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Activity of Cell-Free Supernatant from Sponge-Associated Bacteria against the Growth and Bacterial Adhesion of Microfouling Bacteria

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Abstract. This study was carried out to evaluate the activity of cell free supernatant (CFS) from sponge associated bacteria against the growth and bacterial adhesion of biofilm bacteria. A sponge was collected from the Red Sea by Scuba diving and identified as *Hyrtios erectus*. After washing and cleaning to remove loosely attached bacteria, endophytic bacteria were isolated from the sponge by serial dilution method using Zobell Marine Agar (ZMA) as growth medium. Pure culture of the bacteria obtained were then identified by 16s rRNA gene sequencing method. The cell free supernatant (CFS) from two strains of bacteria isolated from the sponge were subjected to bacterial growth inhibition and bacterial adhesion inhibition assay using bacterial growth inhibition assay method and microtiter plate assay method respectively against four microfouling bacteria. The bacteria are *Halomonas* sp., *Psychrobacter* sp., *Pseudoalteromonas shioyasakiensis*, and *Vibrio alginolyticus*. The two organisms were identified as *Idiomarina* sp. MS-E2 and *Staphylococcus edaphicus* MS-E4. Both organisms show potential inhibition activity against the tested biofilm bacteria by inhibiting their growth and adhesion ability. This is an indication of the role of the sponge associated bacteria in producing compounds with potential activity against bacterial adhesion to surfaces.

Keywords: Sponges, antimicrobial, antibiofilm, bacterial adhesion, sponge-bacteria association.

1. Introduction

Sponges are benthic sessile invertebrates and members of the phylum Porifera. They are always found attached to substrates in water and among the earliest and simplest groups of animals that are multicellular filter feeders that actively pump volumes of water through their pore system (Alexander 2015). Sponges feed on free-living bacteria and larger phytoplankton cells that are drawn into pores where they are captured and phagocytosed (Leys *et al*. 2011). The water flow ensures a supply of oxygen for respiration via diffusive oxygen uptake. They are an ancient and diverse group of ubiquitous, sessile, filter-feeding marine animals that can absorb and withhold fluids through their pores (Larsen and Riisgård 2022). Sponges have been recognized as a rich source of natural products with diverse chemical structures and biological activities, including antifouling, anticancer, antimicrobial, and antiviral properties (Carroll

et al. 2021). The chemical diversity of sponge-derived natural products is largely due to the unique and complex microbial communities that inhabit these animals (Anteneh *et al*. 2021). Sponges host diverse communities of microorganisms, including bacteria, archaea, fungi, and viruses, that are responsible for the production of many of the bioactive compounds found in these animals (Liu *et al*. 2019). These microorganisms may have evolved in close association with sponges, benefiting from the nutrient-rich environment created by the sponge and providing the sponge with chemical defenses against predation and microbial infection (Balskus 2014).

The bioactive compounds produced by sponge-associated microorganisms are typically small molecules, ranging from simple amino acids and peptides to complex terpenes, alkaloids, and polyketides that exhibited potent biological activities, making them promising candidates for drug discovery and development (Alsed *et al*. 2016). Natural products derived from sponge associated microbes have also shown potential in the development of new antifouling agents for the prevention of biofouling on marine surfaces (Satheesh *et al*. 2016). Sponge-associated microbes have been found to produce bioactive compounds that can inhibit bacterial adhesion and biofilm formation (Rosenthal *et al*. 2014; Rashiya *et al*. 2021; Rizzo *et al*. 2021). This can help in mitigating bacterial adhesion to surfaces and biofilm formation. Bacterial adhesion is a critical step in the process of biofilm formation, which can lead to the biofouling of surfaces (Karygianni *et al*. 2020). Studies have shown that bacteria found in association with sponges can produce bioactive compounds in form of secondary metabolites that can interfere with bacterial adhesion and biofilm formation (Rashiya et al. 2021). The use of such compounds may provide a more environmentally friendly and cost-effective alternative to traditional antifouling agents that are applied to surfaces to prevent biofilm adhesion. However, there is need to explore and identify sponge-associated microbes that inhibit bacterial adhesion and to identify and characterize the specific bioactive compounds responsible for this activity to explore the chemical diversity and their biotechnological potential.

