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ORIGINAL ARTICLE

Bioaccumulation of cerium and neodymium by *Bacillus cereus* isolated from rare earth environments of Chavara and Manavalakurichi, India

E. S. Challaraj Emmanuel · V. Vignesh · B. Anandkumar · S. Maruthamuthu

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Abstract Rare earth elements (REEs) are among the common minerals in the Rare earth environment that are very precious and also enhance soil properties. The aim of this present study is to evaluate the accumulation of REEs by bacterial isolates of rare earth environment. Morphological and biochemical characterization were done for 37 bacterial isolates and also molecular studies were carried out using 16S rRNA sequencing method. The assessment of REEs composition in soil samples of Chavara and Manavalakurichi analyzed using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) showed the abundance of Cerium and Neodymium among lanthanides. The bioaccumulation study of rare earth elements by *Bacillus cereus* were accomplished employing FT-IR spectrum and ICP-OES analysis. The significant accumulation of rare earth elements especially Cerium and Neodymium was noticed in *Bacillus cereus* isolated from rare earth environment.

Keywords Rare earth elements · Bioaccumulation · FT-IR and ICP-OES

E. S. Challaraj Emmanuel (✉) · V. Vignesh
Department of Microbiology, Sourashtra College, Pasumalai, Madurai 625004, India
e-mail: emmyesc@yahoo.com

B. Anandkumar
Corrosion Science and Technology Division, Indira Gandhi Centre for Atomic Research, Kalpakkam 603102, India

S. Maruthamuthu
Corrosion Protection Division, Central Electro Chemical Research Institute, Karaikudi 630001, India

The Indian Rare Earths (IRE) has processing plants at Manavalakurichi, (Kanyakumari district, Tamil Nadu), Chavara, (Kollam district, Kerala) and Orissa Sand Complex (OSCOM, Matikhalo, Ganjan district, Orissa). In India, IRE, a Government of India Undertaking under the administrative control of Department of Atomic Energy and Kerala Minerals and Metals Ltd. (KMML), a Kerala State Government Undertaking are actively engaged in mining and processing of beach sand minerals from placer deposits. Several REEs (Rare Earth Elements) are not very "rare" and accurately dispersed in a variety of forms, especially as accessory minerals in granites, pegmatites, gneisses and related common types of rocks [1]. Chavara (Kerala State, India), having depth varying from 1 to 10 m Zircon, constituting 9.2% of the beach sands with 65% ZrO_2 and 33% SiO_2. Manavalakurichi (MK)-25 km north of Kanyakumari, the southernmost tip of the India, annually produces about 90,000 tonnes (t) of ilmenite of 55% TiO_2 grade, 3500 t of rutile and 10,000 t of zircon in addition to 3000 t of monazite and 10,000 t of garnet [2].

Variovorax paradoxus and *Comamonas acidovorans* could particularly adsorb light rare earth elements such as La, Ce, and Pr but not heavy rare earth elements such as Tm, Yb and Lu [3]. The amount of rare earth elements accumulated by Gram positive bacteria *Micrococcus luteus* and *Arthrobacter nictinae* are much higher than those by Gram negative bacteria, fungi and yeasts. These bacteria have high ability to accumulate thorium ions and uranyl ions. Adsorption of several actinide and lanthanide ions by *Mycobacterium smegmatis* take place by the partial release of magnesium from cell wall indicating exchange reactions occurred at magnesium binding sites [4]. There are at least two binding sites on Gram positive and Gram negative bacterial cell surface; they are carboxylate and phosphate group binding sites.

No studies have been reported on microbial analysis of rare earth environment and bioaccumulation of rare earth elements by bacterial species in India. Hence in the present study, soil samples from rare earth environment of Chavara and Manavalakurichi have been selected for microbial analysis. Partially processed rare earth soil samples from Manavalakurichi were taken for evaluation of rare earth elements and accumulation of the same by the bacterial isolates.

