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Pigment-Producing Marine Bacteria from Visakhapatnam Coastal Areas: Isolation, Screening, and Molecular Identification

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ABSTRACT
Marine bacterial pigments exemplify nature's untapped chemical ingenuity. They act as ecological tools for the microbes that produce them and also offer valuable leads for developing novel therapeutic agents and industrial bioproducts. This study focuses on isolating and identifying pigmented bacteria from water and sediment samples collected from the Visakhapatnam coastal region in Andhra Pradesh, India. A total of 23 pigmented bacterial isolates were obtained, 15 from water and 8 from sediment, using Zobell marine agar medium. Among them, 2 isolates with the most intense pigmentation were selected for detailed characterization through morphological, physiological, biochemical, and 16S rRNA sequencing methods. The sediment isolate was identified as a *Rhodococcus pyridinivorans* that produced carotenoid pigments, while the water isolate was identified as *Salinicoccus roseus*. The bacterial cultures were grown in Zobell marine broth at 25°C with shaking at 120 rpm for five days. Pigment extraction was carried out with methanol and repeated centrifugation. These findings suggest that marine *Rhodococcus pyridinivorans* and *Salinicoccus roseus* from Visakhapatnam's coastal waters and sediments may serve as potential sources of bioactive carotenoid pigments for pharmaceutical and biotechnological use.

1. INTRODUCTION:

Among the three major biosphere habitats, the marine realm, covering 70% of the Earth's surface, provides the most significant inhabitable space for living organisms, particularly microbes. Marine microbes thrive not only in the surface waters of the sea, but also in the lower and abyssal depths from coastal to the offshore regions, and from the general oceanic to the specialised niches like the blue waters of coral reefs to the black smokers of hot thermal vents at the sea floor (Qasim *et al*., 1999).

The marine environment is the prime reservoir of biological diversity, and marine microorganisms are recognised to be rich sources of novel compounds. In India, about 1000 natural products were derived from marine microbes (Suthindhiran K *et al*., 2010)

Marine bacterial communities possess enormous potential to produce diverse bioactive molecules such as pigments. On the usual microbial culture media, several marine Gram-positive and Gram-negative bacteria appear to produce an array of pigments. Production of these pigments by microbes appears to be mediated by the quorum-sensing mechanism (Grossarte *et al*., 2009). It is estimated that about 1% of the microbial world is being explored for pigment production.

Pigmentation is widespread in bacteria and consists of carotenoids and many other pigments (Kirti *et al*., 2014). The pigments produced by microorganisms include carotenoids, melanins, flavins, quinones, and, more specifically,

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monascins, violacein, and indigo, which showed distinct antibacterial effects against many pathogenic bacteria (Dufossé, L. *et al*., 2005). A main characteristic of marine bacteria is that most of them are pigmented (Arun, G. *et al*., 2012).

The pigment-producing potential of marine bacterial strains holds significant promise. These strains, often identified from diverse marine ecosystems, exhibit a wide array of activities, including antimicrobial, anticancer, antioxidant, anti-inflammatory, and anti-allergic properties. Such bioactive attributes position marine bacteria as potential sources for the development of novel medicinal applications. The pharmaceutical industry can benefit greatly from harnessing the bioactive compounds produced by these microorganisms. Bacterial pigment production is now an emerging field of research demonstrating its potential for various industrial applications (Ramesh, C. *et al*., 2019).

2. MATERIALS AND METHODS:

2.1 Sample collection and isolation of bacteria

Surface seawater was collected from a distance of 500 metres away from the coastal line, and sediment from a depth of 40 centimetres was collected from multiple sites along the Visakhapatnam coastal region in sterile bottles and zip-lock covers and transported immediately to the laboratory under iced conditions. The water samples were directly spread over the surface of Zobell marine agar medium plates, and the sediment samples were diluted using the serial dilution method ($10^{-}4$ to $10^{-}7$) and then spread over the surface of Zobell marine agar medium plates. The bacteria were cultivated on Zobell marine agar medium containing (per liter): Peptone 5.0g, Yeast extract 1.0g, Ferric citrate 0.1g, Sodium chloride 19.45g, Magnesium chloride 8.8g, Sodium sulphate 3.2g, Calcium chloride 1.8g, Potassium chloride 0.55g, Sodium bicarbonate 0.16g, Potassium bromide 0.08g, Strontium chloride 34.0mg, Boric acid 22.0mg, Sodium silicate 4.0mg, Sodium fluorate 2.4mg, Ammonium nitrate 1.6mg, Disodium phosphate 8.0mg, and Agar 15.0g. The pH was adjusted to 7.6-7.8. After incubation at 25°C for three days, all colonies were screened, and those with distinct pigmentation and morphology were isolated.

