

## Antibacterial Activity of Red Pigment Produced by *Halolactibacillus alkaliphilus* MSRD1—an Isolate from Seaweed

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**Abstract** The present study was carried out with the aim to isolate an antibacterial pigment from seaweed-associated bacterium. The bacterium was identified as *Halolactibacillus alkaliphilus* MSRD1 by 16S rRNA sequencing. The isolated bacterium was cultured in 50 % Luria-Bertani seawater broth (LB-SWB) with 1 % glycerol. The pigment was extracted with 99 % ethanol and analyzed by UV-Vis spectroscopy at 490 nm. The candidate bacterium was optimized with various NaCl concentrations from 5 to 20 %. The results inferred that the bacterium produce maximum pigment at 5 % NaCl level. The candidate bacterium *H. alkaliphilus* MSRD1 was found to be producing the maximum pigment during the 120-h incubation. The protein content of the pigment was found to be maximum of 72 % at the end of the 120-h incubation. The extracted pigment was stable up to 80 °C, pink at acidic pH (1 to 5) and orange at basic pH (8 to 12). The isolated pigment was fractionated by silica gel column chromatography. Fractionated pigment was characterized by TLC, FT-IR, and SDS-PAGE. In the antibacterial context, the pigment was highly inhibited *Staphylococcus aureus* and *Salmonella typhi* with the zone of inhibition 16 and 14 mm, respectively. According to SDS-PAGE, the size of the pigment was approximately 80 kDa. The *H. alkaliphilus* MSRD1 has high capacity to produce the pigment with antibacterial properties. This could be effectively used in the future.

**Keywords** *Halolactibacillus alkaliphilus* MSRD1 · Seaweed · Antibacterial · LB-SWB · SDS-PAGE

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## Introduction

Marine bacteria attract the researchers because of its nature to produce compounds with uncommon biological properties [6]. Marine *Streptomyces*, *Pseudomonas*, *Pseudoalteromonas*, *Bacillus*, *Vibrio*, and *Cytophaga* isolated from different marine environments such as seawater, sediments, algae, and marine invertebrates are known to produce bioactive pigments. They are able to produce indole derivatives (quinines and violacein), alkaloids (prodiginines and tambjamines), polyenes, macrolides, peptides, and terpenoids. A novel phenazine derivative with antibiotic activity, identified as 5,10-dihydrophencomycin methyl ester, along with (2-hydroxyphenyl)-acetamide, menaquinone MK9 (II, III, VIII, IX-H8), and phencomycin, was isolated from an unidentified marine *Streptomyces* sp. [15]. The dark green pigmented *P. tunicate* isolated from marine environment exhibits the most active and broadest range of inhibitory activity when compared to other strains from this genus [8].

Recently, isolation of astaxanthin from the marine bacterium *Paracoccus haeundaensis* has been reported [12]. Astaxanthin is one of the carotenoids that have commercial value as food supplement for humans and as food additives for animals and fish. A carotenoid biosynthesis gene cluster for the production of astaxanthin has been isolated from the marine bacterium *Agrobacterium aurantiacum* [13]. Phenazines are redox-active, small nitrogen-containing aromatic compounds produced by a diverse range of bacterial genera, including *Streptomyces*, *Pseudomonas*, *Actinomycetes*, *Pelagibacter*, and *Vibrio* under the control of quorum sensing [14, 24]. These compounds were subjected to extensive studies due to their broad spectrum of antibiotic activities against other bacteria, fungi, plant and animal tissues [7, 22]. Therefore, the aim of this study was to isolate and characterize phenotypically and phylogenetically cultivable seaweed-associated bacteria from the coast of Kanyakumari, India, able to inhibit the growth of drug-resistant bacteria.

## Materials and Methods

### Collection of Seaweeds and Isolation of Pigmented Bacteria

The seaweed samples *Gracilaria corticata*, *Ulva lactuca*, and *Padina tetrastratica* were collected from Arockiyapuram, Kanyakumari District, Tamilnadu, India, with latitude of 8° 3' 5.694" N and longitude 77° 32' 30.4656" E and brought to the laboratory immediately. The collected seaweeds were washed thoroughly with sterilized seawater before going for the isolation. Three types of media were used for the isolation of associated bacteria to produce pigments namely Zobell Marine agar (ZMA) with 1 % glycerol, Luria-Bertani seawater agar (LB-SWA) with 1 % glycerol, and brain heart infusion seawater agar (BHI-SWA) with 1 % glycerol. The washed seaweeds were cut into small pieces individually and crushed with the help of mortar and pestle. The crushed samples were serially diluted and appropriate dilution was spread on the abovementioned media individually. Then, the inoculated plates were incubated at 27 °C for 5 days. After incubation, the colored colonies with identical morphology were isolated and stored same medium for the future study.

