Indian Journal of Geo Marine Sciences
Vol. 50 (08), August 2021, pp. 620-634

Characterization and antibacterial activity of violacein producing deep purple pigmented bacterium *Pseudoalteromonas luteoviolacea* (Gauthier, 1982) isolated from coral reef ecosystems

V S Jayasree[a,b], K S Sobhana*[,a], P Priyanka[a,b], K R Keerthi[a,b], S Jasmine[a], L Ranjith[a], S Ramkumar[a], R Saravanan[a], H J Kingsley[a], K R Sreenath[a], R M George[a], K K Joshi[a] & A Gopalakrishnan[a]

[a]ICAR-Central Marine Fisheries Research Institute, P. B. No. 1603, Ernakulam North P. O., Kochi, Kerala – 682 018, India

[b]Cochin University of Science and Technology, Cochin University P. O., Kochi, Kerala – 682 022, India

*[Email: sobhanapradeep11@gmail.com]

Received 22 October 2020; revised 04 May 2021

The present study describes the isolation, characterization and antibacterial activity of two strains of the deep purple pigmented bacterium *Pseudoalteromonas luteoviolacea* designated P42 (Accession no. MN647538) and M64 (Accession no. MN647537), isolated from two coral reef regions *viz*. Minicoy Lagoon in the Lakshadweep Sea and Palk Bay, off Olaikuda village in Tamil Nadu, India. Ultrastructural examinations were done using scanning and transmission electron microscopy. Presence of violacein pigment was qualitatively analysed by chemical methods; UV-VIS spectrophotometry and FTIR spectroscopy. The strains, P42 and M64 were found to have antibacterial activity against 13 out of the 36 marine bacterial species tested. Antibiogram analysis revealed the sensitivity of both the strains to all the antibiotics tested, except for the resistance of P42 to Penicillin G and Trimethoprim. This study reports the isolation and characterization of *P. luteoviolacea* for the first time from India. Since both the isolated strains have shown clear evidence for the presence of violacein as well as antibacterial activity against selected marine bacteria, both P42 and M64 can be suggested as potent antibacterial agents.

[**Keywords**: Antibacterial activity, Coral reef bacteria, FTIR spectroscopy, MALDI-TOF MS, Ultrastructure]

Introduction

Coral reefs hold an immense and diverse array of flora and fauna testifying its glory as the rain forests of the ocean. The heterogeneity of microorganisms in coral holobiont exert significant roles in coral reef functioning, survival, health and resilience *via.* processes like pathogen defence, provision of nutrients, metabolism of waste or toxic products, nutrient cycling and acclimatization. These microorganisms thrive and flourish in varying climatic conditions, with astonishing diversity producing structurally complex and potent natural products. But the marine world is still largely unexplored with only a small fraction of microorganisms been investigated for bioactive metabolites.

Microbially derived pigments are of rising demand due to the increasing side effects caused by the synthetic colors. To add up, marine pigmented bacteria have been reported to have great potential for use in medical as well as biotechnological fields. Among the wide array of marine bacterial bioactives studied, violacein [3-(1,2-dihydro-5-(5-hydroxy-1*H*-indol-3-yl)-2- *oxo*-3*H*-pyrrol-3-ilydene)-1,3-dihydro-2*H*-indol-2-one], a bisindole metabolite with a deep purple hue holds a significant position. Violacein pigment shows high solubility in ethanol or acetone or their mixture in water, whereas it is insoluble in water, chloroform and benzene. In ether, it is sparingly soluble forming light violet color with intense reddish tinge than in ethanol. Violacein pigment was reported to be used in dyeing of cotton fabrics and lead detecting whole-cell biosensors. Antileishmanial property of violacein is also well established. Researchers from Ulsan National Institute have developed an antibacterial fabric from violacein extracted from *Chromobacterium violacea* sp. Industrial level production of violacein was established by Sigma-Aldrich using *Janthinobacterium lividum*. Studies have also reported significant antiviral and antitumor properties of violacein.

Violacein was reported to be produced by various strains of bacteria such as *Alteromonas (= Pseudoalteromonas) luteoviolacea, Chromobacterium violaceum, J. lividum, Pseudoalteromonas ulvae, Pseudoalteromonas amylolytica* and *Collimonas* sp.[1].

Antimicrobial activity of violacein present in *P. luteoviolacea* has already been reported[2]. A broad range of Gram positive and Gram negative bacteria were reported to be effectively inhibited by violacein from *J. lividum* and *C. violaceum*[1]. Susceptibility of phytopathogenic fungi *Rosellinia necatrix* to violacein suggests its potent fungicidal activity. There are evidences on the inhibition of virulent *Mycobacterium tuberculosis* by violacein producing Antarctic bacteria *Janthinobacterium* sp. and *Flavobacterium* sp. Violacein from *Janthinobacterium* sp. SMN 33.6, isolated from Antarctic showed potent inhibition of a multi drug resistant bacteria. A comparative antifungal activity to that of bavistin and amphotericin B was reported for violacein extracted from *Chromobacterium* sp., thus proving its competency with the existing synthetic antifungal compounds[1]. Inhibitory activity of violacein against *Aspergillus flavus*, *Fusarium oxysporum*, *Penicillium expansum* and *Rhizoctonia solani* is well established[1]. Use of violacein in an anticancer chemotherapy drug, cisplatin for the treatment of cervix cell carcinoma and antiproliferative activity against HeLa cells highlights the potency of the pigment. Strains of *P. luteoviolacea* were reported to produce an *L*-amino acid oxidase having antimicrobial activity[2], antibiotic compound pentabromopseudilin[3] and a non-violacein antibiotic compound, indolmycin. Certain *P. luteoviolacea* strains also produce a straw yellow pigment which is insoluble in ethanol[4].

Gauthier[4] characterised 16 violet pigmented Gram negative marine bacteria and a novel species named *Alteromonas luteo-violaceus* sp. nov. was described with *A. luteoviolacea* CH130 (ATCC 33492) as type strain. Gauthier *et al.*[5] splitted the genus *Alteromonas*, on the basis of phylogenetic analysis of small subunit ribosomal DNA sequences and proposed a new genus *Pseudoalteromonas* comprising *Pseudoalteromonas luteoviolacea* comb. nov., with the type species as *Pseudoalteromonas haloplanktis* (ATCC 14393). The newly proposed *Pseudoalteromonas* genus accommodated 11 species from genus *Alteromonas* and one from genus *Pseudomonas.* Subsequently, the genus *Pseudoalteromonas* gen. nov. was divided into four monophyletic taxa *viz*. (i) *P. denitrificans*; (ii) two pigmented species, *P. citrea* and *P. aurantia*; (iii) another three pigmented species, *P. piscicida*, *P. rubra* and *P. luteoviolacea* and (iv) all non-pigmented species of *Pseudoalteromonas*[5]. Strictly aerobic bacteria of the genus *Pseudoalteromonas* have DNA guanine plus cytosine (G+C) content of 37-50 mol%[5]. The genus *Pseudoalteromonas* comprises Gram negative, heterotrophic, aerobic marine bacteria coming under the order *Alteromonadales* and class *Gamma proteobacteria*. Members of *Pseudoalteromonas* are prominent producers of antimicrobial metabolites such as alkaloids, polyketides, peptides and proteins[6]. Their association with seawater macro-organisms are critical in maintaining holobiont homeostasis[6-7]. A mutualistic relationship is being shared between coral host and *P. luteoviolacea*[8]. The various stimulatory compounds released by the bacteria induce coral metamorphosis[8] and confer resistance against pathogen attack. The bacteria in turn get nutrients and a substratum to adhere on.

This paper describes the isolation, characterization and antibacterial activity testing of deep purple pigmented marine bacterium, *P. luteoviolacea* from 2 distinct coral reef ecosystems in India. *P. luteoviolacea* was previously isolated from corals[9] and sponges[10]. However, no published reports are available so far from Indian coral reef ecosystems. Therefore, this work presents the first time data on isolation and characterization of *P. luteoviolacea* from India.

Materials and Methods

Sample collection

Coral mucus samples were collected as per the procedure described by Guppy & Bythell[11] with slight modifications. Collected samples were suspended in 15 ml sterile seawater and processed further for bacterial isolation. Ambient seawater samples were also collected and processed separately (in triplicates) as done for mucus samples.

P42 was collected from *Porites lutea* in Palk Bay (79°53’13.5” E, 09°42’51” N), Tamil Nadu, India during the pre-monsoon period (August 2016) and M64 from *Acropora bushyensis* in Minicoy Islands (73.05°00′00″ E, 8.27°00′00″ N) of Lakshadweep Sea, during post-monsoon period (December 2015).

