Studies on the distribution of bacterial isolates in rare earth environment

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Abstract

Rare earth soil is precious, but very common across Arabian coast especially in Chavara, (Quilon district, Kerala) south west coast of India. They are widely distributed but usually occur in small amounts and enhance the soil properties. In the present study, 18 different bacterial isolates were identified from three different samples such as soil and biofilm formed on metal surfaces from the rare earth environment of Chavara using 16S rDNA gene sequencing. The accumulation of rare earth elements (REE) by microbes was studied using FT-IR analysis. In the FTIR spectrum of the test system, a peak at 1548 and 1449 cm^{-1} indicates the presence of aromatic nuclei (carboxylic acid), while C=C stretch for C-O-C group was noticed at 1237 cm^{-1}. Thus significant variations in the peak position confirm the presence of carboxyl group and thus it was confirmed that rare earth elements induce the bacteria to produce carboxylic acid and thereby accumulate rare earth elements.

Introduction

Rare earth elements (REE) are not only of interest because of their usefulness for industry but also because they have raised a discussion whether these might be essential for plants, animals and even humans (Richter and Schermanz,2006). The Chavara plant is based on the mineral deposit of 23 km. long stretch of land in the belt of Neendankara and Kayamkulam which is well known as the richest and the single largest ilmenite deposit in the country(Jeya *et. al*., 2008). The total amount of REE in soils usually is in the range of 108-480 mg g^{-1} with an average of 196 mg g^{-1} (Jeya *et. al*, 2008). It has been reported that the functioning of soil-plant systems can be highly affected by agents which suppress or adversely affect soil organisms or change the quality or quantity of organic matter either in the short or long-term (Tang *et al.,* 2004). The rare earth soils are used as manure in China, and studies on the distribution of bacteria and their affinity between rare earth facilitate our understanding of their association (Ozaki *et al.,* 2006). Some studies show that in the presence of rare earth soil, microorganism produces more organic compound, especially carboxylic acids than in their absence (Gu *et al*., 2001).Chinese studies show that rare earth soil has a significant effect on the growth of plants (Liang *et. al*., 2008). So far no study has been carried out on the relationship between rare earth environment and bacteria.

The bioavailability of REE depend largely on the physico chemical and biological characteristics of the soil. The objective of the present study was to analyze the bacterial distribution in the REE and

bioaccumulation of the REE by the bacterial isolates.

Materials and Methods

Sample collection: Mild steel plates and coal tar epoxy coated plates were immersed in rare earth pond and biofilm samples were scrapped by sterile knife and collected in sterile polythene bottles. Soil sample were also collected from the same site in sterile polythene bottle. The samples were brought to the Central Electro Chemical Research Institute Microbiology Laboratory in an icebox for the microbiological and molecular characterization of the bacterial isolates.

Total viable bacterial count: The soil samples were serially diluted using 9 ml sterile saline. The scrapping from plate samples were serially diluted using 9 ml sterile saline. Total viable bacterial counts were enumerated by pour plate method, using nutrient agar medium (Hi-Media, Mumbai, India) in 50% sea water. One ml aliquot of appropriate dilution was pipetted out in to the sterile petriplates and 20 ml of nutrient agar was added into each petriplate. The sample was mixed thoroughly by rotating the plate clockwise and anticlockwise direction and allowed to solidify. Then the inoculated plates were incubated at 37°C, duplicate plates were also maintained. Petriplates with 30-300 colonies were selected after 24 -48 hr incubation and the total viable bacterial counts were enumerated. The bacterial population was expressed as number of colony forming units CFU mg^{-1} of soil sample and CFU cm^{-2} of biofilm samples (Eashwar *et al.*, 1995).

Morphologically dissimilar and well-isolated colonies were randomly selected and streaked on the nutrient agar media. After noting the colony morphology along with colour, pigmentation, shape, consistency *etc.*, the selected pure colonies were subcultured in nutrient agar slant. Subcultures of bacterial strains were made once in 30 days to keep the bacterial strain viable. The bacterial strains isolated from all the samples were identified up to generic level by employing the standard morphological and biochemical characteristics described in Bergey's manual of systematic bacteriology (Holt *et al.*, 1994).