Materials and Methods

Sample Collection

The soil samples (SOC and SOM) were collected from rare earth environment of Chavara and Manavalakurichi. The samples were rich in rare earth elements and also enriched with numerous microorganisms. Previously the samples were collected in sterile containers and brought to the laboratory in an icebox to avoid microbial contamination and proliferation during transport.

Isolation and Biochemical Characterization

The samples were serially diluted using 9 ml sterile saline and total viable bacterial counts were enumerated by pour plate method technique, using the Nutrient agar medium. Triplicate plates were also maintained. Morphologically dissimilar and well-isolated colonies were randomly selected and streaked onto the Nutrient agar medium to obtain pure cultures. After noting the colony morphology along with color, 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 bacterial strains isolated from soil samples were identified up to generic level by employing the standard morphological and biochemical characteristics described in Bergey's Manual of Systemic Bacteriology [5].

Molecular Identification

Genomic DNA Isolation

Bacterial isolates were sub-cultured in Luria–Bertani broth and genomic DNA was isolated by employing Lysozyme, SDS and Phenol–Chloroform method [6].

PCR Amplification

16S rRNA genes of the bacterial isolates were amplified with genomic DNA isolates as template and 8F and 1490R primers [7] in the following composition; 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 amplification 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 s, 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). A final extension at 72°C for 20 min was included after the last cycle.

Cloning, Sequencing and Sequence analysis

The PCR products were purified by QIAquick PCR purification kit 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.

Phylogenetic Analysis of the Isolates

The sequences obtained were analyzed with BLAST search version 2.2.20 [8] and tools of Ribosomal Database Project II Release 10 (http://rdp.cme.msu.edu) for taxonomic hierarchy of the sequences. Multiple sequence alignments were performed using CLUSTAL X2 [9] with a collection of taxonomically related sequences obtained from National Center for Biotechnology Information (NCBI) Taxonomy Homepage (http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/) and Ribosomal Database Project-II Release 10 (http://rdp.cme.msu.edu). Phylogenetic and similitude analyses were done with the common 16S rRNA gene regions and all alignment gaps were treated as missing data. The paired similitude and pairwise distance calculations using the transversion/transition weighting (R = s/v) and the Kimura-2-parameter model [10] were performed with the MEGA version 4.1 program [11]. The phylogenetic trees were constructed (neighbor-joining method) and 1000 bootstrap replications were carried out to validate internal branches [12].

ICP-MS Analysis of Soils

An adopted method [13] was used for the analysis of REEs in the soil samples. The known weight of collected processed sterile rare earth soil samples from Manavalakurichi (SOM1 and SOM2) were acid digested with 3:1 ratio of HCl and HNO_3 respectively. It was made up to 10 ml with distilled water. Digested soil samples were analyzed for the evaluation of REEs concentration using ICP-MS (Perkin Elmer Sciex ELAN DRC II).

Bioaccumulation Studies

FTIR Analysis

Sterile nutrient broth was prepared in 500 ml conical flask and 1 g of processed sterile rare earth soils (SOM1) was added to all the conical flasks. One of the conical flasks was kept as control where the other two were inoculated with 10^6 cells of *Bacillus cereus* (EU693500) cultures isolated and bacterial isolates with soil samples. All the flasks were incubated without shaking at 37°C overnight. After incubation the control and broths with bacterial cultures were centrifuged to 5,000 rpm for 10 min. All the air dried pellet 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).

ICP OES Analysis

The presence of REEs was measured in bacterial cultures after acid digesting the cell pellet [14]. Accumulation of REEs (Cerium and Neodymium) in bacterial cell samples was analyzed with rare earth soil (SOM1) using ICP-OES (Optima 5300 DV).

The 16S rRNA sequences of the bacterial isolates in the present study were deposited in Genbank under accession numbers EU693495–EU693521, GQ243727–GQ243736.

Results and Discussion

The bacterial colonies were enumerated in the soil samples of Chavara ($126 \pm 2 \times 10^5$) and Manavalakurichi ($87 \pm 1 \times 10^5$) as colony forming units (CFU) per gram of soil. This shows the microbial load with standard deviations and the presence of microbial flora in Chavara soil (SOC) was higher than Malavalakurichi soil (SOM).

