



# ISOLATION, SCREENING, AND MOLECULAR CHARACTERIZATION OF ANTIMICROBIALS-PRODUCING MARINE *BACILLUS STRATOPHERICUS* FROM DAHANU COASTAL WATERS, INDIA

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## Abstract

The marine ecosystem represents a vast and largely untapped reservoir of biologically active microorganisms capable of producing novel antimicrobial compounds. The present study focuses on the isolation, screening, and characterization of marine bacteria from seawater samples collected at Dahanu beach, located along the western coast of Maharashtra, India. A total of twenty marine water samples were collected and were subjected to successive enrichment using Zobell Marine Broth 2216 to enhance the growth of marine bacterial populations. Following three cycles of enrichment, serial dilution and plating techniques were employed for isolation, resulting in thirty-two distinct bacterial isolates.

Primary screening for antimicrobial activity was conducted using agar well diffusion assay against selected test pathogens. Among the isolates, isolate no. 10 demonstrated significant antimicrobial activity and was selected for further studies. Secondary screening involved solvent extraction using chloroform, ethyl acetate, and methanol to obtain crude bioactive compounds. Ethyl acetate extracts exhibited the highest antimicrobial activity.

The potent isolate was characterized using morphological, microscopic, biochemical, and molecular methods. Based on 16S rRNA gene sequencing, the isolate was identified as *Bacillus stratosphericus*. The study highlights the potential of marine-derived *Bacillus* species as a promising source of novel antimicrobial compounds, emphasizing the importance of marine ecosystems in drug discovery.

**IndexTerms:** Marine bacteria, antimicrobial activity, *Bacillus stratosphericus*, Dahanu beach, solvent extraction, 16S rRNA

## 1. Introduction

The emergence of multidrug-resistant pathogens has posed a significant threat to global public health, necessitating the discovery of novel antimicrobial agents (Michael et al., 2014). Marine environments, characterized by extreme and diverse ecological conditions, harbor unique microorganisms capable of producing structurally diverse and biologically active secondary metabolites. These metabolites have gained considerable attention due to their potential applications in pharmaceutical, agricultural, and industrial sectors (Bérdy, 2012; Bucar et al., 2013).

Marine bacteria, in particular, have evolved specialized metabolic pathways that enable them to survive under high salinity, pressure, and varying nutrient conditions. These adaptations often result in the production of unique bioactive compounds not found in terrestrial microorganisms (Thomas et al., 2010; Zheng et al., 2005). Among marine bacteria, members of the genus *Bacillus* are widely recognized for their ability to produce a wide range of antimicrobial substances, including peptides, lipopeptides, and polyketides (Bibi et al., 2020).

Dahanu beach, located along the western coast of Maharashtra, represents a relatively unexplored marine habitat with potential for harboring novel microbial diversity. The present study aims to isolate marine bacteria from this region, screen them for antimicrobial activity, and identify potent strains capable of producing bioactive compounds (Abdi et al., 2022).

## 2. Materials and Methods

### 2.1 Sample Collection

A total of twenty marine water samples were collected from Dahanu beach at a distance of 50 meters from the shoreline. Sterile sampling containers were used to avoid contamination. Samples were coded for identification and transported to the laboratory under refrigerated conditions (4°C) (Gulve & Deshmukh, 2011).

### 2.2 Enrichment of Marine Bacteria

The collected samples were subjected to successive enrichment using Zobell Marine Broth 2216. For the initial enrichment, 1 ml of seawater sample was inoculated into 25 ml of sterile broth and incubated at room temperature with shaking at 120 RPM for 7 days. Growth was monitored by comparing turbidity with uninoculated controls. Subsequent enrichment cycles were carried out by transferring 1 ml of turbid culture into fresh broth under identical conditions. A total of three enrichment cycles were performed to enhance microbial populations (Hayashida-Soiza et al., 2008).

