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Prevalence of *Bacillus* sp. among the biofilm forming community on Ti surface in marine environment

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Abstract

Prevalence of bacterial species involved in biomineralization of manganese on titanium (Ti) surfaces in marine environment was revealed in this research work. This study involves one year sea water exposure of Ti and their periodical biofilm characterization was carried out to quantify the manganese oxidizing bacterial (MOB) presence in the biofilm formed on titanium surfaces. The total viable count study of Ti coupons exposed to sea water for one year resulted in 60% of the MOB in overall biofilm population. The biochemical characterization of MOB isolates were performed for the genus level identification of the seven bacterial isolates. Further, the seven strains were subjected to 16S rRNA gene sequencing. Evolutionary analysis was performed using MEGA 7 to obtain closely related strains within the groups. The manganese oxidizing ability of the bacterial isolates were determined with Leucoberbelin Blue Assay (LBB) and Atomic Absorption Spectroscopy studies (AAS). The results show that among the isolated marine MOB species, *Bacillus* sp. and *Leptothrix* sp. have the maximum Mn oxidizing property. The microtitre plate assay was performed to determine the biofilm forming ability of the isolated marine MOB species. All the results have confirmed the prevalence of *Bacillus* sp. among the biofilm colonizers on Ti surfaces when exposed in sea water.

Key words: biomineralization, titanium surfaces, manganese oxidizing bacteria, 16S rRNA gene sequencing, Leucoberbelin Blue Assay, Atomic Absorption Spectroscopy

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1 Introduction

In aquatic systems, bacteria grow as multi-species communities attached to submerged surfaces by self-produced matrix of extracellular polymeric substances (EPS) called biofilm (Kolari, 2003). The consequence of these bacterial adhesion and biofilm formation leads to biofouling on engineered surfaces which results in unsatisfactory performance or reduced lifetime of the equipment (Characklis, 1984) and it is a universal problem (Brankevich et al., 1988, Satpathy, 1990). Biomineralization is an additional problem reported to develop as a consequence of biofouling. The incorporation of inorganic products formed by the metabolic activities of certain microbes into the biofilm known as biomineralization (Dhami et al., 2012). Marine microorganisms, especially manganese oxidizing bacteria (MOB) colonise on Ti surfaces can mineralise the manganese dissolved in the seawater and the manganese dioxide gets incorporated into the biofilm. Biomineralization on Ti surfaces has been reported to further reduction of heat transfer properties as well as make the biofilm refractory to treatment regimes (Sarvamangala et al., 2008). Our previous papers, suggested that the incorporation of nanoparticles which have bactericidal effects would decrease the bacterial surface attachment and increases the lifetime of the equipments used in marine environment (Priya et al., 2014, 2016).

Once Ti is exposed to sea water environment, it is subjected to biomineralization of manganese on its surfaces. This research work points out the prevalence of bacterial species involved in that processes. In the overall biofilm population formed on the Ti surface for a period of one year in sea water environment, 60% accounts for MOB species. The predominant marine MOB bacteria isolated from the biofilm sample includes *Bacillus* sp., *Flavobacterium* sp., *Pseudomonas* sp., *Micrococcus* sp. and *Leptothrix* sp. The biochemical characterization and 16S rRNA gene sequencing of MOB isolates were performed and the accession number was found to be HQ197382, HQ603747, DQ079003, DQ514307, U70977, DQ448712 and Z18533. Evolutionary analysis was performed using MEGA 7 to obtain closely related strains within the groups. Leucoberbelin Assay (LBB) and Atomic Absorption Spectroscopy studies (AAS) were carried out to determine the manganese oxidizing ability of the bacterial isolates. The results reveal that *Bacillus* sp. and *Leptothrix* sp. have the maximum Mn oxidizing property among the isolated marine MOB species. Further the biofilm forming ability of the isolated marine MOB species was also checked with microtitre plate assay. All the results have confirmed the prevalence of *Bacillus* sp. among the biofilm colonizers on Ti surfaces when exposed in sea water.

