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Studies on siderophore producing halophilic and halotolerant bacteria adhered to mangrove plant litter

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ABSTRACT

Mangroves are unique ecosystems due to their ability to thrive in salt and saline water. In this ecosystem the nutrient recycling occurs consistently with the formation of detritus. Microbes play a major role in recycling and therefore factors affecting their growth influence the rate of mineralization. One of the factors is the availability of iron, the availability of which is limited. Under such conditions bacteria produce iron sequestering ligands called siderophores which help to get iron in soluble form that can be used by the bacterial cell for its cellular and metabolic needs. A total of 34 isolates were obtained by plating on Zobell marine agar and NaCl-tryptone-yeast extract agar. Amongst these 68% were found to be halophilic and 32% were halotolerant bacteria based on their ability to grow in varied salt concentrations. Further screening of the selected isolates for production of siderophores using CAS medium showed that 97% produced siderophores. The 4 best isolates were further identified based on biochemical and molecular characterization and two were identified to be *Halobacillus sp*, and the other two as *Acinetobacter schindleri* and *Brevibacterium casei*.

KEYWORDS
Mangroves, adherence, siderophores, halophiles, halotolerant.

Introduction

Mangroves are unique coastal ecosystems that are constantly under the influx of tides and thus experience a constant fluctuation in salinity. Under such conditions halophilic and halotolerant bacteria are said to be predominant in this ecosystem. Due to the sweeping action of the tides the free living bacteria get washed out into the sea and it has been reported in turbid ecosystems, such as estuarine and mangroves, that most of the degradation is brought about by the attached or adhered bacteria (Tang *et al.* 2012, Crump *et al.* 1998 Griffith *et al.* 1994).

Bacteria require many elements for their growth and metabolism. Limitation of any element will hamper its growth and survival and in turn affect the rate of degradation and mineralisation in the mangrove ecosystem. Though iron is required in trace amounts, it is an important part of many enzymes and metabolic activities of the bacteria. Despite iron being the most abundant elements in the earth's crust, its bioavailability is limited by the low solubility of Fe (III) ion. It mostly occurs in mineral phases such as iron oxides and hydroxides which cannot be readily utilized by the organisms (Wittenwiler, 2007). Microorganisms like bacteria thus release siderophores which are iron binding ligands that have a high affinity to Fe (III) ion and form soluble siderophore-Fe (III) complexes which are then taken up by the bacterial cell by active transport mechanisms (Balagurunathan and Radhakrishnan, 2007, Neilands, 1995, Messenger and Barclay, 1983).

The present study was focused on studying the halophiles and halotolerant bacteria adhered to mangrove plant litter in Goa and their ability to produce siderophores. The selected bacterial isolates were further identified using morphological, cultural, biochemical and molecular characterization.

Materials and methods

Sample collection

Plant litter samples were collected from the mangrove areas during low tides (Fig. 1). The samples were put into sterile petriplates and processed immediately upon reaching the lab.

Isolation of adhered bacteria

10 g of mangrove plant litter sample was added to 100 ml of normal saline and kept on shaker at 150 rpm for overnight at room temperature. This was centrifuged at 1500 rpm for 10 mins and the plant litter pellet was rinsed thrice with 50 ml sterile normal saline. The pellet was then ground with 20 ml of 0.1 M EDTA solution using mortar and pestle and centrifuged at 1500 rpm for 10 mins (Chart *et al.* 1997). Dilutions of the supernatant were prepared and plated on Zobell marine agar (ZMA) and NaCl- tryptone- yeast extract (NTYE) agar to obtain isolated colonies of bacteria.

Classification of the bacterial isolates based on their ability to grow at different salt concentrations

The bacterial isolates were plated on solid media such as nutrient agar (NA) and NTYE agar and the growth was monitored upto 96 hrs. Based on the ability to grow on media with different salt concentrations these isolates were categorized based on the classification of Horikoshi *et al.* 2011.

Figure 1. Map of Goa showing the sampling sites.

