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Anti-quorum Sensing Activity and Bioactive Components of Marine-derived Bacteria

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Abstract

Understanding and harnessing quorum sensing activity and identifying bioactive substances produced by marine-derived bacteria are essential for exploring their potential applications in various fields, including biotechnology, pharmaceuticals, and environmental management. This research aims to investigate the quorum-sensing activities employed by these bacteria and to characterize the bioactive compounds they produce, to unlock their therapeutic, industrial, and ecological potentials. This study focuses on screening, isolation and characterization of marine bacteria from Thoothukudi Harbour Beach, India, and potential antibacterial and anti-quorum sensing activities of their respective spent media against biofilm forming pathogens. Three soil samples were collected and processed for bacterial isolation. Seventeen different bacterial isolates were obtained and identified after prior culture. Antibacterial activity was evaluated against four pathogenic bacteria, with some isolates demonstrating significant inhibition. Additionally, biofilm inhibition assays were conducted, revealing the ability of certain isolates to inhibit the formation of biofilms. The secondary metabolites present in the ethyl acetate fraction of I.B 6 isolate exhibiting relatively high antibacterial and antibiofilm properties were identified by GC-MS. Anti-quorum sensing activity was also investigated using swarming assay and the MIC was determined accordingly for the ethyl acetate fraction. Hence, these marine bacteria hold for producing bioactive compounds with potential pharmaceutical and industrial applications. Finally, the positive organism is subjected to 16S rRNA sequencing for identification and was found to be *Bacillus thuringiensis*.

Keywords: Marine-derived Bacteria, Anti-quorum Sensing, Secondary Metabolites, Biofilm and Antibacterial Activity

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INTRODUCTION

The fastest-growing problem of antibiotic resistance in modern bacteria and the increasing number of resistant bacterial species are the biggest threats to public health.[1] Quorum sensing (QS), which regulates a number of physiological functions within the bacteria as well as their interactions with one another, is primarily responsible for bacterial drug resistance. Bacterial biofilms that are resistant to external stimuli and safeguard the colonized bacterial cells are eventually formed with the assistance of the QS mechanism.[2] The quorum sensing system, which is regulated by the production and recognition of several small signaling molecules produced mostly by gram-positive and gram-negative bacteria, is responsible for controlling the expression of many genes independently of bacterial cell density.[3] Thus, bioactive substances produced from secondary metabolites and other quorum sensing inhibitors (QSIs) may serve some beneficial purposes by potentially targeting and inhibiting the action of transcription factors to regulate expression and stop the formation of biofilms.[4] It is a novel approach to treat bacterial infections with QSIs since these drugs can interfere with QS's normal activity, preventing pathogens from also producing virulence factors. Furthermore, QSIs minimize antimicrobial resistance since they do not directly target the crucial pathogen's growth mechanism and do not place the same lethal selection pressure on the bacterium as conventional antibiotics do. Furthermore, quorum sensing inhibitors can be useful research tools in determining the various roles that bacterial quorum sensing plays within the bacterial population. Consequently, efforts to develop quorum-sensing inhibitors that are isolated from natural sources such phytochemicals and marine compounds have increased.[5] In order to prevent microbial pathogenicity, a great deal of research has focused on inhibiting QS or biofilm growth. The idea is neither bacteriostatic nor bactericidal because it neither stops the growth of the bacterium nor kills it. Antibiotics alone are not always effective in treating bacterial infections that form biofilms because these illnesses are naturally resistant to many different kinds of drugs. The global cycle of carbon and nutrients, feedback mechanisms, and the movement of organic matter to the bottom all depend on marine microbes. In this regard, it can be hypothesized that the metabolic regulation of marine organisms can produce a characteristic mix of secondary metabolites having various biochemical activities *in vitro*.[6] Thus, we have isolated some of the marine-derived bacteria and extracted their metabolites to assess their anti-quorum sensing activity through antibacterial and biofilm inhibition assays in this study.