Isolation and characterization of bacterial strains

Processed mucus samples were aseptically inoculated in Difco Marine Agar (MA) medium and incubated at 28±2 °C for 72 h. From the large number of colonies grown on the plates, dominant deep purple pigmented colonies were selected and purified by multiple rounds of streaking on MA plates. The purified colonies were subcultured routinely at 28±2 °C on MA medium and preserved by cryostorage in marine broth having 50 % glycerol at -80 °C and also by mineral oil overlay method[12].

Phenotypic characterization

Biochemical/phenotypic characteristics of the strains were studied following Bowman and McMeekin[13]. For taxonomic analysis, characteristics of the type strain *P. luteoviolacea* ATCC 33492 were included for comparison. All the biochemical tests used MA as the basal medium. The strains were allowed to grow in MB with different percentages of agar concentrations to determine the agar dependence of the strains.

Molecular and phylogenetic analysis

DNA extraction, PCR amplification and sequencing of 16S rDNA

Standard Phenol Chloroform Extraction Method[14] was employed for bacterial genomic DNA isolation. The universal primer pair 27F (5' AGA GTT TGA TCM TGG CTC AG 3') and 1492 R (5' TAC GGY TAC CTT GTT ACG ACT T 3')[15] was used for 16S rDNA amplification. The PCR cocktail (25 µl) comprised both forward and reverse primers (10 pmol each), DNA template (50-100 ng) and 23 µl of 1x PCR Master Mix (DreamTaq, Thermo Fischer). Amplification of 16S rDNA was performed in a Thermal cycler (Applied Biosystems). The cycling conditions comprised initial denaturation at 94 °C (5 min); subsequently 35 cycles of denaturation (94 °C; 60 s), annealing (58 °C; 60 s) and elongation (72 °C; 75 s); followed by a final extension at 72 °C (10 min)[16]. The amplified PCR products were analysed by agarose (1.5 %) gel electrophoresis, and sequenced with the same set of primers at the sequencing facility, in an Applied Biosystems AB 3730 capillary sequencer. BioEdit sequence alignment editor version 7.0.5.2[(ref. 17)] was used to edit the raw DNA sequences. Sequence homology analyses used the National Centre for Biotechnology Information (NCBI) GenBank (using the Basic Local Alignment Search Tool; BLAST); Ribosomal Database Project (RDP) and EzBioCloud 16S (www.ezbiocloud.net) databases. Species level confirmation was accepted for those strains having similarity score ≥ 99 % with the reference sequence of a classified species. A phylogenetic tree was constructed by neighbor-joining method using MEGA X software[18].

MALDI-TOF MS analysis

MALDI-TOF MS is a low cost, fast and robust, automatable tool for bacterial identification. A valid and reliable data on identity of bacterial species can often be elucidated from MALDI spectra[19]. HCCA matrix solution (*α-Cyano-4-hydroxycinnamic acid*) was prepared by adding HCCA with 250 µl of standard solvent containing Acetonitrile (ACN), water and Trifluoroacetic acid (TFA), which was then vortexed until the solution became clear. A thin bacterial film was smeared directly onto MALDI target plate, overlayed with HCCA solution; air dried along with a bacterial standard (Bruker Daltonik GmbH, Germany) and then loaded into AUTOFLEX speed (Bruker Daltonik GmbH, Germany).

Peptide mass fingerprint spectra were acquired in a linear positive ion extraction mode at a laser frequency of 200 Hz within a mass range of 2,000 – 20,000 Da. The voltages for ion source 1, 2 and the lens were maintained respectively at 19.5 kV, 18.2 kV and 7 kV, with an extraction delay of 240 ns. The standard calibration mixture (*Escherichia coli* extracts) with additional proteins RNase A and myoglobin (Bruker Daltonik GmbH, Germany) were used for external calibration of spectra. MALDI biotyper software 3.0 (Bruker Daltonik GmbH, Germany) was used for identification of the isolate and visualisation of mass spectra.

Fourier Transform Infrared Spectroscopy (FTIR) analysis

Eighty microliters of 72 h old bacterial suspension in distilled water was aliquoted on to ZnSe optical plate of the ATR attachment of FTIR spectrometer (Thermo Avtar 370 DTGS) and vaccum dried (0.1 bar) for 45 min to get a transparent bacterial films. Absorption spectra over a range of 4000 – 400 cm^{-1} were acquired using the software EZOMNIC (Thermo fisher), at spectral resolution of 4 cm^{-1} with an interferogram of 32 scans.

Electron microscopy

Scanning Electron Microscopy (SEM)

After pelletizing (3,800 × *g*, 10 min), the bacterial samples were rinsed with sterile Phosphate Buffered Saline (PBS) before smearing onto adhesive carbon tape attached to the brass stub. The samples were then gold coated for 10 s using a sputtering unit (Model: JFC1600) at 10 mA current. The samples were then placed in the SEM chamber (Jeol JSM 6390LA) and digital images were captured.

Transmission Electron Microscopy (TEM)

Slightly turbid bacterial suspension (in distilled water) was placed on a carbon-coated grid with 200 meshes. The sample was then air dried and fixed in the specimen holder of a Jeol JEM 2100 transmission electron microscope (at 200 kV). Digital images were recorded using Gatan Camera.

Antibiogram analysis

Antibiotic susceptibility of the strains was determined using 17 commercial antibiotic discs (Hi Media, India) by modified Kirby-Bauer disc diffusion technique[20] with basal medium as MA.

Violacein pigment production

Addition of 1 % soluble starch (w/v) to the growth medium was reported to have an elevated response on violacein synthesis by *P. luteoviolacea*[4]. Bacterial samples were grown (for 72 h) in MA supplemented with 1 % soluble starch, suspended in 96 % ethanol and centrifuged at 3,800 × *g* for 15 min at 4 °C. Presence of violacein in the ethanolic extract of violacein was confirmed by a color test using different chemicals *viz*. 10 % KOH, 50 % HNO_3, 50 % H_2SO_4, Glacial acetic acid, 2M HCl and 10 % H_2O_2 and characteristic color changes were noted[21]. Violacein content in the ethanolic extract was also quantified spectrophotometrically (Nanodrop 1C; Thermo Scientific, USA) by measuring the absorbance at 585 nm[4].

Antagonistic activity against marine bacteria

Antagonistic activity of P42 and M64 were tested against 36 test bacterial species collected from various coral reef ecosystems.

Both P42 and M64 were spotted on a mat culture of the test strains (10^6 CFU ml^{-1}, in sterile PBS) on MA plate, incubated for 72 h at 28±2 °C and monitored for zone of inhibition if any. The test was done in triplicates. Diameter of the inhibition zones were recorded (in mm). Identity of the test strains were confirmed by molecular techniques using 16S rRNA gene sequencing and by homology comparison in NCBI GenBank, EzBioCloud and RDP databases.

Results and Discussion

Phenotypic characterization

On 72 h incubation in MA medium, both P42 and M64 formed round (3 – 5 mm diameter) opaque smooth convex regular colonies with entire margin and deep purple pigmentation (Figs. 1a, b).

However, M64 was found to form slightly bigger colonies, with a waxy surface and a pale white outer edge of variable width. Pinpointed colonies started appearing after incubation at 28±2 °C for 48 h and subsequently colonies started intensifying in size and color for up to 96 h. Both the strains P42 and M64, were characterized as *Pseudoalteromonas luteoviolacea*, on the basis of phenotypic and biochemical features. In the present study, *P. luteoviolacea* strains were obtained only from mucus samples and not from ambient seawater, which confirmed the origin of the strains as coral mucus. The biochemical properties of the strains and their comparison with type strain are given in Table 1.