2 Meterials and methods

2.1 *Materials*

Commercially pure Ti (Grade 2) coupons of size 30 mm×20 mm×3 mm were used for exposure studies. These Ti coupons were tied with titanium frame and exposed to sea water at Co-operative society of Kalpakkam Township, Kalpakkam. The expos-

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ure studies were carried out for a period of one year.

2.2 *Isolation of manganese oxidizing bacteria (MOB) from biofilm samples on Ti surface*

The coupons were taken out periodically and sonicated in the sterile phosphate buffer (0.042 5 g KH_2PO_4, 0.19 g $MgCl_2$ per liter) by ultrasonication for 10 min. The period of sonication for optimum recovery of cells was found to be 10 min (Gopal et al., 2008). The resulting buffer solution was used as bacterial suspension. The obtained bacterial suspension was serially diluted and plated on artificial sea water nutrient agar (ASWNA) by the pour plate technique. Artificial sea water nutrient agar medium (ASWNA) composed of NaCl 28.13 g/L, KCl 0.07 g/L, $CaCl_2{\cdot}2H_2O$ 1.60 g/L, $MgCl_2{\cdot}6H_2O$ 4.80 g/L, $NaHCO_3$ 0.11 g/L, $MgSO_4{\cdot}7H_2O$ 3.50 g/L, Peptone 5 g/L, Beef extract 3 g/L. The medium was autoclaved at 121°C (15 psi) for 15 min. Before the media has been poured on the plates, it was amended with filter sterilized solution of 100 mg/L $MnCl_2$ (Gopal et al., 2008). After inoculation, the plates were incubated at 37°C for 48 h.

2.3 *Determining and quantification of manganese oxidizing ability of isolated marine bacteria*

In solid media, the formation of visibly brown color colonies on a solid agar plate containing Mn^{2+} reflected biological manganese oxidation (Gopal et al., 2008). Similarly the presence of Mn oxides in liquid culture can be detected by reacting with calorimetric dye Leucoberbelin blue (LBB). LBB is a redox dye that is known to be oxidized by a single electron transfer reaction with Mn (III).

$$Mn^{3+}+\text{LBB (reduced)} \rightarrow Mn^{2+}+\text{LBB (oxidized)}.$$

Oxidized LBB is blue in colour. The degree of colouration is a function of the number of electrons transferred in the reaction to the LBB and therefore can be used to quantify the amount of Mn oxides being reduced. The strong redox potential of LBB means only very strong oxidizing agent like Mn oxides can react with it. Therefore, a positive LBB reaction in a sterile medium can be an inference caused by Mn (III) (DePalma, 1993).

Briefly, the bacterial culture was mixed with 0.04% LBB solution in the ratio 1:3 and incubated in darkness for 15 min, followed by recording the Optical density at 620 nm using UV-VIS spectrometer. The results were plotted against $KMnO_4$ standard curve. Simultaneously, the unreacted Mn^{2+} ions present in liquid culture were measured using AAS (Gopal et al., 2008), where the culture suspension was prepared by centrifugation for 20 min at 15 000 r/min and analysed using AAS comparing with standard solution of $MnCl_2$ with concentration ranging from 0.5 to 5.0 mg/L.

2.4 *Biofilm quantification assay of marine MOB isolates*

Crystal violet assay which is widely used to quantify the biofilm content was followed for the isolated marine MOB species. Crystal violet is a basic dye which binds to negatively charged molecules, including cell surfaces and Extracellular Polymeric Substances (EPS) which provides structure and protection to the biofilm community. Briefly, the cultures were grown at 37°C in nutrient broth at pH 7. One millileter of the overnight culture was used to inoculate 100 mL of Luria-Bertani (LB) media (1:100 dilutions). 200 μL of 1:100 dilution of the overnight culture in LB medium at pH 7 was added to each well of the microtitre plate and was incubated at 37°C for 24 h without shaking. For reproducibility concern, three replicate wells were kept for each culture. After incubation, the culture was destrained and gently rinsed twice to remove the unattached cells and media components and also significantly lowers background staining. Following this the wells were stained with 200 μL of 0.01% crystal violet and incubated in room temperature for 20 min and rinsed with sterile water. Finally the dye was solubilised by adding 200 μL of 30% acetic acid in water (O'Toole, 2011). The intensity of the color which is directly proportional to the biofilm quantity was determined using the GENios Multi-Detection Multi-plate Reader at 550 nm using 30% acetic acid in water as the blank.