Most of the isolates were inferred as Gram positive and related species except SM3, SM10, SM14 and SM16. Biochemical characteristics (data not given) of the isolates were found to be rod shaped Gram positive and negative, catalase positive, sporulating and non sporulating species. The biochemical characters show similarity with the BLAST analysis (Table 1) 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 trees (Figs. 1, 2) show the relationship between the 16S rRNA sequences of firmicutes and gamma proteobacteria related sequences. The branching patterns having a dominant clad containing *B. cereus* was noticed in the phylogenetic tree.

Among the 37 bacterial species identified in the rare earth environments, *Bacillus cereus* was found to be predominant (8 isolates) and the accumulation studies were carried using the same (EU693500).

The soil samples were processed for ICP-MS analysis. Among the 12 lanthanides analyzed Cerium, Neodymium,

Table 1 Organisms identified with BLAST analysis. The dominant *Bacillus cereus* among the other isolates in the soil samples have been given in *bold* face letters

Isolate name	BLAST results	GenBank accession number
SC1	*Bacillus fusiformis*	EU693495
SC2	*Lysinibacillus* sp.	EU693496
SC3	*Bacillus flexus*	EU693497
SC4	*Bacillus megaterium*	EU693498
SC5	*Lysinibacillus boronitolerans*	EU693499
SC6	***Bacillus cereus***	EU693500
SC7	*Bacillus thuringenesis*	EU693501
SC8	*Bacillus* sp.	EU693502
SC9	*Exiguobacterium* sp.	EU693503
SC10	*Lysinibacillus sphaericus*	EU693504
SC11	***Bacillus cereus***	EU693505
SC12	*Bacillus pumilus*	EU693506
SC13	***Bacillus cereus***	EU693507
SC14	*Bacillus macroides*	EU693508
SC15	*Bacillus subtilis*	EU693509
SC16	*Bacillus firmus*	EU693510
SC17	***Bacillus cereus***	EU693511
SC18	*Bacillus licheniformis*	EU693512
SC19	*Bacillus* sp.	EU693513
SC20	*Brevibacillus brevis*	EU693514
SM1	*Bacillus sphaericus*	EU693515
SM2	*Brevibacillus brevis*	EU693516
SM3	*Pseudomonas* sp.	EU693517
SM4	***Bacillus cereus***	EU693518
SM5	*Bacillus* sp.	EU693519
SM6	***Bacillus cereus***	EU693520
SM7	*Lysinibacillus sphaericus*	EU693521
SM8	*Bacillus subtilis*	GQ243727
SM9	*Lysinibacillus fusiformis*	GQ243728
SM10	*Klebsiella* sp.	GQ243729
SM11	*Lysinibacillus* sp.	GQ243730
SM12	***Bacillus cereus***	GQ243731
SM13	*Paenibacillus* sp.	GQ243732
SM14	*Pseudomonas* sp.	GQ243733
SM15	***Bacillus cereus***	GQ243734
SM16	*Pseudomonas* sp.	GQ243735
SM17	*Bacillus* sp.	GQ243736