The description of *Alteromonas luteo-violaceus* sp. nov.[4] (later redesignated as *Pseudoalteromonas*

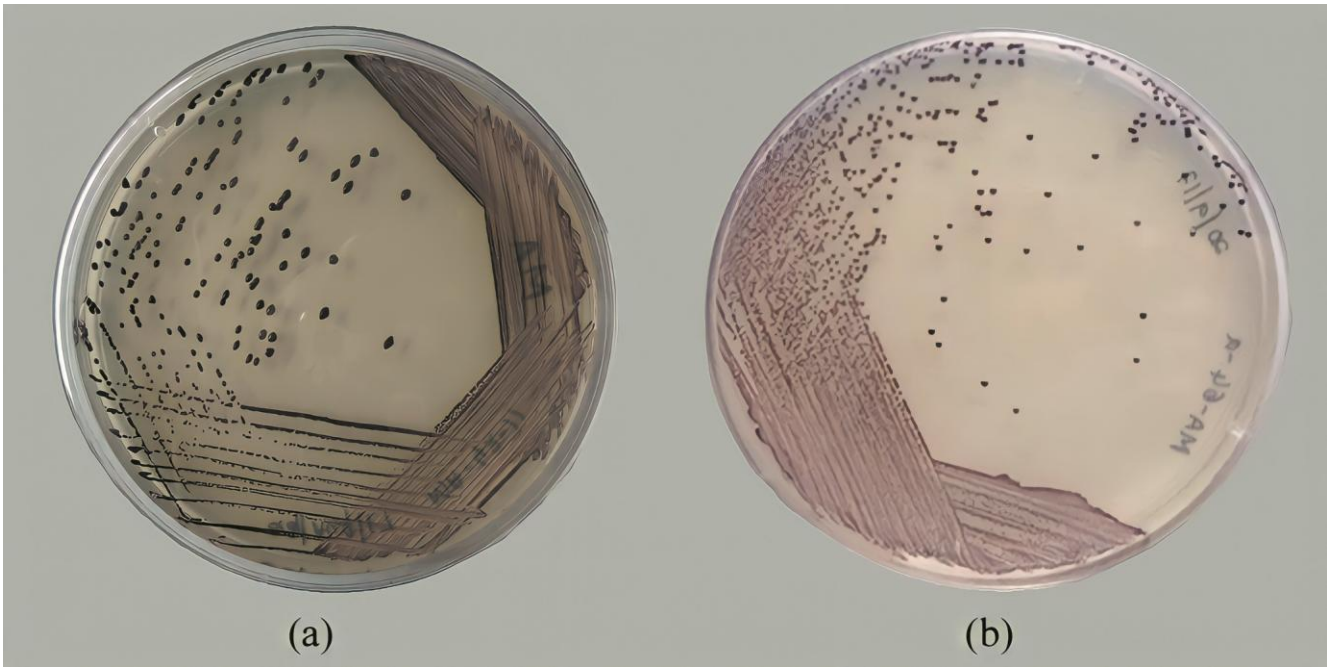

Fig. 1 — Colonies of *P. luteoviolacea* (a) P42 and (b) M64 strains grown on marine agar medium

Table 1 — Biochemical characteristics of *P. luteoviolacea* P42 and M64 strains in comparison with type strain *P. luteoviolacea* ATCC 33492

Tests	Type strain *(P. luteoviolacea* ATCC 33492)	*P. luteoviolacea* P42	*P. luteoviolacea* M64
Gram reaction	-ve	-ve	-ve
Cell morphology	Rod	Rod	Rod
Growth on TCBS	-	-	-
Luminescence	-	-	-
Swarming on solid media	-	-	-
Motility	+	+	+
0/129 sensitivity :			
10 µg	-	-	-
150 µg	-	-	-
Growth in % NaCl :			
0	-	-	-
1	-	+	+
2	+	+	+
4	-	+	+
6	-	+	+
8	-	-	-
10	-	-	-
Catalase	-	-	Weak +
Oxidase	+	+	+
ONPG	-	-	-
Production of violacein pigment	+	+	+
Growth at (°C) :			
4	-	-	-
20	+	+	+
30	+	+	+
42	-	-	-
Growth at pH6	-	+	+
Reduction of nitrate	-	-	-
Esculin hydrolysis	-	-	-
Indole production	-	-	-
H_2S production	-	-	-
Citrate	-	-	-
Urease	-	-	-
Metabolism (O/F)	Oxidative	Oxidative	Oxidative
Acid from :			
Glucose	-	Slight +ve	Slight +ve
Maltose	+	+	+
Trehalose	+	+	+
Enzyme activity :			
Amylase	+	+	+
Gelatinase	+	-	-
Caseinase	+	+	+
Lipase	+	-	-
DNase	+	+	+
Amino acid decarboxylation :			
Arginine	-	-	-
Lysine	-	+	+
Growth in % agar :			
0	-	-	-
0.1	+	+	+
0.4	+	+	+
0.8	+	+	+
1.0	+	+	+

+ : Positive reaction; - : Negative reaction

luteoviolacea comb. nov.[3]) was similar to that of the type strain *P. luteoviolacea* ATCC 33492 reported by Gauthier. Both P42 and M64 are Gram negative aerobic slightly curved rods[5-6] with growth shown only in NaCl concentrations ranging from 0.9 – 6.0 % *i.e*. 0.2 – 1 M (Na^+). This narrow fastidious demand for Na^+ ions indicates their incapability to propagate in freshwater medium that is deficit in sodium ions. *P. luteoviolacea* was reported to have salt tolerance up to 0.6 M (*i.e*. 3.6 %), with optimum growth at 0.4 M (2.3 %)[4]. However, both P42 and M64 strains were found to grow up to 1M Na^+ levels. Catalase activity was generally absent in *Pseudoalteromonas* genus but weak catalase effect[4] was shown by M64 which would probably be due to the presence of polyanionic antibiotic[22]. Also the catalase activity for the *Pseudoalteromonas* genus, were reported as "weak or irregular"[6] rather than "absent". In agreement to the report by Gauthier[4], P42 had no catalase enzyme activity while both the strains P42 and M64, were oxidase positive[4,6]. In contradiction to the type strain, growth at pH 6 and slight acid formation from D-glucose were observed for both the strains. Similarly, both strains exhibited lysine decarboxylation activity. None of the strains were found to hydrolyse lipid, gelatin and DNA unlike amylase and casein. Neither of the strains had a constitutive arginine dihydrolase system nor did they utilise citrate[4,6] and produce H_2S from thiosulfate. Growth was shown by both isolates at 20 °C while no growth observed at 4, 37 and 42 °C. Both cultures showed resistance to vibriostatic disc (Difco) O/129 in both 10 µg and 150 µg concentrations. Both the strains produced acid from only D-glucose, trehalose and maltose. They also exhibited oxidative metabolism as determined by Hugh-Leifson method[23]. Inability of the bacterium to grow in marine broth media, unlike the type strain *P. luteoviolacea* ATCC 33492 and in general purpose nutrient (both agar and broth media) even with extra supplemented NaCl, implies the essentiality of a seawater based solid medium for its survival[5]. Probing into the minimum requirement of agar to facilitate bacterial growth, it was found that a concentration as low as 0.1 % was found to be sufficient, but is absolutely essential for its survival.

The physiological and biochemical properties of both P42; M64 and the type strain were observed to be significantly similar. However, slight variations encountered might represent strain variations, as they were isolated from geographically distinct locations and different host species. The microbiota from different ecosystems and host species would genetically acclimatise in response to various biotic and abiotic factors[24]. The minor morphological differences observed between the bacterial strains are thus justifiable. However, the inability of both strains to grow in liquid medium should be specifically mentioned. Sixteen strains of *P. luteoviolacea*, each showing alterations from the type strain in one or other way were already reported[25]. Thus, different strains of *P. luteoviolacea* are expected to show differences in their characteristics. Appendix 1 shows the details of other bacteria isolated along with *P. luteoviolacea*.

Molecular characterization and phylogenetic analysis

Molecular analysis and homology search identified the strains P42 and M64 as *Pseudoalteromonas luteoviolacea* with similarity scores of 99.85 and 99.93 %, respectively with the type strain *P. luteoviolacea* ATCC 33492. Sequences were deposited in NCBI GenBank with Accession numbers MN647537 and MN647538 for M64 and P42, respectively. Phylogenetic comparison of the two strains (Fig. 2) was performed by neighbor-joining method using MEGA X software. Distance matrices were calculated using Kimura's 2-parameter correction and robustness of grouping were checked by performing bootstrap analysis (1000 replicates).

MALDI-TOF MS analysis

Both P42 and M64 strains were characterised using MALDI-TOF MS. Good quality MS spectra were generated for both strains (Figs. 3a, b).