MATERIALS AND METHODS

Collection of samples

Selected marine soil samples were taken from the beach at Thoothukudi Harbor, which is situated on the Bay of Bengal at latitude 8.7429°

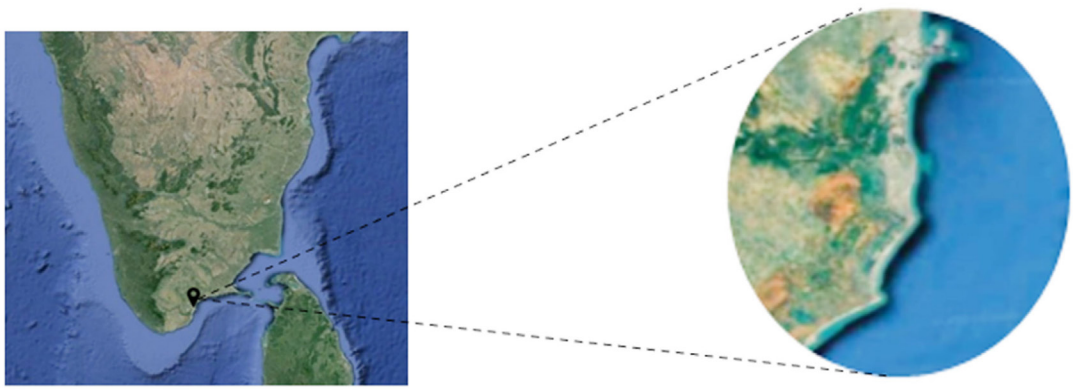

Figure 1. Sample collection and location (source: http://earth3dmap.com)

N and longitude 78.1675° E (Figure 1). Following their extraction, the samples were stored in three different sterile containers and were taken from 10 to 15 cm below the surface. Before being used again, samples were stored at 4°C.

Bacterial isolation and preservation

To facilitate spore formation and lower the bacterial count, the samples were pre-treated for 15 minutes at 50°C in a water bath. To generate a homogeneous suspension, the 1 gram pre-treated samples were combined with 10 mL of autoclaved sterile saline solution (0.9% NaCl) one at a time. This mixture was then serially diluted to produce 10^{-3} and 10^{-4} dilutions. After shaking, 100 µl of each diluted solution was applied using an L-rod to the Zobell Marine agar medium, and it was then incubated for two to three days at 30°C. Single colonies of 17 distinct isolates (I.B. 1 to I.B. 17) were isolated and placed on recently made Zobell Marine agar plates following incubation.

Biofilm formation test tube assay

The microbial cell community coming together to form a biofilm. Six harmful bacterial strains (*Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa, Streptococcus pyogenes,* and *Proteus mirabilis*) were resurrected overnight and put into individual tubes holding 5 mL of sterile nutrient broth. The tubes were then placed in a shaking incubator and incubated at 37°C and 125 rpm. After being incubated for 72 hours, the cultures from each tube were discarded, and the tubes were dried for 10 minutes at 37°C. Finally, by adding 5 mL of 0.1% crystal violet solution, the optical densities at 570 nm was determined using a spectrophotometer. To estimate the antibacterial activity, only the bacterial species that showed considerable biofilm formation out of the six inoculated were considered.

Antibacterial activities of crude spent media against biofilm forming bacteria

Using the Agar-well diffusion method, the antibacterial properties of the crude spend medium containing metabolites were assessed against *Enterococcus faecalis, Pseudomonas aeruginosa, Streptococcus pyogenes,* and *Proteus mirabilis,* which are harmful bacteria that form biofilms. Using sterile cotton swabs, these bacterial cultures were plated on distinct nutrient agar plates. The wells were then bored and filled with individual crude spend medium from 17 distinct bacteria that were isolated from coastal soil. Using a ruler, the zone of inhibition (ZI) was measured in millimeters following a 24 hour incubation period at 30°C.

