Research Paper

Studies of Two Pigment Producing Halophilic Bacteria from Karnataka Mangrove Soil

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Thirty halophilic bacteria were isolated from the soil of Karwar mangrove regions, Karnataka. These were examined for the production of pigments, antibiotic and proteolytic activities. KA16SPiv produced fluorescent green pigment and KA16SK2HS produced pink pigment. The optimal pigment production was at pH 8, 37°. Pigments are extractable in chloroform and methanol. Absorption spectra suggested phenazine skeleton for green and prodigiosin skeleton for pink pigment. A polyphasic taxonomic study showed KA16SPiv to be *Pseudomonas aeruginosa* whereas KA16SK2HS to be *Salinicoccus roseus* with accession no: MF377544 and MF377542, respectively.

Key words: Halophilic bacteria, Karwar mangrove, Pigments

Pigments are the inseparable components of human life, which are associated with almost all articles being used today. It is a vital constituent, as a color is probably one of the first characteristics to be perceived by senses. Synthetic pigments are often carcinogenic and non-biodegradable. These limitations of synthetic pigments have caused the need for a fresh, enthusiastic look out for natural pigments, which are found in plants, insects, and microbes. Microbes after plants are the second largest[1,2] source for obtaining natural color.

Bacteria among all the microbes have immense potential to produce diverse pigments. The microbial pigments are of industrial interest as they are more stable and with a high solubility factor than those of plant and animal origin[3]. Besides having controllable and predictable yield these microbial pigments are season-independent, often of food grade may also exhibit medicinal properties. Various pigments like carotenes, melanins, flavones, quinines and violacein[4-6] are produced by different bacterial genera like *Serratia*, *Streptomyces*, *Pseudomonas*, *Bacillus*, *Vibrio* and *Cytophyga* that play an important role in their molecular and physiological processes and help taxonomist to identify and classify the bacteria, as they differ in chemical structure with specific chromophores. Carotenoids such as bacterioruberin, which plays a role as a biological pigment and in bacterial metabolite have been isolated from *Halorubrum* sp., *Haloarcula* sp. from the western coast of India by Raghavan and Furtado; Kibilan *et al.*[7,8]. In humans, these pigments act as protective agents against oxidative damage[9] and demonstrate pro-vitamin A function that protects from lifestyle-related disorders such as cardiovascular disease and age-related muscular degeneration[10].

The present study was taken up with an objective to isolate industrially useful pigments from mangrove microbes of Karwar mangrove region. Terrestrial freshwater and marine halophilic environments existing together in the mangrove forests create an extremophilic environment and make the microbes different from those of either environment. Two pigment producing isolates identified as *P. aeruginosa* and *Salinicoccus roseus* with accession numbers MF377544 and MF377542 were taken up for the study of various parameters to optimize pigment production.

MATERIALS AND METHODS

UV spectrophotometer (Carry 60, Agilent Technology) was used for spectrophotometric studies. PCR (T100, Bio-Rad), horizontal electrophoresis- gel unit (Bio-Rad), universal hood II- Gel-Doc (Bio-Rad) were

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used. All media were of HiMedia and all chemicals used were of analytical grade. Molecular analysis was carried out in Trans-Disciplinary University of Health Science and Technology and Eurofins Genomics India, Bangalore. All solvents used were of analytical grade and HPLC grade methanol (HiMedia, purity 99.7 %), chloroform of Fisher Scientific (purity 99.7 %).

Isolation of bacteria:

The bacterial strains were isolated from the sediments collected during post-monsoon (October-November) season from the coastal Mangrove region of Karwar, Karnataka (Latitude- 14.84587° and Longitude- 74.11872°). Sediment samples were collected at a depth of 5 and 40 cm from the root region of various species of trees like *Sonneratia alba, Kandelia candel*. The sediment samples were incubated in HiMedia halophilic broth (M591-500G) for 12 d for enriching and isolation of extreme halophiles. Following ten-fold serial dilution technique in halophilic agar plates, halophilic bacterial isolation was carried out[11] by incubating aerobically at 37° for 24-48 h[12]. A greenish color was observed in KA16SPiv and a pink color in KA16SK2HS cultures. Pure cultures were obtained by repeated streaking over the nutrient agar plates. Then the isolates were preserved in glycerol at –20°[13] and on NA slants at 4° for further use.

