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SCREENING AND IDENTIFICATION OF SIDEROPHORE PRODUCING MARINE BACTERIA

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ABSTRACT
Siderophores are low molecular weight compounds that are secreted by bacteria under conditions of low iron. The levels of soluble iron are comparatively very low in the marine environment. Therefore, marine bacteria secrete diverse siderophores which help them to compete for iron in the dilute environment. The present study reports the isolation of siderophore producing bacteria from marine environment. Out of 48 isolates obtained, 03 isolates namely S31, S34 and W16 were found to be potent siderophore producers. The siderophore production was confirmed using CAS assay. The morphological, biochemical and molecular identification of these isolates reveal them to be from the genus *Pseudomonas* and *Bacillus*.

KEY WORDS: 16 s RNA, iron chelation, CAS assay, *Pseudomonas*, MegaBlast

INTRODUCTION

Iron is an essential element and the second most abundant metal after Aluminium in the earth's crust. However, at biological pH and under aerobic condition iron is oxidised to its insoluble form which makes it unavailable for microorganisms (Raval and Desai, 2015). To overcome this limitation, microorganisms produce low molecular weight compounds that bind ferric iron with an extremely high affinity. These compounds known as siderophores play an important role in sensing and uptake of iron (Bindu and Nagendra, 2016). They are produced under iron limiting conditions and they change iron to a soluble form making it available for the cell (Sujatha and Ammani, 2013). The mechanism involves scavenging iron from mineral phases by formation of soluble Fe^{+3} complexes that can be taken up by energy dependent membrane transport mechanism and thus bind it for transport into bacterial cells. Thus, siderophore act as solubilising agents for iron from minerals or organic compounds under conditions of iron limitation (Gamit and Tank, 2014). Depending on the functional group present, siderophores have been divided into three main families namely, catecholates, hydroxamates and carboxylates (Radhakrishnan *et al.*, 2014). Siderophores have wide applications in weathering soil minerals and soil formation, enhancing plant growth as PGPRs, biocontrol of pathogens, nuclear fuel processing, bioremediation of pollutants, recycling of iron in the ocean, bio-bleaching of pulps and as biosensors for iron detection (Maurya *et al.*, 2015). Most of the siderophores till date have been reported from a variety of organisms inhabiting diverse environments. Out of these siderophores, a majority of them have been isolated from terrestrial counterparts more commonly the soil rhizosphere (Dimpka, 2016). The reports for siderophores from the aquatic environment have been restricted to fresh water only (Christinia *et al.*, 2015) and for marine counterparts to marine based phytoplanktons and sediments (Trivedi *et al.*, 2016). Hence, the present study was carried out to screen for potential siderophore producing marine bacteria followed by their identification using conventional microbial methods and 16sDNA based analysis.

MATERIALS AND METHODS

Screening of siderophore producing bacterial isolates

Marine water samples from different parts of India were collected in sterile bottles at surface and at 10m depths. These were from the coastal regions of a) Kerala (Kovalam beach, Latitude 8.4004° N, Longitude 76.9787° E) b) Diu and Daman (Jampore beach, Latitude 20.3809° N, Longitude 72.8239° E) and c) Maharashtra (Ganpatipule, Latitude 17.1489° N, Longitude 73.2727° E). Soil samples around the shore were collected in sterile plastic bags and all the samples were stored under sterile conditions at 4°C for preventing bacterial cross contamination until further use (Balraj *et al.*, 2014). The subsequent isolation of the bacterial strains was carried out within 48hrs by spread plate technique on sterile. Zobells agar medium. Well isolated colonies were selected, purified and maintained on Zobell agar slants. To check for the presence of siderophore, isolates were streaked on Chrome Azurol S (CAS) plates. CAS agar plates were prepared by overlaying the blue dye on basal medium comprising of sterile Zobell's agar. The blue dye was prepared by dissolving 60.5mg CAS in 50ml distilled water (Solution 1). Solution II was prepared by dissolving 1mM ferric chloride ($FeCl_3.6H_20$) in 10mM HCl. Solution I and II were mixed and to this solution 72.9mg of hexadecyl trimethyl ammonium bromide (HDTMA) dissolved in 40ml D/W was added by constant stirring. The resultant blue dye was autoclaved separately and added to molten N/A butts for preparation of CAS plates.

