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Antibiofilm activity of secondary metabolites of sponge-associated bacterium *Alcanivorax* sp. from the Red Sea

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Sponge-associated bacteria are a well-known source of unique and diverse natural products with potential biological activities. In this study, a rarely occurring sponge-associated bacterium from the genus *Alcanivorax* was isolated from the sponge *Siphonochalina siphonella* and subjected to extraction of metabolites. Metabolites were extracted from the culture supernatant and biomass of the bacterium using the solvent extraction method. Extracts were evaluated for antibacterial and antibiofilm activities against five different biofilm-forming bacteria isolated from the microfouling assemblage. The crude extracts were subjected to chemical profiling to identify their composition using gas chromatography-coupled mass spectrometry (GC-MS). The results obtained show mild to strong antibacterial activity of the extracts against the biofilm-forming bacteria with the minimum inhibitory concentration (MIC) of 3.125 mg ml⁻¹. The percentage inhibition of biofilm formation ranged from 46% to 71%. Compounds detected in GC-MS analysis are mostly fatty acids and their derivatives that include compounds with known antibiofilm activity such as tetradecanoic acid, dodecanoic acid, and hexadecanol from the culture supernatant extract. Eicosane; hexadecanoic acid, methyl ester; and hexadecanoic acid, ethyl ester were detected from the biomass extract. Molecular docking analysis of the compounds from the bacterial extracts confirms their potential antibiofilm properties. The result indicated that *Alcanivorax* sp. associated with the sponge possess relevant antibiofilm activity and may serve as a novel source of secondary metabolites with the application as antibiofilm agents.

KEYWORDS

sponge, symbiotic bacteria, biofilm, biofouling, antibiofilm, bioactive compounds, *alcanivorax*

Introduction

The attachment of microbial communities and the formation of a structured assemblage embedded in the extracellular matrix are called biofilms (Karygianni et al., 2020). In the marine environment, biofilm formation on artificial materials is a cause of concern due to its economic significance. Bacterial biofilm is the fundamental cause of biofouling development in the marine environment on artificial and natural surfaces (Unabia and Hadfield, 1999). When an object is submerged in the sea, the biofouling process is initiated by the attachment of biofilm bacteria that then attract larger organisms (Dobretsov and Rittschof, 2020). When this occurs on marine infrastructure and installations, it will have negative environmental and economic impacts on marine transport, aquaculture, fisheries, tourism, and mariculture (Bannister et al., 2019; Cruz, 2020). Biofouling results in an increase in frictional resistance of ship hulls and subsequent increase in fuel consumption. There is also an increase in biodiversity threat *via* the transfer of invasive microorganisms (Molnar et al., 2008).

Many antibiofilm and antifouling strategies are used to control biofouling on artificial materials (Satheesh et al., 2016). One of the most common methods to get rid of biofilm in water is the use of biocides (Bhoj et al., 2021). Biocides are expensive and have toxicity toward non-target organisms (Schulte et al., 2005). The biofilm bacteria could also develop resistance to the biocides, making them ineffective (Kocot and Olszewska, 2017). Sustainable antibiofilm strategies are required to control biofilms on marine installations and structures. Secondary metabolites from marine bacteria have been found to be sources of novel and unique antimicrobial compounds that could overcome the problem of antimicrobial resistance (Proksch et al., 2002; Kijjoa and Sawangwong, 2004; Vignesh et al., 2011; Mondol et al., 2013; Kumar et al., 2014; Santos-Gandelman et al., 2014; Malve, 2016; Mayer et al., 2021). The natural compounds from marine bacteria and other organisms are considered potential antifouling compounds (Satheesh et al., 2016).

The marine environment has rich biodiversity and covers a major portion of the biosphere, making it one of the largest unexplored sources of bioactive compounds (Malve, 2016). Among marine organisms, sponges are one of the most important organisms due to its worldwide distribution and bioprospecting potentials. Bioactive compounds from sponges are enormous compared with other organisms and represent about 30% of marine natural products (MNPs) reported between the years 2001 and 2010 (Mehub et al., 2014). The sponge harbors diverse microorganisms that are living in symbiotic association with the sponge host. Microbes make up 25% of its biomass and contribute to its survival (Fahmy and Abdel-Tawab, 2021). Through the association, most of the sponge MNPs are produced as by-products of metabolism (Santos-Gandelman et al., 2014). The MNPs are produced by the

sponge and its symbionts and have antagonistic activity against other microbes (Srinivasan et al., 2021). The microbes produce compounds as secondary metabolites that enable them to provide some form of chemical defense to the host through chemical interactions, while the host provides the microbes with nutrients necessary for their growth (Zhang et al., 2022). Some of the bacterial symbionts are unique because they have not been described previously elsewhere, making them potential sources of bioactive compounds (Wang, 2006; Caruso et al., 2018; Rizzo et al., 2020). As sources of diverse and new compounds, bacteria associated with sponges have been explored for many activities including as antifouling compounds (Satheesh et al., 2016; Wang et al., 2017; Liu et al., 2020). Compounds from the sponge and its associated bacteria are chemically diverse, covering a range of chemical classes such as fatty acids, polyketides, alkaloids, benzenoids, amino acids, steroids, and many others (Fusetani, 2004). Antibiofilm compounds reported from these organisms with promising activity include polyethers (Dash et al., 2011), butenolide (Yin et al., 2019), phorbaketol B, and phorbaketol C (Kim et al., 2021).

The Red Sea is home to diverse marine organisms; many of them are endemic to the environment (Berumen et al., 2019). The organisms are the repository of diverse bioactive secondary metabolites, with over 700 compounds reported in the last decade alone (El-Hossary et al., 2020). It is also a source of novel marine microbial secondary metabolites due to its harsh environmental conditions, making it a model in the search for microbial species that can produce promising antibiofilm compounds (Behzad et al., 2016). However, to date, only a few antibiofilm metabolites have been reported from the Red Sea, making it one of the underexplored environments. Hence, in this study, the antibiofilm activity of the bacteria associated with the sponge species *Siphonochalina siphonella* collected from the Red Sea was assessed using laboratory assays and *in silico* methods against the biofilm-associated protein (BAP). The results of this study will be useful for the development of novel natural product antibiofilm compounds from marine microbes.

