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# Antagonistic Activity of Marine Bacteria from Cochin Backwater of Arabian Sea

*[1]M. Sethukkarasi, [2]N.K. Asha Devi, [1]B. Vasuki,*
*[1]A. Nishanthini, [1]Falaq Naz and [1]R. Thirumurugan*

[1]Department of Animal Science, School of Life Sciences,
Bharathidasan University, Tiruchirappalli-620 024, Tamilnadu, India
[2]Department of Zoology, Thiagarajar College (Autonomous), Madurai-625009, Tamilnadu, India

**Abstract:** In the present study, antimicrobial property of marine bacteria was assessed in order to find novel metabolites. Fifty three bacteria were isolated from different regions of Cochin backwater namely, Munambam, Vypin, Thevara, Fort Kochi and Kalavathy. All isolates were subjected to primary screening against test organisms and further carried out to secondary screening selectively using well diffusion assay. Many selected bacteria showed some promising antagonistic activity against test organisms. In FTIR and NMR analysis, the compound which might have one hydroxyl methyl group, primary or secondary amines or amides and -CH-OH (Hydroxyl methylenes) were linked to each other by ether linkages. It predicted that the antimicrobial compound probably have a straight chain polyhydroxy polyether compound with a single double bond possessing complex ring structure. Therefore, these metabolites can be used as potential bioactive products.

**Key words:** Backwater · Bacteria · FTIR · NMR · Antimicrobial activity

## INTRODUCTION

Natural products have been the source of most of the active ingredients of medicines. This is widely accepted to be true when applied to drug discovery in 'olden times' before the advent of high-throughput screening and the post-genomic era: more than 80% of drug substances were natural products or inspired by a natural compound [1].

Natural products show a diversity of chemical structures that are not accessible even by the most sophisticated synthetic concepts. Moreover, natural products have often opened up completely new therapeutic approaches. They have contributed to identifying and understanding novel biochemical pathways and proved to make not only valuable drugs available but also essential tools in biochemistry and molecular cell biology [2].

In the era of antibiotic resistance development, many pathogenic bacterial and fungal organisms are gaining resistance by curious mechanisms due to the indiscriminate use of the antibiotics. In order to prevent various infectious diseases, search of new bioactive compounds produced by microorganisms is needed. These bioactive substance though chemically diverse show antagonistic activity against varied bacterial and fungal pathogens [3]. While comparing with earlier available antibiotics, the search for new, safe and broad spectrum antagonistic compounds with great potency has been increasing in order to combat against drug resistance [4].

The world oceans compose over 70% of the earth's surface and over 90% of the volume of its crust. Microbiologically, the oceans represent indeed the most diverse resource of life with huge dimensions and extreme variations in pressure, salinity and temperature. These extreme conditions require unique adaptation strategies leading to new natural products, which differ from products known for terrestrial organisms [5].

The search for novel metabolites has got importance because of increased occurrence of multi-drug resistant pathogens. Many clinically relevant microbes have raised resistance resulting not only from the exposure to sub lethal concentrations of antibiotics in hospital environment but also in animal farms where antibiotics are used as growth enhancers [6]. Marine bacteria have been recognized as an important resource for novel bioactive compounds. The chemical compounds of marine

**Corresponding Author:** R. Thirumurugan, Department of Animal Science, School of Life Sciences,
Bharathidasan University, Tiruchirappalli - 620024, Tamil Nadu, India.
Mob: +91-94430 94199.

microorganisms are less well known than those of their terrestrial counterparts. However, in the last decade several bioactive compounds have been isolated from marine bacteria and are new resources for the development of medically useful compounds [7, 8].