The log score obtained for both the strains were 2.42 and 2.39, respectively which confirms the species level identity of the strains[26]. In MALDI-TOF Mass Spectrometry, a genus-specific peak for *Pseudoalteromonas* was reported by Emami *et al*.[27] at *m/z* 4233±2. Both P42 and M64 showed peaks at *m/z* 4252 which was in agreement to this report. Close similarities in mass-spectral patterns of P42 and M64 further confirms their identity as strains of the same species. The major peaks presented by M64 were at *m/z* 3287, 4252, 4905, 5625, 6571, 7246, 9804 and 11238 and by P42 at *m/z* 3455, 4252, 5294, 5874, 7246, 9802 and 11237. The peaks at *m/z* 9804±2, *m/z* 11238±2, and *m/z* 4252, *m/z* 7246 are characteristic to *P. luteoviolacea*[27]. Slight differences among mass spectra of the strains could be attributed to their

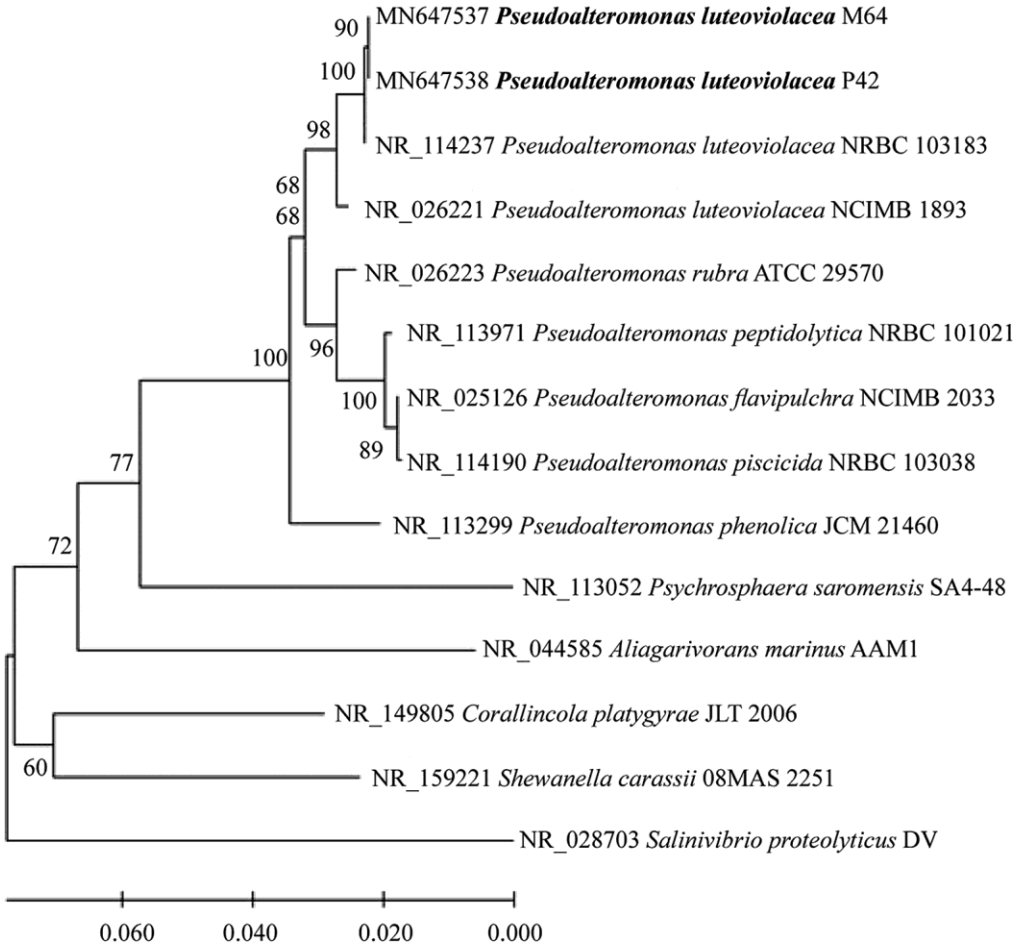

Fig. 2 — Neighbor-joining phylogenetic tree based on the 16S rRNA gene sequences of *P. luteoviolacea* P42 and M64 strains. Evolutionary distances were computed using Kimura's 2-parameter method with 1000 bootstrap replicates in MEGA X. Scale bar indicates mean number of substitutions per base. Bootstrap values are displayed at branch points

spatially and genetically distinct origin in terms of both location and host species. According to Hazen *et al.*[28], even organisms belonging to the same species showed variations in their spectral patterns owing to spatial and temporal variations.

Analysis of FTIR spectra

Infra-red spectrum of both the strains (Figs. 4a, b) exhibited all the signals that are characteristic to violacein pigment[29] which further substantiates the presence of violacein in both P42 and M64. Spectral peaks corresponding to the absorbance frequencies of organic molecules and primary molecular vibrations were observed between 4,000 and 400 cm^{-1}. The bands at 1638 cm^{-1} (P42) and 1636 cm^{-1} (M64) corresponded to the carbonyl (C=O) group present in violacein[30]. The indole nucleus present in the violacein holds an -NH group which forms a peak at 3432 cm^{-129} which were demonstrated in the spectra of P42 and M64 at 3473 and 3543 cm^{-1}, respectively.

The polysaccharide region is represented between 1,200 and 950 cm^{-130} which describes the C–O–C and C–O–P stretching of diverse polysaccharide groups.

The spectra of both the strains showed almost identical patterns. Strain P42 showed strong absorption bands in regions 3600 – 3400 cm^{-1}, 2100 – 2000 cm^{-1}, 1638 cm^{-1}, 1200 – 900 cm^{-1} and 600 – 400 cm^{-1}. M64 has absorption bands in regions 3700 – 3200 cm^{-1}, 2200 – 2000 cm^{-1}, 1636 cm^{-1} and 700 – 400 cm^{-1}.

Highly specific IR patterns can be reliably used for deciphering bacterial identity. The differential absorbance of various biomolecules present in the bacteria including cell wall components *viz.* peptidoglycan layer, lipoproteins, phospholipids, proteins and lipopolysaccharides causes discrete absorbance in the mid-infrared region producing

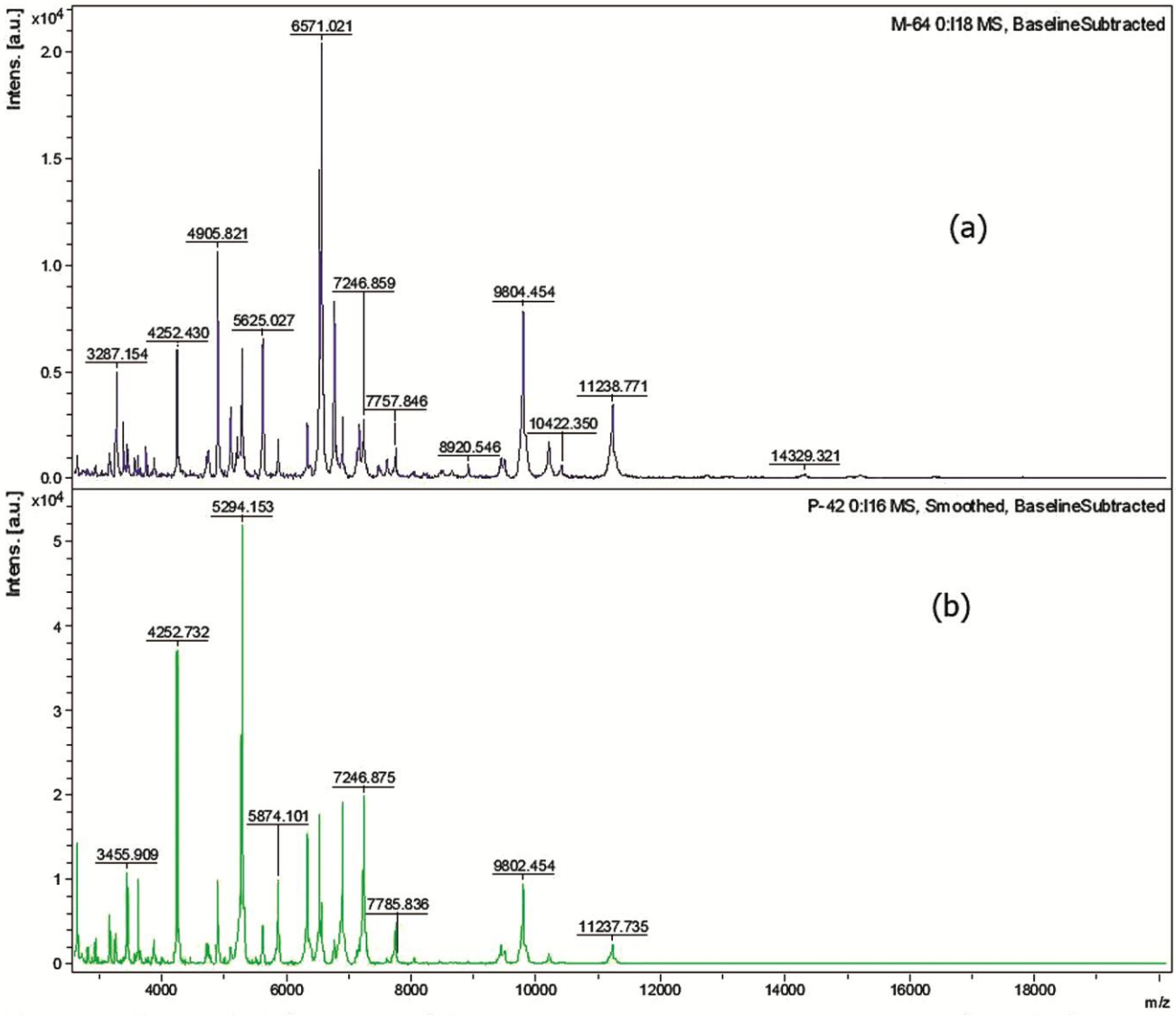

Fig. 3 — MALDI-TOF MS spectra of *P. luteoviolacea* (a) M64 and (b) P42 strains

unique and reproducible spectral patterns. The straightforwardness and flexibility of FTIR makes it a resourceful technique for speedy differentiation and taxonomic classification of microorganisms.

