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Antifouling activity of bacterial extracts associated with soft coral and macroalgae from the Red Sea

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Abstract

In marine environments where biofouling occurs and has an impact on the maritime economy and environment, compounds that inhibit the attachment, growth and survival of microorganisms in a biofilm complex as well as settlement of larvae are considered potential antifouling compounds. In this study, the extracellular metabolites from two surface- associated bacteria isolated from soft coral and macroalga were evaluated for antibiofilm and antisettlement activity. The bacteria were identified using 16S rRNA gene sequencing, and the culture supernatant extract of each strain was evaluated for antibiofilm activity. The compounds present in the extracts were analysed using GC-MS. The two bacterial strains were identified as *Bacillus licheniformis* MBR1 and *Vibrio alginolyticus* MBR4 for the isolates from soft coral and macroalgae, respectively. The extracts inhibited the growth of biofilm-forming bacteria, biofilm formation and barnacle larval settlement. The GC-MS analysis of the extract detected the presence of compounds such as tetrapentacontane, octadecanoic acid, 2,3-dihydroxypropyl ester, hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester and 17-pentatriacontene. The results of the study show that extracellular metabolites of the bacteria associated with marine organisms could be used as natural antifouling compounds to control biofouling.

Key words: antifouling, antibiofilm, antisettlement, marine bacteria, bioactive compounds, Red Sea

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1. Introduction

Biofouling is the accumulation of unwanted biological matter on surfaces. Microorganisms produce biofilms and macroorganisms produce macroscale biofouling (Bhushan 2016). It causes harmful environmental effects due to increased harmful emissions and loss of biodiversity (Subba Rao 2005). The process of biofouling occurs as a sequence, beginning with biofilm-forming organisms attaching and initially colonising surfaces; invertebrates are then attracted and their subsequent attachment to the hull's surface is supported (Vinagre et al. 2020). Antifouling systems using antifouling paint coatings and other means have been used for decades to overcome biofouling in the maritime sector (Yebra et al. 2004). One of the most effective and successful paints commonly used to prevent biofouling is a self-polishing copolymer paint containing the biocide tributyltin (TBT). However, its application has a negative effect due to its extreme toxicity towards non-target organisms, such as reproduction failure and shell deformity in molluscs; bivalves and gastropods are especially sensitive to TBT (Alzieu 1998). Its use was therefore banned by the International Maritime Organization in 2003, leading to the need for TBT-free paints in antifouling applications (Chambers et al. 2006). To ensure environmental sustainability in the treatment of biofouling, TBT and other organotin-based paints need to be replaced by antifouling compounds that are highly effective, yet less toxic to the environment (Qian et al. 2009).

Natural products have been used as new, effective and nontoxic sources of antifouling compounds to replace TBT-based paints and other toxic products (Wang et al. 2017). Natural products from marine organisms are good candidates for antifouling due to the organisms' ability to survive biofouling through various strategies. The secondary metabolites they produce have bioactive properties that prevent predation and colonisation of their surfaces by fouling organisms (Wahl et al. 2014). Most of the secondary metabolites produced by marine organisms have inhibitory activity against biofouling organisms, which makes them a source of antifouling compounds (Carroll et al. 2021). Soft corals and macroalgae can resist colonisation of their surfaces by fouling organisms. Surface-associated marine bacteria are a unique source of new compounds with diverse chemical structures and bioactive properties, including antifouling (Wang et al. 2022). They are currently being explored as new sources of antifouling compounds due to their broad spectrum of activity (Gomez-Banderas 2022). They are considered to be the source of the next generation of bioactive compounds in the near future due to the many effective new compounds that are being discovered in them in different studies around the world, as reviewed recently (Srinivasan et al. 2021). Many diverse natural products of these organisms have been reported, and many more chemically unique compounds are being discovered (Blockley et al. 2017). The bacteria perform several roles necessary for the survival of their host, such as providing nutrients like carbon and nitrogen (van de Water et al. 2018) and chemical defences that protect the host against colonisation and predation (Krug 2006; Kamat et al. 2022). Potent antifouling compounds have been isolated from the bacteria associated with the living surfaces of marine organisms such as soft corals and macroalgae (Satheesh et al. 2016). Many compounds from the bacteria found in association with these organisms have been reported to have antifouling activity (Holmström et al. 2002; Ma et al. 2009).

Soft coral-associated bacteria have been described as a rich source of bioactive compounds with antifouling activity (Modolon et al. 2020). Macroalgae-associated bacteria are another important source of bioactive compounds, with several bacteria having been reported to show antifouling activity (Egan et al. 2001). Tackling biofouling will require compounds with multiple activities against the different group of organisms that attach and colonise on the surface to form the fouling structure. The compounds should have an inhibitory effect on bacterial growth, biofilm formation and larval settlement. To date, only a few studies have evaluated compounds for multiple inhibitory activity, especially antisettlement activity. In this study, surface-associated bacteria from the soft corals and macroalgae from the Red Sea were evaluated for antibiofilm activity against biofilm-forming bacteria and antisettlement activity against barnacle larvae. A study of this kind will reveal the bioprospecting potential of these Red Sea organisms as important sources of antifouling compounds.