Adherence of selected bacterial isolates to hydrocarbons using the BATH assay

The ability of the 16 selected bacterial isolates to adhere to hydrocarbons was seen using the bacterial adhesion to hydrocarbon (BATH) assay (Qiao *et al*, 2012, Tuleva *et al.* 2002, Rosenberg, 2006, Rosenberg, 1991). The isolates were grown on Zobell marine broth (ZMB) and NTYE broth overnight at room temperature in shaker conditions of 150rpm. These were then centrifuged at 6000 rpm for 10 mins to obtain pellet and supernatant. The supernatant was discarded and the pellet of each isolate was dissolved in 5ml of 9.6 mM sterile phosphate buffered saline (PBS). 0.5 ml of hexadecane was added to 2ml of this PBS suspended pellet. The tubes were vortexed for one minute and the initial absorbance (A_0) was measured at 450 nms. The tubes were allowed to stand for 30 minutes and the final absorbance (A_1) was measured of the lower layer at the same wavelength. Adhesion percentage (A%) was calculated using the formula

$$A\ \% = \frac{A_0 - A_1}{A_0} \times 100$$

Screening of the isolates for production of siderophores

The 16 Selected isolates were spot inoculated on CAS (Chrome Azurol sulphonate) agar (Gaonkar *et al.* 2012, Leong and Neilands, 1976). CAS with $FeCl_3$ was used as the negative control for siderophore. The plates were incubated at room temperature and observed for a yellowish- orange zone around the bacterial colonies.

Characterization and identification of the selected isolates

The cultural, morphological and biochemical characteristics of the 4 bacterial isolates showing adhesion and siderophore production were determined and the isolates were tentatively identified upto the genus level based on Bergey's Manual of Systematic Bacteriology. These 4 isolates were further subjected to partial sequencing of 16S rRNA gene. The genomic DNA was extracted by cetyl trimethyl ammonium bromide (CTAB)- proteinase K method as described by Ausubel *et al.* 1995. After the extraction, the concentration and purity of the DNA was measured by NanoDrop spectrophotometer (Thermo Fisher Scientific). 16S rRNA gene was amplified using standard universal primer U1 5'-CCAGCAGCCGCGGTAATACG-3' and U2 5'-ATCGG (C/T)TACCTTGTTACGACTTC-3'. The derived 16S rRNA gene sequence was compared with sequences in the GenBank database using BLAST.

Results and Discussion

Isolation of adhered bacteria from mangrove plant litter

A total of 34 isolates were obtained from the treated plant litter sample, out of which 73% were isolated on ZMA, 21% on 15% NTYE agar and 6% on 25% NTYE agar (Fig. 2). The results indicated that most of the isolated adhered bacteria were halophilic in nature. Studies with such plant litter samples have shown presence of *Chromobacterium violaceum*, *Flexibacter*, *Flavobacterium*, *Cytophaga*, *Achromobacter* and *Pseudomonas* species (D'Costa *et al.* 2004, Das et al. 2007). Further some halophilic bacteria such as as *Halomonas maura, Marinobacter sp, Vibrio sp, Pseudoalteromonas, Sagittula stellata, Hyphomonas strain MHS-3* isolated from marine and estuarine ecosystem have also shown ability to adhere to substratum in the natural environment (Gonzalez *et al.* 1997b, Quintero *et al.* 1998, Gulig *et al.* 2005).

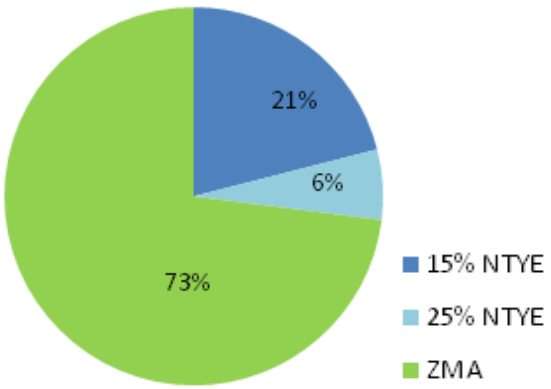

Figure 2. Total viable count (TVC) of adhered bacteria on different isolation media.