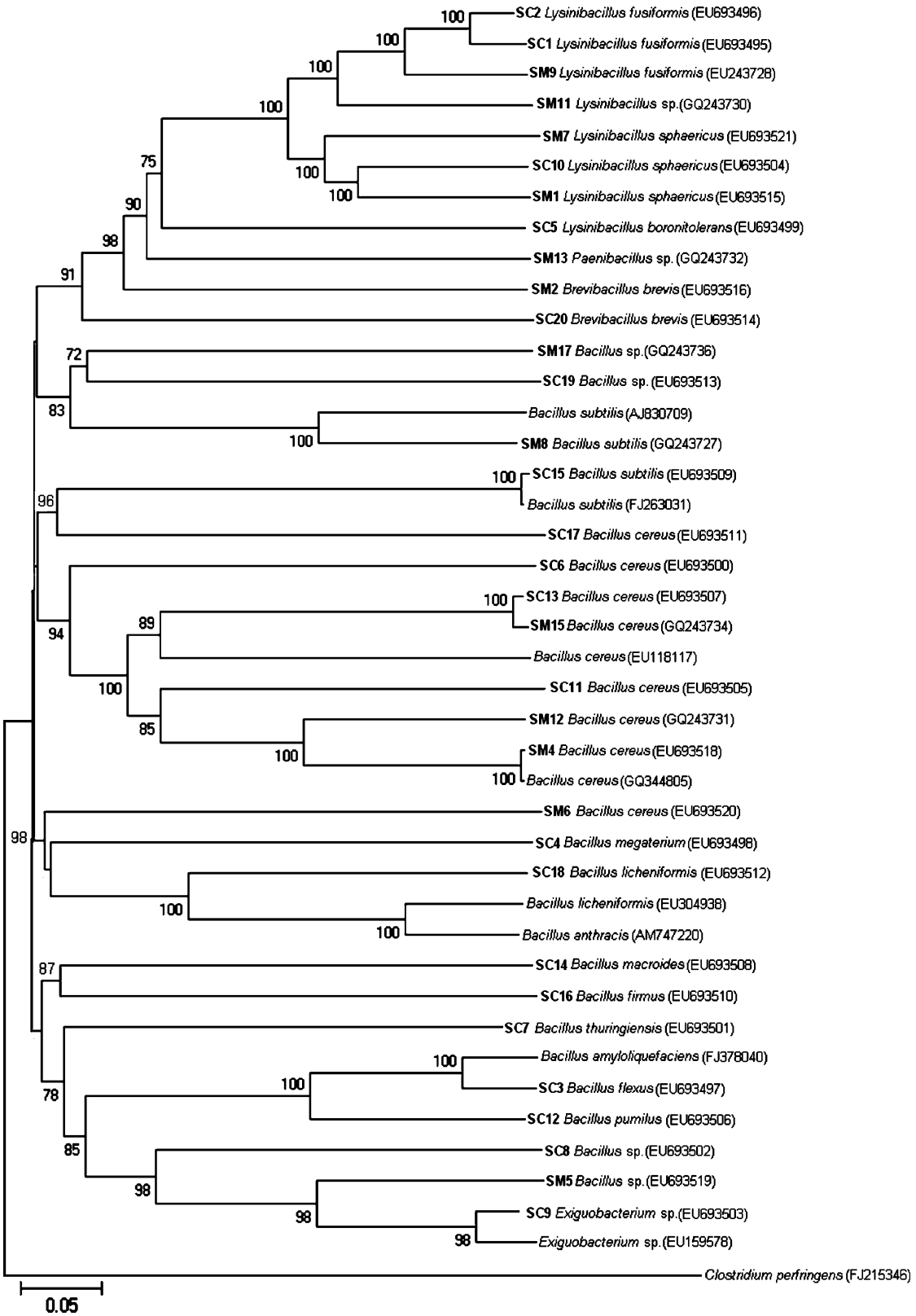

Fig. 1 Neighbor-joining tree based on 16S rRNA gene sequences, showing phylogenetic relationships between sequences of the Phylum Firmicutes. *Clostridium perfringens* was used as the out group sequence. Numbers at nodes indicate bootstrap values >50% from 1,000 replicates. *Bold face* numbers indicate isolate names of the present study. GenBank accession numbers are given in *parentheses*. The *scale bar* indicates sequence divergence