2.5 *Physiological and biochemical characterization of marine manganese (II) oxidizing bacteria (MOB)*

Physiological characterization like colony morphology, Gram's staining and biochemical anlaysis, such as Catalase test, Oxidase test, Indole test, Starch hydrolysis test, Nitrate reduction test, Citrate utilization test, Carbohydrate utilization test, Motility test (Cappucino and Sherman, 1996; Aneja, 2003) was performed at the genus level of identification of the biofilm colonizers.

2.6 *16S rRNA gene sequencing and phylogenetic analysis*

Pure cultures of manganese oxidizing bacteria isolated from the biofilm sample grown on the Ti surface were subjected to 16S rRNA gene sequencing analysis. The retrieved sequences were compared with other bacterial sequences by using NCBI BLAST search for their pairwise identities among the closest relative species. The phylogenetic analyses of all the seven isolates were performed using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

3 Results and discussion

3.1 *Prevalence of MOB on Ti coupons exposed to seawater*

Table 1 reveals the prevalence of MOB among the biofilm forming microorganisms with 60% of total biofilm population on the Ti surface when exposed to sea water for a period of one year. The total viable count of marine bacteria and MOB in the biofilms on titanium surfaces was observed to increase with exposure time. Figures 1a and b show the FE-SEM and Epifluorescence microscopic images of one month exposed Ti surfaces. The images reveal the presence of biofilm with bacterial species adhering to the Ti surface.

3.2 *Identification and quantification of Mn oxidizing ability of isolated MOB from biofilm sample*

Figure 2 shows the formation of visibly brown colonies on a solid agar plate containing 100 mg/L of Mn (II) reflected that the bacteria is capable of oxidizing Mn II were identified as manganese oxidizing bacteria (MOB). The brown coloration is due to the presence of insoluable Mn oxides which is formed by the reaction of these bacteria with Mn (II). Since this is the qualitative assay, these MOB isolates were further quantitatively character-

Table 1. TVC result of marine bacterial and MOB isolates from one year exposed Ti coupon

Ti coupon exposure period in seawater	General bacterial count/CFU·cm^{-2}	Marine MOB isolates/CFU·cm^{-2}
One year	(7.8±2.0)×10^5	(4.6±1.3)×10^5

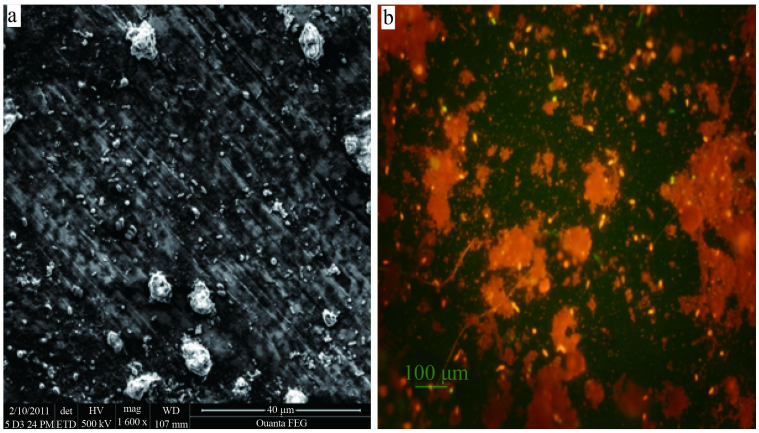

Fig. 1. FE-SEM image of control Ti coupon exposed to sea water for a period of 1 month (a) and epifluorescence microscopic image of control Ti coupon exposed to sea water for a period of 1 month (b).

