Fermentative Production of Pyranone Derivate I from Marine *Vibrio* sp. SKMARSP9: Isolation, Characterization and Bioactivity Evaluation

P. Shiva Krishna · B. Sudheer Kumar ·
P. Raju · M. S. R. Murty · T. Prabhakar Rao ·
M. A. Singara Charya · R. S. Prakasham

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Abstract Pyranone derivative **I** was isolated from fermented broth of isolated marine bacterial strain *Vibrio* sp. SKMARSP9. The compound **I** was characterized, and evaluated for its antimicrobial properties. The isolated strain was identified based on 16S rRNA based phylogenetic analysis. The molecular analysis data suggested that this strain is closely related to *Vibrio ruber*, *Vibrio* sp. MSSRF10 and *Vibrio rhizosphaerae*. The best fermentative growth of this isolate was achieved under halophilic conditions and grew efficiently at 30 °C in the presence of 12 % NaCl. The compound **I** production by this strain is associated with growth. The unpurified extract is hydrophobic in nature, and released only during late growth phase. The extract was purified and characterized by spectral data using NMR, DEPT, and ESI–MS. The purity of **I** was 97 % which was confirmed by HPLC. The pyranone derivative **I** exhibited >50 % antioxidant activity and

broad spectrum antimicrobial properties against gram negative and gram positive strains. Molecular docking analysis revealed that this pyranone derivative **I** may be a potential candidate at pharmaceutical sector.

Keywords Pyranone derivative · Antioxidant activity · Antibacterial activity · NMR · ESI–MS

Introduction

Natural products have been sources for new pharmaceuticals and drug research. In recent years, many researchers have focused on identifying lead compounds from marine sources as the oceans of the world cover more than 70 % of the earth's surface and are considered as a rich source for organisms with novel traits and biochemical properties. Marine microbes are prolific but underexploited producers of novel secondary metabolites with pharmaceutical potential and differ from that of terrestrial ecosystems in many aspects. Bacteria living in the sea must be able to survive and grow in an environment with low nutrition, high salinity, and high pressure. Hence, they are of special interest to researchers in understanding their metabolism and associated unique potential to produce compounds with exceptional biological properties [1]. Until now, several marine microbial strains belonging to the genera *Streptomyces*, *Pseudomonas*, *Pseudoalteromonas*, *Bacillus*, *Vibrio*, and *Cytophaga* isolated from seawater, sediments, algae, or marine invertebrates are known to produce novel metabolites of biomedical importance. Marine derived antibiotics are often more efficient at fighting microbial infections than those from terrestrial bacteria mainly due to resistance related issues. In addition, a marine source for microorganisms was more attractive to researchers, since the resource of this

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P. Shiva Krishna · M. A. Singara Charya
Department of Microbiology, Kakatiya University,
Warangal 506009, India

B. Sudheer Kumar · R. S. Prakasham (✉)
Bio Engineering & Environmental Sciences, CSIR - Indian
Institute of Chemical Technology, Hyderabad 500007, India
e-mail: prakashamr@gmail.com; prakasam@iict.res.in

P. Raju · M. S. R. Murty
Medicinal Chemistry & Pharmacology, CSIR - Indian Institute
of Chemical Technology, Hyderabad 500007, India

T. Prabhakar Rao
Centre for NMR & Structural Chemistry, CSIR - Indian Institute
of Chemical Technology, Hyderabad 500007, India

kind for new drug discovery is more reachable with higher hit rate and can be easily reproduced in large scale in industry because of their unexplored nature.

Natural products represent one of the key sources of chemical diversity and possess a high potential for commercial applications. Many secondary metabolites produced by microbial strains are used in the pharmaceutical sector. Pigment production by microbial strains continues to intrigue microbiologists, clinical doctors and bioprocess engineers. The synthesis of any metabolite has an impact on the physiological state of the producing strain. Understanding of the functional implications would be one of the important contributions towards commercialization. Studies associated with microbial bright red pigment suggested their functional role as a bioactive agent [2]. Pigmentation is widespread among marine heterotrophic bacteria and includes carotenoids, flexirubin, xanthomonadine, and prodigiosin [2]. Hence, among marine microbiota, pigment producing bacteria are of special interest to science because they may produce many potential pharmaceutical compounds with various biological properties.

