RESEARCH PAPER

Isolation And Identification Of Bioactive Secondary Metabolites From The Marine Bacteria Shewanella Algae And Their Biological Activity

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ABSTRACT

Background: Marine bacteria are a prolific source of bioactive secondary metabolites with significant pharmaceutical potential, particularly in addressing antibiotic resistance and chronic diseases. **Aim:** This study aimed to isolate and identify bioactive secondary metabolites from the marine bacterium Shewanella algae, and to evaluate their antioxidant activity. **Methodology:** Marine sediment samples were collected from the coastal region of Ramanathapuram (latitude: 9.319078°, longitude: 79.330245°). Bacterial isolates were obtained via serial dilution. Pure colonies were isolated using the streak plate method. Phenotypic identification involved Gram staining and a series of biochemical tests, while genotypic identification was performed by 16S rRNA gene sequencing and phylogenetic analysis. Bioactive compounds were extracted from the biomass using ethyl acetate. Purification through medium-pressure liquid chromatography (MPLC) and thin-layer chromatography (TLC). Structural characterization was conducted using FT-IR, NMR, and LC-MS. Antioxidant activity was assessed using the DPPH assay, with results compared to ascorbic acid. **Results:** Shewanella algae was successfully isolated and identified, exhibiting a distinct metabolic profile (citrate and glucose positive; indole, methyl red, and several sugars negative). The primary purified compound was identified as 9-isopropyl-3,6-dimethoxy-9-methyl-9H-fluorene, with a purity of 90.55% (RT: 1.12 min, LC-MS m/z: 283.4 [M+1]). The ethanolic extract demonstrated notable antioxidant activity with an IC_{50} of 8.82 µg/mL (R^2 = 0.8512), compared to ascorbic acid (IC_{50} = 0.8 µg/mL, R^2 = 0.8963). **Conclusion:** Shewanella algae from marine sediments produce a novel fluorene derivative with significant antioxidant activity. These findings highlight the potential of marine bacteria as a source of new bioactive compounds for pharmaceutical development.

Keywords: Shewanella algae, Secondary metabolites, Isolation, Structural characterization, Antioxidant activity, Marine bacteria. Email: norhashimah@utem.edu.my

How to cite this article: Padmavathi R, Muralidhara Rao D, Ashokkumar N., Isolation And Identification Of Bioactive Secondary Metabolites From The Marine Bacteria Shewanella Algae And Their Biological Activity. Int J Drug Deliv Technol. 2026;16(2s): 935-943; DOI: 10.25258/ijddt.16. 935-943

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

Marine bacteria represent a vast and largely untapped reservoir of biologically active secondary metabolites, offering immense potential for drug discovery and biotechnological applications.[1] Thriving in extreme environments characterized by high salinity, pressure, and temperature fluctuations, these microorganisms have evolved unique metabolic pathways to produce structurally diverse compounds with potent bioactivities. Secondary metabolites from marine bacteria, including alkaloids, peptides, polyketides, and terpenoids, exhibit antimicrobial, anticancer, antiviral, and anti-inflammatory properties, addressing critical challenges in medicine such as antibiotic resistance and chronic diseases.[2] Marine Actinobacteria, Firmicutes, and Proteobacteria are particularly prolific, accounting for over 93% of antimicrobial compounds identified in recent decades.[3] Cyanobacteria contribute peptide-based molecules like dolastatins and apratoxins, which have inspired anticancer drugs such as Brentuximab vedotin. Beyond direct pathogen inhibition, these metabolites exhibit ecological roles in quorum-sensing disruption and biofilm inhibition, enhancing their utility against persistent infections.[4] Despite their promise, only a fraction of marine bacterial diversity has been cultivated, with an estimated 3.7 × 10^30 microbial species remaining unexplored. Advances in metagenomics, synthetic biology, and high-throughput screening are overcoming historical challenges in cultivation and compound characterization, enabling the identification of novel agents. With microbial secondary metabolites constituting 70% of known bioactive compounds, and marine environments covering 70% of Earth's surface, sustained investment in marine bioprospecting could revolutionize pharmaceutical development, offering solutions to emerging global health crises while preserving marine biodiversity through sustainable harvesting techniques.[5,6] A recent study focused on the isolation, structural characterization, and *in vitro* bioactivity assessment of bioactive secondary

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metabolites from the marine bacterium *Shewanella algae*.