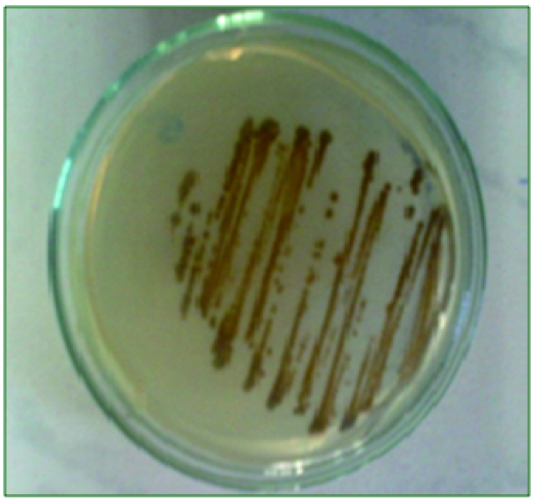

Fig. 2. Photograph image of Mn oxidizing bacterial growth on the ASWNA plate with brown color colonies.

ized to know their level of Mn oxidizing ability among the other isolates.

3.3 *Leucoberbelin blue assay*

Manganese with a higher state of oxidation is associated with the cell exopolymer matrix which was removed from the culture sample when the cells were pelleted by centrifugation. Mn with an oxidation state higher than 2^+ would result in a color change to dark blue in reaction with LBB depending on the concentration of Mn oxides as seen in Figs 3a and b. Figure 4 shows Mn^{3+} concentration profile of seven Marine MOB isolates using LBB dye. The results revealed the varying levels of positive LBB reaction within the marine MOB isolates. Among the isolated marine MOB species, *Bacillus* and *Leptothrix* sp. have the maximum Mn oxidizing property.

3.4 *Atomic absorption spectrophotometer analysis*

The unreacted Mn ions in the culture media were measured using AAS. The culture supernatant was analysed with AAS and results in Fig. 5 was obtained by plotting a graph against the standard $MnCl_2$ solution. The decrease in Mn (II) concentration in the supernatant solution confirms the manganese oxidizing ability of the species. From the graph it was clear that the manganese oxidizing ability varies between the MOB species.

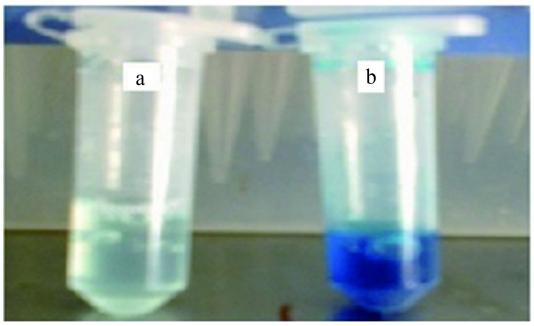

Fig. 3. Control LBB solution (a) and Mn^{3+} oxides reacting with LBB dye resulting (b) in dark blue colour solution.

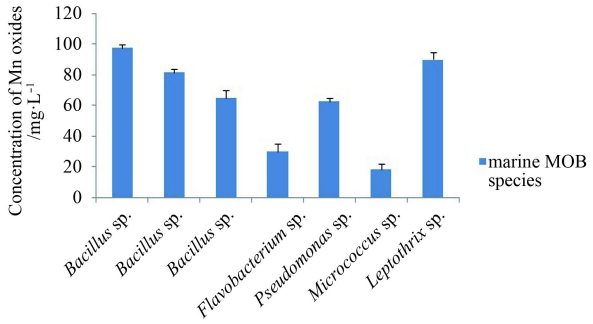

Fig. 4. Mn^{3+} concentration profile of marine MOB isolates using LBB dye.

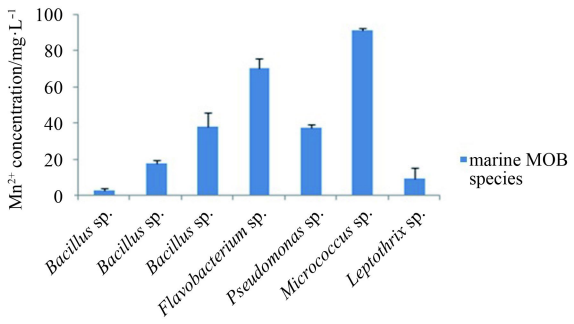

Fig. 5. Mn^{2+} concentration profile of marine MOB isolates using AAS.