Different pigment producing microbial strains belonging to *Serratia marcescens*, *Vibrio psychroerythrus*, *Streptomyces griseoviridis* and *Hahellache juensis* have been isolated due to their potential antibacterial, antimycotic, immune-modulating, anti-tumor and anti-malarial properties [3]. For several decades, the red pigment produced by these strains has been identified as prodigiosin. Several other strains such as *Pseudomonas magnesorubra*, and other *eubacteria* producing this novel prodigiosin compound have been isolated and the compound recognized as having a broad cytotoxicity [2]. Hence, the scientific community is constantly making efforts to exploit the marine environment for new antitumor drugs with unique structure and excellent bioactivity. In the present investigation a novel pigment producing marine microbe with the potential to produce a broad range antibacterial compound was isolated and characterized at the molecular level. The pigment production process as well as an isolated pigment potential as bioactive agent with respect to antimicrobial and antioxidant properties was evaluated.

Materials and Methods

Isolation of Marine Bacteria

Seawater samples were collected in the intertidal zone at coastal locations of Nellore Krishnapatnam, India. The samples were spread over the entire surface of marine agar plates consisting of g l⁻¹: peptone 5.0, yeast extract 1.0, ferric citrate 0.1, sodium chloride 19.45, magnesium chloride 8.8, sodium sulphate 3.24, calcium chloride 1.8, potassium

chloride 0.55, sodium bicarbonate 0.16, potassium bromide 0.08, strontium chloride 0.034, boric acid 0.022, sodium silicate 0.004, sodium fluoride 0.0024, ammonium nitrate 0.0016, disodium phosphate 0.008, agar 15.0. After incubation at 25 °C for 48 h, all colonies were screened and those with different pigmentation and morphology were isolated.

Biochemical Characterization

Different tests were performed to characterize the biochemical properties of the isolated strain, including those for establishing temperature tolerance, pH tolerance and response to NaCl concentrations (minimum, optimum and maximum). Specific tests such as Gram staining, spore staining, motility, indole production, MR-VP test, gelatin hydrolysis, citrate utilization, triple sugar iron agar, oxidase activity, catalase production, nitrate reduction, urease production and starch hydrolysis were performed according to standard methodology. The ability to ferment the sugars such as arabinose, sucrose, glucose, fructose, rhamnose, xylose, raffinose and mannitol as sole carbon sources was also evaluated for the isolated strains.

Molecular-Based Characterization

The molecular identification of 16S rRNA was performed at the Microbial Type Culture Collection Centre, IMTECH, Chandigarh, India. DNA was extracted from the culture and its quality was evaluated by agarose (1.2 %) gel electrophoresis. A fragment of the 16S rDNA gene was amplified by PCR and purified. Forward and reverse DNA sequencing reactions were carried out with primers 8F and 1492R using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Standard nucleotide BLAST searches using the identified 16S rRNA sequence as a query were performed against NCBI's 16S ribosomal RNA sequences (Bacteria and Archaea) database. Additional searches were conducted against NCBI genomes, whole genome shotgun contigs, and the non-redundant nucleotide collection databases also available on NCBI Gene bank (www.ncbi.nlm.nih.gov). Sequences were aligned using the online version of MAFFT [4, 5]. The multiple sequence alignment was trimmed and manually adjusted where necessary. A phylogenetic tree displaying the evolutionary relationships between sequences was obtained with the maximum likelihood method implemented in RAxML [6]. Node support was estimated with 1000 rapid bootstrap replicates.

Scanning Electron Microscopy

The micro- and external morphology of the strain was studied by scanning electron microscopy (SEM analysis) with a Hitachi-S520 (Japan; Oxford Link ISIS-300 UK).

Fermentation process

A loopful of culture was transferred to pre-sterilized 50 ml Zobell marine broth in 250 ml flask. The flasks were incubated in stationary condition at 28 °C for 16–18 h or until the absorbance of the culture reached optical density of 1.0 at 600 nm. One ml of the inoculum was transferred to the production medium (100 ml Zobell marine broth) in a 250 ml Erlenmeyer flask. The flask was incubated at 28 °C for 72 h.