Methodology

Isolation and identification of sponge-associated bacteria

The sponge-associated bacterium (strain AA8) was isolated from a marine sponge. The sponge was collected from the Central Red Sea coast in Jeddah (N21°42.562' E39°05.764') and identified as *S. siphonella* using the method described previously (Lieske et al., 2004). The isolation of bacteria associated with the sponge was done by washing the organism (sponge) with 0.22- μ m filter-sterilized seawater (FSW), cut into pieces, and then immersed in an FSW-containing flask. The flask was kept in a rotary shaker for 4 h, and the contents were serially

diluted (10-fold serial dilution). An aliquot of 100 μ l of the serially diluted sample was inoculated in Zobell marine agar (ZMA) (HiMedia, India) plates using the spread plate method and incubated at 28°C for 72 h or until visible colonies appeared on the plates. Individual colonies were selected following subcultures to obtain pure culture. It was identified by the partial 16S rRNA method as reported previously (Abo-State et al., 2018). The PCR product of the 16S rRNA gene was commercially sequenced at Macrogen Inc., Korea. The gene sequence of the selected organism was deposited in the NCBI database (accession number: ON514280). This was followed by phylogenetic tree construction using MEGAX neighbor joining method to determine the phylogenetic position of the strain and its evolutionary relatedness (Kumar et al., 2018).

Extraction of metabolites from the culture supernatant and biomass extract

The solvent extraction method was applied for the extraction of secondary metabolites from the sponge-associated bacterium (strain AA8). The growth of the organism was optimized for conditions such as temperature and incubation time required for reaching the exponential phase. The pH of the media was maintained as 7.4 throughout the experiment. The temperature was varied from 20°C to 35°C (i.e., 20°C, 25°C, 30°C, and 35°C) to select the best growth temperature by measuring the optical density (OD) at 600 nm (OD_{600}) using a spectrophotometer (Shimadzu, Japan) according to an earlier reported method (Selvin et al., 2009). The best growth was observed at 30°C, which has the highest OD_{600} value. The time required to reach the exponential phase was determined using a previously described method with modifications (Ortega-Morales et al., 2008). The incubation time was varied from 12 h to 24 h at a temperature of 30°C with the observation of the OD at hourly intervals until the exponential phase was attained. The time taken to reach the exponential phase was found to be at the 18th hour. To extract the metabolites, the organism was inoculated in 10 ml of Zobell Marine Broth (ZMB) (HiMedia, India) overnight at 30°C until it reaches the exponential phase. From the overnight culture, 5 ml was transferred into a 500-ml-capacity Erlenmeyer flask containing 250 ml of ZMB. It was incubated with constant shaking (180 rpm) at 30°C for 7 days. After that, centrifugation was carried out at 8,000 \times g for 20 min at 4°C. Following that, the supernatant and culture biomass (pellet) were collected separately for the extraction of metabolites. For the cell-free culture supernatant, ethyl acetate was added in equal volume and agitated for 24 h. For the biomass, the cells recovered were washed in phosphate-buffered saline (PBS). Metabolites were extracted from the biomass using an ice-cold solvent mixture of acetonitrile and methanol in a ratio of 1:1. This was followed by shaking, filtration, and evaporation of the solvents as described

previously (Nadeem et al., 2020). Each of the crude extracts obtained was weighed and solubilized in dimethyl sulfoxide (DMSO) to a concentration of 50 mg ml⁻¹. The extracts were stored at refrigerated temperature until required for further use.

Determination of the antibacterial activity of the extracts

The well diffusion method was applied to test the antimicrobial susceptibility of the extracts as described previously by Al-Daghistani et al. (2021). The antibacterial activity of the crude extracts obtained from the sponge-associated bacterium was evaluated against selected biofilm bacteria. The target biofilm-forming bacteria were obtained from the culture collections maintained in our laboratory (Faculty of Marine Sciences, King Abdulaziz University). The biofilm bacteria selected for this study were *Pseudoalteromonas* sp. IMB1 (NCBI: ON003955), *Halomonas* sp. IMB2 (NCBI: ON415519), *Vibrio alginolyticus* IMB11 (NCBI: ON003958), *Pseudoalteromonas gelatinilytica* IMB14 (NCBI: ON003961), and *P. gelatinilytica* IMB15 (NCBI: ON003962). These biofilm bacteria were isolated from the microfouling assemblage developed on artificial materials submerged in the Obhur Creek, Jeddah coast (Abdulrahman et al., 2021). For the biofilm bacteria, colonies from the bacterial culture were inoculated in 10 ml of ZMB and incubated overnight. This was followed by centrifugation at 5,000 \times g for 5 min. The supernatant obtained after centrifugation was discarded, and the pellets were washed twice with PBS to remove any leftover supernatant. PBS was added to the biomass, and the suspension was adjusted to 0.5 McFarland standard (1.0×10^6 CFU/ml) using a spectrophotometer at OD_{600} . The surface of freshly prepared ZMA plates was uniformly spread with the suspension with a sterile cotton swab, and wells were perforated with a sterile cork borer that was 6 mm in diameter each. The extract (50 μ l) was added to each well, and 100% DMSO was used as control. The plates were incubated at 28°C for 24 h. Antibacterial activity was evaluated by measuring the diameter of the zone of inhibition (ZI) formed around the well that indicates the absence of growth. All assays were repeated in triplicate, and the average was recorded. The result was categorized as strong (ZI \geq 20 mm), moderate (ZI = 14–19 mm), weak (ZI \leq 13 mm), and resistant (when there is no zone of inhibition).

Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) for each extract against the target biofilm bacteria was determined with the help of resazurin dye according to a previously described

broth microdilution method with modifications (Elshikh et al., 2016). The MIC was determined in a 96-well microtiter plate. For each well, 100 μ l of ZMB was added. In the first column, 100 μ l of the stock solution of the extract was added to the first well in the row containing 100 μ l of ZMB and mixed with a pipette tip. A 100- μ l aliquot from the well was pipetted to the next well in the column. Serial dilution was carried out until the 10th well in the column. Negative and positive controls were used per experiment. Negative control contains only media without extract and culture in column 11, and positive control contains 200 μ l of bacterial suspension without extract in the 12th column. The first column has the highest concentration of 25 mg/ml. The standardized culture of each bacterium was prepared and adjusted to a final concentration of 1×10^6 CFU/ml. A 100- μ l standardized culture of the tested strain was suspended in each of the wells except for column 11 (media control). Each experiment was repeated three times, and the plates were incubated at 28°C for 24 h under static conditions (Sarker et al., 2007). After incubation, the plates were observed and 30 μ l of resazurin solution (0.02%) was added to each well and then incubated statically at 28°C for 2–3 h. Color change was observed visually after the incubation in the wells, and MIC values were recorded as the lowest concentration where the color changes from the resazurin color (purple) to red. Wells that were observed without any color change were regarded as above MIC values. A color change to pink in the growth control (positive) well was the indication of proper growth of the bacteria, while no change in color of the negative control well was considered the absence of contamination (Teanpaisan et al., 2017).