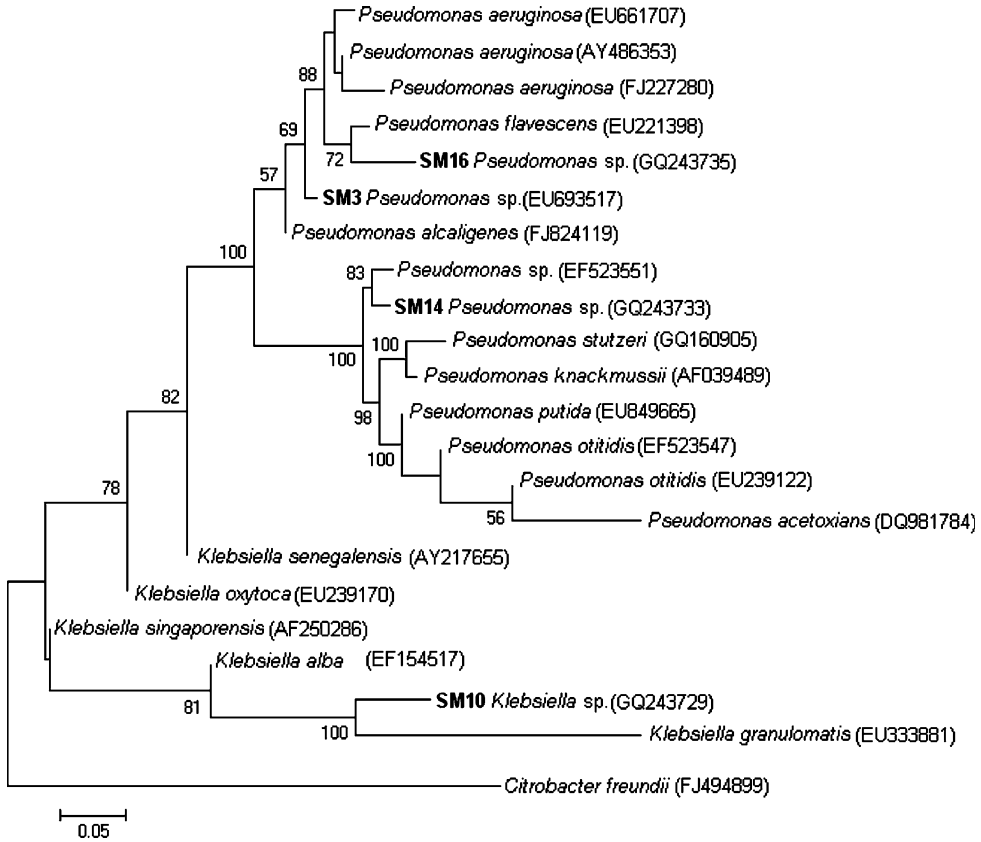

Fig. 2 Neighbor-joining tree based on 16S rRNA gene sequences, showing phylogenetic relationships between sequences of the Phylum Gamma Proteobacteria. *Citrobacter freundii* was used as the out group sequence. Numbers at nodes indicate bootstrap values >50% from 1,000 replicates. *Bold face* numbers indicate isolate names of the present study. GenBank accession numbers are given in *parentheses*. The *scale bar* indicates sequence divergence

Table 2 ICP MS for SOM 1 and SOM 2. The presence of Cerium and Neodymium more than 200 ppm among other rare earth elements have been highlighted in *bold* face

Analyte	Mass	Conc. Mean (ppm)	
		SOM1	SOM2
Pr	141	55.885 ± 0.002	36.493 ± 0.002
Yb	172	23.726 ± 0.001	02.820 ± 0.002
Eu	151	00.330 ± 0.002	01.019 ± 0.001
Ce	140	**478.219 ± 0.002**	**296.586 ± 0.002**
Nd	146	**201.618 ± 0.002**	**127.609 ± 0.001**
Sm	147	43.117 ± 0.001	21.223 ± 0.002
Gd	157	40.810 ± 0.002	14.152 ± 0.001
Tb	159	00.003 ± 0.002	01.484 ± 0.001
Dy	163	41.723 ± 0.002	05.766 ± 0.002
Er	166	20.368 ± 0.001	02.581 ± 0.002
Tm	169	04.543 ± 0.002	00.510 ± 0.001
Ho	165	10.803 ± 0.002	01.305 ± 0.002

Samarium and Gadolinium are present in higher proportions in both soil samples. When compared to soil sample SOM1, the sample SOM2 contains three fold increase in the element composition given in Table 2.