Amplification, cloning and sequencing of 16S rRNA gene analysis: Genomic DNA was extracted according to Ausubel *et al.,* (1988). Amplification of gene encoding for small subunit ribosomal RNA was carried out using eubacterial 16S rDNA primers (forward primer 5' AGAGTTTGATCCTGGCTCAG 3' (*E. coli* positions 8 to 27) and reverse primer 5'ACGGCTACCTTGTTACGACTT3' (*E. coli* positions 1494 to 1513). Polymerase chain reaction (PCR) was performed with a 50 µl reaction mixture containing 2 µl (10 ng) of DNA as the template, each primer at a concentration of 0.5 µM, 1.5 mM MgCl$_2$ and each deoxyribonucleoside triphosphate at a concentration of 50 µM as well as 1µl of *Taq* polymerase (1unit) and buffer as recommended by the manufacturer (MBI Fermentas). The PCR was carried out with a Mastercycler Personal (Eppendorf) with the following program: initial denaturation at 95°C for 1 min; 40

cycles of denaturation (3 min at 95°C), annealing (1 min at 55°C), and extension (2 min at 72°C); followed by a final extension (at 72°C for 5 min). The amplified product was purified using GFXTM PCR DNA and Gel Band Purification kit (Amersham Biosciences) and cloned in pTZ57R/T vector according to the manufacturer's instruction (InsT/A clone^{TM} PCR Product Cloning Kit, MBI Fermentas) and transformants were selected on Luria Bertani medium (LB) medium containing ampicillin (100 µg ml^{-1}) and X-gal (80 µg ml^{-1}). 16S rRNA gene sequencing for all the isolates were carried out with isolated plasmids from the clones using ABI PRISM 310 Genetic Analyzer (Applied Biosystems). For sequencing reaction, Big Dye Ready Reaction-Dideoxy Terminator Cycle Sequencing kit (Perkin-Elmer) was employed. The sequences obtained were searched in GenBank of National Center for Biotechnology Information (NCBI) using Basic local alignment search tool (BLAST) (Altschul *et al.*, 1997) to obtain the best homologous sequences.

Multiple sequence analysis was carried out using ClustalX (Thompson *et al.,* 1997) and Phylodraw (Choi *et al.*, 2000) were employed for constructing phylogenetic tree. To validate the reproducibility of the branching pattern, a bootstrap analysis was performed.

Bioaccumulation study of rare earth elements: This experiment was done to check the effect of rare earth soil on microorganism. Sterile nutrient broth was prepared in two 500 ml conical flask and 1 g of sterile rare earth soil was added in a conical flask. One of the conical flasks was kept as control where the mixed bacterial cultures were inoculated without rare earth soil. In another system, mixed bacterial cultures were inoculated in nutrient broth along with rare earth soil as experimental system. These systems were maintained at room temperature for five days incubation. Then the bacterial cultures were taken and centrifuged at 10,000 rpm for 10 min to pellet out from both the systems (Rubio *et al.,* 2006). All the pelleted samples were analyzed by Fourier transform infrared spectroscopy (FTIR) spectrum for knowing the physiological changes in the above samples and characterization was done by employing model NDXUS-672. The spectrum was taken in a mixed IR 400 -4000 cm^{-2} with 16 scan speed and was recorded using attended total reflectometer (ATR).