Antibiofilm activity of the extracts

The crude extract was evaluated for antibiofilm activity against the five biofilm-forming bacteria. Each of the tested organisms was prepared for the assay, and the inoculum was standardized using the procedure described by Skogman et al. (2016) with some modifications. Briefly, the selected biofilm organism undergoes preculture by culturing in tubes containing modified nutrient broth (MNB) that was composed of nutrient broth (HiMedia, India) and filtered seawater (0.22 μ m) and incubated for 16–18 h at 28°C at 180 rpm in a shaker incubator. The biofilm bacteria culture was diluted 100 times in Luria-Bertani (LB) broth and incubated using the same conditions as above until it reaches exponential growth (OD range: 0.2–0.3 at OD₅₉₅). The biofilm bacteria culture was further diluted 100 times with LB to arrive at 10^6 CFU ml⁻¹. Microtiter biofilm inhibition assay was carried out to test the antibiofilm activity. The assay was done in a round-bottom 96-well microtiter plate

to determine the ability of each of the extracts to reduce or inhibit biofilm formation using a previously described method (Nunes et al., 2021). The plates were first moistened with sterile filtered seawater (SFSW) for conditioning. The diluted biofilm bacterial culture was added (175 μ l) to the microtiter well of 96-well plates. This was followed by the addition of 25 μ l of the extract. Each extract was evaluated against all of the biofilm-forming bacteria in replicate (n = 4). For the control, 200 μ l of the diluted bacterial culture only was added to the well without extract. Inoculated plates were incubated statically and aerobically at 28°C for 48 h. The whole unattached cells were removed carefully using a multichannel pipette after incubation, and the wells were washed with PBS (three times). The bacteria that attached on the wells were stained by adding 200 μ l of 0.2% crystal violet (CV) into the wells and allowed to stay statically at ambient temperature for 15 min. After that, the CV stain was removed from the wells carefully by inverting the plates and washing thrice in PBS. The plate was allowed to air-dry, and 200 μ l of 96% ethanol was added to the wells for the solubilization dye from the attached bacterial cells. The plates were incubated with shaking for 20 min at 28°C in a shaker incubator after which the content was carefully transferred to fresh 96-well plates. The OD was measured at 570 nm (OD₅₇₀) with a multiplate reader (Synergy, Biotek). The experiment was repeated independently in duplicate, and the average for six values with the standard deviation (SD) was considered. The biofilm inhibition was calculated as a percentage using the following formula:

$$BI = \left(\frac{OD_{CONTROL} - OD_{SAMPLE}}{OD_{CONTROL}} \right) 100$$

BI = Biofilm Inhibition

OD control = Absorbance of control at 570 nm

OD sample = Absorbance of test sample at 570 nm

Gas chromatography-coupled mass spectrometry analysis of the extracts

Gas chromatography-coupled mass spectrometry (GC-MS) using GCMS-QP2010 system (Shimadzu, Japan) was used to analyze the chemical composition of the crude extracts. The sample was dissolved in iso-octane ($\geq 99\%$, Sigma-Aldrich, Germany) and filtered through a syringe filter prior to injection. The column was programmed, and the analysis was carried out as previously described (Pugazhendi et al., 2017). Identification of volatile compounds from the extracts was done by comparing the retention time and mass spectra with those of mass spectral library search of the National Institute of Standards and Technology library using the similarity index

(<92%). Also, the GC-MS data were compared with those of the antibiofilm agent resource database “aBiofilm” (Rajput et al., 2017) and other relevant literature to identify compounds with known antibiofilm activity.

Collection and preparation of test and reference ligands

The compounds identified from the extract of the strain AA8 through GC-MS were tested for antibiofilm activity against the BAP using an *in silico* approach. For that, the molecular target BAP (PDB ID-7C7U) was downloaded from the protein databank. Following this, the water molecules and hetero-atom (hetatm) associated with the BAP were removed using the software Biovia Discovery Studio Visualizer (BDVS) (Figure 1). Also, the structures of the identified compounds and the reference ligand were downloaded from PubChem. The details of the identified compounds and the reference are given in Table 1 and in Supplementary Figures S1–S3.

Docking analysis

The docking software PyRx was used to analyze the antibiofilm property of the compounds identified in the bacterial extract against the BAP. In brief, the target protein and the test ligands were carried over to the docking (PyRx) software and converted into PDBQT files. Thereafter, the ligand-binding locations on the receptor protein were marked with the grid box, and the docking was run. Once the docking was over,

the docking scores were noted, and the necessary files (receptor and ligands) were carried over to BDSV to visualize the interaction of the ligands with the receptors.

Results

Identification of sponge-associated bacterium

The sponge-associated bacterium isolated was described as a Gram-negative rod and motile. The colonies appear as non-pigmented white colonies that are transparent, circular, and raised with an entire margin (Supplementary Figure S4). The biochemical reaction of the organism to oxidase was negative while that to catalase reagent was positive. Analysis of the 16S rRNA gene sequence with BLAST (restricted to sequence from type material) revealed close similarity with members of the *Alcanivorax* genus. It shows close identity similarity with *Alcanivorax diesolei* (99.33%), *A. xenomutans* (99.54%), and *A. balearicus* (98.45), and the next nine members of the genus *Alcanivorax* have identity similarities between (93.33%–94.88%). The phylogenetic tree of the organism (*Alcanivorax* sp. AA8) was constructed with other similar species (Figure 2).

Antibacterial activity of the extracts

The crude extracts obtained from *Alcanivorax* sp. AA8 associated with the sponge *S. siphonella* exhibited antibacterial activity against the tested biofilm bacteria. The crude extracts

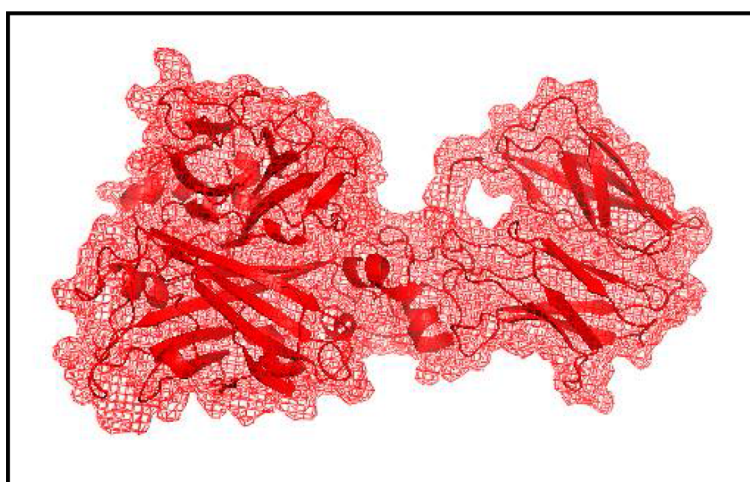


FIGURE 1

The structure of the molecular target, the biofilm-associated protein (BAP) after the removal of water molecules and HETATM. The BAP was downloaded from RCSB protein data bank (PDB ID: 7C7U, Ma et al. 2021a; Ma et al. 2021b) and modified structure was created using Biovia Discovery Studio Visualizer.