## Production of Red Pigment

### Cultivation of the Red Pigment Producing Bacterium

LB-SWB was used for the cultivation of the bacterium which contains 10 g of tryptone, 5 g of yeast extract in 1 l of seawater adjusted to pH 8.0 with 5 M NaOH solution, and additionally, the medium was added with 1 % glycerol. Seawater was filtered through filter paper before being used. The above setup was sterilized at 121 °C for 15 min before usage. The procedure for the cultivation of the *Halolactibacillus alkaliphilus* MSRD1 bacterial strain to produce the red pigment consisted of the following steps: first, a single colony from the agar plate was transferred into the test tube containing 50 % of 5 ml LB-SWB and incubated overnight with rotation at 27 °C. From this culture, a sample of approximately 1 % of total volume was transferred to a flask also containing LB-SWB and cultivated with intensive shaking at 130 rpm for 4 to 5 days. The change of the clear culture medium to a red color view was indicative of the sufficient bacterial growth.

### Extraction of the Red Pigment

Dark-red-colored bacterial culture broth was first centrifuged at 10,000 rpm for 15 min at 4 °C to precipitate the bacterial cell with the red pigment. The obtained supernatant and pellet with the red pigment was taken and treated two times with ethanol to remove the red pigment. The pigment collected was then dried using a rotary evaporator. To remove the remained salt in the red pigment, it was again dissolved in pure acetone and centrifuged at 10,000 rpm for 10 min at 4 °C.

### Identification of Pigments by Analytical Methods

#### *UV-Visible Spectrometry*

UV-Visible spectrum of the extracted pigment in ethanol was recorded using UV-Visible spectrophotometer (Schimadzu 160 A). Pigment in acetone was analyzed by scanning the absorbance in the wavelength region of 200–800 nm using UV-Vis scanning spectrophotometer (UV 2101 Shimadzu). The total pigment content in solvent extract was estimated by measuring the absorbance at 490 nm.

### Optimization of Culture Condition

Determination of optimum time for the growth and maximum pigment production was carried out by inoculating the selected strain in 100-ml LB-SW broth in a 250-ml Erlenmeyer flask and incubating on a rotary shaker at 120 rpm for 5 days at 25 °C. Growth and pigment production were determined by taking OD value at 6-h interval. Likewise, parameters such as NaCl concentration was adjusted to 5, 10, 15, and 20 % [2].

### Protein Estimation

Protein extraction was done according to Rausch [17]. An aliquot of 2 ml sample was centrifuged, and 1 ml of 0.5 N NaOH was added to the supernatant and extracted at 80 °C for 10 min. After cooling centrifugation, the supernatant was transferred to

a new tube and the extraction was repeated three times. The final repeat was heated at 100 °C for 10 min for complete extraction of residual proteins. All the three extractions were pooled and mixed well before analysis. Total protein concentration was determined using the method of Bradford [4] with bovine serum albumin (BSA) as standard. To every milliliter of supernatant, 4 ml of Bradford reagent (0.01 % Coomassie blue, 4.7 % ethanol, and 8.5 % phosphoric acid), prepared just prior to the assay, was added and allowed to stand for 5 min at room temperature. The absorbance of the solution was then read at 595 nm.

### Characterization of the Extracted Pigment

The extracted red pigment was tested for its stability by incubating the extracted crude pigment at various pH and temperature ranging from extremely acidic 1 to highly basic 13 and 20 to 100 °C, respectively. Ten grams of the ethanol extracted pigment was subjected to column chromatography using silica gel (60–120 mesh) and eluted stepwise ethanol and ethyl acetate (2:1).

The column fractions collected were spotted on silica gel coated TLC plates. The plates were developed in ascending direction at 12- to 15-cm height. Hexane and ethyl acetate (2:1 v/v) were used as the mobile phase solvent. The plates were air-dried to locate the spots. The spot was scrapped and dissolved in acetone for the future analysis.