After spot inoculation, the CAS agar plates were incubated at room temperature (RT) for 24 - 48 hrs. Development of yellow / orange zones around the growth was considered positive for siderophore production (John and Thangavel, 2015).

Quantitative estimation of siderophore

The quantitative estimation of siderophore was done by CAS shuttle assay. The medium used for siderophore estimation was sterile Zobell's broth (Cabaj and Kosakowska, 2009). The isolates were inoculated in sterile Zobell's broth and incubated at RT for minimum 72 hours. Siderophore production was monitored at an interval of 24 hours upto 72 hours. Following each interval of incubation, the broth was centrifuged at 10,000 rpm for 15 minutes and the cell free supernatant (CFS) was determined for siderophore production using CAS shuttle assay. Equal volume of CFS was mixed with CAS reagent and incubated at RT for 30 minutes. The absorbance was measured at 630nm using a UV spectrophotometer (UV-1700 Schimadzu, Japan) against a reference containing equal volume of uninoculated medium and CAS reagent. The amount of siderophore in the CFS was calculated using the formula:

$\% SU = (A_r – A_s / A_r) \times 100$

Where %SU = % Siderophore Units, A_r = absorbance of reference at 630nm and A_s = absorbance of sample at 630nm (Ghosh *et al.*, 2015).

Characterization and identification of potential siderophore producing isolates

The isolates exhibiting maximum siderophore production as estimated by CAS shuttle assay were subjected for identification.The cultural, morphological and biochemical characteristics of potent siderophore producers were determined and the isolates were identified based on Bergey's Manual of Systemic Bacteriology. In addition to biochemical analysis, the isolates exhibiting maximum siderophore production were subjected to molecular characterization by partial sequencing of 16s rRNA gene. The genomic DNA was extracted using the Bangalore Genei Kit for DNA isolation. The extracted DNA was confirmed for purity using agarose gel electrophoresis. The concentration of DNA was measured using UV spectrophotometer (UV-1700 Schimadzu, Japan). The A260/A280 ratio was calculated to confirm the purity of isolated DNA (Srividya *et al.*, 2011). The isolated DNA was sent for sequencing at Eurofins India Pvt. Ltd. 16s rRNA gene was amplified using standard universal forward primer (5'AGAGTTTGATCATGGC TCA3') and standard universal backward primer (5'TAC GGTTACCTTGTTACGACTT3'). The derived 16s rRNA gene sequence of the potential siderophore producing bacteria was subjected to homology search sequence in the GenBank database (NCBI) using the BLAST search program and aligned by using the multiple alignment Clustal X program (Gaonkar *et al.*, 2011).

RESULTS & DISCUSSION

A total of 48 isolates were obtained on Zobell agar medium out of which only 4 isolates exhibited significant biological activity. These 4 isolates were further screened for siderophore production and were denoted as S31, S34, W15 and W16.

Screening of potential siderophore producing isolates and quantitative estimation of siderophore:

The siderophore producing isolates exhibited yellowish orange growth on CAS agar plates after 48-72 hours of incubation as shown in Figure. 1. Results were visually distinct in terms of halo formation against the blue medium. The basic principle underlying the test is that when a strong ligand like siderophore is added to a highly coloured Fe^{+3}- dye complex, the iron-ligand complex is formed and the release of free dye is accompanied by colour change (Sasirekha and Srividya, 2016).

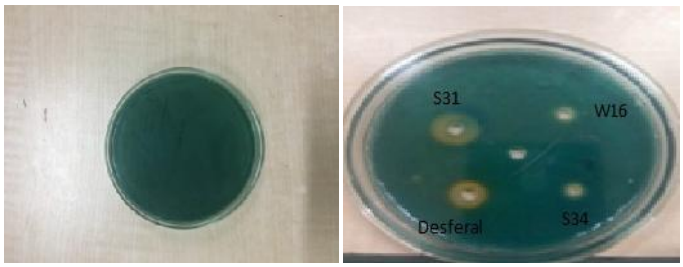

FIGURE 1: From left: CAS agar plate (control) and siderophore positive isolates (right) exhibiting yellow zone.