Strain identification

Totally 17 strains were analyzed for antibacterial and antibiofilm activities, among which I.B 6 strain has the maximum antibacterial activity against the biofilm forming pathogens. I.B 6 isolate was selected for strain identification by 16S rRNA sequencing. The selected bacterial isolate was cultured in Zobell marine medium and the genomic DNA was extracted using the SK20-UNIQ-10 DNA extraction kit following the manufacturer's protocol. The universal primer pairs, 27F (AGAGTTTGATCATGGCTCA) as forward and 1492R (TACGGTTACCTTGTTAGCACTT) as reverse primers were used. The thermal cycling conditions were set as follows: 5 minutes at 94°C for initial denaturation; 31 cycles of 30 seconds at 95°C, 1 minute at 54°C, 2 minutes at 72°C, and a final extension of 5 minutes at 72°C. The PCR amplicon of about 1500 bp were resolved by electrophoresis in 1% (w/v) agarose gel to validate the result. Single-pass Sanger sequencing method was adopted for identifying the original sequence of the I.B 6 bacterial isolate. The final 16S rRNA gene sequence produced in this investigation was aligned with NCBI sequences using BLAST for strain identification. Finally, the neighbor-joining method was employed to construct a phylogenetic tree with the help of MEGA6 software.

Secondary metabolites extraction

A little quantity of culture is added to nutrient broth and allowed to incubate for three to five days. Following the incubation, a 1:1 ratio of ethyl acetate and methanol is added to the production media as solvents. To enable the produced compounds to dissolve into the solvent, the incubation medium and solvent are

maintained in a shaker. The synthesis of secondary metabolites is indicated by a change in the color of the medium.

Separation of secondary metabolites

The metabolites present in the crude spent medium of I.B 6 bacterial isolate was used for secondary metabolite characterization. The spend medium was dissolved in ethyl acetate solvent, shaken well, and allowed to settle for a few minutes. Two separate layers were formed within the separating funnel from which only the ethyl acetate fraction was collected for investigation.

Characterization of secondary metabolites

Using a SHIMADZU GCMS QP 2010 PLUS instrument, the ethyl acetate fraction comprising the dissolved metabolites of the marine-derived bacterial strain I.B 6 was examined. The analytes were separated using a Rxi-1ms non-polar column. The temperatures of the injection and the column were adjusted to 250°C and 70°C, respectively. The column flow was 1 mL/min and the overall flow was 50 mL/min with a linear velocity of approximately 36.7 cm/sec.

Anti-quorum sensing assay

The ethyl acetate fraction was subjected to a biofilm inhibition assay (also known as a swarming assay) utilizing a microtiter plate against four pathogens that generate biofilms. In a 96-well microtiter plate, 190 µL of Zobell marine bacteria was applied to each well for this purpose. Each well received 10 µL of the four bacterial cultures that were used for inoculation. The ethyl acetate fraction was then added to the corresponding wells at five progressively escalating

Table 1. List of metabolites identified by GC-MS

Peak	Retention time (min)	Area (%)	Compound
1	3.042	9.68	Ethyl acetate
2	3.150	4.86	Butanoic acid, 2-methyl-
3	5.314	0.80	Decane
4	8.374	1.38	3-Tridecene, (Z)-
5	11.250	1.01	1-Dodecene
6	11.394	0.70	Hexadecane
7	12.705	2.11	Decane, 3,7-dimethyl-
8	13.892	1.63	Hexadecane
9	15.238	1.67	Octadecane
10	15.623	6.30	Octadecanoic acid
11	15.700	1.00	Hexadecane
12	16.123	2.37	Nonadecane
13	16.254	4.28	Isopropyl myristate
14	17.497	1.65	Hexadecane
15	17.665	2.87	n-Hexadecanoic acid
16	18.136	1.10	Nonadecane
17	19.534	0.84	Octacosane
18	19.978	0.93	Nonadecane
19	20.840	0.74	Eicosane, 7-hexyl-
20	21.671	1.68	Tetracosane
21	22.469	1.99	Tetracosane
22	22.580	2.01	Bis (2-ethylhexyl) phthalate
23	23.244	3.60	Hexatriacontane
24	24.081	4.67	Tetracosane
25	34.347	17.23	1,3-Benzenedicarboxylic acid, Bis (2-ethylhexyl) ester
26	25.041	5.48	Heptacosane
27	25.192	1.26	Supraene
28	26.161	6.38	Hexatriacontane
29	27.501	5.00	Tetracosane
30	29.117	4.76	Heptacosane

Figure 2. Streaking of different bacterial strains isolated from marine soil sample

concentrations (20-100 µL), and sterile distilled water was used to raise the total volume to 400 µL.