Biochemical characterization of bacterial isolates:

Morphological and physiological properties of the isolates were investigated on the basis of colony characteristics on the nutrient agar medium and Gram's reaction. The isolates were subjected to various biochemical tests followed by enzymatic and sugar utilization test as per Bergy's manual of determinative bacteriology[14]. The online ABIS tool-based on these results helped in identifying the close prokaryotic relative of the isolates[15].

16S rRNA sequence analysis:

Further identification was carried out using 16S rRNA gene sequencing. Bacterial genomic DNA was extracted[16]. The DNA was used as a template for PCR using universal primers. These purified products are a template in cycle sequencing[17]. The amplified 16S rRNA gene was purified with Qiagen Inc. kit and electrophoreses on 1 % agarose gel sequencing was carried out in Eurofins[18-20]. BLAST program was used to access the DNA similarities and multiple sequence alignment and molecular phylogeny were performed using BioEdit software.

Growth parameters:

Overnight grown bacterial cultures were inoculated in different Erlenmeyer flasks; each containing nutrient broth with different salt concentration (0, 5, 10, 15, 20, 25, 30 and 35 %) for a period of 24 h and streaked on to nutrient agar plates with the same concentration of salts as above. After a period of 24 h, the tubes without visible growth were kept incubated for a period of another 72 h for confirmation. The organisms were set incubated with varying pH environments (i.e. 2, 4, 6, 8 and 10) for a period of 24 h. pH was adjusted with the help of 1 N HCl, 1 N NaOH. 5 ml of bacterial suspensions from each of the flasks was pipetted out and analyzed at 620 nm.

Pigment production and extraction:

The pigment was extracted following the method described by Frank and DeMoss[21] for blue-green pigment and by Maekel and Kester[22] for pink pigment. The selected bacterial isolates *P. aeruginosa* and *S. roseus* were cultured in nutrient broth at pH 7±0.1 and temperature 37°. The colour of nutrient broth has changed to dark green colour, which was inoculated with KA16SPiv at the end of the incubation period and the extraction of pigment was carried out using chloroform by centrifuging it at 8000×g for 10 min at 4° (the process was repeated three times). The pink pigment from KA16SK2HS was found in the cell biomass, which was separated from the medium by centrifugation at 6000×g for 10 min at 4°. Methanol was added to the pellet and centrifuged at 6000× g for 7 min at 4°. Hexane, ethyl acetate and n-butanol, proved ineffective in extracting these pigments. Salting out of the pigment was also found to be ineffective. The crude pigments extracted were primarily identified by measuring their OD in a range of 200-800 nm.

Antibacterial assay of the pigment:

The assay was carried out by well diffusion technique for both green and pink pigments against clinical pathogenic strains such as *Staphylococcus aureus*, *Bacillus subtilis* (Gram-positive bacteria), *Proteus mirabilis*, *Escherichia coli*, *Salmonella typhimurium* (Gram-negative bacteria), *Candida glabrata*, *C. albicans*, *C. parasilosis* and *Fibulobasidium inconspicuum* (fungal strains), chloroform and methanol were kept as control. The isolates were swabbed on to the surface of the Muller-Hinton agar plates with wells dug into it. 10 µl each of the pigments were added to the wells and incubated for 24 h at 37°. Standard antimicrobial were

taken as comparison by implementing as disc diffusion method[23]. The antibacterial activity of the pigments was determined by measuring the zones of growth inhibition around the well.

Screening for protease activity:

The proteolytic activity of the bacterial isolates was tested by using skimmed milk medium supplemented with 5 % NaCl and 1 % casein. The plates were incubated overnight at 37°. *P. aeruginosa* was found to produce protease as identified based on clear zone formation on the plates. *S. roseus* proved negative for proteolytic activity.

Assay of proteolytic activity:

Fresh inocula of protease producing strain were added to the nutrient broth containing 5 % NaCl and 1 % casein at pH 7 and incubated in a shaker incubator in 150×g at 37° for 24 h. After incubation, the cultures were centrifuged at 10 000×g for 15 min at 4°. The cell-free supernatant was used for protease assay by Lowry method used by Kembhavi *et al*.[24]. The reaction mixture containing 1 ml of the cell-free supernatant was added to 1 ml of casein (1 % w/v in 50 mM potassium phosphate buffer, pH 7.5) and incubated for 10 min at 37°. 2 ml of 10 % trichloroacetic acid reagent was added to terminate the reaction, kept for 30 min at room temperature and centrifuged at 10 000×g for 15 min at 4°. Five millilitres of 500 mM sodium carbonate solution was added to the 2 ml of filtrate and absorbance was taken at 570 nm. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µmol of tyrosine per min under the defined assay conditions.

