Indian Journal of Natural Products and Resources
Vol. 16(4), December 2025, pp 656-666
DOI: 10.56042/ijnpr.v16i4.16033

Isolation of pigmented marine bacteria and evaluation of their antibacterial and antioxidant activities against multidrug-resistant bacterial pathogens

V Ramabhai[1], S Sivaranjani[2], A Sarumathi[2], A Anusuya[3], D K Usha[2] and A Gejalakshmi[4]*

[1]Department of Food Processing Technology, AMET Deemed to be University, Chennai 600040, Tamil Nadu, India

[2]Department of Biotechnology, Bon Secours College for Women (Autonomous), Affiliated to Bharathidasan University, Thanjavur 613006, Tamil Nadu, India

[3]Indian Biotrack Research Institute, Thanjavur 613005, Tamil Nadu, India

[4]Faculty of Science, Department of Microbiology, Thiruvalluvar University, Serkadu, Katpadi, Vellore 632006, Tamil Nadu, India

Received 30 December 2024; revised received 11 August 2025; accepted 13 October 2025

The present study focused on isolating pigmented marine bacteria and evaluating their antibacterial and antioxidant activities. A total of 12 bacterial strains were isolated from marine sediment samples, among which only PB4, PB5, PB9, PB10, and PB11 produced pigments. Primary antibacterial screening revealed that PB5 and PB10 exhibited antagonistic activity against multidrug-resistant bacterial pathogens. In secondary screening, the cell-free supernatant of PB5 exhibited the highest inhibition zone (18 mm) against all tested pathogens at a concentration of 100 µL. Consequently, PB5 was selected for further morphological, biochemical, and molecular characterisation. Gram staining and biochemical tests identified the strain as belonging to the genus *Bacillus*, and 16S rRNA sequencing confirmed it as *Bacillus gibsonii* (GenBank accession no. OQ608089). Pigment extraction using organic solvents such as methanol, ethanol, ethyl acetate and petroleum ether of varying polarity revealed methanol as the most effective, yielding an orange pigment with the highest antibacterial activity. Methanolic crude pigment showed maximum inhibition against *Staphylococcus aureus* (24±1.53 mm) and *Escherichia coli* (22±0.58 mm) at 100 µL. Antioxidant analysis by DPPH assay demonstrated significant radical scavenging activity (229.46±0.3%) with an IC_{50} value of 45.6 µg/mL. These findings suggest that the orange pigment from *Bacillus gibsonii* possesses strong antibacterial potential against multidrug-resistant pathogens and notable antioxidant properties.

Keywords: DPPH, Marine bacteria, Molecular profiling, Sediment, Strains

IPC code; Int. cl. (2021.01)− A61K 35/00, A61P 31/00, A61P 39/00

Introduction

Mangroves are trees or large shrubs that thrive in the intertidal zones of tropical and subtropical regions, exhibiting unique adaptations that enable them to survive in saline and waterlogged conditions. The term *mangrove* refers not only to individual plant species capable of tolerating saltwater environments but also to the entire ecosystem they form, commonly known as a *mangal*[1]. These forests are among the most productive ecosystems on Earth, enriching coastal waters, providing valuable forest resources, protecting shorelines from erosion, and sustaining coastal fisheries. Despite their ecological importance, mangroves endure extreme environmental conditions, including high salinity, fluctuating tides, intense winds, high temperatures, and oxygen-poor muddy soils[2].

Marine ecosystems, such as mangroves, remain a largely unexplored reservoir of novel microorganisms with the capacity to produce bioactive secondary metabolites[3]. The continuous rise of multidrug-resistant pathogens and the resulting decline in antibiotic efficacy have intensified the global search for new antimicrobial agents derived from such unique habitats. Significant advancements have been achieved in both chemical synthesis and engineered biosynthesis of antibacterial compounds. Nevertheless, nature continues to be the most abundant and promising source for the discovery of new antibiotics[4]. Antimicrobial resistance is one of the emerging public health problems every day. Some pathogenic multidrug-resistant clinical bacteria are capable of causing diseases. *Staphylococcus aureus* is a frequent

*Correspondent author
Email: arunranjani1402@gmail.com

antibiotic-resistant bacterial pathogen, and it causes various infections. Antibiotic-resistant bacterial pathogens such as *Escherichia coli* and *S. aureus* have developed resistance to the existing antibiotics used to treat microbial disease[5]. The World Health Organisation (WHO) published a report in 2017 on bacterial infections and human health, recommending the development of new antibiotics to combat the resistance of microorganisms. The demand for new antibiotics continues to grow due to the rapid emergence of multiple antibiotic-resistant pathogens, which cause life-threatening infections.

At present, significant advancements are being made in the areas of chemical synthesis and engineered biosynthesis of antibacterial compounds. However, nature continues to be the richest and most versatile source for the discovery of new antibiotics.

The chemicals that absorb light in the visible spectrum are known as pigments. The chromophore, a molecule-specific structure that absorbs solar energy and stimulates electrons to move into higher orbitals, is responsible for the colour that results. Non-absorbed solar energy is then refracted or reflected, allowing it to be picked up by the eye. Plants, animals, algae, and microorganisms all contain natural pigments, which are safer, healthier, biodegradable, renewable, and environmentally beneficial[6,7].

Microbial pigments are safe, cost-effective, independent of seasonal variations, capable of high yields, consistent in production, and easily modified through genetic engineering[8]. Bacterial pigments are characterised by using spectral characterisation, UV–Vis Spectrometry, Gas chromatography-mass spectrometry (GC-MS), Nuclear magnetic resonance (NMR), and Fourier transform-infrared spectroscopy (FT-IR).

The majority of bacterial pigments, on the other hand, are still in the research and development stage. As a result, efforts to develop marine bacterial pigments should be intensified to explore new pigments with distinct biological features and make them commercially available. The main objective of the present study is to isolate bioactive pigments from marine bacteria in the mangrove region and screen them for potential pharmacological applications[8].

