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# A preliminary investigation on the antibacterial activity of marine bacteria

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Original Article

## Abstract

The marine environment is a prolific resource for the isolation of less exploited microbes. Owing to the nature of this environment, there is a strong competition among microbes for space and nutrients that endows them to produce natural products possessing medical and industrial value. The present study was undertaken to discover novel antibacterial compounds from marine bacterial sources for the treatment of ever-increasing drug-resistant infections. A total of 150 isolates obtained from different marine sources were screened for their antibacterial activity using the agar well diffusion method. Nine of the isolates were found to exhibit antibacterial activity. The current data reports the preliminary screening of the antimicrobial effect of the Cell-Free Supernatant (CFS) of these nine isolates against six pathogenic microbes. This study is an attempt to isolate diverse marine bacteria that exhibit significant biological activity that could be an important lead in the search for novel natural products of clinical importance.

***Keywords****: Antibacterial activity, marine, CFS, Agar well diffusion method*

## Introduction

The marine environment is increasingly appreciated as a reservoir for diverse habitats which serve as novel sources for the production of natural bioactive compounds. Many such compounds exhibit structural and chemical features that are not found in terrestrial natural products (Biswas *et al.*, 2016). The low content of active compounds in marine plants and animals and the limitations of bioresource supply have made marine microorganisms gain more attention as powerful resources for the discovery of such compounds (Jeganathan *et al.*, 2013). Marine bacteria thrive in a competitive biological environment with unique conditions of salinity, pressure, nutrition, temperature and complex relations with their different counterparts (Mazalan *et al.*, 2012). The high stress levels and possible lack of nutrients trigger these bacteria to produce unique secondary metabolites. A major class of these metabolites include antibiotics, bacteriocins or bacteriocin-like inhibitory substances (BLIS), lysozymes, siderophores, proteases, hydrogen peroxide and organic acids (Bindiya and Bhat, 2016). These metabolites help marine bacteria to compete for nutrients and space and also act as defence mechanisms for their survival (Shukla, 2016). A variety of novel compounds with relevant biological activities from marine microorganisms have been reported in the past (Chen *et al.*, 2014). Bacteriocins and BLIS have been isolated from marine strains of *Brevibacter* sp., *Vibrio* sp. and *Bacillus* sp. (Bindiya and Bhat, 2016; Sharma *et al.*, 2014). With the advent of new technologies like deep seawater pumping and other available types of equipment, investigation of the marine environment has become extremely popular for both academic and industrial applications (Soliev *et al.*, 2011). The present study was aimed to isolate bacteria from the marine environment (seawater and soil samples) and assess their potential for the production of antimicrobial compounds.

## Material and methods

### *Sample collection*

Water samples were collected in sterile bottles from different parts of the Indian seas at the surface and 10m depths. The locations of the collection were from the coastal regions of a) Kerala (Kovalam beach, Latitude 8.4004° N, Longitude 76.9787° E), b) Diu and Daman (Jampore beach, Latitude 20.3809° N, Longitude 72.8239° E) and c) Maharashtra (Ganpatipule, Latitude 17.1489° N, Longitude 73.2727° E). Soil samples from the shores were collected in sterile plastic bags and all the samples were stored under sterile conditions at 4 °C for preventing bacterial cross-contamination until further use (Balraj *et al.*, 2014). The subsequent isolation of the bacterial strains was carried out within 48 h.

### *Isolation of marine bacteria*

Marine bacteria from the water/soil samples were isolated using the spread plate method on different media like Zobell's medium, MRS medium (Mann Rogosa Sharpe agar), Nutrient agar with 3.5% NaCl and nutrient agar medium (Bibiana and Nithyanand, 2014). The plates were incubated at room temperature (RT) for 48-72 h. The isolated colonies developed after incubation were labelled and maintained by repeated subculture on respective media.

### *Preparation of Cell-Free Supernatant (CFS)*

One ml saline culture of the isolates in the log phase (Optical density adjusted to 0.1 at 660nm) was inoculated in respective media for 72 h under static conditions (Alghazeer *et al*., 2017). The isolates growing on the nutrient agar with 3.5% NaCl were checked for growth on the Nutrient agar to eliminate the inhibitory effects of NaCl during the assay. After incubation, the broth was centrifuged at 10,000 rpm for 15 minutes (Rizvi *et al*., 2014). The crude supernatant was used as a source of CFS for further screening.

