Research Article

GC-MS, FTIR and ^{1}H, ^{13}C NMR structural analysis and identification of secondary metabolites from seawater bacterial population

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Abstract

Objectives: The present study was undertaken to provide information about bioactive compounds of *Pseudoalteromonas luteoviolacea* in the sea water samples collected from Rameswaram. **Materials and Methods:** 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. **Results:** In this study, over all 2 secondary metabolites / bioactive compounds were extracted from marine bacterial strains *Pseudoalteromonas luteoviolacea*. The *Pseudoalteromonas luteoviolacea* secrete 2 different bioactive compounds (4-(benzyloxy)-benzaldehyde and Propyl-4-Hydroxybenzoate) in the lab-scale fermentation broth. **Conclusion:** This study has been proven to be a profound resource on the development of natural product chemistry and upon the medical sciences. The improvement of natural products-based screening, rather than relying on synthetic sources, has been the aim of current pharmaceutical research and development. Alternative strategies to consider include the identification of potential new antibiotics from commercial crude bacterial fermentations.

Keywords: Marine bacteria, *Pseudoalteromonas luteoviolacea*, biomolecule, FTIR, GC-MS, ^{1}H and ^{13}C NMR

Introduction

The study of marine bacteria and their potential role in the production of metabolites is becoming a new topic for research. Several investigations have supplied an increasing number of biologically active and structurally unique compounds. Bacteria and other micro-organisms are ubiquitous in the marine drugs. They are taxonomically diverse, biologically active, and colonize all marine habitats, from the deep oceans to the shallowest estuaries. It has been estimated that the majority of bacteria in natural aquatic ecosystems are organized in biofilms (Bultel-Ponce et al., 1999). Approximately 30,000 structurally diverse natural products with a vast array of bioactivities have been discovered from marine organisms including microbes, algae and invertebrates. Invertebrates alone comprise approximately 60% of all marine animals and were described as the source of almost 10,000 new natural products since 1990 with a pronounced increase to about 1,000 compounds per year in more recent years By the turn of the 21st century larger percentages of bioactive NCEs were reported for marine organisms in comparison to terrestrial organisms, but nevertheless, marine chemical ecology is still several decades behind its terrestrial counterpart specifically compared natural products from terrestrial and marine sources. 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 (Burgess et al., 1999; Proksch et al., 2002; Schupp et al., 2016). 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 (Carte et al., 1996; Kohler et al., 1999; Osinga et al., 2001; Rinehart 2000; Sponga et al., 1999). Because of the low content of active compounds in marine animals and plants, as well as limitation of bioresource supply, more and more

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researches have been focused on marine microorganisms as sustainable resources (Bultel-Ponce et al., 1999; Hentschel et al., 2001; Holmstrom et al., 2002; Ivanova et al., 1998). 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 (Armstrong et al., 2001).

Many antimicrobial, antifouling substances have been found among these kinds of bacteria due to the specialized role they play in their respective hosts (Burkholder et al., 1966; Holler et al., 2000; Holmstrom et al., 2002). 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 (Burkholder et al., 1966; Isnansetyo et al., 2003; Kohler et al., 1999). 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

FTIR spectra of 4-(benzyloxy) benzaldehyde (F2a) are such as 2830 (Diazo,RCH=N=N Stretching), 1688 (Diazo, RCH=N=N Stretching), 1601 (Nitrate, O-NO2 Stretching asymm), 1395 (Monametric, O-H plane bending), 1260.7 (Formats, Acetates, propionate and higher ester, C-O-C stretching) and 734.91 (C-S, R-C-CH3 stretching for sulphur compounds), cm^{-1}. FTIR spectra of propyl - 4- hydroxy benzoate (F2b) revealed absorptions at 2982 (Alcohols & Phenols, O-H(Free) 1917.6 (Isothio-cyanates, Aromatic N=C=S stretching), 1676.8 (β-diketone (enolic form), C=O), 1395.3 (Alkanes, CH3 symmetric bending, R-CH3) and 773.99 (Chloro compounds, C-Cl stretching) . Wherein some pronounced absorbance was recorded in the region between 4000 and 400 cm^{-1}. (Figure 1).

