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ORIGINAL ARTICLE

Isolation and Screening of Marine Bacterial Metabolites against Beta-Lactamase-Producing Bacteria

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ABSTRACT

Background: The emergence of antibiotic resistance underscores the urgent need for new therapeutic sources.

Objective: In this study, seven marine bacterial isolates from seawater were screened for bioactive metabolites active against β-lactamase–producing pathogens.

Methodology: Each isolate was tested against four clinical strains—*Escherichia coli*, *Proteus*, *Klebsiella*, and *Bacillus*—using cross-streak and agar well diffusion assays.

Result: In the cross-streak assay, inhibition was strongest against *Klebsiella* and weakest against *Bacillus*. In the agar well diffusion assay, isolates 3 and 5 produced clear inhibition zones against *E. coli* and *Proteus*, whereas the remaining isolates showed no activity.

Conclusion: These results demonstrate that certain marine bacteria can generate metabolites effective against β-lactamase producers.

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INTRODUCTION

Marine bacteria inhabit diverse habitats, including sediments, open oceans, and surfaces of marine organisms. They represent a largely untapped reservoir of bioactive compounds[1-3]. In competitive ecological niches, bacteria synthesize secondary metabolites (e.g., toxins, inhibitory enzymes, and antimicrobial agents) to suppress rival species[4-7]. A global rise of antibiotic-resistant pathogens, especially β-lactamase–producing strains, has been reported. Therefore, there is a strong need to isolate the marine-derived bacteria and use their metabolites as novel antimicrobials.

Historically, antibiotics microbial secondary metabolites have revolutionized infection control. Penicillin (from *Penicillium*), streptomycin (active against *Mycobacterium tuberculosis*), and newer β-lactams (e.g., cephalosporins) provided broad-spectrum coverage, but resistance rapidly emerged[8-12]. Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA) now undermine β-lactam and glycopeptide efficacy[13-15]. Similarly, *Klebsiella pneumoniae* isolates frequently produce extended-spectrum β-lactamases (ESBLs), rendering third-generation cephalosporins ineffective[16,17].

Marine microorganisms also yield antitumor agents such as didemnin (from a *Caribbean tunicate*) and metabolites active against melanoma and leukemia[18]. Moreover, certain marine bacteria produce L-asparaginase, an enzyme with anticancer properties[19]. Thus, bioprospecting marine bacteria could address both antibiotic resistance and cancer-therapy gaps. This study explores marine bacterial isolates for secondary metabolites with antimicrobial and anticancer potential—seeking new therapeutics to combat resistant infections and malignancies.

MATERIALS AND METHODS

Bacterial Strains

Escherichia coli, *Proteus, Klebsiella*, and *Bacillus subtilis*, were used in this study.

Sample Collection

This study was performed on various isolated samples of sea-living bacteria; samples of water were stored at 3-5°C. These samples were collected from four different places in the Arabian Sea (Karachi Sea). Different marine samples, such as sponges' biofilms and sediments, were collected from different sampling points of ocean water reservoirs. Then transferred to the laboratory aseptically for the isolation of bacteria.

Isolation of Marine Bacteria

One mL seawater sample and 9 mL distilled water samples were mixed for serial dilution. After this, 0.5 mL of the liquid sample was poured on the petri plate containing nutrient agar. The inoculated plates were incubated at 37°C for 4 days. After incubation, visible colonies of growing bacteria were observed. Streaking was performed to obtain a pure sample. Four isolates were identified based on their color, size, growth pattern, and growth margins. After getting the successful growth of the colonies on agar plates, the seawater agar plates were used for re-streaking.

Screening for Antimicrobial Activity

Cross-Streak Assay

Marine bacterial isolates were evaluated for antagonism against four pathogenic test strains. Each marine isolate was streaked vertically on the surface of tryptic soy agar (TSA) plates and incubated at 30 °C for 24 h to establish growth. The pathogenic strains were then streaked horizontally, perpendicular to the marine isolates, stopping 2 mm short of the marine streak. Plates were incubated at 37 °C for 24 h. Inhibition was recorded when pathogen growth did not cross the intersection zone.