The first report on antimicrobial activity of *Stenotrophomonas* strains isolated from deep sea invertebrates came in 2008, stating that remarkable fungal inhibitory activity was observed in six *Stenotrophomonas* strains isolated from sponge, sea urchin and ophiura specimens. Although negligible activity was observed against *Candida albicans*, these strains could substantially inhibit Gram-positive microorganisms. It is worth noting here that *S. maltophilia* is an opportunistic pathogen known for its bio-controlling capabilities [9]. There are very few reports on antagonistic activity of marine microorganisms from backwaters of Arabian Sea. Hence, the aim of this work was to carryout detailed studies on the bioactive microorganisms of bacteria collected from Cochin backwaters of Arabian Sea, India.

## MATERIALS AND METHODS

**Area of Study and Collection of Samples:** The Cochin backwaters situated between 09°58' - 10°10N and 76°25' E is a shallow semi enclosed body of brackish water running parallel to the coastal line located in the tropical zone. Five water samples were collected from Cochin back waters, Kerala namely Munambam, Vypin, Thevara, Fort Kochi and Kalavathy.

**Isolation of Marine Bacteria:** The five water samples were serially diluted upto $10^{-8}$ using 0.85% saline and 100 microlitre from each dilution was spread over the surface of marine agar medium prepared in sea water to enhance the isolation of marine bacteria. The plates were then incubated at 28±2°C and the colonies were observed from 24 hours onwards for 5 days. Bacterial colonies were picked out and purified by repeated streaking on marine agar medium. The pure cultures of the bacterial colonies were inoculated into nutrient agar slants and preserved at 4±2°C. The potent bacterial strains were selected from primary screening and processed for obtaining cell free extract.

**Filtrate of Marine Strains and its Activity:** The culture filtrate activities of bacterial strains were tested against 15 test organisms (bacterial pathogens and pathogenic fungi) using the well assay method. The strains were centrifuged at 10,000rpm for 20 min. After swabbing the pathogens on the plates, 100 µl of cell free culture broth of bacterial strains was poured into the well and plates were incubated at 37°C for 48 h. The culture filtrate of bacterial strains inhibiting the growth of pathogen around the well after two days was assessed by the inhibition zone around the well [10]. The assay was carried out in duplicate against all the test organisms.

**Susceptibility Test:** The isolated fish pathogens were subjected to find their susceptibility pattern to a group of selected antibiotics discs by Kirby - Bauer method [11]. The following commercial antibiotics were used for this assay erythromycin (E15), bacitracin (B10), gentamicin (G10), tetracycline (T10), amoxycillin (Am10), ampicillin (Ap10), chlorampenicol (C30), amikacin (Ak30), rifampicin (R5) and doxycycline hydrochloride (Dos25), Streptomycin (S10), Penicillin (P10), Polysporin (Cr30) and cephalexin (Ce30).

**FTIR Studies:** Based on the earlier investigations, the selected bacterial extracts which have maximum antagonistic activity were analyzed by Fourier Transform Infra Red Spectroscopy (FTIR) (NEXUS-672 model).

The spectrum was taken in the mid IR region of 400 - 4000 $cm^{-1}$. The spectrum was recorded using ATR (Attenuated Total Reflectance) technique. The sample was directly placed in the Sodium crystal and the spectrum was recorded in the transmittance mode.

## RESULTS

After the primary screening, 10 bacterial strains were selected, of which antibiotic activity varied from 12 to 22 mm zone of inhibition against test organisms (bacterial and fungal pathogens).

Totally 15 test organisms including Gram positive (Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus mutans, Micrococcus luteus) and Gram negative (Enterobacter aerogenes, Escherichia coli, Klebsiella pneumonia, Proteus vulgaris, Pseudomonas aeruginosa, Vibrio cholera, Vibrio parahaemolyticus, Shigella flexneri, Serratia marcescens, Salmonella typhi) were used.

The antagonistic activity of the ten isolated strains was determined against the entire set of the test organisms. It was found that few of them were not active against Bacillus subtilis, Enterobacter aerogenes, Klebsiella pneumonia, Pseudomonas aeruginosa and Staphylococcus epidermidis.