TABLE 1 Details of the ligands (reference compound and the compounds identified from the extract of the bacterium *Alcanivorax* sp. AA8) downloaded from PubChem.

S. No.	Ligands	Molecular weight	Molecular formula	PubChem ID
1	Butenolide	84.07	C ₄ H ₄ O ₂	10341
2	Heptane, 2,5-dimethyl-	128.25	C ₉ H ₂₀	16662
3	Dodecanoic acid	200.32	C ₁₂ H ₂₄ O ₂	3893
4	Cyclopropaneoctanoic acid, 2-octyl-, methyl ester	310.5	C ₂₀ H ₃₈ O ₂	543406
5	1-Hexadecanol	242.44	C ₁₆ H ₃₄ O	2682
6	Octadecanoic acid, 3-hydroxypropyl ester	342.6	C ₂₁ H ₄₂ O ₃	168316
7	Tetradecanoic acid	228.37	C ₁₄ H ₂₈ O ₂	11005
8	Cis-11-Eicosenamide	309.5	C ₂₀ H ₃₉ NO	5365374
9	Hexadecanoic acid, ethyl ester	284.5	C ₁₈ H ₃₆ O ₂	12366
10	Eicosane	282.5	C ₂₀ H ₄₂	8222
11	n-Pentadecanol	228.41	C ₁₅ H ₃₂ O	12397
12	Pentadecanoic acid, methyl ester	256.42	C ₁₆ H ₃₂ O ₂	23518
13	Hexadecanoic acid, methyl ester	270.5	C ₁₇ H ₃₄ O ₂	8181
14	l-(+)-Ascorbic acid 2,6-dihexadecanoate	652.9	C ₃₈ H ₆₈ O ₈	54722209
15	Heptadecanoic acid, methyl ester	284.5	C ₁₈ H ₃₆ O ₂	15609

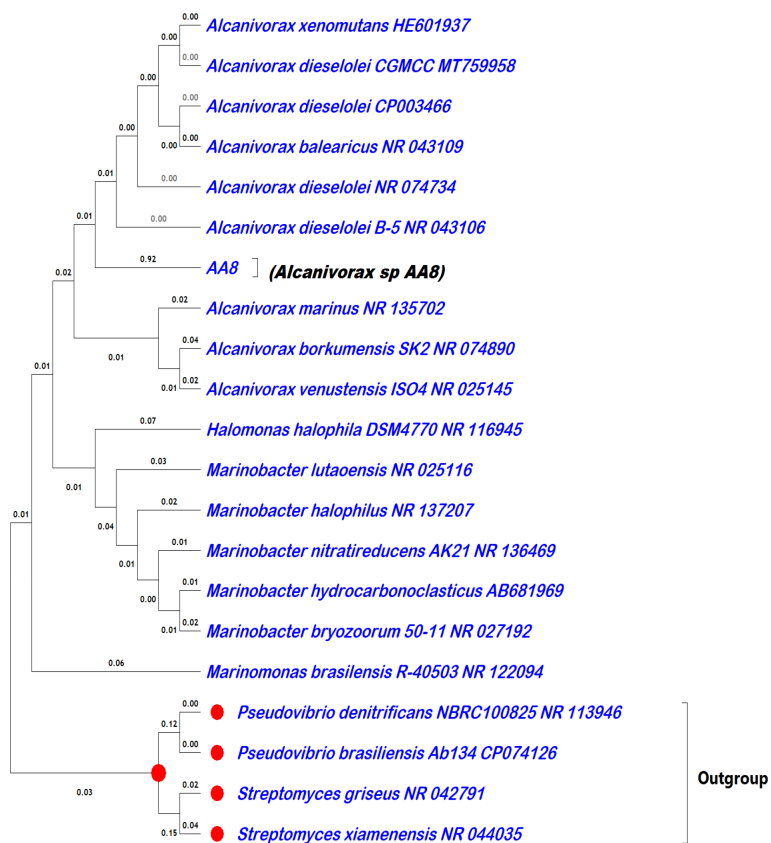


FIGURE 2

Phylogenetic tree constructed using MEGAX. The organism isolated in this study was labeled as AA8 with the full name of the organism in brackets.

TABLE 2 Zone of inhibition and minimum inhibitory concentration (MIC) of extracts from *Alcanivorax* sp. AA8 against biofilm bacteria.

Organism	Zone of inhibition (mm)		MIC (mg ml ⁻¹)	
	AE1	AE2	AE1	AE2
<i>Pseudoalteromonas</i> sp. IMB1	18 ± 2	20 ± 1	12.5	12.5
<i>Halomonas</i> sp. IMB2	16 ± 2	19 ± 0	12.5	3.125
<i>Vibrio alginolyticus</i> IMB11	8 ± 1	14 ± 0	6.25	6.25
<i>Pseudoalteromonas gelatinilytica</i> IMB14	16 ± 0	18 ± 1	12.5	12.5
<i>Pseudoalteromonas gelatinilytica</i> IMB15	11 ± 2	16 ± 2	12.5	6.25

Key: IMB1, *Pseudoalteromonas* sp. IMB1 (ON003955); IMB2, *Halomonas* sp. IMB2 (ON415519); IMB11, *Vibrio alginolyticus* IMB11 (ON003958); IMB14, *Pseudoalteromonas gelatinilytica* IMB14 (ON003961); IMB15, *Pseudoalteromonas gelatinilytica* IMB15 (ON003962). AE1, extract of the culture supernatant; AE2, biomass extract.

obtained from *Alcanivorax* sp. (AA8) were labeled as *Alcanivorax* extract 1 (AE1) and *Alcanivorax* extract 2 (AE2) respectively for the extract of the culture supernatant and biomass extract. The results of the antibacterial activity of extracts with their respective MIC against the five biofilm bacteria are presented in Table 2. The results showed that the extracts have varying activities from weak (mild) activity to strong activity against the tested organisms. All of the strains were sensitive to the extracts differently. The extracts recorded 70% moderate activity, 20% mild or weak activity, and 10% strong activity. AE1 recorded moderate activity toward all of the strains except *Pseudoalteromonas* sp. IMB1 and *V. alginolyticus* IMB11, while AE2 demonstrated the highest activity with a wider zone of inhibition against all of the tested organisms. Extracts from the biomass culture (AE2) recorded higher activity than extracts from the culture supernatant (AE1). *Halomonas* sp. IMB2, *V. alginolyticus* IMB11, *P. gelatinilytica* IMB14, and *P. gelatinilytica* IMB15 strains have mild to moderate sensitivity to the extracts, while *Pseudoalteromonas* sp. IMB1 has mild, moderate, and strong sensitivity to the extracts. Strong activity was recorded only by AE2 against *Pseudoalteromonas* sp. IMB1. The extracts recorded MIC values of 3.125, 6.25, and 12.5 mg ml⁻¹. The least MIC recorded for AE1 was 6.25, while the least MIC for AE2 was 3.125. The AE2 has better MIC than AE1, maintaining similar trends with the result of antibacterial activity based on the zone of inhibition.