Utilizing cell-free supernatant from bacteria associated with sponges is a promising strategy for evaluating prospective antibiofilm or antifouling agents (Selvin *et al*. 2009). This approach entails extracting secondary metabolites produced by the bacteria using appropriate fermentation conditions. The use of cell-free supernatant is cost effective and can reduce certain risk in the use of live bacteria such as toxicity and environmental pollution and may also offer a more economical and scalable option (Pelyuntha *et al*. 2020). Nevertheless, it is necessary to optimize extraction process for cell-free supernatant and identify the bioactive compounds presence in the cell free supernatant. Hence, in this study, an attempt has been made to evaluate the activity of cell free supernatants from the bacteria against the growth and adhesion of microfouling bacteria. The main objective of this study was to understand the bioactivity of cell free supernatant of the bacteria associated with sponge. The results obtained in this study will be relevant in establishing the role of secondary metabolites produced by marine bacteria associated with sponges.

2. Materials and Methods

2.1 Media and Chemicals

All media used were purchased from Himedia, India. Chemicals from Honeywell, USA. Molecular kits for molecular microbial studies were purchased from Invitrogen (U.S.A) and Qiagen (Germany).

2.2 Sample Collection

The sponge sample was collected by Scuba diving from the Obhur creek, Jeddah part of the Central Red Sea coast (N21°42.562′ E39°05.764′) at a depth of 1 – 15 m and stored at -20 ^{o}C. It was identified using previously existing keys for identification of Red Sea sponges based on observable characters as reported previously (Myers and Fiedler, 2004). A voucher of the specimen was deposited in the Department of Marine Biology, King Abdulaziz University, Jeddah, Saudi Arabia. The organism is an erect finger sponge described and identified as *Hyrtios erectus*.

2.3 Isolation of Sponge- Associated Bacteria

The study aimed to isolate bacteria associated with the sponge *Hyrtios erectus*. Bacteria associated with the sponge *Hyrtios erectus* were isolated by initially washing the sponge several times with 0.22 µ filtered sea water (FSW) to remove loosely attached microbes, cut into pieces, and then immersed in FSW for serial dilution. The samples were inoculated in Zobell marine Agar (ZMA) (Himedia, India) and incubated at 28^{o}C. The resulting colonies were sub-cultured to obtain pure culture and identified morphologically and by 16S rRNA method.