Table 1: Secondary Screening of Antimicrobial activity of Bacterial Isolates against Test Organisms

| | Marine Bacteria (MB) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Test organisms | 2 | 3 | 15 | 17 | 19 | 22 | 24 | 27 | 32 | 43 |
| Gram positive organisms | | | | | Zone of inhibition in mm | | | | | |
| *Bacillus subtilis* | - | 18 | - | - | 24 | - | 14 | 12 | 20 | 11 |
| *Staphylococcus aureus* | 10 | 14 | 14 | 11 | 20 | 10 | 10 | 18 | 14 | 10 |
| *Staphylococcus epidermidis* | - | 26 | - | 10 | 18 | - | 11 | - | 28 | 14 |
| *Streptococcus mutans* | 10 | 18 | 14 | 10 | 14 | 10 | 14 | 14 | 16 | 12 |
| *Micrococcus luteus* | 10 | 18 | 11 | 10 | 15 | - | 12 | 14 | 10 | 14 |
| Gram negative organisms | | | | | Zone of inhibition in mm | | | | | |
| *Enterobacter aerogenes* | 10 | 16 | - | 12 | 17 | - | 14 | 14 | 14 | 11 |
| *Escherichia coli* | 10 | 14 | - | 14 | 16 | - | 12 | 14 | 16 | 13 |
| *Klebsiella pneumoniae* | - | 15 | - | 12 | 14 | 10 | 12 | 12 | 14 | 10 |
| *Proteus vulgaris* | 10 | 14 | 12 | 10 | 15 | - | 14 | 12 | 10 | 10 |
| *Pseudomonas aeruginosa* | 10 | 12 | - | - | 18 | - | 14 | - | 14 | 14 |
| *Vibrio cholerae* | 14 | 18 | 10 | 10 | 16 | 11 | 12 | 14 | 12 | 12 |
| *Vibrio parahaemolyticus* | 12 | 16 | 12 | - | 14 | 12 | 14 | 12 | 14 | 10 |
| *Shigella flexneri* | 12 | 20 | 10 | 12 | 12 | 14 | 14 | 12 | 14 | 13 |
| *Serratia marcescens* | 12 | 20 | 22 | - | 18 | 20 | 14 | 15 | 14 | 12 |
| *Salmonella typhi* | 10 | 14 | 10 | 14 | 19 | 10 | 14 | 12 | 14 | 15 |

Table 2.1: Optimization Studies (pH) for the selected Bacterial Extract No. 19

| S. No. | Conditions | Bacterial culture | Test Organisms | Zone of inhibition in mm |
|---|---|---|---|---|
| 1 | pH9 | 19 | *Staphylococcus aureus* | 15 |
| | | | *Staphylococcus epidermidis* | 16 |
| | | | *Micrococcus luteus* | 14 |
| | | | *Bacillus subtilis* | 12 |
| | | | *Streptococcus mutans* | 16 |
| | | | *Escherichia coli* | 15 |
| | | | *Salmonella typhi* | 14 |
| | | | *Proteus vulgaris* | 13 |
| | | | *Serratia marcescens* | 12 |
| | | | *Vibrio cholera* | 15 |
| | | | *Candida albicans* | 11 |

Table 2.2: Optimization Studies (NaCl Concentration) for the Selected Bacterial Extract No. 19

