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Research Article

INVESTIGATING THE MICROBIOTA OF SOLAR SALTERN OF MULUND, MUMBAI, INDIA

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ABSTRACT
Solar salterns, located in tropical and subtropical areas worldwide, are artificial shallow ponds for the production of halite (NaCl) from seawater. They are often built as multipond systems. Multi-pond solar salterns have been widespread environments for studies on halophilic microorganisms. In the present study Archaeal and bacterial diversity of the salterns located at Mulund (19°10'12''N, 72°57'18''E), Mumbai was investigated. Three Halophiles were isolated from the brine samples collected. All isolated archaea were shown intensely orange pigmentation. These salt tolerant organisms were characterized using morphological, biochemical and 16S rRNA analysis. Phylogenetic analysis of these sequences revealed a range of identities to several group of bacteria. The clones fell into major lineages the Actinobacteria and firmicutes. Presence of industrially important carotenoids producing halophiles were also recorded.

Keywords: Diversity, Carotenoids, Phylogeny, Solar saltern, 16S rRNA.

1. INTRODUCTION
Multi-pond solar salterns have always been popular environments for studies on halophilic microorganisms. It represents a wide range of salinities, from seawater to halite saturation. The microbial community densities encountered are generally high, and the ponds are often easily accessible [1, 2]. Solar salterns serve as a good model for studying the changes in biodiversity over salinity, thereby providing us with information on extremes of life. As diversity studies on hypersaline environments are gaining momentum since the past decade, the chances of obtaining novel isolates are relatively high when compared with any other ecosystem. Novelty of isolates is not limited to bacteria and archaea but also includes eukaryotes like fungi, protists and algae [3]. Many reports have focused on the occurrence of halophilic archaea in hypersaline regions [4-9]. Microorganisms originating of salterns possess biotechnological prospective for the production of hydrolytic enzymes, exopolysaccharide, carotenoid pigments etc. Besides this, compatible solutes produced in response to an osmotic stress by halophiles are equally important. Carotenoids are used by food and feed industry, as well as in pharmaceutics and cosmetics products, and their demand is growing not only due to their utilization as food colorants, but also on account of their biologic and physiologic roles. Most of the chemical compounds used to color food are produced by chemical synthesis as it is economically feasible. However, the negative perception of the synthetic colorants by consumers has increased the demand of natural pigments, such as carotenoids. Besides, carotenoids are involved in numerous metabolic functions [10], and epidemiological studies support their protective role in prevention of certain diseases.

Large numbers of microbes present in the environment remain uncultured due to a lack of ambient culturable conditions. Therefore, biodiversity studies employing only culture dependent techniques provide limited insight [11]. Culture-independent techniques employing 16S rRNA fingerprinting have provided us with valuable and diverse information since the past two decades [12].

The current study focuses on isolation screening and characterization of halophilic bacteria with the use of morphological, biochemical, and molecular approaches from solar saltern of Mulund, Mumbai, for identifying potential carotenoids producers. These ponds are situated along western coast in Arabian Sea. As there is scarcity of literature on community structure, we aim to investigate the characterization of prokaryotic

biodiversity of these ponds. We have characterized the isolates from these salt pans using a polyphasic approach. As a result of the halophiles' potential, this research was conducted to determine the maximum salt tolerance, investigate, and analyze their carotenoids production.

2. MATERIAL AND METHODS

2.1. Sample collection and isolation of bacteria from solar saltern

The samples were collected from marine solar slatterns of Mulund (E) (19°10'12''N, 72°7'18''E) Mumbai from different locations around the area. All seven samples were mixed in equal proportion and used for isolation of halophiles. They were mixed to get an adequate representation of the local micro-flora. Aliquots of 100µL composite hypersaline samples were plated on solidmedia. Plates were incubated at 37°C for 14 days, number of colonies appeared and colony characteristics were recorded. Among the seven media used for cultivation the medium that showed highest diversity and supported faster growth was selected in further investigation. Using the selected medium minimum salt requirement for growth was determined by varying NaCl concentration in the range of 0 to30%. Distinct colonies were observed and colony characters were recorded. Gram staining of isolates was performed using Dussalt's method [13].