GC-MS spectra

The fractions exhibit pure distinct five bands were isolated

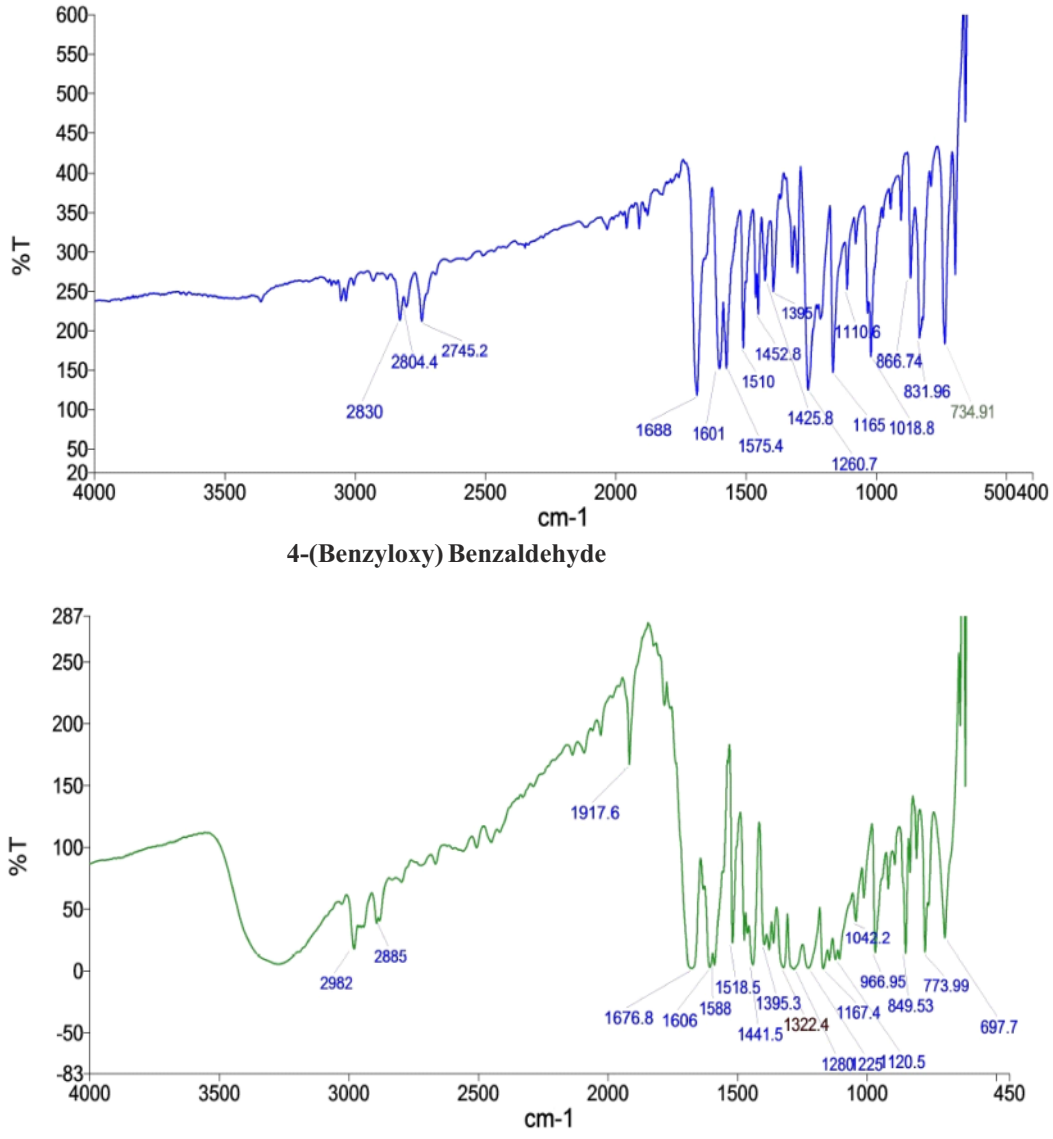

Figure 1. FT-IR spectrum of bioactive compounds from *Pseudoalteromonas luteoviolacea.*