### 2.3 Isolation of Marine Bacteria

Following enrichment, serial dilutions up to  $10^{-8}$  were prepared using reduced seawater. Aliquots were plated onto Zobell Marine Agar 2216 and incubated at room temperature for 7 days. Colonies were selected based on morphological differences (Wiese et al., 2011).

### 2.4 Purification and Preservation

Selected colonies were purified through repeated streaking on marine agar plates. Pure cultures were preserved on Zobell marine agar slants at 4°C and sub cultured monthly. Glycerol stocks were prepared and stored at -20°C (Wiese et al., 2011).

### 2.5 Primary Screening for Antimicrobial Activity

Each isolate was cultured in Zobell marine broth at a concentration of  $10^8$  CFU/ml and incubated for 7 days at 120 RPM. Cell-free supernatants were obtained by centrifugation at 5000 RPM for 10 minutes and reduced to 5 ml. Agar well diffusion assays were performed against selected test organisms. Zones of inhibition were measured, and assays were conducted in triplicate (Nishanthini et al., 2012; Palomo et al., 2013).

### 2.6 Secondary Screening and Solvent Extraction

The most active isolate (Isolate No. 10) was selected for secondary screening. Culture volume was scaled up to 350 ml. Cell-free supernatants were extracted using chloroform, ethyl acetate, and methanol in a 1:1 (v/v) ratio. The organic layers were collected and concentrated using a rotary vacuum evaporator. Extracts were tested for antimicrobial activity using agar well diffusion assays (Mehta et al., 2018; Mohan et al., 2016).

### 2.7 Identification of Potent Isolate

The selected potent isolate (Isolate No.10) was subjected to detailed phenotypic and genotypic characterization. Classical identification included macroscopic colony morphology, microscopic

examination, and a battery of biochemical tests. Molecular identification was carried out using 16S rRNA gene sequencing (Anand et al., 2006).

### 3. Results

#### 3.1 Isolation and Diversity of Marine Bacteria

A total of thirty-two bacterial isolates were obtained following successive enrichment and plating. Colonies exhibited diverse morphological characteristics, including variations in size, color, texture, and elevation.

#### 3.2 Primary Screening

It was performed to select the isolate with antimicrobial potential. Among the thirty-two isolates, several exhibited antimicrobial activity. Isolate no. 10 demonstrated the highest zone of inhibition and was selected for further studies.

Table No. 3.1. Primary screening

| Isolate No. | Zone of inhibition in mm |           |           |           |           |           |           |           |           |
|-------------|--------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
|             | <i>Ec</i>                | <i>Bb</i> | <i>Sa</i> | <i>Kp</i> | <i>St</i> | <i>Pv</i> | <i>Vc</i> | <i>Af</i> | <i>Ca</i> |
| 1           | 10 ± 2                   | -         | -         | -         | -         | 7 ± 2     | -         | -         | -         |
| 2           | -                        | -         | -         | 10 ± 1    | -         | -         | -         | -         | 9 ± 2     |
| 3           | 11 ± 1                   | -         | -         | -         | -         | -         | -         | -         | -         |
| 4           | -                        | -         | -         | -         | 10 ± 1    | -         | -         | -         | -         |
| 5           | -                        | -         | -         | -         | -         | -         | 11 ± 2    | -         | -         |
| 6           | -                        | 9 ± 1     | -         | -         | -         | -         | -         | -         | -         |
| 7           | -                        | -         | -         | -         | -         | 10 ± 1    | -         | -         | -         |
| 8           | 10 ± 1                   | -         | -         | -         | -         | -         | -         | -         | -         |
| 9           | -                        | -         | -         | 10 ± 1    | -         | -         | -         | -         | -         |
| 10          | 11 ± 2                   | -         | 12 ± 1    | -         | -         | 13 ± 2    | -         | -         | 11 ± 1    |
| 11          | -                        | -         | 10 ± 1    | -         | -         | -         | -         | -         | -         |
| 12          | -                        | -         | -         | -         | 9 ± 2     | -         | -         | -         | -         |
| 13          | -                        | -         | -         | -         | -         | -         | -         | 10 ± 2    | -         |
| 14          | -                        | -         | 10 ± 1    | -         | -         | -         | -         | -         | -         |
| 15          | -                        | -         | -         | -         | -         | 9 ± 2     | -         | -         | -         |
| 16          | 10 ± 1                   | -         | -         | -         | -         | 9 ± 2     | -         | -         | -         |
| 17          | -                        | -         | 9 ± 2     | -         | -         | -         | -         | -         | -         |
| 18          | -                        | -         | -         | -         | -         | -         | -         | 11 ± 2    | -         |
| 19          | -                        | -         | -         | 11 ± 2    | -         | -         | -         | -         | -         |
| 20          | -                        | -         | -         | -         | -         | -         | -         | -         | 9 ± 2     |
| 21          | 9 ± 2                    | -         | -         | 11 ± 2    | -         | -         | -         | -         | -         |
| 22          | -                        | -         | -         | -         | -         | 10 ± 1    | -         | -         | -         |
| 23          | -                        | 9 ± 2     | -         | -         | -         | -         | 9 ± 2     | -         | -         |
| Isolate No. | Zone of inhibition in mm |           |           |           |           |           |           |           |           |
|             | <i>Ec</i>                | <i>Bb</i> | <i>Sa</i> | <i>Kp</i> | <i>St</i> | <i>Pv</i> | <i>Vc</i> | <i>Af</i> | <i>Ca</i> |
| 24          | 9 ± 2                    | -         | -         | -         | -         | -         | -         | -         | 10 ± 1    |