2.4 Isolation of genomic DNA, PCR amplification and sequencing

The genomic DNA of the isolates was extracted using a DNA extraction kit. Genomic DNA from each of the bacterial strain was extracted by first harvesting the bacterial cells through centrifugation for 10 min. at 5000g. The next step was the resuspension of the pellet in 180 µL of digestion solution, then addition of proteinase K solution and mixed thoroughly by vortexing to obtain a uniform suspension. This was then followed by the subsequent addition of 20 µL of RNase A solution, mixed by vortexing and incubated for 10 minutes at room temperature. Next was the addition of 200 µL of lysis solution to the sample and then addition of 400 µL of 50% ethanol and mixed by vortexing to obtain the lysate. The prepared lysate was then purified using GeneJET Genomic DNA Purification kit (ThermoFisher) according to the manufacturer's instruction. The extracted and purified DNA of the isolates was then confirmed using agarose gel electrophoresis, viewed with UVP 310 GelDoc-It Imager. The 16S rRNA gene of each bacterium was amplified by PCR with universal primers and confirmed by gel electrophoresis. The DNA was subjected to PCR to amplify the 16S rRNA gene using universal primers: 27F (5' - AGAGTTTGATCMTGGCTCAG - 3') and 1100R (5' - GGGTTGCGCTCGTTG - 3') in a Multigene thermal Cycler (Labnet Inc). The PCR conditions are as follows: one cycle of 95°C for 5 min. followed by 28 cycles of 95°C for 45 sec., and annealing at 58°C for 45 sec. with extension at 72°C for 1m 45sec, and a final extension step at 72°C for 10 min. The amplified DNA fraction was confirmed with agarose gel electrophoresis as described above. The PCR products were purified using GeneJET PCR Purification Kit according to manufacturer's instructions. For further studies (sequencing) of amplified 16S rRNA genes, samples of purified PCR product were sent to Macrogen Inc., South Korea. Taxonomic groups were assigned using the RDP classifier (http://rdp.cme.msu.edu/). BLAST programs available at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with search limited to sequences from type materials was applied to select the type strain with the highest BLAST score and nearest match found in GenBank. The threshold adopted between the isolates and the type strain was 97%. Phylogenetic tree construction using Molecular Evolutionary Genetics Analysis (MEGA) neighbor-joining method was performed to determine the evolutionary relatedness of the strains.

2.5 Phylogenetic Analysis

Phylogenetic analysis was performed from the BLAST results as follows: The sequences of the 16s rRNA gene were first aligned using the web version of muscle program by setting the output as ClustalW (Edgar 2004) and then the result was trimmed using trimAL web version to remove poorly aligned regions (Capella-Gutiérrez *et al*. 2009) to obtain a qualitative phylogenetic analysis. The output file was converted to MEGA file format using readAL v 1.3 available on Phylemon 2.0 for evolutionary history and phylogenetic tree construction (Sánchez *et al*. 2011). Neighbor-Joining method developed by Saitou and Nei, (1987) was used to infer the evolutionary history using the Maximum Composite Likelihood method reported by Tamura *et al*. (2004). The evolutionary analysis was done in MEGA X standalone application (Kumar *et al*. 2018). The sequences for the two strains were used with 2 outgroups.

2.6 Cell Free Supernatants from the Bacteria

After isolating the bacteria, they were cultured in 10 ml tubes of Zobell marine broth (ZMB) and incubated for 24 hours at 28°C with shaking. The culture was then standardized to a turbidity of 0.2 at OD600 for uniformity using a spectrophotometer. Zobell marine broth (ZMB) was prepared in 50 ml tubes containing 25 ml of the media. The tubes were inoculated by transferring 1 ml of the culture and incubated at 28 °C for 7 days with agitation of 150 rpm in a shaker incubator. To separate the cell pellets from the fermented medium, the culture was then centrifuged at 6000 rpm for 10 minutes to separate the supernatants from the cell's biomass. The resulting supernatants were filtered using a cellulose filter paper with a pore size of 0.2 µm. To confirm the absence of cells in the filtrates, 100 µL was cultured on ZMA plates at 35 °C for 24 hours. The absence of growth on the plates indicates that the supernatant is free from cells, as described by Sayem *et al*. (2011).

2.7 Antibacterial Bioassay

Cell Free Supernatant from each of the marine bacteria associated with the sponge were used to evaluate the growth inhibition of four different microfouling bacteria involved in biofilm formation on surfaces submerged in marine environment. The tested bacterial strains are *Halomonas* sp. (ON415519), *Psychrobacter* sp. (ON003956), *Pseudoalteromonas shioyasakiensis* (ON003957) and *Vibrio alginolyticus* (ON003958). Bacterial growth inhibition assay using spectrophotometer as described by Ba-akdah & Satheesh (2021) was adopted. The overnight culture of each isolate was adjusted to a standard value of 0.2 at 600 nm with a spectrophotometer by diluting in a fresh media. A 1 ml aliquot of the standardized culture of the tested bacteria was transferred to tubes containing 8 ml ZMB. Next, CFS was added to each tube excluding the control. Sterilized distilled water was added to the control instead. The OD was measured at 600 nm immediately (initial OD) and again after incubation for 8 h (Final OD) at 30°C. The bacterial growth inhibition was calculated as a percentage from equation 1 below. Experiment was carried out in triplicates while the average and the standard deviation was recorded.