and mixed with dichloromethane for GC-MS analysis respectively. F2 active fraction was observed two peaks with retention time 5.0 mins ((benzyloxy) benzaldehyde) and 7.5 mins (propyl - 4- hydroxy benzoate) (Figure 2). The peak separation data were given in the (Figure 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. (Figure 4) show the ^{13}C NMR and ^{1} H NMR spectrum, respectively.

Discussion and conclusion

In this study, analysis of the compounds was revealed that F2 (*Pseudoalteromonas luteoviolacea*) had mixture of two compounds. F2 fractions were content of (benzyloxy) benzaldehyde (F2a) and propyl-4-hydroxy benzoate (F2b). 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 bioactive compounds from *Pseudoalteromonas luteoviolacea. P. luteoviolacea* excreted only 2 different bioactive compounds such as 4-(benzyloxy)-benzaldehyde and propyl-4-Hydroxybenzoate. It is worth giving serious consideration to the exploitation of marine microbial life and the associated secondary metabolites, technical studies aided by genomic analyses, applying metabolic approach and employing combined biomedical and biotechnological

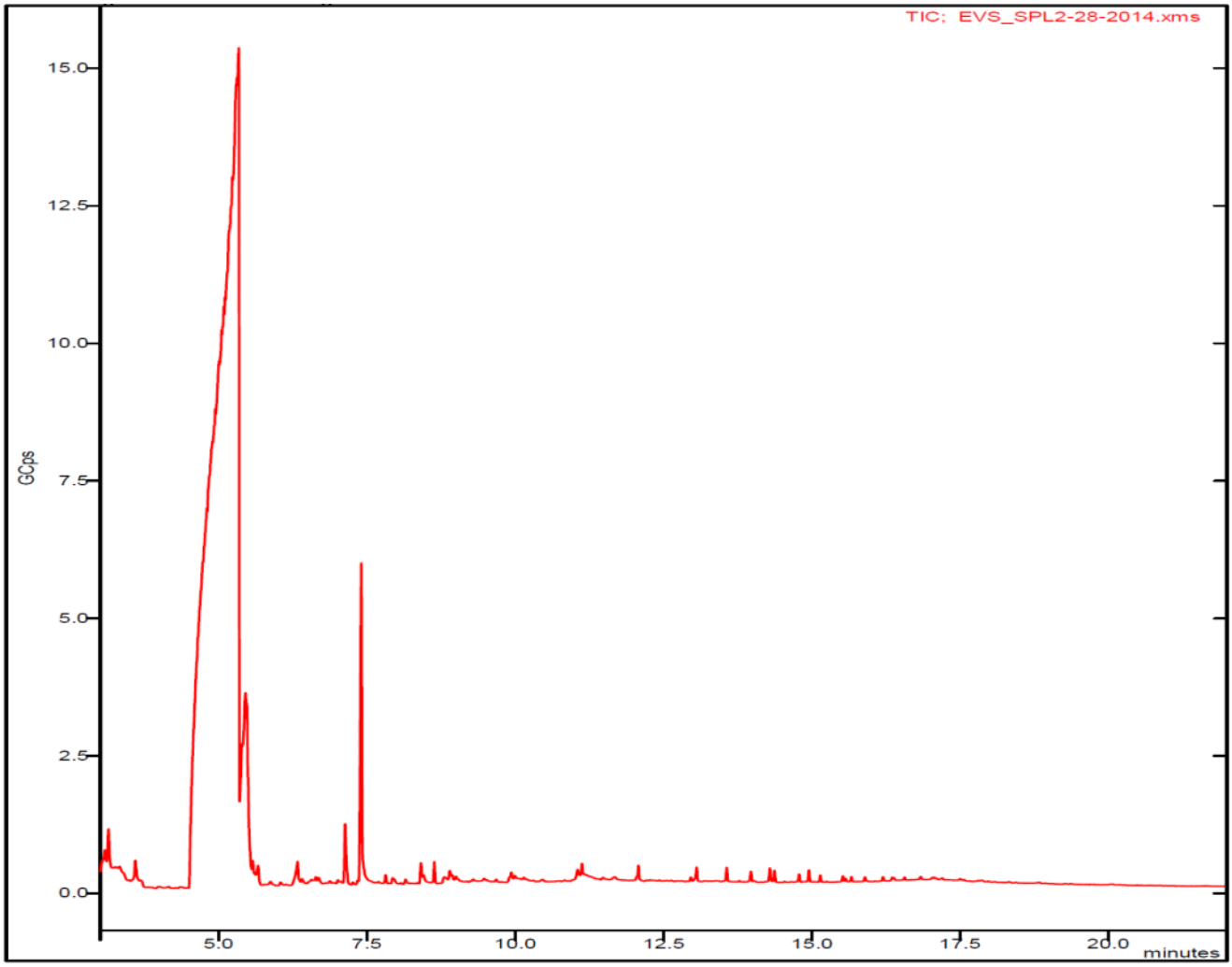

Figure 2. GC-MS spectrum for *Pseudoalteromonas luteoviolacea.*

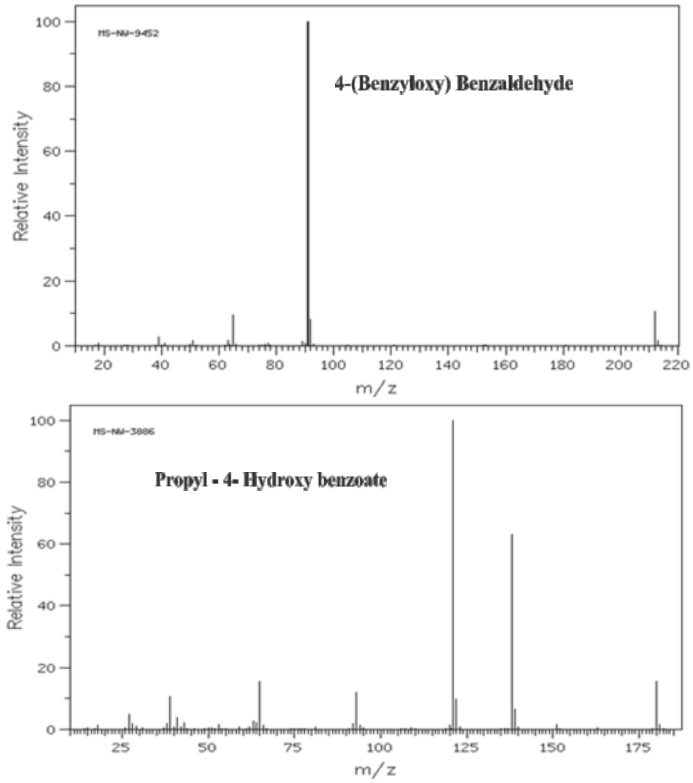

Figure 3. GC-MS Peak separation of bioactive compounds from *Pseudoalteromonas luteoviolacea*

