The 17 rare earth elements are Cerium(Ce), dysprosium (Dy), erbium (Er), europium (Eu), gadolinium (Gd), holmium (Ho), lanthanum (La), lutetium (Lu), neodymium (Nd), praseodymium (Pr), promethium (Pm), samarium (Sm), scandium (Sc), terbium (Tb), thulium (Tm), ytterbium (Yb), and yttrium (Y). Scandium and yttrium are considered REE since they tend to occur in the same ore deposits as the lanthanides and exhibit similar chemical properties. All REE occur in nature but not in pure metal form, although Promethium, the rarest, only occurs in trace quantities in natural materials as it has no long-lived or stable isotopes **[1]**. Presently these metals have become very useful in modern technologies ranging from cell phones and televisions to LED light bulbs and wind turbines **[2]**.

Significant rare earths minerals found in India include ilmenite, Sillimanite, garnet, zircon, monazite and rutile, collectively called Beach Sand Minerals (BSM). India has almost 35 percent of the world's beach sand mineral deposits. Their importance lies in their unique electronic, optical and magnetic characteristics, which cannot be matched by any other metal or synthetic substitute **[3]**. The only place on Earth where this reaction can occur on a large scale is the marine environment. Sea water is believed to be a major contributor of REE where fractionation takes place, resulting in significant variations in REE distributions. This results in the precipitation and depletion of certain REE in sea water **[4]**. Hence, for geochemical studies related to all kinds of rock formations, the REE data is vital in providing information on the geochemical processes within the mantle and crust.

Several studies have been conducted for the identification of REE in Beach washings. The Laser-induced breakdown spectroscopy (LIBS) is an emerging and promising tool for rapid and efficient elemental analysis. LIBS technique was used to analyze the raw monazite sands collected from beaches **[5]**. The significant accumulation of rare earth elements especially Cerium and Neodymium was noticed in Bacillus cereus isolated from rare earth environment **[6]**. In comparison with the performance of inductively coupled plasma atomic emission spectrometry (ICP- AES) with the inductively coupled plasma mass spectrometry (ICP-MS) used for the determination of fourteen lanthanides and yttrium in Monazite **[7].** The introduction of ICP-MS in the 1980s brought a remarkable change in REE analysis because of its very low detection levels, sensitivity, large dynamic range, limited interference effects and simplified sample preparation procedures **[8]**.

Lanthanides occur only in trace amount in organisms. A biological role for Ln is not known, and their interaction with bacterial cells has yet been the subject of detailed studies. It is assumed that lanthanides can bind to the external surfaces of microorganisms and that they are not transported into the cytoplasm of bacteria, algae, or yeasts. Generally, the teichoic acid polymers in gram-positive bacteria definitely confer a strong negative charge on the surface of the cell wall because of their high content of ionized phosphate groups, although little if any teichoic acid is found in gram-negative bacteria. Between the binding of metal and cell wall strongly depends on teichoic acid in *Bacillus subtilis* and *Lactobacillus buchneri*. Therefore, it is tentatively considered that the chelate formation between the cell surface of gram-positive bacteria and metal ion becomes stronger than that of gram-negative bacteria and metal ion. Consequently, it is reasonable the amount of metal accumulated by gram positive bacteria is larger than that of gram-negative bacteria. Gram-positive bacteria are expected to have different REEs accumulating features from a mixed solution containing REEs **[9]**. The term 'Bioaccumulation' has been proposed for the sequestering of metal ions by metabolically mediated processes (living microorganisms), and the term 'biosorption' for the sequestering by non - metabolically mediated process **[10]**. The two processes can coexist and can also function independently as, for example, in the case where a consortium of microorganisms is exposed to metal bearing solutions.

MATERIALS AND METHODS

Sample Collection

The Bulk beach-placer samples (R1 & R2) were collected from the beach washings of Panambur Beach, Mangalore, Karnataka, along the South West coast of India. The samples were collected during December of a calendar year. The samples were collected in sterile polythene cover and brought to the laboratory for analysis.

