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Extraction and Identification of Bioactive Compounds from Marine Bacteria *Pseudoalteromonas tunicata* through FTIR, GC-MS and NMR Studies

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Abstract

Natural products are of special interest because of the dazzling diversity and uniqueness of the creatures that make the sea their home. One reason marine organisms are so interesting to scientists is because in adapting to the various ocean environments, they have evolved fascinating repertoires of unique chemicals to help them survive. Interestingly, more than 120 drugs available today derive from land-based microbes. But, scientists see marine-based microbes as the most promising source of novel medicines from the sea. In all, more than 20,000 biochemical compounds have been isolated from sea creatures since the 1980s. In this study, over all 3 secondary metabolites / bioactive compounds were extracted from marine bacterial strains *Pseudoalteromonas tunicata*. The *Pseudoalteromonas tunicata* secrete 3 different bioactive compounds (Salicylic acid, Isatin and Ethyl vinyl sulfone) in the lab-scale fermentation broth. The bioactive compounds were screened by various methods such as Gas Chromatography – Mass Spectroscopy (GC-MS), Fourier Infra-Red Spectroscopy (FT-IR) and Nuclear Magnetic Resonance (NMR) spectrum activity.

Keywords

Marine bacteria, Ethyl acetate, Biomolecule, FTIR, GC-MS, NMR.

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INTRODUCTION

Natural products from the unique environments of sea water and oceans represent a largely unfamiliar source for isolation of new microbes, which are potent producers of secondary bioactive metabolites. These unique life-forms from the marine ecosphere have served as an important source of drugs since ancient times and still offer a valuable resource for novel findings by providing remedial treatments. Therefore, it can be expected that many naturally bioactive marine microbial compounds with novel structures and bioactivities

against those from terrestrial environments may be found among marine metabolites. Compared with terrestrial organisms, the secondary metabolites produced by marine organisms have more novel and unique structures owing to the complex living circumstance and diversity of species, and the bioactivities are much stronger [2, 14, 16]. Furthermore, along with the deep studies of marine natural products biosynthesis, some evidence indicates that many bioactive compounds previously found in marine animals and plants were in fact produced or metabolized by associated microorganisms [5, 12, 13, 15, 17]. Because of the low content of active compounds in marine animals and plants, as well as limitation of bio resource supply, more and more researches have been focused on marine microorganisms as sustainable resources [3, 6, 7, 11]. Competition among microbes for space and nutrient in marine environment is a powerful selection pressure that endows marine microorganisms to produce many natural products possessing medical and industrial values [1].

Many antimicrobial, antifouling substances have been found among these kinds of bacteria due to the specialized role they play in their respective hosts [4, 8, 7]. It is suggested that the primary role of these antibiotic substances could be related to ecological competition. If this were true, we would expect the antibiotic producing bacteria associated with some particular hosts to be proportionally higher than others. However, few investigations have been conducted to study and compare the antibiotic activities of marine bacteria isolated from different origins [4, 10, 12]. In the present work, different coastal areas of Tamilnadu these bacteria were then compared with marine bacteria isolated from seawater producing secondary metabolites with antimicrobial activity. These marine bacteria were expected to be potential resources of natural antibiotic products. It can be concluded that isolation of Marine bacterial samples can offer a numbers of microbial strains for sources of new biomolecules from Marine sources.

MATERIALS AND METHODS

Isolation of marine bacteria

The isolation and enumeration of marine bacteria was carried out by using the pure culture method. The collected samples were serially diluted and plated on Zobell marine agar (ZMA) medium. The petri dishes were then incubated at room temperature and the colonies were observed up to 3 days. The colonies were counted and expressed as colony forming units (CFU). Single strains of marine bacteria were picked out and purified by repeated streaking on ZMA medium. The pure cultures were transferred to ZMA slants and preserved at 4^0C consistently and morphologically different bacterial strains were selected from seawater and sediment for further analysis.

Identification of marine bacteria

For identification of marine bacteria, the bacterial strains were inoculated into Rapid Microbial Limit Test kits recommended for diagnostic microbiology supplied by Hi-media Laboratories Limited and using biochemical tests.