Agar Well Diffusion

Marine isolates were cultured in 5 mL Zobell marine broth at 30 °C, 180 rpm, for 72 h. Cultures were centrifuged at 5,000 × g for 10 min, and supernatants were filtered (0.22 µm). Pathogens were grown overnight in nutrient broth at 37 °C, 180 rpm, then adjusted to 0.5 McFarland. Mueller–Hinton agar plates were lawn-inoculated with each pathogen. Wells (6 mm diameter) were cut, and 100 µL of each marine supernatant was dispensed into separate wells. Plates were incubated at 37 °C for 24 h. Zones of inhibition were measured in millimeters.

Disc Diffusion on Crude Extracts

Twenty milliliters of marine broth culture (30 °C, 180 rpm, 72 h) were centrifuged at 6,000 × g for 15 min at 4 °C. The supernatant (10 mL) was mixed with an equal volume of ethyl acetate, vigorously shaken for 5 min, and allowed to separate in a separatory funnel. The organic (ethyl acetate) phase was collected and evaporated under reduced pressure to yield crude extract. Dried extract was dissolved in 3 mL dimethyl sulfoxide (DMSO). Sterile 6 mm paper discs were each loaded with 20 µL of extract solution and air-dried. Pathogen lawns were prepared as above, discs placed on agar, and plates incubated at 37 °C for 24 h. Inhibition zones were recorded.

Sequential Screening for Antibiotic Production

Marine isolates were spot inoculated in TSA supplemented with 1% NaCl and incubated at room temperature for 5 days. Pathogenic test strains were then streaked perpendicular to each marine colony and incubated for an additional 24 h at 37 °C. Inhibition of pathogen growth indicated antibiotic production.

Each marine isolate was inoculated into 5 mL Zobell marine broth and incubated at 25 °C for 6 days. Cultures were centrifuged at 4,800 × g for 10 min to pellet cells; the supernatant was retained. Cell pellets were resuspended in 200 µL sterile seawater and incubated 24 h at 25 °C, then centrifuged at 4,800 × g for 5 min. Supernatants were tested by disc diffusion: 100 µL was spotted onto Whatman No. 1 discs (5 mm). Discs were placed on pathogen-inoculated agar and incubated 24 h at 37 °C.

Marine isolates were each grown in 100 mL Zobell marine broth (25 °C) for 5–7 days. Cultures were extracted with an equal volume of ethyl acetate, shaken for 30 min, and allowed to separate. The organic phase was collected and evaporated to dryness. The crude extract (dissolved in DMSO) was applied to 6 mm discs (20 µL/disc) and assayed against pathogens by disc diffusion as described above. Marine strains exhibiting the largest inhibition zones were identified to genus level using Bergey's Manual (biochemical tests).

Cross-Cell Induction to Enhance Antimicrobial Production

Each marine isolate was cultured in four 20 mL Zobell marine broth sets. The control was marine isolate only. Heat-killed induction includes marine isolate plus heat-inactivated pathogen (autoclaved, 121 °C, 15 min). Live-pathogen induction represents the marine isolate plus live pathogen (1:1 v/v, ~10^6 CFU/mL). Marine broth only (no inoculum) was taken as a blank. All tubes were incubated at 25 °C, 290 rpm, for 72 h. Supernatants were harvested by centrifugation (4,800 × g, 10 min), and crude extracts were prepared via ethyl acetate as in section 2.4.4. Extracts were tested by disc diffusion against the corresponding pathogen. Enhanced inhibition zones indicated induced antibiotic production.

Identification of Bacterial Strains

Gram Staining

A loopful of each isolate was heat-fixed on a glass slide, stained with crystal violet (60 s), Gram's iodine (60 s), decolorized with 95% ethanol (5 s), and counterstained with safranin (30 s). Slides were rinsed, air-dried, and examined by light microscopy (1000× oil immersion).

Biochemical Profiling

Marine isolates (overnight, 35–37 °C, marine broth) were standardized to ~10^6 CFU/mL. Fifty microliters of each suspension were inoculated into wells of a rapid biochemical identification kit (*Bacillus* panel). Plates were incubated at 35–37 °C for 18–24 h. The manufacturer's reagents were added according to instructions to reveal positive/negative reactions. Results were interpreted following the kit's guidelines for genus-level identification.