### Antibacterial Activity of the Extracted Pigment by Disk Diffusion Method

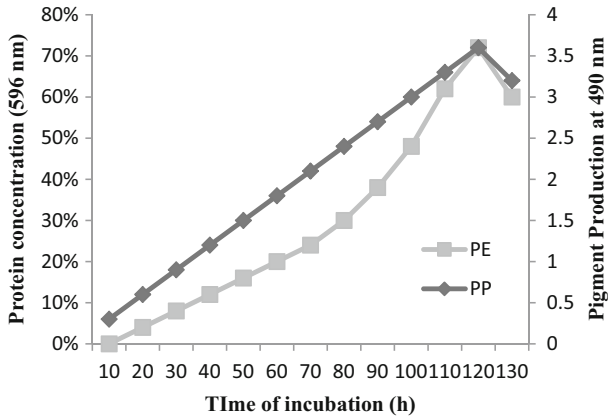
The antibacterial activity of the purified pigment by TLC was evaluated by disk diffusion assay [3] against *Escherichia coli*, *Klebsiella pneumonia*, *Salmonella paratyphi*, *Salmonella typhi*, and *Staphylococcus aureus*. Of the overnight pathogen broth, 0.1 ml was inoculated onto Muller-Hinton (MH) agar plates containing Whatman filter paper disks (wet strengthened, 0.5 cm in diameter) which were previously impregnated with 50  $\mu$ l of the purified red color acetone extract. Inhibition zones were recorded after overnight incubation at 37 °C.

### Determination of the Size of Pigment Protein

To extract the pigment protein the supernatant was saturated by 0.2 N ammonium sulfate. The isolated pigment protein fraction was dialyzed overnight and separated by centrifugation at 1000 rpm for 20 min at 5 °C. Then, the incorporated pigment protein fractions were resuspended in the minimal volume bidistillate. The pigment was partially purified by dialysis, and the molecular weight of the pigment was determined by SDS-PAGE analysis, and the result was compared with the standard marker of 10 to 100 kDa [11].

### FT-IR Spectral Analysis

FT-IR analysis of the fractionated red pigment was carried out as follows: the ethanolic extracted sample (1 mg) was ground with 200 mg of KBr (spectroscopic grade) in a mortar before pressed into 10-mm diameter disks less than 6 t of pressure. FT-IR spectra were obtained on a FT-IR 8300, Shimadzu spectrometer. The analysis conditions used were 16 scans at a resolution of 4  $\text{cm}^{-1}$  measured between 472.63 and 3811.34  $\text{cm}^{-1}$ .



**Fig. 1** Phylogenetic tree of the pigment producing species with other *Halolactibacillus* species

## 16S rRNA Sequencing

The extraction of genomic DNA of the candidate bacterium was performed according to the method [16]. PCR method based on the detection of 16S ribosomal RNA (rRNA) genes using the following primers: forward 5'-GAGTAACGCGTGGGGAATCT-3' and reverse 5'-CCGTCCCTTCTGGGTAGTT-3'. The DNA sequence was initially denatured by 94 °C for 1 min and coupled to 35 cycles of denaturation (94 °C for 10 s), annealing (57 °C for 30 s), and extension (72 °C for 40 s), and to a final extension step at 72 °C for 15 min and addition of 5 µl of enzyme solution containing 1 µl of Taq DNA polymerase in the 1× reaction buffer. The PCR product was sequenced using the genetic analyzer (Applied Biosystems, USA). The comparison of 16S rRNA gene sequence of the candidate strain and the 16S rRNA sequences of other *Halolactibacillus* species was done by using the NCBI-BLAST database; then, the respective gene sequence of the candidate bacterium was submitted in NCBI, and the accession number (KJ541806) was obtained. The reference gene sequences were retrieved from NCBI GenBank database. All the sequences were aligned using the multiple sequence alignment program CLUSTAL-X 2.0.12 [23]. Phylogenetic tree was constructed using MEGA 5.2 program by following the method of neighborhood joining [20] (Fig. 1).

## Results

### Isolation, Identification, and Cultivation of the Red-Pigment-Producing Bacteria

Among the seaweeds employed, the red-pigment-producing bacteria *H. alkaliphilus* MSR D1 isolated from *Padina tetrastratica*, and the bacterium was grown on LB-SWA whereas no colored colonies observed on the other two media were noticed. The strain isolated is a slow-growing, small-sized white-colored colony. It is Gram positive, rod shaped, catalase positive, oxidase negative, and not motile. The 16S rRNA partial sequence analysis (1488 bp) showed that it was closely related to the *Halolactibacillus*, with a 100 % sequence similarity to *Halolactibacillus alkaliphilus* (GenBank accession no. AB682143). The isolated bacterium produced red pigment with 1 % glycerol.