After 24 hours of incubation at 37°C, the biofilm biomass was measured on the plate using an ELISA reader using 0.1% crystal violet solution, as previously mentioned. Based on the acquired data, the Minimum Inhibitory Concentration (MIC) of the antibiofilm activity of the I.B. 6 isolate spend medium ethyl acetate extract was determined.

RESULTS AND DISCUSSION

Bacterial isolates

A total of 17 different bacterial isolates were obtained from marine soil samples collected

Table 2. Inhibition zone of antibacterial activity of marine bacteria (+++) high inhibition zone, (++) medium inhibition zone, (+) less inhibition zone and (-) no inhibition zone

Bacterial strain	Zone of inhibition					
	Gram-positive bacteria			Gram-negative bacteria		
	Staphylococcus aureus	*Streptococcus pyogenes*	*Enterococcus faecalis*	*Proteus mirabilis*	*Pseudomonas aeruginosa*	*Escherichia coli*
I.B 1	++	-	-	-	-	+
I.B 2	-	+	-	-	++	-
I.B 3	-	-	+	++	-	-
I.B 4	-	-	-	-	-	-
I.B 5	+	-	-	-	-	-
I.B 6	-	+++	+++	++	+++	++
I.B 7	-	-	-	-	-	-
I.B 8	++	-	+	-	-	-
I.B 9	-	-	-	++	+	-
I.B 10	-	++	-	-	-	-
I.B 11	-	-	-	-	-	+
I.B 12	-	-	-	++	-	+
I.B 13	-	++	++	-	-	++
I.B 14	++	-	-	-	-	+
I.B 15	-	-	-	-	++	-
I.B 16	-	+++	++	+	-	+++
I.B 17	-	-	-	-	++	-

(Abbreviation I.B – Isolated Bacteria)

Table 3. Crystal violet test tube assay for biofilm formation

Bacteria strain	Variant hours	Colony colour	Inference
Streptococcus pyogenes	Control	Dark Blue	Heavy Biofilm Formation
	72 hrs	Dull Blue	Biofilm Formation
Enterococcus faecalis	Control	Dark Blue	Heavy Biofilm Formation
	72 hrs	Dull Blue	Biofilm Formation
Escherichia coli	Control	Dark Blue	Absence of Biofilm Formation
	72 hrs	Blue	Absence of Biofilm Formation
Staphylococcus aureus	Control	Dark Blue	Absence of Biofilm Formation
	72 hrs	Blue	Absence of Biofilm Formation
Pseudomonas aeruginosa	Control	Dark Blue	Heavy Biofilm Formation
	72 hrs	Dull Blue	Biofilm Formation
Proteus mirabilis	Control	Dark Blue	Heavy Biofilm Formation
	72 hrs	Dull Blue	Biofilm Formation

Table 4. The result of antibacterial activity against biofilm-forming bacteria and inhibition zone in mm

Bacterial strain	Inhibition zone in mm			
	Gram-positive bacteria		Gram-negative bacteria	
	Streptococcus pyogenes	*Enterococcus faecalis*	*Pseudomonas aeruginosa*	*Proteus mirabilis*
Control	25	25	23	23
I.B 1	-	-	-	-
I.B 2	10	-	15	-
I.B 3	-	10	-	15
I.B 4	-	-	-	-
I.B 5	-	-	-	-
I.B 6	25	20	20	15
I.B 7	-	-	-	-
I.B 8	-	10	-	-
I.B 9	-	-	10	13
I.B 10	12	-	-	-
I.B 11	-	-	-	-
I.B 12	-	-	-	18
I.B 13	14	18	-	-
I.B 14	-	-	-	-
I.B 15	-	-	14	-
I.B 16	21	15	-	10
I.B 17	-	-	13	-

at different locations in Thoothukudi, Tamil Nadu, India. Marine bacteria are being recognized as a possible source of novel chemicals, including enzymes, antibiotics, and other secondary metabolites with significant industrial value.[7] The Zobell marine medium was found to be more suitable for culturing marine-derived bacteria from which single colony forming units were easily obtained as it serves as a selective medium for marine microorganisms (Figure 2).