2. Materials and methods

2.1. Isolation of bacteria associated with soft corals and macroalgae

The soft coral *Sarcophyton trocheliophorum* and the brown macroalga *Dictyota dichotoma* were collected from Obhur Creek (latitudes 21°42'11" and 21°45'24" and longitudes 39°05'12" and 39°08'48"E) in Jeddah, Saudi Arabia. The samples were transferred to the

laboratory in a sterile container with filtered (Millipore, 0.45 µm) and sterilised (autoclaved) seawater (FSW). In the laboratory, they were rinsed thoroughly with FSW to remove the loosely attached microbes and other associated epifauna. Cotton swab sticks were used to thoroughly swab the surface of the organisms and were then placed in test tubes containing 5 ml of FSW; this was followed by vigorous agitation of the test tubes with a vortex mixer. The swabs were removed from the tubes while the agitated seawater was serially diluted. Then, 0.1-ml aliquots of 10^6 diluent were transferred to Zobell marine agar plates (ZMA; HiMedia, India) via the spread plate method, and incubated at 30°C for 24–48 h. Bacterial colonies observed on the agar plates were subcultured individually and purified using the streak plate method. The purified bacterial colonies were kept in agar slants at 4°C for further studies. A preliminary screening of the isolated bacterial strains' antagonistic activity against biofilm-forming bacteria was performed using the agar overlay method (Hockett & Baltrus 2017). Two strains (MBR1 and MBR4) were selected for further analysis based on their preliminary activity against the biofilm bacteria.

2.2. Extraction of secondary metabolites from the culture supernatant

Loopfuls from the agar slants were transferred to Zobell marine broth (ZMB; Himedia, India) in 10-ml tubes and incubated in a shaker incubator at 30°C overnight. Aliquots of the overnight cultures were transferred to conical flasks containing ZMB and were incubated at 30°C for 3 days in an incubator shaker. The cultures were centrifuged for 15 min at 5000 rpm. The supernatant was collected in a tube and filtered through a filter paper (Whatman no. 1) to remove the cells. An equal volume of ethyl acetate was added to the cell-free supernatant collected and agitated for 4 h at 28°C (Rajan & Kannabiran 2014). A separating funnel was used to separate the mixture and the organic phase was evaporated under reduced pressure using a vacuum evaporator to get the culture supernatant extract (CSE). The crude CSE was used for antibiofilm and antisettlement assays as well as GC-MS analysis.

2.3. Bacterial growth inhibition assay

A growth inhibition assay of CSE from the two bacterial isolates was performed against two biofilm-forming bacterial strains: *Vibrio harveyi* (NCBI: KY266820) and *Planomicrobium* sp. (NCBI: KY224086). The bacterial strains were isolated from the aquaculture cage nets submerged in Obhur Creek, as previously reported (Balqadi et al. 2018). The biofilm-forming ability of these bacterial strains and barnacle larval settlement-inducing activity had previously been tested under laboratory conditions (Siddik & Satheesh 2019). The bacteria were grown overnight in ZMB. The OD of the overnight culture was adjusted to a cell density of 0.4 at 600 nm and used for the experiments. The CSE was adjusted to three different concentrations (5, 10 and 20 µg ml^{-1}) and the method of Ba-akdah & Satheesh (2021) was followed for the bacterial growth inhibition assay using the spectrophotometric method at 600 nm. In brief, each target bacterium was grown overnight and adjusted to an OD of 0.4 with a spectrophotometer at 600 nm by diluting the culture in ZMB. Next, 3 ml of the adjusted cultures were transferred to new tubes followed by the addition of the extract. Three different concentrations (5, 10 and 20 µg ml^{-1}) were used for each target bacterium. Separate tubes without the bacterial extract were used as controls. The OD was measured at 600 nm at baseline and again after 5 h of incubation at 30°C. The bacterial growth inhibition was calculated as a percentage following Equation 1 below.

$$Bacterial\ growth\ inhibition\ (\%) = \frac{final\ OD\ -\ initial\ OD}{initial\ OD} \times 100$$

Final OD is the final optical density at 600 nm after 5 h of incubation, while initial OD is the initial optical density at 600 nm at baseline.