Biofilm inhibition activity

The biofilm inhibition activity of the extracts shows their ability to reduce/inhibit biofilm development of the target organisms. Extracts of the culture supernatant (AE1) recorded a higher biofilm inhibition activity than the biomass extract (AE2) against all of the strains except *P. gelatinilytica* IMB15 as shown in Figure 3. The extracts recorded 71% as the highest inhibition activity and 47% as the lowest across the five biofilm-forming strains. The highest was recorded by AE1 against *P.*

gelatinilytica IMB15, while the lowest was recorded against *P. gelatinilytica* IMB14 by AE1.

Chemical composition of the extracts

GC-MS results (Figure 4) detected several volatile organic compounds from the extracts (Table 1). Some of the detected compounds that have been reported in the literature with antibiofilm properties are presented in Table 3. Compounds detected in the extracts with a known antibiofilm activity include tetradecanoic acid; 1-hexadecanol; dodecanoic acid; and heptadecanoic acid, methyl ester from the culture supernatant extract (AE1) and eicosane and hexadecenoic acid, methyl ester from the biomass extract (AE2).

Docking analysis

The docking analysis results of the test ligands (identified compounds) and the reference ligand (butenolide) with the molecular target BAP are given in Tables 4 and 5. The binding affinity of the reference butenolide with the target BAP was -3.9 kcal/mol. Whereas the binding affinities of the compounds identified in the extract of the culture supernatant of the bacterium *Alcanivorax* sp. AA8 such as heptane, 2,5-dimethyl; dodecanoic acid; cyclopropanoic acid, 2-octyl-, methyl ester; 1-hexadecanol; octadecanoic acid, 3-hydroxypropyl ester; tetradecanoic acid; cis-11-eicosenamide; and hexadecanoic acid, ethyl ester were -4.7, -5.1, -5, -4.5, -4.7, -5.5, -5.7, and -5.7 kcal/mol, respectively. Similarly, the binding affinity of the compounds identified in the biomass extract of the bacterium *Alcanivorax* sp. AA8 including eicosane; n-pentadecanol; pentadecanoic acid, methyl ester; hexadecanoic acid, methyl ester; l-(+)-ascorbic acid 2,6-dihexadecanoate; and heptadecanoic acid, methyl ester were -4.1, -4.5, -4.5, -4.3, -5.2, and -4.5 kcal/mol, respectively.

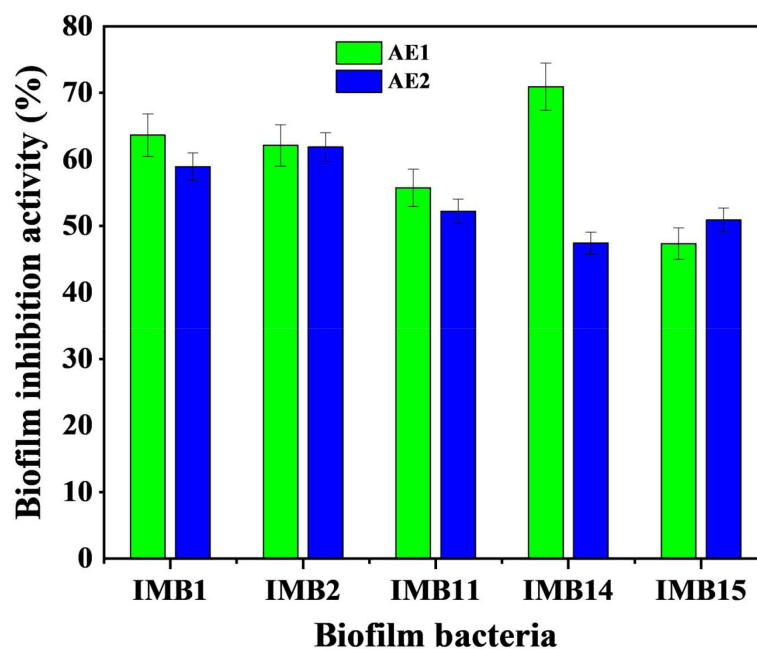


FIGURE 3

Biofilm inhibition activity of crude extracts from *Alcanivorax* sp. AA8 bacteria against five biofilm bacteria. Each data point represents an average of eight replicates and SD. Key: IMB1, *Pseudoalteromonas* sp. IMB1 (ON003955); IMB2, *Halomonas* sp. IMB2 (ON415519); IMB11, *Vibrio alginolyticus* IMB11 (ON003958); IMB14, *Pseudoalteromonas gelatinilytica* IMB14 (ON003961); IMB15, *Pseudoalteromonas gelatinilytica* IMB15 (ON003962).

Visualization of ligand–protein interaction

According to the data obtained from BVDS, the ligands were found to make interactions with various amino acid residues of the BAP with H-bond, Alkyl, Pi-Alkyl, and Pi-Sigma bonding (Tables 4, 5). The reference ligand butenolide interacted with amino acid residues (THR722) of the receptor BAP, whereas the number of amino acid residues that interacted with the test ligands ranged from 2 to 6. Overall, a total of 43 amino acid residues of the receptor protein BAP were found to be involved with the ligands (compounds identified in the bacterial extract) (Figures 5, 6).

Discussion

Isolation of *Alcanivorax* sp. from sponge host

Alcanivorax sp. was isolated from the sponge *S. siphonella* collected from the Red Sea. The bacteria *Alcanivorax* is widely distributed in marine environments occupying different depths and habitats as free-living or in association with other marine organisms. It is found on the surface water and surface

sediments, deep seawater, and deep-sea sediments (Lai et al., 2013; Barbato et al., 2015; Lai et al., 2016; Karthick and Mohanraju, 2018; Song et al., 2018; Liu J. et al., 2019; Liao et al., 2020). It has been reported to be living in a symbiotic relationship with several organisms in the marine environment such as seaweeds and microalgae (Karthick and Mohanraju, 2018; Chernikova et al., 2020). Only rare reports exist on the presence of the *Alcanivorax* genus in association with sponges such as its association with sponges collected from lower mesophotic reefs (Indraningrat et al., 2019).