Materials and Methods

Sample Collection

The mangrove sediment samples were collected at a depth of 10 cm, 20 meters near the mangrove region in Pichavaram, Cuddalore district, Tamil Nadu, between North latitudes 11.4226°N and 79.7748°E East longitudes. The mangrove samples were also collected from the location mentioned earlier. The sediment sample was collected at random in sterile polythene bags and stored in a refrigerator for further use. The samples were used to isolate microorganisms, and a portion was deep-frozen in a sterile polythene container at -4°C for future use.

Isolation of marine bacteria

One gram of serially diluted sediment samples was plated on Zobel marine agar (pH range: 7–10) for the isolation of marine bacteria. The plates were incubated at 37°C for 24 hours. After the incubation, the pigmented bacteria were picked and purified. This process was repeated several times until pure cultures of the isolates were obtained. The purified colonies were subcultured onto Zobel marine agar slants and stored at 4°C for further studies[9].

Antibacterial activity studies

Collection of clinical pathogens

Multidrug-resistant bacterial strains, including *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922), were procured from the American Type Culture Collection (ATCC), United States. Pathogens were stored in agar slants at 4ºC for further use.

Primary screening

The pigmented bacteria were screened for antagonistic activity using the cross-streak method[10]. In this method, a single streak of the pigmented bacteria was made on the surface of Mueller-Hinton agar medium and incubated at 37ºC. After incubation, the growth of halophilic bacteria on the petri plates, the pathogenic bacteria were streaked at right angles to the original streak of the isolated marine bacteria and incubated at 37ºC. The antagonistic marine bacteria were chosen after 24–48 hours based on the presence or absence of an inhibition zone[11].

Extraction of cell-free supernatant

The selected antagonistic isolates were inoculated into Zobell Marine Broth separately and incubated at 37°C in a shaker at 100–150 rpm (It is standard for zobel marine broth) for 3 days. After incubation, the broths were filtered through Whatman No. 1 filter paper. Then the filtrates were centrifuged separately at 6000 rpm for 10 min to extract the supernatant. It was transferred aseptically into screw capped bottles and stored at 4°C for further assay[11].

Secondary screening

The activity of the cell-free supernatant was assessed against two multidrug-resistant pathogenic bacteria (*S. aureus* and *E. coli*) by the agar well diffusion method. The antibacterial assay was performed on sterile Mueller-Hinton agar plates. Pathogenic bacterial strains were inoculated in muller hinton broth and incubated overnight for 24 hours at 37°C. After solidification of the media, 6 mm diameter wells were made in the agar plates using a cork borer and seeded with bacterial pathogens. Each well was loaded with 100 µL of pigmented bacterial isolate supernatant and incubated at 28±2°C for 24 hours. Finally, the zone of inhibition was measured by the antibiotic[12].

Genus-level identification of pigmented marine bacteria

The potential bacterial isolates were subjected to Gram staining, microscopic observation, and biochemical tests used for preliminary tests at the genus-level identification was performed by[13].

Morphological characterisation

The potential bacterial isolates were grown on Zobel marine agar medium at 37°C for 72 h. The plates were observed daily for bacterial growth, and the morphological characteristics were documented. The bacterial colony characteristics, such as shape, colour and margin of the colonies on plates, were observed[14].

Microscopical characterisation

Gram's staining

The smear of the culture was made on a clean glass slide and heat fixed. It was flooded with crystal violet solution and allowed to remain for one minute. Then it was washed with water, flooded with iodine solution and left for one minute. It was then drained and decolourised with 95% ethanol, washed gently in running water and counterstained for one minute with Safranin, followed by washing with distilled water. The water-drained smear was observed under the microscope. Purple-coloured cells that retained crystal violet were considered Gram-positive bacteria. Pink colored cells that have lost the primary stain and picked up safranin colour and were considered as Gram-negative bacteria[14].

Motility test

A drop of bacterial suspension was placed on the centre of the cover slip, and wax or soft paraffin was applied over the corners of the cover slip. A glass slide was gently placed over the cover slip and held upside down in a manner that allowed the bacterial suspension to hang between the cover slip and the glass slide. The specimen was examined under the microscope[14].

Biochemical tests

Indole production test

Indole is one of the metabolic degradation products of the tryptophan amino acid. The test strains were inoculated into tryptone broth and incubated at room temperature for 24–48 hours. After incubation, 0.2 mL of Kovac's reagent was added to the test tubes. The development of the cherry red colour ring indicates the production of indole.

Tryptophan → Indole + Pyruvic acid + Ammonia

p-Dimethylaminobenzaldehyde + Indole → Quinoidal red-violet complex

Methyl Red Test

The methyl-red (MR) test is employed to identify bacteria capable of performing mixed acid fermentation. Test strains are inoculated into MR-VP broth and incubated at 37°C for 24–48 hours. After incubation, a few drops of methyl red indicator are added to each culture.

- The appearance of a red colour indicates a positive MR test, confirming the production of stable acids that lower the pH to 4.4 or below.
- A yellow colour indicates a negative MR test, where the pH remains around 6.0, showing the absence of mixed acid fermentation.

Reaction:

Glucose → Pyruvic acid → Mixed acids + CO_2

Methyl red: Yellow at pH 6.0 → Red at pH 4.4 or lower

Voges-Proskauer (VP) test

The Voges-Proskauer test was performed to detect the acetyl methyl carbinol (Acetoin). The test strains were inoculated into MR-VP broth and incubated at 37ºC for 24–48 hours. After incubation, 0.6 mL of 5% alpha naphthol and 0.2 mL of 40% potassium hydroxide were added. The tubes stand for 15 minutes, and a pink colour indicates a positive result, while the absence of pink colour indicates a negative result.

