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Isolation and identification of exopolysaccharide producing bacteria from Someshwar beach of Dakshina Kannada, Mangalore

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Abstract
The exopolysaccharides (EPS) derived from marine bacteria have shown to exhibit versatile applications in diverse biotechnological segments. Therefore a study was done to bio prospect marine bacteria from surface sea water, associated with algae and sand obtained from the coastal area of Someshwar, Mangalore and was screened for exopolysaccharide production. Out of the twenty colonies isolated; two colonies that produced prominent mucoid colonies were selected, designated as YUI6-DR3A and YU17-DR6A, identified using 16S rRNA gene sequencing and the EPS produced by them in Zobell marine broth and MY broth was evaluated using standard spectrophotometric methods. The phylogenetic analyses of the isolates done on the basis of 16S rRNA gene sequence revealed the isolates YU16-DR6A and YU16-DR3A shared 97.54% and 100% similarity respectively to *Pseudoalteromonas shioyasakiensis sp.* SE3^{T}. The strains produced appreciable amount of EPS in MY media. The expolysaccharides of these newly isolated strains can be further explored for its applicability in biotechnological industries.

Keywords: *Pseudoalteromonas*, exopolysaccharide, 16S r RNA sequencing, phylogeny

Introduction
Marine environs cover almost three quarters of earth and contain a vast biological diversity. Marine habitats are highly complex and are the largest continuous ecosystem that consists of extremely variable temperature, salinity, pressure and nutritional conditions. In the oceans, bacteria constitute the most abundant and diverse members of the microbial world. Many reports describe the efficiency of marine bacterial strains in biotechnological applications including human and environmental benefits. The bacteria isolated from these environments are potent sources of various industrially important bioactive compounds such as enzymes, exopolysaccharide (EPS), biosurfactants, antibacterial, antiviral, anticancer compounds with distinctive and diverse compositions. Marine bacteria secrete different compounds based on their habitat and their ecological functions. Many marine bacteria produce exopolysaccharides (EPS) as a strategy for growth, binding to the substratum, to survive unfavorable conditions and also to intra- and inter-specific communication and competition (Corinaldesi *et al*., 2017) [7]. The bacterial polysaccharides are particularly gaining its importance because of its versatile applicability and easier downstream processing (Senugupta *et al*., 2017) [19]. Currently many research works are focused on isolating new EPS producing bacteria from marine environments as the EPS has found to possess important biotechnological applications in the field of food, textile, cosmetic, pharmaceutical and biomedical industries. The EPS produced by marine bacteria such as *Agrobacterium* sp., *Alcaligenes faecalis*, *Xanthomonas campestris*, *Bacillus* sp., *Alteromonas macleodii*, and *Pseudoalteromonas* sp. have been used as emulsifying, thickening, absorption and gel formation and anti-wrinkle agents (Corinaldesi *et al*., 2017) [7]. The EPS extracted from *Bacillus licheniformis*, *Paenibacillus polymyxa*, *Ochrobactrum* sp. HG16, *Thermal B*acillus sp. *Pantoea* sp., Marine sulfate-reducing bacteria, and *Alteromonas macleodii* subsp. *Fijiensis* have found application as ecofriendly biomaterial for bioremediation of heavy metals (Mohite *et al*., 2017) [14]. These unique functional properties of EPS has shown its efficacy as anti-cancer, antioxidant and immune stimulating and immunomodulating agent that made its applicability in treating cancer and also used in regenerative medicine and tissue engineering (Abdelnasser *et al*., 2017) [1]. Hence, the present work was carried out to isolate potential EPS producing bacteria from the marine surface water of Someshwar region located in Karnataka, India.

Also to identify them using 16S rRNA gene sequencing and to construct phylogenetic relationship of selected isolates with their closest members.