| S. No. | Optimization condition | Bacterial culture | Test Organisms | Zone of inhibition in mm |
|---|---|---|---|---|
| 1 | 5% NaCl | 19 | *Staphylococcus aureus* | 15 |
| | | | *Streptpcoccus mutans* | 14 |
| | | | *Micrococcus luteus* | 14 |
| | | | *Bacillus subtilis* | 12 |
| | | | *Staphylococcus epidermidis* | 13 |
| | | | *Escherichia coli* | 13 |
| | | | *Salmonella typhi* | 12 |
| | | | *Proteus vulgaris* | 10 |
| | | | *Serratia marcescens* | 10 |
| | | | *Vibrio parahaemoluticus* | 11 |
| | | | *Enterobacter aerogenes* | 12 |
| | | | *Candida albicans* | 11 |
| 2 | 7% Nacl | 19 | *Staphylococcus aureus* | 17 |
| | | | *Streptpcoccus mutans* | 13 |
| | | | *Micrococcus luteus* | 12 |
| | | | *Bacillus subtilis* | 15 |
| | | | *Staphylococcus epidermidis* | 13 |
| | | | *Escherichia coli* | 10 |
| | | | *Salmonella typhi* | 15 |
| | | | *Proteus vulgaris* | 11 |
| | | | *Serratia marcescens* | 16 |
| | | | *Vibrio parahaemoluticus* | 15 |
| | | | *Enterobacter aerogenes* | 11 |
| | | | *Aspergillus niger* | 13 |

Table 3: Standard Antibiotic Discs Assay against Test Organisms

| S.No. | Test organisms | Zone of inhibition in mm | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | A10 | Ak30 | Am10 | C30 | Ce30 | Cr30 | E15 | G10 | P10 | R5 | S10 | T10 |
| 1 | *Bacillus subtilis* | - | 18 | 16 | 30 | 26 | 26 | 20 | 18 | - | - | 10 | 26 |
| 2 | *Staphylococcus aureus* | 14 | 16 | 28 | 26 | 30 | 30 | 18 | 18 | 36 | 26 | 16 | 24 |
| 3 | *Staphylococcus epidermidis* | - | 22 | - | - | 16 | - | 10 | 22 | - | 10 | - | 15 |
| 4 | *Streptococcus mutans* | - | 28 | 28 | 28 | 30 | 30 | 28 | 26 | 32 | 20 | - | 28 |
| 5 | *Micrococcus luteus* | 10 | 22 | - | 32 | 26 | 18 | 16 | 18 | 16 | 16 | 14 | 24 |
| 6 | *Escherichia coli* | - | 20 | 12 | 20 | 18 | -+ | 16 | 19 | 14 | - | - | T |
| 7 | *Enterobacter aerogenes* | - | 22 | 12 | 27 | 18 | 14 | 10 | 18 | 8 | 10 | 14 | 20 |
| 8 | *Klebsiella pneumoniae* | - | 15 | 16 | 24 | 16 | T | 14 | 16 | 14 | - | 12 | 12 |
| 9 | *Proteus vulgaris* | 14 | 10 | 20 | 22 | 18 | 18 | 19 | 18 | 30 | 22 | 16 | 26 |
| 10 | *Pseudomonas aeruginosa* | - | 16 | - | 12 | 20 | - | T | 18 | 12 | - | - | - |
| 11 | *Salmonella typhi* | - | 14 | 18 | 14 | 30 | 14 | 10 | 14 | - | 12 | - | - |
| 12 | *Serratia marcescens* | - | 17 | - | 22 | 24 | - | - | 15 | - | - | - | - |
| 13 | *Shigella flexneri* | - | 18 | 12 | 22 | 16 | 14 | 13 | 17 | - | 14 | 14 | 18 |
| 14 | *Vibrio cholerae* | - | 12 | 12 | 14 | 14 | 14 | - | - | - | - | 12 | 14 |
| 15 | *Vibrio parahaemolyticus* | - | 22 | - | - | T | T | - | T | - | 12 | - | 18 |