Bacterial growth inhibition activity of extracts of *Alcanivorax* sp.

The extracts from the sponge-associated *Alcanivorax* sp. demonstrated good antibacterial activity against the biofilm bacteria. There is a broad spectrum of activity across the organism due to the large size of the zone of inhibition against the growth of the tested organisms. The strong antibacterial activity of the extract of *Alcanivorax* sp. isolated in this study could be attributed to the bioactive compounds present in the extract that inhibited the growth of the tested organisms. Antibacterial compounds such as n-tridecan-1-ol were observed in AE1, while n-tridecan-1-ol and n-pentadecanol

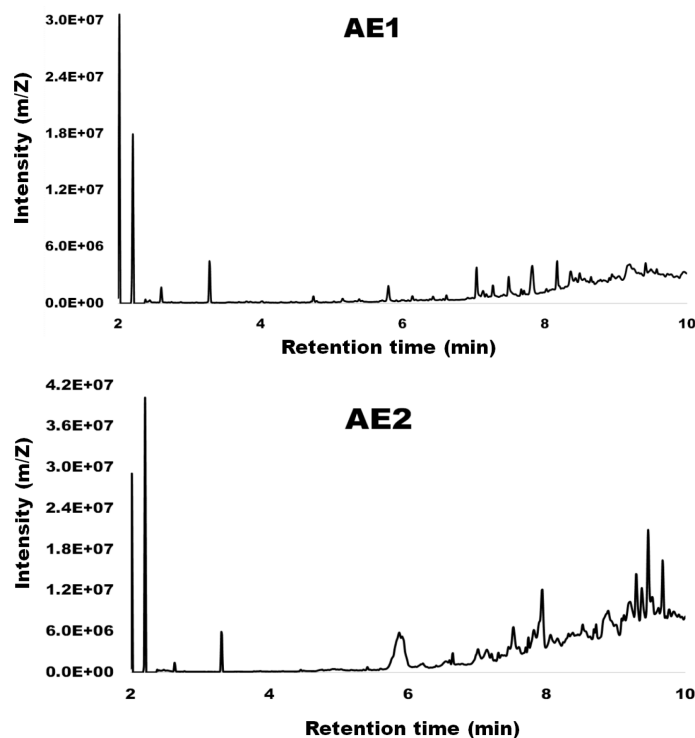


FIGURE 4

Spectrum of compounds detected by gas chromatography-coupled mass spectrometry (GC-MS) from the culture supernatant extract (AE1) and biomass crude extract (AE2) of *Alcanivorax* sp. AAS.

were detected in AE2. Both compounds are long-chain fatty alcohols with previously reported antibacterial activity (Togashi et al., 2007). The AE2 extract has more antibacterial compounds than AE1, which might have contributed to the higher antibacterial activity it recorded in the assay. Members of the *Alcanivorax* genus are well-known as hydrocarbooclanistic bacteria associated with petroleum hydrocarbon degradation (Qiao and Shao, 2010; Barbato et al., 2015; Chernikova et al., 2020). However, they produced enzymes that extend their

activity beyond oil degradation by performing other activities such as polyester degradation (Zadjelovic et al., 2020). Bioactive compounds have been reported from members of the *Alcanivorax* genus (Debnath et al., 2007). The chemical defense role of marine symbiotic members of the *Alcanivorax* genus was reported previously with antimicrobial activity against different bacteria (Pereira et al., 2017). A high antimicrobial activity was reported earlier from symbiotic *Alcanivorax dieselolei* that was found in association with seaweeds

TABLE 3 Antibiofilm compounds detected from *Alcanivorax* sp. AAS extracts.

Compounds	Molecular formula	Molecular weight	Peak area	Retention time	Type of compound	Activity	Reference
Dodecanoic acid ¹	C ₁₂ H ₂₄ O ₂	200.3178	5.29	7.492	Free fatty acid	Antibiofilm	(Jin et al., 2021)
Eicosane ²	C ₂₀ H ₄₂	282.5475	4.24	9.575	Straight-chain alkane	Antibiofilm	(Beema Shafreen et al., 2022)
Hexadecanoic acid, methyl ester ²	C ₁₇ H ₃₄ O ₂	270.4507	13.98	9.255	Fatty acid esters	Antibiofilm	(Dusane et al., 2011)
Hexadecanoic acid, ethyl ester ²	C ₁₈ H ₃₆ O ₂	284.5	4.24	9.575	Fatty acid esters	Antibiofilm	(Wang et al., 2018)
1-Hexadecanol ¹	C ₁₆ H ₃₄ O	242.44	6.65	8.172	Fatty acid alcohol	Antibiofilm	(Manilal et al., 2020)
Tetradecanoic acid ¹	C ₁₄ H ₂₈ O ₂	228.3709	6.68	8.49	Free fatty acid	Antibiofilm	(Rajalaxmi et al., 2016)

Key: 1, extracts from the culture supernatant; 2, biomass extract.

TABLE 4 The docking result of the compounds identified in the culture supernatant extract of the bacterium *Alcanivorax* sp. AA8 carried out using the software PyRx.

S. No.	Ligands	Binding affinity	Amino acids involved	Interaction Bond
1	Butenolide	-3.9	THR722	H-bond
2	Heptane, 2,5-dimethyl-	-4.7	LEU528, LEU720	Alkyl
3	Dodecanoic acid	-5.1	ARG357, ILE419, HIS423, ASP452	H-bond, Alkyl, Pi-Alkyl
4	Cyclopropaneoctanoic acid, 2-octyl-, methyl ester	-5	ALA373, LEU376, TYR400, LEU472, M ET576, PRO660	H-bond, Alkyl, Pi-Alkyl
5	1-Hexadecanol	-4.5	TYR767, VAL850	Alkyl, Pi-Alkyl, Pi-Sigma
6	Octadecanoic acid, 3-hydroxypropyl ester	-4.7	TYR370, PHE372, ALA373, TYR400, LEU472	H-bond, Alkyl, Pi-Alkyl
7	Tetradecanoic acid	-5.5	PRO356, GLY358, SER359	H-bond, Alkyl, Pi-Alkyl
8	Cis-11-Eicosenamide	-5.7	ALA373, TYR400, LEU472, TYR613	Alkyl, Pi-Alkyl, Pi-Sigma
9	Hexadecanoic acid, ethyl ester	-5.7	LEU364, LYS415, PHE508, ASP701, ALA726	H-bond, Alkyl, Pi-Alkyl

(Karthick and Mohanraju, 2018). This is an indication of the role of *Alcanivorax* associated with sponge as the ideal source of antibacterial compounds.