## Antibacterial Activity of the Isolated Pigment

Interestingly, pigment produced by *H. alkaliphilus* MSRD1 showed strong growth inhibition against *S. aureus* and *S. typhi* and the zone of inhibition was 16 and 14 mm, respectively. The results are presented in Table 1.

## Optimization of the Pigment Production

The growth curve and pigment production curve of *H. alkaliphilus* MSRD1 was characterized by taking OD value at 600 and 490 nm. Pigment production by this strain was observed after 3 days of incubation, as the culture became slightly orange in color. Growth and pigment production increased to a maximum during 4–5 days of incubation (Fig. 2) followed by a decrease in both growth, and pigment production was noticed after 5 days.

## Characterization of the Extracted Pigment

The color intensity of the red pigment produced by the candidate bacterium was changed at different pH values. At extremely low pH (2–4), the red pigment appeared pink while in highly basic condition (8–12), the pigment was orange.

## Protein Estimation

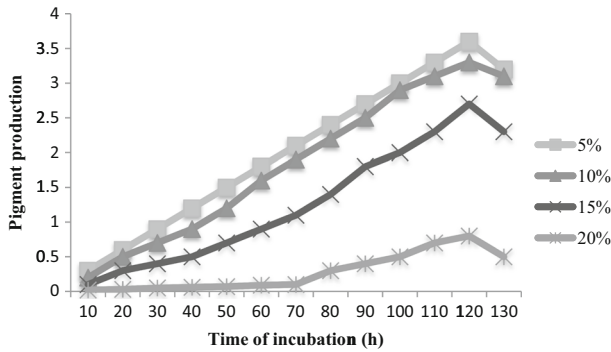
The protein content of *H. alkaliphilus* MSRD1 species was estimated with the fused methodology. The pigment production curve and percentage protein content of *H. alkaliphilus* MSRD1 was found to be 72 % at the end of cultivation (Fig. 3).

## Purification and Determination of the Size of Pigment Protein

About 20 fractions were collected and 7th to 15th fractions were selected depending upon color intensity and pooled together for future characterization. The pooled fractions were concentrated by evaporation under reduced pressure. The TLC plate after development with hexane-ethyl acetate (1:1) and ethanol-hexane (7:3) showed only one red color band when viewed under ultraviolet light (250 and 350 nm). Rf

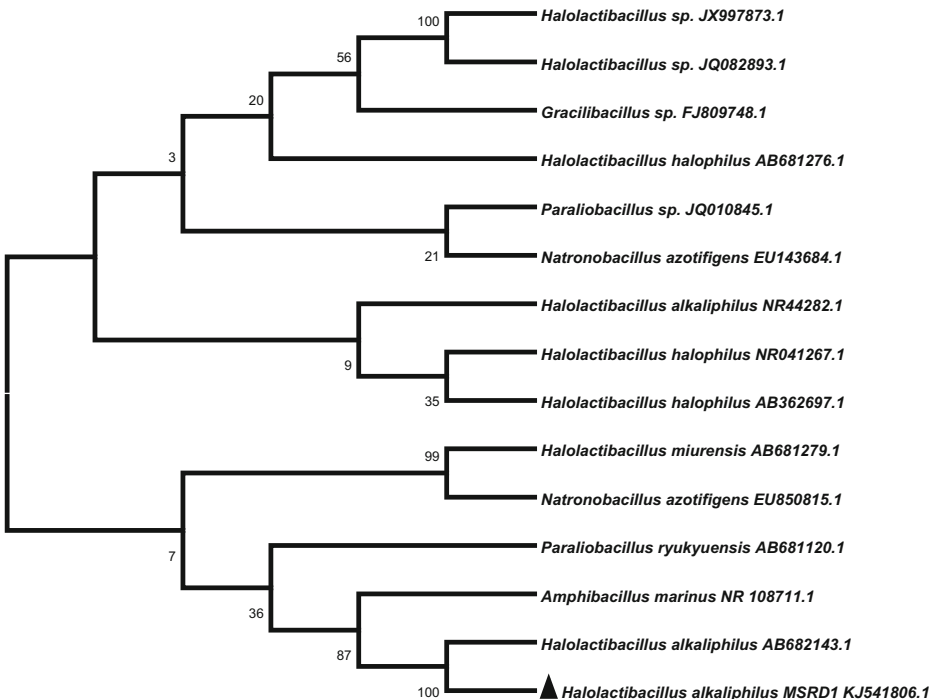
**Table 1** Antimicrobial activity of the red pigment produced by *Halolactibacillus alkaliphilus* MSRD1