2.2. Biochemical tests

Out of screened microorganisms, 3 salt resistant microorganisms were selected for further analysis. Indole production was detected by growing cells in medium supplemented with 1% (w/v) tryptophan and adding Kovacs' reagent to the culture supernatant. To determine amylase production test, starch agar plates were inoculated and grown cultures were flooded with iodine solution. Production of catalase was determined by adding a 3% (w/v) H_2O_2 solution to colonies on agar. Gelatinase production was detected on agar plates supplemented with 1.5% (w/v) gelatin. Grown cultures were examined for the presence of clear zones around the colonies by flooding the plates with 15% $HgCl_2$ in 20% HCl. Presence of oxidase was determined by observing the formation of a violet colour by inoculating culture on a piece of filter paper moistened with 1% tetramethyl-p-phenylenediamine dihydrochloride. Protease production was determined by observing the formation of clear zones around colonies on agar media amended with 2% (w/v) casein. Formation of turbid zones around colonies on agar medium supplemented with 0.1% (v/v) Tween 80 and 0.01%$CaCl_2.2H_2O$ were considered as positive lipase test [14]. Appropriate positive and negative controls were usedinallthese tests [15].

Effect of *p*H was determined by inoculating screened selected isolates in nutrient broth having various *p*H in the range of 6, 7 and 8. *p*H showing highest growth was selected for further investigation. Effect of temperature was determined by inoculating isolates in nutrient broth and incubating at temperatures in the range of 30°C-40°C. Temperature showing highest growth was selected for further investigation. Optimum incubation time at predetermined *p*H and temperature was calculated by incubating culture and recording optical density afterevery12 hours up to168 hours.

Antibiotic susceptibility was tested by disc diffusion method using disc containing Bacitracin (10 U/disc), Polymyxin (300 µg/disc), Ciprofloxacin (5 µg/disc), Gentamycin (10µg/disc), and Tetracycline (30µg/disc), (Himedia Mumbai, India) [15].

2.3. 16S rRNA sequencing of selected isolates

Three potential cultures which showed favorable growth, and coloration were selected for16s rRNA identification. Glycerol stock of the cultures was revived. After the cultures reached to an optical density of 2.0, 3 mL of culture was centrifuged. CTABlyses buffer (1mL) was added to pellet to form a homogeneous suspension. Proteinase K (2 µL) was also added. Samples were incubated at 60°C for 30 mins. After incubation, equal volume of chloroform:isoamyl alcohol (24:1) was added and centrifuged. Chilled absolute alcohol (2.5 vols.) was added slowly and mixed till the DNA started spooling. To the spooled DNA, 300 µL of 70% chilled alcohol was added and centrifuged. To the pellet, 300 µL of absolute alcohol was added and centrifuged. The sample was air dried and dissolved in 150 µL TE buffer to be stored at 4°C.

For visualization of gDNA, samples were loaded onto 0.7% agarose gel stained with 0.1µg/mL ethidiumbromide. Once the gDNA's integrity was examined on the gel, the samples were quantified with Nanodrop (Thermo Scientific).Template concentration for amplification was standardized using 10-100 ng of gDNA. Samples were diluted to the given conc. using TE buffer.

The annealing temperature was set at 55°C as per the Tm values provided by the manufacturer. The cycling conditions of PCR amplification of the 16s rRNA region was set as follows: Initial denaturation at 96°C for 5

mins followed by 35 cycles of denaturation at 95°C for 45 secs, annealing at 55°C for 45 secs and extension at 72°C for 60 secs followed by final extension at 72°C for 5 mins and hold at 4°C. The reaction volume for PCR was fixed at 50µL. The final conc. of components in the reaction volume was as follows: 2.5 mM $MgCl_2$, 1 X Taq Buffer, 0.2 mM dNTPs, 0.5 µM primers, 5 U Taq polymerase, standard concentrations of the template DNA, diluted to a final volume of 50 µL using nuclease free D/W. The amplicon was observed on 1% agarose gel. A 900 bp amplicon was observed under UV transilluminator. The corresponding band was carefully excised, and the amplicon was purified using QiagenPCR purification kit as per the protocol mentioned by the manufacturer.