2.2 Screening for selection of potential strain

The potential strains were selected based on the intensity and stability of pigment production. The cultures that showed bright pigmentation were selected, and the bacterium that produced the most intense pigmentation on Zobell marine agar medium was selected for further studies. The isolated pigmented bacterial strains were maintained on Zobell agar slants at 4°C for further use.

2.3 Molecular Characterisation and Identification

2.3.1 Morphological, phenotypic, and biochemical characterisation

The Morphological, phenotypic, and biochemical characterisation of the selected strains was performed using Bergey's Manual of Determinative Bacteriology. The morphological and physiological properties of the isolated strains include Colony morphology, Gram staining, cell shape, and motility. Various tests were conducted to characterise the biochemical properties, including indole production, methyl red-Voges Proskauer (MR-VP) test, gelatin hydrolysis, citrate utilisation test, catalase production, nitrate reduction, urease production, and starch hydrolysis, were performed according to standard microbiological techniques. (S. M. Reddy and S. Ram Reddy, *Microbiolog y: A Laboratory Manual*, Third Revised Edition)

.2.3.2 Molecular identification

The isolates with the most intense pigmentation were selected for molecular identification. To emphasise the evolutionary relationships of microbial taxa, the 16S rRNA sequence is usually considered the definitive standard for taxonomy (Weose, 1987). In addition to enabling identification at finer taxonomic levels such as species and subspecies, 16S rRNA gene sequence analysis provides a reliable means of differentiating organisms at the genus level across a variety of bacterial phyla (M.Z. El-Fouly *et al*., 2015). RNA was isolated from the cultures. Its quality was evaluated on a 1.0% Agarose Gel, and a single band of high-molecular-weight RNA was observed. PCR amplified a fragment of the 16S rRNA gene. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose. The PCR amplicon was purified to remove contaminants. Forward and reverse RNA sequencing reaction of the PCR amplicon was carried out with 27F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyser. A consensus sequence of the 16S rRNA gene was generated from forward and reverse sequence data using aligner software. The 16S rRNA gene sequence was used to carry out BLAST with the NCBI GenBank database. Based on the maximum identity score, the first ten sequences were selected and aligned using the multiple alignment software program Clustal W. A distance matrix was generated using the RDP database, and the phylogenetic tree was constructed using MEGA 4.

2.4 Production and extraction of Pigment

The selected isolates were cultured in Zobell marine broth and incubated in a shaker at 120 rpm for 5 days at 28°C. After incubation, the culture

Journal of Molecular Science
Volume 35 Issue 3, Year of Publication 2025, Page 989-994
DoI-10.004687/1000-9035.2025.133

broth was centrifuged at 10,000 rpm for 10 min at 4°C to obtain the bacterial pellet. The obtained pellet was washed twice with sterile distilled water. For pigment extraction, the cell pellet was subjected to three freeze-thaw cycles to facilitate cell lysis. The lysed cells were then extracted with methanol by repeated centrifugation. The methanolic extract was concentrated using a rotary evaporator under reduced pressure. The concentrated pigment extract was stored for further analysis.

2.5 Test for Carotenoids:

The characteristic of the extracted pigments was tested. Both VJW_9 and VJS-1 isolated sample pigments were treated with a few drops of 85% Sulphuric acid, and the results were observed. The appearance of blue colour indicated the presence of carotenoids (Vora *et al.*, 2014)

3. Results

3.1 Isolation and Screening

In the present study, 23 pigmented bacterial isolates were obtained from water and sediment samples collected at various sites along the Visakhapatnam Sea coast. The isolates exhibited diverse pigmentation patterns ranging from yellow, orange, red, to pink colouration. Based on the intensity of pigmentation, one isolate from the water sample (10^{-4} dilution plate) and one isolate from the sediment sample (10^{-7} dilution plate), designated as VJW-9 and VJS-1, showed the most prominent orange-red pigmentation and were selected and subcultured for detailed study (**Figures 1, 2 (A, B), 3(A, B)**).