Classification of isolates based on their ability to grow at different salt concentrations.

Classification of bacteria based on their ability to grow in presence of different salt concentrations of NaCl is well studied and reported by Horikoshi *et al.* 2011 and Kushner (Dworkin *et al.* 2006). The preliminary classification of halophilic and halotolerant has now been revised and presented into six categories based on the concentration of NaCl ranging from1%- 32% (Horikoshi *et al.* 2011).

The isolates obtained during the present study were also classified into categories based on their ability to grow on different concentrations of NaCl in the solid medium. It was interesting to note that out of 34 isolates 68% were halotolerant bacteria and 32% were halophiles (Fig. 3). Although reports on free living bacteria in the mangroves have shown the presence of halotolerant bacteria such as *Micrococcus, Microbacterium* (Yateem and Sharrah, 2011), *Halococcus* (Sahoo and Dhal, 2009), *Microbulbifer* (Britto *et al.* 2006). However comparatively few reports are available on halophilic and halotolerant bacteria from mangrove plant litter (D'Costa *et al.* 2004). Significantly 45% of the halophiles were found to be extreme halophiles as they showed growth in media containing more than 15% NaCl. Earlier studies by Ventosa *et al.* 1998 and Rodriguez- Valera *et al.* 1979, have reported extreme halophiles such as *Salinivibrio costicola*, *Halomonas halodenitrificans* and *Halococcus sp.*

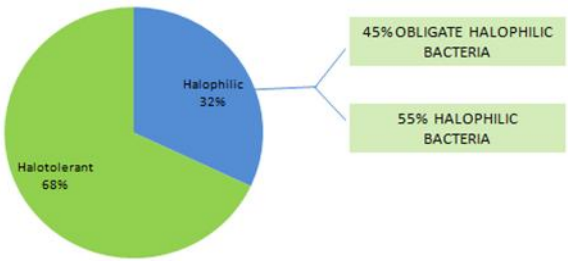

Figure 3. Distribution of halophilic and halotolerant bacteria among the isolates.

Adherence of selected bacterial isolates to hydrocarbon using BATH assay.

Mechanism of adhesion has shown that hydrophobic surface of bacteria is more conducive to adhesion than hydrophilic cell surface (Katsikogianni and Missirlis, 2004). In order to understand the nature of their cell surface the 16 selected isolates were subjected to BATH assay.

Among the halophiles 67% showed adherence while almost all halotolerant bacteria were capable of adhering to the hydrocarbon. This indicated that the cells had a hydrophobic cell surface that helped them to adhere to the substrate. Maximum adherence was shown by giving a reduction in turbidity of almost 36% by isolate no. MXM-8 which was identified as *Acinetobacter schindleri MXM-8.*

Screening of the bacterial isolates for siderophore formation

Significance of siderophore producing bacteria has been well documented (Saha *et al.* 2013, Essen *et al.* 2007, Chaiharn *et al.* 2009). Such bacteria are known to be useful not only for scavenging iron but also in bioremediation and phytoremediation processes (Gaonkar and Bhosle, 2013). Such bacteria are important in nutrient deficient ecosystems such as sand dunes (Gaonkar *et al.* 2012, Nayak *et al.* 2013, Godinho and Bhosle, 2013).

In iron deficient environments such as marine ecosystems the survival of such adhered bacteria is dependent on their ability to scavenge iron. In the present study therefore the selected isolates were screened for siderophore production using CAS agar. It was observed that all halophilic isolates produced siderophores as evident from the zone of yellow- orange colouration (Fig. 4). Amongst the halotolerant bacteria 97% produced siderophores depicting iron deficiency of available iron for growth in the mangrove ecosystem. Earlier studies have shown siderophore producing halophilic and halotolerant bacteria in mangrove and coastal ecosystems (Selvam and Kathiresan, 2010, Kannapiran and Ramkumar, 2011). Some of the well known siderophore producing bacteria are *Pseudomonas* (Matthijs *et al.* 2004, Gaonkar *et al.* 2012), *Escherischia coli, Bacillus subtilis* (Gaonkar *et al.* 2012).