Optimization of growth conditions

Optimization of culturing conditions was carried out for each bacterial strain depending on the screening results. Incubations were performed at the following temperatures: 4 °C, 10 °C, 21 °C, 28 °C, 35 °C and 37 °C. In order to optimize the salt content, different concentrations of marine salts mixture were used (0 g, 10 g, 50 g and 100 g per litre) and the cultures were incubated at 21 °C and 35 °C. Whenever Zobell's marine agar was used for bacterial cultivation, the strain was simultaneously cultivated in the same medium under the same conditions.

Lab-scale fermentation of the selected bacterial strains

The selected bacterial strains were inoculated into 5 liters of Zobell's agar broth, and incubated in a shaker at 120 rpm for 48 hrs.

Extraction of secondary metabolites

After incubation period the broth culture was centrifuged at 5000 rpm for 15 min. The supernatant was extracted twice with equal volume of Hexane, Ethyl acetate, Chloroform and n-Butanol. The solvent phases were then separated using separating funnel and concentrated by evaporation. The extractions were studied by FTIR, GC-MS and NMR analysis.

Fourier Transform-Infra Red (FT-IR) Spectroscopy

The analysis of functional groups of the chemical agents (bioactive compounds from bacterial strains) was measured by FT-IR. After the reaction, a small aliquot of the concentrated reaction mixture was measured in the transmittance mode at 400 to 4000 cm^{-1}.

GC-MS Analysis

The bacterial extracts were subjected to GC-MS analysis with suitable solvents. GC-MS was carried out on a HP 5890 GC system coupled to a Quadrupole Mass Detector. Helium was used as carrier gas in the constant flow mode at 1ml/min. The initial temperature of the column was 70°C which was gradually increased by 10°C up to 280°C. The instrument was set to an initial temperature of 70°C,

and maintained at this temperature for 2 min. At the end of this period, the Oven temperature was raised up to 280°C, at the increased rate of 5°C/min, and maintained for 9 min. Injection port temperature was ensured as 250°C and helium flow rate as 1 ml/min. The ionization voltage was 70eV. Separation was achieved by RTS-volatile column about 30 m long. Quadruple Mass Detector was employed to detect compounds when they were vented from the column. Temperature of the detector was 300°C. Using computer searches MS data library and comparing the spectrum obtained through GC–MS compounds present in the bacterial samples were identified.

NMR Analysis

The active isolated compound fractions were characterized using nuclear magnetic resonance (NMR) of C^{13} and H^{1} spectroscopy. NMR [1H, 13C, homonuclear correlation spectroscopy (COSY), heteronuclear single quantum coherence spectroscopy (HSQC), and heteronuclear multiple bond correlation spectroscopy (HMBC)] spectra were recorded on a Varian INOVA 400 spectrometer (Varian Inc, Palo Alto, CA, USA), operating at 400 MHz for 1H and 100 MHz for 13C, respectively, with tetramethylsilane (TMS) as internal standard.

RESULTS

Fourier Transform Infra-Red Spectroscopy

Spectroscopic studies were carried out for the five bioactive samples were derived from TLC. Wherein some pronounced absorbance was recorded in the region between 4000 and 400 cm^{-1}. (Fig.4.15 and 4.16) The FTIR spectrums of first compound Ethyl vinyl sulfone (F1a) spectrum values are include 3058.7(secondary amine, free, N-H asymmetric stretching), 2946.2 (Tertiary amine salt, -NH+ stretching), 2885.2 (Diazo, RCH=N=N Stretching), 1612.8 (Nitrate, O-NO2 Stretching asymm) and 630.27 (C-S, R-C-CH3 stretching for sulphur compounds), cm^{-1}.

The FTIR spectrum values of the Isatin (F1b) are 3167.8 (secondary amine, free, N-H asymmetric stretching), 2786.6 (Tertiary amine salt, -NH+ stretching), 1757.8 (Diazo, RCH=N=N Stretching), 1613.3 (Nitrate, O-NO2 Stretching asymm), 1388.7 (Monametric, O-H plane bending), 1222 (Formats, Acetates, propionate and higher ester, C-O-C stretching) and 687 (C-S, R-C-CH3 stretching for sulphur compounds), cm^{-1}.