Pathogens	Medium	Temperature (°C)	Solvent	Antimicrobial activity by red pigment (mm)
<i>Escherichia coli</i>	LB-SW	37	Ethanol	6
<i>Staphylococcus aureus</i>	LB-SW	37	Ethanol	16
<i>Klebsiella pneumoniae</i>	LB-SW	37	Ethanol	7
<i>Salmonella paratyphi</i>	LB-SW	37	Ethanol	8
<i>Salmonella typhi</i>	LB-SW	37	Ethanol	14

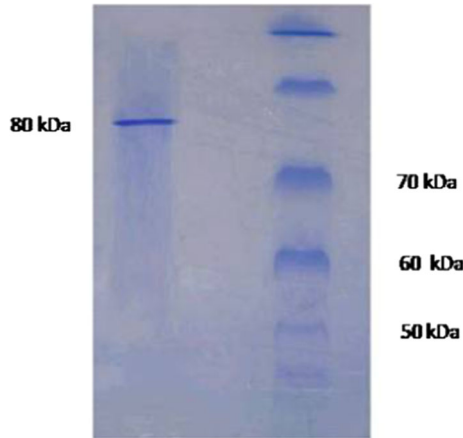


**Fig. 2** Growth curve and pigment production by *Halolactibacillus alkaliphilus* MSR D1. Strain growth (OD<sub>690 nm</sub>), pigment (OD<sub>490 nm</sub>)

value for red-colored pigment was calculated to be 0.443. The size of the molecules could be characterized by SDS-PAGE with standard marker of molecular weight 10–100 kDa. Our results could be interpreted as a complex pigment protein by molecular weight of 80 kDa (Fig. 4).



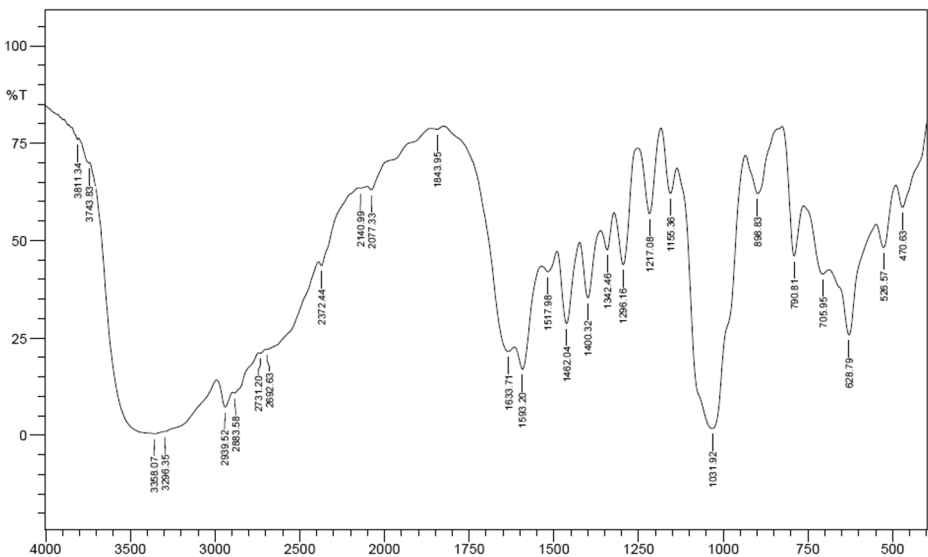
**Fig. 3** Pigment production and protein estimation from *Halolactibacillus alkaliphilus* MSR D1 strain (OD<sub>490 nm</sub>)



**Fig. 4** SDS-PAGE of purified red pigment

### FT-IR Spectral Analysis

From the spectrum obtained (Fig. 5), crude ethanolic extract of the red pigment showed a broad band at  $3358\text{--}3000\text{ cm}^{-1}$  which corresponds to O–H bond that also overlapped with N–H bond of a secondary amide at  $2883.58\text{ cm}^{-1}$ . A band at  $2372\text{--}2731\text{ cm}^{-1}$  corresponds to O–H bond belongs to carboxylic acids. A broad band at  $1600\text{--}1500\text{ cm}^{-1}$  to  $\text{NO}_2$  belongs to nitro groups. A sharp band observed at  $1463\text{--}1031\text{ cm}^{-1}$  corresponds to C–N bond belongs to amines. A broad band observed at  $898\text{--}526\text{ cm}^{-1}$  corresponds to C–H bond belongs to phenyl ring substitution.