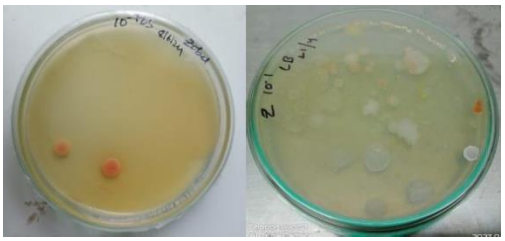


Figure 1: Plates showing Isolated colonies

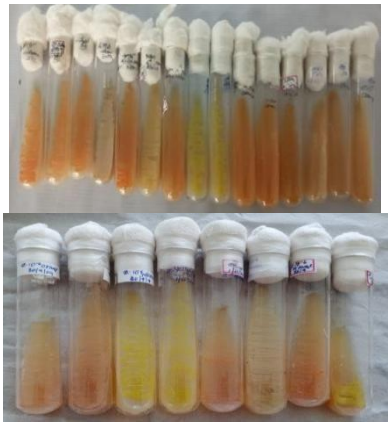


Figure 2: A. Pure cultures of bacteria isolated from water

B. Pure cultures of bacterial isolates from sediment

Figure 3: A. Pure culture from sediment (VJS-1) B. Pure culture from water (VJW-9)

3.2 Morphological and Biochemical Characterisation

The selected isolates, VJW-9 and VJS-1, appeared to produce an intense orange-red pigmentation (**Figures 1, 3**). The Morphological, Physiological, and Biochemical properties of marine isolates were presented in **Table 1**. Both VJS-1 and VJW-9 were observed as Gram-positive microscopically, as shown in **Figure 4 (A, B)**.

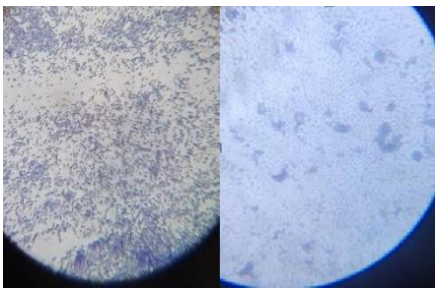


Figure 4: A. VJS-1 Microscopic image
B. VJW-9 Microscopic image

Table 1: Morphological, Physiological, and Biochemical Properties of Marine isolates

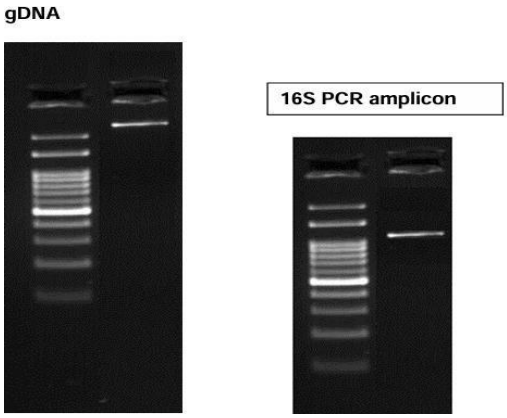
Characteristic	VJW-9	VJS-1
Colony Morphology	Orange pigmented, opaque, flat circular	Orange pigmented, opaque, rough circular
Cell shape	Coccus	Rods to cocci (polymorphic)
Motility	-ve	-ve
Gram staining	+ve	+ve
Starch hydrolysis	+ve	-ve
Indole	-ve	-ve
Methyl Red	+ve	-ve
Voges proskaeur	-ve	+ve
Citrate utilisation	-ve	+ve
Catalase	+ve	+ve
Urease test	+ve	+ve
Nitrate Reduction test	+ve	+ve
Arabinose	-ve	-ve
Mannitol	-ve	-ve

3.3 Molecular Identification

The 16S rRNA gene sequence analysis revealed

Journal of Molecular Science
Volume 35 Issue 3, Year of Publication 2025, Page 989-994
DoI-10.004687/1000-9035.2025.133

99% similarity with *Salinicoccus roseus* species, which was isolated from seawater, and the 16S rRNA gene sequence of the strain isolated from the sediment analysis revealed 99% similarity with the *Rhodococcus pyridinivorans* species. Phylogenetic analysis confirmed the isolate's placement within the *Salinicoccus roseus* and *Rhodococcus pyridinivorans* genera, specifically showing the closest relationship to *Salinicoccus roseus* strain D5 16S ribosomal RNA gene, and *Rhodococcus pyridinivorans* strain LS2. The sequence of VJW-9 has been deposited in GenBank with the accession number PV345634. The gel images of VJW-9 and VJS-1 are depicted in **(Figure 5(A, B),** and the phylogenetic tree of VJW-9 and VJS-1 with sequence alignments is shown in **Figure 6 (A,B, C, D).**