FTIR spectra of the salicylic acid (F1c) revealed absorptions at 1666.5- (Nitrate, O-NO2 Stretching asymm), 1324.9 (Monametric, O-H plane bending), 1209.8 (Formats, Acetates, propionate and higher ester, C-O-C stretching), 697.65 (C-S, R-C-CH3 stretching for sulphur compounds), cm^{-1} and 659.73 (Sulphur compounds, C-S stretching). (Fig. 1)

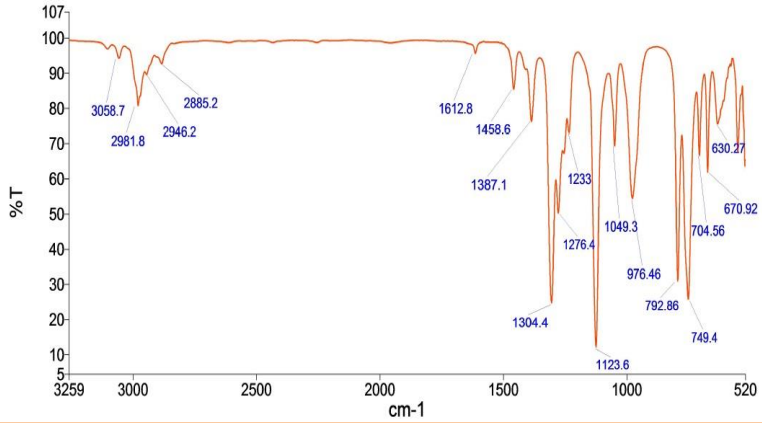
GC-MS spectra

The fractions exhibit pure distinct five bands were isolated and mixed with dichloromethane for GC-MS analysis respectively. The result of GC-MS was revealed F1 fraction derived compounds such as ethyl vinyl sulfone (F1a), Isatin (2, 3-indolinedione) (F1b), and Salicylic acid (F1c). The F1 active fraction was revealed (Fig.2) three peaks were observed with retention time 4.5 min (ethyl vinyl sulfone), 5.3 min (Isatin) and 7.1(Salicylic acid) (Fig. 3).

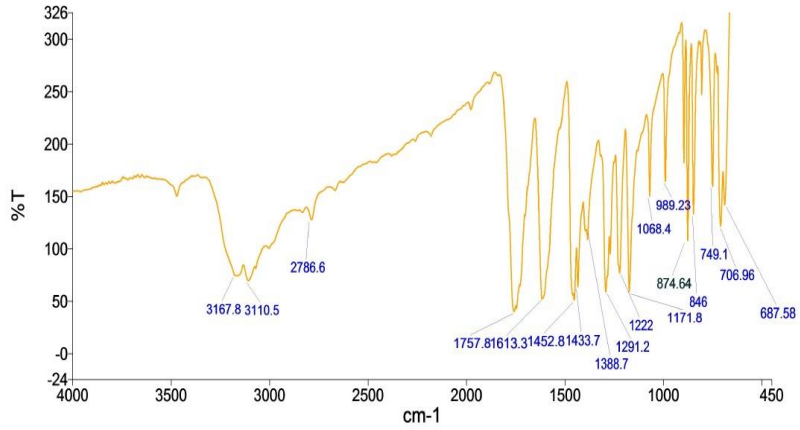
Nuclear magnetic resonance (NMR)

The ^{1}H NMR is quantitative, which means that the area under each peak is proportional to the number of protons giving rise to the resonance. The peaks in this spectrum were integrated, by setting one peak as a reference peak. The integrals were normalized on the peak of the identical hydrogen atoms adjacent to the nitrogen atom peak, corresponding to nine protons. They have the intensity of nine hydrogen atoms. When compared with the hydrogen atoms on the methyl- tail to the ω- carbon the ratio should be 9:3, if this sample was pure. 1H NMR showed some common main signals in the 0.9–2.4 ppm regions indicating the presence of alkyl groupings. The signals at 6.9 ppm (doublet) and 7.7 ppm (doublet) indicate the presence of *p*-disubstituted benzenic ring. The difference among these molecules was a value corresponding to CH2 succession. (Fig.4) show the ^{13}C NMR spectrum, respectively.