Pigments as food and textile colorant:

As per the spectrophotometer analysis, a single peak was seen in the case of pink pigment while multiple peaks were recorded in the case of the green pigment. The pink pigment was further analysed for colour retaining ability test for food and textiles. Test for textile grade colour was carried out by taking several 5 cm of white cotton cloth strips and treating each of them with 2 ml of pink pigment and drying them under laminar airflow for 90 min. Once dried, the cloth was boiled in 20 ml of water. Food colour grade test was carried out by taking 2 ml of the pink colour crude extract and diluting it with 10 ml of distilled water mixed with 1.5 % agar and heated till boiled then cooled for solidification. These tests were made to ensure the stability of the pigment and its colouring ability[25].

RESULTS AND DISCUSSION

Thirty halophiles were isolated from the mangrove sediments, 2 of them showed pigmented characters over NA plates, which were selected for further study. After studying the morphological characters followed by staining procedure and observation under the microscope, the 2 bacterial strains were found to be different, were subjected to biochemical tests as shown in Table 1 in order to determine their physiology. Below 5 % NaCl no growth could be found; luxuriant growth was observed within a range of 5-15 % of NaCl concentration, whereas in 20-30 % of NaCl mild growth was seen and above 35 % NaCl no growth was observed. Optimum pH is shown in fig. 1, which was observed at 620 nm.

P. aeruginosa was found positive towards proteolytic activity via clear zone formation. *S. roseus* did not display protease activity. *P. aeruginosa* was further subjected to the protease assay. The observation was

TABLE 1: BIOCHEMICAL TEST OF THE ISOLATES

Test conducted	Isolate designation	
	KA16SPiv	KA16SK2HS
Shape	Rod	Coccus
Gram stain	Negative	Positive
Oxidase	Positive	Positive
Indole	Negative	Negative
Methyl red	Positive	Negative
VP test	Negative	Negative
Citrate	Positive	Positive
Hydrogen sulfide Production	Negative	Negative
Urease	Positive	Positive
Motility	Positive	Negative
Gelatine liquefaction	Positive	Negative
Nitrate reduction	Negative	Positive
Catalase	Positive	Positive
Amylase	Negative	Negative

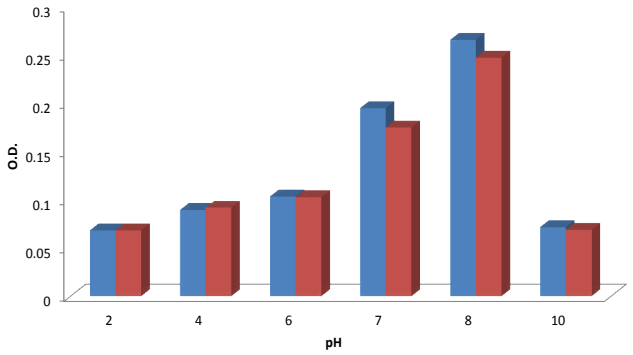

Fig. 1: pH tolerance
pH tolerance of (■) *P. aeruginosa* and (■) *S. roseus*. OD is optical density

made based on absorbance value at 570 nm quantifying the amount of protease to be 5.266 U/mg.

Extraction of green pigment was carried out by using chloroform and methanol was used for pink. In our study crude extract containing pink pigment from the bacterial isolate gave maximum absorption spectra at 535 nm, which is comparable to prodigiosin skeleton whereas green gave maximum absorption at 280, 270, 260, 365 and 310 nm as shown in fig. 2A and B, which are comparable to phenazine skeleton present in 1-hydroxyphenazine, phycocyanin, and oxychlororaphine.