2.4. Microtiter biofilm inhibition assay

The biofilm inhibitory activity of the CSE was tested against the two target bacteria, *V. harveyi* and *Planomicrobium* sp., using the microtiter plate method described previously by O'Toole (2011) with some modifications. The experiment was conducted in 96-well microtitre plates. The overnight culture for each strain was adjusted to a cell density of 0.4 OD at 600 nm. To each well of the plate, 500 µl of overnight biofilm-forming bacterial culture and 500 µl of CSE was added at a concentration of 5 µg ml^{-1} in triplicate (n=3). The control wells contained culture only without the addition of CSE (n=3). The microtiter plates were incubated statically at 37°C for 24 h in an incubator. After that, the contents of the wells were decanted gently by turning the plates downwards; they were then rinsed with phosphate-buffered saline (PBS) to remove the unattached cells and other materials from the culture broth. The biofilm-forming bacterial cells attached to the wells of the microtitre plate were stained with crystal violet solution (0.4%) for 5 min.

After that, the wells were rinsed gently with PBS to remove the excess stain. Next, the wells were air-dried at room temperature and ethanol (96%) was added. They were incubated at room temperature while being shaken for 10 mins. The contents were transferred to new plates and the absorbance of the dye eluted from the wells after the addition of ethanol was measured at 630 nm in a microplate reader (Biotek). The percentage of biofilm inhibition was calculated using Equations 1 and 2 below:

$$PI = 100 - PF \quad \text{Eq. 1}$$

$$PF = \frac{BFC \times 100}{C} \quad \text{Eq. 2}$$

where
PI is the percentage of biofilm inhibition,
PF is the percentage of biofilm formation,
BFC is the result of biofilm formed in the presence of the extract and
C is the biofilm formed by the pure strain without the addition of extract.

2.5. Collection of barnacles and larval rearing

The adult barnacles (*Amphibalanus Amphitrite* = *Balanus amphitrite*), along with the attached substratum, were collected from Obhur Creek and brought to the laboratory. The method described by Salama et al. (2018) was used to keep the adults and rear barnacle larvae. In the laboratory, the barnacles were kept in a tank containing FSW. The tank was supplied with moderate aeration, and the salinity and temperature of the tank water were maintained during the experiment at 39 PSU and 27°C, respectively. The adult barnacles were fed a diet of *Artemia* nauplii and microalga (*Chaetoceros calcitrans*). The nauplii released by the adults were collected using a hand net and transferred to small glass tanks. In the nauplii stage, the larvae were fed a microalgal diet (*Chaetoceros calcitrans*) and were reared in the laboratory until stage III for the toxicity assay. The nauplii were also reared to the cypris stage (settling stage) for the antisettlement assay.

2.6. Toxicity assay

A toxicity assay was conducted in order to study the effects that the secondary metabolites present in the CSE had on the survival of the barnacle larvae. Six-well plates were used for the toxicity assay. Each well was filled with 5 ml of FSW and 25 nauplii (stage III) were added to the wells using a Pasteur pipette. Three different concentrations (5, 10 and 20 µg ml^{-1}) of CSE were added to the treatment wells. The control wells were maintained without the addition of CSE. The 6-well plates were kept at 28°C in an environmentally controlled chamber (walk-in type). The number of dead/alive nauplii in the wells was observed periodically for 96 hours. The toxicity assay was repeated in triplicate (n=3 for each concentration) and the mean ± SD values were recorded.

2.7. Antisettlement assay

An antisettlement assay was conducted to understand the settlement-inhibiting activity of the CSE against the settlement of barnacle larvae. The nauplii larvae were reared to the cypris stage in the laboratory. This experiment was also conducted in 6-well plates, with 25 cypris larvae in each well. The CSE (5 µg ml^{-1}) was added to the treatment wells. The control wells were maintained without the addition of CSE. The 6-well plates were kept at 28°C in an environmentally controlled chamber (walk-in type) under dark conditions. The plate was observed periodically under a stereomicroscope for the settlement of larvae. The experiment was conducted in triplicate using different batches of barnacle larvae for a period of 48 h.

2.8. Identification and phylogenetic analysis of surface-associated bacteria

The bacterium isolated from the soft coral was labelled as MBR1, while the one from macroalga was labelled as MBR4. They were identified based on 16S rRNA gene sequencing. Previously outlined methods (Satheesh et al. 2012; Balqadi et al. 2018) were followed for the isolation of DNA and sequencing of the 16S rRNA. In brief, a loopful of bacterial culture was inoculated in marine broth in a conical flask and kept in an incubator at 30°C. The overnight bacterial culture was used for DNA extraction using a genomic DNA isolation kit (InstaGene™). The isolated DNA from the bacterial strains was confirmed using agarose gel electrophoresis. Universal 16S rRNA gene primers (27F:5' - AGAGTTTGATCMTGGCTCAG - 3' and 1100R: 5' - GGGTTGCGCTCGTTG - 3') were used for the amplification of the isolated DNA in a multigene thermal cycler (Labnet Inc). The PCR conditions were as follows: initial cycle at 95°C for 5 min, followed by 28 cycles at 95°C for 45 sec, annealing at 58°C for 45 sec, with extension at 72°C for 1 min 45 sec and a final extension step at 72°C for 10 min. The amplified PCR products were purified using a GeneJET PCR Purification Kit according to the manufacturer's

instructions and then confirmed by agarose gel electrophoresis. The amplified PCR products were sent to Macrogen Inc. in South Korea for sequencing of the 16S rRNA gene. The resulting sequences were analysed and identified using the RDP classifier (http://rdp.cme.msu.edu/) and the Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi/), with the search option limited to type material in order to select the nearest match and using 99% as the threshold for identification at the species level, as reported previously (Abdulrahman et al. 2022a). The 16S rRNA gene sequences of all the strains were submitted to NCBI Genbank for the assignment of accession numbers. A phylogenetic tree was constructed with related strains from Genbank using MEGAX (Tamura et al. 2021) and annotated using Itol software, web version (Letunic & Bork 2021).

