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Biosurfactant and Heavy Metal Resistance Activity of *Streptomyces* spp. Isolated from Saltpan Soil

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Abstract: Actinomycetes were isolated from the marine soil samples collected at the Ennore saltpan and were screened for biosurfactant and heavy metal resistance activity. Biosurfactant activity was evaluated by haemolysis, drop collapsing test and lipase production. Similarly heavy metal resistance was determined by tube method and agar diffusion method. Among them, two actinomycetes isolates VITDDK1 and VITDDK2 exhibited significant biosurfactant and heavy metal resistance activity. Based on the Hideo Nonomura's key for classification of actinomycetes, the isolate VITDDK1 was similar to *Streptomyces orientalis* and VITDDK2 to *Streptomyces aureomonopodiales.* However molecular phylogeny based on neighbour-joining method showed 99% similarity of VITDDK1 with *Streptomyces* sp. A403Ydz-QZ and 93% similarity of VITDDK2 with *Streptomyces* sp. strain 346.

Key words: Bioremediation, biosurfactant, Hideo Nonomura's classification, *Streptomyces* spp. VITDDK1, *Streptomyces* spp. VITDDK2

INTRODUCTION

Actinobacteria are exciting structures inhabiting almost all possible niches (Deepika and Kannabiran, 2009a). They are gram-positive firmicutes with high GC content (Deepika and Kannabiran, 2009b; Deepika *et al*., 2009; Stackebrandt *et al*., 1997). These prokaryotes are filamentous in nature and are considered as an intermediate group between bacteria and fungi (Pandey *et al*., 2004). Actinobacteria especially *Streptomyces* are free-living saprophytes found predominantly in the soil (Mohan and Vijayakumar, 2008; Kavitha and Vijayalakshmi, 2007). *Streptomyces* constitutes 50% of the total actinomycetes population (Piret and Demain, 1988).

Biosurfactants are microbial surface active agents produced by certain microorganisms during their growth phase (Zajic and Panchal, 1976). They may be extracellular or intracellular in nature (Chen *et al*., 2007). Substrates for biosurfactant production are sugars, oils, alkanes and waste materials (Lin, 1996). Biosurfactants are amphiphilic, non-toxic and biodegradable molecules with high specificity (Zajic and Panchal, 1976; Cooper and Zajic, 1980). They are highly stable at extremities of temperature, pH and salt concentration (Desai, 1987; Drouin and Cooper, 1992). These molecules have the ability to decrease the surface tension, critical micelle concentration and interfacial tension (Banat, 1995). Biosurfactants are thus used as an alternative for chemical surfactants. They are highly useful in agriculture, food, health care and cosmetics industries (Kokare *et al*., 2004).

Heavy metals are released as by-products of industries such as tanneries, leather industries, sugar mills, fertilizer industries and textiles (Maria *et al*., 1998). Heavy metal contamination of the water bodies affects the micro and macro flora and fauna inhabiting the water bodies thereby disturbing the whole ecosystem (Joseph *et al*., 2009). Releases of these metals are of major concern since they cause a serious threat to the health and well-being of human and animals (Joseph *et al*., 2009). The present study was undertaken with the view of exploring marine actinomycetes to address the environmental problems. Hence in the present study *Streptomyces* strains isolated and maintained aseptically under laboratory conditions were evaluated for their efficiency as potential biosurfactant agents and for their ability to bioremediate heavy metals.

MATERIALS AND METHODS

Sample collection: Ennore saltpan (Lat. 13º 14'N and Long. 80º 22' E) soil samples (100-500 g) were collected in sterile polythene bags during December 2007, transported to the laboratory aseptically and stored at ambient temperature for further use (Deepika and Kannabiran, 2009b). Isolation of *Streptomyces,* biosurfactant and heavy metal resistance activity of the isolate was carried out in Biomolecules research laboratory at VIT University.

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Isolation of actinobacteria: Soil samples were serially diluted upto 10^{-6} dilution using sterilized sea water. From each dilution, 1 ml was plated on Starch Casein agar by pour plate technique (Deepika *et al*., 2009). The plates were incubated at 30ºC for 7-10 days. Morphologically different colonies were identified and purified by streak plate technique.