RESULT

Cross Streak Method

The cross-streak technique was used against four pathogenic strains of marine bacteria, namely *E. coli, Proteus, Klebsiella*, and *Bacillus*. *Klebsiella* indicated good inhibition. The least inhibition was shown by *Bacillus* (Figure **1** & Table **1**).

Agar Well Diffusion Method

E. coli indicated significant inhibition, followed by *Proteus*. The *Bacillus* and *Klebsiella* indicated the least activity (Figure **2** & Table **2**).

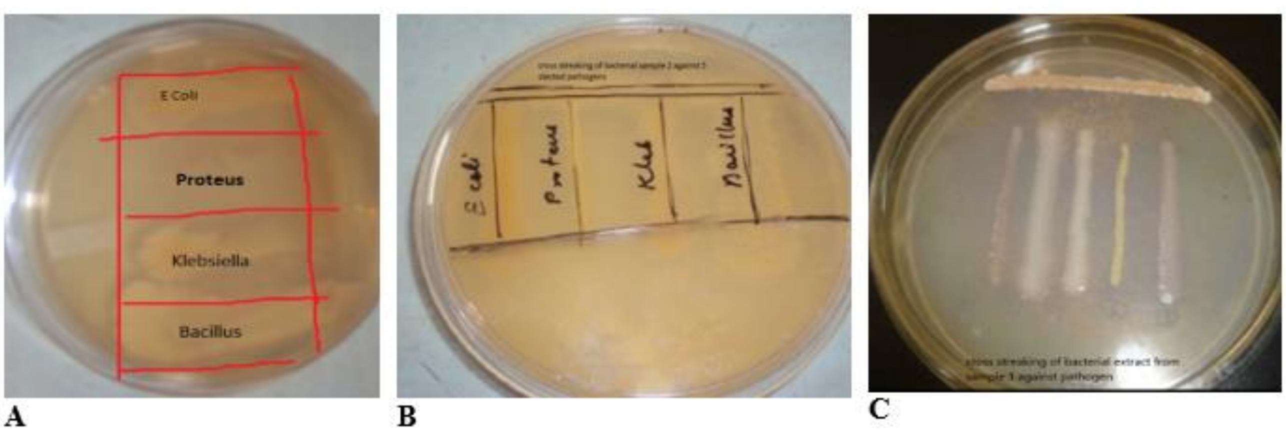

Figure 1. Cross streaking of bacterial strain against four different pathogens (**A**). Cross streaking of bacterial strain against pathogens (**B**). Cross streaking of bacterial extract from sample 1 against pathogen (**C**).

Table 1. Inhibition Shown by Four Marine Bacteria Against Other Testing Strains.

Strains	*E. coli*	*Proteus*	*Bacillus*	*Klebsiella*
Bacterial strain 1	+	-		+
Bacterial strain 2	-	-	-	+
Bacterial strain 3	-	-	-	-
Bacterial strain 4	+	-	-	+
Bacterial strain 5	-	-	-	-
Bacterial strain 6	-	-	-	+
Bacterial strain 7	-	-	-	+