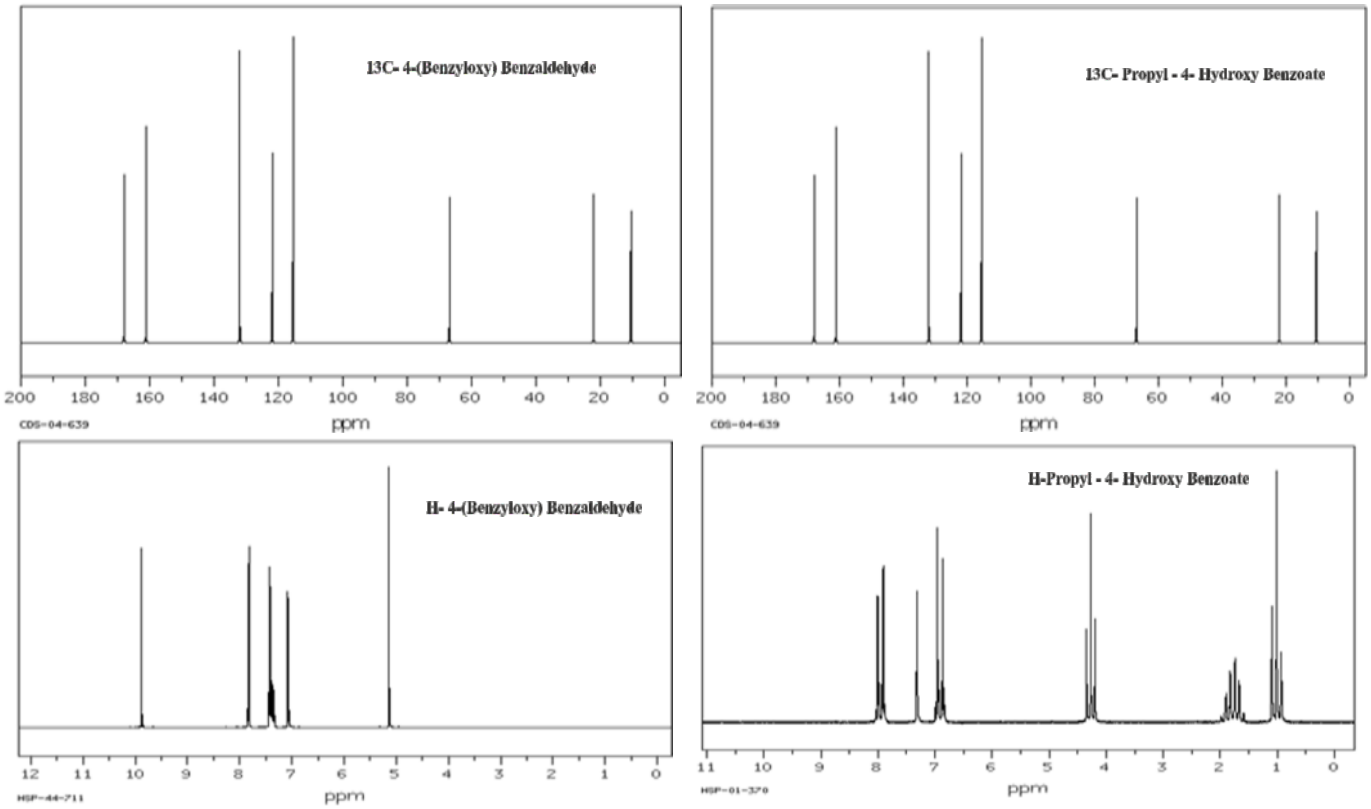

Figure 4. NMR spectrum of bioactive compounds from *Pseudoalteromonas luteoviolacea*

efforts, which would lead to discovery of some novel, lead compounds of a varied degree of bioactivity. The novel bioactive metabolites isolated and characterized from marine microbes would be useful in controlling human diseases and protecting human health by solving tribulations associated with antibiotic resistance. Certain bioactive metabolites may

also be beneficial in ensuring environmental hygiene (antifouling compounds). The development of more automated and more affordable techniques for isolating and characterizing marine microbial bioactive metabolites would definitely make marine microbial natural product extracts more accessible to natural products chemists and make life more disease free and worth living for mankind.

Conflict of interest

There is no conflict of interest regarding the publication of the article.

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