Materials and Methods

Marine Sample Collection and Isolation of Functional Bacteria

Marine sediment soil, Sponge, and plant samples (MS1, MS2, and MS3) were collected from the ground sediment of the coastal region of Ramanathapuram (Figure 1) (latitude: 9.319078°, longitude: 79.330245 °) using a core sampler. Samples were collected at 0-10 cm; the surface sediment was muddy sand, ash, and black.[7]

Isolation of functional Bacteria from marine sediment

Sediment samples were serially diluted twice in sterile water. 1 mL of sample from each dilution was spread on different bacterial culture media (Himedia, India) plates using the spread plate technique and incubated at room temperature under anaerobic conditions for seven days. Colonies producing inhibition zones were isolated using the crowd-plate method.[8]

Isolation of pure colonies by the streak plate method

The streak plate method is a fundamental microbiological technique used for isolating pure colonies of microorganisms from a mixed culture. In this process, a sterilized inoculation loop is used to collect a small amount of the microbial sample, which is then streaked across the surface of an agar plate. The loop is dragged in a series of zigzag patterns, starting from one section of the plate and gradually diluting the sample as it moves across different sections. This technique reduces the density of the microorganisms, allowing individual cells to be spatially separated. When incubated, these isolated cells form distinct colonies, which can be further analysed or used for subsequent experiments. The streak plate method is precious for obtaining pure cultures and essential for identifying and studying microbial species in detail.[9]

Phenotypic Identification

The isolated bacteria were subjected to Gram's staining and biochemical tests: indole, methyl red, Voges-Proskauer's, citrate, glucose, adonitol, arabinose, lactose, sorbitol, mannitol, rhamnose, sucrose, and starch hydrolysis, by using a rapid biochemical identification test kit (KB001- Himedia, India).

Genotypic identification

To identify and determine the phylogenetic relationships of S-2, the bacterial 16S rRNA gene was amplified by PCR using 16S rRNA gene universal primers. The purified PCR products were sequenced using the commercial services of Biokart India Pvt Ltd research laboratory (Bangalore, India).[10]

Isolation and identification of bioactive compound from Shewanella Algae marine bacteria using spectroscopic methods.

The recovered biomass layer from the sample was mixed and lyophilized. The resulting powder was extracted thrice with ethyl acetate (3 × 0.5 L), providing a 500 mg dry extract sample. This extract was dissolved in a minimum volume of ethyl acetate, loaded onto 10 g dry silica gel, then subjected to medium-pressure liquid chromatography (MPLC) and eluted with a stepwise gradient of 20, 50, 80 and 100% ethyl acetate in hexane at 20, 40, 62 and 90 min, respectively (Figure 1). The fractions were collected based on (TLC) monitoring to yield one major fraction (F1). The TLC eluting solvent ratio is Ethyl acetate: hexane (4:1). Fraction F1 (125 mg) was subjected again to the MPLC system and eluted with a stepwise gradient of 10, 20, 30 and 50 ethyl acetate in cyclohexane to yield. The mixture yielded a colourless solid compound.[11]

Antioxidant activity of bioactive fraction

Initially, stock solutions of the extract were prepared at a concentration of 1.0 mg/ml and then serially diluted to obtain final concentrations of 100, 200, 300, and 400 µg/ml. For each dilution, 2.5 millilitres of the sample solution was mixed with 1 millilitre of 0.3 mM DPPH prepared in methanol. The mixtures were incubated at room temperature for 30 min to allow the reaction to occur. Following incubation, the absorbance of each sample was recorded at 517 nm using an ELISA reader. The percentage of antioxidant activity (AA) was calculated from these absorbance values, providing a quantitative
measure of the extract's antioxidant capacity. Antioxidant activity (I%) = Abs. Blank- Abs. Sample/Abs. Blank x 100.

RESULTS

Isolation of functional bacteria *Shewanella algae* from marine sediment

Figure 1 shows the isolation of marine bacteria Shewanella algae on nutrient agar slants. Distinct bacterial growth is visible, indicating successful cultivation under laboratory conditions for further study.