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

Where: Final OD is the final optical density at 600nm while initial OD is the initial optical density at 600nm.

2.8 Bacterial Adhesion Inhibition Bioassay

Bacterial adhesion inhibition bioassay was carried out to evaluate the activity of the supernatants against surface adhering bacteria, microfouling bacteria. The four microfouling bacteria involved in biofouling were grown overnight on ZMB and adjusted to an optical

density of 0.2 at 600 nm. Then, 200 µL of the bacterial suspension was inoculated in each well using a multichannel pipette (n = 8). The CFS (50 µl) was added to 6 wells while the remaining 2 served as control. Wells containing 200 µl of media only were used as positive control. The covered plate was incubated for 48 h at 35 °C using horizontal shaking (150 rev min^{-1}). The non-adhered bacteria were eliminated by 5 washings with PBS, and the adhered bacteria were stained with 200 µL crystal violet solution for 45 min. After staining, the plates were washed with sterile distilled water five times. The quantitative analysis of biofilm production was performed by adding 200 µL of 95% ethanol. The OD level of crystal violet staining present in the solution was measured at 570 nm in a microplate reader. The results were expressed as the percentage of inhibition of bacterial adhesion using the equation below.

$$BAIA = [(OD\ control - OD\ test)/OD\ control] \times 100$$

Where BAIA is Bacterial adhesion inhibition activity, OD control is the absorbance of control at 600 nm while the OD test is the absorbance of test sample at 600 nm.

3. Results and Discussion

3.1 Identification of Bacteria associated with the Sponge

The organisms were identified based on the colony morphology on Zobell marine agar plates, cell morphology from microscopy and 16s rRNA sequencing. All the organisms grow under aerobic conditions at temperature of 25 – 30^{o}C. The organisms show different colonial morphology as presented in Fig. 1. The species names were assigned based on the closest relative with the highest score from the BLAST tool as shown in Table 1 using the phylogenetic tree constructed as was presented in Fig. 2. The two organisms were identified as *Idiomarina* sp MS-E2 and *Staphylococcus edaphicus* MS-E4.

Idiomarina is a genus of bacteria and a member of the Gammaproteobacteria class, phylum pseudomonadata. The organism was reported previously from the marine environment and were found in association with sponges and play important roles in the sponge's ecosystem, as they contribute to the sponge's overall health and functioning (Brettar *et al*. 2003; Thomas *et al*. 2010). They can be involved in nutrient cycling, the production of bioactive compounds, and even the defense mechanisms of the sponge against pathogens (Bibi *et al*. 2017). The diversity, specific characteristics and functions of *Idiomarina* species from marine sponges varies depending on the species of bacteria and the sponge they are associated with as was reported previously from the Red Sea sponges (Lee *et al*. 2011).

Staphylococcus edaphicus is known to adapt to extreme environment and was first reported from Antarctica (Pantůček *et al*. 2018). Staphylococcal species have been isolated from a diverse array of human and environmental sources; however, few have been isolated from extreme environments such as Antarctica. The report of this bacterium from sponge is an indication of its presence in the marine environment and can survive in association with marine invertebrates. This is a new and unique information that requires further confirmation. The organism can have a role on the sponge host and will have played a role in the survival of the sponge and produce bioactive secondary metabolites with potential anti-colonization activity.