The amplicon was quantified on Nanodrop, and its quality was examined by estimating the A260/280ratio. Next, the amplicon was sequenced on Beckman Coulter CEQ8000+. Sanger's sequencing reaction was setup as follows: 100 fM of template DNA (amplicon) was taken and 11 µL Premix (provided in the kit) was added to it. Forward (8F) and reverse (907R) primers at a conc. Of 1.6µM were added to the reaction mix and the volume was adjusted to 20 µL using nuclease free D/W. Controls were set using pUC vector provided in the kit. The cycling conditions for the Sanger's PCR was set as follows: 30 cycles of denaturation at 96°C for 20 secs, followed by annealing at 50°C for 20 secs and extension at 60°C for 4mins and a final hold at 4°C .Stop solution was prepared as follows : 2µl of 3M Sodium acetate (dissolve 2.46 g sodium acetate in 10mL D/W), 2 µl of 100 mM disodium EDTA (dissolve 0.372 g disodium EDTA in10mLD/W) and 1µl of 20 $mgmL^{-1}$ glycogen (provided in the quick start kit). The stop solution was added to the PCR product followed by cold 95% ethanol and centrifuged at 14,000 rpm for 15 mins at 4°C. The pellet was air dried and resuspended in 40µL of sample loading solution (provided in the kit). The resuspended pellet was put in the sample tray and sample loading buffer was added in the buffer tray. The capillaries, wetting tray, sample plate, buffer plate and gel cartridge were installed. On the software, "test sequence" method was selected and the samples were run. Chromatograms were generated and the raw data was analyzed using the Multalin software and BLAST feature provided by NCBI. Sequenced samples showing maximum homology to the cultures deposited in the database were selected and its genus and species name was assigned to the isolated cultures [16]. The phylogenetic tree was constructed in MEGA11 using the neighbor joining (NJ) method along with the Boot strap Phylogeny test with number of boot strap replications set at 1000 and the Tamura-Neimodel [17].

2.4. Accession numbers

The 16S rRNA sequences determined in this study were deposited in the Genbank sequence database under the accession numbers: OP268236.1, OP268237.1 and ON062192.1 (Fig.1a-c).

3. RESULTS AND DISCUSSION

3.1. Isolation and screening of halophilic bacteria

The traditional seasonal salt pond of Mulund, Mumbai, was sampled. Water samples were pale yellow in color and viscous. Total: 1 shows halophilic organisms that were isolated and designated as SC-14, SC-15 and SC-16. Amongst the 3 isolates, 2 were pigmented and intense coloured colonies. Colonies of all selected isolates were circular, orange to yellow in colour, small in size having raised elevation and entire margin. Out of all isolates two developed orange colonies while one isolate has developed yellow color colony. All isolates were Gram negative and circular in shape. All isolates showed luxuriant growth at 5% salt concentration at 30°C temperature.

SC-14 used glucose, sucrose, maltose, fructose and trehalose as a carbon source and SC-15 has used all sugars as a carbon source except sorbitol, whereas, SC-16 has used only glucose fructose and trehalose as energy source. However, none of the isolates has used sorbitol as carbon source. Enzyme profile of isolates showed that out of 3, SC-15 and SC-16 have secreted extracellular amylase. All the isolates showed positive oxidase test.

All the three isolates showed remarkable growth at pH 7, temperature 30°C and salt conc. at 5 %. All the 3 isolates were tested for antibiotic susceptibility isolates. Both SC-15 and SC-16 were sensitive to polymyxin B, ciprofloxacin, Tetracyclin and resistant to gentamycin. Pattern of sugar utilization, enzyme profile and antibiotic sensitivity of isolates is given in Table1.