Materials and Methods

Collection of samples

Seawater samples were collected from offshore of Someshwar, Dakishna Kannada, India (12.7862° N, 74.8535° E).The samples were collected from different areas such as rock –scrappings from shore, water and sand of Someshwar region and stored in sterile containers later transported to laboratory under cold conditions

Isolation and Screening of exopolysaccharide producing marine bacteria

After transporting to lab conditions, the sample were serially diluted to prepare aliquots ranging from $10^{-2}$to 10^{-6} using normal saline as diluent. 100 µl from each aliquot were spreaded onto MY agar plate and Zobell marine agar plate (Himedia, India). Zobell Marine Agar was formulated by Zobell and has a composition that mimics seawater and thus helps the marine bacteria to grow abundantly (Zobell, 1940; Lynman, 1941) [22, 12]. Based on the mucoid phenotypic appearance of the colonies, strains were selected as exopolysaccharide producing bacteria (Ng and Hu, 1989) [15].The selected isolates were pure cultured and preserved in 30% (*V*/*V*) glycerol at -80^0 C.

Morphological and molecular characterization of bacterial Isolates:

Colony morphology

The bacterial colonies were observed for the morphological characteristics such as shape, size, margin, pigmentation, elevation, surface, consistency and opacity.

Gram's staining

Gram staining was done according to the procedure (Cappuccino and Sherman, 2002) [5].The isolate was smeared in the slide and heat fixed. The crystal violet dye was added, kept for 1 minute and washed in running water. Gram's iodine was added, kept for 1 minute and washed in running water then the smear was decolorized with 90% ethanol and finally the counter stain safranin was added and after a minute washed in running water. It was observed under the light microscope (Olympus clinical microscope model CH20i, Japan)

Molecular characterization of the bacterial isolates

Sequencing and phylogenetic analysis

Bacteria were grown in Zobell marine broth for 24 hrs at 32^0 C. Genomic DNA was extracted and purified using a commercial kit (QIAGEN KIT). Electrophoresis on 0.8% Agarose gel of extracted DNA was done and the bands formed were visualized on a Gel Documentation System. After the purity check of DNA (Colibri, Nanodrop spectrophotometer), the 16S rRNA gene was amplified by PCR using universal primers 27F and 1492R. An aliquot of PCR product of isolates was directly sequenced using an ABI PRISM 310 instrument using the same primers mentioned above. Sequence data was aligned and compared with available standard sequence. The acquired 16S rRNA gene sequences were submitted to National Center of Biotechnology Information (NCBI) GenBank (http://www.ncbi.nlm.nih.gov/). By using sequenced data a phylogenetic tree was constructed using the Jukes–Cantor model and the neighbor-joining method using Clustal W software, in Molecular Evolutionary Genetics Analysis Tool (MEGA X) (Kumar *et al*., 2018) [11].

Growth optimization of the isolates

The screened isolates were further evaluated for exopolysaccharide production. The inoculum was prepared by transferring bacterial colony into 250 ml conical flask containing 50 ml of Zobell Marine (ZM) broth and MY broth with slight modifications in glucose compositions. Inoculated flasks were incubated on shaker at 100 rpm for 168 h at 32^0 C. At regular intervals (24, 48, 72, 96, 120h) culture broth was harvested and the yield of the EPS was noted. The strains that yielded maximum EPS were selected for the further study. The broth was made cell free by centrifugation (8000×g) for 15 minutes. Chilled absolute ethanol was added to supernatant in ratio 1:3 (v/v) and kept at 4^0 C for 24 hours (Kim and Yim, 2007) [10] to precipitate EPS. The precipitated EPS were recovered by centrifugation (8,000×g) and purified by washing with Milli Q water. Finally, the exopolysaccharide was re-precipitated by 1:3 volume of cold absolute alcohol and was separated by centrifugation and dialysed (MWCO 12,000, HiMedia, India) against distilled water for 48 h. The dialysed EPS was lyophilized and subjected for the determination of carbohydrate content by Phenol sulphuric acid method (Dubois et al., 1956) [8] to ensure the presence of EPS.

Statistical analysis

Data was reported as the mean of the experiments standard errors (SEM). Each experimental condition was performed in triplicate (n=3). One way analysis of variance (ANOVA) was used to analyze data. p<0.05 was considered as statistically significant.