Biosurfactant assay: Biosurfactant activity of the isolates was evaluated by the following methods. Haemolytic activity was tested using blood agar plate. Blood agar was prepared with human blood (5%) and blood agar base. The blood agar base was sterilized by autoclaving at 121ºC at 15lbs pressure for 15 min. Prior to pouring blood was added and allowed to solidify. The isolates were streaked on the blood agar and the plates were incubated at 28ºC for 7 days. The plates were then observed for zone of clearance around the colonies (Carillo *et al*., 1996).

Drop collapsing test: Mineral oil (2 µl) was added to 96-well microtitre plate and equilibrated for 1 h at 37ºC. The culture supernatant (5 µl) was added to the surface of the oil in the well. The shape of drop on the oil surface was noted after 1 min. The culture supernatant that collapsed the oil drop was indicated as positive and the culture supernatant which failed to collapse the oil drop was indicated as negative. Distilled water was used as negative control (Youssef *et al*., 2004).

Lipase production: Tributyrin agar plates were prepared using Actinomycetes Isolation Agar (AIA) and tributyrin (1%). The pH of the medium was adjusted to 7.3-7.4 using 0.1N NaOH. The cultures were streaked on the tributyrin agar plates and incubated at 28ºC for 7 days. The plates were then examined for zone of clearance around the colonies (Gandhimathi *et al*., 2009).

Screening for heavy metal resistance: Heavy metal standards, mercury, lead, cadmium, zinc and copper were used for evaluating the heavy metal resistance of the isolates. Similarly heavy metal salt solutions, cadmium sulphate, nickel sulphate, copper sulphate, zinc sulphate, mercuric chloride, lead acetate, potassium dichromate, cobalt nitrate and sodium arsenate used for evaluating the heavy metal resistance of the isolates. The concentration of the standard heavy metal solutions was 1000 ppm whereas the concentration of heavy metal salt solutions ranged from 10, 50, 100, 500, 1000, 5 and 10 mM. The salt solutions were prepared with phosphate buffer saline, PBS (pH 6.8). The heavy metal standards and salt solutions were sterilized by autoclaving for 15 min at 121ºC.

Tube method: ISP1 broth was dispensed in test tubes and sterilized at 121ºC for 15 min. To each tube, 500 µl of the appropriate metal standard or salt solutions was added and incubated at 28ºC for 7 days (Konopka and Zakharova, 1999). The tubes were examined for turbidity. The tubes with turbidity were recorded as resistant and the ones without growth as sensitive to the particular metal standards and salt solutions.

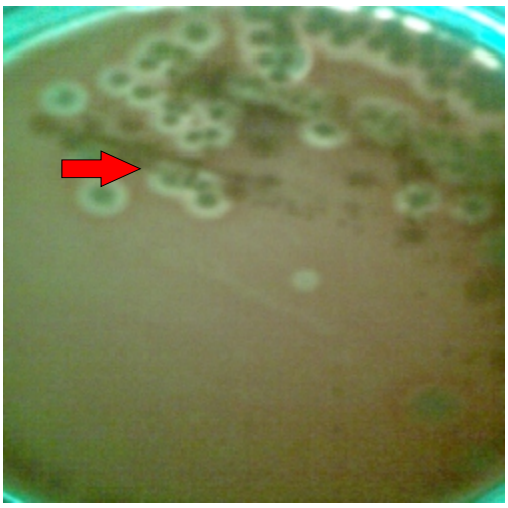
Agar diffusion method: The isolates raised in ISP 1 broth (International Streptomycetes Project) for 7 days were swabbed on Starch Casein Agar (SCA). Using a sterile cork borer (7 mm width), wells were made on the surface of SCA initially seeded with the isolates. To each well 500 µl of the standard metal standards or the salt solutions were added and incubated at 28ºC for 7 days. The inhibition area (mm) was measured as described earlier (Hassen *et al*., 1998).

Identification of the potential isolates: The potential isolates VITDDK1 and VITDDK2 were inoculated on Oat meal agar (ISP3) and the aerial spore mass colour and the production of reverse side pigment and soluble pigment other than melanin were studied. Melanin pigment production was studied by inoculating the strains on Peptone yeast extract iron agar (ISP6). Spore chain surface and spore orientation was studied using scanning electron microscope (S3400, 20 KV, 5.00 µm). Assimilation of the sugars namely as arabinose, xylose, inositol, mannitol, fructose, rhamnose, sucrose and raffinose as sole carbon source was determined by inoculating the isolates in modified Bennets' broth with the respective carbon source. The inoculated tubes were incubated for 7 days at 30ºC (Nonomura, 1974).