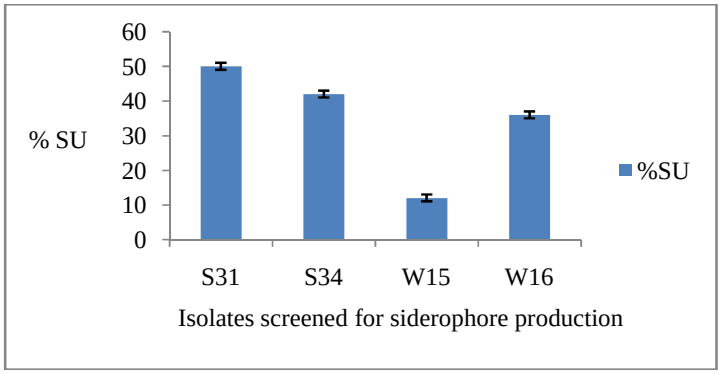

FIGURE 2: Siderophore production by marine isolates

Quantitative production of Siderophores:
The amount of siderophore produced in the CFS was determined by CAS shuttle assay. Figure 2 depicts the amount of siderophore produced by each isolate in marine broth at room temperature. Of the four siderophore producing isolates, S31 was found to be most efficient in producing siderophore (50 ±1.0% SU) while W15 was found to be least efficient (12 ± 0.5 % SU).

Identification of potent siderophore producing isolates:
The isolates S31, S34 and W16 were selected for further identification as they exhibited more than 35%SU production. The isolates were identified based on their morphological and biochemical characteristics using the HiMedia kit for identification. Table 1 indicates the morphological characteristics of these marine isolates. Both S31 and S34 were Gram negative rods while W16 depicted Gram positive rods in chain.

TABLE 1: Colony characteristics of the siderophore producing isolates on Zobell's agar plate after 72 hrs of incubation at room temperature

Colony characters	S31	S34	W16
Size	1mm	1mm	2 mm
Shape	Circular	Circular	Circular
Colour	Greenish blue	Yellowish	Yellow
Margin	Undulate	Entire	Entire
Elevation	Elevated	Elevated	Elevated
Opacity	Translucent	Opaque	Opaque
Consistency	Mucoid	Mucoid	Mucoid
Grams nature	Gram negative rods	Gram negative rods	Gram positive rods in chain
Motility	+	+	+
Spore staining	-	-	+

TABLE 2: Biochemical characterization of the siderophore producing isolates using Hi-Assorted kit for Gram negative rods and Hi-Bacillus identification kit

Tests	S31	S34	W16
Citrate	+	+	+
Lysine	-	-	-
Ornithine	-	-	-
Urease	-	Weakly +	+
TDA	+	+	-
Nitrate	+	+	+
H_2S	-	-	-
Glucose	+	+	+
Adonitol	-	-	-
Lactose	-	-	-
Arabinose	-	-	-
Sucrose	-	-	-
Maltose	-	-	-
Galactose	-	-	-
Mannitol	Weakly +	-	+
Sorbitol	-	-	-
Catalase	+	+	+
Gelatinase	-	-	+
Oxidase	+	+	-

Key: ' +' indicates positive result and '–' indicates negative result

The biochemical characterization of all the three isolates was in accordance to the results obtained by Gram staining. Based on the results of biochemical characterization, the isolates S31 and S34 were identified to be in genus *Pseudomonas*. The results of the biochemical tests (as depicted in Table 2), suggest that the isolate S31 could be *Pseudomonas aeruginosa*, S34 as *Pseudomonas simiae* and the isolate W16 could be *Bacillus paralicheniformis*. Based on morphological and biochemical characterization, further molecular characterization using 16s RNA sequencing was performed. The extracted DNA was isolated and checked for purity before sequencing. The gel electrophoresis image of the isolated DNA is presented in Figure. 3. DNA isolated from the three isolates showed O.D in the range of 0.566, 0.557 and 0.591 for S34, W16 and S31 respectively. The A260 / A280 ratio for the isolates S34, W16 and S31 was found to be 1.72, 1.8 and 1.85 respectively confirming the purity of the DNA.

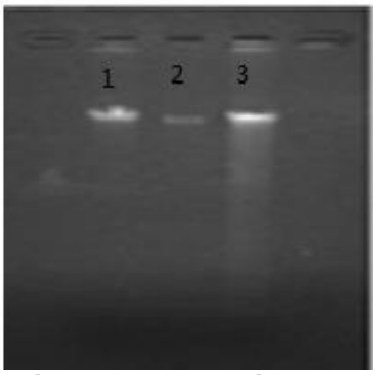

FIGURE 3: Gel electrophoresis of isolated DNA (Lanes 1, 2 and 3 contain DNA isolated from S34, W16 and S31 respectively).