3.5 *Biofilm quantification assay*

The biofilm forming ability of the isolated marine bacterial species was characterized by microtitre plate assay. Based on the intensity of the dye read by UV spectrophotometer, it was clear that among the other MOB isolates, the three *Bacillus* sp. predominates in the biofilm formation followed by *Leptothrix* sp. and *Pseudomonas* sp. Figure 6 shows the biofilm quantification profile of the isolated marine MOB species.

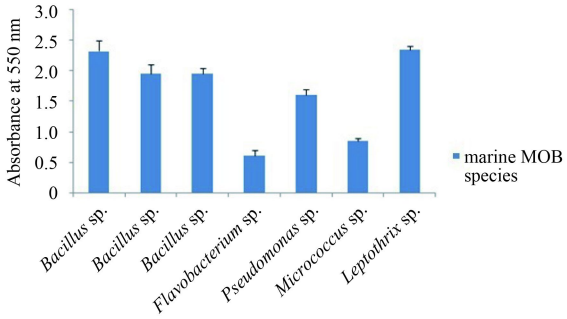

Fig. 6. Microtitre plate assay showing biofilm formation ability of different MOB isolates.

3.6 *Physiological and biochemical characterization of marine MOB isolates*

Mn oxidizing bacteria are abundant and distributed widely (Johnston and Kipphut, 1988). In our study, totally seven MOB species were isolated from biofilm formed on titanium surfaces. The biochemical characterization was carried out for the isolated MOB species and it was presented in Table 2. The results showed that among the seven Mn oxidizing bacterial species four are gram-positive and three are gram negative bacteria.

3.7 *16S rRNA gene sequencing analysis of marine MOB isolates*

The isolated MOB strains were sequenced using 16S rRNA gene sequencing method. The obtained sequences were aligned and compared with other bacterial sequences in the NCBI by BLAST search. The 16S rRNA gene sequences of the seven strains were deposited to NCBI database and the accession numbers were obtained. The accession numbers of seven MOB strains were HQ197382, HQ603747, DQ079003, DQ514307, U70977, DQ448712 and Z18533. Initial results of comparative 16S rRNA gene sequences of isolates revealed that the strains belong to three bacterial phyla, Firmicutes, Actinobacteria and Alphaproteobacteria. All these isolated strains are able to oxidize manganese, and the results are in accordance with other findings, that these groups are manganese oxidizers (Bargar et al., 2005; Palmer and Turekian, 1986).

3.8 *Phylogenetic analysis of marine MOB isolate*

Figure 7 represents the molecular phylogenetic analysis of *Bacillus* sp. strain RV.B2.90, *Bacillus* sp. GZT and *Bacillus* sp. GB02-39A with the highest log likelihood (–3 130.724 4). The analysis involved 13 nucleotide sequences. There were a total of 1 291 positions in the final dataset. Figure 8 represents the molecular phylogenetic analysis of *Pseudomonas putida*, *Leptothrix discophora*, *Micrococcus* sp. CNJ719 PL04 and *Flavobacterium* sp. BSs20191 with the highest log likelihood (–5 956.108 4). The analysis involved 16 nucleotide sequences. There were a total of 1 271 positions in the final dataset. For both the analysis codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated and the tree was drawn to scale, with branch lengths measured in the number of substitutions per site.

In this study, all the predominant marine bacteria listed by Zobell (1946) in sea water like *Bacillus*, *Flavobacterium*, *Pseudomonas*, *Micrococcus*, *Leptothrix* were also represented. Throughout the study it was observed that gram positive bacteria *Bacillus* were dominant Mn(II) oxidizing bacteria isolated from the biofilm on titanium surfaces. Gram-negative bacteria such as *Pseudomonas*, *Flavobacterium* and *leptothrix* were also present. During the exposure studies, *Bacillus* sp. were abundant in the biofilm compared to any other species, which is contrary with the result of Judy (2006) shows that *Pseudomonas* was predominant from the biofilm on titanium surfaces.