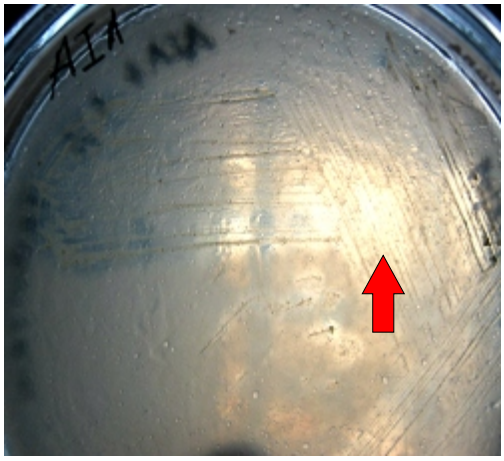
Molecular taxonomy: DNA was isolated from the two strains VITDDK1 and VITDDK2 using the protocol as described by Kieser *et al*. (2000). PCR amplification was carried out using Actino specific forward and reverse primers as designed described earlier (Stach *et al*., 2003). The PCR conditions were followed as described by Farris and Olson (2007). The PCR product was ligated into the cloning vector pTZ57R/T. The recombinant plasmid was sent for sequencing to Macrogen (Seoul, South Korea). The 16S rRNA partial sequences were subjected to BLAST search engine available in the NCBI data bank. The query sequences and the homologous sequences were aligned using ClustalW (DDBJ) and phylogenetic tree was constructed by neighbor joining method (MEGA 4.0.2 software) (Saitou and Nei, 1987). The 16S rRNA sequences was then submitted to the GenBank, NCBI, USA.

RESULTS AND DISCUSSION

Serial dilution of soil sample and subsequent plating on Starch caesin agar resulted in isolation of 100 colonies of actinobacteria. On blood agar plate, only two isolates VITDDK1 (Fig. 1a) and VITDDK2 (Fig. 2a) produced a

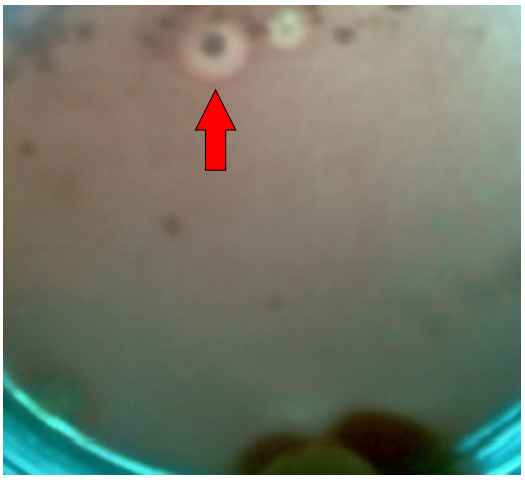


(a)



(b)

Fig. 1: Biosurfactant activity of *Streptomyces* spp.VITDDK1. (a) A clear zone around the colonies on blood agar plate indicates haemolysis of the blood cells. (b) A clear zone around the colonies on tributyrin agar indicates the production of lipase



(a)

(b)

Fig. 2: Biosurfactant activity of *Streptomyces* spp.VITDDK2. (a) A clear zone around the colonies on blood agar plate indicates haemolysis of the blood cells. (b) A clear zone around the colonies on tributyrin agar indicates the production of the enzyme lipase

clear zone around the colonies causing lysis of the blood. In the drop-collapsing test both the strains collapsed the oil drop thus producing a flat drop. On Tributyrin agar plate the two strains VITDDK1 (Fig. 1b) and VITDDK2 (Fig. 2b) produced a clear zone around the colony indicating lipase production. All these results confirmed the ability of the two strains VITDDK1 and VITDDK2 to produce surface active molecules. Based on lipase production and emulsification test, the strain *Streptomyces* sp. S1 isolated from marine sediments collected from the Goa coastal region was reported to possess biosurfactant activity (Kokare *et al*., 2007). Recently the actinomycetes *Nocardiopsis alba* MSA 10 was identified to possess biosurfactant activity. Reportedly this actinomycete produced lipopeptide surface active agent (Gandhimathi *et al*., 2009). *Streptomyces* sp. isolated from the soil sample recovered from the Chiang Mai Province, Thailand was evaluated by drop collapsing test to possess biosurfactant activity (Thampayak *et al*., 2008). Marine actinobacteria *Brevibacterium aureum* MSA13 associated with the marine sponge *Dendrilla nigra* was collected from the southwest coast of India by SCUBA diving at 10-15 m depth was found to produce lipopeptide type of biosurfactant (Kiran *et al*., 2009).