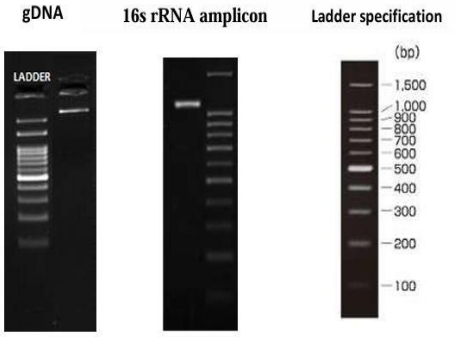


Figure 5: A. Gel image of VJW-9 isolate
B. Gel image of VJS-1 isolate

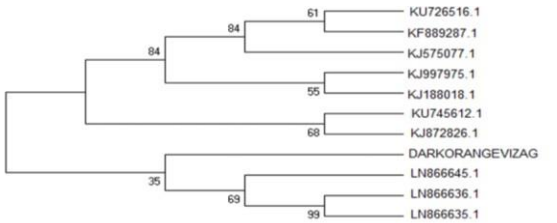


Figure 6A: Phylogenetic tree evolutionary relationships of the VJW-9 isolate by the Neighbor-Joining method

Description	Max score	Total score	Query coverage	E value	Max ident	Accession
Salinicoccus roseus strain D5 16S ribosomal RNA gene, partial sequence	1114	1114	100%	0	99%	KU745612.1
Salinicoccus roseus strain KV3 16S ribosomal RNA gene, partial sequence	1114	1114	100%	0	99%	KU726516.1
Salinicoccus sp. JC397 partial 16S rRNA gene, strain JC397, isolate Gj21	1114	1114	100%	0	99%	LN866645.1
Salinicoccus sp. JC388 partial 16S rRNA gene, strain JC388, isolate Gj10	1114	1114	100%	0	99%	LN866636.1
Salinicoccus sp. JC387 partial 16S rRNA gene, strain JC387, isolate Gj9	1114	1114	100%	0	99%	LN866635.1
Salinicoccus sp. M 16S ribosomal RNA gene, partial sequence	1114	1114	100%	0	99%	KJ997975.1
Salinicoccus roseus strain KVD-HS42 16S ribosomal RNA gene, partial sequence	1114	1114	100%	0	99%	KJ872826.1
Salinicoccus roseus strain NIOT-bflm-S12 16S ribosomal RNA gene, partial sequence	1114	1114	100%	0	99%	KJ575077.1
Salinicoccus sp. L21-PYE-C40 16S ribosomal RNA gene, partial sequence	1114	1114	100%	0	99%	KJ188018.1
Salinicoccus sp. BAB-3250 16S ribosomal RNA gene, partial sequence	1114	1114	100%	0	99%	KF889287.1

Figure 6B: Sequences producing significant alignments of DARKORANGEVIZAG (VJW-9)

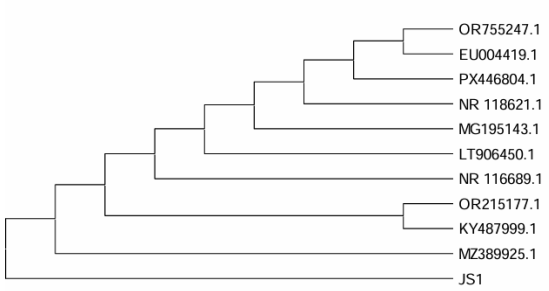


Figure 6C: Phylogenetic tree evolutionary relationships of the VJS-1 isolate by the Maximum Likelihood method

Journal of Molecular Science
Journal of Molecular Science
Volume 35 Issue 3, Year of Publication 2025, Page 989-994
DoI-10.004687/1000-9035.2025.133

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Rhodococcus pyridinivorans strain LS2	2736	2736	100%	0	99.87%	PV474117.1
Rhodococcus sp. strain CNS16	2734	2734	100%	0	99.93%	MH879823.1
Rhodococcus sp.	2730	2730	100%	0	99.87%	KX500189.1
Rhodococcus pyridinivorans strain GX12401	2730	2730	100%	0	99.87%	OM980344.1
Rhodococcus pyridinivorans strain AAa3	2730	2730	100%	0	99.87%	PX492091.1
Rhodococcus sp. NAM81	2730	2730	100%	0	99.87%	KF150201.1
Rhodococcus sp.	2730	2730	100%	0	99.87%	KY458569.1
Rhodococcus sp. T104	2730	2730	100%	0	99.80%	AY286493.3
Rhodococcus sp. strain BN02	2730	2730	100%	0	99.87%	PP389875.1
Rhodococcus pyridinivorans strain GX1036	2730	2730	100%	0	99.87%	OP223729.1