3.2. 16S rRNA sequencing

Partial sequences of 16S rRNA fragments were amplified from 5' terminus. Phylogenetic analysis of these sequences revealed arrange of identities to several group of bacteria (Table 2). The clones fell into major lineages the Actinobacteria and firmicutes. Our study describes not only the existence of bacterial and archaeal diversity of solar salterns of Mulund, but also indicates presence of

industrially important cultures which could be used as efficient producers of carotenoids, hydrocarbon. Most of our isolates from solar saltern of Mumbai are related to halophilic and haloalkalophilic organisms reported earlier from solar saltern of Tamilnadu [18], Bhavnagar [19], Puerto Rico [20], Almeria and Tunisian multi pond solar salterns [21].

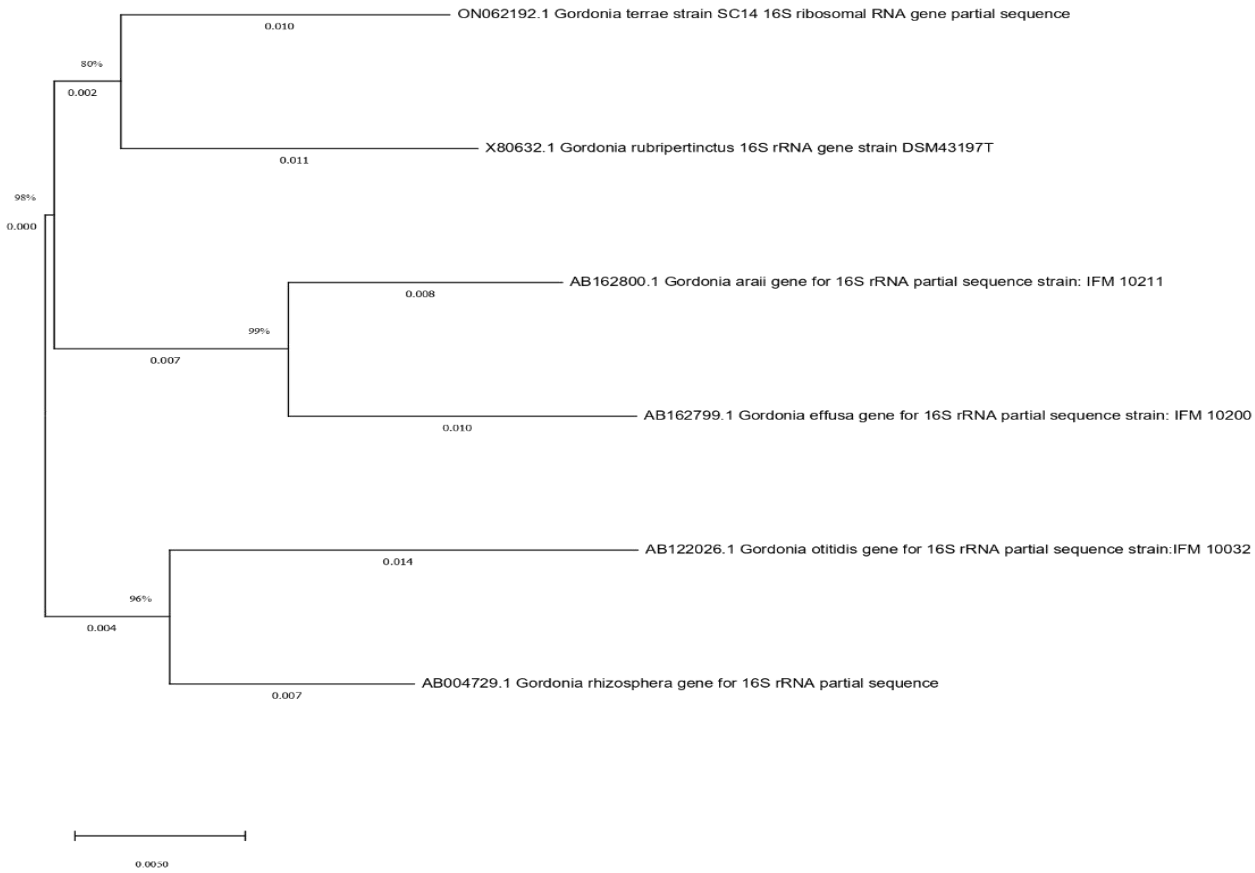
Table1: Biochemical characteristics o fisolated microorganisms