2.9. GC-MS analysis of CSE

The secondary metabolites present in the CSE were analysed using gas chromatography-mass spectrophotometry (GCMS-QP2010 Plus, Shimadzu, Japan). The protocol outlined by Arulazhagan and Vasudevan (2009) was followed for the analysis. In brief, the capillary column (30 m × 0.25 mm; ID = 0.25 µm) was programmed as follows: the initial column temperature was set at 100°C for 1 min, then increased up to 160°C at the rate of 15°C min^{-1}. The column temperature was then set for 7 min at 300°C, the final temperature, at a rate of 5°C min^{-1}. Subsequently, with helium as the carrier gas at 1.0 ml min^{-1}, the GC injector was held isothermally at 280°C flow rate while the temperature at the GC/MS interface was maintained at 280°C for an uninterrupted period of 3 min. The mass spectrometry was set to operate in electron impact ionization mode with 70 eV electron energy. The possible metabolites were identified with the National Institute of Standards and Technology library using the similarity index (<92%) by comparing their retention index.

2.10. Statistical analysis

The inhibitory activity against bacterial growth of different concentrations of CSE was tested using the Kruskal–Wallis H test. The antibiofilm assay results and the anti-larval settlement data were tested using one-way analysis of variance (ANOVA) in order to determine the difference between the controls and the treatments. Furthermore, the barnacle larval toxicity of CSE was tested using two-way ANOVA. Concentration and bacterial strain were used as the factors for two-way ANOVA. A post hoc Tukey HSD test was carried out to determine the variation between the controls and the treatment groups in the antibiofilm and antisettlement assays. All the statistical tests were conducted in STATISTICA (ver. 13) and $p<0.05$ was set as the level of significance.

3. Results

3.1. Isolation and identification of surface-associated bacteria from macroalga and soft coral

After the bacteria were screened using the agar overlay assay, one bacterium each was selected from soft coral and macroalgae. Based on its morphologic features and a comparison with the closest strain from the NCBI database, MBR1 had close similarity with more than 15 strains of *Bacillus licheniformis* and 99.72% with the type strain *B. licheniformis* ATCC 14580^{T}. MBR4 had similarity with *Vibrio alginolyticus* and a similarity of 99.91% and recorded the highest BLAST score with the type strain *V. alginolyticus* NBRC 15630^{T}. These strains MBR1 (from soft coral) and MBR4 (from macroalga) were identified as *Bacillus licheniformis* MBR1 (NCBI: OP379261) and *Vibrio alginolyticus* MBR4 (NCBI: OP379262), respectively. A phylogenetic tree showing the strains and other related organisms is presented in Figure 1.

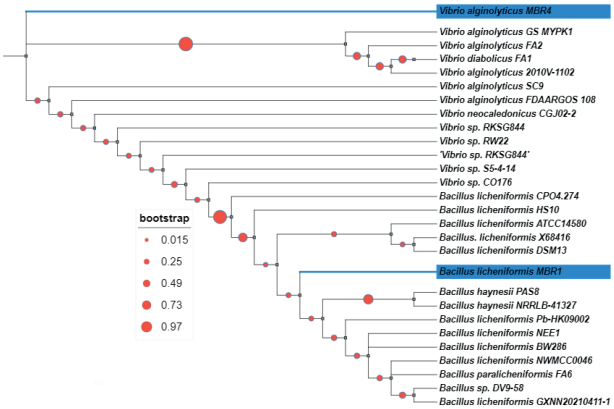

Figure 1

Phylogenetic tree showing the two organisms with other related species. The organisms from this study are highlighted in blue, while the red symbol was used to show the bootstrap values.

3.2. Bacterial growth inhibitory activity of CSE

The CSE from the two bacterial strains inhibited the planktonic growth of the biofilm-forming bacteria *V. harveyi* and *Planomicrobium* sp. The CSE extracted

from MBR1 exhibited strong inhibitory activity against *V. harveyi* (Figure 2), whereas the CSE of MBR4 strongly inhibited the planktonic growth of both *V. harveyi* and *Planomicrobium* sp. (Figure 3). Among the concentrations of the CSE tested, no significant differences were observed in growth inhibiting activity.