3.2 Antibacterial Activity

The cell free supernatant from the two bacterial strains inhibited the growth of the microfouling bacteria that forms adhesion on surfaces in the marine environment. The results revealed that the CFS isolated from the two bacteria associated with sponges affect the growth of the microfouling bacteria. Since the

CFS concentration is uniform across all the organisms, the activity cannot be said to be concentration dependent from the results. The activity of CFS from strain MS-E4 range from 72 to 83% while CFS from strain MS-E2 is from 47% to 81% as shown in Fig. 3. More activity of the CFS was recorded against *Psychrobacter* sp. by both extracts at above 80% which is the highest in both cases. This is an indication that the organism is the most susceptible amongst the microfouling bacteria tested. Least activity was recorded by MS-E2 CFS against *Halomonas* sp. (47%). This indicates that the bacteria associated with the sponge have antimicrobial properties. Moderate antimicrobial activity of bacteria associated with sponge has been reported previously (Retnowati and Katili, 2023). In a previous study by El Samak *et al*. (2018), it was reported that bacteria associated with sponges from the Red Sea have high biosynthetic potential to produce bioactive compounds due to the detection of biosynthetic gene clusters in most of its diverse strains. Abd El-Moneam *et al*. (2017) reported the ability of the sponge, *Hyrtios erectus* from the Red Sea to harbor highly significant bacteria that can serve as reservoir for bioactive compounds with multiple activities due the presence of biosynthetic cluster genes for secondary metabolites production. (Abd El-Moneam *et al*. 2017). Antimicrobial activity of sponge bacteria such as members of the genus *Idiomarina* have been reported widely in many studies (Thomas *et al*. 2010; Elhady *et al*. 2021; Amelia *et al*. 2022). The bacteria can provide protection to their host during growth by producing compounds that have antagonistic properties against other bacteria such as pathogens and other invading bacteria. This is an indication that the bacteria isolated from Red Sea sponge can serve as source of potential novel bioactive compounds with several activities including antimicrobial activity against microfouling bacteria.

3.3 Bacterial Adhesion Inhibition Activity

The CFS from the two sponge associated bacteria recorded very low activity against the microfouling bacteria. As presented in Fig. 4, the activity for the CFS from strain MS-E4 range from 0% (against *Halomonas* sp.) to 14 % (*Pseudoalteromonas shioyasakiensis*) where it recorded less than 5% against *V. alginolyticus* and *Psychrobacter* sp. In contrast, strain MS-E2 CFS shows no activity against *Halomonas* sp. and *V. alginolyticus* while displaying activity of 14% against *Psychrobacter* sp. and 5% against *Pseudoalteromonas shioyasakiensis.* The bacterial adhesion inhibition activity demonstrated by the CFS of the two sponge associated bacteria is an indication that the bacteria produce compounds during growth that can interfere with the bacterial adhesion process. Sponge associated bacteria have been reported with the ability to produce antibiofilm compounds. Marine bacteria associated with sponges are reservoir for bioactive compounds that can inhibit the adhesion of bacteria to surfaces (Liang *et al*. 2023).

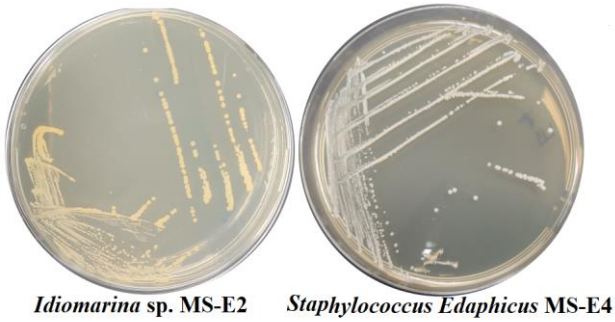

Fig. 1. Bacteria isolated from the Red Sea sponge, *H. erectus.*

Table 1. Morphological and 16S rRNA features for the identification of bacteria isolated from the Red Sea sponge, *H. erectus.*