Analysis of SEM and TEM images

Ultrastructural examination of the bacterial cells was made by TEM and SEM analysis. Both P42 and M64 were found to be slightly curved short thick single rods with rounded ends[4] averaging in size about 458.12 – 489.39 nm to 464.28 – 500 nm, respectively. TEM images of *P. luteoviolacea* strains P42 and M64 showed capsulated cells with single polar flagellum (Figs. 5a, b) which is in agreement with previous reports[4-5]. Also, the strains were found to be motile when grown in marine agar medium containing 0.4 % bacto agar.

P. luteoviolacea was reported to have 500 – 800 nm width and 1200 – 3000 nm length[5]. Both curved and straight rods were reported for *P. luteoviolacea*. Strains of *P. luteoviolacea* exhibiting either single polar flagellum or occasionally multiple polar flagella or some with even sheathed flagella have been reported. Filamentous forms were observed in cultures after 10 days of incubation[4]. However, a decrease in motility was reported after 3 – 4 days at room temperature[4].

Antibiotic susceptibility

Table 2 depicts the antibiotic susceptibility profile of P42 and M64 in comparison with the type strain

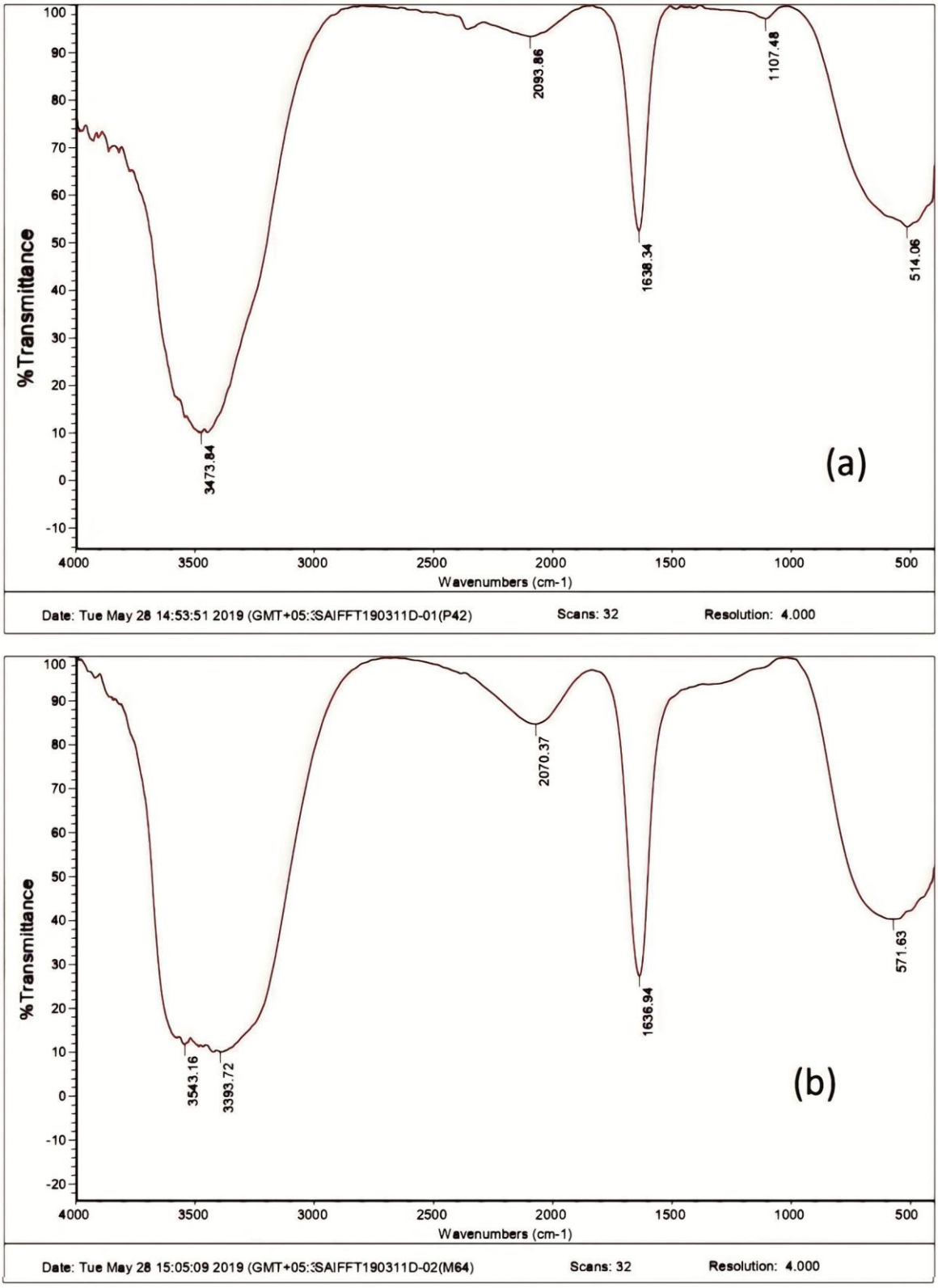

Fig. 4 — Attenuated total reflection type (ATR)-FTIR Spectrum showing peak assignments from 4000 – 400 cm^{-1} of *P. luteoviolacea*: (a) P42, and (b) M64

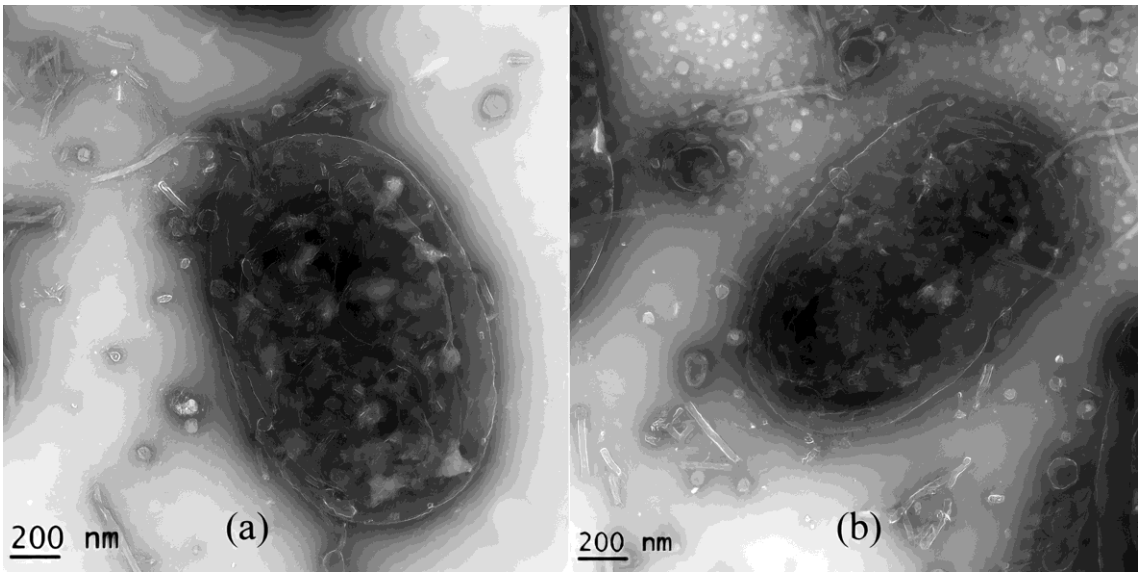

Fig. 5 — Transmission Electron Microscopic images of *P. luteoviolacea*: (a) P42, and (b) M64

Table 2 — Antibiotic susceptibility profile of *P. luteoviolacea* P42 and M64 strains in comparison with type strain *P. luteoviolacea* ATCC 33492

Antibiotic	*P. luteoviolacea* M64	*P. luteoviolacea* P42	Type strain (*P. luteoviolacea* ATCC 33492)
Streptomycin (10 µg)	***S***	***S***	R
Carbenicillin (100 µg)	S	S	-
Sulfafurazole (300 µg)	S	S	-
Nitrofurantoin (300 µg)	S	S	-
Amikacin (30 µg)	S	S	-
Amoxyclav (30 µg)	S	S	-
Ciprofloxacin (50 µg)	S	S	-
Kanamycin (30 µg)	***S***	***S***	R
Nalidixic Acid (30 µg)	S	S	-
Tobramycin (10 µg)	S	S	-
Vancomycin (30 µg)	S	S	-
Tetracycline (30 µg)	***S***	***S***	R
Erythromycin (15 µg)	S	S	S
Trimethoprim (5 µg)	S	R	-
Rifampicin (5 µg)	S	S	S
Penicillin G (10 µg)	***S***	R	R
Chloramphenicol (30 µg)	S	S	S

S: Sensitive; R: Resistant; and -: Data not available. Those given in bold italics show variations from type strain

P. luteoviolacea ATCC 33492. M64 was found sensitive to all the tested antibiotics while P42 was sensitive to all antibiotics except Trimethoprim and Penicillin G.