Glucose →pyruvic acid → acetoin +CO_2

Diacetyl +α naphthol +guanidine group → pink to

red-complex

Citrate utilisation test

The citrate utilisation test determines the ability of an organism to utilise sodium citrate as the carbon source. The test strains were streaked on sterile Simmons' Citrate agar slant and incubated at 37°C for 24 hours. After the incubation, the development of a deep Prussian blue colour indicates the citrate-positive results.

Sodium citrate → NH3 + NH4OH

Bromothymol blue Blue (pH 7 →Bromothymol blue)Green (pH 8 or high)

Triple sugar iron test

To differentiate the sugar fermentation, anaerobic respiration, and hydrogen sulphide production. The test strains were inoculated on TSI slant and then incubated at 37°C for 24-48 hours. After incubation Acid butt and Alkaline slant) yellow butt and red slant – (glucose has been fermented but not lactose and sucrose. Acid butt and Acid slant) yellow butt and yellow slant – (lactose and/or sucrose have been fermented. Alkaline butt and Alkaline slant) red butt and red slant – (neither glucose, lactose, nor sucrose has been fermented. Gas production has been indicated by gas bubbles in the butt, and agar may be broken into H2S production, which is indicated by the blackening of the butt due to the reaction of H2S with ferrous ammonium sulphate to form black ferrous sulphide.

The reaction was modified as follows:

1. Cysteine Desulphurase Reaction

Cysteine → Pyruvic acid + H_2S + NH_3

2. Thiosulphate Reductase Reaction

$S_2O_3^{2-} + 4H^+ + 4e^- \rightarrow 2H_2S + 2SO_3^{2-}$

3. Detection Using Ferrous Ammonium Citrate

$H_2S + Fe^{2+} \rightarrow FeS \downarrow$ (black precipitate)

Urease test

Urea hydrolysis was detected by the urease test. Urea is a diamide of carbonic acid. The test strains were inoculated in sterilised Christensen's Urease agar slant with phenol red indicator (pH 6.8). The tubes were incubated at 37°C for 24 hours. After the incubation, the development of pink or red colour indicated a positive reaction. The reaction was modified as follows:

1. Urease Reaction

Urea → 2 NH_3 + CO_2

2. Ammonium Carbonate Formation

$2 NH_3 + CO_2 + H_2O \rightarrow (NH_4)_2CO_3$

3. Phenol Red Indicator (pH Change)

Yellow at pH ≤ 6.8

Pink/Red at pH ≥ 8.2

Nitrate reduction test

The nitrate reduction test is essential for identifying both Gram-positive and Gram-negative species. The test strains were inoculated into nitrate broth and incubated for 48 hours. After incubation, a drop of sulfanilic acid and alpha-naphthylamine mixture (1:1) was added. The appearance of a deep pink colour indicated a positive result.

Catalase test

The catalase enzyme acts as a catalyst in the breakdown of hydrogen peroxide into oxygen and water. Place a drop of H_2O_2 on a slide, and one loopful of test strains was mixed. The immediate liberation of air bubbles indicates a catalase-positive result.

Catalase reaction $2H_2O_2 \longrightarrow 2H_2O + O_2$

Oxidase test

The sterile Hi-media oxidase disc Tetramethyl para-phenylenediamine dihydrochloride was placed on a clean petri dish. The test strains were smeared on the disc. The presence of purple colour within 10-15 seconds indicates the suspected organisms are oxidase positive.

Molecular profiling

Genomic DNA extraction

The genomic DNA of the bacterial strains were isolated using NucleoSpin® Tissue Kit (Macherey-Nagel) following the manufacturer's instructions.

A pure culture is taken in a microcentrifuge tube. 180 µL of T1 buffer and 25 µL of proteinase K were added, and the mixture was incubated at 56°C in a water bath until the cells were completely lysed. After lysis, 5 µL of RNase A (100 mg/mL) was added and incubated at room temperature for 5 minutes. 200 µL of B3 buffer was added and incubated at 70°C for 10 minutes. 210 µL of 100% ethanol was added and mixed thoroughly by vortexing. The mixture was pipetted into a NucleoSpin® Tissue column, placed in a 2 mL collection tube and centrifuged at 11000 x g for 1 minute. The NucleoSpin® Tissue column was transferred to a new 2 mL tube and washed with 500 µL

of BW buffer. The Wash step was repeated using 600 µL of B5 buffer. After washing, the NucleoSpin® Tissue column was placed in a clean 1.5 mL tube, and DNA was eluted using 50 µL of BE buffer.

Agarose gel electrophoresis for DNA quality and quantity check

The quality of the DNA isolated was checked using agarose gel electrophoresis. 1 µL of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5 µL of DNA. The samples were loaded onto a 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/mL ethidium bromide. Electrophoresis was performed using 0.5X TBE as the electrophoresis buffer at 75 V until the bromophenol dye front had migrated to the bottom of the gel. The gels were visualised in a UV transilluminator (Genei) and the image was captured under UV light using a Gel documentation system (Bio-Rad).

PCR amplification of 16S rRNA

The PCR amplification was performed using a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

Primers used

Target	Primer Name	Direction	Sequence (5' -3')
16S rRNA	27F	Forward	5′AGAGTTTGATC CTGGCTCAG-3′
	1492R	Reverse	5′-GGTTACCT TGTTACGACTT-3′

Agarose Gel electrophoresis of PCR products

The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/mL ethidium bromide. 1 µL of 6X loading dye was mixed with 4 µL of PCR products and loaded. Electrophoresis was performed at 75V with 0.5X TBE as the electrophoresis buffer for approximately 1-2 hours, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was a 2-log DNA ladder. The gels were visualised in a UV transilluminator (Genei) and the image was captured under UV light using a Gel documentation system (Bio-Rad).