Results and Discussion

Pseudoalteromonas is a genus of obligate marine Gram-negative heterotrophic bacteria that includes several ecologically and industrially important strains (Zhao *et al*., 2014) [21]. They are one among the wide varieties of EPS-producing marine bacteria (Hayashida-Soiza 2008) [9]. *Pseudoalteromonas* species are commonly found in association with eukarytotic hosts, surface waters and sediments of the marine environment. *Pseudoalteromonas* bacteria play an important role in marine environments owing to their abundance and high metabolic activities (Al Khudary *et al*., 2008) [2]. In the present study, we isolated EPS producing *Pseudoalteromonas* sp. from marine surface water from Someshwar coast, India. The spread plate method was used for the isolation of the mucoid colonies. Based on the efficiency of EPS production, the strains were selected for the further study.

The ZMA plates showed luxuriant growth of the bacterial colonies. Out of the twenty colonies isolated; only two colonies that produced prominent mucoid colonies were selected. The culture plates were incubated at 32℃ for 96 h under static conditions in an inverted position. The two isolates were designated as YUI6-DR3A (Fig.1) and YU17-DR6A (Fig.2) and were used to evaluate exopolysaccharide production in Zobell marine broth and MY broth that has been used for the EPS production from the marine bacterial strains (Priyanka *et al*., 2015) [17]. Maximum production of EPS was observed in the isolate *Pseudoalteromonas* sp.YU16-DR3A.

In MY broth at pH 7.4, 32^0 C after 72 h of incubation period the EPS yield was maximum for both the isolates when compared with ZMA broth. However, the yield of the EPS decreased in the subsequent growth period. The strain YU16-DR3A and YU16-DR6A was able to produce 2.2 g/L and 0.9 g/L of EPS respectively in ZM broth whereas in MY broth they produced 2.9 g/L and 1.69 g/L of EPS respectively. It has been reported that most bacteria release the highest quantity of EPS in the stationary phase of growth (Wietz *et al*., 2010) [20]. This result might be justified by the decrease in the yield of EPS-DR3A after the stationary phase of growth. The depletion of nutrients as well as the presence of polysaccharide degrading enzymes in batch culture may also lower the EPS yield. However, the yield of the EPS by the two isolates were higher than that of the EPS yield (0.2 g/ L and 0.7 g/L) produced by *Pseudoalteromonas* sp.MER144 (Caruso *et al*., 2018) [4] and *Pseudoalteromonas* sp.MB-16 (Senugupta *et al*.,2017) [19] respectively. The two isolates were fast growing strains and the 48 h old cultures exhibited all the colony characteristics that are described in table 1.

Table 1

Morphological characteristics	YU16-DR6A	YU16-DR3A
Gram reaction.	Gram negative	Gram negative
Shape and Arrangement	Straight rods	Straight rods
Colonial characteristics		
Shape	Round	Round
Colony Size	Intermediate	Intermediate
Margin	wavy	Smooth
Pigmentation	Non-Pigmented	Non Pigmented
Elevation	Raised	Raised
Surface	Mucoid	Mucoid
Consistency	Moist	Moist
Opacity	Opaque	Opaque

Fig 1

Fig 2

The presence of carbohydrates in the EPS was confirmed by the amount of sugars quantified in the test. The standard spectrophotometric assay carried out for the determination of carbohydrate content in the extracted EPS from the two isolates showed positive results. The carbohydrate content of the EPS-DR3A and EPS-DR6A was found to be 69.79 ± 0.15% and 17.42 ± 0.19% respectively.