Table 4: Spectral Data for Bacterial Extracts

| Samples | IR |
|---|---|
| *Marinonascens* sp. (MB3) | 3432.09 - Amides or Amines |
| | 2088.76 - Alkynes |
| | 1641.31 - Aliphatic amines (C=N) groups |
| | 520.74 - Alkyl halides (Bromo alkane or chloro alkane) |
| *Bacillus* sp.(MB19) | 3432.09 - Primary amines (Amides or Amines) |
| | 2324.06 and 2358.78 - Triple bonds N-H bond C=N |
| | 2087.8 - Alkynes |
| | 1640.35 - Aliphatic amines (C=N) |
| | Below 600 - alkyl halides |
| *Mesophilobacter* sp. (MB24) | 3432.09 - Primary amines (N=H) |
| | 2325.03 and 2359.74 - Triple bonds N-H bond C=N |
| | 2088.76 - Primary amines |
| | 1641.31 - Aliphatic amines |
| | Below 600 - alkyl halides (Bromo alkane or chloro alkane) |
| *Alteromonas* sp. (MB32) | 3432.09 - Primary amines (N=H) |
| | 2087.8 - Alkynes |
| | 1641.31 - Aliphatic amines |
| | Below 600 - Alkyl halides (Bromo alkane or chloro alkane) |
| *Marinococcus* sp.(MB43) | 3404.71 - Primary or Secondary amines (N-H stretch) |
| | 1620.88 - Primary amines |
| | 1117.55 - Aliphatic amines |
| | 797.42 - Alkyl halides |
| | 668.21 - Primary or Secondary amines |
| | Below 600 - Alkyl halides (Iodo alkane or Bromo alkane) |

The selected ten isolates were incubated in Nutrient broth for 30 days and the cell free extract was used for further analysis. Among the 10 selected isolates, 5 bacterial strains (MB3, MB19, MB24, MB32 and MB43) were selected randomly (Table 1) and identified as *Marinonascens* sp. (MB3), *Bacillus* sp. (MB19), *Mesophilobacter* sp. (MB24), *Alteromonas* sp. (MB32) and *Marinococcus* sp. (MB43). .

While screening, *Bacillus* sp. (MB19) showed maximum antagonistic activity up to 14 to 24 mm against all the test organisms when compared with other four selected bacterial strains. While optimizing, the selected isolate *Bacillus* sp. (MB19) showed antimicrobial activity for most of the target organisms at pH 9, 7% NaCl and 9% NaCl (Table 2.1 & 2.2)). The antibiotic susceptibility assay was carried out for all the test organism using standard antibiotic discs (Table 3).

FTIR spectrum for all the experimental samples exhibited absorption bands at 3432.09 $cm^{-1}$, 2088.75 $cm^{-1}$, 520.74 $cm^{-1}$ or below 600 $cm^{-1}$which indicates primary or secondary amides, hydroxyl groups and alkyl halides respectively. The spectrum at 1641.31$cm^{-1}$ showed the