Chemical composition of soil samples

The major elements in the collected soil sample were analyzed by gravimetric method (Soluble) and the other elements (insoluble) were measured using Wet chemical analysis **[11]**. The soil samples were first converted to a solid by precipitation with an appropriate reagent. (2N HNO_3). The compound was precipitated by the reagent that yields a sparingly soluble product that has a known composition or can be converted to such a substance. The precipitate was then collected by filtration, washed, dried to remove traces of moisture from the solution, and weighed. The amount of analyte in the original sample can then be calculated from the mass of the precipitate and its chemical composition. Soils having larger particles are usually heavier in weight per unit volume than those having smaller particles. True density of a soil is based on the individual densities of soil constituents and according to their proportionate contribution. The bulk density or apparent density is the weight per unit volume of dry soil as a whole i.e. particle and pore space and hence it is lower than the true density. The relationship between the true density (T) and the apparent density (A) and the pore space (P) is as follows:

$$P\% = (T-A) \times 100/T$$

Acid digestion and REE analysis of soil samples

The soil was air-dried at room temperature and sieved through a 2-mm sieve to remove large rocks, roots, and other large particles. The dry soil was then ground and passed through l-mm sieve. The known weight of collected soil samples was acid digested with 3:1 ratio of HCl and HNO_3 respectively, and volume was made up to 10 ml with distilled water **[12]**. The digested soil samples were analyzed for the evaluation of REEs concentration using Inductively Coupled Plasma-Mass Spectrometry (Perkin Elmer Sciex ELAN DRC II.ICP-MS). (Courtesy –Indian Institute of Technology; IIT, Chennai).

Serial dilution and Total viable bacterial count

The samples were serially diluted using 9 ml of sterile saline to reduce the number of viable cells. 100 µl of aliquot was pipetted onto nutrient plate and evenly spread and they were incubated at 37 °C for overnight. Plates with grown colonies were taken and the isolated colonies were picked and re inoculated in 50 ml of sterile LB broth and glycerol stock was prepared for these cultures. The soil samples were serially diluted using 9ml sterile saline. And total viable bacterial counts were enumerated by pour plate method technique, using the Nutrient agar medium. One ml aliquot of appropriate dilution was pipetted out in to the sterile petri plates and 20 ml of Nutrient agar, was added into each petri plate. The sample was mixed thoroughly by rotating the plate clockwise and anti -clockwise direction and allowed to solidify. Then the inoculated plates were incubated at 37°C. Duplicate plates were also maintained. The bacterial counts were made after 24-48 hours incubation. Petri plates with 30-300 colonies were selected and the total viable bacterial counts were made. The bacterial population was expressed as number of colony forming units (CFU) per ml of soil sample and (CFU) per ml of water sample.

Morphological and biochemical characterization

After noting the colony morphology along with colour, Pigmentation, shape, consistency etc., the selected pure colonies were sub cultured in Nutrient agar slants. Sub cultures of bacterial strains were made once in 30 days to keep the bacterial strain viable. The characterization study of the bacterial isolates was performed following Bergey's Manual of Determinative Bacteriology. Bacterial cultures were subjected to microscopic and various physiological tests. The isolated bacterial cultures were identified up to genus level by their morphological and biochemical characterization viz. Gram staining, motility, IMViC, Urease, Catalase, Nitrate reduction test etc., **[13]**.

Molecular Identification

Genomic DNA isolation and PCR amplification, cloning and sequencing of 16S rRNA genes

Bacterial isolates were sub-cultured in Luria Bertani broth and genomic DNA was isolated by employing Lysozyme, SDS and Phenol- Chloroform method followed by Wawer and Muyzer 1995 **[14]**. 16S rRNA genes of the bacterial isolates were amplified with genomic DNA isolates as template and 8F and 1490R primers **[15]** in the following composition and amplification cycle. Each reaction mixture contained 2 µl of template DNA (100 ng), 0.5 µM of two primers, and 25 µl of Enzyme Master Mix (Bioron). The PCR program consisted of an initial denaturation step at 94°C for 5 min, followed by 30 cycles of DNA denaturation at 92°C for 30 sec, primer annealing at 50°C for 1 min, and primer extension at 72°C for 2 min was carried out in Thermal Cycler (Thermo Hybaid). After the last cycle, a final extension at 72°C for 20 min was added. The PCR products were purified by QIAquick PCR purification kit as described by the manufacturer and cloned using QIAGEN PCR cloning plus kit as described by the manufacturer. Clones were selected and isolated plasmids with insert were sequenced with M13 Sequencing Primers using ABI Biosystems automated sequencer.

Sequence analysis

Nucleotide database was searched with the sequences obtained with NCBI BLAST (Blastn) tool (http://www.ncbi.nlm.nih.gov/BLAST) **[16]**. Multiple sequence analysis was carried out using CLUSTALW **[17]**. A bootstrap analysis was performed to validate the reproducibility of the branching pattern.