Extraction and Measurement of Produced Pigment

Pigment was extracted according to Slater [7]. Briefly, 10 ml of the culture was removed from the broth and centrifuged at 100×100 g for 10 min. Pigment extraction was performed by adding methanol to the pellet and incubating at 60 °C for 20 min followed by centrifugation (100×100 g, 10 min). The colored supernatant was analyzed in UV–visible spectrophotometer for detecting the absorption maximum (λ max) by scanning in the range of 400–600 nm and absorbance peak (λ max) was used for further investigation.

Antibacterial Activity

Antibacterial activity was assayed by the agar well diffusion method using both gram positive and gram negative bacterial strains (*Klebsiella pneumoniae*, *Proteus vulgaris*, *Salmonella paratyphi*, *Salmonella typhi*, *Escherichia coli*, *Bacillus cereus*, *Micrococcus luteus*, *Bacillus subtilis*, *Bacillus stearothermophilus* and *Staphylococcus aureus*) as target organisms. Sterilized nutrient agar plates were first inoculated with test organisms by spreading them uniformly. Wells of 6 mm diameter were loaded with 60 μ l of pigment solution (10 mg ml⁻¹). Streptomycin was used as positive control with similar concentration. After 24 h incubation at 37 °C, the zone of growth inhibition of above test organism was measured.

DPPH Antioxidant Activity

The stable 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) was used to determine the free radical-scavenging (antioxidant) activity of the extracts [8]. The samples are kept incubation for 20 min and readings were recorded at 517 nm. Percent inhibition of antioxidant activity was calculated by using the following formula and readings of test sample are compared with that of ascorbic acid (Vitamin C) (Positive control).

$$\% \text{ inhibition of DPPH} = \frac{[(\text{Control OD} - \text{Test OD}) / \text{Control OD}] \times 100}{}$$

Protein–Ligand Docking

The docking of ligands to the pdb 2ITO and 1JNX was performed using AutoDock 4.0 software (<http://autodock.scripps.edu/>). AutoDock is reported to be the most popular docking program and is reliable. Using the software, polar hydrogen atoms were added to the pigment molecule and its non-polar hydrogen atoms were merged, whereas for the ligand, non-polar hydrogen atoms were merged and Gasteiger charges were added. All rotatable bonds of ligands were set to be rotatable. All calculation for protein-fixed ligand–flexible docking was done. The grid box with a dimension of $60 \times 60 \times 60$ points and 0.375 Å grid spacing was used around the catalytic triad to cover the entire pigment molecule binding site and accommodate ligands to move freely. After the docking searches were completed, clustering histogram analysis was performed based on an RMSD (root mean square deviation) of not more than 1.5 Å. The best conformation was chosen from the most populated cluster with the lowest docked energy.

Molecular Docking Simulation

In order to carry out the docking simulation, AutoDock 4.0 suite molecular-docking tool was used and the methodology. The ligand was manually docked into functional sites respective protein individually and the docking energy was monitored to achieve a minimum value. AutoDock 4.0 is widely distributed as public domain molecular docking software which performs the flexible docking of the ligands into a known protein structure. The default parameters of the automatic settings were used. Each docking experiment consisted of 10 docking runs. The Auto Dock results indicated the binding position and bound conformation of the peptide, as well as a rough estimate of its interaction. The docked conformation which had the minimum binding energy was selected to analyze the mode of binding according to [9]. The binding results could be displayed by scoring ligand poses and several scoring functions were used for measuring the goodness of a docking study to find a top ranked pose for ligands.