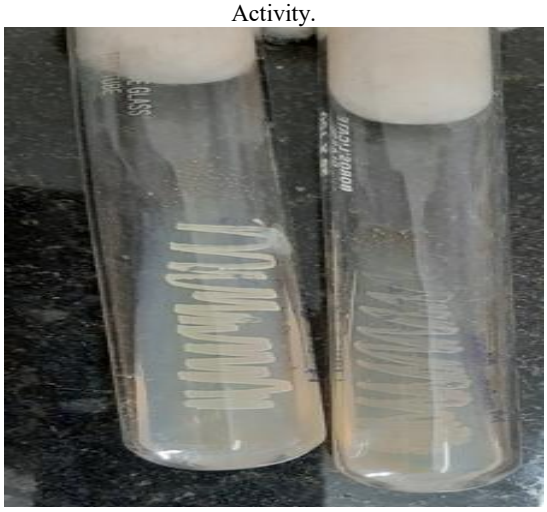

Figure 1: Isolation of marine bacteria *Shewanella algae*

Phenotypic Identification

Table 1 reveals that Shewanella algae exhibits a distinct metabolic profile. The bacterium's ability to utilize citrate and ferment glucose reflects metabolic adaptability, while negative results for indole, methyl red, Voges-Proskauer, and several sugar utilization tests indicate restricted substrate metabolism. This unique biochemical signature differentiates Shewanella algae from other bacterial species and aids in its identification as a marine isolate within the Shewanella genus. The combination of positive and negative test outcomes offers important insights for taxonomic classification and enhances our understanding of the physiological characteristics and metabolic potential of *Shewanella algae*.

Table 1. Phenotypic characteristics of *Shewanella algae*.

Test	**Results**
Indole test	-
Methyl red	-
Voges Proskauer's	-
Citrate	+
Glucose test	+
Adonitol test	-
Arabinose test	-
Lactose test	-
Sorbitol test	-
Mannitol test	-

Genotypic identification

The phylogenetic tree presented in the image demonstrates the close evolutionary relationship of the isolated Shewanella sp. (Shewanella algae) with other members of the Shewanella genus, particularly *Shewanella haliotis* and *Shewanella upenei*. The isolate clusters closely with MN559295.1_Shewanella_algae, confirming its taxonomic placement within the Shewanellaceae family and the Gamma Proteobacteria class (Figure 2). The distinct branching and low genetic distance values further support the accurate identification of the isolate as *Shewanella algae*. This phylogenetic analysis not only validates the molecular identification but also highlights the evolutionary diversity within the genus, providing valuable insights for future ecological and biotechnological studies involving marine Shewanella species.

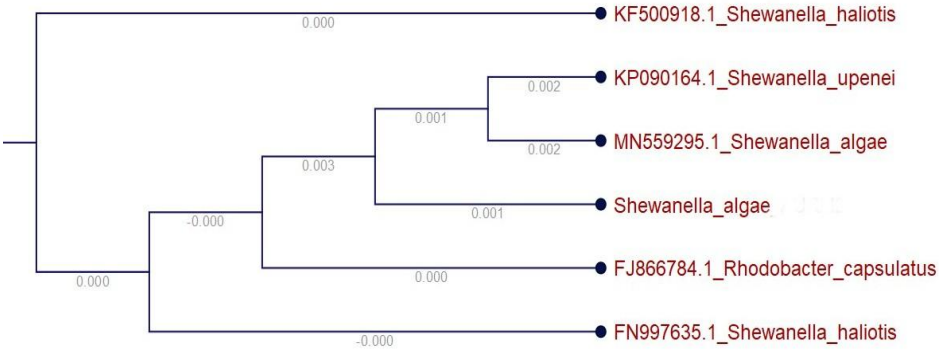

Figure 2: Phylogenetic analysis of Shewanella algae.

Isolation and identification of bioactive compound from *Shewanella algae* marine bacteria using spectroscopic methods.

The bioactive compound from *Shewanella algae* was purified through a sequential liquid-liquid extraction using ethyl acetate, followed by medium-pressure liquid chromatography (MPLC). Chromatographic separation was tracked via thin-layer chromatography (TLC) with an ethyl acetate:hexane (4:1) solvent system, resulting in the collection of a major fraction (F1). Subsequent purification steps ultimately yielded a colorless solid, which was identified as 9-isopropyl-3,6-dimethoxy-9-methyl-9H-fluorene.

9-isopropyl-3,6-dimethoxy-9-methyl-9H-fluorene structure characterization.