|    |   |        |   |        |        |        |   |   |       |
|----|---|--------|---|--------|--------|--------|---|---|-------|
| 25 | - | -      | - | 10 ± 1 | -      | -      | - | - | -     |
| 26 | - | 10 ± 1 | - | -      | -      | -      | - | - | -     |
| 27 | - | -      | - | -      | -      | 10 ± 1 | - | - | -     |
| 28 | - | 9 ± 1  | - | -      | -      | -      | - | - | -     |
| 29 | - | -      | - | -      | 11 ± 2 | -      | - | - | -     |
| 30 | - | 9 ± 1  | - | -      | -      | -      | - | - | -     |
| 31 | - | -      | - | -      | -      | -      | - | - | 9 ± 1 |
| 32 | - | -      | - | -      | 9 ± 1  | -      | - | - | -     |

*Ec* – *E. coli* NCIM 2576, *Bb* – *Bacillus brevis* NCIM 2533, *Sa* – *Staphylococcus aureus* NCIM 2540, *Kp* – *Klebsiella pneumoniae* NCIM 5082, *St* – *Salmonella typhimurium* NCIM 2501, *Pv* – *Proteus vulgaris* NCIM 20207, *Vc* – *Vibrio cholerae* NCIM 5316, *Af* – *Aspergillus fischeri* NCIM 517, *Ca* – *Candida albicans* NCIM 3471

### 3.3 Secondary Screening

It was performed to select the potent organic solvent extract. Solvent extraction revealed that ethyl acetate extracts showed maximum antimicrobial activity compared to chloroform and methanol extracts.

Table No. 3.2. Secondary screening

| Organic solvent | Zone of inhibition in mm |           |           |           |           |           |           |           |           |
|-----------------|--------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
|                 | <i>Ec</i>                | <i>Bb</i> | <i>Sa</i> | <i>Kp</i> | <i>St</i> | <i>Pv</i> | <i>Vc</i> | <i>Af</i> | <i>Ca</i> |
| Chloroform      | 17 ± 2                   | -         | -         | -         | -         | 14 ± 2    | -         | -         | -         |
| Ethyl acetate   | 15 ± 2                   | -         | 15 ± 3    | -         | -         | 19 ± 1    | -         | -         | 18 ± 1    |
| Methanol        | 11 ± 1                   | -         | -         | -         | -         | -         | -         | -         | -         |