In comparison to the type strain, P42 and M64 showed difference in susceptibility to certain antibiotics *viz*. Streptomycin, Kanamycin, Tetracycline and Penicillin G. Researchers have proved environment as an important component in determining antibiotic resistance[31]. Increasing anthropogenic influences on coral reef ecosystem could also have contributed to this differing antibiotic susceptibility profile.

Estimation of violacein pigment production

Characteristic color changes were observed on addition of various reagents to the ethanolic extracts of the bacterial strains P42 and M64. Addition of 10 % KOH produced a green color which later turned to red and subsequently to brown in a few minutes, 50 % HNO_3 resulted in a yellowish orange color, while development of an emerald green color was observed with 50 % H_2SO_4. A stable blue and green color was produced on reaction with glacial acetic acid and 2M HCl, respectively. Addition of 10 % H_2O_2 resulted in no color change at 20 °C and the solution remained violet but decolorized at 80 °C. All the color changes were characteristic to violacein and were in accordance with the report of Sneath[21] (Fig. 6).

The presence of violacein pigment was spectrophotometrically verified in both the strains. The UV-VIS absorption spectra obtained for the ethanolic extract of the pigment were found to be in accordance with already published reports[32]. A

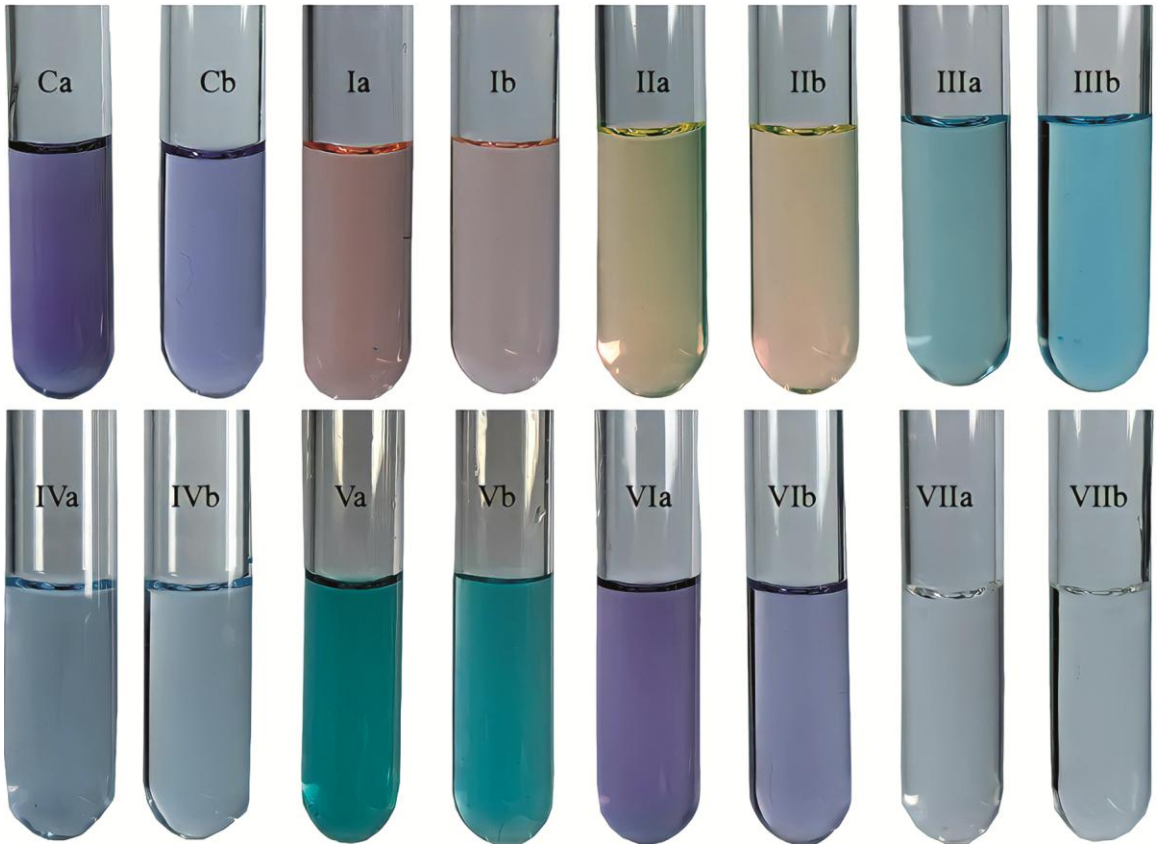

Fig. 6 — Characteristic color changes shown by ethanolic extract of violacein pigment from *P. luteoviolacea*: (a) P42, and (b) M64 strains in response to addition of various chemical reagents (C: Control; I: KOH; II: HNO_3: III: H_2SO_4: IV: Glacial acetic acid; V: HCl; VI: H_2O_2 at 20 ℃ and VI: H_2O_2 at 80 ℃)

violacein specific absorption peak was observed at 585 nm. Both the strains showed similar absorption peaks at 585 nm inferring the presence of violacein pigment. However, the intensity of light absorption differed between the two strains with M64 and P42 showing respective OD values of 0.55 and 0.90, respectively. Difference in the OD value reflects the difference in the concentration of violacein pigment produced by the strains. The intensity of pigment extracted from P42 was found to be higher than that of M64. However, the impurities in the crude pigment could also alter the absorption maximum in the ultraviolet region[32]. In this study, both P42 and M64 exhibited peaks similar to that obtained for purified violacein as published by Gilman[32] for *C. violaceum.*

Antagonistic activity against marine bacterium

Antagonistic activity of *P. luteoviolacea* strains P42 and M64 were tested against 36 marine bacterial strains of which 13 were found to be susceptible (Table 3).

Both P42 and M64 strains were found to have antagonistic activity against all the test strains except *Vibrio neocaledonicus* K3.11.B2 and *Vibrio harveyi* KP.12.3A which were found resistant to M64.

Studies on inhibitory interactions of coral-associated microorganisms unveil the list of secondary metabolite-producing bacteria[33]. P42 was found to have comparatively better antagonistic activity against the tested strains. The antagonistic activity exhibited by these strains could be the cumulative effect of the various antimicrobial products present in the strains of *P. luteoviolacea viz.* violacein pigment, antibiotic compounds like indolmycin, pentabromopseudilin[3], antimicrobial *L*-amino acid oxidase[2] and other antibacterial proteins[32]. Presence of these compounds is strain dependent which explains the reason for the better antibacterial properties of P42. For instance, *P. luteoviolacea* S4054 produces antimicrobial compounds violacein and indolmycin, whereas antimicrobial activity of *P. luteoviolacea* S4060 was conferred by violacein and pentabromopseudilin[33]. According to Sneath[21],

Table 3 — Antagonistic activity of *P. luteoviolacea* P42 and M64 against marine bacterial strains isolated from coral reef ecosystems

Bacterial strain	NCBI GenBank Accession #	Diameter of zone of inhibition (mm)	
		P42	M64
Vibrio corallilyticus K3.4B	MT020410	7	7
Photobacterium rosenbergii K2.2.1	MT020411	7.5	7
Vibrio neocaledonicus KP3.14B	MT020412	8	8
Vibrio neocaledonicus K3.11.B2	MT020413	8	0
Vibrio diabolicus K2.7.2B	MT020414	7	7
Vibrio sp. K1.57	-	8	7
Vibrio sp. K3.27A	-	8	7
Vibrio alginolyticus K2.17.1A	MT020415	8	7
Vibrio neptunius K3.19A	MT020416	7	7
Vibrio alginolyticus K3.56B	MT020417	8	8
Bacillus cereus KP.31.1A	MT020418	7.5	8
Vibrio harveyi KP.12.3A	MT020419	8	0
Vibrio rotiferianus KP.40.3	MT020420	8	7.5

violacein which is not soluble in water and soluble in acetone as well as ethanol shows a higher antibacterial activity in the presence of catalase.