Characteristics	SC-14	SC-15	SC-16
Morphology	Circular	Circular	Circular
Gram nature	-	-	-
Cell size (µm)	2mm	2mm	2mm
Motility	Motile	Motile	Motile
Margin	Entire	Entire	Entire
Colony pigmentation	Orange	Yellow	Orange
Opacity	Opaque	Opaque	Opaque
NaCl Range for growth (%)	5-20	5-20	5-20
Temp. optimum (°C)	30	30	30
pH Optimum	+	+	-
Utilization of			
Glucose	+	+	-
Sucrose	+	+	+
Arabinaose	-	+	-
Lactose	-	+	-
Maltose	+	+	-
Fructose	+	+	+
Mannitol	-	+	-
Xylose	-	+	-
Sorbitol	-	-	-
Trehalose	+	+	+
Galactose	-	+	-
Indole production	-	-	-
Enzyme Profile			
Catalase	+	+	+
Amylase	-	+	+
Gelatinase	-	-	-
Urease	-	-	-
Protease	-	-	-
Cellulase	-	-	-
Oxidase	+	+	+
Antibiotics susceptibility			
Bacitracin (10 unit/disc)	ND	ND	ND
Polymyxin (300mcg/disc)	ND	S	S
Ciprofloxacin (5mcg/disc)	ND	S	S
Gentamycin (10mcg/disc)	ND	R	R
Tetracyclin (30mcg/disc)	ND	S	ND
Identified as	*Gordonia Terrae*	*Rothia kristinae*	*Nialliaalba*

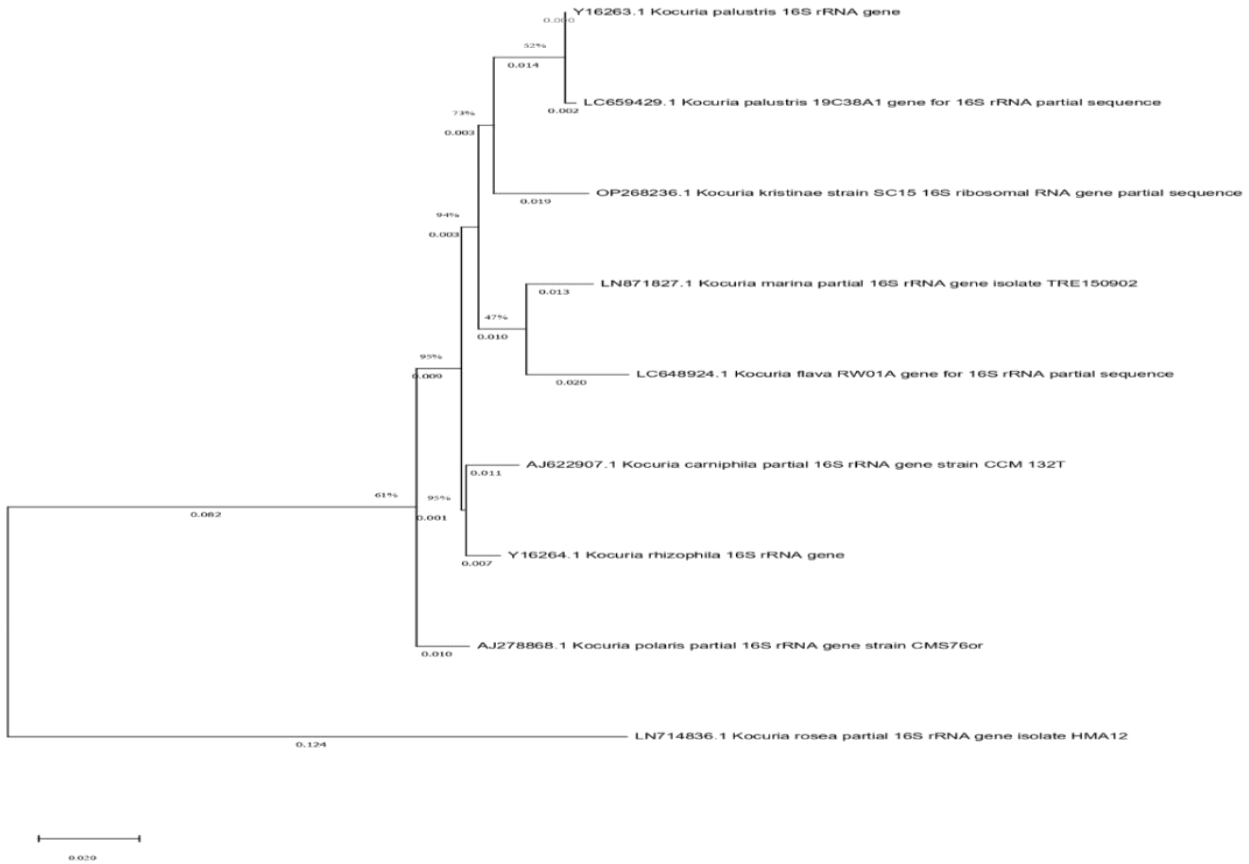
ND=Not detected, S=Sensitive R=Resistant