ExoSAP-IT Treatment

ExoSAP-IT (USB) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for removing unwanted primers and dNTPs from a PCR product mixture, with no interference in downstream applications. Five microliters of PCR product are mixed with 0.5 µL of ExoSAP-IT and incubated at 37°C for 15 minutes, followed by enzyme inactivation at 85°C for 5 minutes.

Sequencing using Big Dye Terminator v3.1

The sequencing reaction was performed in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) according to the manufacturer's protocol.

The Sequencing PCR mix consisted of the following components

Component	Volume
D/W	6.6 µL
5X Sequencing Buffer	1.9 µL
Forward Primer	0.3 µL
Reverse Primer	0.3 µL
Sequencing Mix	0.2 µL
Exosap treated PCR product	1 µL

Post Sequencing PCR Clean up

Component	Volume
D/W	5 µL
3M Sodium Acetate	1 µL
EDTA	0.1 µL
100% Ethanol	44 µL

PCR Procedure

A precipitation mix was prepared using distilled water (D/W), 125 mM EDTA, 3 M sodium acetate (pH 4.6), and absolute ethanol. This mixture was thoroughly vortexed to ensure homogeneity. A volume of 50 µL of this precipitation mix was added to each well of the sequencing plate containing the PCR products. The plate was vortexed briefly to mix and incubated at room temperature for 30 minutes to allow complete precipitation. Following incubation, the plate was centrifuged at 3700 rpm for 30 minutes to pellet the DNA. The supernatant was carefully decanted, and 50 µL of 70% ethanol was added to each well to wash the pellet. The plate was centrifuged again at 3700 rpm for 20 minutes. The ethanol wash step was repeated once more to ensure the removal of residual salts and contaminants. After the final wash, the supernatant was decanted, and the DNA pellet was air-dried completely. The cleaned and air-dried PCR products were then

subjected to Sanger sequencing using the ABI 3500 DNA Analyser (Applied Biosystems).

Sequence merging and BLAST analysis

The forward and reverse ab1 files of sequences were opened with Bioedit software. The reverse complement sequence was constructed for both forward and reverse sequence files. Both sequences were aligned using the Pairwise alignment tool. The aligned sequence was used to create a consensus sequence[13]. The created consensus sequence was used to BLAST in NCBI to retrieve similar sequences. The top 15 sequences from the hit table were chosen to construct a phylogenetic tree in MEGA software[15].

Phylogenetic analysis

The phylogenetic analysis of the aligned file was performed with MEGA software. The MEGA Program was used with an aligned file containing the top 15 highly similar sequences. The phylogenetic tree was constructed using the Neighbor-Joining (NJ) test method[16].

GenBank submission

The 16S rRNA sequences of actinomycete strains have been deposited in GenBank (NCBI) to obtain the corresponding accession numbers.

Extraction of pigment

The selected potential bacteria were further inoculated in zobell marine broth and incubated at 37°C in a shaker at 100 – 150 rpm for 24 hours. After 24 h, the medium with the bacteria were centrifuged at 5000 rpm for 10 minutes. The bacterial cell pellet obtained was mixed with 20 mL of methanol and centrifuged at 10,000 rpm for 10 minutes. The supernatant was collected in a beaker, and the pellet was resuspended in 30 mL of methanol, ethanol, ethyl acetate, and petroleum ether sequentially. After each solvent addition, the mixture was centrifuged to separate the components. The supernatants thus collected were pooled, and the procedure was repeated until the bacterial cell pellet became colourless. The supernatant contains the extracted bacterial pigment, which was then filtered using Whatman filter paper. The solvent was allowed to evaporate at 80°C for 24 hours. The dried pigment was weighed and stored at 4°C until further use[17].

Antibacterial activity of crude pigment

Crude pigment was tested against bacterial pathogens. For this, bacterial pathogens were inoculated in nutrient broth and incubated for 12 hours before the antibacterial assay. All the bacterial strains were individually spread on the Muller-Hinton agar plates. Wells were made in the plates at 6 mm using a cork borer. The different concentrations of crude extracts were added to the wells and incubated for 24 hours[18]. The assay was carried out in triplicate. The zone of inhibition was measured in mm after the completion of the incubation period.

Antioxidant activity

DPPH radical scavenging activity

The scavenging effect of the crude methanolic extract and its solvent-partitioned fractions—methanol, ethanol, ethyl acetate and Petroleum ether content was determined following the method described by reference[18]. Briefly, 2.0 mL of 0.16 mM DPPH solution (prepared in methanol) was added to 2.0 mL of each test sample in a test tube. The mixture was vortexed for 1 minute and incubated at room temperature in the dark for 30 minutes to allow the free radical scavenging reaction to occur. The absorbance of each sample was then measured at 517 nm using a UV-Vis spectrophotometer. The percentage of DPPH radical scavenging activity was calculated to assess the antioxidant potential of each fraction. The scavenging effect (%) was calculated using the formula provided.

Scavenging effect (%) = $\mathbf{1-(A_{sample}-A_{lank})/A_{ontrol}}\times 100$

Where: $A_{(Control)}$ = Absorbance of the control (DPPH solution without the sample)

A_{sample} is the absorbance of the test sample (DPPH solution+Test sample). A_{blank} is the absorbance of the sample only (sample without DPPH solution)

Results

Collection of soil samples

Sediment samples were collected from various locations within the Pichavaram mangrove region (11.4226°N, 79.7748°E) in the Cuddalore district using sterile zip-lock bags. The samples were obtained from a depth of approximately 10 cm below the surface, an area known to be rich in organic matter.

Isolation of marine bacteria from mangrove soil samples

Mangrove soil samples were collected from a depth of 10 cm in the Pichavaram region, Cuddalore District, Tamil Nadu. The isolated marine bacteria were named according to the area as P1. (Table 1). A total of 12 colonies were counted based

on colony morphology. Out of these, six colonies were producing pigments. The isolates PB4, PB5, PB9, PB10, and PB11 were found to produce pigments. All pure cultures were maintained on Zobell Marine Agar slants and stored at 4°C.