Similarly the isolates were also tested for their ability to remove heavy metals and their respective salts. Preliminary screening was carried out by tube method. The isolates VITDDK1 and VITDDK2 grew well in the presence of cadmium, sodium arsenate, zinc sulphate,

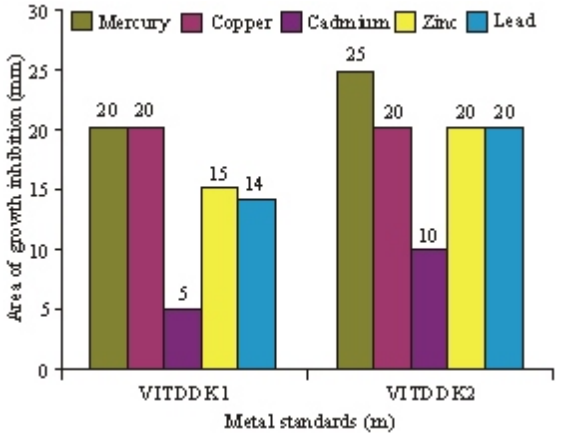


Fig. 3: Heavy metal resistance exhibited by *Streptomyces* spp. VITDDK1 and *Streptomyces* spp. VITDDK2 against metal standards by agar diffusion method. An inhibition zone of 10mm is considered as the tolerance limit

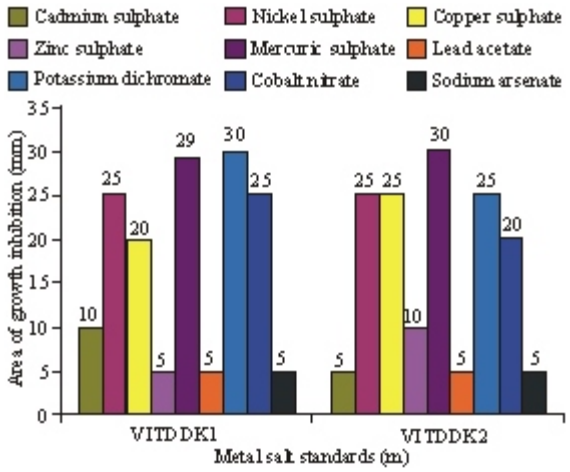


Fig. 4: Heavy metal resistance exhibited by *Streptomyces* spp. VITDDK1 and *Streptomyces* spp. VITDDK2 against metal salt solutions by agar diffusion method. An inhibition zone of 10 mm is set as the arbitrary tolerance limit

lead acetate and cadmium sulphate. Based on the results obtained secondary screening was carried out by agar well diffusion method. VITDDK1 produced an inhibition zone of 5 mm and VITDDK2 produced 10 mm for cadmium (Fig. 3). From the results it is clear that both the strains are resistant to the heavy metal cadmium. However the degree of inhibition for cadmium produced by the strain VITDDK2 was greater than that produced by the strain VITDDK1. Similar studies related to heavy metal resistance was already reported, a chromium resistant actinomycete, *Streptomyces* sp. VITSVK5 was isolated from Marakkanam sediments (Saurav and Kannabiran, 2009). Heavy metal tolerant actinomycetes were isolated from the polluted areas in the Sali River, Argentina (Maria *et al*., 1998). Similar studies on heavy metal

Table 1: Hideo and Nonomura's key for classification of *Streptomyces* spp.VITDDK1 and *Streptomyces* spp.VITDDK2

S. No.	Characteristics	*Streptomyces* spp. VITDDK1	*Streptomyces* spp. VITDDK2
a.	Aerial spore mass colour	White	White
b.	Pigmentation		
	• Reverse side pigment	-	-
	• Soluble pigment	-	-
	• Melanin pigment	-	Dark brown pigment
d.	Spore surface	Smooth	Smooth
e.	Spore chain orientation	Rectiflexibles	Rectiflexibles
f.	Sugars		
	• Arabinose	+	+
	• Xylose	+	+
	• Inositol	+	+
	• Mannitol	+	+
	• Fructose	+	+
	• Rhamnose	+	+
	• Sucrose	+	+
	• Raffinose	+	+

resistance by marine bacteria associated with marine sponge *Fasciospongia cavernosa* have been reorted (Joseph *et al*., 2009). Both VITDDK1 and VITDDK2 produced a zone of 5-10 mm for sodium arsenate, zinc sulphate, lead acetate and cadmium sulphate (Fig. 4). From the results it is clear that both the strains are resistant to the metal salt solutions sodium arsenate, zinc sulphate, lead acetate and cadmium sulphate.