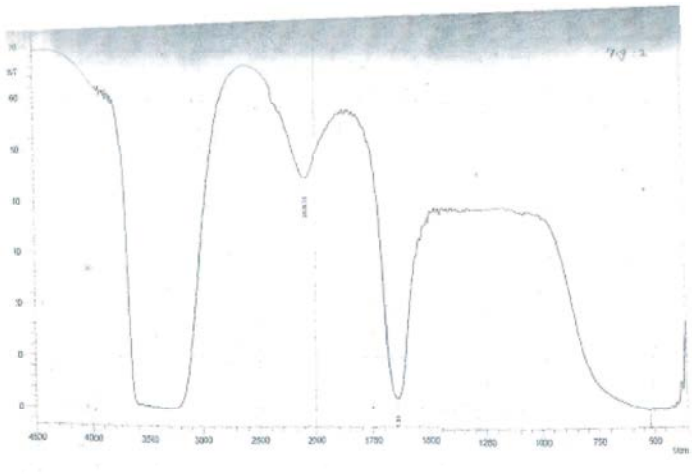

Fig. 1: IR spectra of Marine Bacterial metabolites MB 3-*Marinonascens sp.*

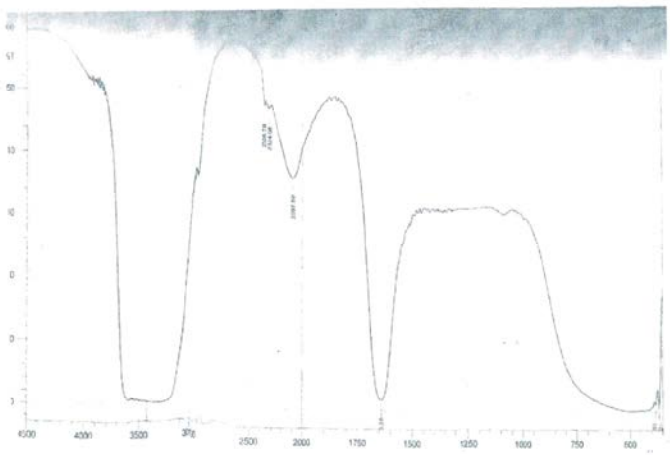

Fig. 2 : IR spectra of Marine Bacterial metabolites MB 19-*Bacillus sp*.

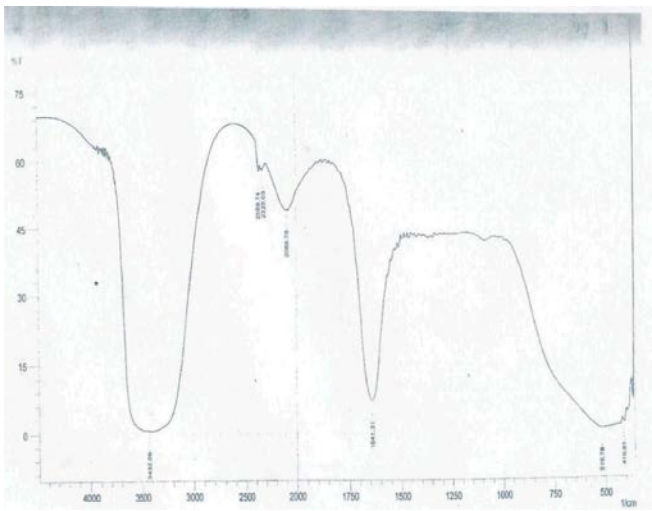

Fig. 3: IR Spectra of Marine Bacterial Metabolites MB 24-*Mesophilobacter sp*.

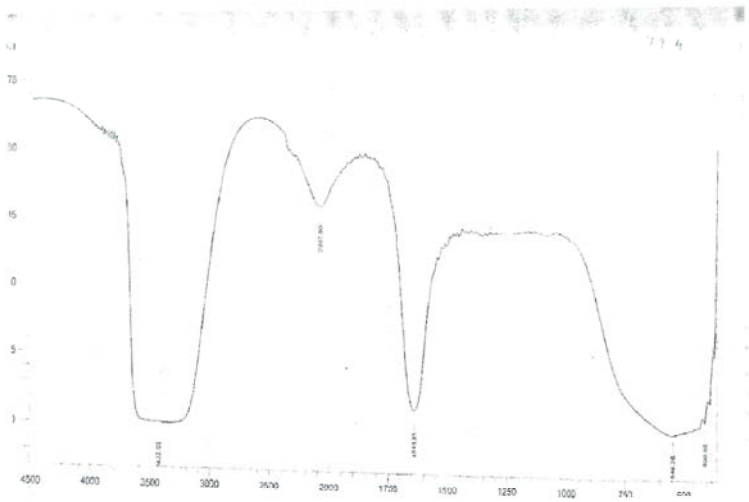

Fig. 4: IR Spectra of Marine Bacterial Metabolites MB 32-*Alteromonas sp*.