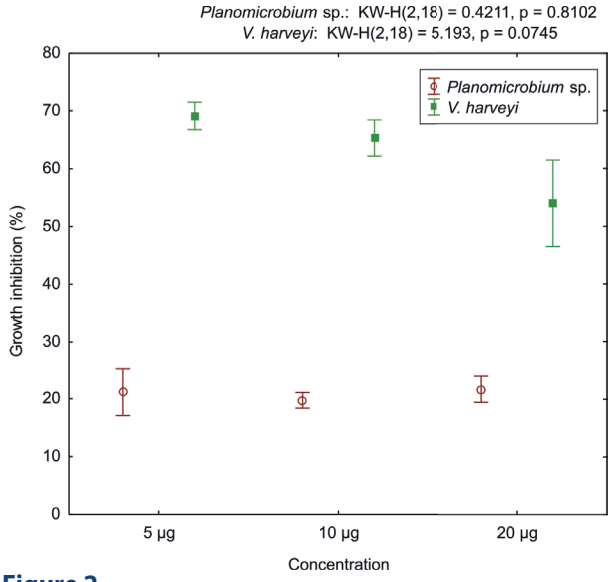

Figure 2

Bacterial growth inhibitory activity of the CSE of MBR1

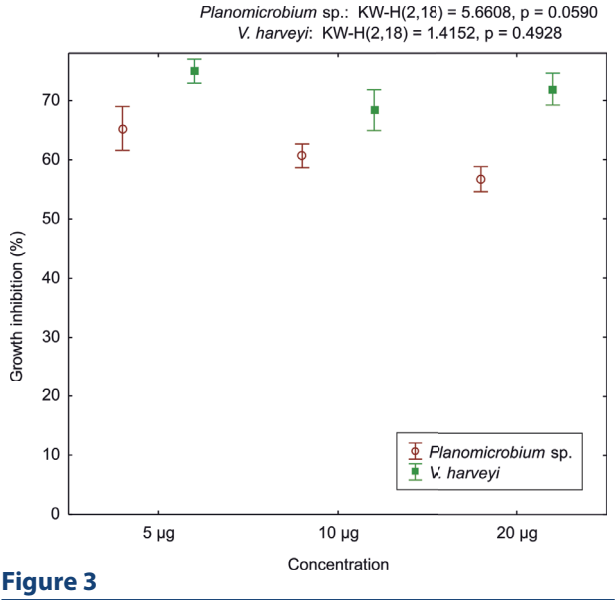

Figure 3

Bacterial growth inhibitory activity of the CSE of MBR4

3.3. Biofilm inhibitory activity

The percentage of biofilm inhibition from the extract against the test strains ranged from 45% to 78%, as presented in Figure 4. Both the lowest and high activity was recorded by the extract from MBR1, and the two different extracts demonstrated higher inhibition activity towards *V. harveyi* than *Planomicrobium* sp. The extracts of both bacterial strains significantly ($p<0.05$) inhibited the attachment of biofilm-forming bacteria (*V. harveyi*: F=38.65; df=3,20; $p<0.05$; *Planomicrobium* sp: F=14.83; df=3,20; $p<0.05$). Post hoc analysis indicated significant variation in biofilm-inhibiting activity between the extract treatment and control (Table 1).

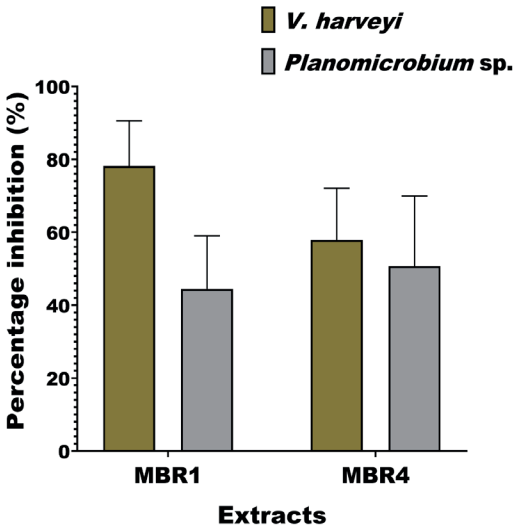

Figure 4

Percentage of biofilm inhibition activity of the extracts from MBR1 and MBR4 against the biofilm bacteria

3.4. Toxicity of CSE on barnacle larvae

The CSE treatment did not show much effect on the survival of the nauplius larvae during the 96-h toxicity study (Figure 5). The two-way ANOVA results

Table 1

Post hoc Tukey test results (approximate probabilities) for the antifouling activity of CSE extracted from three bacterial strains against biofilm-forming bacteria and barnacle larvae

Bacterial extract		Antibiofilm activity		Antilarval settlement activity
		V. harveyi	*Planomicrobium* sp.	Cyprid larvae
Control	MBR1	<0.001	<0.01	<0.001
	MBR4	<0.001	<0.001	<0.001
MBR1	MBR4	0.067	0.808	0.942