Bioaccumulation study of rare earth elements

FTIR Analysis

Sterile nutrient broth was prepared in 500ml conical flask and 1g of sterile soil sample (R1) was added to all the conical flasks. One of the conical flasks was kept as control where the other was inoculated with bacterial culture *(Bacillus aerius*) isolated. All the flasks were maintained at 37 °C and incubated for five days. Then the broth with the bacterial culture and control were taken and centrifuged at 10,000 rpm for 10 minutes, to pellet out. All the pelleted samples were analyzed by FTIR spectrum for knowing the physiological changes in the above samples and characterization was done by employing model NDXUS-672 model. The spectrum was taken in a mixed IR 400 -4000 cm-1 with 16 scan speed and was recorded using ATR (Attended Total Reflectometer) **[18]**.

ICP -OES Analysis

Sterile 100 ml of LB broths were prepared in 250 ml conical flasks. To each of these broths sterile 1 g of rare earth soil (R1) was added and the bacterial isolate *(Bacillus aerius*) was inoculated. Separate controls were maintained for rare earth soil and bacterial isolates. All the flasks were incubated at 37 °C for overnight. The broth with bacterial culture was taken and was subjected to centrifugation. The pellet containing bacterial cells was dried and processed for ICP-OES analysis. The bacterial samples were digested with the mixture of 3:1 HCl: HNO_3 in a 50-ml beaker. The digested solution was evaporated to near dryness. The residue was dissolved with dilute HCl and transferred into a 10 ml flask, then diluted to volume with deionized water. REE concentrations in the samples were determined by ICP-OES **[19]**.

RESULTS AND DISCUSSION

The bacterial colonies were isolated and enumerated in the soil samples of obtained from Panambur beach washings, Mangalore. It was found to be 126 $X10^5$ and 114X 10^5 colony forming units (CFU) respectively in per gram of soil (R1 & R2) shown in **Table 1**. This shows the microbial load and the presence of microbial flora in Panambur soil.

Soil as predominantly made up of Silica (SiO_2 90.12% & 87.04%) respectively in the soil samples. Second significant mineral was Aluminium (Al_2O_3) with 2.02% &4.10%. The third significant mineral found most in the soil sample Iron Fe_2O-1.16% & 3.02%). The other minerals that were found by gravimetric analysis of the soil were Magnesium, Calcium Potassium and Phosphorous in negligible quantities. Plant nutrients like Ca, Mg, K, Na, P and S are present in the minerals and in the soil solution. O_2, Si, and Al occur as constituents of minerals and as oxides In the present study the Soil samples collected at four different locations reveal a classical chemical composition of soil containing oxides **(Table 2)**.

The soil samples were processed for ICP-MS analysis. Among the 12 lanthanides analyzed in the soil sample **Cerium, Samarium** were found to be abundant in different proportions in both the soil samples (R1& R2) given in **Table 3**. Cerium and Samarium constituted the greatest fraction with highest concentrations. Nevertheless, concentrations of other rare earth elements were found in very small quantities. In most soils, rare earths Ce, Nd, Sm and Sc were consistent of the order of mg/kg.

Five different organisms were isolated and their morphological and colony characteristics were analysed. Most of the isolates were inferred as Gram positive and related species. Biochemical characteristics of the isolates were found to be rod shaped Gram positive and negative, catalase positive, sporulating and non sporulating species. Out of which one Gram positive organism was selected for the accumulation studies.

The isolate was Gram-positive bacilli having sub-terminal spores. The PCR products were purified and loaded on agarose gel and band without any primer band was observed. They were sent for sequencing. The sequence showed similarity with the BLAST analysis **(Table 4)** of the sequences showing the similarity of the sequences of 16S rRNA gene sequences of the database and the bacterial species were identified with the same. The phylogenetic tree **(Figure 1)** show the relationship between the 16S rRNA sequences of firmicutes and gamma proteobacteria sequences. The phylogenetic analysis showed the relationship between the 16S rRNA sequences of bacterial samples and the boot strap analysis (90 % and 95 %) validated the tree with 0.5 nucleotide change per position. The branching patterns having a dominant clad containing *B. cereus* was noticed in the phylogenetic tree. Based on these characteristics and sequence analysis, the isolate was identified as *Bacillus aerius.*

Infrared spectroscopy revealed that rare earth elements induce the bacteria for the production of excess carboxylic acid **(Figure 2)**. Most of the soil bacteria convert the insoluble REEs in soil into organic compounds such as carboxylic acids and carry out accumulation in the form of soluble complexes. . In the test system when compared with control system, strong peaks of –OH and –C=C and carbonyl peak C=O were observed in cultures and elements system. In the control C=O carbonyl peaks were not observed. In the test system -OH and –NH intense peaks were observed in cultures with elements **(Table 5)**.