Figure 6D : Sequences producing significant alignments of JS_1 (VJS-1)

3.4 Test for Carotenoids

A Blue colour layer formed in the pigment extracts after the addition of a few drops of 85% sulphuric acid, confirming the presence of carotenoids. The presence of polyene pigments was indicated by the formation of a blue ring when a pigment extract was combined with concentrated sulphuric acid, as shown in **Figure 7 (A, B)**. (I.A. Ajayi *et al*.,2011; F. Shla and Hyolai,2013).

Figure 7: A. Pigment extract of VJS-1 B. Positive test for carotenoids of VJS-1

4. DISCUSSION:

This study successfully isolated and characterised the pigment-producing marine bacteria from Visakhapatnam coastal waters and sediment, identified as *Salinicoccus roseus* and *Rhodococcus pyridinivorans* producing carotenoid pigments. The marine environment of Visakhapatnam, with its unique physicochemical conditions, appears to harbour diverse pigmented bacteria with biotechnological potential.

The identification of the isolates as *S. roseus* and *Rhodococcus pyridinivorans* is significant as members of this genus are known for their metabolic versatility and ability to produce various bioactive compounds.

Salinicoccus roseus, a halotolerant Gram-positive coccus commonly isolated from saline soils and hypersaline ecosystems, is known for its remarkable osmoadaptation mechanisms and pigment production. These physiological traits enable the species to withstand high salt fluctuations and oxidative stress, supporting its stability in challenging niches. Recent reports also suggest that members of *Salinicoccus* spp. synthesise carotenoid pigments and compatible solutes, which contribute to membrane protection and enhance survivability, indicating a possible role in bioactive compound production and biotechnological applications (Ventosa *et al*., 2015).

Rhodococcus pyridinivorans is significant for its ability to synthesize intracellular carotenoid pigments, which contribute to the characteristic orange–red coloration observed in many *Rhodococcus* species. These pigments play a crucial role in protecting the cells against oxidative stress, desiccation, and fluctuations in environmental conditions. Carotenoid biosynthesis in *Rhodococcus* is often associated with enhanced membrane stability and resilience to organic solvents, which may support the organism's survival in contaminated or industrial ecosystems (Martínková & Uhnáková, 2011). Although pigment studies specific to *R. pyridinivorans* are limited, available evidence indicates that strains of this species produce carotenoid-type pigments similar to those found in related *Rhodococcus* taxa, suggesting a conserved protective function (Pham *et al*., 2020). Moreover, marine-derived *Rhodococcus* strains often exhibit distinctive physiological traits, including pigment production, enhanced stress tolerance, and the ability to utilize varied carbon sources, which support their survival in fluctuating saline conditions (Baskaran *et al*., 2021). The presence of these pigments may also correlate with improved tolerance to high light intensity and reactive oxygen species, further reinforcing its ecological adaptability. Understanding the pigment profile of *R. pyridinivorans* could therefore provide valuable insights into its stress physiology and potential applications in antioxidant or biocolorant production.

Journal of Molecular Science
Volume 35 Issue 3, Year of Publication 2025, Page 989-994
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Marine-derived carotenoids are gaining attention due to their enhanced stability and bioactivity compared to their terrestrial counterparts. The harsh marine environment often leads to the production of unique structural variants of common carotenoids with improved functional properties (Galasso *et al.*, 2017).

5. CONCLUSION:

In the current study, the successful isolation of a carotenoid-producing *Salinicoccus rosues* and *Rhodococcus pyridinivorans* bacteria from Visakhapatnam coastal waters and sediment highlights the potential of Indian marine ecosystems as sources of bioactive compounds. This finding contributes to the growing body of knowledge on marine microbial diversity and its biotechnological applications.

Future studies should focus on optimising culture conditions for enhanced pigment production, purification, characterisation, and exploring the antimicrobial and antioxidant properties of the extracted pigment, and evaluating its potential applications in food, pharmaceutical, and cosmetic industries. Additionally, scale-up studies for commercial production and detailed toxicological assessments would be valuable for industrial applications.

6. ACKNOWLEDGEMENTS:

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7. CONFLICT Of INTEREST

Authors disclose no conflict of interest.

8. FUNDING

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