* $p<0.05$ indicates statistical significance

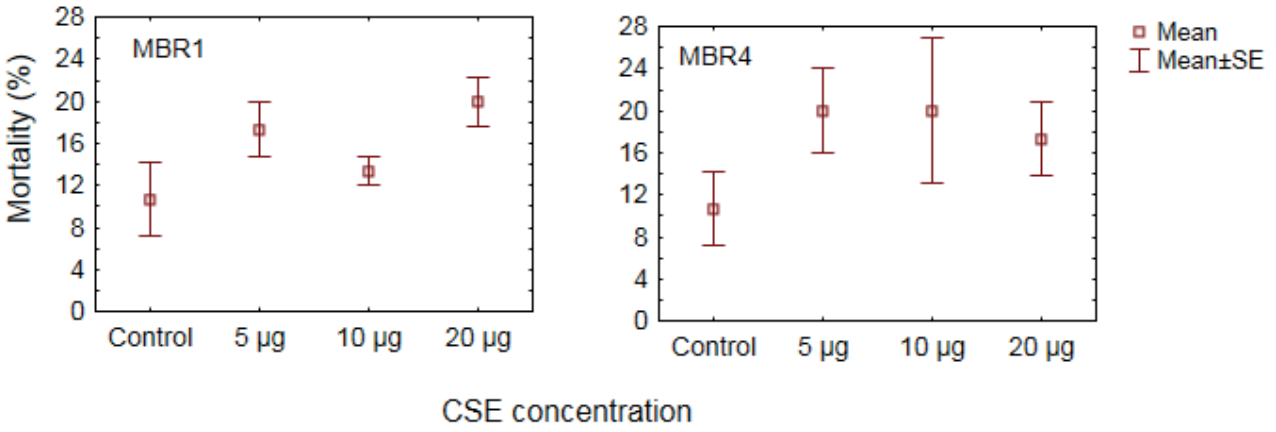

Figure 5

Toxicity of CSE extracted from the bacterial strains against barnacle larvae

also confirmed the weak toxicity of the extracts against barnacle larvae, as evidenced by the insignificant variation (F=2.32; df=3,24; p=0.1) in mortality between the concentrations of the extracts.

3.5. Barnacle larval settlement inhibiting activity of CSE

The results indicated reduced settlement of cypris larvae on the 6-well plates due to CSE treatment. The CSE of strain MBR4 exhibited higher settlement inhibition than the extract of MBR1 (Figure 6). The one-way ANOVA results indicated a significant variation in cyprid settlement between the control and CSE treatment groups (F=25.86; df=3,8; p<0.05). Further, the post hoc Tukey test revealed that settlement of larvae in the wells with CSE was reduced significantly in comparison with the control (Table 1).

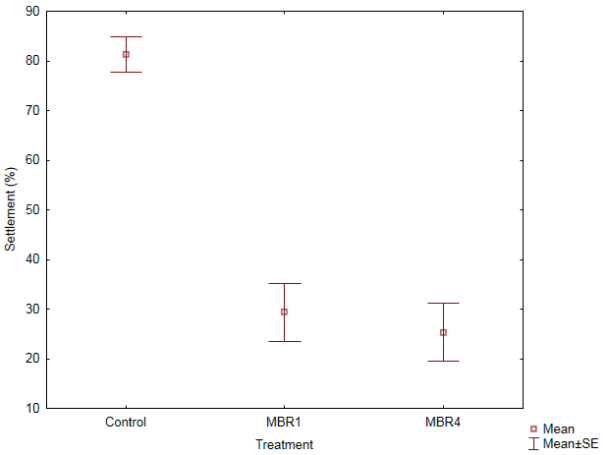

Figure 6

Antilarval settlement activity of CSE against cyprid-stage larvae of barnacles

3.6. GC-MS analysis

The GC-MS spectra of the CSE extracted from the two bacterial strains are presented in Figures 7 and 8. The secondary metabolites present in the CSE of MBR1 included n-tridecanol, 3-chloropropionic