IR (υmax, cm^{-1} , KBr): 3307 cm^{-1}, 2933 cm^{-1} (CH_3O, Strong, broad) (Figure 2). ^{1}H NMR (400 MHz): δ 0.72 (3H, d, *J* = 6.94 Hz), 2.42 (1H, pd, *J* = 6.94, 3.42 Hz), 3.61 (3H, s), 4.40 (1H, d, *J* = 3.42 Hz), 7.03 (1H, dd, *J* = 8.80, 1.46 Hz), 7.18-7.34 (2H, 7.23 (dd, *J* = 1.46, 0.51 Hz), 7.28 (dd, *J* = 8.80, 0.51 Hz)), 0.72 (3H, d, *J* = 6.94 Hz), 3.61 (3H, s), 4.40 (1H, d, *J* = 3.42 Hz), 7.03 (1H, dd, *J* = 8.80, 1.45 Hz), 7.26 (2H, m). (Figure 3, Figure 4, Figure 5) ^{13}C NMR (101 MHz): δ 18.60 (1C, s), 30.00 (1C, s), 55.70 (1C, s), 71.10 (1C, s), 102.20 (1C, s), 110.70 (1C, s), 122.40 (1C, s), 127.05 (1C, s), 143.60 (1C, s), 155.47 (1C, s), 18.60 (1C, s), 30.00 (1C, s), 55.70 (1C, s), 71.10 (1C, s), 102.20 (1C, s), 110.70 (1C, s), 122.40 (1C, s), 127.05 (1C, s), 143.60 (1C, s), 155.47 (1C, s) (Figure 6). The purity of the isolated compound is 90.55% (RT:1.12), which is confirmed by LC-MS analysis (Figure 7). The calculated molecular weight of $C_{19}H_{22}O_2$ is 282.3 m/z. It was confirmed in LC-MS analysis: 283.4 [M+1] m/z (Figure 8). Finally, the IUPAC name of the isolated compound is 9-isopropyl-3,6-dimethoxy-9-methyl-9H-fluorene (Figure 10).

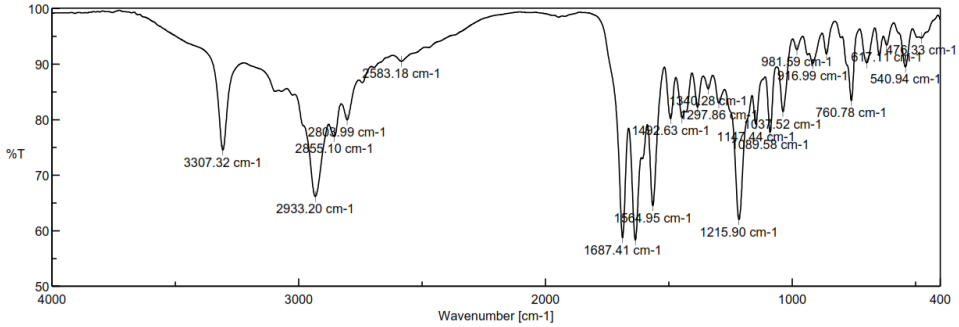

Figure 3: FT-IR analysis of isolated compound 9-isopropyl-3,6-dimethoxy-9-methyl-9H-fluorene from Shewanella Algae.

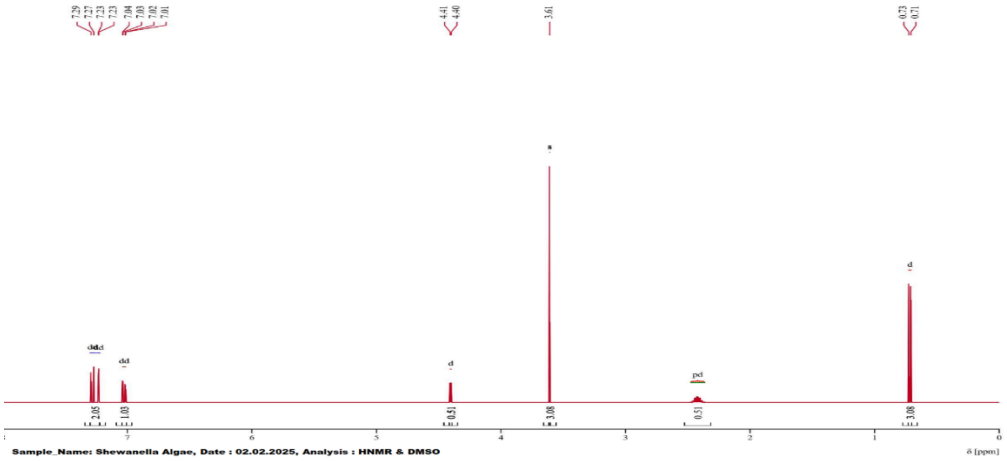

Figure 4: [1]HNMR analysis of the isolated compound 9-isopropyl-3,6-dimethoxy-9-methyl-9H-fluorene from Shewanella Algae.