Inhibition of biofilm formation

Extracts from the sponge-associated bacterium *Alcanivorax* sp. recorded a good biofilm inhibition activity against all of the tested biofilm bacteria. Antibiofilm agents from sponge holobionts have proven to be good candidates for tackling biofilm formation (Santos-Gandelman et al., 2014). Many of the sponge-associated bacteria are considered as the source of antibiofilm agents that can inhibit biofilm formation through its multiple targets (Rajput et al., 2017). The good antibiofilm activity recorded in this study is a confirmation of the role of the symbiotic bacteria in protecting the sponge host against invading biofilm bacteria. The promising results obtained from this study are interesting due to the recalcitrant nature of biofilm bacteria and its negative economic impact on the affected industries (Cámara et al., 2022). To overcome the biofilm challenge, inhibition of biofilm on surfaces is a requirement for sustainable growth of the affected industries. More antibiofilm agents were identified through GC-MS analysis from the AE1 extract than AE2, which could be one of the possible reasons for the higher antibiofilm activity recorded by

AE1. *Alcanivorax* sp. is known to produce biosurfactants and many enzymes that have contributed to its ability to produce bioactive compounds (Zadjelovic et al., 2020). *Alcanivorax* sp. therefore has a great potential application as source of new compounds with multiple biological activities. This suggests that sponges can attract diverse microorganisms with the ability to produce compounds that can protect against predators and colonization.

Chemical compounds from *Alcanivorax* sp. extracts

From the extracts, several compounds were detected through the GC-MS analysis. The result further confirms the potential of the *Alcanivorax* sp. to produce secondary metabolites with bioactive properties. The compounds detected in the extracts are mostly fatty acids and their derivatives. Fatty acid-derived compounds are secondary metabolites produced by diverse marine symbiotic organisms with antibacterial, antibiofilm, and antifouling properties (Kaspar et al., 2019; Supardy et al., 2019; Ashitha et al., 2020; Kai, 2020). Fatty acids affect the cell membrane of the bacterial cell and cause disruption by solubilizing the membrane due to their detergent properties (Desbois and Smith, 2010). Fatty acid activity can be either antimicrobial activities in high doses or antibiofilm activities in

TABLE 5 The docking result of the compounds identified in the biomass extract of the bacterium *Alcanivorax* sp.

S. No.	Ligands	Binding affinity	Amino acids involved	Interaction Bond
1	Butenolide	-3.9	THR722	H-bond
2	Eicosane	-4.1	ALA373, TYR400, LEU472, TYR613	Alkyl, Pi-Alkyl
3	n-Pentadecanol	-4.5	ALA373, SER374, TYR400, LEU472, THR662	H-bond, Alkyl, Pi-Alkyl
4	Pentadecanoic acid, methyl ester	-4.5	VAL355, GLY358, SER359, PRO418,	H-bond, Alkyl, Pi-Alkyl
5	Hexadecanoic acid, methyl ester	-4.3	ALA373, TYR613, ASN656	H-bond, Alkyl
6	l-(+)-Ascorbic acid 2,6-dihexadecanoate	-5.2	LYS775, PRO793, PRO868, VAL877, GLN938, ILE939, THR940	H-bond, Alkyl
7	Heptadecanoic acid, methyl ester	-4.5	LYS502, TYR526, ASP532, GLN596, LYS599, VAL639	H-bond, Alkyl

The docking result of the compounds identified in the biomass extract of the bacterium *Alcanivorax* sp. AA8 carried out using the software PyRx.

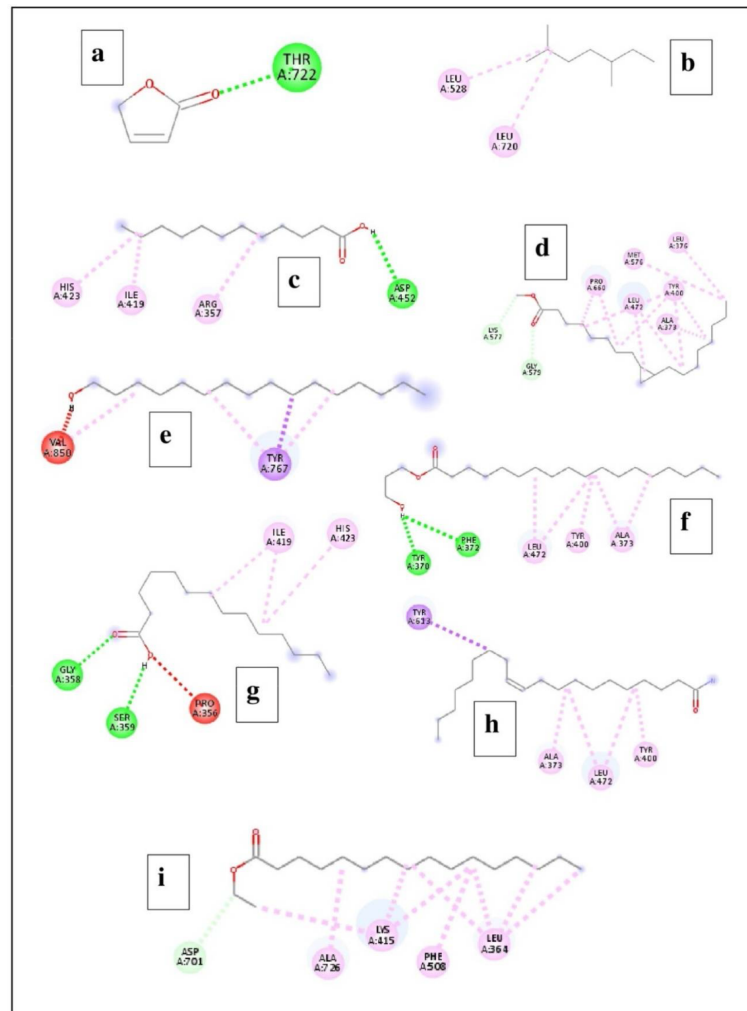


FIGURE 5

The interaction of compounds identified in the extract from the culture supernatant of *Alcanivorax* sp. AAB with the target biofilm-associated protein (BAP). (A) Butenolide; (B) Heptane, 2,5-dimethyl; (C) Dodecanoic acid; (D) Cyclopropaneoctanoic acid, 2-octyl-, methyl ester; (E) 1-Hexadecanol; (F) Octadecanoic acid, 3-hydroxypropyl ester; (G) Tetradecanoic acid; (H) Cis-11-eicosenamide; and (I) Hexadecanoic acid, ethyl ester.