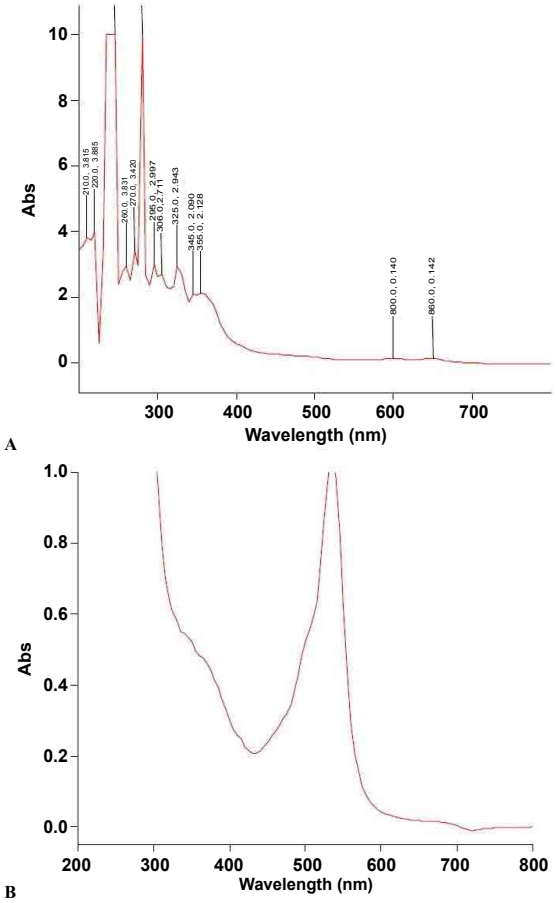

Fig. 2: UV absorbance spectra of green pigment and pink pigment

UV absorbance spectra of A. green pigment from *Pseudomonas aeruginosa* and B. pink pigment from *Salinicoccus roseus.* Wavelength range used 200-800 nm

The antimicrobial activity exhibited by the pigments was compared with that of the standard clinically-used antimicrobial drugs against bacterial and fungal pathogens. The selected bacterial strains, KA16SPiv, and KA16SK2HS belonged to the genus *Pseudomonas* and *Salinicoccus* as revealed from ABIS analysis. These purified products are a template in cycle sequencing. 16S rRNA gene sequences from the isolates were used to determine the closest prokaryotic relative using NCBI BLASTN, which was found to be *P. aeruginosa* and *S. roseus*. Sequences deposited in GenBank under accession number MF377544 and MF377542, respectively. Sequence alignments were carried out using Bioedit (version 7.2.6). The evolutionary history was inferred using the UPGMA method with a tree evaluation by bootstrap analysis for 3000 replication in MEGA 6.0 (Molecular Evolutionary Genetics Analysis)[26,27], which provides confidence estimation through phylogenetic tree topologies that the isolates are *P. aeruginosa* and *S. roseus*. 16S rRNA sequences from major bacterial clusters obtained from GenBank were included in the phylogenetic analysis, which is represented in fig. 3A and B. The *P. aeruginosa* and *S. roseus* isolate have shown similarities with ATCC 10145 and DSM 5351 respectively[25,28] (Tables 2-4).

Microbial fibrinolytic protease is used by the detergent and pharmaceuticals industry and in medical diagnosis for resolving serious medical threats like disorders of blood clotting and fibrinolysis[16,17]. Skimmed milk agar[29] and other enzyme-substrate been incorporated in solid media[27-30] are widely used as a screening agent. Proteolytic isolate screening was carried out with skimmed milk media. Tri-chloroacetic acid[30] and tannic acid[31] applied by several authors to determine the enzyme activity. It was observed that the *P. aeruginosa* has a protease, an activity of 5.266 U/mg. These results showed similarity with those reported by Morihara *et al.,* who have isolated a protease from *Pseudomonas* sp*.,* which inactivated S human plasma alpha1-proteinase[32,33]. The intracellular fibrinolytic protease (2.5 U/g) was found

TABLE 2: ANTIBACTERIAL ASSAY

Bacterial strains	Zone of inhibition (mm)				
	Ampicillin (30 µg)	Tetracycline (10 µg)	*Streptomycin* (10 µg)	Green pigment (1 mg/ml)	Pink pigment (1 mg/ml)
S. aureus	25	28	23	18	14
B. subtilis	26	24	21	16	17
P. mirabilis	21	10	18	10	10
E. coli	17	15	20	19	15
S. typhimurium	24	15	21	11	10