Results and Discussion

The counts of bacteria from soil and biofilm samples from mild steel plates and coated mild steel plate showed variation. Soil bacteria were in the range between 7.7 x 10^6 and 5.6 x 10^{10} CFU mg^{-1}. The count of bacteria on mild steel was in the range between 6.4 x10^5 and 8.3 x 10^6 CFU cm^{-2}. The count of bacteria on coated plate was 7.4 x 10^6 CFU cm^{-2}. The biochemical characterization of the collected isolates from three different samples was tabulated in Table 1a,b and c, respectively. The discrete band was observed in 1% agarose gel on UV illumination after loading the genomic DNA sample isolated. The sequence obtained was searched in nucleotide

Table - 1a : Biochemical characteristics of soil isolates

Test performed	Observation					
	SI6	SM1	SM3	SN1	SN2	SN3
Morphology	Rod	Rod	Rod	Cocci in pairs	Rod	Rod
Gram staining	+	+	+	+	+	+
Motility	−	−	+	−	+	+
Indole test	−	−	+	−	+	+
Methyl red test	+	−	−	−	−	+
Voges proskauer test	−	−	+	+	+	−
Citrate test	+	+	+	+	+	−
Starch hydrolysis	+	+	−	+	−	−
Gelatin hydrolysis	−	−	−	+	−	+
Lipid hydrolysis	−	−	−	−	−	−
Catalase	+	+	−	+	−	−
Oxidase test	+	+	+	+	+	−
Acid production	+	+	−	+	−	−
Gas production	−	−	−	−	−	−

+ = Present, - = Absent

Table - 1b: Biochemical characteristics of mild steel plate isolates

Test performed	Observation							
	PI1	PI2	PI4	PM2	PM3	PM5	PS2	PS3
Morphology	Rod	Rod	Rod	Rod	Rod	Cocci in chain	Cocci in chain	Rod
Gram staining	−	+	−	+	+	+	+	−
Motility	−	−	+	−	−	+	+	−
Indole test	−	−	+	−	−	−	−	−
Methyl red test	+	−	−	+	+	+	+	−
Voges proskauer test	−	+	−	−	−	−	−	+
Citrate test	+	+	−	+	+	+	+	+
Starch hydrolysis	+	+	−	+	+	+	+	+
Gelatin hydrolysis	−	+	+	−	−	+	+	+
Lipid hydrolysis	−	−	−	−	−	+	+	−
Catalase	+	+	−	+	+	−	−	+
Oxidase test	+	+	+	+	+	+	+	+
Acid production	+	+	+	+	+	+	+	+
Gas production	−	−	+	−	−	+	+	-

+ = Present, - = Absent

Table - 1c: Biochemical characters of coated steel plate isolates

| Test performed	Observation			
	CFI1	CFI5	CFI6	CFM3
Morphology	Rod	Rod	Rod	Rod
Gram staining	+	−	−	+
Motility	+	−	+	+
Indole test	+	−	−	+
Methyl red test	−	+	+	−
Voges proskauer test	−	−	−	−
Citrate test	+	+	+	+
Starch hydrolysis	+	+	+	+
Gelatin hydrolysis	−	−	−	−
Lipid hydrolysis	−	−	−	−
Catalase	+	+	+	+
Oxidase test	+	+	+	+
Acid production	+	+	+	+
Gas production	−	−	−	−

+ = Present, - = Absent

database using BLAST software in NCBI server. BLAST results showed that the sequences were having similarity with listed microorganisms and the sequences were deposited in Genbank using Bankit software and assigned accession numbers. (Table-2). The phylogenetic tree (Fig. 1) showed the relational information between the sequences of the isolates. Though the bacterial species were diverged as shown in the tree, they formed three clusters among them due to variations in 16S rRNA gene sequences.

Centrifuged bacteria pellets from the systems, microorganisms grown in nutrient broth with rare earth soil and without rare earth soil were used for FTIR analysis. In the control system, OH stretch (3319 cm^{-1}), C=C conjugated diene (1636 cm^{-1}), CH- deformation for CH_3 group (1465 and 1361 cm^{-1}), C=O stretch for COC group (1222 cm^{-1}) and chloride peak (66 cm^{-1}) were noticed. In the rare earth soil system, a peak at 3312 cm^{-1} indicates OH stretch. Peaks at 2924 and 2852 cm^{-1} indicate the CH aliphatic stretch and a peak at 1638 cm^{-1} indicates the C=C conjugated diene.