### 3.4 Identification of Isolate No. 10

#### 3.4.1 Macroscopic and Microscopic Characteristics

On Zobell Marine Agar 2216, the isolate produced well-defined colonies with typical *Bacillus*-like features. Colonies were observed to be opaque, irregular to slightly circular, with a dry to slightly rough surface and creamy-white pigmentation. Margins were undulate to irregular with a raised elevation. These macroscopic traits are consistent with members of the genus *Bacillus*. Microscopic examination revealed Gram-positive, rod-shaped cells arranged singly and in short chains. Endospore staining using the Schaeffer–Fulton method confirmed the presence of endospores, indicating the organism's ability to withstand adverse environmental conditions. Motility testing using the hanging drop method showed actively motile cells.

#### 3.4.2 Biochemical Characterization

Table No. 3.3 A comprehensive biochemical profile of Isolate No. 10 is summarized below.

| Test Category | Biochemical Test   | Medium / Method                       | Result        | Remarks                                      |
|---------------|--------------------|---------------------------------------|---------------|--|
| Morphological | Gram staining      | Gram staining                         | Positive      | Gram-positive rods                           |
| Morphological | Endospore staining | Schaeffer–Fulton method               | Positive      | Endospore forming                            |
| Physiological | Motility           | Hanging drop method                   | Positive      | Motile cells observed                        |
| Enzymatic     | Catalase test      | 3% H <sub>2</sub> O <sub>2</sub> test | Positive      | Bubble formation indicates catalase activity |
| Enzymatic     | Oxidase test       | Oxidase reagent (TMPD)                | Weak positive | Presence of cytochrome oxidase               |
| Enzymatic     | Starch hydrolysis  | Starch agar + iodine                  | Positive      | Clear halo around colonies                   |
| Enzymatic     | Casein hydrolysis  | Skim milk agar                        | Positive      | Casein degradation                           |

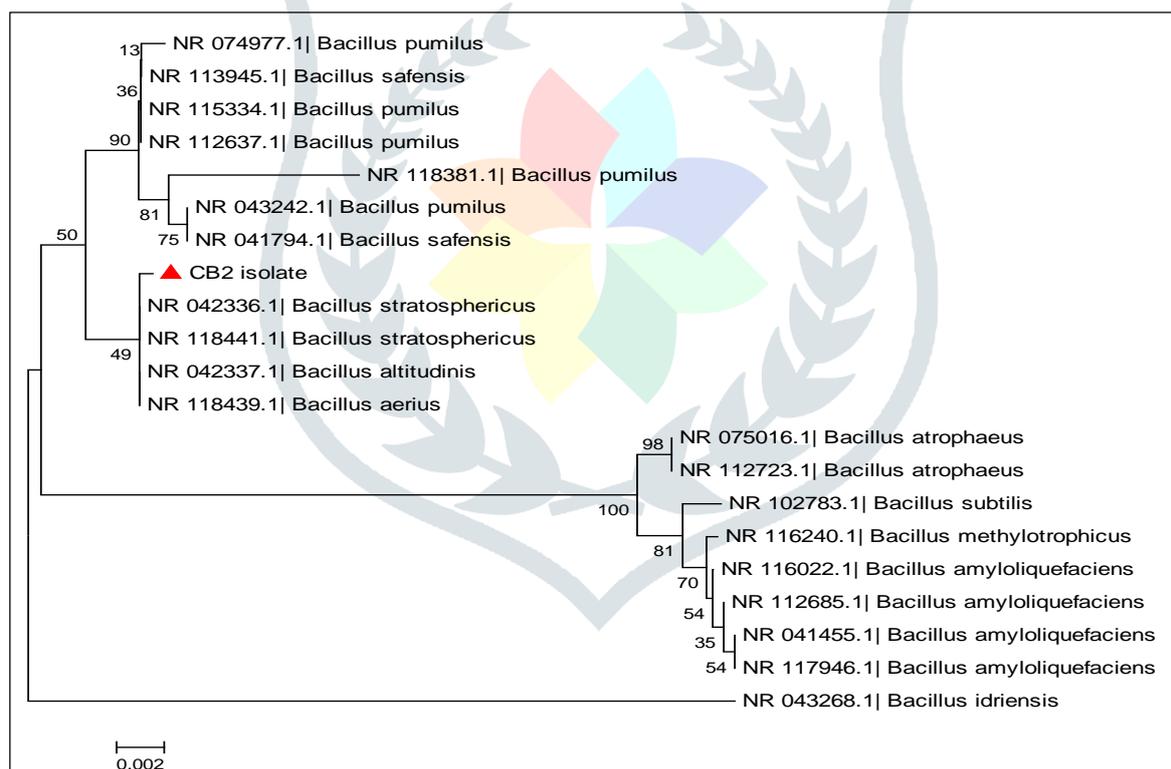
| Test Category | Biochemical Test     | Medium / Method      | Result   | Remarks                           |
|---------------|----------------------|----------------------|----------|-----------------------------------|
|               |                      |                      |          | observed                          |
| Enzymatic     | Gelatin hydrolysis   | Nutrient gelatin     | Positive | Gelatin liquefaction              |
| Metabolic     | Nitrate reduction    | Nitrate broth        | Positive | Nitrate reduced to nitrite        |
| Metabolic     | Citrate utilization  | Simmons citrate agar | Positive | Growth with blue coloration       |
| Metabolic     | Indole production    | Tryptone broth       | Negative | No red ring after Kovac's reagent |
| Metabolic     | Methyl Red test      | MR-VP broth          | Negative | No stable acid production         |
| Metabolic     | Voges-Proskauer test | MR-VP broth          | Positive | Acetoin production                |