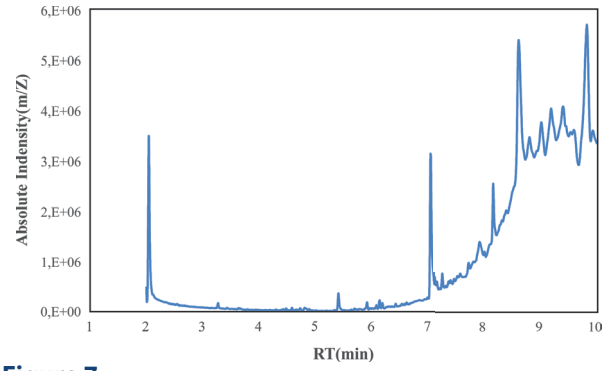

Figure 7

GC-MS spectrum of CSE extracted from the bacterial strain MBR1

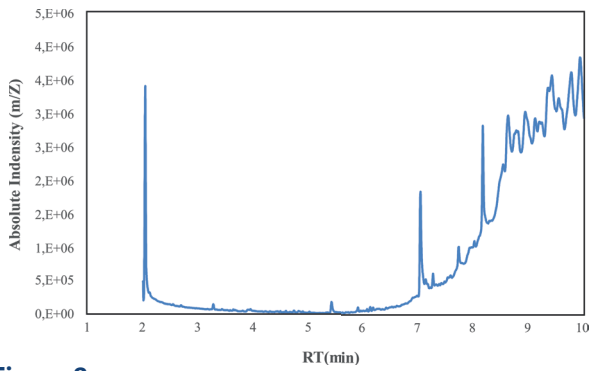

Figure 8

GC-MS spectrum of CSE extracted from the bacterial strain MBR4

acid heptadecyl ester, hexadecanoic acid ester, hexacontane and tetrapentacontane (Table 2). The GC-MS analysis results of the CSE obtained from MBR4 showed the presence of compounds such as cyclopropane, octadecanoic acid ester, cis-anti-cis-tricyclo (2,6) dodecane -7,8-diol, 7-hexyltridecanol, tetrapentacontane, pentatriacontane, 2H-Pyran-2-one and 5-ethylidenetetrahydro-4-(2-hydroxyethyl) (Table 3).

Table 2

Compounds detected in the CSE of MBR1

Compound name	RT (min)	Peak area
Hexane, 2,2-dimethyl-	2.043	5682084
n-Tridecan-1-ol	7.046	5873147
3-Chloropropionic acid, heptadecyl ester	8.176	2029795
Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	8.627	13557111
Hexacontane	8.808	5252629
Tetrapentacontane, 1,54-dibromo-	9.027	2603872
Tetrapentacontane, 1,54-dibromo-	9.2	4136789
Tetrapentacontane	9.412	1932874
Tetrapentacontane	9.832	13033013

Table 3

Compounds detected in the CSE of MBR4

Compound name	RT (min)	Peak area
Pentane, 2,2,4-trimethyl-	2.04	4716273
Cyclopropane, 1-(1-hydroxy-1-heptyl)-2-methylene-3-pentyl-	8.542	3254671
Octadecanoic acid, 2,3-dihydroxypropyl ester	8.633	5949198
(7R,8R)-cis-anti-cis-Tricyclo[7.3.0.0(2,6)]dodecane-7,8-diol	8.783	6609750
Tetrapentacontane, 1,54-dibromo-	8.945	7398782
7-Hexyltridecan-1-ol	9.258	3273783
9,12,15-Octadecatrienoic acid, 2-phenyl-1,3-dioxan-2-yl ester, (Z,Z,Z)-	9.35	4608587
Tetrapentacontane, 1,54-dibromo-	9.426	4100361
17-Pentatriacontene	9.549	3437915
2H-Pyran-2-one, 5-ethylidenetetrahydro-4-(2-hydroxyethyl)-	9.777	3956877

4. Discussion

The aim of this study was to investigate the antifouling activity of extracts of culture supernatant of surface-associated bacteria from soft coral and macroalgae collected from the Red Sea. The CSE from the isolated organisms showed antifouling activity in the form of potential antibiofilm and antisettlement activities against biofilm bacteria and barnacle larvae, respectively. Many previous studies have found that bacteria living on the surface of marine organisms such as sponges, corals and seaweeds produce compounds that protect the host from fouling organisms (Satheesh et al. 2016). The bacteria isolated from the surface of the marine organisms identified as *Bacillus licheniformis* MBR1 (from soft coral) and *Vibrio alginolyticus* MBR4 (from macroalgae) are among the organisms known to be dominant in the marine environments. *B. licheniformis* is of great interest due to its broad biotechnological applications and production of bioactive compounds that can be used for many purposes (Muras et al. 2021b). It forms symbiotic relationships with many marine invertebrates and survives hostile conditions due to spore production. *B. licheniformis* belongs to the *Bacillus subtilis* subgroup and is relevant in the search for bioactive compounds (Kaspar et al. 2019). Several studies have reported high antifouling activity potential in bioactive compounds from *B. licheniformis* (Ortega-Morales et al. 2008; Aguila-Ramírez et al. 2014; Eduok et al. 2015; Muras et al. 2021a). Many species have been isolated from soft corals with a demonstrated ability to produce compounds with antifouling activity (Pham et al. 2016; Hou et al. 2019; Abdulrahman et al. 2022a; Abdulrahman et al. 2022b). Members of the genus *Vibrio* exist as epibiotic bacteria living on the surfaces of other organisms living in the marine

environment. Several members have been reported to form a chemical relationship with macroalgae and to produce bioactive compounds (Goecke et al. 2010). *Vibrio* sp., associated with macroalgae surfaces, has been reported as a relevant source of new compounds with bioactive properties, including inhibitory activities against fouling organisms (Tilmann et al. 2004, Ba-akdah & Satheesh 2021). It has been isolated from the surface of a macroalga and has demonstrated good antifouling activity through the production of relevant compounds (Tilmann et al. 2004). *Vibrio alginolyticus* is significant for the defence of macroalgae against biofouling because it has been shown that macroalgae rely on the bacterium to produce secondary metabolites for its protection (Dobretsov & Qian, 2002).