Laboratory code of the Strain	Colony morphology	Cell Morphology	Closest NCBI Relative	Identification based on nearest type strain	Assigned Accession number
MS-E2	Yellowish, circular, smooth, convex, raised medium colony	Gram negative, motile, rod shaped	*Idiomarina sp.* TPS4- (97.06%) (KM407747.1)	*Idiomarina sp* MS-E2	OQ568869
MS-E4	Circular, smooth, convex, and white, raised, medium colony	Gram positive, motile, rod shaped	*Staphylococcus edaphicus* CCM 8730 (99.5%) (NR_156818.1)	*Staphylococcus edaphicus* MS-E4	OQ568872

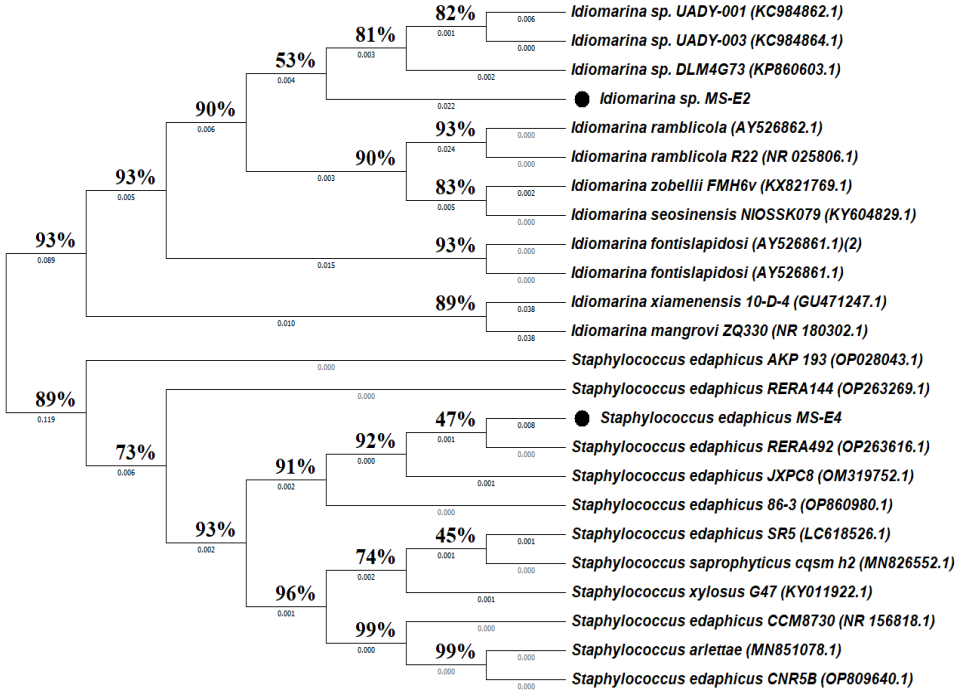

Fig. 2. Phylogenetic tree for the two bacteria isolated from the Red Sea sponge, *H. erectus* and other related species using neighbor joining method in MEGAX.

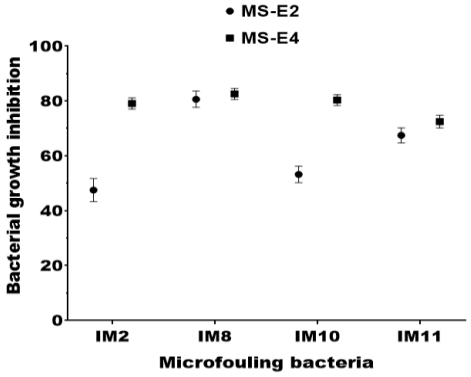

Fig. 3. Antimicrobial activity of CFS from two bacteria isolated from the sponge *H. erectus against* microfouling bacteria. [MS-E2: *Idiomarine* sp. MS-E2, MS-E4: *Staphylococcus edaphicus* MS-E4, IM2: *Halomonas* sp (ON415519), IM8: *Psychrobacter* sp. (ON003956), IM10: *Pseudoalteromonas shioyasakiensis* (ON003957), and IM11: *Vibrio alginolyticus* (ON003958)].