Based on earlier ICP-MS analysis seven elements were considered for further bacterial accumulation studies viz., Pr, Sm, Ce, Eu, Gd, Nd and Y. The accumulation of REEs in the bacterial isolate was carried out by ICP-OES after acid digestion of cell pellets. Significant levels of accumulation of Samarium & Cerium 20.41 and 8.10 mg/L (µmol /gm of dry weight of cells) respectively were observed with the isolate **(Table 6) w**hereas very less amount of other five elements were found to be accumulated in the isolate. Concentrations of other elements in the isolate were found to be below the detectable limit.

It has been shown that Lanthanum, Europium and Terbium were accumulated during growth, between inner and outer membrane of the cell envelope (periplasmic space) of *Escherichia coli* **[20]**, 1991). Biosorption encompasses the uptake of metals by the whole biomass (living or dead) through physico-chemical mechanism such as adsorption, ion exchange or surface precipitation. The process takes place on the cell wall with rapid kinetics.

The distribution of *Klebsiella* sp. and *Bacillus* sp were noticed in Goa sediment and reported as phosphate solubilizers by Desouza in 2000 **[21]**. These species were also reported in Chavara Waters. It can be assumed that alkaline phosphatase production and ability to solubilize inorganic phosphate may be due to the above microbes in phosphorites sediment. The influence of cations ($A1^{3}$+, Ca^{2-}, Na, K) and anions ($N0_3^-$, $S0_3^{2-}$, Cl) in the solution on the biosorption performance has been studied. Aluminium was noticed as more inhibitive ion for the fixation of rare earth element namely Europium, Lanthanum and Ytterbium.

The industrial use of low cost biosorbents like microorganism has been of increasing interest in environmental remediation. The optimization of the biosorption conditions, the location of rare earth element binding sites and the studies of the sorption capacities of immobilized cells are good argument for using biosorption in the industrial removal of heavy metal from solutions. *Staphylococcus* sp*.*, *Staphylococcus epidermidis, Pseudomonas aeroginosa* were used for the bio adhesion to Zirconium **[22]**. They demonstrated that these three bacteria preferred the zirconium than SS and suggested that the adsorption depends upon the surface of the material.

In the present study the influence of rare earth elements on bacterial reveals that rare earth enhances the production of acid, and aromatic nuclei which can be noticed in FTIR spectrum. It can be assumed that rare earth Ziercem and Ilmnate induces the bacteria for the production of carboxylic acid. Scientists in 2004 **[23]** also noticed promotion of indole alkaloid production in *Catheranthus roseus* cell cultures by rare earth elements.

Hence, the present investigation suggests that the interaction between bacteria and rare earth elements are needed for future technology development in agriculture. On the other hand, literature information on the environmental effects, bioaccumulation and bioavailability of REEs has received scant attention until very recent years. Starting from the late 1990s, increasing effort has been directed to the environmental effects of REEs, and some of the recent reports include the study of REE accumulation in plant **[24]**. Hence as preliminary study, bacteria were enumerated and identified in the soil sediment. Infrared spectroscopy revealed that rare earth elements induce the bacteria for the production of excess carboxylic acid and it is expected that the production of carboxylic acid chelate the available phosphorites in rocks as $P0_4$ and supplies to the plant, which will be useful for future research for developing countries like India and also that the base line data will be useful to investigate on the development of manure from rare earth environment.

Table 1: Enumeration of Bacteria in the soil samples

S. No.	Samples	No. of CFU/ml in 10^{-4} dilution
1	R1	126×10^5
2	R2	114×10^5

Table 2: Chemical composition of Soil

Soil sample	Chemical composition in %								
	SiO_2	Al_2O_3	Fe_2O	MnO	CaO	MgO	K_2O	Na_2O	P_2O_5
R1	90.12	2.02	1.16	-	0.22	0.65	0.14	0.02	0.07
R2	87.04	4.10	3.02	-	0.94	0.68	0.13	0.01	0.09

Plate 1: Gram Positive (Purple coloured) rod shaped microorganism

Table 3: Rare Earth Element Concentration in the collected soil samples as Measured on ICP-MS

S. No.	Analyte	Type of REE	Mass	R1 Conc. Mean (ppm)	R2 Conc. Mean (ppm
1.	Pr	Light	141	51.22	41.82
2.	Yb	Heavy	172	11.14	11.01
3.	Eu	Heavy	151	0.37	0.24
4.	Ce	Light	140	571.21	418.20
5.	Nd	Light	146	31.10	22.13
6.	Sm	Light	147	296.12	222.04
7.	Gd	Heavy	157	12.00	11.75
8.	Tb	Heavy	159	0.00	0.00
9.	Dy	Heavy	163	11.19	14.01
10.	Er	Heavy	166	16.25	17.42
11.	Tm	Heavy	169	3.50	2.82
12.	Ho	Heavy	165	1.20	1.52