In FTIR analysis of the pellets of the system with rare earth soil SOM1 and SOM2, O–H of carboxyl stretch (3633 cm^{-1}), 1,240 and 1,077 cm^{-1} attribute to the phosphate peak, chelate compounds at 2628 cm^{-1}, peak at 1631 and 1591 cm^{-1} indicates C=C conjugated diene. A peak at 1442, 1409 cm^{-1} indicates the presence of aromatic nuclei (Carboxylic acid) while C=C stretch for C–O–C group has been noticed at 1353 cm^{-1} in the system with the culture *Bacillus cereus* (Fig. 3). The spectral analysis of the biomass reveals the major role of the carboxyl and phosphate groups in REEs binding by the bacterial biomass.

REEs are comprising of Light REEs (LREEs) and Heavy REEs (HREEs) including all the lanthanides and actinides [15]. The communication [16] also reported the presence of Ce and Nd (LREEs) in monazite sample was comparatively higher than other REEs in the samples of both Chavara and Manavalakurichi. Hence in the present study, Ce and Nd were considered for their accumulation by bacteria. Based on earlier ICP-MS analysis, four elements were considered for further bacterial accumulation studies viz., Cerium, Neodymium, Samarium and Gadolinium (Table 3). The accumulation in bacterial isolate with element SOM1 shows increased levels of Cerium and Neodymium (3.02 and 1.40 μmol/g of dry weight of cells) whereas the concentration of other two elements namely Samarium and Gadolinium was below the detectable limit. The accumulation in bacterial isolate with element SOM2 also shows increased levels of Cerium, Neodymium and Samarium (7.05, 3.17 and 0.10 μmol/g of dry weight of

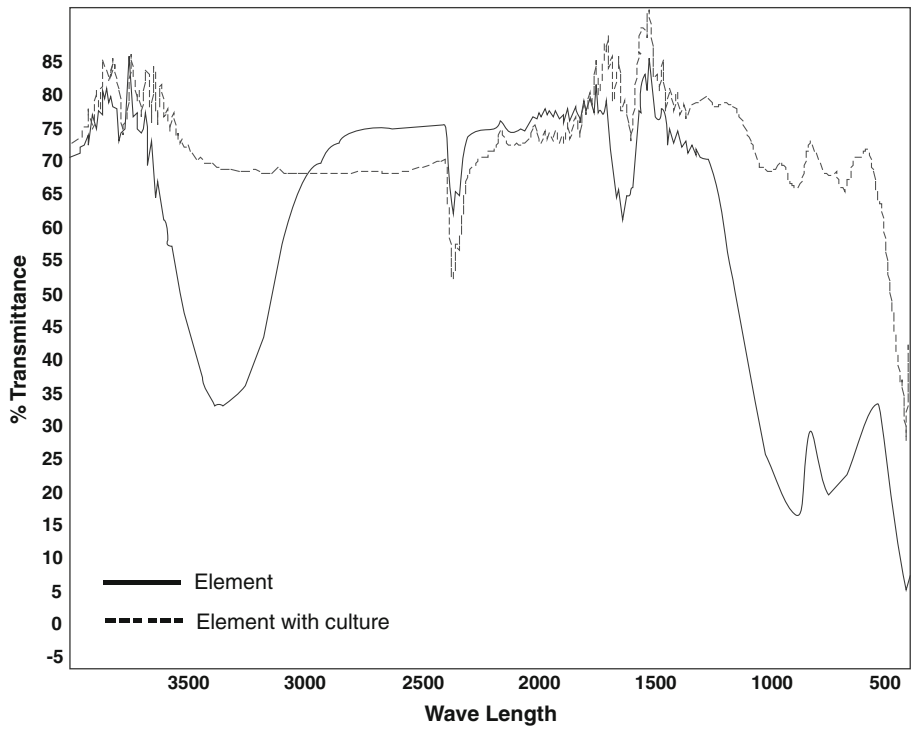

Fig. 3 FTIR analysis (Element control and with cultures {*Bacillus cereus*})