**Fig. 5** FT-IR spectrum for ethanol extract of red pigment



## Discussion

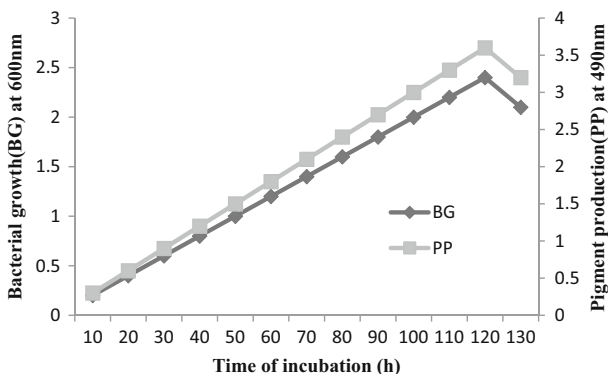
Indian coast is a hot spot of diverse marine floral and faunal assemblages particularly sponges, seaweeds, sea anemones, sea cucumber, sea urchin, and soft corals. Most of these pristine resources have not been explored for bioprospecting studies. The seaweed-microbial association is a potential chemical, ecological phenomenon, which provides sustainable source of supply for developing novel pharmaceutical leads. The isolated bacterium was considered as a highest pigment-producing strain on the basis of high growth and production Calo et al. [5]. In the antibacterial context, the candidate bacterium raises the possibility of using the red pigment as a source of antibacterial compounds for controlling the pathogenic bacteria *S. aureus* and *S. typhi*. Similarly, August et al. [1] reported that violet pigment produced by *Chromobacterium violaceum* has a broad spectrum antibacterial property against both Gram-positive and Gram-negative pathogens.

Normally, the pigment production correlates positively with increase in growth rate. In the present study, the pigment production and growth rate was maximum at 120-h cultivation whereas it slowly decreased after 120-h cultivation. A similar report was extended by Ryazantseva et al. [19] who reported that both growth and prodigiosin production from *Serratia marcescens* was equal.

The growth and pigment production by *H. alkaliphilus* MSRD1 was maximum when the NaCl concentration was 5 % (Fig. 6). Conversely, the growth and pigment production by the candidate bacterium did not occur when the concentration of NaCl exceeded 5 %. Likewise, similar report was extended by Khanafari et al. [9] who reported that the growth and pigment production by *Halorubrum sodomense* did not occur when the concentration of NaCl was exceeded 15 %.

The decrease in color intensity at high pH values can be attributed to the deprotonation of nitrogen by NaOH from the three conjugated rings at the pigment structure. This resulted in electron conjugations in the ring structure that gives it stability. Similar observation was previously reported by Konzen et al. [10] who reported that the electron conjugations in the ring structure that gives the stability of the violet pigment.

The protein content was found to be maximum at the end of the 120-h incubation, and the percentage was 72 %. The maximum pigment production was corresponds to the maximum protein concentration. The size of the pigmented protein molecules could be characterized



**Fig. 6** Effect of NaCl concentration on pigment production ( $OD_{490\text{nm}}$ )

using molecular mass 10–100-kDa standard markers. Our results could be interpreted as a complex of pigment and protein by molecular mass as smaller than 100 kDa, and it was found to be 80 kDa. A similar observation was also reported by Ryazantseva et al. [19] who stated that the prodigiosin from *Serratia marcescens* as pigment protein complex by diameter of particles less than 100 kDa.

In this study, the FT-IR spectra of the red pigment produced by *H. alkaliphilus* MSR1 showed several degrees of similarity to the spectra of prodigiosin pigment produced by *Serratia marcescens* [21, 18].

## Conclusion

The present study provides the baseline for the production and characterization of proteinaceous red pigment and its pharmacological importance. Such molecules have a wide variety of biologically active properties and continue to provide promising avenues for both fundamental sciences and applied biomedical research. For instance, this is the first time we have reported the novel bacterium *H. alkaliphilus* MSR1 from marine environment especially from seaweed with pigment-producing capability.

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**Conflict of Interest** We declare that we have no conflict of interest.

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