The biochemical profile strongly supports the identification of the isolate as a member of the genus *Bacillus*. The combination of Gram-positive rod morphology, endospore formation, catalase positivity, and hydrolytic enzyme production is characteristic of *Bacillus* species. Additionally, positive citrate utilization, nitrate reduction, and Voges-Proskauer test further corroborate this classification

### 3.4.3 Molecular Identification

For definitive identification, 16S rRNA gene sequencing was performed. The obtained sequence was compared with sequences available in public databases, revealing high similarity with *Bacillus stratosphericus*. Thus, Isolate No. 10 was conclusively identified as *Bacillus stratosphericus*.

Figure 3.1 Phylogenetic tree of *Bacillus stratosphericus* (Labelled as CB2 isolate)



## 4. Discussion

The present study demonstrates the effectiveness of successive enrichment using Zobell Marine Broth 2216 in recovering diverse marine bacterial isolates from Dahanu coastal waters. The isolation of 32 morphologically distinct strains highlights the microbial richness of this marine ecosystem and supports previous reports that enrichment-based techniques enhance the recovery of metabolically active and low-abundance marine bacteria (Fenical & Jensen, 2006). Primary screening revealed that several isolates exhibited antimicrobial activity, with isolate no. 10 showing the highest inhibitory potential. The production of antimicrobial compounds in the cell-free supernatant indicates extracellular secretion of bioactive metabolites, a common trait among marine bacteria that utilize such compounds as competitive survival strategies in nutrient-limited environments (Fenical and Jensen, 2006). Secondary screening revealed that ethyl acetate extract exhibited superior antimicrobial activity compared to chloroform and methanol

extracts, suggesting that the active compounds are likely semi-polar in nature (Abad et al., 2008; Bibi et al., 2020; Senbagam et al., 2013). This is consistent with earlier studies reporting ethyl acetate as an efficient solvent for extracting microbial secondary metabolites, including lipopeptides and polyketides (Graça et al., 2013; Li et al., 2012; Offret et al., 2016). Biochemical characterization and 16S rRNA sequencing identified the potent isolate as *Bacillus stratophericus*, a member of a genus well-known for producing broad-spectrum antimicrobial compounds such as surfactins and iturins (Ongena and Jacques, 2008). The findings reinforce the potential of marine *Bacillus* species as promising candidates for antimicrobial drug discovery and highlight the importance of exploring coastal ecosystems as reservoirs of novel bioactive compounds.