Computational Analysis

The nucleotide sequence samples of these isolates were analysed using Bioinformatics tools. Initially, the sequences were screened for vector contamination using VecScreen tool (https://www.ncbi.nlm.nih. gov/tools/ vecscreen/) and no vector regions were reported. The reverse complimentary sequence of the reverse primer sequence was generated using BioEdit v7.0 (Hall, 1999), followed by merging with the forward primer sequence using Emboss merger (Rice *et al.*, 2000). The merged sequences were analysed using the online tool Megablast with default parameters against all genomic databases (Morgulis *et al*, 2008). The MegaBlast result was further confirmed using EzBioCloud- EzTaxon- a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. The identify service of EzBioCloud provides proven similarity-based searches against quality-controlled databases of 16S rRNA sequences (Kim *et al*, 2011). The result of the same is presented in Figure 4. The results confirm the findings that W16 retrieved a top hit similarity of 92.92% with *Bacillus paralicheniformis*, S34 retrieved a high similarity of 88.96% with *Pseudomonas simiae* and S31 retrieved highest similarity of 93.89% with *Pseudomonas aeruginosa*. All the sequences were submitted to GenBank and the accession numbers for the isolates S31 and W16 were obtained as MF511820 and MF511907 respectively. Our results are in accordance with Peek *et al* (2012) reporting siderophore production by *Pseudomonas aeruginosa*. Berendson and colleagues (2015) have also reported the siderophore activity of *Pseudomonas simiae* and its application as plant growth promoter. The siderophore producing activity of *Bacillus sp*. has also been reported by Nabti *et al* in 2013. However, all these studies have reported siderophore activity from microbes of terrestrial origin. Very few reports have mentioned the siderophore producing ability of bacterial isolates of marine origin. This study will thus provide an important insight in the study of siderophores of marine origin.

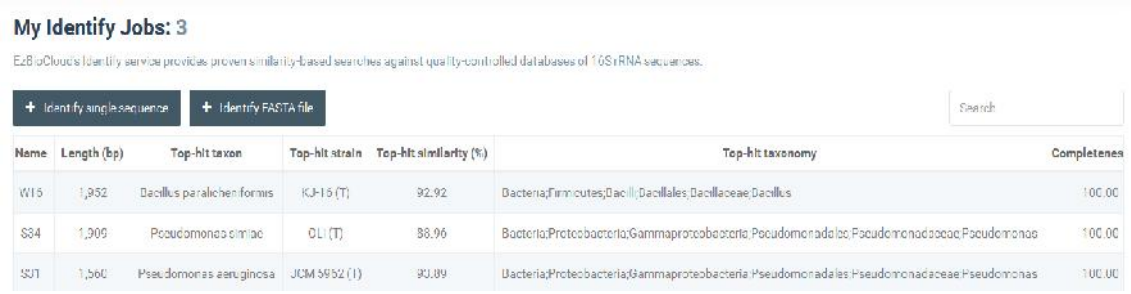

FIGURE 4: EzBioCloud Ez-Taxon result for the sample S31, S34 and W16

CONCLUSION & RECOMMENDATIONS

The rich mineral contributions from land and rivers are often responsible for high iron concentrations in the coastal regions. However, the biological ability of iron in marine environment is very low. The tendency of ferric ion to get hydrolysed at the pH of sea water makes it unavailable for bacterial growth (Dengg *et al.*, 2001). In response to these conditions, bacteria secrete low molecular weight compounds known as siderophores that scavenge iron and make it available for bacteria thus providing a model for co-operative yet competitive interaction. However, the relationship between siderophore and microbial structure in an environment with low iron availability such as the oceans still remains less explored (Ahmed and Holmstrom, 2014). The current study thus provides a useful lead in studying siderophore production in marine environment. Our studies provide strong evidence that marine bacteria produce siderophores to satisfy their needs of iron. Due to their iron chelating ability, marine siderophores play a significant role in aquatic ecosystems by maintaining the iron cycle. These siderophores can also contribute to the development of therapeutic drugs and also in the development of biosensors and biofilms (Carvalho and Fernandez, 2010). This investigation has attempted to isolate and identify potent siderophore producing marine bacteria, which can

find application as growth promoters for plants in saline-sodic coastal areas.

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