Results

Screening and Isolation

Marine samples collected from Nellore marine area were used to isolate pigment producing microbial strains using Zobell marine agar plates. A total of 70 different pigmented bacterial colonies were isolated upon incubation at

30 °C, purified and preserved. Among the isolated strains, the strain which produced a red pigment was selected for further studies and designated as SKMASRSP9. Several microbial strains producing coloured pigments (yellow to violet pigments by *Alteromonas* sp. and yellow, orange or red by family members of the *Microbacteriaceae*) have been reported [3]. Antibacterial activity in marine bacteria is a well-known phenomenon and has been demonstrated in a number of studies [1, 10]. Hence, the isolated strain, SKMASRSP9, was evaluated to understand the antimicrobial nature and noticed that this isolate revealed antibacterial activity against both gram positive and gram negative bacteria as bio-pigment producing microbial strains. Although several microbial strains belonging to *S. marcescens* and *Vibrio gazogenes* had been evaluated for the production of pigments, the isolation of red pigment producing marine bacterial species has been rarely reported in literature [11, 12].

Identification of Isolated Strain

Morphology

The shape and external morphology of the isolated strain was confirmed by scanning electron microscopy (SEM). The scanning electron micrograph of the isolated bacteria which revealed comma shape indicating that the isolated red pigmented bacteria may belong to the genus *Vibrio*. Further characterization of the isolated strain was done by biochemical studies and 16S rRNA sequencing studies.

Physiological and Biochemical Characterization

Physiological tests revealed that the isolated strain grows in the wide pH range from 6.0 to 10.0 pH. The isolate was able to grow in medium containing up to 12 % NaCl concentration indicating its halophilic nature. Evaluation of the growth temperature profile suggested that this organism does not grow in wide temperature range. Maximum growth was noticed at 30 °C and any variation in incubation temperature negatively influenced growth. Carbon source utilization studies suggested that the isolated bacterium can grow in the presence of starch, cellulose, and lipids as sole source of carbon and energy, establishing its ability to produce amylase, cellulase, and lipase during its growth. In addition, the bacterium expressed a positive test for catalase, urease and oxidase reactions; however, it showed negative result for H₂S, gelatin liquefaction, MRVP and indole tests. Based on various morphological and biochemical characteristics the isolated strain SKMASRSP9 strain could be identified as either *Serratia* sp. or *Vibrio* sp (Online Resource 1). Because the colony

characteristics and morphological features recorded for this bacterium matched better with those reported for different *Vibrio* strains isolated from diverse sources [13], thus establishing to the above identity of the red bacterial strain may require further investigation.

Molecular Identification

The taxonomic position of the isolated marine strain was determined with molecular methods. A single discrete PCR amplicon band of 1500 bp was observed by agarose gel electrophoresis. A consensus sequence of 1507 bp length for the 16S rDNA gene was generated from forward and reverse sequence data using Auto Assembler software, version 2.1. Blast searches against NCBI Gene bank did not retrieve a perfect match (100 % identity) to our query, indicating that this strain may represent a new taxonomic entity [14]. With the closest match, *Vibrio* sp. BL-102, it shares 99 % identity between the 16S rRNA sequences. All hits retrieved from Gene bank belong to the *Vibrionaceae* and share at least 93 % similarity in the 16S rRNA. The phylogenetic tree (Online Resource 1) gives a more detailed picture about the taxonomic position within the *Vibrionaceae*.

Growth and Bio-Pigment Production Profile

The bulk of the novel compound is produced in association with secondary metabolism mainly observed during the stationary growth period. Hence, two parameters (growth of isolate and bio-pigment production) were analyzed during fermentation by growing the isolate in Zobell marine broth at 30 °C. Biomass and pigment production were quantified spectroscopic method over 72 h by analyzing the sample every 6 h at 640 and 535 nm, respectively. Biomass and pigment production are correlated and increased during the first 48 h of fermentation time (Fig. 1a, b). After 48 h both total biomass and amount of pigment decreased, suggesting that the bio-pigment production by this bacterial strain is strongly associated with growth. This is evidenced from the fact that the pigment concentration increased with increase in incubation time from 6 to 48 h and approximate increase of 150 % was noticed in 42 h of growth (Fig. 1b). Red pigment producing microbial strains [2, 15] were previously reported and are assumed to have enormous potential for commercial exploitation due to their tripyrrole chemical structure exhibiting antibacterial, antimycotic, immunomodulating, anti-tumor and anti-malarial properties [3, 15]. In view of above, the isolated strain requires further investigation to identify the nature of compound and may be effective for commercial exploitation.