Table 2: Samples color and identification by 16S rRNA gene analysis of 3 strains isolated from marine solar slatterns of Mulund (E) (19°10'12"N, 72°7'18"E) Mumbai

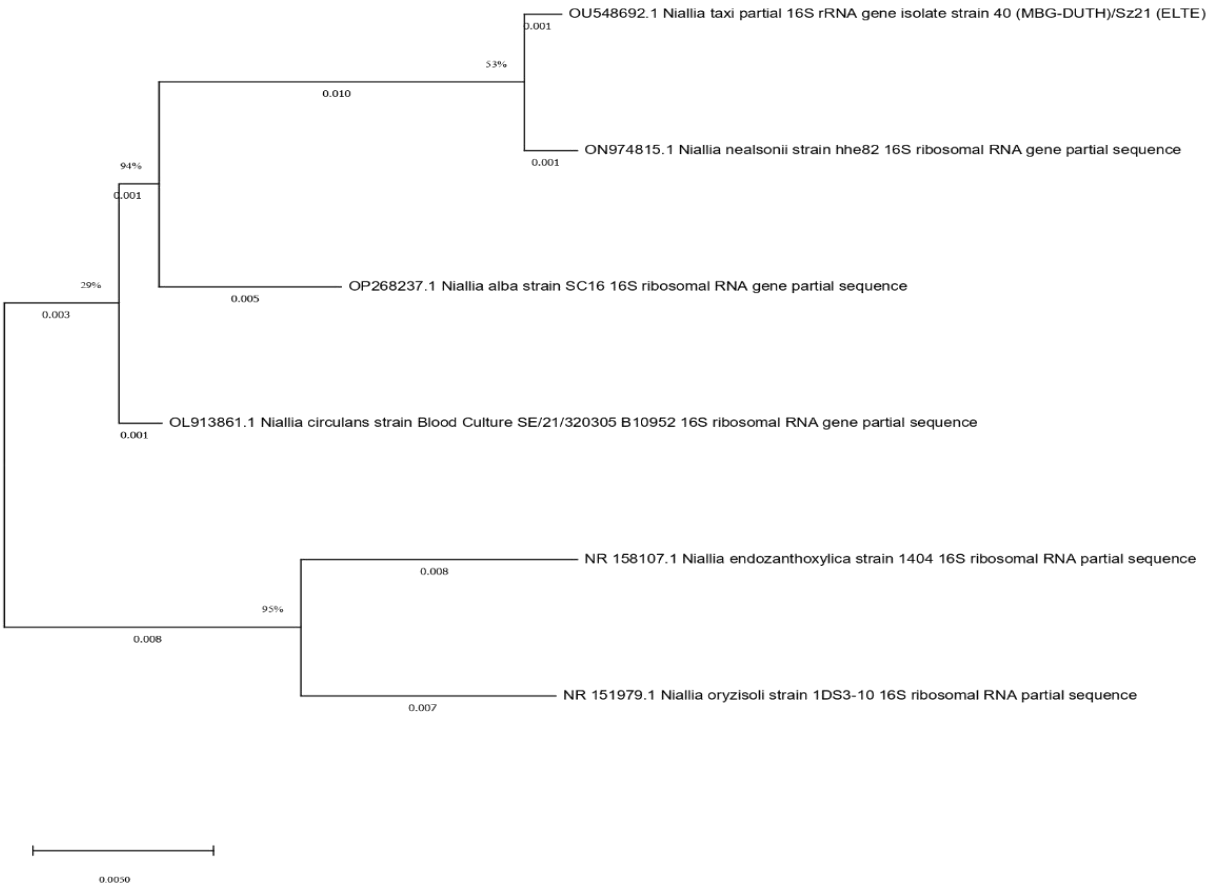
Name	**Accession Number**	**Color**	**Database microorganism**
SC-14	ON062192.1	Orange	*Gordonia Terrae*
SC-15	OP268236.1	Yellow	*Rothia Kristinae*
SC-16	OP268237.1	Orange	*Nialliaalba*