Excluding *Photobacterium rosenbergii* K2.2.1 and *Bacillus cereus* KP.31.1A, all the susceptible test strains belonged to the genus *Vibrio*, which comprises several opportunistic marine pathogens. Since certain species of *Vibrios* are major etiological agents causing coral destruction[34], the anti-vibrio effect of both P42 and M64 is of significance. *Vibrio corallilyticus* was reported to impact coral species belonging to *Pocillopora damicornis*, *Montipora* spp. and *Acropora* spp. leading to their mortality by tissue lysis and bleaching. It is also an etiological agent in fishes and shellfishes[34]. *V. alginolyticus* was reported to cause yellow band/blotch in *Acropora* spp. and *Fungia* spp.[35]. Coral diseases are largely caused by bacteria with members of *Vibrio* genus representing a prominent share[35]. Growth inhibition of *V. harveyi* by *P. luteoviolacea* was already reported by Radjasa *et al.*[9]. The secondary metabolite, Non-Ribosomal Peptide Synthetase (NRPS) produced by *P. luteoviolacea* effectively inhibits *V. harveyi* which substantiates the potential role of *P. luteoviolacea* in controlling shrimp disease[9]. Studies have delineated the role of *P. rosenbergii* in coral bleaching[36]. The antibacterial activity shown by *P. luteoviolacea* strains P42 and M64 could be attributed to the cumulative effect of various antibacterial compounds present in the strains.

Major representation of the antimicrobial metabolites from *Pseudoalteromonas* is from alkaloids, of which non-halogenated antimicrobial alkaloid from *P. luteoviolacea viz.* indolmycin, violacein, oxy-violacein and deoxy-violacein were found inhibitory to *Staphylococcus aureus*, *Bacillus subtilis*, *B. megaterium*, *Photobacterium* sp. and various fungi[37]. Among the halogenated antimicrobial alkaloid metabolites, tetrabromopyrrole was demonstrated to be vulnerable to *Candida albicans*, *Enterobacter aerogenes*, *E. coli*, *Photobacterium fisheri*, *Photobacterium mandapamensis*, *Photobacterium phosphoreum*, *Pseudomonas aeruginosa* and *S. aureus*[38]. Likewise, hexa-bromo-2,2′-bipyrrole and 4′- (3,4,5-tribromo-1H-pyrrol-2-yl) methyl phenol were found to be the antimicrobial compounds acting against *Photobacterium* sp. and *S. aureus*, respectively[39]. *S. aureus* and *P. phosophoreum* were inhibited by pentabromopseudilin[40]. 2,4,6-tribromophenol and 2,6-dibromophenol produced by *P. luteoviolacea* were reported to have inhibitory effect against bacteria and fungi[41]. 4-hydroxy benzaldehyde, n-propyl-3-hydroxybenzoate[42], thiomarinols A, B, C, D, E, F, G and xenorhabdin[43] of violacein were found inhibitory to Gram positive and Gram negative bacteria[44]. Several human pathogens and marine bacteria were reported to be effectively inhibited by tetrabromopyrrole, an yellow autoinhibitory pigment produced by *Chromobacterium* sp.[44]. *Vibrio parahaemolyticus* and *Vibrio anguillarum* were also reported to be inhibited by *P. luteoviolacea*[45].

Representatives of the genus *Pseudoalteromonas* possess remarkable potential for producing antimicrobial metabolites, however industrial level prospective of this genus is still underutilized. The fact that almost all these anti-microbially active isolates have originated from healthy marine macro organisms, proposes their participation in the host's homeostasis. Their role in maintaining microbial consortia and host health should also be specifically mentioned. Undoubtfully, in the list of antimicrobial

Appendix 1 — GenBank Accession numbers of pigmented and non-pigmented bacterial strains isolated along with *P. luteoviolacea*

S. No.	Strain ID	GenBank Accession No.	Species
1.	P35.85	MZ054427	*Oceanimonas doudoroffii*
2.	P27.68	MZ054428	*Shewanella indica*
3.	P49.123	MZ054429	*Micrococcus* sp.
4.	P48.102	MZ054430	*Vibrio nereis*
5.	P14.27	MZ054431	*Bacillus* sp.
6.	P16.34	MZ054432	*Vibrio alginolyticus*
7.	P17.31	MZ054433	*Vibrio alginolyticus*
8.	P3.18G	MZ054434	*Pseudoalteromonas* sp.
9.	P3.28.40	MZ054435	*Staphylococcus haemolyticus*
10.	M15.B	MZ054436	*Brevibacterium sediminis*
11.	M29.B	MZ054437	*Brevibacterium* sp.
12.	M31.A	MZ054438	*Salinicoccus sediminis*
13.	M55.A	MZ054439	*Cytobacillus kochii*
14.	M39.B	MZ054440	*Bacillus* sp.
15.	M51.A	MZ054441	*Vibrio* sp.

providers among marine microbiota, the genus *Pseudoalteromonas* will find a remarkable position and offer potential candidates for the next generation marine antibiotics. Further, their use as probiotics in aquaculture also needs to be investigated.

Conclusion

This study reports the isolation and characterization of deep purple pigmented marine bacterium *P. luteoviolacea*, for the first time from India. Also this is the first time report on isolation of *P. luteoviolacea* from coral reef ecosystem. *P. luteoviolacea* exhibits inhibitory effect on bacteria of the genera *Bacillus*, *Photobacterium* and *Vibrio*. Both strains of *P. luteoviolacea*, P42 and M64, reported here were found to be potential source of violacein pigment and promising antibacterial agents.

Acknowledgments

The first author thankfully acknowledges ICAR-CMFRI, Kochi for providing research facilities. The first author also expresses gratitude to Cochin University of Science and Technology, Kochi for granting admission for Ph.D and to Department of Science and Technology, New Delhi for providing financial support as DST-INSPIRE Fellowship.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualisation, formal analysis and writing: VSJ & KSS; Funding acquisition: VSJ, KSS, KKJ, & AG; Investigation: VSJ, KSS, SJ, PP, KRB, LR, SR, RS, HJK, KRS, RMG &KKJ; Resources: VSJ, KSS, SJ, LR, RMG & KKJ; and Supervision: KSS, RMG, KKJ & AG.

References

1 Park H, Park S, Yang Y H & Choi K Y, Microbial synthesis of violacein pigment and its potential applications, *Crit Rev Biotechnol*, 41 (6) (2021) 879-901. doi: 10.1080/07388551.2021.1892579

2 Gomez D, Espinosa E, Bertazzo M, Lucas Elio P, Solano F, *et al.*, The macromolecule with antimicrobial activity synthesized by *Pseudoalteromonas luteoviolacea* strains is an L-amino acid oxidase, *Appl Microbiol Biotechnol*, 79 (6) (2008) 925-930. https://doi.org/10.1007/s00253-008-1499-x

3 Hanefeld U, Floss H G & Laatsch H, Biosynthesis of the marine antibiotic pentabromopseudilin. Part 1. The benzene ring, *J Org Chem*, 59 (13) (1994) 3604-3608. https://doi.org/10.1021/jo00092a020.

4 Gauthier M, Morphological, physiological and biochemical characteristics of some violet-pigmented bacteria isolated from seawater, *Can J Microbiol*, 22 (2) (1976) 138-149. https://doi.org/10.1139/m76-019.

5 Gauthier G, Gauthier M & Christen R, Phylogenetic analysis of the genera *Alteromonas*, *Shewanella* and *Moritella* using genes coding for small-subunit rRNA sequences and division of the genus *Alteromonas* into two genera, *Alteromonas* (emended) and *Pseudoalteromonas* gen. nov. and proposal of twelve new species combinations, *Int J Syst Evol Microbiol*, 45 (4) (1995) 755-761. https://doi.org/10.1099/00207713-45-4-755.

6 Bowman J P, Bioactive compound synthetic capacity and ecological significance of marine bacterial genus *Pseudoalteromonas*, *Mar Drugs*, 5 (2007) 220–241.

7 Modolon F, Barno A R, Villela H D M & Peixoto R S, Ecological and biotechnological importance of secondary metabolites produced by coral-associated bacteria, *J Appl Microbiol*, 129 (2020) 1441-1457. https://doi.org/ 0.1111/jam.14766

8 Alker A T, Delherbe N, Purdy T N, Moore B S & Shikuma N J, Genetic examination of the marine bacterium *Pseudoalteromonas luteoviolacea* and effects of its metamorphosis-inducing factors, *Env Microbiol, 22* (2020) 4689-4701. https://doi.org/10.1111/1462-2920.15211

9 Radjasa O K, Martens T, Grossart H P, Sabdono A, Simon M, *et al.*, Antibacterial property of a coral-associated bacterium *Pseudoalteromonas luteoviolacea* against

shrimp pathogenic *Vibrio harveyi* (In Vitro Study), *Hayati J Biosci*, 12 (2) (2005) 77-81. https://doi.org/10.1016/S1978-3019(16)30329-1

10 Yang L H, Xiong H, Lee O O, Qi S H & Qian P Y, Effect of agitation on violacein production in *Pseudoalteromonas luteoviolacea* isolated from a marine sponge, *Lett Appl Microbiol*, 44 (2007) 625-630. https://doi.org/10.1111/j.1472-765X.2007.02125.x

11 Guppy R & Bythell J C, Environmental effects on bacterial diversity in the surface mucus layer of the reef coral *Montastraea faveolata*, *Mar Ecol Prog Ser*, 328 (2006) 133–142.