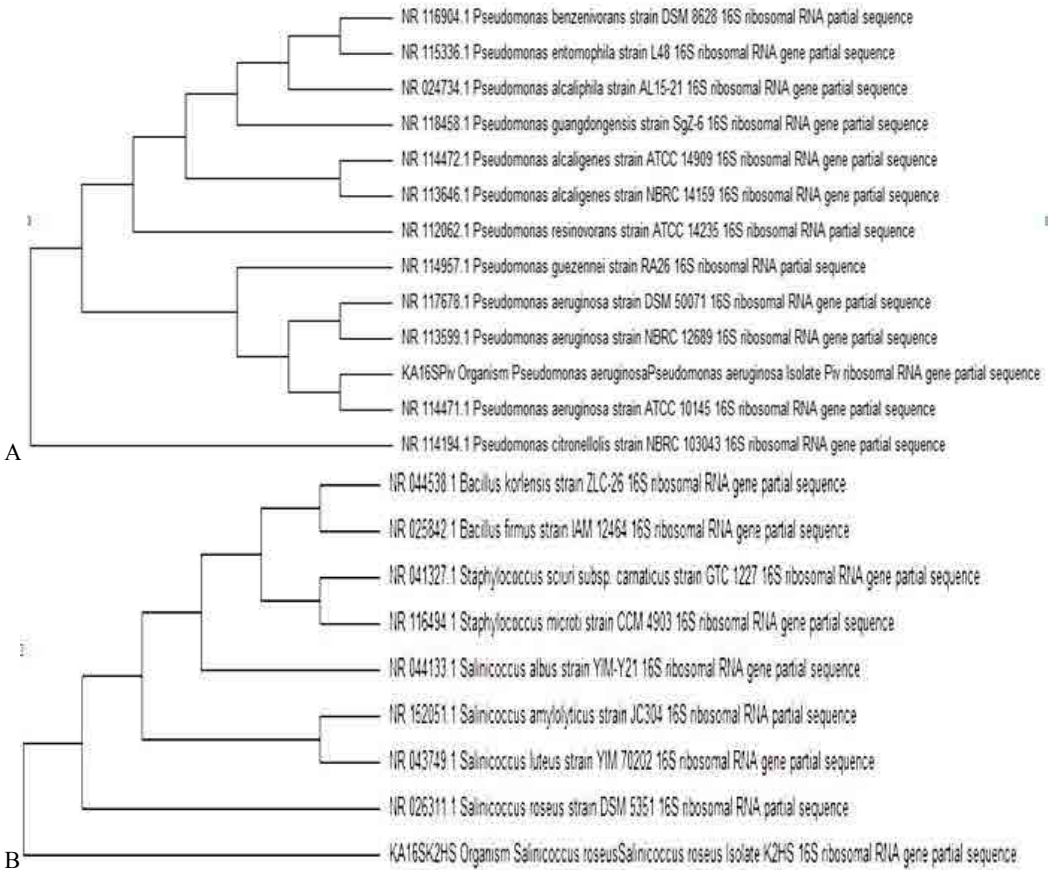

Fig. 3: (A) KA16SPiv-*Pseudomonas aeruginosa* and (B) KA16SH2HS- *Salinicoccus roseus* phylogenetic tree analysis

TABLE 3: ANTIFUNGAL ASSAY

Fungal strains	Zone of inhibition (mm)				
	Ciprofloxacin (10 µg)	Ketoconazole (10 µg)	Fluconazole (10 µg)	Green pigment (1 mg/ml)	Pink pigment (1 mg/ml)
Candida glabrata	28	10	10	18	12
Candida albicans	10	29	30	12	17
Fibulobasidium inconspicuum	31	10	10	16	12

TABLE 4: SALT TOLERANCE TEST

Test Isolates	NaCl Concentration (%)							
	0	5	10	15	20	25	30	35
P. aeruginosa	Nil	+++	+++	+++	+++	++	+	Nil
S. roseus	Nil	+++	+++	+++	+++	++	+	Nil

in case of *G. lucidum* isolated by Kumaran *et al.*[34]. *B. subtilis* isolated from the marine environment by Rym *et al.* produced 20.72-292.36 U/mg protease activity when tested under various conditions and changing the media[26]. The production of microbial pigments is affected by varying pH[35,36]. The optimum pH of the bacterial isolate was found to be at an alkaline pH i.e. 8.0 and growth was reduced at pH 10. An acidic pH has an adverse effect on both of the isolates. Results of the present investigation showed similarities to the results reported by Tibor[37] and Aberoumand[38], who observed optimum temperature and pH for growth of pigmented bacterial isolates to be 37° and pH 7.