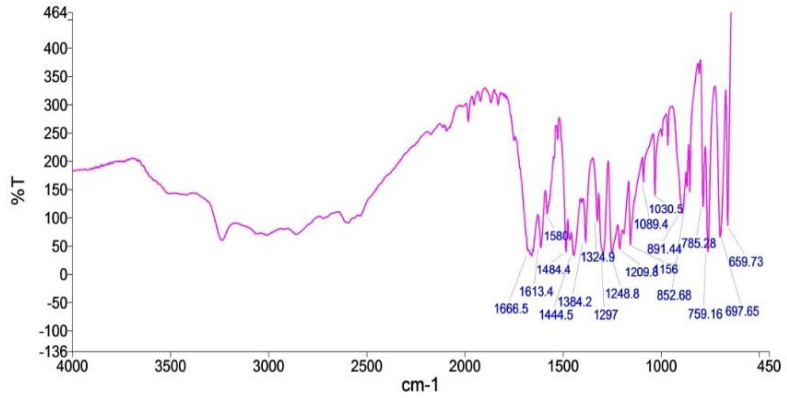
Figure.1: FT-IR spectrum of bioactive compounds from *Pseudoalteromonas tunicate.*



ETHYL VINYL SULFONE



ISATIN *(2, 3-INDOLINEDIONE)*

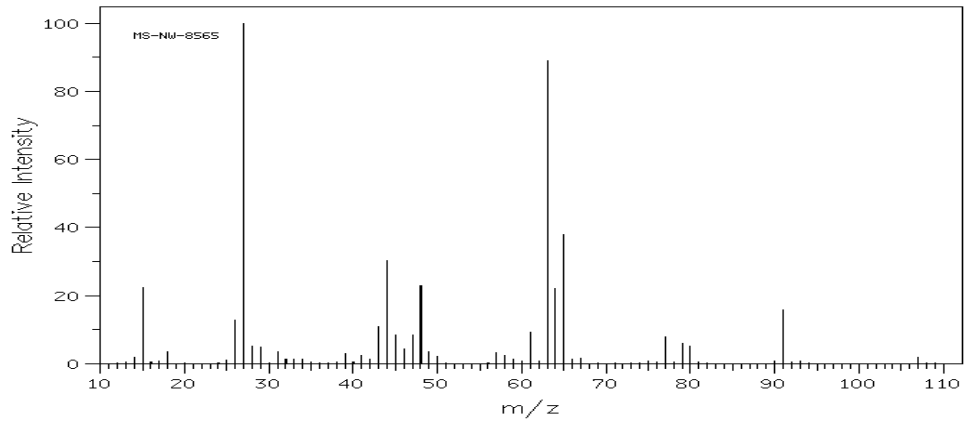


SALICILIC ACID

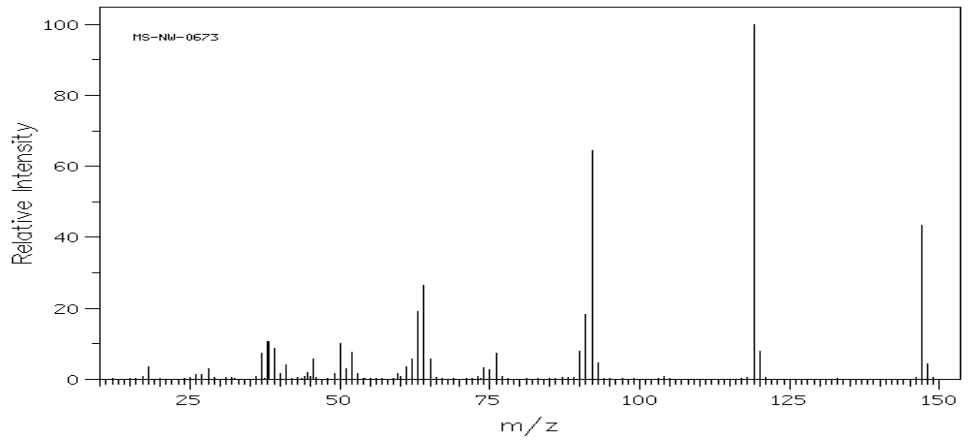
Figure.2: GC-MS spectrum for *Pseudoalteromonas tunicate.*