low doses or synergistic effects with both antimicrobial and antibiofilm activities (Park et al., 2022). Fatty acids are an important component of the bacterial cell that is involved in cell signaling and bacterial adaptation, which is an important requirement in biofilm signal control (De Carvalho and Caramujo, 2018). Fatty acids could be responsible for the inhibition of the biofilm formation under static conditions. Fatty acids can control biofilm development against diverse microbes through many targets, especially by reducing the biomass of the biofilm bacteria, suppressing the cell-to-cell communication expression or quorum quenching (Kumar et al., 2020). Fatty acid compounds have the potential to enable symbiotic bacteria to protect their host against colonization by

biofilm bacteria on its surface due to their ability to interfere with the biofilm formation ability (Khadke et al., 2021). Fatty acids have been reported from several marine bacteria with biofilm inhibition activity against biofilm bacteria including Gram-negative bacteria (Rajput et al., 2017). For instance, the biofilm biomass of *Vibrio* has been reduced significantly by palmitic acid, a free fatty acid, by blocking the formation of biofilm through the suppression of cell-to-cell communication (Santhakumari et al., 2017). Free fatty acids such as tetradecanoic acids and dodecanoic acids that were detected in this study were reported to play key roles in providing protection to hosts against opportunistic organisms (Karimi et al., 2015). Another compound, eicosane, detected in the extract AE2 has been reported with significant

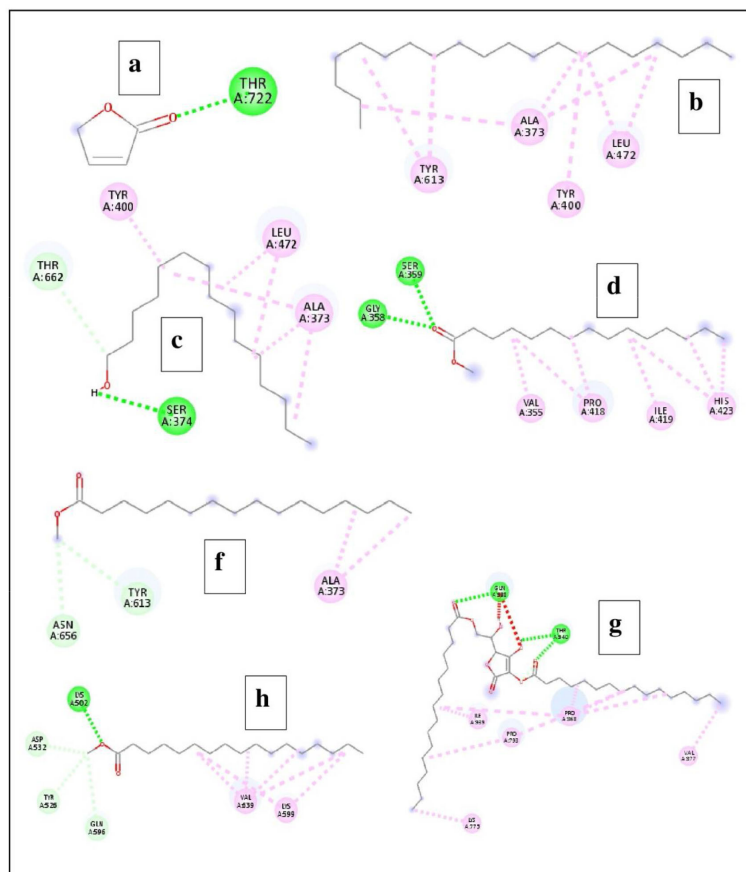


FIGURE 6

Illustrate the interaction of compounds identified in the biomass extract of the bacterium *Alcanivorax* sp. AA8 with the target biofilm-associated protein (BAP). (A) Butenolide; (B) Eicosane; (C) n-Pentadecanol; (D) Pentadecanoic acid, methyl ester; (E) Hexadecanoic acid, methyl ester; (F) l-(+)-Ascorbic acid 2,6-dihexadecanoate; and (G) Heptadecanoic acid, methyl ester.

antibiofilm activity (Beema Shafreen et al., 2022). Also, hexadecanoic acid, methyl ester and hexadecanoic acid, ethyl ester detected respectively in AE1 and AE2 have antibiofilm potentials and are associated with biosurfactant formation in an endophytic bacterium (Ashitha et al., 2020). Biosurfactant production is an important characteristic of *Alcanivorax* sp., which is an essential requirement for inhibiting the biofilm formation and biofouling growth (Qiao and Shao, 2010; Alemán-Vega et al., 2020; Mishra et al., 2020). The detection of fatty acids in the extracts along with strong antibiofilm activity in this study indicated the potential of sponge-associated *Alcanivorax* as a good source of antibiofilm compounds.

In silico analysis

The *in silico* analysis of the compounds detected from the two extracts of *Alcanivorax* sp. AA8 showed significant

antibiofilm properties. The identified compounds strongly interacted with the molecular target, the BAP. The BAP is a surface protein that plays an important role in primary adhesion, intercellular adhesion, and biofilm formation (De Gregorio et al., 2015). The BAP is generally present in both Gram-positive and Gram-negative bacteria (Cucarella et al., 2004). Therefore, it is assumed that the interaction of the compounds with the target BAP can interfere with the function of the BAP, which has an important role in biofilm formation. Notably, the binding affinity of the identified compounds with the target BAP was better than the binding affinity of the reference ligand butenolide made with the target BAP. Butenolide is a lactone that has broad-spectrum antibiofilm activity (Yin et al., 2019). Comparatively, the compounds present in the bacterial extracts showed better antibiofilm activity than the antibiofilm agent butenolide. Both the *in silico* and the experimental results demonstrated the ability of the bacterium to produce compounds with antibiofilm properties.

Conclusion

This study revealed the antibiofilm activity of the *Alcanivorax* sp. AA8 associated with a sponge from the Red Sea. The potential secondary metabolites detected in the extract of the bacterium may serve as promising antibiofilm compounds to tackle the attachment of biofilm bacteria to surfaces. Though further studies are required to identify the compounds responsible for the observed antibiofilm activity, the results demonstrated that bacteria associated with marine sponges could be used as a prospective source for natural antibiofilm and antifouling compounds to control biofouling.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/[Supplementary Materials](#).

Author contributions

SS designed the study, analysed the data and conducted *in silico* analysis, MJ and SS performed the experiments and wrote the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2022.980418/full#supplementary-material>

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