SUMMARY
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The emergence of drug resistant pathogens as well as the increase in diseases affecting the human immune system greatly intensified the need to investigate new bioactive metabolites of natural origin for potential pharmaceutical and industrial applications. Microorganisms are capable of carrying out a tremendous variety of reactions and can adapt to a range of environments allowing them to be transplanted from nature to the laboratory where they can be grown on inexpensive carbon and nitrogen sources to produce valuable compounds. Because of their biological activity, secondary metabolites of microbial origin are extremely important to our health and nutrition and have a tremendous economic importance. The screening of microbial natural products continues to represent an important route to the discovery of novel chemicals, for development of new therapeutic agents and for evaluation of the potential of lesser-known or new bacterial taxa.

Among the potential sources of natural products, bacteria have proven to be a predominantly prolific resource with a surprisingly small group of taxa accounting for the vast majority of compounds discovered. Among them, bacteria belong to the Order Actinomycetales (commonly called actinobacteria) account for more than fifty percent of the compounds reported in the Dictionary of Natural Products. They also produce other bioactive secondary metabolites including anticancer and immunosuppressive agents apart from their major role in recycling of organic matter.

The terrestrial habitat has been the most predominant and widely exploited source. A very little is known about the microbial diversity of marine sediments which is an inexhaustible resource that has not been properly exploited.
A number of biologically active compounds with varying degrees of action such as anti-tumor, anti-cancer, anti-microtubule, anti-proliferative, cytotoxic, photo protective as well as antibiotic and antifouling properties have been isolated to date from marine sources. Since marine organisms live in a significantly different environment from those of the terrestrial organisms, it is reasonable to expect that their secondary metabolites will differ considerably.

The exploitation of marine actinomycetes as a source for the discovery of novel secondary metabolites is still at its infancy and several novel metabolites have been isolated during the past few years including Abyssomicin C, Diazepinomicin and Salinosporamide A.

Indian marine environment is believed to have rich microbial diversity. However, the wealth of indigenous Indian marine microflora has not been fully explored. In view of the significance of marine actinomycetes as potential producers of bioactive compounds, the present study is mainly aimed to isolate and identify the actinomycetes from marine habitats and characterize the bioactive metabolites produced by the potent strain.

**The main objectives of the present investigation include:**

- Isolation of actinomycete strains from marine soil samples collected from Bheemili beach, Visakhapatnam, India.
- Screening the strains for antimicrobial activity to select the potent one.
- Cultural, morphological, physiological, biochemical and molecular studies of the selected strain.
- Optimization of nutritional and physiological parameters for improved production of secondary metabolites by the strain identified as *Rhodococcus* sp. VLD 10.
Extraction, purification and structural confirmation of bioactive compounds produced by *Rhodococcus* sp. VLD 10.

Minimum Inhibitory Concentration of the bioactive compounds produced by *Rhodococcus* sp. VLD 10 against Gram positive and Gram negative bacteria as well as fungi.

Marine soil samples were collected from Bheemili beach, Visakhapatnam, India for the isolation of potent actinomycete strains. The samples were initially analysed for physico-chemical properties such as moisture content, pH, organic carbon and total nitrogen content. The pretreated samples were plated on YMD and SC agar media for the isolation of actinomycetes. A total of 35 actinomycete strains designated as VLD 1 – VLD 35 were isolated.

The growth pattern and antimicrobial activity of isolates were initially screened using agar well diffusion method and the inhibition zones were recorded. The bacteria tested include *Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Escherichia coli, Klebsiella* sp., *Pseudomonas aeruginosa, P. solanacearum, Salmonella typhi, Shigella flexneri, Staphylococcus aureus, Vibrio cholerae* and *Xanthomonas campestris*, while *Alternaria* sp., *Aspergillus niger, Botrytis cinerea, Candida albicans, Fusarium solani, Fusarium oxysporum* and *Verticillium alboatrum* were used as test fungi. Among the strains tested for antimicrobial activity, the strain designated as VLD 10 exhibited high antimicrobial activity against the test microorganisms, hence selected for further study. Cultural, morphological, physiological and biochemical characteristics along with genomic analysis were employed for the identification of the strain.
Based on the cultural, morphological, physiological and molecular analysis, the strain VLD10 was identified as *Rhodococcus* sp. VLD10. The optimal cultural and nutritional conditions affecting the production of bioactive metabolites by *Rhodococcus* sp. VLD10 were recorded by changing different parameters *viz.*, incubation period, initial pH, temperature, culture media and carbon and nitrogen sources.