Fig. 1 Isolated marine *Vibrio* sp. biomass and pigment production pattern. **a** Growth and **b** pigment production with function of fermentation time

Extraction of Pigment

Different solvents such as ethanol, methanol, distilled water, chloroform, ethyl acetate, petroleum ether and acetone have been tested to find the most suitable solvent for pigment extraction (Online Resource 1). The concentration of extracted pigment was estimated by measuring the absorbance at 535 nm. The data revealed that the solvent plays a significant role in extraction of pigment from *Vibrio* sp. biomass. Methanol was the most and petroleum ether was the least efficient solvent for the extraction of the pigment. Chloroform, ethanol, ethyl acetate and acetone were intermediate in extracting the pigment. No traces of the pigment were detected in distilled water further supporting the hydrophobicity of the pigment.

Isolation and Chemical Characterization of Pure Compound

The resulting hydrophobic crude material (120 mg) was collected and by thin layer chromatography (20 % ethyl acetate and *n*-hexane) it was observed the presence of three compounds. The three compounds were separated by column chromatography using gradient elution method using *n*-hexane and ethyl acetate solvents. The column was packed by choosing the column width 8 cm and height 25 cm. The column was loaded up to 12 cm with silica gel (100–200 mesh) and 120 mg crude material was also loaded on the column. First pure compound, 8 mg was obtained with *n*-hexane as eluent solvent. The second pure compound 15 mg was obtained with 6 % ethyl acetate and *n*-hexane solvent mixture as elution solvent. The third compound 85 mg was obtained with 50 % ethyl acetate and *n*-hexane solvent mixture as eluent. Of the three compounds the second pure compound was obtained as a red colour liquid and was found to exhibit significant biological actions. The structural elucidation of the pure isolated compound **I** was determined by ^1H NMR, ^{13}C NMR, DEPT, IR and MS (ESI) mass spectral data and was elaborated below. The compound was confirmed to be 97.18 % pure by HPLC (Online Resource 1).

Structural Elucidation of the Pure Compound

In the ^1H NMR Spectrum (Fig. 2), two types of aromatic protons were observed at $\delta = 7.76\text{--}7.70$ (m, 2H), $7.57\text{--}7.50$ (m, 2H). In the aliphatic region six types of aliphatic protons were observed at $\delta = 5.3$ (t, $J = 5.4$ Hz, 1H) corresponding to valero lactone methine, $\delta = 4.37$ (q, $J = 7.1$ Hz, 2H) oxymethylene, $\delta = 2.34$ (t, $J = 7.3$ Hz, 2H) from valero lactone methylene at $\delta = 2.07\text{--}1.96$ (m, 2H), $\delta = 1.70\text{--}1.56$ (m, 2H). The three protons at $\delta = 1.37$ (t, $J = 7.1$ Hz, 3H) were due to methyl group.

The ^{13}C NMR spectrum (Fig. 3) the signals, $\delta = 179.82$ and 167.60 were due to carbonyl carbons. The signals at $\delta 132.19$, 130.87 and 128.73 were ascribable to three aromatic carbons; whereas the signal at $\delta 61.58$ was attributed to oxy methylene carbons. The peaks at 29.63 , 29.01 , and 24.63 were due to valero lactone methylene and 14.05 was corresponding to methyl carbon.

In DEPT Spectrum (Online Resource 1) $\delta = 130.87$, 128.78 appeared at positive region and these two signals are referred to aromatic $-\text{CH}$ groups. The peaks at 61.5 , 29.63 , 29.01 , and 24.63 from methylene groups appeared at negative region and the peak at 14.05 appeared at positive region which was ascribable to methyl group. In IR spectroscopy the bands observed at 1735 , 1718 corresponding to ester and keto groups respectively. The MS (ESI) mass spectrum (Online Resource 1) showed peak at 277 was due to $\text{M} + 1$ ion and was further confirmed the structure.

These spectroscopic analyses led to the assignment of structure for the new compound as pyranone benzoate derivative i.e., Ethyl 4-(6-oxo tetra hydro-2H-pyran-2-carbonyl) benzoate. The structure of the compound was shown below and all the spectral and analytical chromatograms were provided.