Table 4: Isolate identified with BLAST analysis

S. No.	Sample Name	BLAST Results	GenBank Accession Number
1.	R2	***Bacillus aerius***	MT043812

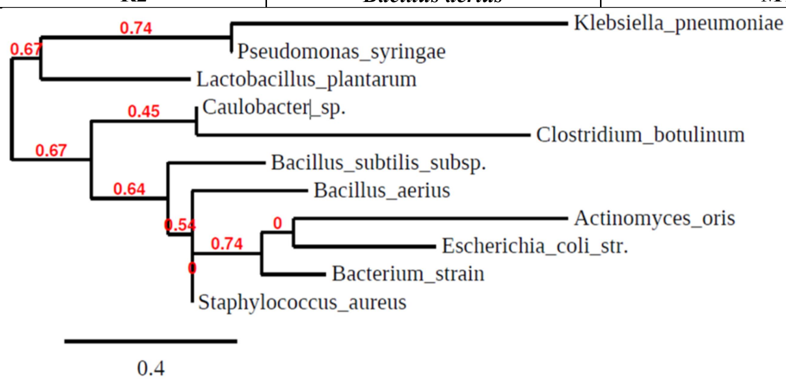
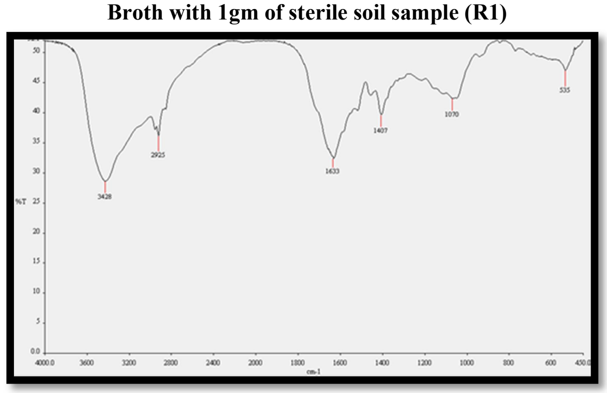


Figure 1: Phylogenetic Analysis of the *Bacillus aerius*

Neighbor-joining tree based on 16S rRNA gene sequences, showing phylogenetic relationships between sequences of the Phylum Firmicutes. *Clostridium botulinum* was used as the out group sequence

<u>Control system</u>

Broth with 1gm of sterile soil sample (R1)



<u>Test system</u>

Broth with 1gm of srterile soil sample (R1) inoculated with *Bacillus aerius*

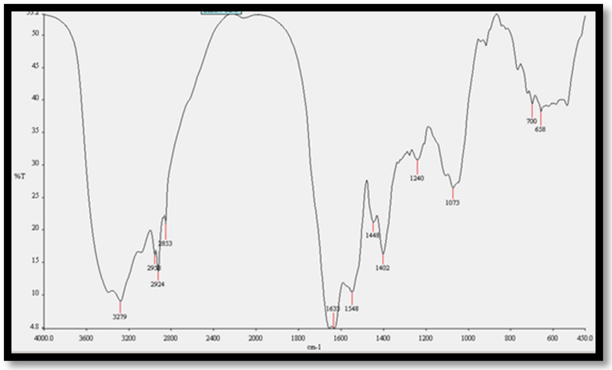


Figure 2: FTIR Peaks

Table 5: FTIR-Analysis (Accumulation of REEs by *Bacillus aerius*)

S. No.	Contents of sample	FTIR Spectrum Peaks Observed
1	Soil sample without bacterial isolate	No significant peaks observed1070-1630cm^{-1}
2	Bacterial isolate (*Bacillus aerius*)with soil sample	Presence of C=C bond, 1548-1633cm-^{1}
		Strong OH bonds, presence of C≡C1402-1448cm^{-1}
		Weak N-H, strong CH3, CH2 and CH2 or 3 bands1448-1548cm^{-1}

Table 6: ICP OES analysis of the isolate with soil sample (R1)

S. No.	Analyte	Mass	Concentration
1	Pr	390.844	BDL
2	Sm	359.260	20.41mg/L
3	Ce	418.764	8.101mg/L
4	Eu	381.967	BDL
5	Gd	342.247	BDL
6	Nd	406.109	BDL
7	Y	371.029	BDL

BDL-BELOW DETECTABLE LIMIT (Pr- < 0.0370 mg/L , Eu- < 0.0027 mg/L,Gd-< 0.0140 mg/L,Nd- < 0.0960 mg/L,Y - < 0.0035 mg/L)

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