Characterization of secondary metabolites

Every biological molecule found in the GC-MS result (Figure 3) is non-polar in nature, with 30 peaks we totally obtained in this analysis. Table 1 contains tabulated data on peak, retention time, and corresponding bioactive compounds. The antibacterial properties of natural extracts can be attributed mostly to alkanes, including hexadecane, nonadecane, octacosane, tetracosane, eicosane, and octadecane. Similar antibacterial action to our work has been validated through experimentation with the fatty acids octadecanoic acid and n-hexadecanoic acid.[8] It was reported that 1,3-benzenedicarboxylic acid, Bis (2-ethylhexyl) ester present about 73% in the ethyl acetate extract of *Streptomyces rochei* spent medium was able to selectively inhibit the growth of *P. aeruginosa* and *S. aureus*.[9]

Biofilm forming pathogenic bacteria

As shown in Figure 4, biofilm quantification was carried out using a test tube technique. Using the test tube method, both strains generated significantly more biofilm after 72 hours (Table 2). Only four bacteria—*Streptococcus pyogenes, Enterococcus faecalis, Pseudomonas aeruginosa,* and *Proteus mira*bilis—exhibited biofilm formation along the tube walls out of the six studied (Table 3). It was not observable for the other two bacteria*, Escherichia coli* and *Staphylococcus aureus,* to build biofilms inside the tubes. It can be inferred that both these bacteria are poor in biofilm formation and quorum sensing mechanisms as they require controlled microenvironment.[10,11] All of the above bacteria are the causative agents

which cause skin and urinary tract infections in humans that can form strong biofilms *both in vitro* and *in vivo* making them resistant to external factors.[12-14]

Antibacterial activity of crude spent media against biofilm forming bacteria

A translucent zone that developed around the wells in nutrient agar plates demonstrated the effectiveness of the spend medium obtained from all marine bacteria against pathogenic bacteria that form biofilms. The findings concerning the antibacterial properties of marine bacteria against pathogenic bacteria that form biofilms are shown in Table 4. This allowed the I.B. 6 isolate to demonstrate antagonistic activity against each of the four tested biofilm-forming bacteria. Against each of the four bacterial pathogens, isolates included I.B. 1, I.B. 4, I.B. 5, I.B. 7, I.B. 11, and I.B. 14 did not exhibit any antibacterial activity. Likewise, just one bacterial species was inhibited by I.B. 8, I.B. 10, I.B. 12, and I.B. 17. Comparably, bacteria that have been isolated from marine sponges have been subjected to an evaluation of their antibacterial properties against *E. Coli, P. aeruginosa, S. aureus, S. pyogenes,* and *S. typhimurium* using their spend medium media.[15] Among the bacteria isolated from marine sources, the genus *'Bacillus'* has high antimicrobial activity of others.[16]

Identification of strain I.B 6

It has been established that the strain belonged to the genus *Bacillus* and was most