This might be a result of a molecular adaptation by bacterial isolates as well as increases the activity of an enzyme for optimal growth. At 37°, the isolates were incubated for a period of 7 d to check optimum time to produce the pigment. It was observed that the production starts after 24 h. 72 h of incubation was the optimal time period for pigment production in case of *P. aeruginosa*. In the case of *S. roseus* pigment

production started from 48 h optimal period required is 96 h. The isolates are strictly aerobe since both pigment production and growth increased with agitation.

Maximum growth and pigment production occurred in 100 ml medium per 250 ml Erlenmeyer flask. Increasing the volume of the medium keeping the flask size same decreased the growth and pigment production by the cells, which might be a result of decreased dissolved oxygen volume. Asker and Ohta[39] have reported similar observations. Yokoyama *et al.*[40] reported that varying the volume of the medium controlled the growth of and carotenoid production by *Agrobacterium auranticum*. Various solvents were used to extract the colours, except for chloroform in case of green colour and methanol for pink, those solvents were found ineffective. Both pigment solutions were scanned from 200-800 nm. Spectral data of all microbial pigments were collected. Pigment structures for the two relevant genera were selected. The spectra of the pigments isolated were compared with literature report to arrive at their structural features.

The pink pigment from *S. roseus* showed a single peak with the maximum absorbance at 535 nm, which was clear in speculating the compound to be similar to that of prodigiosin skeleton, whereas the green compound showed various peaks similar to different compounds like 1-hydroxyphenazine, phycocyanin, and oxy-chlororaphine, which are similar to phenazine skeletons. This result was corroborated by many authors[41,42], who observed red colored pigment production using *Serratia* sp., *Streptomyces* sp. and *Salinicoccus* sp. and blue-green pigment from *Pseudomonas* sp corroborated with many reported studies[43-48]. Both the pigments showed a distinct antimicrobial effect against the test pathogens and are effective as antibacterial and antifungal towards a few clinical pathogenic species. Generally, the pigments are ineffective against most fungal pathogens. Results obtained in the present study also corroborated with studies that reported isolation of antifungal compound 1,6-dihydroxy-2-chlorophenazine from *Streptosporangium* sp. and *P. aeruginosa*[49,50]. Similar observation was made from *P. chlororaphis* showing an inhibition zone of 21 mm against *Pythium asphanidermatum* and when the concentration was increased complete inhibition of *Macrophomina phaseolina, R. solani* and *S. rolfsi* was observed[51]. Phenazine from *Pseudomonas* sp. has been reported to be an antibacterial agent[52,53]. Pink pigment isolated by Darah *et al*., from *Serratia marcescens* has completely inhibited *P. aeruginosa* and 13 more bacterial strains[51]. Results of the present study agreed with those reported by Pierson and co-workers[54-56]. The pink pigment produced a good coloration when mixed with agar; it gets deteriorated to light yellowish pink on boiling but had created no negative effect on agar binding property[57]. The cotton cloth treated with pink pigment has shown a lighter shade of pink after 20 min of boiling but has retained its color which can be said effective for textile coloration. Even on exposure to sunlight for 60 min no visible change in colour degradation was found, which was similar to those reported by Shirata and co-workers[58].

The idea behind testing the antimicrobial activity of the pigments against food and waterborne pathogens is to check whether an additional protective coating can be laid to the food and textiles when these are used as a colorant. Not many details of *Salinicoocus* sp. could be found but the isolated pigment has shown many spectrophotometrical similarities with *Serratia* sp., which helped to carry out studies on purification of the pigments through TLC, HPLC method and identifying the compound through NMR, LCMS, and MS-MS for future and also test the antimicrobial effect of the pure compounds. As *P. aeruginosa* is a proteolytic organism, further studies would be based on extraction and purification of protease and test it *in vitro*.

The present study was an attempt to demonstrate the multi-functionality found within the estuarine environment strains isolated from the Mangrove region. The isolate *Pseudomonas* sp. and *Salinicoocus* sp. are potential producers of blue-green and pink colour pigment, which are comparable with 1-hydroxyphenazine, phycocyanin and oxy-chlororaphine and prodigiosin. This report highlights the key role of bacterial pigmentation, growth in varying pH of such hypersaline region, with promising food colorant potential[38]. This was also observed in the present study, that both strains have different performing property and required cheap raw material having the ability of rapid growth rate leading to short fermentation cycle times which is an advantage for industrial production. Thus the mangrove environment seems to be a fruitful environment to explore.

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Conflicts of interest:

The authors have no conflict of interest to declare.

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