GC-MS analysis

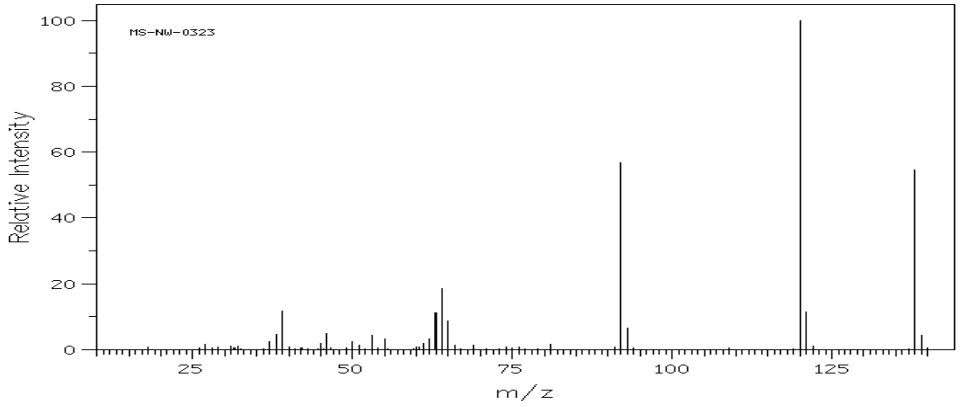
Figure.3: GC-MS Peak separation of bioactive compounds from *Pseudoalteromonas tunicate*