According to the results of 16S rRNA gene sequencing, these two isolates, YU16-DR-3A and YU16-DR6A were shown to be closely related to the genus *Pseudoalteromonas* with 100% and 97.54% similarity respectively to *Pseudoalteromonas shioyasakiensis sp.* $SE3^T$. The partial nucleotide sequencing of *Pseudoalteromonas* sp.YU16-DR3A and *Pseudoalteromonas* sp.YU16-DR6A have been deposited in Gen bank with accession numbers MH191376 and MH392742 respectively. The phylogenetic relationship was obtained using neighbor joining by pair wise comparison among the 16S rRNA gene sequence of selected isolates with their closest members of non-pigmented *Pseudoalteromonas* sp. and the dendrogram was constructed for their phylogenetic relationship (Fig.3). The 16S rRNA gene sequencing, data of the two isolates showed closest resemblance to *Pseudoalteromonas shioyasakiensis* sp. $SE3^T$. The dendrogram revealed that the two isolates were distinctly placed under separate clusters. The two *Pseudoalteromonas* sp. showed exuberant growth in ZM and MY medium and produced appreciable amount of EPS. The colonies were non-pigmented. One of the interesting features of the genus *Pseudoalteromonas* is that it can be divided into two clades: pigmented and non-pigmented species. The non-pigmented species tend to possess unusual and diverse enzymatic activities (carrageenases, chitinases, alginases, cold-active enzymes), broader environmental tolerance ranges (temperature, water activity and pH) and greater nutritional versatility compared to the pigmented species. Some of the non-pigmented strains that are been reported for their biologically important molecules are *Pseudoalteromonas espejiana*, *Pseudoalteromonas undina*, *Pseudoalteromonas carrageenovora*, *Pseudoalteromonas elyak*ovii, *Pseudoalteromonas agarivorans*, *Pseudoalteromonas paragorgieola*, *Pseudoalteromonas aliena*, *Pseudoalteromonas haloplanktis*, *Pseudoalteromonas distincta* and *Pseudoalteromonas nigrefaciens* (Bowman, 2007)[3]. EPS produced by *P. elyak*ovii has been found to display cryoprotection to microorganism cells (Casillo *et al*., 2018) [6]. *Pseudoalteromonas issachenkonii* PAMC 22718 was reported to produce chitinase (β-*N*-acetylglucosaminidase), which was cold-active even at $0\text{-}10^0$ C (Park et al., 2016) [16]. They are also gaining importance as probiotics in aquaculture (Mejias

et al., 2018) [13]. *P. haloplanktis* and *P. undina* strains have been found to provide probiotic benefits (Bowman, 2007) [3]. EPS produced by Antarctic bacterium *Pseudoalteromonas* sp. S-5 was reported to possess anticancer activity (Ramana *et al*., 2017) [18].

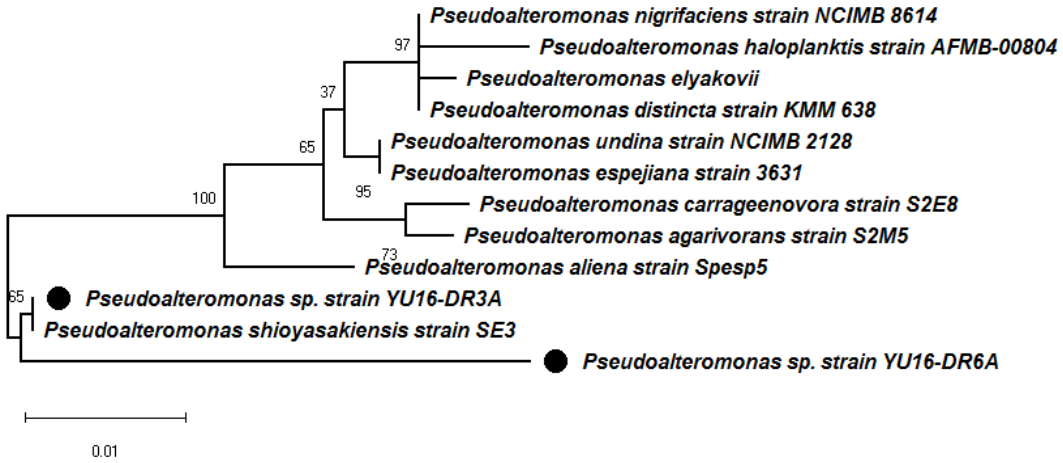

Fig 3

Conclusion

The demand for industrially important and economically useful bacterial strains is increasing to meet the requirements of the human population. Thus, with further exploration into their beneficial aspects it is anticipated that the isolated *Pseudoalteromonas* strains could be utilized in different industries to serve mankind and the environment.

Conflict of Interest

Authors declare that they have no conflict of interest

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