Table - 2: Identified bacterial species with assigned Genbank accession numbers

Sample name	Identification code	BLAST results	Gen bank accession number
Biofilm sample from painted mild steel coupons	CFI1	*Bacillus* sp.	EF532899
	CFI5	*Pseudomonas* sp.	EF532900
	CFI6	*Klebsiella pneumoniae*	EF532901
	CFM3	*Bacillus cereus*	EF532902
Biofilm samples from mild steel coupons	PI1	*Klebsiella* sp.	EF532903
	PI2	*Bacillus megaterium*	EF532904
	PI4	*Klebsiella* sp.	EF532905
	PM2	*Bacillus* sp.	EF532906
	PM3	*Bacillus firmis*	EF532907
	PM5	*Uncultured bacterium*	EF532908
	PS2	*Methylomicrobium*	EF532909
	PS3	*Acenitobacter junii*	EF532910
Soil samples	SI6	*Bacillus* sp.	EF532911
	SMI	*Paenibacillus* sp.	EF532912
	SM3	*Paenibacillus* sp.	EF532913
	SN1	*Staphylococcus* sp.	EF532914
	SN2	*Clostridium* sp.	EF532915
	SN3	*Arthrobacter luteolus*	EF532916
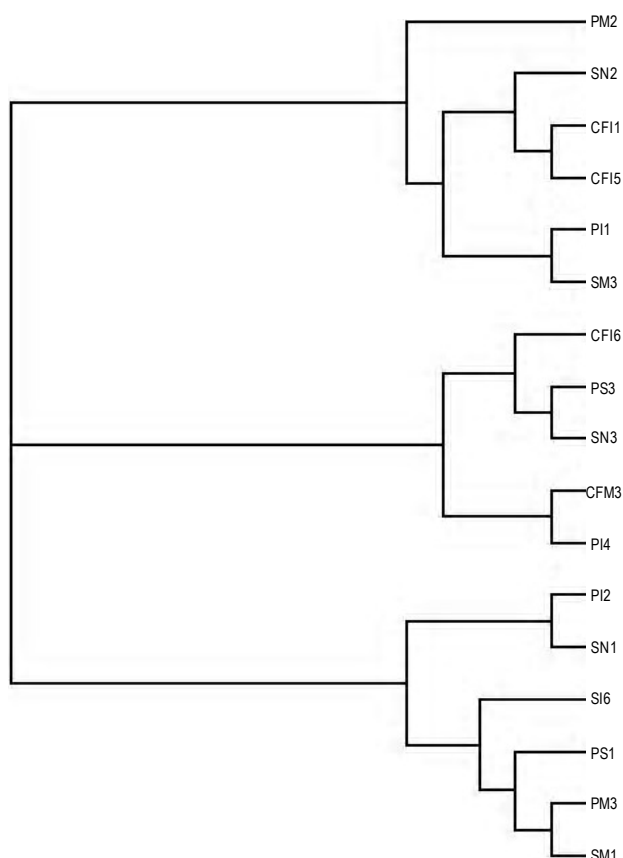
Fig. 1: Phylogenetic tree (Neighbor Joining method) for 16S rRNA sequences of the isolates

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* (Bayer, 1991). On the other hand, they may influence the environment by producing mineral acids, chelating agents such as siderophores or by-product of the metabolism (organic acids).

Rare earth elements are often used in industry for the production of glass additives, fluorescent materials, catalysts, ceramics, lighters, superconductors, magnets or condensers. Some authors (Diatloff *et al.*, 1996) have shown that light rare earth elements lanthanum and cerium could have a negative effect on the root elongation of corn and mungbean. A published review on REE toxicity has reported that cerium could be a potent antiseptic drug for Gram-negative bacteria and fungi (Hirano and Suzuki, 1996). There is no work available on the interaction between microorganisms and rare earth elements. The distribution of *Klebsiella* sp. and B*acillus* sp. were noticed in Goa sediment and reported as phosphate solubilizers by Desouza (2000). The above species were also reported in Manavalakkurichi (Tamil Nadu, India) 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.