### *Assay for antibacterial activity – Agar well diffusion method*

The test cultures used for the assay were Gram-positive organisms like *Corynebacterium diptheriae, Staphylococcus aureus* and *Sarcina lutea* and Gram-negative cultures namely *Escherichia coli*, *Salmonella typhi* and *Klebsiella pneumonia,* procured and maintained at the School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, Navi Mumbai, India. For the antibacterial assay, log phase saline cultures of all test organisms were used. Nutrient agar plates with test organisms were prepared by pour plate method and wells of 8 mm (diameter) were punched using a sterile cork borer. 50 $\mu$l of the neat (undiluted) CFS from each isolate were added in a separate well and the plates were kept at 4 °C for 15 minutes to allow the diffusion of CFS in the medium. The un-inoculated medium was maintained as a control. The plates were then shifted to an incubator at 37 °C for 24 h (Goh and Philip, 2015). After incubation, the zones of inhibition exhibited by the respective CFS of nine isolates against all the test organisms were measured.

### *Characterization of the CFS*

Tests with Proteinase K and Trypsin: The CFS was treated with Proteinase K (SRL, 20 mg/ml) at a final concentration of 1mg/ml. CFS (190 $\mu$l) was mixed with Proteinase K (10 $\mu$l) and incubated at 37 °C for 30 minutes (Balasubramanian *et al*., 2014). After incubation, the tubes were centrifuged (10,000 rpm for 15mins at RT) and the (proteinase K treated) CFS was used to check antibacterial activity by agar well diffusion method. Controls were maintained by replacing 190 $\mu$l of CFS with un-inoculated media (Thuy Pham *et al.,* 2014). The same procedure was also followed with 1mg/ml trypsin.

Catalase test: The isolates and the CFS were checked for the presence of catalase (Yang *et al*., 2012) using 3% $H_2O_2$ (30% obtained from Merck).

Test for acid production: The pH of the CFS was checked after 72 h of incubation. For some isolates, the pH was found to be drastically reduced in the range of 4.0-5.0. For such isolates, the CFS was neutralised to pH 7.0 using sterile 0.1N NaOH (Fossi *et al*., 2017).

## Results

### *Isolation of marine bacteria*

A total of 150 isolates were obtained from different media after 48-72hr of incubation. Of the 150 isolates obtained, 77 isolates were obtained on the Nutrient agar, 48 isolates on Zobells medium, and 15 isolates on Nutrient agar with 3.5% NaCl. 10 isolates were obtained on MRS medium. All the isolates on the MRS medium were colourless, mucoid, exhibiting entire margin and the Grams nature revealed them to be Gram negative rods. Pigmented, as well as colourless colonies, were observed on Zobells and Nutrient agar plates. All the isolates obtained on these media were differentiated based on their colony characteristics and from the sample/source used for screening. The isolates obtained from water samples were designated as 'W' and from soil samples as 'S'.

### *Screening for antibacterial activity*

Out of the 150 isolates screened, 9 isolates exhibited significant antibacterial activity (Fig.1) Isolates S35 and S36 displayed activity against *C. diptheriae, S. lutea*, and *S. aureus,* whereas isolates S34 and W14 were active against *S. lutea* and *S. aureus.* Isolate S31 has a wide spectrum of activity against *S. aureus*, *S. lutea, C. diptheriae* and *E. coli*. Isolate S33 and W16 exhibited specific inhibitory activity against *S. lutea* only. Specific inhibition was exhibited by the isolates W15 and W5 against *S. aureus* and *C. diptheriae* respectively.

### *Characterization of Cell-Free Supernatant (CFS)*

The CFS of individual cultures treated separately with Proteinase K and Trypsin resulted in a clear zone of inhibition. Controls

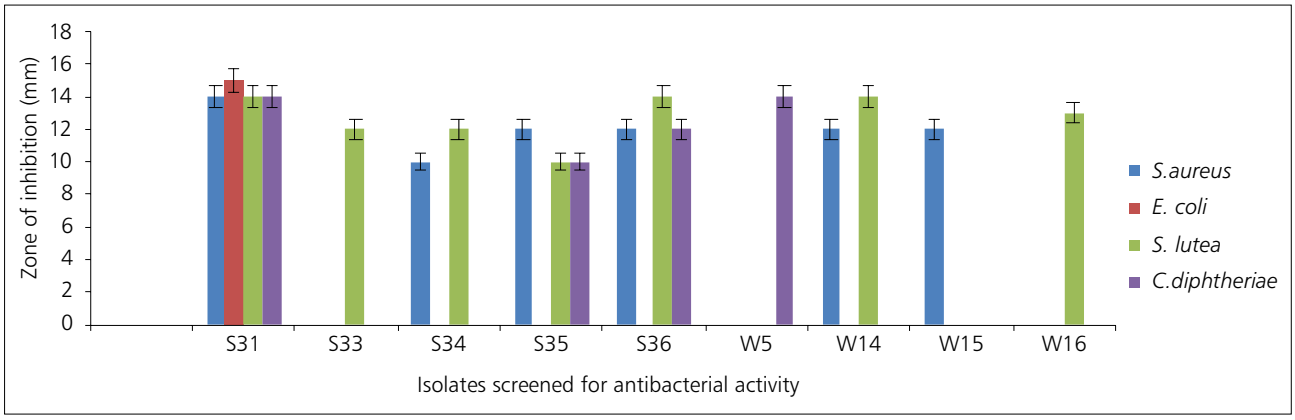


Fig. 1. Isolates screened for the study showing the zone of inhibition against selected test organisms