ON062192.1 Gordonia terrae strain SC14 16S ribosomal RNA gene partial sequence
0.010
80%
0.002
X80632.1 Gordonia rubripertinctus 16S rRNA gene strain DSM43197T
0.011
98%
0.000
AB162800.1 Gordonia araii gene for 16S rRNA partial sequence strain: IFM 10211
0.008
99%
0.007
AB162799.1 Gordonia effusa gene for 16S rRNA partial sequence strain: IFM 10200
0.010
AB122026.1 Gordonia otitidis gene for 16S rRNA partial sequence strain:IFM 10032
0.014
96%
0.004
AB004729.1 Gordonia rhizosphera gene for 16S rRNA partial sequence
0.007
0.0050

(a)


Y16263.1 Kocuria palustris 16S rRNA gene
52%
0.014
LC659429.1 Kocuria palustris 19C38A1 gene for 16S rRNA partial sequence
0.002
73%
0.003
OP268236.1 Kocuria kristinae strain SC15 16S ribosomal RNA gene partial sequence
0.019
94%
0.003
LN871827.1 Kocuria marina partial 16S rRNA gene isolate TRE150902
0.013
47%
0.010
95%
0.009
LC648924.1 Kocuria flava RW01A gene for 16S rRNA partial sequence
0.020
AJ622907.1 Kocuria carniphila partial 16S rRNA gene strain CCM 132T
0.011
61%
0.082
95%
0.001
Y16264.1 Kocuria rhizophila 16S rRNA gene
0.007
AJ278868.1 Kocuria polaris partial 16S rRNA gene strain CMS76or
0.010
LN714836.1 Kocuria rosea partial 16S rRNA gene isolate HMA12
0.124
0.020

(b)



(c)

Fig. 1 (a-c): Phylogenetic tree showing the relationship among 16S rRNA gene sequences of isolate from Solar saltern, Mulund, Mumbai, India obtained in this study. The tree was constructed using neighbor joining tree

4. CONCLUSION

Solar salterns are known to harbor high number of taxonomically diverse halophilic organisms. Understanding of this ecosystem is therefore highly desirable. Present investigation for first time reported presence of halophilic bacteria and archaea from solar saltern of Mulund, Mumbai, India. Different isolates are also reported by different groups from solar salterns distributed worldwide. Microbiota reported in this work includes many of salt stable protease and carotenoid pigment producing Halophiles.

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Conflicting interest

No conflict of interest.

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