Bioactive Profile of Pigment

Antibiotic Property Evaluation

The extracted red pigment produced by isolated marine *Vibrio* sp. was evaluated for its antibacterial activity using the cup plate method with streptomycin as reference standard

Fig. 2 ^1H NMR spectrum of isolated pigment molecule. Spectral data of Ethyl 4-(6-oxo tetra hydro-2H-pyron-2-carbonyl) benzoate: Red colour liquid; ^1H NMR (300 MHz, CDCl_3) : δ = 7.76–7.70 (m, 2H), 7.57–7.50 (m, 2H), 5.3 (t, J = 5.4 Hz, 1H), 4.37 (q, J = 7.1 Hz, 2H), 2.34 (t, J = 7.3 Hz, 2H), 2.07–1.96 (m, 2H), 1.70–1.56 (m, 2H), 1.37 (t, J = 7.1 Hz, 3H)

Fig. 3 ^{13}C NMR spectrum of isolated pigment molecule. ^{13}C NMR (75 MHz, CDCl_3) δ = 179.82, 167.60, 132.19, 130.87, 128.73, 61.58, 29.63, 29.01, 24.63, 14.05

[16]. The antibacterial studies revealed that the pigment has antibacterial activity against both gram positive (*B. cereus*, *M. luteus*, *B. subtilis*, *B. stearothermophilus* and *S. aureus*) and gram negative (*K. pneumoniae*, *P. vulgaris*, *S. paratyphi*, *S. typhi* and *E. coli*) bacteria. However, the raw pigment did not surpass the standard streptomycin in any case, but showed the same level of inhibition against *M. luteus* (Online Resource 1). The inhibition zone around each well amended with pigment extract or streptomycin reveals that the highest antimicrobial activity was observed against *E. coli* (22 mm), followed by *S. paratyphi* (20 mm) and *S. typhi* (16 mm) (Online Resource 1). The lowest activity levels were observed against *P. vulgaris* and *S. aureus* (6 mm each) (Online Resource 1). The antibacterial activity reveals that the produced extract has a broad spectrum activity against gram positive and gram negative bacteria.

Antioxidant Activity Evaluation

DPPH radical scavenging assay was performed to better understand the role of the red pigment as an antioxidant.

DPPH is a relatively stable nitrogen centered free radical that easily accepts an electron by reacting with suitable reducing agents. As a result, the DPPH solution loses its violet color depending on the number of electrons accepted [17]. The observed optical density for red pigment along with DPPH (test sample) was found to be 0.614 while for the negative control (solvent + DPPH) the optical density value was 1.249. The calculated percentage antioxidant activity of biosynthesized red pigment would be approximately 50 % of the Ascorbic acid (ascorbic acid is used as positive control in the present experimentation) (Online Resource 1). The antioxidant activity data suggested that the red pigment produced by *Vibrio* sps. SKMASRSP9 has antioxidant properties.

Molecular Docking Studies

In general, most of the natural products provide an unparalleled access to unique chemical scaffolds which have novel modes of action against a myriad of disease targets. However, natural products arena is the most difficult

choices to explore for understanding their impact at health care or Pharma sectors. Molecular docking is one of the easiest and safest ways to evaluate bioactivity profile of compound especially with respect to human health care. In view of the unique chemical structure of isolated compound, a comparative protein–ligand dock analysis was conducted using two different proteins such as 1JNX (breast cancer protein) and 2ITO (lung cancer protein).