The previous research studies by Dickinson et al. (Dickinson and Lewandowski, 1996; Dickinson et al., 1997) and Rosson and Nealson (1982) also reported the presence of *Leptothrix* sp. from stainless steel surfaces in a fresh water environment which is also confirmed by our study. Widely reported non-conventional manganese oxidizing bacteria are *Bacillus* and *Pseudomonas* sp. Extensive work with these two organisms has revealed that both these bacteria were capable of efficiently oxidizing manganese (II). The result indicates that the presence of *Bacillus* and *Pseudomonas* sp. in the biofilm formed on Ti surface exposed to sea water.

Table 2. Results of biochemical tests to identify marine MOB species isolated from the biofilm sample on Ti surface

Tests	Mn1	Mn2	Mn3	Mn4	Mn5	Mn6	Mn7
Cell shape	rods with spores	filament	big rods	filament	small rods	tetrads cocci	rods
Gram reaction	positive	positive	positive	negative	negative	positive	negative
Pigment	spreading white waxy	opaque white	white	yellow	brownish spindle shape colony	yellow	transparent
Catalase	+	+	+	+	+	+	+
Oxidase	–	–	+	–	+	+	–
Indole production	–	–	–	–	+	–	–
Starch hydrolysis	+	+	–	–	+	+	–
Nitrate reduction	+	+	+	–	+	+	+
Citrate utilization	–	+	+	+	+	–	+
Lactose fermentation	–	–	–	–	+	–	–
Dextrose fermentation	acid	–	–	acid	acid	–	acid
Motility	–	+	+	+	+	–	+
Identified as Genus	*Bacillus* sp.	*Bacillus* sp.	*Bacillus* sp.	*Flavobacterium* sp.	*Pseudomonas* sp.	*Micrococcus* sp.	*Leptothrix* sp.

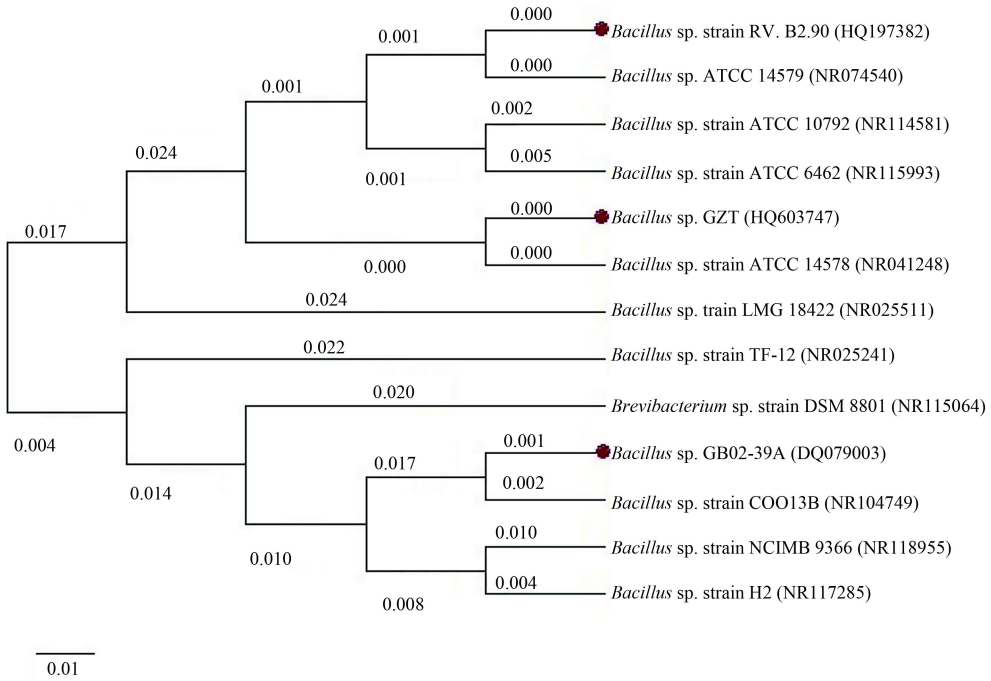

Fig. 7. Molecular phylogenetic analysis of *Bacillus* sp. strain RV.B2.90, *Bacillus* sp. GZT and *Bacillus* sp. GB02-39A by Maximum Likelihood method.