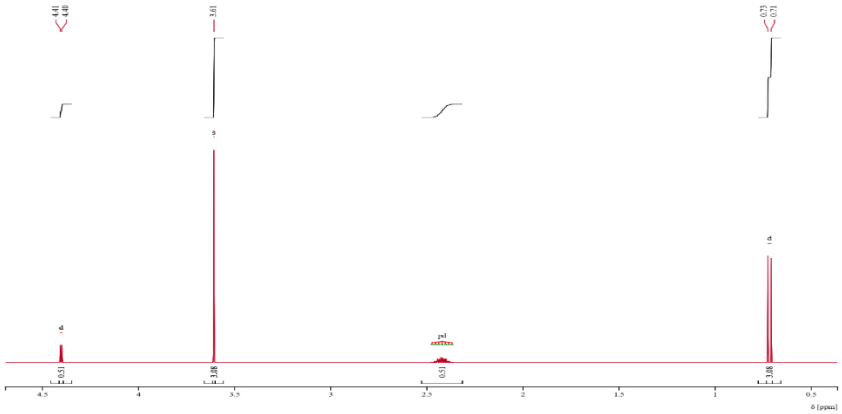

Figure 5: [1]HNMR analysis of the isolated compound 9-isopropyl-3,6-dimethoxy-9-methyl-9H-fluorene from Shewanella Algae (Peak analysis from 0.5 ppm to 4.5 ppm).

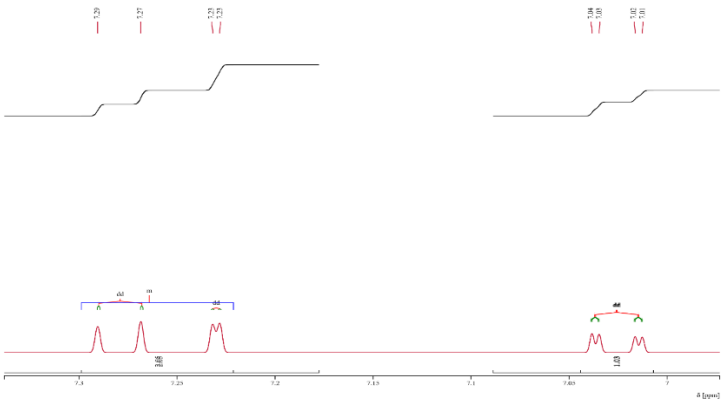

Figure 6: [1]HNMR analysis of the isolated compound 9-isopropyl-3,6-dimethoxy-9-methyl-9H-fluorene from Shewanella Algae (Peak analysis from 7 ppm to 7.3 ppm).

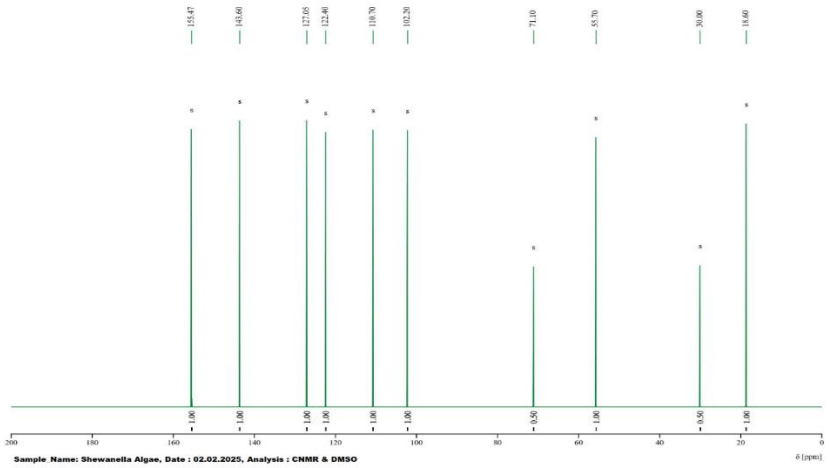

Figure 7: ^{13}C NMR analysis of the isolated compound 9-isopropyl-3,6-dimethoxy-9-methyl-9H-fluorene from Shewanella Algae.