Structure–functional relationship of isolated pigment was evaluated to understand its biological activity against two cancer proteins i.e., 2ITO (lung cancer protein) and 1JNX (breast cancer protein) as target molecules. Analysis of the binding pattern between 2ITO protein and ligand suggested that the binding pattern varied with the nature of the protein (Fig. 4). This could be exemplified based on the observation that the isolated pigment molecule interacted with 2ITO protein amino acid residues of Ser¹⁶⁵⁵, Arg⁶⁹⁹, Thr¹⁷⁰⁰, Leu⁷⁰¹, Lys¹⁷⁰², Phe¹⁷⁰⁴, Met⁷⁷⁵, Arg⁸³⁵, Leu¹⁸³⁹, Leu⁷¹⁸, Val⁷²⁶, Lys⁷²⁸, Ala⁷⁴³, Leu⁷⁹² and Met⁷⁹³ while 1JNX the interaction was observed with only two amino acid residues i.e., Pro794³²¹ and Leu⁸⁴⁴ (Table 1) [18]. This docking data with isolated pigment molecule revealed that the pigment interact with two selected 2ITO and 1JNX differently. This is further confirmed based on the observation that the free energy binding value for 2ITO is -5.25 kcal/mol while the same for 1JNX is -4.03 kcal/mol. Similar variations also noticed in case of total intermolecular energy values (Table 1) indicating the better interaction with ligand molecular was noticed with 2ITO (lung cancer protein) while other 1JNX (breast cancer protein).

Discussion

Microbial species inhabited at marine environment produce a wide variety of pigments that play significant role in

cellular physiology and survival. However, it is not clear in the present study, the isolated compound role on the metabolism of the *Vibrio* sp. The chemical nature of isolated compound further suggested that this compound is specific for this strain and it is produced as one of the metabolite. This is confirmed based on the observation that the isolated compound is the major compound produced by isolated marine strain and several other minor compounds were observed during isolation process (data not shown). Though biological significance of this compound is not known in this strain however, in general most of these natural metabolites produced mostly via the quorum sensing mechanism were found to have antibiotic, anticancer, and immunosuppressive activities at very low concentrations. The observed nature of bio-pigment production by isolated marine bacterial strain along with antimicrobial and antioxidant property suggested the imperative role of isolated strain for effective exploitation for production of bioactive compound. This is further confirmed based on the fact that the marine environment represents one of the hotspots of chemical diversity and known for several biopigments with wide potential for medicinal, synthetic and commercial applications [3, 10]. Despite the enormous difficulty in marine microbial strain isolation and culturing, metabolites from these strains are increasingly attractive especially those with unique color pigments, that play a prominent role in bacterial life as well as several diverse biological properties such as antibiotic and anticancer activities. The latter is of special interest due to the consistent requirement for chemotherapeutic drugs with high selectivity towards malignant cells. Binding of a small molecule with a large molecule is called docking. Docking is the process by which two molecules fit together in 3D space. The objective of computational docking is to determine how molecules will interact which will aid the interaction studies in bio-molecules. Molecular docking is often

Fig. 4 Docked superimposed image showing pigment molecule interacting residues within the proteins

Table 1 Pigment molecule interaction energies and residues with target proteins

Protein id	Free energy of binding	Total intermolecular energy (kcal/mol)	Interacting residues
1JNX	4.03 kcal/mol	−5.45	1655SER, 699ARG, 1700THR, 701LEU, 1702LYS, 1704PHE, 775MET, 835ARG, 1839LEU
2ITO	5.25 kcal/mol	−6.73	718LEU, 726VAL, 728LYS, 743ALA, 792LEU, 793MET, 794PRO, 844LEU

employed to aid in determining how a particular drug lead will interact to form a binding pocket. Molecular docking is the particular arrangement of a ligand and a protein, which can be defined by a set of values describing the translation, orientation, and conformation of the ligand with respect to the protein [9]. The observed variation in binding pattern of this isolated pigment molecule with cancer proteins further indicated that vide analysis is one of the essential requirements for effective utilization of this compound at pharmaceutical sector. The initial studies in this direction revealed that potential exploitation would offer and the efforts in this direction are continuing. All living organisms utilize oxygen to metabolize and use the dietary nutrients in order to produce energy for survival. Though oxygen is one of the most essential components for living, it is also a double edged sword. Oxygen is a highly reactive atom that is capable of becoming part of potentially damaging molecules, free radicals, which have been implicated in the pathogenesis. Protection against reactive oxygen species play a significant role in cellular metabolism as this reactive species is known to react with membrane lipids, nucleic acids, proteins and enzymes, and other small molecules. One of the biological properties attributed to pigments produced by diverse microbial strains is the protection against free radicals. The observed antioxidant activity of red pigment produced by isolated marine strain based on DPPH radical scavenge assay indicates its pharmaceutical role. Such type of DPPH radical scavenge based antioxidant activity was reported by Mensor et al. [17]. Understanding taxonomic position helps in prediction of basic characteristics of any microbial strain and biologists use the characteristics of different organisms to describe specific forms of life and to identify new ones. Microbe's classified in any particular group have certain common characteristics for example *E. coli* cells are rod-shaped and have a Gram-negative cell wall. The organisms as well within taxonomic group's exhibit diversity like variations in size, shape, and ability to form specific structures, such as endospores. Phylogenetic tree helps in understanding the evolutionary relationship between isolate and with literature reported strains. The 16S rRNA based molecular identification of isolated strain suggested that the new isolate may belong to the species *V. ruber*, together with strains BL-102, MSSRF10, and MSSRF 28 and noticed to