Staphylococcus, Staphylococcus epidermidis, Pseudomonas aeruginosa were used for the bioadhesion to zirconium and suggested that the adsorption depends upon the surface of the material by Buczuski *et al.* (2003). Ozaki *et al.* (2006) studied the interaction of rare earth elements between *Pseudomonas sp.* and organic ligands. It was noticed that Eu (III) adsorbs on bacterial cells in the presence or organic ligands with low chelating ability. Merroun *et al.* (2003) also noticed the fixation of lanthanum

Peaks at 1548, 1449, 1409 cm^{-1} indicate the presence of C=C aromatic nuclei (carboxylic acid) while C=C stretch for C-O-C group has been noticed at 1084 and 1237 cm^{-1} (Fig. 2). It has
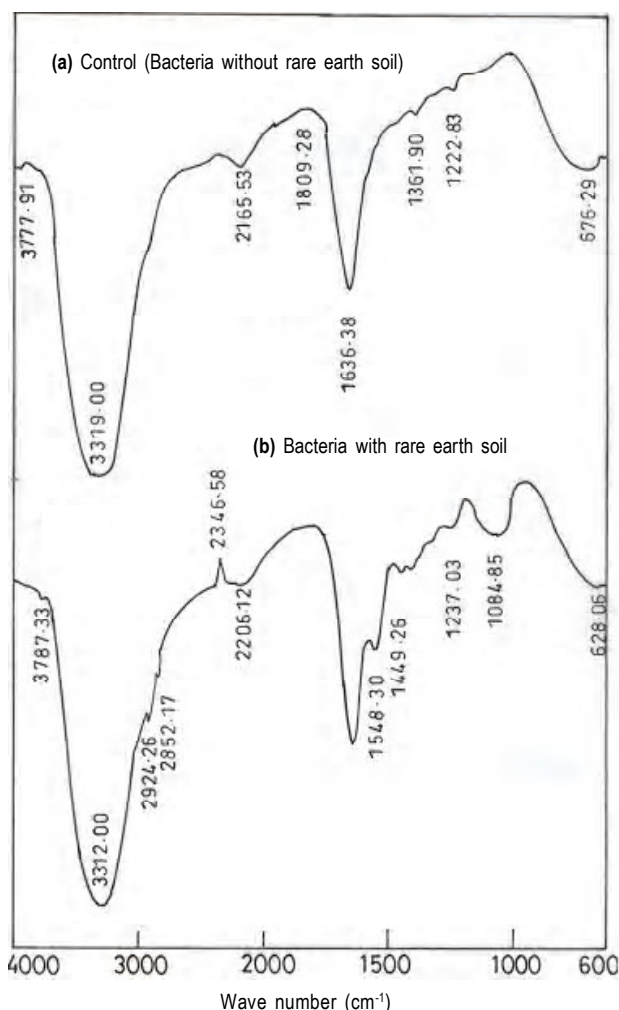
Fig. 2: Fourier Transform-Infra Red spectral observations for bacterial pellets

by *Myxococcus xanthus* in its extra cellular polymeric substances. The fixing of heavy metals was higher and this microorganism could be used as model of bacteria *Pseudomonas* sp. - lanthanide interactions (Kazy and Susanta, 2006). In the present study, the influence of rare earth elements on bacteria reveals that rare earth enhances the production of CH aliphatic stretch and C=C aromatic nuclei which were noticed in FTIR spectrum. It can be assumed that rare earth induces the bacteria for the production of carboxylic acid. It is expected that the carboxylic acid may act as chelating compound and it may enhance the production of nutrients in drought land. Hence, it is possible that the rare earth minerals can be used as manure in rocky or drought areas. Zhao and Zhu (2004) also noticed promotion of indole alkaloid production in *Catheranthus roseus* cell cultures by rare earth elements. 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. It was reported that various microorganisms present a high capacity of absorbing rare earth ions (Andres *et al.,* 2000).

The present study can be concluded that the rare earth element induces the bacteria for the production of carboxylic acid. The strong absorption peaks noticed in FTIR spectrum 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 rare earth elements sorption.

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