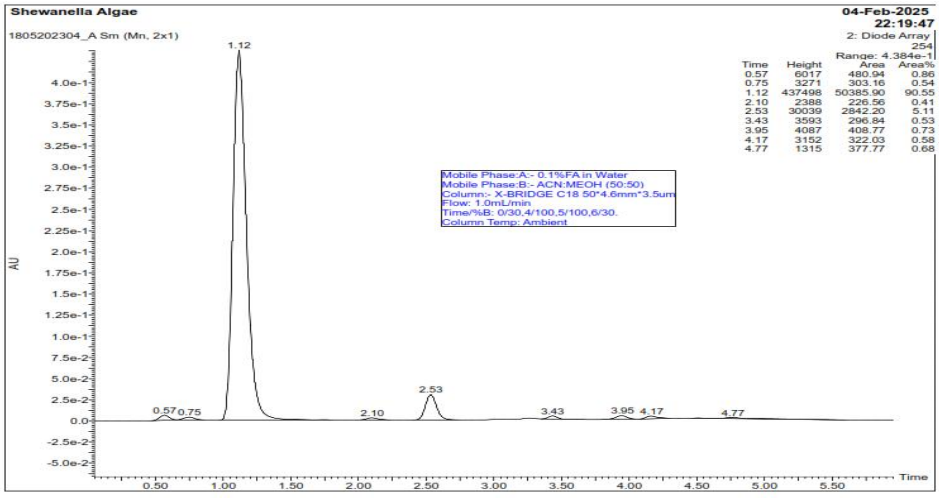

Figure 8: LC-MS purity analysis of the isolated compound 9-isopropyl-3,6-dimethoxy-9-methyl-9H-fluorene from Shewanella Algae.

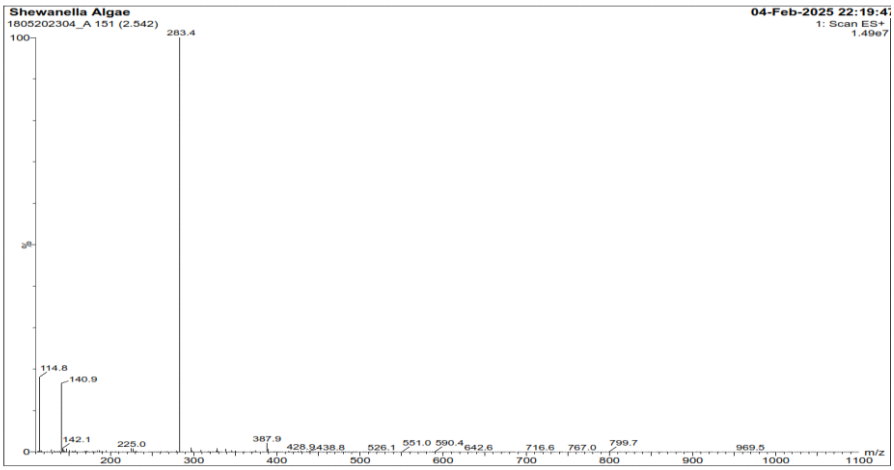

Figure 9: LC-MS mass analysis of the isolated compound 9-isopropyl-3,6-dimethoxy-9-methyl-9H-fluorene from Shewanella Algae.

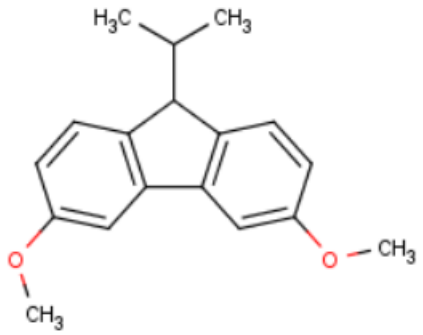

Figure 10: 9-isopropyl-3,6-dimethoxy-9-methyl-9H-fluorene

Antioxidant activity of the bioactive fraction of *Shewanella algae* ethanolic extract.

The inhibitory effects of the ethanolic fraction and ascorbic acid were evaluated using a log(inhibitor) vs. response (three parameters) model. For the ethanolic fraction, the best-fit values were: Bottom = 62.49% inhibition, Top = 13.21% inhibition, $LogIC_{50}$ = 3.331, IC_{50} = 2142, and Span = -49.28. In comparison, ascorbic acid showed a Bottom of 82.48%, Top of 3.969%, $LogIC_{50}$ of 1.249, IC_{50} of 17.74, and Span of -78.51. The 95% confidence intervals (profile likelihood) for the ethanolic fraction were: Bottom (49.76 to 75.20), Top (-3.892 to 33.00), $LogIC_{50}$ (0.9451 to 12.82), and IC_{50} (8.812 to 6.61 × 10^{12}); for ascorbic acid, the intervals were: Bottom (71.49 to 93.54), Top (-222.2 to 34.82), $LogIC_{50}$ (-0.07751 to 3.085), and IC_{50} (0.8366 to 1216). Goodness-of-fit statistics showed degrees of freedom of 6 for both, R^2 values of 0.8512 (ethanolic fraction) and 0.8963 (ascorbic acid), sum of squares of 807.2 and 717.9, and x values of 11.60 and 10.94, respectively. Both analyses included 9 X values and 9 Y values (Figure 11).