## 5. Conclusion

The present study successfully establishes Dahanu coastal waters as a promising source of antimicrobial-producing marine bacteria and identifies *Bacillus stratophericus* as a potent candidate for further investigation. The findings contribute to the growing body of research emphasizing the importance of marine bioprospecting in addressing the urgent need for novel antimicrobial agents. Continued exploration and interdisciplinary research in this field hold significant promise for the development of new therapeutics to combat antimicrobial resistance and improve global health outcomes. However, the present study represents an initial step in the discovery pipeline. Further investigations are required to purify and structurally characterize the active compounds using advanced analytical techniques such as high-performance liquid chromatography (HPLC), liquid chromatography–mass spectrometry (LC-MS), and nuclear magnetic resonance (NMR) spectroscopy. Additionally, evaluating the efficacy of these compounds against clinically relevant multidrug-resistant pathogens, as well as optimizing fermentation conditions for enhanced metabolite production, will be critical for translating these findings into practical applications.

## References

1. Abad, M., Bedoya, L., & Bermejo, P. (2008). Natural Marine Anti-inflammatory Products. *Mini-Reviews in Medicinal Chemistry*, 8(8), 740–754. <https://doi.org/10.2174/138955708784912148>
2. Abdi, G., Karande, V. C., Mohammed, A., Abbasi Tarighat, M., Wen Goh, K., Abdul Kari, Z., Seong Wei, L., Ahmad Mohd Zain, M. R., Mohammadi, M., Ee Lee, G., & Barwant, M. M. (2022). Pharmacological potential of Sargassum sp. of west coast of Maharashtra Kunkeshwar, India. *Frontiers in Marine Science*, 9(December), 1–14. <https://doi.org/10.3389/fmars.2022.1011218>
3. Anand, T. P., Bhat, A. W., Shouche, Y. S., Roy, U., Siddharth, J., & Sarma, S. P. (2006). Antimicrobial activity of marine bacteria associated with sponges from the waters off the coast of South East India. *Microbiological Research*, 161(3), 252–262. <https://doi.org/10.1016/j.micres.2005.09.002>
4. Bérdy, J. (2012). Thoughts and facts about antibiotics: Where we are now and where we are heading. *Journal of Antibiotics*, 65(8), 385–395. <https://doi.org/10.1038/ja.2012.27>
5. Bibi, F., Yasir, M., Al-Sofyani, A., Naseer, M. I., & Azhar, E. I. (2020). Antimicrobial activity of bacteria from marine sponge *Suberea mollis* and bioactive metabolites of *Vibrio* sp. EA348. *Saudi Journal of Biological Sciences*, 27(4), 1139–1147. <https://doi.org/10.1016/j.sjbs.2020.02.002>
6. Bucar, F., Wube, A., & Schmid, M. (2013). Natural product isolation-how to get from biological material to pure compounds. *Natural Product Reports*, 30(4), 525–545. <https://doi.org/10.1039/c3np20106f>
7. Fenical, W., & Jensen, P. R. (2006). Developing a new resource for drug discovery: Marine actinomycete bacteria. *Nature Chemical Biology*, 2(12), 666–673. <https://doi.org/10.1038/nchembio841>
8. Graça, A. P., Bondoso, J., Gaspar, H., Xavier, J. R., Monteiro, M. C., De La Cruz, M., Oves-Costales, D., Vicente, F., & Lage, O. M. (2013). Antimicrobial activity of heterotrophic bacterial communities from the marine sponge *Erylus discophorus* (Astrophorida, Geodiidae). *PLoS ONE*, 8(11), 1–10. <https://doi.org/10.1371/journal.pone.0078992>
9. Gulve, R. M., & Deshmukh, a M. (2011). Antimicrobial Activity of Marine Bacteria Isolated From the Mangalore Coast, West Coast of India. *Recent Research in Science and Technology*, 3(5), 80–83.
10. Hayashida-Soiza, G., Uchida, A., Mori, N., Kuwahara, Y., & Ishida, Y. (2008). Purification and characterization of antibacterial substances produced by a marine bacterium *Pseudoalteromonas haloplanktis* strain. *Journal of Applied Microbiology*, 105(5), 1672–1677. <https://doi.org/10.1111/j.1365-2672.2008.03878.x>