Characterization and identification of the selected isolates

Based on the characteristics, the isolates were identified using Bergey's Manual of Systematic Bacteriology. The 16S rRNA of the 4 isolates were sequenced and the sequences were deposited in the GenBank. Two of the four bacterial isolates were identified as *Halobacillus sp. (MXM-5)*, *Halobacillus sp. (MXM-16)*, one as *Acinetobacter schindleri (MXM-8)* and MXM-7 as *Brevibacterium casei.* Earlier studies have reported siderophores of *Acinetobacter sp.* (Dorsey *et al.* 2004, Okujo *et al.* 1994) and *Brevibacterium sp.* (Noordman *et al.* 2006).

Phylogenetic analysis of the 16S rRNA of isolate MXM-8 which showed the highest percentage in adhesion and siderophore production showed 96% similarity (based on BLAST) to *Acinetobacter schindleri strain XFB-P* and *Acinetobacter schindleri strain ESHS9* while MXM-5 and MXM-16 showed 99% similarity (based on BLAST) to *Halobacillus sp. H-93 Halobacillus sp. MON009* and MXM-7 showed 92% similarity (based on BLAST) to *Brevibacterium casei strain DY40-62* and *Brevibacterium casei strain XFB-P.*

Conclusion

The adhesion ability of bacteria is an important aspect in the attachment of the bacteria to the substrate and its degradation in the mangrove ecosystem. It also provides the bacteria the ability to form biofilms. Halophilic and halotolerant bacteria such as *Halobacillus, Acinetobacter* and *Brevibacterium* from the mangroves bring about degradation

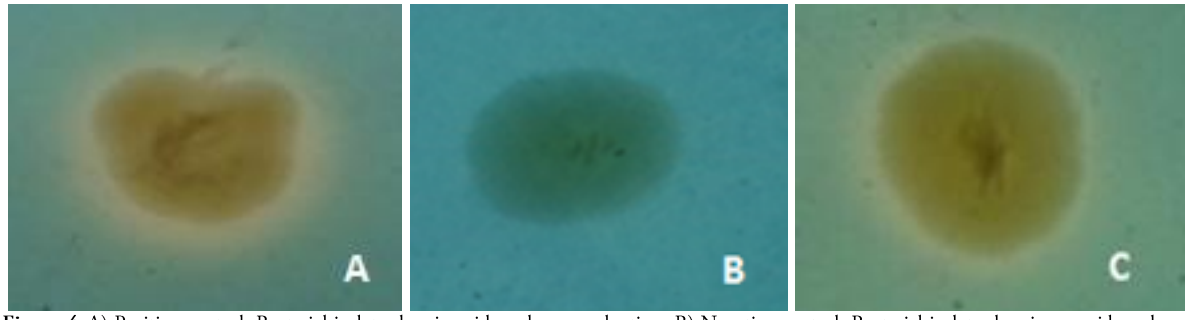

Figure 4. A) Positive control: Bacterial isolate showing siderophore production. B) Negative control: Bacterial isolate showing no siderophore production. C) Bacterial isolate MXM-16 showing siderophore production.

by adhesion to the leaf litter. These heterotrophic bacteria are also capable of producing siderophores for sequestering Fe (III) from the mangrove soil. Siderophores being very important ligands in metal sequesteration are now being looked at as future prospects in metal contaminated soil and water remediation. Thus siderophores from halophilic and halotolerant bacteria can hold tremendous potential in bioremediation of metal contaminated saline environments. Due to their adhesive ability the bacteria remain localized and are thus more efficient in bioremediation and biodegradation processes.

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