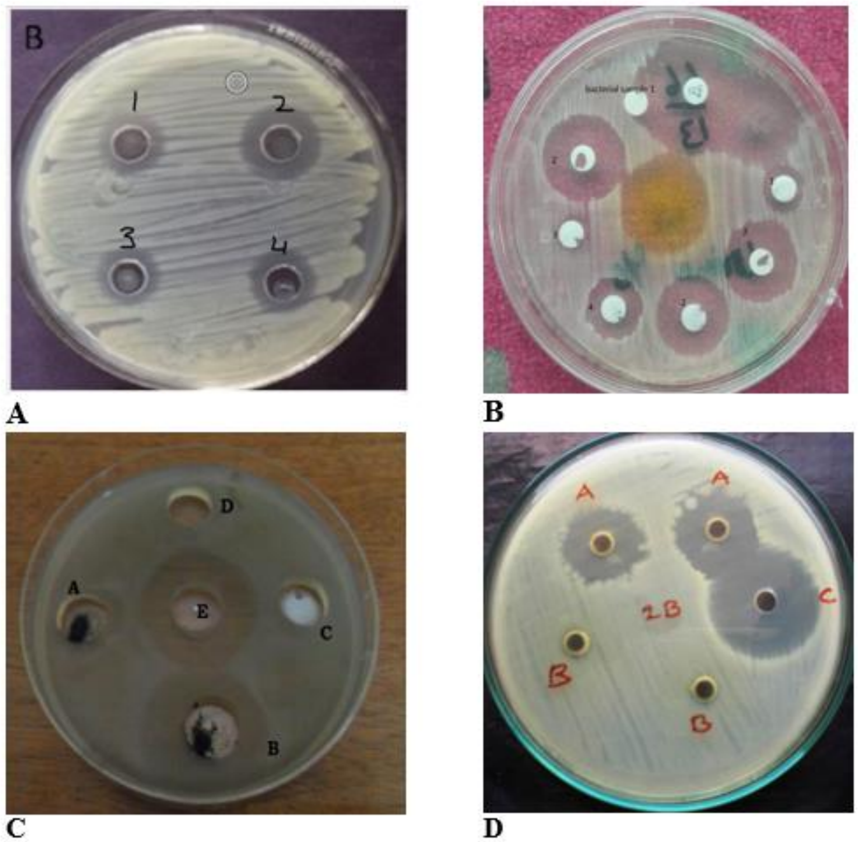

Figure 2. Screening and testing of antibacterial activity by the agar well method (**A**). Testing of extract obtained from different samples against pathogenic bacteria (**B**). Agar well diffusion method of testing antibiotics against different bacterial pathogens (**C, D**).

Table 2. The Antimicrobial Activity Demonstrated by Four Marine Bacterial Strains.

Strains	*E. coli*	*Proteus*	*Bacillus*	*Klebsiella*
Bacterial strain 1	-	-		-
Bacterial strain 2	-	-	-	-
Bacterial strain 3	+	-	-	-
Bacterial strain 4	-	-	-	-
Bacterial strain 5	+	+	-	-
Bacterial strain 6	-	-	-	-
Bacterial strain 7	-	-	-	-

DISCUSSION

Competition among bacteria often triggers or enhances antibiotic production, as limited nutrients and space drive microbes to secrete secondary metabolites that inhibit rivals[20,21]. In this study, isolates 1 and 2 produced greater antimicrobial activity when co-cultured with *Pseudomonas sp.* and *Bacillus sp.,* mirroring findings by Pepper *et al.* (2011). Pepper *et al.* (2011) reported that antibiotic synthesis by marine strains occurred only in the presence of methicillin-resistant *Staphylococcus aureus*[22].

*Pseudomonas s*pecies are well known for solubilizing phosphate and producing targeted antibiotics against organisms such as *Bacillus subtilis* and *Proteus vulgaris*[23]. However, standard antimicrobial assays may miss inducible producers, since some bacteria require interspecies signals to activate biosynthetic pathways. For instance, co-culture has enabled the discovery of pyrrole antibiotics from marine bacteria that were otherwise undetectable in monocultures[24].

Quorum sensing and interspecies interactions play a crucial role: in one soil-derived survey, 34 of 54 isolates (including *Bacillus, Micrococcus, Stenotrophomonas, a*nd *Lysobacter*) increased antibiotic production when exposed to *Streptomyces* coelicolor signals[25]. Similarly, Slattery *et al.* (2001) found that 11 out of 54 marine co-culture partners induced istamycin production by *S. tenjimariensis*[26].

Here, live or heat-killed *Pseudomonas* and *Bacillus cells* were introduced to marine isolates following Harvey *et al.* (2008) and Després *et al.* (2012) protocols[27,28]. As McCully *et al.* (2023) showed for *B. licheniformis*, antimicrobial compounds often appear only under competitive conditions, our results confirm that most marine isolates produce minimal activity in isolation but upregulate antibiotic synthesis when facing bacterial competitors[29]. These findings underscore the importance of co-culture–based screening to reveal inducible antimicrobial metabolites from marine bacteria.

CONCLUSION

Competition between bacterial strains induces antibiotic production. In co-cultures with *Bacillus sp.* and *Pseudomonas sp.,* certain marine isolates synthesized antimicrobial compounds more efficiently, effectively inhibiting those competitors. Both live and heat-killed *Bacillus* and *Pseudomonas* cells provided induction signals. Notably, these marine bacteria produced antibiotics to kill β-lactamase–producing pathogens.

CONFLICT OF INTEREST

None.

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