The isolate VITDDK1 produced small, white, powdery, flat, round and regular colonies whereas VITDDK2 produced large, white, powdery, raised and irregular colonies on ISP3 agar medium. On ISP6 agar surface the isolate VITDDK1 did not produce any kind of pigments whereas VITDDK2 produced a dark brown melanin pigment but no reverse side pigment or soluble pigment. The spore surface of both VITDDK1 as well as VITDDK2 was smooth. Similarly the spore chains of both the strains were arranged in rectiflexibles fashion. Both the strains had the ability to assimilate all the 8 sugars tested. Using the Nonomura's classification key, the strain VITDDK1 was identified as *Streptomyces orientalis* whereas the strain VITDDK2 was identified as *Streptomyces aureomonopodiales* (Table 1). *Streptomyces violachromogenes* isolated from Yemen fresh soil was identified according to Nonomura's key (Ahmed, 2007). Nonomura's key was adapted for the identification of actinomycete LG-10 isolated form forest soil on Mt. Baekun, Chonnam, Korea (Seong *et al*., 2001). Based on Nonomura's key, the actinomycete LG-10 producing L-glutaminase enzyme was identified upto the species level and the strain LG-10 was found to be *Streptomyces rimosus* (Sivakumar *et al*., 2006).

The PCR amplicons of the strains VITDDK1 and VITDDK2 were sequenced and a phylogenetic tree was constructed using 16S rRNA partial gene sequence. The phylogenetic tree constructed based on neighbour-joining method using MEGA 4.0.2 software showed 99%

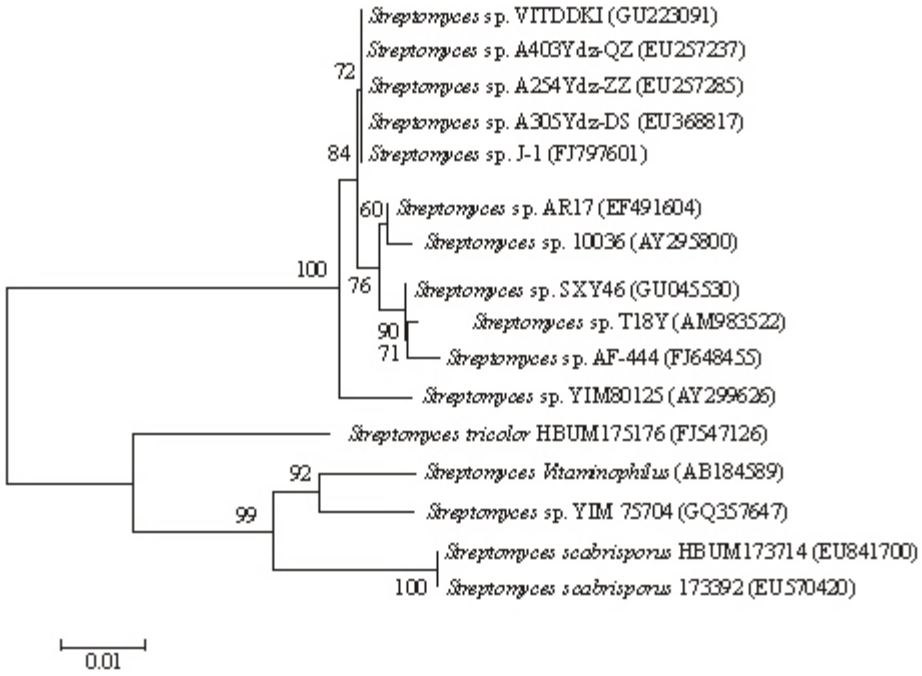


Fig. 5: The taxonomic position of *Streptomyces* VITDDK1 spp. was determined based on 16S rRNA gene sequencing. A phylogenetic tree was constructed based on neighbour-joining method using the MEGA 4.0.2 software. The closest neighbor of *Streptomyces* VITDDK1 spp. in the tree is *Streptomyces* sp. A403Ydz-QZ