The effect of incubation on growth and metabolite production was evaluated by harvesting the culture broth at every 24 h interval for 8 days. The culture broth collected from five day old culture extracted with ethyl acetate exhibited high antimicrobial activity. The strain was cultured on YMD broth with different pH levels ranging from 5-9. With the increase in pH, growth and antimicrobial metabolite production increased and reached maximum at pH 7. Effect of incubation temperature on growth of the strain and antimicrobial metabolite production was tested by incubation of culture at varying temperatures ranging from 20° - 45°C. The optimum temperature for growth and antimicrobial metabolite production was recorded at 35°C.

Attempts were also made to optimize suitable carbon and nitrogen sources and their preferable concentrations in order to improve the growth of the strain and productivity of antimicrobial metabolites. Among the carbon sources tested, lactose at 1% concentration was found to support the growth as well as antimicrobial metabolite production compared to the other concentrations tested. Similarly, of the nitrogen sources studied, tryptone at 1% proved to be highly favored for growth as well as antimicrobial activity. As the strain is isolated from marine environment the sodium chloride (NaCl) tolerance of the strain was also determined. The strain did not
show any growth in the medium without NaCl and above 8% NaCl. Both the growth and secondary metabolite production was maximum in the medium amended with 6% sodium chloride.

*Rhodococcus sp.* VLD 10 demonstrated high antimicrobial activity when cultured on optimized culture medium (lactose @ 1%, tryptone @ 1% and NaCl @ 6%) with initial pH 7 and incubated at 35°C for 120 h.

To enhance the production of bioactive compounds by the strain, 10% of seed broth was inoculated into the optimized production medium. The fermentation was carried out in 1L Roux bottles for 120 h at 35°C. The harvested broth (25L) was extracted with ethyl acetate was concentrated in a Rotovac. The deep brown semi solid compound (3.8 g) obtained was served as the crude antimicrobial compound.

The separation of the crude extract was conducted via gradient elution with Hexane: Ethyl acetate. The eluent was run over the column and small volumes of eluent collected in test tubes were analyzed via thin-layer chromatography (TLC) using silica gel plates (Silica gel, Merck, Mumbai, India) with Hexane: Ethyl acetate solvent system. Compounds with identical retention factors ($R_f$) were combined and assayed for antimicrobial activities. The crude eluent was recuperated in 5-10 ml of ethyl acetate and was further purified.

A total of 11 fractions were eluted, of them 10 fractions were polar and 1 was non polar residue. Antimicrobial activity was tested for all the fractions obtained. All the fractions were rechromatographed using different gradient eluent systems for final elucidation of compounds. The fraction D1 on further purification yielded two compounds in pure form (D1Ba and D1Bb). The second fraction D2 also yielded 2 fractions in pure form namely D2Ba and D2Bb. The fraction D3 was single and obtained in pure form. The structures of these active fractions were analyzed on the
basis of Fourier Transform Infrared (FTIR); model: Thermo Nicolet Nexus 670 spectrophotometer with NaCl optics, Electron Ionization Mass/Electron Spray Ionization Mass Spectrophotometry (EIMS/ESIMS); model: Micromass VG – 7070H, 70 eV spectrophotometer and Nuclear Magnetic Resonance (\(^1\)H NMR and \(^{13}\)C NMR) model: Varian Gemini 200 and samples were made in CDCl\(_3\) with Trimethyl silane as standard. These compounds were identified as

- D1Ba - Benzoic acid
- D1Bb - 2 Nitrobenzaldehyde
- D2Ba - 4 Chlorobenzaldehyde
- D2Bb - Nonadeconoic acid
- D3 - 3 Isopropylhexahydro -1H- pyrido[1,2-a] pyrazine-1,4(6H) -dione
- D4 - Mixture fraction

Minimum inhibitory concentrations of the bioactive compounds were determined by micro-dilution method in microtitreplates. Antibacterial activity of compounds was done by micro-dilution method in 96 well microplates. The compound were active against a wide range of G+ve as well as G-ve bacteria, molds and yeast and the MIC of these compounds ranged from 15-100 µg/ml.

It is clearly evident from the present study that the bioactive compounds produced by *Rhodococcus* sp. VLD 10 exhibited good antimicrobial activity against a wide array of Gram+ve and Gram –ve bacteria as well as fungi. Hence the strain may be recommended for its use in pharmacological developments.