The CSE of the two isolates inhibited the growth of biofilm bacteria in liquid conditions based on turbidity measured with a spectrophotometer. This method is commonly used for determining bacterial growth under various conditions. For instance, Viju et al. (2014) and Ba-akdah & Satheesh (2021) reported the antibacterial activity of compounds obtained from bacteria associated with seaweeds using a similar method. Furthermore, this approach is relevant in easily determining whether a bioactive compound has antibacterial activity (Satheesh et al. 2012). It has been shown that the effect on antibiofilm formation is directly related to the compounds produced by the bacteria (Viju et al. 2014). The CSE tested in this study showed variation in the inhibitory effect against biofilm-forming bacteria and the tested organisms had varying susceptibility to the extracts. For example, the CSE from strain *B. licheniformis* MBR1 exhibited a strong growth inhibition effect against *Planomicrobium* sp., while the CSE extracted from *V. alginolyticus* MBR4 showed a strong growth inhibitory effect against *V. harveyi* and *Planomicrobium* sp. These variations may be due to the sensitivity of the biofilm-forming bacteria to the different secondary metabolites present in the CSE. Screening for antibacterial growth activity is important in determining the antifouling activity of a secondary metabolite from bacteria (Bowman 2007) because the growth of the bacteria occurs before the bacteria adheres to surfaces and begins the biofilm/biofouling process (Adnan et al. 2018).

After the growth of the biofilm bacteria, they adhere to the surface and colonise it to form biofilm. Bacteria are the initial colonisers on any newly exposed surface in marine waters, and they have been shown to influence the settlement of larval invertebrates (Hadfield 2010; Antunes et al. 2019; Dobretsov & Rittschof 2020; Peng et al. 2020; Chang et al. 2021). As a result, the adhesion assay utilising bacteria isolated from biofilms could be useful in developing natural products into antifouling agents. This makes targeting biofilm formation an important strategy for antifouling. The results of the antibiofilm assays conducted in this study indicated biofilm inhibitory activity of the compounds present in the CSE from the two bacterial strains. Both extracts demonstrated good antibiofilm formation activity. Natural antibiofilm compounds are required to tackle biofilm and its devastating effects in order to prevent the resulting substantial economic losses (Cámara et al. 2022).

Under laboratory conditions, the culture supernatant of bacterial strains isolated from a soft coral and a macroalga strongly inhibited the settlement of barnacle larvae without significant toxicity. Preventing settlement of larvae is an important requirement in the development of antifouling agents. The invertebrate larvae are responsible for the visible fouling structure seen on the surface of ship hulls (Vinagre et al. 2020). The mobile nauplius larva undergoes a series of transitions through stages before metamorphosising to the cypris stage, which then settles and attaches to suitable surfaces and finally transits to the sessile adult stage, contributing to biofouling (Kamino, 2016). The results of the study indicate that these extracts have an inhibitory effect on the settlement of the cyprid larvae, preventing metamorphosis to the adult stage and thereby preventing biofouling.

The activities demonstrated by the extracts are attributed to the secondary metabolites produced by the bacteria, as revealed by the GC-MS analysis. The GC-MS analysis of the CSE yielded many compounds with known bioactivities. Compounds detected from the *B. licheniformis* (MBR1) CSE include those previously reported to show antibacterial activity, such as n-Tridecan-1-ol (Togashi et al. 2007) and tetrapentacontane (Dhankhar et al. 2013). Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester was also detected; it has antibiofilm activity because of its ability to interfere with quorum sensing (Venkatramanan et al. 2020). Another compound is 3-chloropropionic acid, heptadecyl ester, which was reported previously as an active compound in a *Streptomyces* extract (Elsayed et al. 2020). From the CSE of *V. alginolyticus*, compounds detected that have antimicrobial properties include octadecanoic acid, 2,3-dihydroxypropyl ester (Sultan et al. 2020) and 17-pentatriacontene (Kumar et al. 2018). These compounds show an inhibitory effect against microorganisms. The presence of these compounds in the bacterial extract indicates the bacteria's ability to

produce compounds that are relevant to antifouling and that explain the activities demonstrated by the extracts.

In conclusion, this study adds to our understanding of the ability of bacteria associated with the surface of marine organisms to produce secondary metabolites with antifouling activities. The diverse nature of the compounds presents in the CSE revealed the ecological functions of microbial association with marine organisms. Furthermore, the results indicate that the culture supernatant can be considered for the extraction of bioactive compounds. Further studies using other fouling organisms and field assays will provide more insights into the antifouling properties of the metabolites present in the culture supernatant.

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