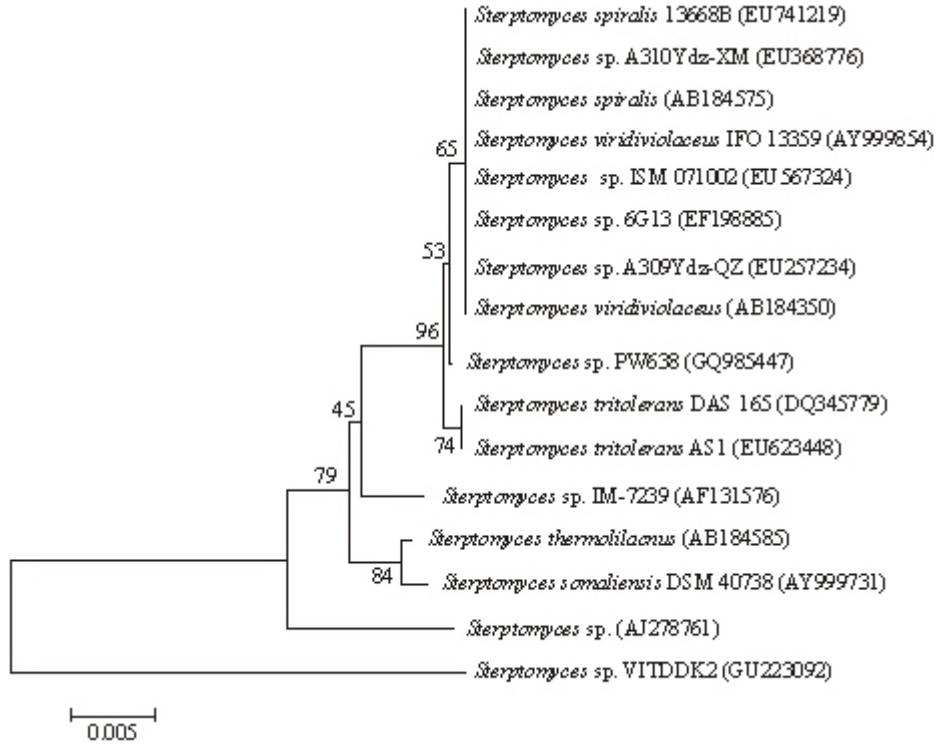


Fig. 6: The taxonomic position of *Streptomyces* VITDDK2 spp. was determined based on 16S rRNA gene sequencing. A phylogenetic tree was constructed based on neighbour-joining method using the MEGA 4.0.2 software. The closest neighbor of *Streptomyces* VITDDK2 spp. in the tree is *Streptomyces* sp. strain 346

similarity of VITDDK1 with *Streptomyces* sp. A403Ydz-QZ (Fig. 5). Similarly VITDDK2 shared 93% similarity with *Streptomyces* sp. strain 346 (Fig. 6). From the above results it can be concluded that though these two strains VITDDK1 and VITDDK2 show phenotypical and biochemical similarity to *Streptomyces orientalis* and *Streptomyces aureomonopodiales* respectively, they bear no genomic relatedness with theses organisms. Due to the non-availability of phenotypic and biochemical data of *Streptomyces* sp. A403Ydz-QZ (EU257237) and *Streptomyces* sp. strain 346 (AJ278761) we are unable to compare these strains with our strains.

Several reports on the antimicrobial activity of actinobacteria isolated from the Eastern Ghats are available whereas only few studies related to the biosurfactant activity and heavy metal resistance were available (Gandhimathi *et al*., 2009; Kokare *et al*., 2007; Joseph *et al*., 2009). Further studies are in progress with respect to the extraction and identification of the lead molecule with biosurfactant and heavy metal resistance activity from the two strains VITDDK1 and VITDDK2.

CONCLUSION

Microorganisms residing in the marine environment are more complex, diverse and rather very unique. Intensive studies are needed to unravel its unexhausted reserve of secondary metabolites. In this paper we have reported the isolation and identification of two strains *Streptomyces* spp. VITDDK1 and *Streptomyces* spp. VITDDK2 with excellent biosurfactant and heavy metal resistance activity.

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