Antibacterial activity

Primary screening of antagonistic activity

The selected pigmented bacterial isolates were screened for antagonistic activity against multidrug-resistant bacterial pathogens by the cross-streak method. A total of six isolates were screened for antagonistic activity; out of these, PB5 and PB10 were the only isolates that exhibited antagonistic activity against the two bacterial pathogens. The results are summarised in Table 2.

Secondary screening of cell free supernatant of pigmented marine strains

The selected antagonistic isolates, PB5 and PB10 supernatants, were tested against the selected clinical bacterial pathogens using the agar well diffusion method. In secondary screening, the PB5 supernatant exhibited a maximum zone of inhibition against all tested bacterial pathogens at a concentration of 100 µL. The results are tabulated (Table 3).

Identification of pigment-producing marine bacteria

Based on the primary and secondary screening of antibacterial activity results, PB5 strains were selected for morphological, microscopic, cultural, and biochemical tests.

The pure culture PB5 strain was grown in zobell marine agar at 37°C for 24 h. After incubation, the PB5 strain showed small, shiny, circular and orange-coloured colonies (Fig. 1a and 1b).

Morphological characterisation of pigmented marine strains

Microscopical observation of PB5 Strain

The Pure culture of potential strain PB5 was observed under compound microscopy by using the Gram stain

Table 1 — Antagonistic activity against MDR bacterial pathogens

S. No	Isolates	Multi-drug resistant bacterial pathogens	
		S. aureus	*E. coli*
1	PB4	-	+
2	PB5	++	++
3	PB9	+	-
4	PB10	++	+
5	PB11	-	-

- : No inhibition, + : moderate inhibition, ++ : high inhibition

Table 2 — Antibacterial activity of supernatant

S. No	Isolates	Multi-drug resistant bacterial pathogens	
		S. aureus	*E. coli*
1	PB4	-	+
2	PB5	++	++

Table 3 — Physiological characteristics of the PB5 strain

S. No	Test	PB5
1	Gram staining	+
2	Motility	+
3	Shape	Rod
4	Colony form	Circular
5	Colony appearance	Shiny
6	Melanin pigment	+
7	Growth pH value	7.5
8	Growth temperature	37°C

+Present

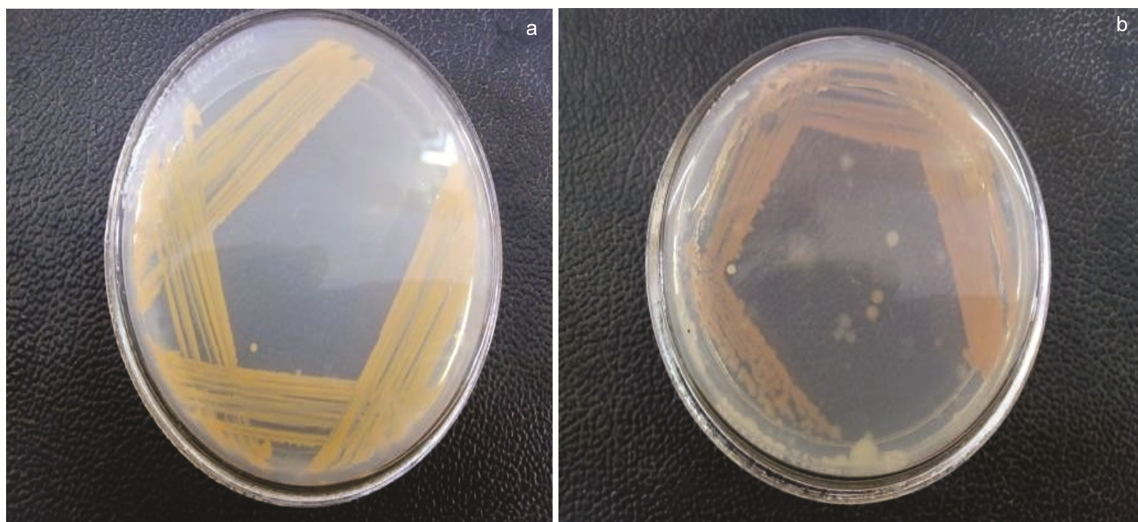

Fig. 1 — a) Pure culture of PB5 strain, and b) Pure culture of PB10 strain.

technique. In light microscopy, the PB5 strain showed Gram-positive, rod-shaped bacteria under microscopical observation. In the motility test, the potential strain was observed to be motile (Table 4 and Fig. 2).

Biochemical tests

Based on morphological, microscopic, cultural, biochemical tests, a sugar test, and hydrolysis, the PB5 strain was confirmed as *Bacillus* sp. The results were summarised (Table 5).

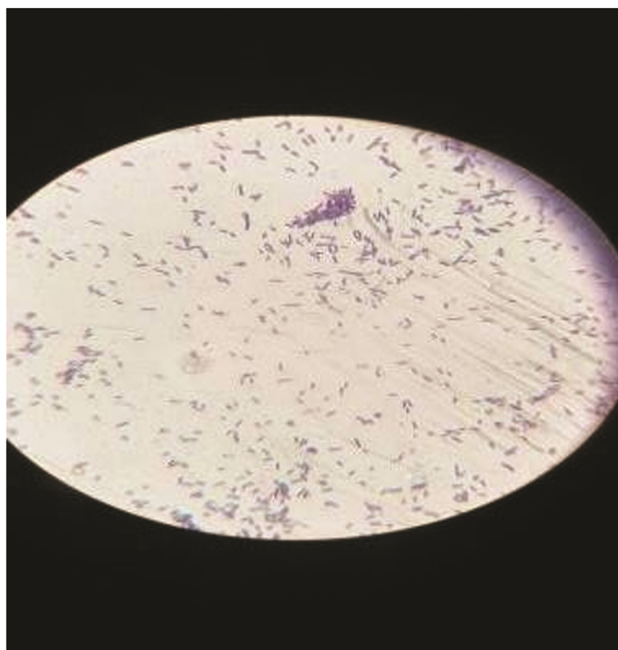

Fig. 2 — Gram staining (100 X).