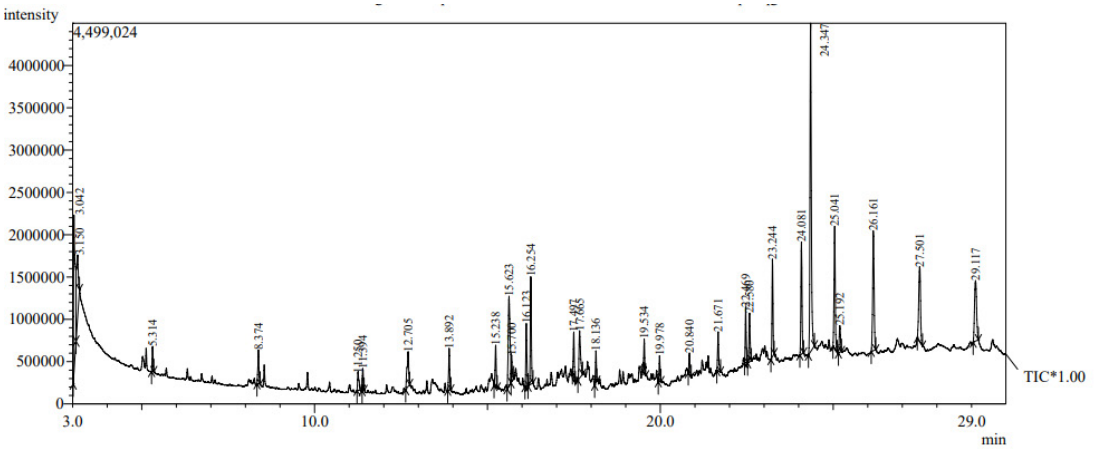

Figure 3. GC chromatogram of ethyl acetate extract of *Bacillus thurinegiensis*

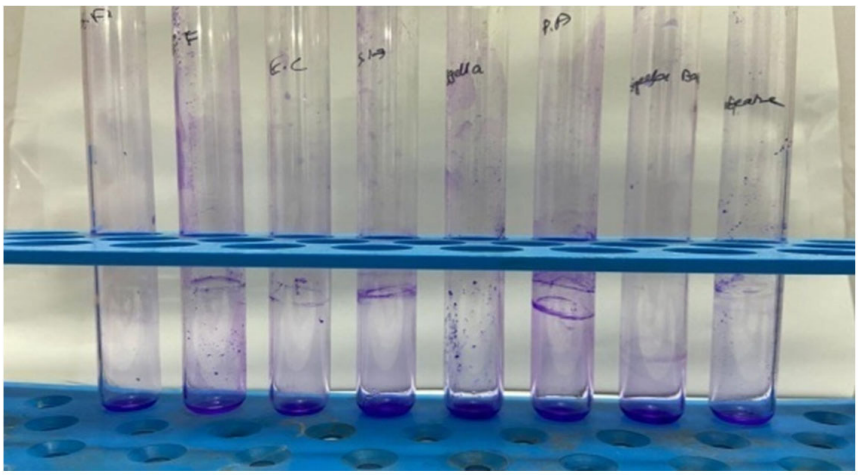

Figure 4. Test tube assays for biofilm-forming different pathogenic selected microbes

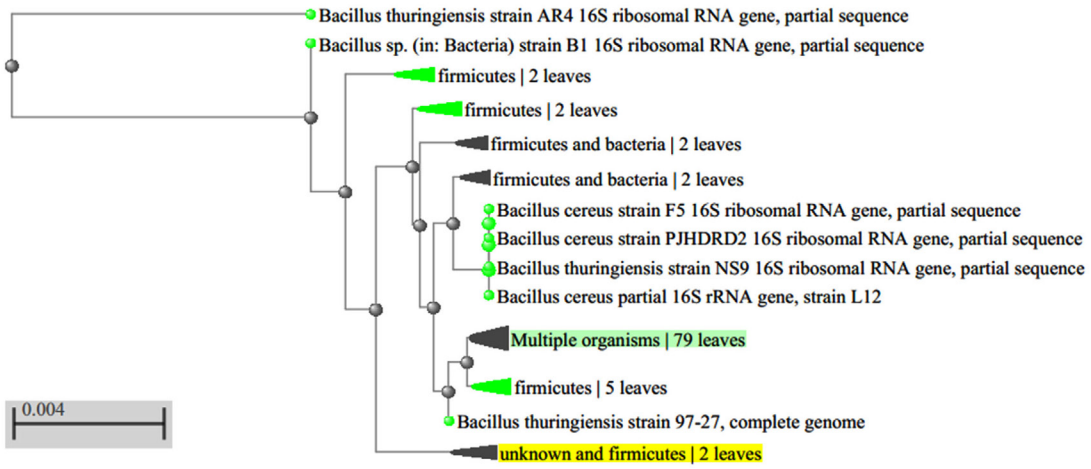

Figure 5. IB6 strain phylogenetic trees were analyzed using MEGA 5, and the neighbor-joining (NJ)method was used to build the trees