Table 3 ICP OES for SOM 1 and SOM 2

Sample composition	Observation (μmol/g of dry weight of cells)			
	Ce	Nd	Sm	Gd
1% SOM 1 + *Bacillus cereus*	3.02 ± 0.02	1.40 ± 0.01	BDL	BDL
1% SOM 2 + *Bacillus cereus*	7.05 ± 0.01	3.17 ± 0.01	0.10 ± 0.01	BDL

BDL below detectable limit (Ce <0.0480 mg/L and Nd <0.0960 mg/L)

cells) whereas the concentration of the other element Gadolinium was below the detectable limit.

Though accumulation of LREEs by Gram negative bacterial species have been reported [3], the accumulation by Gram positive species in the present study has been observed. Initial concentration of REEs in soil had been taken account for the accumulation studies with *B. cereus*. 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* [17]. On the other hand, they may influence the environment by producing mineral acids, chelating agents such as siderophores, or by-products of the metabolism (organic acids etc.). For example, the interaction between a mycobacterial siderophore (mycobactin) and Europium [18] ions have been shown by or Spectrophotometric approach. Moreover, some siderophores such as ferrioxamine B could deplete europium fixation by goethite or boehmite [18]. 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.

REE toxicity, has been reported that Cerium could be a potent inhibitor for Gram-negative bacteria and fungi [19] supporting that Gram Positive strains show higher absorption towards cerium. Moreover, some Lanthanide ions are produced in nuclear fission and could be dispersed in the environment like^{140} La or^{141} Ce/^{143}Ce in the case of the Chernobyl accident in 1986 [18] FTIR and ICP-OES analysis showed the accumulation of REEs by *B. cereus*.

In India, there is no work available on the interaction between biology and rare earth elements. The distribution of *Klebsiella* sp. and *Bacillus* sp. were noticed in Goa sediment and reported as phosphate solubilizers [20]. These species were also reported in Manavalakkurichi 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 (Al^{3+}, Ca^{2+}, Na^{+}, K^{+}) and anions ($N0^{3-}$, Cl^{-}) in the solution on the biosorption performance has been studied [18].

Accumulation of rare earth elements by *Bacillus* have been extensively studied [21]. 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 and they preferred the zirconium than others and suggested that the adsorption depends upon the surface of the material [22].

The interaction of rare earth elements between *Pseudomonas* sp. and organic ligands were noticed [23]. They noticed Eu(III) adsorbs on bacterial cells in the presence or organic ligands with low chelating ability. The fixation of heavy metal lanthanum by *Myxococcus xanthus* by extra Cellular polymeric substances was noticed as model of bacteria–lanthanide interactions [24].

FT-IR spectroscopy revealed strong involvement of cellular carboxyl and phosphate groups in lanthanum binding by the bacterial biomass of *Pseudomonas* sp. [25]. 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 has also been suggested [26] that carboxylate and phosphate sites are mainly responsible for the adsorption of cations onto *B. subtilis.* The strong absorption peaks confirm the presence of the carboxyl groups in the bacterial polysaccharide structure; after metal binding by the biomass, the significant variations in the peak positions these regions strongly support the involvement of the carboxyl groups in REEs sorption. It can be assumed that rare earth induces the bacteria for the production of carboxylic acid.

In conclusions, significant cerium accumulation by *Bacillus cereus* species was observed. It also reveals that accumulation by Gram positive organism shows that REE may be inhibitory to Gram negative bacterial strains. Thus most of the organisms isolated from Chavara and Manavalakurichi are Gram positive forms. The above study may give additional information on accumulation of rare earth element by microorganisms which have been reported only in few studies. The present study can be concluded that the production of carboxylic acids due to REEs accumulation in bacteria may enhance the fertility of soil. Therefore the accumulation of Nd and Ce in *B. cereus* may be due to the physico-chemical binding with the cell components which may be studied in the future.

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