Table 4 — Biochemical identification of PB5 Halophilic strains

S. No	Biochemical Test	PB5
1	Indole	Positive
2	Methyl- Red	Positive
3	Voges-Proskaeur	Negative
4	Citrate	Positive
4	Triple Sugar Iron	H2S
5	Urease	Negative
6	Nitrate	positive
7	Catalase	positive
8	Oxidase	positive

Molecular identification of pigmented bacterial strains

The species-level identification of the PB5 strain by using 16S rRNA sequence analysis. The strains are amplified by the PCR technique using 27F and 1492R primers. Following this, a phylogenetic analysis was performed to compare the obtained sequence with similar sequences retrieved from GenBank. The BLAST sequence revealed that PB5 is related to *Bacillus gibsonii.* The partial sequences were deposited in the NCBI GenBank database with accession number PB5 (OQ608089) (Fig. 3 and Fig. 4).

Extraction of pigment

The pigmented bacterial isolate PB5 was used for extracting pigments. In this present study, Methanol, Ethanol, Ethyl acetate, and Petroleum ether solvents were used for the extraction of pigments. The orange colour pigment produced by PB5 (*Bacillus gibsonii*).

Antibacterial activity of crude pigment

The crude pigments were tested against clinical bacterial pathogens by the agar well diffusion method. Among the four different solvents, the methanol solvent extract showed the maximum zone of inhibition against *S. aureus* (19±1.53 mm) and *E. coli* (18±0.58 mm) at a 100 µL concentration (Table 5).

Pharmacological activity of pigment

Antibacterial activity

The different concentrations of 25, 50, 75, and 100 µL of crude pigment were screened for antibacterial activity against clinical bacterial pathogens. The maximum zone of inhibition (24±1.53) was observed for *S. aureus* followed by *E. coli* (22±0.58) at 100 µL concentration (Table 6 and Fig. 5).

Antioxidant activity

DPPH radical scavenging activity

The ability of the purified pigment to scavenge free radicals was assessed by using DPPH radical as the substrate. The DPPH$^{\cdot}$ radical scavenging activity of

Table 5 — Antibacterial activity of crude pigment PB5

S. No	Clinical pathogens	Zone of inhibition (mm)			
		Methanol	Ethanol	Ethyl acetate	Petroleum ether
1	*Staphylococcus aureus*	24±1.53	14±1.00	9±1.53	7±0.58
2	*Escherichia coli*	22±0.58	-	9±1.53	8±0.58

Value represents mean ±SD; n=3, - No zone

Bacillus gibsonii_MB5
ACCTACCTTATCGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATCTAGCACCTCCT
GGTGCCGGATTAAAAGAGGGCTTCTTGCTCTCACGATGAGATGGGCCCGCGGCGCATTAGCTAGTTGC
AG
AGGTAACGGCTCCCCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACT
GAGA
CACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGA
GCAA
CGCCGCGTGAGTGATGAAGGGTTTCGGCTCGTAAAGCTCTGTTATGAGGGAAGAACACGTACCGTTC
GAA
TAGGGCGGTACCTTGACGGTACCTCATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAA
TAC
GTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGCCTTTTAAGTCTGAT
GTG
AAATCTTGCGGCTCAACCGCAAGCGGCCATTGGAAACTGGGAGGCTTGAGTACAGAAGAGGAGAGT
GGAA
TTCCACGTGTAGCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGT
CTG
TAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGT
AAA
CGATGAGTGCTAGGTGTTAGGGGTTTCGATGCCCGTAGTGCCGAAGTTAACACATTAAGCACTCCGCC
TG
GGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGTGGAGCATG
TGGT
TTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACTCTGGAGACAGAGC
TTC
CCCTTCGGGGGCAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA
AGT
CCCGCAACGAGCGCAACCCTTGACCTTAGTTGCCAGCATTTAGTTGGGCACTCTAAGGTGACTGCCG
GTG
ACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTG
CTA CAATGGATGGTACAAAGGGTTGCGAAGCCGCGAGGTGAAGCC.

Fig. 3 — Partial sequence of PB5.

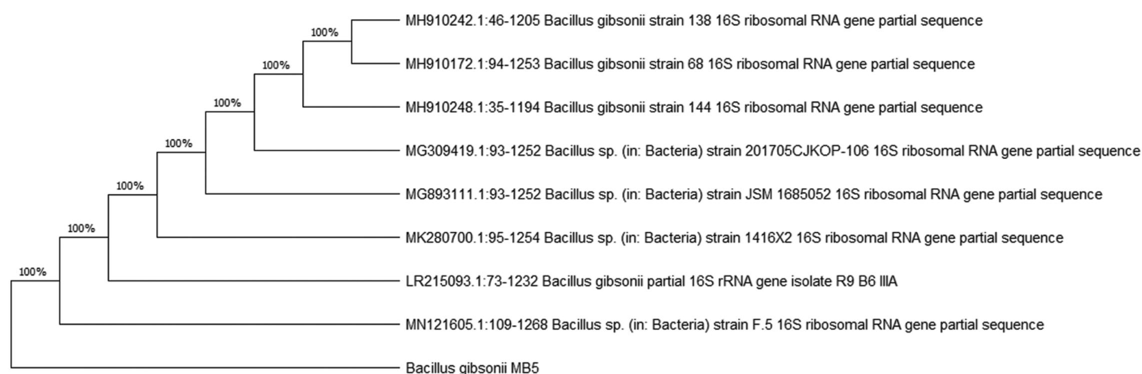

Fig. 4 — Phylogenetic tree of PB5.