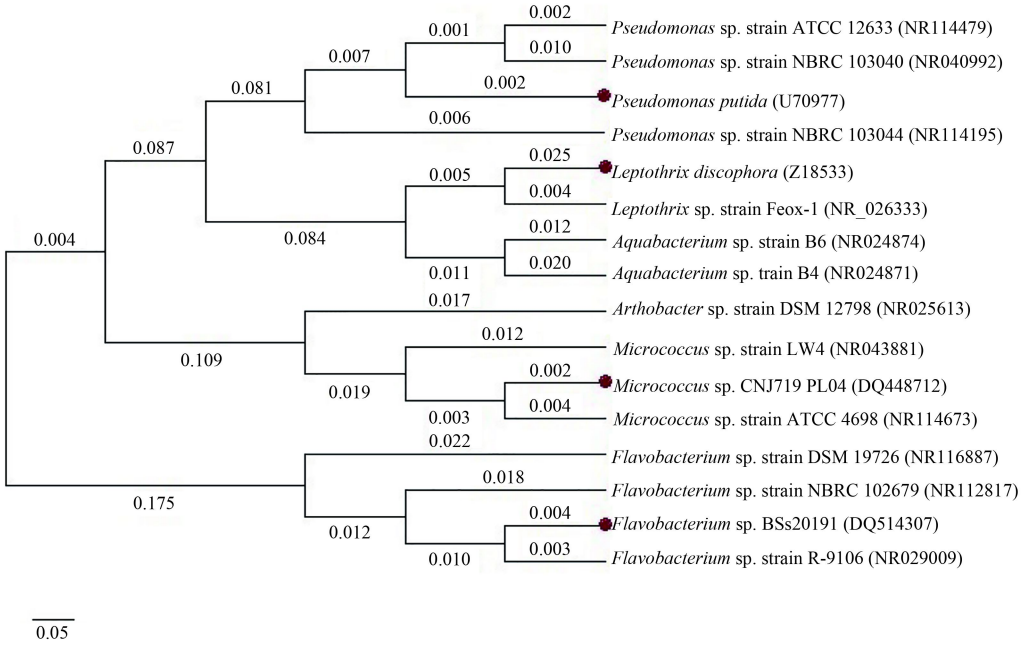

Fig. 8. Molecular phylogenetic analysis of *Pseudomonas putida*, *Leptothrix discophora*, *Micrococcus* sp. CNJ719 PL04 and *Flavobacterium* sp. BSs20191 by Maximum Likelihood method.

Gopal et al. (2008) has already revealed the importance of biomineralization of Mn(II) on titanium surfaces exposed to sea water (Judy, 2006). A similar enrichment of Mn in biofilms formed on PVC and stainless steel exposed to sea water in Tuticorin has been reported by Palanichamy et al. (2002). Hence the characterization of MOB in biofilms is important and that cannot be ruled out. Leucoberbelin blue assay and Atomic Absorption Spectroscopy results (AAS) of present study shows that among the other MOB isolates, *Bacillus* sp. and *Leptothrix* sp. involves in maximum Mn oxidation. So this study emphasis that *Bacillus* sp. is more prevalent among the other marine bacterial species in the biofilm formed on Ti surface when exposed to sea water.

4 Conclusions

In this study, the prevalence of marine MOB among the biofilm forming community in sea water environment was checked. As a result, it was found that *Bacillus* sp. is predominant among the isolated MOB species and also oxidizes the Mn in high content which leads to biomineralization. Since biofilm formation and subsequent biomineralization are both unfriendly process in any objects submerged in sea water are needed to be

avoided. So the process which deploys the attachment of MOB, especially *Bacillus* sp. would result in increased lifetime of the Ti which is widely used as condenser material in sea water environment.

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