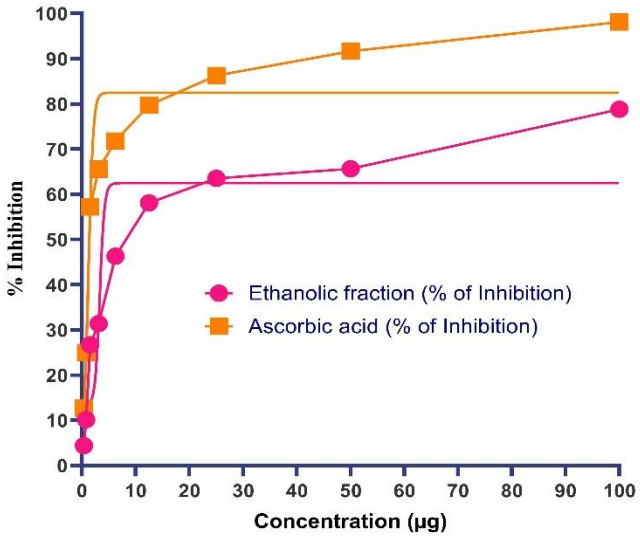

Figure 11: Antioxidant activity of the bioactive fraction of *Shewanella algae* ethanolic extract.

DISCUSSION

The present study focused on the isolation, identification, and characterization of the functional marine bacterium Shewanella algae from marine sediment, followed by the extraction and analysis of its bioactive compounds and assessment of their antioxidant activity. The initial isolation process was conducted using nutrient agar slants, where distinct and robust bacterial growth was observed (Figure 1), confirming the successful cultivation of Shewanella algae under laboratory conditions. This foundational step was crucial as it provided pure cultures for downstream phenotypic and genotypic analyses. Phenotypic identification, as detailed in Table 1, revealed that Shewanella algae possesses a unique metabolic profile characterized by its ability to utilize citrate and ferment glucose, while showing negative results for the indole, methyl red, Voges-Proskauer, and various sugar utilization tests. This restricted metabolic versatility, combined with the positive results for citrate and glucose, serves as a distinguishing biochemical signature, setting Shewanella algae apart from other marine bacteria and aiding in its accurate taxonomic classification. Such a metabolic fingerprint not only supports its identification but also provides insights into its physiological capabilities and ecological adaptability within marine environments. For instance, *Streptomyces seoulensis* A01 produces streptoseomycin, effective against *Helicobacter pylori* and methicillin-resistant *Staphylococcus aureus* (MRSA) with

MIC values as low as 2 µg/mL, while *Streptomyces* sp. CB02980 yields pyrroloformamides C and D showing activity against MRSA and *Klebsiella pneumoniae*.[13]

Genotypic identification was carried out through phylogenetic analysis, which further validated the taxonomic placement of the isolate. The phylogenetic tree (Figure 2) demonstrated that the isolated Shewanella sp. clusters closely with other members of the Shewanella genus, particularly *Shewanella haliotis* and *Shewanella upenei*, and is most closely related to MN559295.1_Shewanella_algae.[14] The distinct branching and low genetic distance values observed in the tree confirm the accurate identification of the isolate as Shewanella algae, placing it firmly within the Shewanellaceae family and the Gamma Proteobacteria class. This molecular approach not only corroborates the phenotypic findings but also highlights the evolutionary diversity and relationships within the genus, which is valuable for future ecological and biotechnological research involving marine Shewanella species.