having un-inoculated media with Proteinase K or Trypsin did not exhibit any zone of inhibition (Fig. 1 and 2). The nine isolates and their respective CFS were checked separately for the presence of the catalase enzyme. All nine isolates and their respective CFS were found to be catalase positive. The CFS from the nine isolates was also checked for the production of acid after 24, 48 and 72 h of incubation. No significant change in pH was observed in the CFS of any of these nine isolates (Table 1).

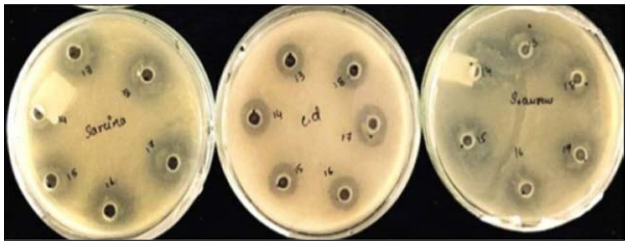

Fig. 2. CFS of the isolates S31 and S35 exhibiting zone of inhibition against test organism a) *S. lutea* b) *C. diptheriae* and c) *S. aureus* and their further characterization. Key: Well No 13–CFS of S31, 14–CFS of S31 + Proteinase K, 15–CFS of S31 + Trypsin, 16–CFS of S35, 17–CFS of S35 + Proteinase K, 18–CFS of S35 + Trypsin

Table 1. pH of the crude CFS of the 9 isolates selected for study

| Isolate No. | pH of the CFS | | |
|---|---|---|---|
| | 24 h | 48 h | 72 h |
| S31 | 7.0 | 7.0 | 7.03 |
| S33 | 6.50 | 6.40 | 6.37 |
| S34 | 6.40 | 6.33 | 6.30 |
| S35 | 6.0 | 6.0 | 6.00 |
| S36 | 6.0 | 6.0 | 6.29 |
| W5 | 7.0 | 7.0 | 6.90 |
| W14 | 7.0 | 7.10 | 7.30 |
| W15 | 7.0 | 6.92 | 6.80 |
| W16 | 7.0 | 7.0 | 6.90 |

## Discussion

The rich diversity and the extreme physical conditions make the marine environment an ideal source for providing novel drug leads. The coasts of South West India harbour rich and diverse microbes that can produce different bioactive compounds. Though the percentage of cultivable microbes is less, they can provide valuable insights into the potency of microbes in such extreme conditions (Sinimol *et al.*, 2016). A wide variety of bacteria have been isolated from the southern and the western coastal regions of India (Mishra *et al.*, 2017). However, few regions of the Konkan coast have still not been fully exploited for the novel microorganisms. Hence, in the present work, 150 bacterial isolates of marine origin were obtained from different marine sources like the coastal areas of Ganpatipule, Jampore beach and Kovalam beach. Of these150 different isolates, only 6% exhibited antibacterial activity. The reasons for exhibiting antibacterial activity could be due to the production of antibiotics, or antimicrobial proteinaceous compounds like bacteriocins or BLIS. Apart from these compounds, the inhibition could also be due to the production of acids or toxic reactive intermediates like hydrogen peroxide (Oldak *et al.*, 2017). The crude CFS when treated with Proteinase K and Trypsin individually gave a zone of inhibition suggesting the antibacterial compound not to be proteinaceous in nature or the compound to be resistant to the action of proteases (Fig. 1 and 2). A positive catalase test of the isolates and their respective CFS also eliminates the possibility of inhibition due to the production of hydrogen peroxide. All nine isolates considered for the study exhibited no drastic change in pH confirming that the inhibition was not due to the production of acid. All these results indicate that the antimicrobial studies in this direction have also led us to identify a few siderophores producing organisms (GenBank accession numbers-MF511820 and MF51190) with antibacterial activity (Uchgaonkar *et al.*, 2018) compound could be a class IV bacteriocin that is resistant to proteases or a BLIS (Leite *et al.*, 2016). The results suggest

that these isolates could be an important lead in the search for novel natural products of clinical importance. Further studies on the purification of CFS and characterization of the molecule could prove a powerful alternative to combat the ever-increasing threat of drug resistance in bacteria.

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