12 Hartsell S E, The preservation of bacterial cultures under paraffin Oil, *Appl Microbiol*, 1 (1953) 36–41.

13 Bowman J P & McMeekin T, Pseudoalteromonas, In: *Bergey's manual of systematic archaea and bacteria*, edited by Whitman & B William, (John Wiley & Sons, Inc. and Bergey's Manual Trust), 2015. https://doi.org/10.1002/9781118960608.gbm01098

14 Sambrook J & Russell D W, Purification of nucleic acids by extraction with phenol:chloroform, *CSH Protoc*, 1 (2006) 4455. doi: 10.1101/pdb.prot4455

15 Lane D J, 16S/23S rRNA sequencing, In: *Nucleic acid techniques in bacterial systematics*, edited by E Stackebrandt & M Goodfellow, (John Wiley, Chichester, United Kingdom), 1991, pp. 115-175.

16 Karlsen C, Ellingsen A B, Wiik Nielsen C, Winther Larsen H C, Colquhoun D, *et al.*, Host specificity and clade dependent distribution of putative virulence genes in *Moritella viscosa*, *Microb Pathog*, 77 (2014) 53 – 55. https://doi.org/10.1016/j.micpath.2014.09.014

17 Hall T A, BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, *Nucleic Acids Symp Ser*, 41 (1999) 95-98.

18 Kumar S, Stecher G, Li M, Knyaz C & Tamura K, MEGA X: Molecular evolutionary genetics analysis across computing platforms, *Mol Biol Evol*, 35 (2018) 1547-1549.

19 Karger A, Current developments to use linear MALDI-TOF spectra for the identification and typing of bacteria and the characterization of other cells/organisms related to infectious diseases, *Proteomics Clin Appl*, 10 (2016) 982-993. doi: 10.1002/prca.201600038

20 Bauer A W, Kirby W M, Sherris J C & Turck M, Antibiotic susceptibility testing by a standardized single disc method, *Am J Clin Pathol*, 45 (4) (1966) 493-496.

21 Sneath P H, Cultural and biochemical characteristics of the genus *Chromobacterium*, *J Gen Microbiol*, 15 (1) (1956) 70-98. https://doi.org/10.1099/00221287-15-1-70

22 Gauthier M J, Modification of bacterial respiration by a macromolecular polyanionic antibiotic produced by a marine *Alteromonas*, *Antimicrob Agents Chemother*, 9 (3) (1976) 361-366. https://doi.org/10.1128/aac.9.3.361

23 Hugh R & Leifson E, The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram negative bacteria, *J Bacteriol*, 66 (1) (1953) 24 – 26.

24 Agler M T, Ruhe J, Kroll S, Morhenn C, Kim S T, *et al.*, Microbial hub taxa link host and abiotic factors to plant microbiome variation, *PLoS Biol*, 14 (1) (2016) e1002352. https://doi.org/10.1371/journal.pbio.1002352

25 Gauthier M, Validation of the name *Alteromonas luteoviolacea*, *Int J Syst Evol Microbiol*, 32 (1) (1982) 82-86. https://doi.org/0020-7713/82/010082-05$02.001

26 Freiwald A & Sauer S, Phylogenetic classification and identification of bacteria by mass spectrometry, *Nat Protoc*, 4 (5) (2009) 732-742. https://doi.org/10.1038/nprot.2009.37

27 Emami K, Askari V, Ullrich M, Mohinudeen K, Anil A C, *et al.*, Characterization of bacteria in ballast water using MALDI-TOF mass spectrometry, *PLoS One*, 7 (6) (2012) e38515. https://doi.org/10.1371/journal.pone.0038515

28 Hazen T H, Martinez R J, Chen Y, Lafon P C, Garrett N M, *et al.*, Rapid identification of *Vibrio parahaemolyticus* by whole-cell Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry, *Appl Environ Microbiol*, 75 (21) (2009) 6745-6756. https://doi.org/10.1128/AEM.01171-09

29 Subramaniam S, Ravi V & Sivasubramanian A, Synergistic antimicrobial profiling of violacein with commercial antibiotics against pathogenic micro-organisms, *Pharm Biol*, 52 (1) (2014) 86 - 90. https://doi.org/10.3109/13880209.2013.815634

30 Ojeda J J & Dittrich M, Fourier Transform Infrared Spectroscopy for molecular analysis of microbial cells, *Methods Mol Biol*, (2012) 187-211. https://doi.org/10.1007/978-1-61779-827-_8

31 Silvia B, Sabine K & Jens S, Antibiotic resistance and the environment - There and back again: Science & Society series on Science and Drugs, *EMBO reports*, 2014, pp. 15. 10.15252/embr.201438978

32 Gilman J P, Studies on certain species of bacteria assigned to the genus *Chromobacterium*, *J Bacteriol*, 65 (1) (1953) 48-52.

33 Krediet C J, Ritchie K B, Paul V J & Teplitski M, Coral-associated micro-organisms and their roles in promoting coral health and thwarting diseases, *Proc R Soc B: Biol Sci*, 280 (2013) 20122328. https://doi.org/10.1098/rspb.2012.2328

34 Ben Haim Y, Zicherman Keren M & Rosenberg E, Temperature-regulated bleaching and lysis of the coral *Pocillopora damicornis* by the novel pathogen *Vibrio coralliilyticus*, *Appl Environ Microbiol*, 69 (7) (2003) 4236-4242. https://doi.org/10.1128/aem.69.7.4236-4242.2003

35 Cervino J M, Thompson F L, Gomez Gil B, Lorence E A, Goareau T J, *et al.*, The *Vibrio* core group induces yellow band disease in Caribbean and Indo-Pacific reef-building corals, *J Appl Microbiol*, 5 (2008) 1658-1671. https://doi.org/10.1111/j.1365-2672.2008.03871.x

36 Thompson F L, Thompson C C, Naser S, Hoste B, Vandemeulebroecke K, *et al.*, *Photobacterium rosenbergii* sp. nov. and *Enterovibriocoralii* sp. nov., vibrios associated with coral bleaching, *Int J Syst Evol Microbiol*, 55 (2) (2005) 913-917.

37 Wang H, Wang F, Zhu X, Yan Y, Yu X, *et al.*, Biosynthesis and characterization of violacein, deoxyviolacein and oxyviolacein in heterologous host, and their antimicrobial

activities, *Biochem Eng J*, 67 (2012) 148–155. doi: 10.1016/j.bej.2012.06.005

38 Tebben J M C A, Tapiolas D, Thomas Hall P & Harder T, Coralline algal-associated bacterium, *Pseudoalteromonas* Strain J010, yields five new korormicins and a bromopyrrole, *Mar Drugs*, 12 (2014) 2802–2815. https://doi.org/10.3390/md12052802

39 Gribble G W, The diversity of naturally occurring organobromine compounds, *Chem Soc Rev*, 28 (1999) 335–346. https://doi.org/10.1039/a900201d

40 Feher D, Barlow R, Mc Atee J & Hemscheidt T K, Highly brominated antimicrobial metabolites from a marine *Pseudoalteromonas* sp., *J Nat Prod*, 73 (2010) 1963–1966, https://doi.org/10.1021/np100506z

41 Zsolnai T, Attempts to discover new fungistatika-I: Phenol-derivate, *Biochem Pharmacol*, 5 (1-2) (1960) 1–19. https://doi.org/10.1016/0006-2952(60)90002-2

42 Offret C, Desriac F, Chevalier P L, Mounier J, Jegou C, *et al.*, Spotlight on antimicrobial metabolites from the marine bacteria *Pseudoalteromonas*: Chemodiversity and ecological significance, *Mar Drugs*, 14 (2016) 129. https://doi.org/10.3390/md14070129

43 Qin Z, Huang S, Yu Y & Deng H, Dithiolopyrrolone natural products: Isolation, synthesis and biosynthesis, *Mar Drugs*, 11 (10) (2013) 3970-3997. https://doi.org/10.3390/md11103970

44 Andersen R J, Wolfe M S & Faulkner D J, Autotoxic antibiotic production by a marine *Chromobacterium*, *Mar Biol*, 27 (1974) 281–285. https://doi.org/10.1007/BF00394363

45 Sakata T, Sakaguchi K & Kakimoto K, Antibiotic production by marine pigmented bacteria, 2: Purification and characterization of antibiotic substance of *Alteromonas luteoviolacea*, *Memoirs of Faculty of Fisheries-Kagoshima University*, 1986, pp. 243-250.