Following identification, the study proceeded to isolate and characterize a bioactive compound from Shewanella algae. The extraction process involved sequential liquid-liquid extraction with ethyl acetate, followed by purification using medium-pressure liquid chromatography (MPLC). Thin-layer chromatography (TLC) with an ethyl acetate: hexane (4:1) solvent system facilitated the collection of a major fraction (F1), which was further purified to yield a colorless solid identified as 9-isopropyl-3,6-dimethoxy-9-methyl-9H-fluorene. Comprehensive spectroscopic analyses were employed for structural elucidation. FT-IR analysis revealed characteristic absorption bands at 3307 cm^{-1} and 2933 cm^{-1}, indicating the presence of methoxy groups (Figure 3). The ^{1}H NMR spectrum (400 MHz) displayed distinct chemical shifts and coupling patterns consistent with the proposed structure (Figures 4–6), while ^{13}C NMR (101 MHz) further confirmed the carbon framework (Figure 7). The compound's purity was determined to be 90.55% by LC-MS analysis (Figure 8), and the molecular weight ($C_{19}H_{22}O_2$, 282.3 m/z) was confirmed by the observed [M+1] peak at 283.4 m/z (Figure 9). The IUPAC name of the isolated compound was thus established as 9-isopropyl-3,6-dimethoxy-9-methyl-9H-fluorene.

The antioxidant activity of the bioactive fraction from the ethanolic extract of Shewanella algae was evaluated and compared to ascorbic acid using a log(inhibitor) versus response model. The ethanolic fraction exhibited an IC_{50} value of 2142, with a bottom inhibition of 62.49% and a top inhibition of 13.21%. In contrast, ascorbic acid demonstrated a much lower IC_{50} of 17.74, with higher inhibition values, indicating its superior antioxidant potency. Statistical analysis showed good model fit for both samples, with R^2 values of 0.8512 for the ethanolic fraction and 0.8963 for ascorbic acid. These results suggest that while the bioactive compound from *Shewanella algae* exhibits moderate antioxidant activity, it is less potent than ascorbic acid. Nevertheless, the isolation and characterization of this novel compound highlight the potential of marine bacteria as a source of unique bioactive molecules with possible applications in biotechnology and pharmaceuticals. The integration of phenotypic, genotypic, and chemical analyses in this study provides a comprehensive framework for exploring marine microbial diversity and their functional metabolites.

CONCLUSION

This study successfully isolated and identified *Shewanella algae* from marine sediment, confirming its unique phenotypic and genotypic characteristics. A novel bioactive compound, 9-isopropyl-3,6-dimethoxy-9-methyl-9H-fluorene, was purified and structurally characterized using advanced spectroscopic techniques. The compound demonstrated moderate antioxidant activity, highlighting the potential of marine bacteria as valuable sources of bioactive molecules. These findings contribute to our understanding of marine microbial diversity and underscore the biotechnological promise of Shewanella algae-derived compounds for future pharmaceutical and industrial applications. Further research may reveal additional functional properties and expand their potential uses.

CONFLICT OF INTEREST: Nil

FUNDING: Nil

ACKNOWLEDGEMENT: The authors gratefully acknowledge the Department of Biochemistry and Biotechnology, Annamalai University, Annamalai Nagar, Tamil Nadu, India, for providing the facilities necessary to carry out this research.

DATA AVAILABILITY: Data generated while conducting the research are available to the corresponding author upon reasonable request.

Ethical Approval Statement: Not applicable (No Human and Animal studies conducted in this research).

AUTHORS CONTRIBUTION: Rudravaram Padmavathi, Dowlathabad Muralidhara Rao, and Natarajan Ashokkumar conceptualized and designed the study. Rudravaram Padmavathi conducted the analysis, interpreted the results, and drafted the manuscript. All authors reviewed and approved the final version of the manuscript for publication.

ABBREVIATIONS

MIC: Minimum Inhibitory Concentration
MRSA : Methicillin-Resistant *Staphylococcus aureus*
TLC: Thin Layer Chromatography
MPLC: Medium-Pressure Liquid Chromatography
FESEM: Field Emission Scanning Electron Microscope
PCR: Polymerase Chain Reaction
rRNA: Ribosomal Ribonucleic Acid
FT-IR: Fourier Transform Infrared Spectroscopy
NMR: Nuclear Magnetic Resonance
LC-MS: Liquid Chromatography-Mass Spectrometry
ELISA: Enzyme-Linked Immunosorbent Assay
AA: Antioxidant Activity
IC_{50}: Half Maximal Inhibitory Concentration

Ppm: Parts Per Million
m/z: Mass-to-Charge Ratio
s: Singlet (NMR notation)
d: Doublet (NMR notation)
dd: Doublet of Doublets (NMR notation)
pd: Pseudo Doublet (NMR notation)
RT: Retention Time.

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