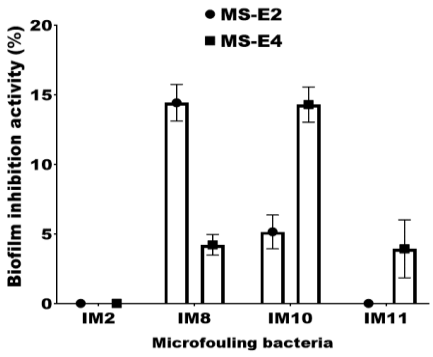

Fig. 4. Bacterial adhesion inhibition activity of CSF from two bacteria isolated from the sponge *H. erectus* against microfouling bacteria. [MS-E2: *Idiomarine* sp. MS-E2, MS-E4: *Staphylococcus edaphicus* MS-E4, IM2: *Halomonas* sp (ON415519), IM8: *Psychrobacter* sp. (ON003956), IM10: *Pseudoalteromonas shioyasakiensis* (ON003957), and IM11: *Vibrio alginolyticus* (ON003958)].

4. Conclusions

The sponge, *H. erectus* widely reported from the Red Sea is associated with diverse bacteria which are important for its survival. These bacteria play significant role in providing the sponge with protection against invading pathogens and fouling organisms. They achieved this by producing bioactive compounds. The bacteria are potential sources of novel bioactive compounds that can inhibit the growth of microfouling bacteria that attach to surfaces when submerged in the marine environment. The bacteria can also produce compounds that can inhibit the growth of invading bacteria and their ability to attach to surfaces and forms biofilm on the surface through their adhesion properties.

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نشاط المستخلصات الأيضية المستخرجة من البكتيريا المرتبطة بأحد أنواع الإسفنج ضد نمو والتصاق الحشف الحيوي البكتيري

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المستخلص. تم إجراء هذه الدراسة لتقييم نشاط المركبات الايضية الثانوية من البكتيريا المرتبطة بأحد أنواع الإسفنج ضد نمو الحشف الحيوي البكتيري والتصاق البكتيريا. تم جمع أحد أنواع الإسفنج (*Hyrtios erectus*) من البحر الأحمر بواسطة الغوص. بعد الغسيل والتنظيف لإزالة البكتيريا السطحية بشكل واسع، تم عزل البكتيريا الداخلية من الإسفنج بطريقة التخفيف المتسلسل باستخدام Zobell Marine Agar (ZMA) كوسط نمو. بعد ذلك تم التعرف على البكتيريا المعزولة بواسطة طريقة تسلسل الجينات (rRNA 16s). تم إخضاع المادة المستخلصة الخالية من الخلايا (CFS) من سلالتين من البكتيريا المعزولة من الإسفنج للاختبارات البيولوجية لتثبيط النمو البكتيري وقياس تثبيط الحشف البكتيري ضد أربعة أنواع من بكتيريا الحشف الحيوي، وهي: (*Halomonas* sp.)، (*Psychrobacter* sp.)، (*Pseudoalteromonas shioyasakiensis*) و (*Vibrio alginolyticus*). تم التعرف على أنواع البكتيريا المعزولة، وهي: (*Idiomarina* sp. MS-E2) و (*Staphylococcus edaphicus* MS-E4). أظهر كلا النوعين من البكتيريا نشاطًا مثبطًا محتملاً ضد بكتيريا الحشف الحيوي المختبرة عن طريق تثبيط نموها وقدرتها على الالتصاق. ويعتبر هذا مؤشراً على دور البكتيريا المرتبطة بالإسفنج في إنتاج مركبات ذات نشاط محتمل ضد الالتصاق البكتيري بالأسطح.

الكلمات المفتاحية: الإسفنج، مركبات، الالتصاق البكتيري، مضاد الميكروبات، البكتيريا المرتبطة بالإسفنج.