11. Li, Y., Xu, Y., Liu, L., Han, Z., Lai, P. Y., Guo, X., Zhang, X., Lin, W., & Qian, P. Y. (2012). Five new amicoumacins isolated from a marine-derived *Bacterium bacillus subtilis*. *Marine Drugs*, *10*(2), 319–328. <https://doi.org/10.3390/md10020319>
12. Mehta, P. K., Yadav, A. K., & Kumar, D. (2018). Secondary metabolites from microorganisms. *Secondary Metabolite and Functional Food Components: Role in Health and Disease*, *74*(1), 153–177.
13. Michael, C. A., Dominey-Howes, D., & Labbate, M. (2014). The antimicrobial resistance crisis: Causes, consequences, and management. *Frontiers in Public Health*, *2*(SEP), 1–8. <https://doi.org/10.3389/fpubh.2014.00145>
14. Mohan, G., Thangappanpillai, A. K., & Ramasamy, B. (2016). Antimicrobial activities of secondary metabolites and phylogenetic study of sponge endosymbiotic bacteria, *Bacillus* sp. at Agatti Island, Lakshadweep Archipelago. *Biotechnology Reports*, *11*, 44–52. <https://doi.org/10.1016/j.btre.2016.06.001>
15. Nishanthini, a, Naz, F., & Thirumurugan, R. (2012). Antagonistic Activity of Marine Bacteria from Cochin Backwater of Arabian Sea. *International Journal of Microbiological Research*, *3*(1), 16–23. <https://doi.org/10.5829/idosi.ijmr.2012.3.1.56180>
16. Offret, C., Desriac, F., Le Chevalier, P., Mounier, J., Jégou, C., & Fleury, Y. (2016). Spotlight on antimicrobial metabolites from the marine bacteria *Pseudoalteromonas*: Chemodiversity and ecological significance. *Marine Drugs*, *14*(7), 1–26. <https://doi.org/10.3390/md14070129>
17. Palomo, S., González, I., De La Cruz, M., Martín, J., Tormo, J. R., Anderson, M., Hill, R. T., Vicente, F., Reyes, F., & Genilloud, O. (2013). Sponge-derived *Kocuria* and *Micrococcus* spp. as sources of the new thiazolyl peptide antibiotic kocurin. *Marine Drugs*, *11*(4), 1071–1086. <https://doi.org/10.3390/md11041071>
18. Senbagam, D., Gurusamy, R., & Senthilkumar, B. (2013). Physical chemical and biological characterization of a new bacteriocin produced by *Bacillus cereus* NS02. *Asian Pacific Journal of Tropical Medicine*, *6*(12), 934–941. [https://doi.org/10.1016/S1995-7645\(13\)60167-4](https://doi.org/10.1016/S1995-7645(13)60167-4)
19. Thomas, T. R. A., Kavlekar, D. P., & LokaBharathi, P. A. (2010). Marine drugs from sponge-microbe association - A review. *Marine Drugs*, *8*(4), 1417–1468. <https://doi.org/10.3390/md8041417>
20. Wiese, J., Ohlendorf, B., Blümel, M., Schmaljohann, R., & Imhoff, J. F. (2011). Phylogenetic identification of fungi isolated from the Marine Sponge *Tethya aurantium* and identification of their secondary metabolites. *Marine Drugs*, *9*(4), 561–585. <https://doi.org/10.3390/md9040561>