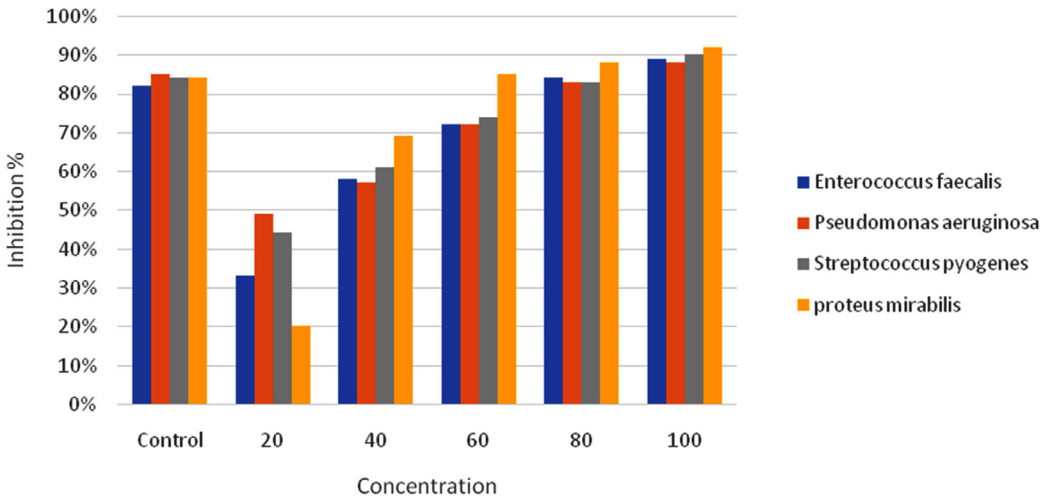

Figure 6. The result of anti-quorum sensing activity in swarming assay [MIC] of bacterial crude extract on biofilm-forming pathogen bacteria at different concentrations

closely related to *Bacillus thuringiensis* in terms of DNA sequence based on comparisons between the 16S rRNA gene sequences and use of the nucleotide standard BLAST algorithm. The MEGA5 program was used to align the sequences, analyze the phyletic lineage, and construct the tree (Figure 5). It was found that most of the bacteria isolated along the coastal and marine regions often contain at least one isolate of *Bacillus* species.[17] The biological activity of *B. thuringiensis* is well known and their formulations are frequently employed as biocontrol agents.[18]

Anti-quorum sensing activity

For determination of anti-quorum sensing activity, (swarming assay) was determined for the ethyl acetate fraction of *Bacillus thuringiensis*, isolated marine bacteria (I.B 6). The Minimum inhibitory Concentrations (MIC) of the compounds soluble in the ethyl acetate fraction against four pathogenic biofilm forming bacteria were determined through spectrophotometer at 570 nm. The result of inhibition percentages are presented in (Figure 6). The findings suggest that there was a dose-dependent reduction in the bacterial growth

in the ethyl acetate fraction. *Proteus mirabilis* was maximally suppressed by the fraction, although *Streptococcus pyogenes* and *Enterococcus faecalis* were similarly inhibited. Although total inhibition of growth was not achieved in our experiment, the tested bacteria's ability to form biofilms may be completely prevented at concentrations greater than 100 µL of the same ethyl acetate fraction. The anti-quorum sensing and the antibiofilm activities of crude ethyl acetate extracts of bacteria extracted from various marine resources were tested against *Chromo bacterium violaceum* and pathogenic bacterium, specifically *Aeromonas hydrophila, Vibrio alginolyticus,* and *Pseudomonas aeruginosa*. The most effective isolate out of 188 was sequenced and identified as *Bacillus* species.[19] Furthermore, the usage of natural and marine chemicals is more pharmacologically active than synthetic antibacterial medications against the formation of biofilms, according to molecular docking studies.[5, 20-23]

CONCLUSION

In this work, we isolated and characterized the marine-derived bacteria *Bacillus thuringenesis.* By utilizing the spent media of all the bacterial isolates, we were able to considerably suppress the development of four harmful bacteria: *Enterococcus faecalis, Streptococcus pyogenes, Pseudomonas aeruginosa, and Proteus mirabilis.* The identified *B. thuringiensis* strain's secondary metabolites were also effectively extracted using ethyl acetate solvent, and the compounds included in that solvent were shown to be able to inhibit the *in vitro* production of biofilms. In conclusion, it can be said that marine microorganisms, particularly bacteria derived from the marine soil, have the ability to produce metabolites with exceptional antimicrobial and antibiofilm qualities. These compounds can be used in place of synthetic drugs and aid in the fight against antimicrobial resistance after careful examination.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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