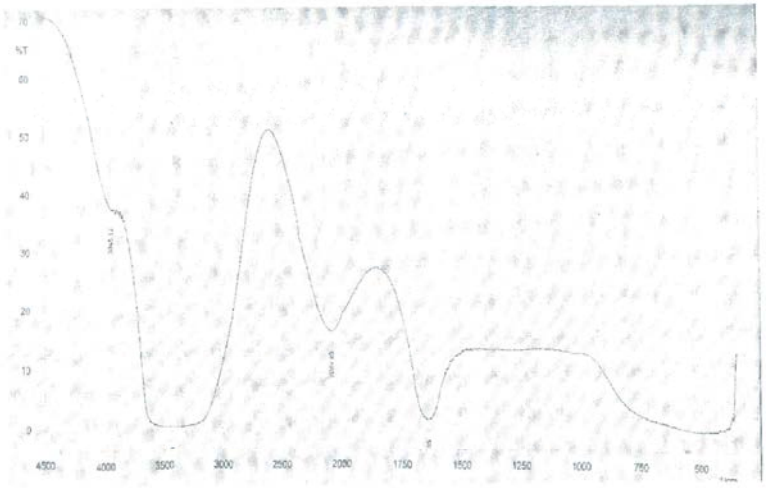

Fig. 5: IR Spectra of Marine Bacterial Metabolites MB 43-*Marinococcus sp*.

presence of alkenes in the cell free extract of *Bacillus* sp. MB19 (Table 4). The other peaks were indicated in Table 5 (Fig. 1-5).

## DISCUSSION

Emerging new infectious diseases and resistant pathogens for which no effective therapies are available represent a serious problem for the human life [12, 13]. In the recent years, marine microorganisms are able to produce molecules w ith novel structures and biological activities [14, 15]. Marine organisms as model systems offer the potential to understand and develop treatments for diseases based on the normal physiological role of their secondary metabolites [16, 17]. *Alteromonas rubra*, the organisms under investigation, is one such organism that potentially contains an antibiotic effective against resistant strains of bacteria.

Infectious diseases and drug resistance phenomena cause 17 million living beings affected every year [18]. Certain *Staphylococcus aureus* strains showed no response even against the aminoglycopeptide, vanocomycin. For this reason, there is a worldwide research to look for new medicine sources as well as continuing international focus exploring the new antibiotics sources and especially pharmaceutically active microbial products as well as synthetic approaches. But in the present study, the identified five bacterial strains such; *Marinonascens sp*. *Bacillus sp*. *Mesophilobacter sp*. *Alteromonas sp.* and *Marinococcus sp.* showed antimicrobial activity against *Staphylococcus aureus*.

Where the selected bacterial isolate *Bacillus sp.* (20 mm) showed potent antimicrobial activity against *Staphylococcus aureus*.

Out of five bacterial isolates, *Bacillus sp.* (MB19) showed the maximum zone of inhibition (18 mm) against *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* is an opportunistic pathogen that is important in the etiology of many infectious diseases of humans and the bacterium is commonly isolated from clinical specimens (wounds, burns and urinary tract infections) [19]. Normally, *Pseudomonas* is capable of growth in some antibiotics such as quaternary ammonium compounds. Their resistance to many antibiotics has also been a source of medical concern.

In the present case, it can be concluded from the present investigation the extracts obtained at 37°C, pH 7 and 3% NaCl concentration showed activity against all the test organisms. Exceptionally the bacterial strain *Bacillus sp.* showed equality of antimicrobial activity against all the target organisms at pH 9 and 5 & 7% NaCl while comparing with optimum conditions.

The IR absorptions could be seen as several distinct peaks like 3400 $cm^{-1}$ that might contain hydrogen bonded O-H stretch. The antibacterial compounds produced by these strains probably possessed non polar structure and consisted of several active components. Based on the results obtained for FTIR and NMR spectrum analysis, the compound probably having one hydroxyl methyl group and the rest being - CH-OH (hydroxyl methylenes) linked to each other by ether linkages.

Thus the antimicrobial compound probably is a straight chain polyhydroxy poly ether compound with a single double bond. In addition to that, the spectrum of MB19 possessed peaks for complex ring structure which was linked to other substituted groups like alcohols, amides and amines. This complex structure of MB19 might help them to show increased antagonistic activity when compared to other samples.

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