be positioned in a clade with *V. gazogenes*, *V. rhizosphaerae*, *V. mangrove*, *V. ruber*, and *Vibrio* sp. strains Y47, BL-102, MSSRF10 and MSSRF28 which receives 100 % bootstrap support (Online Resource 1). However, more detailed studies will be necessary to delimit species boundaries in this clade. Previous phylogenetic analyses of the *Vibrio*'s based on single genes were suitable to delimit family boundaries but were not able to resolve species-level relationships with confidence [19–23]. Typically, high support values are limited to species pairs and are missing on deeper nodes. In this regard our study is in sync with previous studies. Recently, the use of concatenated multi-gene analyses has led to considerable advances unravelling the phylogeny of *Vibrio*'s [19, 24, 25]. The 16S rRNA sequence of this strain has been deposited at EMBL under the accession number HE798514. Microbial growth and metabolism mediated compound production pattern mainly influenced by their respective environmental conditions. Marine microbial strains possess unique ability to survive and grow in the water environment with low nutrition, high salinity, and high pressure. Understanding the pattern of metabolite production is one of the basic requirements for exploitation of isolated strain in any industrial sector. Analysis of the red bio-pigment production pattern by this isolate revealed that the pigment is produced continuously during exponential growth. However, further increase in incubation period beyond 48 h the pigment concentration dropped slightly despite considerable reduction in growth of the microbial strain (Fig. 1) unlike the production of many compounds through microbial fermentation which tend to be restricted to either exponential or stationary phase. This indicated that the biopigment production is linked to cellular processes and that the pigment is not released into the medium during growth. This is further confirmed by the observation that based on the fact that when the culture biomass was separated from broth during growth period, the supernatant did not show any coloration (data not shown). The biopigment may thus be associated with the cell wall vesicles of the bacteria as has been reported by Sundaramoorthy et al. [12]. However, association of microbial red pigment with spore membranes of *Bacillus megaterium* also reported [3]. When culture incubated under growth conditions beyond 48 h, the pigment is seen on the surface of the fermentation broth

suggesting release of pigment only at stationary or fourth phase of microbial growth (data not shown). Such a release may be attributed to higher cell death rates during late growth phases i.e., stationary and death phases. The absence of pigment in the fermentation broth during exponential phase of growth, its association with cell biomass during active cell growth, the appearance of biopigment on the surface during late growth phases denote that this pigment is hydrophobic in nature. Non-appearance of pigment during exponential growth phase on the surface of broth and uniform distribution of colour during this phase in the medium may be attributed to produce pigment attachment to the cell wall of the isolated microbial strain.

Conclusion

A novel pigmented compound was isolated from marine *Vibrio* species, purified and characterised to structural level using different analytical tools such as NMR, DEPT, ESI-MS and HPLC and observed to be a pyranone benzoate derivative i.e. ethyl 4-(6-oxo tetra hydro)-2H-pyron-2-carbonyl benzoate. The production of this compound is associated with growth of the isolated microbe and released to medium during late exponential phase. The purified compound revealed an anti-oxidant and an anti-bacterial activity against different strains of gram positive and gram negative bacteria. Molecular docking analysis against two cancer proteins i.e. 1JNX (breast cancer protein) and 2ITO (lung cancer protein) denoted the isolated compound has potential for its utilization at pharmaceutical sector.

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Conflict of interest The authors declare that they have no conflict of interest.

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