ETHYL VINYL SULFONE

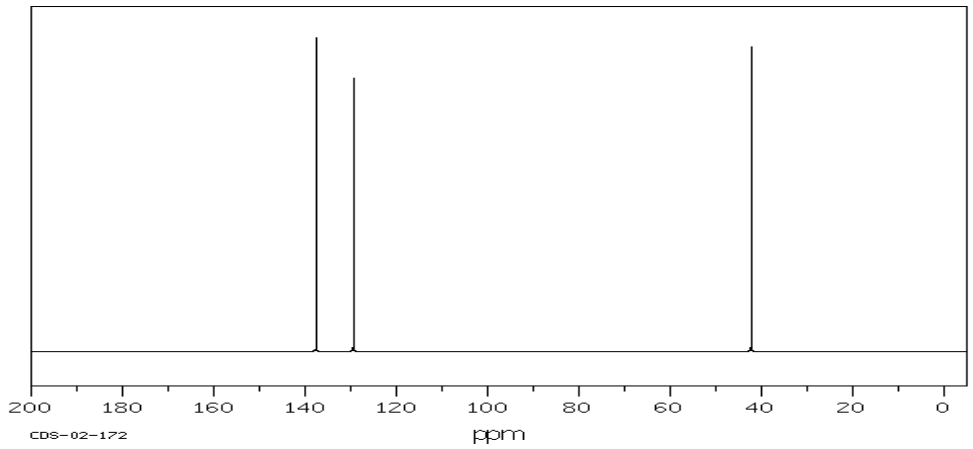


ISATIN *(2, 3-INDOLINEDIONE)*

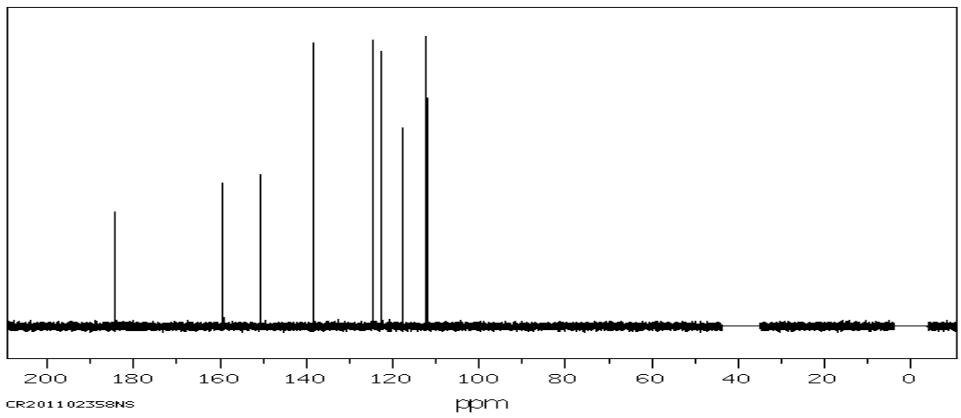


SALICILIC ACID

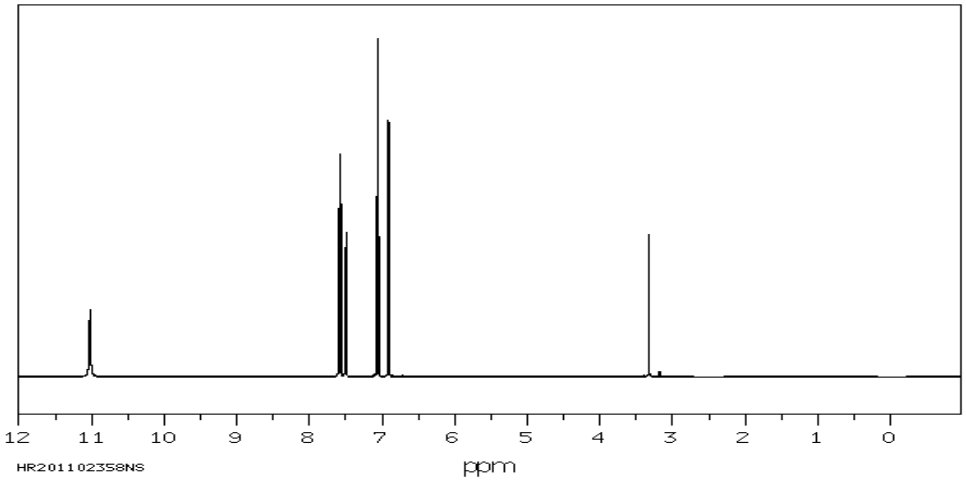
Figure.4: NMR spectrum of bioactive compounds from *Pseudoalteromonas tunicate*



13C-ETHYL VINYL SULFONE



13C-ISATIN (2, 3-INDOLINEDIONE)



H- ISATIN (2, 3-INDOLINEDIONE)

DISCUSSION

In this study, analysis of the compounds was revealed that F1 (*Pseudoalteromonas tunicata*) had mixture of three compounds The result of GC-MS was revealed F1 fraction derived compounds such as ethyl vinyl sulfone (F1a), Isatin (2, 3-indolinedione) (F1b), and Salicylic acid (F1c). In FT-IR, some pronounced absorbance was recorded in the region between 4000 and 400 cm^{-1} for detecting the functional groups of the particular compounds. The NMR was also used to help the structural elucidation of these bioactive compounds Based on this analysis we confirmed that the *Pseudoalteromonas tunicata* secrete 3 different bioactive compounds such as salicylic acid, isatin and ethyl vinyl sulfone.

CONCLUSION

Collection of fractional crude compounds were subjected to FT-IR, NMR and GC-MS studies. In FT-IR studies, the spectrums of first compound ethyl vinyl sulfone spectrum values are include 3058.7 (secondary amine, free, N-H asymmetric stretching), 2946.2 (Tertiary amine salt, -NH+ stretching), 2885.2 (Diazo, RCH=N=N Stretching), 1612.8 (Nitrate, O-NO2

Stretching asymm) and 630.27 (C-S, R-C-CH3 stretching for sulphur compounds), cm^{-1}. Similar to this type of analysis, other four compounds were analyzed. The NMR was also used to elucidate the structure of bioactive compounds from marine bacteria. The result of GC-MS was revealed F1 fraction derived compounds such as ethyl vinyl sulfone (F1a), Isatin (2, 3-indolinedione) (F1b), and Salicylic acid (F1c). It can be concluded that isolation of Marine bacterial samples can offer a numbers of microbial strains for sources of new biomolecules from Marine sources. This study indicated that certain strains of bacterial it could be induced to produce antibiotics.

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