Table 6 — Antibacterial activity of pigment

Bacterial pathogens	Zone of inhibition mm					
	25 µL	50 µL	75 µL	100 µL	Positive	Negative
Staphylococcus aureus	10±1.00	14±1.00	20±1.00	24±1.53	30±1.00	-
Escherichia coli	14±1.00	16±1.00	18±0.58	22±0.58	26±1.53	-

Value represents mean ±SD; n=3, - No zone

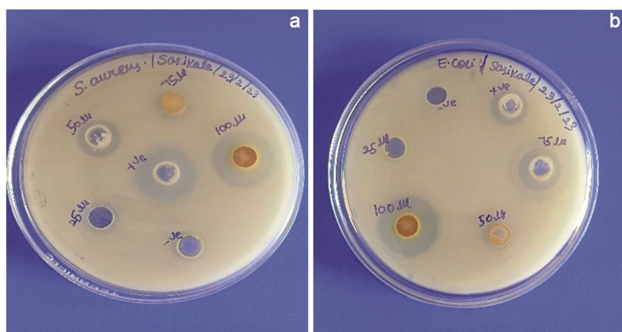

Fig. 5 — Antibacterial activity of pigment, a) *Staphylococcus aureus* and, b) *Escherichia coli*.

Table 7 — Antioxidant scavenging activity of PB5 crude pigment

Concentration (µg/mL)	Ascorbic acid	Scavenging activity of PB5 (µg/g)
20	219.15 ± 3.1	128.43±0.1
40	237.25 ± 2.9	146.05±0.1
60	240.34 ± 3.5	174.65±1.7
80	315.78 ± 2.5	202.32±1.5
100	322.40 ± 1.6	**229.46±0.3**

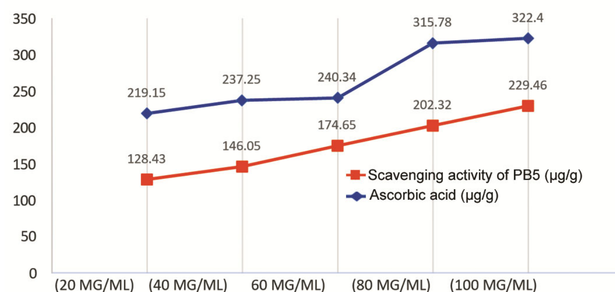

Fig. 6 — Antioxidant scavenging Assay of PB5 Crude Pigment.

crude pigment was shown to be 229.46±0.3 at 100 µg/mL concentration (Table 7 and Fig. 6).

Discussion

Mangrove forests are among the most productive ecosystems in the world, enriching coastal waters, providing valuable forest products, protecting shorelines, and supporting diverse fisheries. These unique environments thrive under challenging conditions, including high salinity, extreme tides, strong winds, elevated temperatures, and muddy, anaerobic soils[2].

Sediment samples were collected from mangrove regions of Saudi Arabia, from which a total of ten marine bacterial isolates were obtained from the sediments[19]. In the present study, twelve colonies were identified based on distinct morphological characteristics, among which six colonies were found to produce pigments[20]. Screening for antagonistic activity among the marine Pseudomonas species revealed that, out of the six isolates tested, PB5 and PB10 exhibited strong antibacterial activity against two multidrug-resistant bacterial pathogens[21]. During secondary screening, the supernatant from isolate PB5 demonstrated the maximum zone of inhibition against all tested bacterial pathogens at a concentration of 100 µL. Pigmented marine bacterial isolates were identified at the genus level using Gram staining and biochemical tests, while species-level identification was performed through 16S rRNA gene sequencing[22]. Based on the primary and secondary antibacterial screening results, the MHB5 strain was selected for detailed morphological, microscopic, cultural, and biochemical characterization[23]. Microscopic observation of the pure PB5 culture under a compound microscope revealed Gram-positive, rod-shaped bacteria[24]. The motility test confirmed that the PB5 strain was motile. BLAST analysis of the 16S rRNA sequence indicated that PB5 was closely related to Bacillus gibsonii. The partial sequence was submitted to the NCBI GenBank database under the accession number PB5 (OQ608089). The study's findings indicate that coastal regions harbour marine bacteria capable of producing antibacterial compounds with potential medicinal value. The antibacterial activity of the crude pigment extract showed maximum inhibition zones against *S. aureus* (21 mm), *E. coli* (16 mm), *Streptococcus pyogenes* (18 mm), *Enterococcus faecalis* (18 mm), *Enterobacter sp.* (22 mm) and *Serratia marcescens* (17 mm).

According to Ishwarya *et al.*, the methanolic extract of the crude pigment exhibited the highest inhibitory effect (24±1.53 mm) against *S. aureus*, followed by *E. coli* (22±0.58 mm) at a 100 µL concentration. Similarly, the orange pigment produced by the marine bacterium *Paracoccus haeundaensis* SAB E11 was identified as a potential natural antioxidant source. In the present study, the extracted light-orange crude pigment was evaluated for antioxidant activity using the DPPH assay, showing a significant antioxidant potential of 229.46±0.3 µg/mL at a 100 µg/mL concentration.

Conclusion

The orange pigment extracted from *Bacillus gibsonii* demonstrated significant antibacterial activity against life-threatening multidrug-resistant (MDR) bacterial

pathogens, along with notable antioxidant potential. These findings suggest that the pigment possesses considerable pharmaceutical value, warranting its exploration as a potential therapeutic agent. In light of its promising bioactivities, the crude pigment was subjected to detailed characterisation through various spectral analysis techniques to determine its structural and chemical properties. Furthermore, the pigment was systematically screened for additional pharmacological activities to assess its broader biomedical applicability and potential for drug development.

References

1 Spalding M, Blasco F and Field C, *World mangrove atlas, Okinawa, Japan: The international society for Mangrove Ecosystem*, 178 State Forest Report, (Forest Survey of India, Dehra Dun), 2010, doi: 10.1126/science.317.5834.41b.
2 Kathiresan N K and Bingham B L, Biology of mangroves and mangrove ecosystems, *Adv Mar Biol*, 2001, **40**, 81-251.
3 Baskaran R, Vijayakumar R and Mohan P M, Enrichment method for the isolation of bioactive actinomycetes from mangrove sediments of Andaman Islands, India, *Malays J Microbiol*, 2011, **7**(1), 1-7.
4 Kpehn F E and Carter G T, The evolving role of natural products in drug discovery, *Nat Rev Drug Discov*, 2005, **4**, 206-220, doi: 10.1038/nrd1657.
5 Blair J M, Webber M A, Baylay A J, Ogbolu D O and Piddock L, Molecular mechanisms of antibiotic resistance, *Nat Rev Microbiol*, 2015, **13**(1), 42-51, doi: 10.1038/nrmicro3380.
6 Aruldass C A, Dufossé L and Ahmad W A, Current perspective of yellowish-orange pigments from microorganisms—a review, *J Clean Prod*, 2018, **180**, 168–182, doi: 10.1016/j.jclepro.2018.01.077.
7 Shatila F, Yusef H and Holail H, Pigment production by *Exiguobacterium aurantiacum* FH, a novel Lebanese strain, *Int J Curr Microbiol Appl Sci*, 2013, **2**(12), 176-191.
8 Vora J U, Jain N K and Modi H A, Extraction, characterisation and application studies of red pigment of halophile *Serratia marcescens* KH1R KM035849 isolated from Kharaghoda soil, *Int J Pure Appl Biosci*, 2014, **2**(6), 160-168.
9 Sanders E R, Aseptic laboratory techniques: Plating methods, *J Vis Exp*, 2012, **63**, e3064, doi: 10.3791/3064.
10 El-Mokhtar M A, Hetta H F, Abd El-Hamid M I, El-Masry H A, Elmahallawy E K, *et al.*, Antibacterial and antibiofilm activities of probiotic Lactobacillus isolates against multidrug-resistant *Staphylococcus aureus* and *Escherichia coli*, *Int J Immunopathol Pharmacol*, 2020, **34**, 1–12, doi: 10.1177/2058738420922996.
11 Cappuccino J G and Sherman N, Microbiology: *A Laboratory Manual*, 10th ed., (San Franscisco Pearson), 2014.
12 Quandri I, Hassani I I, Haridon S, Chalopin M, Hacène H, *et al.*, Characterisation and antimicrobial potential of extremely halophilic archaea isolated from hypersaline environments of the Algerian Sahara, *Microbiol Res*, 2016, **186**, 119-131, doi: 10.1016/j.micres.2016.03.003.
13 Bergey D H, Buchanan R E and Gibbons N E, *American society for microbiology, Bergey's manual of determinative bacteriology*, (Baltimore: Williams and Wilkins Co.), 1974.
14 Koneman E W, Allen S D, Janda W M, Schreckenberger P C and Winn W C, *Diagnostic microbiology. The nonfermentative gram-negative bacilli*, (Philedelphia: Lippincott-Raven Publishers), 1997, 253-320.
15 Hall T A, BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, In Nucleic acids symposium series, *Information Retrieval Ltd*., 1999, vol 41, 95-98.
16 Madden T, The BLAST Sequence Analysis Tool, edited by McEntyre J, Ostell J, in *The NCBI Handbook, Bethesda (MD)*, (National Center for Biotechnology Information (US)), 2002.
17 Kumar S, Stecher G, Li M, Knyaz C and Tamura K, MEGA X: molecular evolutionary genetics analysis across computing platforms, *Mol Biol Evol*, 2018, **35**(6), 1547, doi: 10.1093/molbev/msy096.
18 Sasidharan P, Raja R, Karthik C, Ranandkumar S and Indra A P, Isolation and characterisation of yellow pigment producing Exiguo bacterium sps, *J Biochem Technol*, 2013, **4**(4), 632-635.
19 Conde-Martínez N, Acosta-González A, Díaz L E and Tello E, Use of a mixed culture strategy to isolate halophilic bacteria with antibacterial and cytotoxic activity from the Manaure solar saltern in Colombia, *BMC Microbiol*, 2017, **17**(1), 1-12, doi: 10.1186/s12866-017-1034-2.
20 Liyana-Pathiranan S and Shahidi F, Antibacterial and antioxidant activities of *Adiantum pedatium* L, *J Food Sci*, 2005, **31**, 26-32, doi: 10.1111/j.1365-2621.2005.tb11530.x.
21 Qureshi K A, Seroor M, Al-Masabi A, Saykhan M A, Mutairi Y A, *et al.*, Bio-characterisations of some marine bacterial strains isolated from mangrove sediment samples of four major cities of Saudi Arabia, *J Environ Biol*, 2020, **41**(5), 1003-1012.
22 Radjasa O K, Martens T, Grossart H P, Brinkhoff T, Sabdono A, *et al.*, Antagonistic activity of a marine bacterium *Pseudoalteromonas luteoviolacea* TAB4. 2 associated with coral *Acropora sp*, *J Biol Sci*, 2007, **7(2)**, 239–246.
23 Ishwarya R, Tharani J, Geetha M and Sangeetha D, Isolation, identification and antibacterial activity of pigmented bacteria isolated from marine soil, *Indian J Nat Sci*, 2023, **13**(76), 52264-5227.
24 Velho-Pereira S and Kamat N M, Antimicrobial screening of actinobacteria using a modified cross-streak method, *Indian J Pharm Sci*, 2011, **73**(2), 223-228, doi: 10.4103/0250-474x.91566.