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The anti-settlement activity of extracts of marine bacteria associated with soft corals against barnacle larvae

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ABSTRACT

Surface colonization by barnacles is responsible for the high maintenance cost, the increase in fuel production and greenhouse gas emission associated with marine biofouling. Barnacle larvae metamorphose into sessile juvenile stage before settling on submerged surfaces to cause biofouling. Targeting the larval settlement and metamorphosis of larval barnacles is an important step in antifouling assays. In this study, extracts from soft coral-associated bacteria from two Red Sea soft corals were screened for antifouling activity using the cyprid larvae of the barnacle, *Balanus amphitrite*. The larvae of the barnacles were reared from the nauplius to the cyprid stage. Extracellular and intracellular extracts obtained from the six bacterial strains were subjected to anti-larval settlement assay against the cyprid larvae and nauplius toxicity assay using the nauplii (stage III). The results showed that most of the extracts (7 out of 12) inhibited cyprid larval settlement either strongly or moderately, while the remaining five extracts showed no activity. The extracellular extract of *Bacillus* sp. (IAB5^{T}) inhibited the settlement of cyprid larvae completely. The toxicity of some extracts was mild while that of others was as high as 100%. The results indicate the ability of the bacteria to produce compounds that will prevent cyprid settlement on surfaces.

INTRODUCTION

Biofouling is a problem affecting shipping and naval activities in the marine environment. It is caused by the growth and colonization of fouling organisms on the surface of any submerged objects, including ships, boats, oil pipelines and aquaculture nets **(Rittschof *et al*., 2003; Schultz *et al*., 2011)**. Biofouling alters the surface roughness of submerged objects, causing corrosion, frictional and powering resistance **(Lorite *et al*., 2011; Holm, 2012)**. When it occurs on ships and boats, it reduces its performance causing a reduction in boat speed and an increase in fuel consumption **(Cruz, 2020)**. In addition, it causes a significant loss to the blue marine economy and has environmental consequences **(Mathew *et al*., 2021)**. The biofouling is a sequential process where

biofilms start the biofouling process and provide cues for the subsequent attachment of macrofouling organisms; however, it is the macrofouling community colonization that makes it highly undesirable, and it is the most visible component **(Almeida & Vasconcelos, 2015)**.

Among macrofouling communities, barnacles cause serious problem to submerged surfaces. After attachment, their accumulation change the roughness of the surfaces **(Schultz, 2007, Demirel *et al*., 2017)**. Barnacles are distributed worldwide with a highly specialized larval settlement stages among benthic invertebrates **(Fertl & Newman, 2018)**. They possess a hard, opaque shell just like other crustaceans, but they are sessile, and their hard shell is important in their life cycle. The shell is constantly shed and regenerated continuously while supporting the growth, metamorphosis, and development of barnacles **(Zhang *et al*., 2015)**. Barnacles undergo a radial growth, adhere permanently to surfaces using secreted adhesives underwater called 'cement' **(Burden *et al*., 2012)**. The cement glues them to surfaces, making the underwater attachment firm and unique, facilitating settlement and permanent bonding **(Kamino, 2013, Kamino, 2016)**. The complex life cycle of barnacles consists of multiple stages; planktonic, free swimming nauplii stage, a cyprid larval stage and a sessile juvenile/adult stage permanently attached to surfaces **(Maruzzo *et al*., 2012)**. The nauplius metamorphoses to cypris stage which then searches and carefully selects site or substrate for the settlement of the juvenile stage using different environmental cues **(Kamino, 2016)**. The attachment to substrates is necessary for their adult life stage and survival because their motile larvae must metamorphose to sessile juveniles before settling on suitable surfaces **(Maréchal & Hellio, 2011)**. Many barnacles exist worldwide; however, the common organism used as model in laboratory bioassays is *Balanus Amphitrite amphitrite* Darwin (=*Amphibalanus amphitrite*) **(Clare and Høeg, 2008)**. It is a major test organism widely used for the laboratory screening of marine natural compounds for antifouling activity **(Lau & Qian, 2000; Qian & Xu, 2012)**. Its life cycle and continuous reproduction throughout the year and completion of larval development within days makes it easy and convenient for rearing, managing and control under laboratory conditions **(Maréchal & Hellio, 2011)**.

An antifouling (AF) compound is expected to prevent larval attachment; hence, there is a need for barnacle attachment assays using the cyprids larvae of *Balanus Amphitrite* **(Price *et al*., 1992)**. Marine organisms comprising of microorganisms and macroorganisms are excellent resource for natural products with antifouling potential **(Clare, 1996; Liu *et al*., 2020)**. Marine invertebrates are serving as reservoir for many bioactive compounds that can tackle biofouling **(Qi & Ma, 2017)**. The invertebrates have survived biofouling, and their surface is free from colonization making them a reliable source of compounds that will prevent attachment and colonization of surfaces **(Almeida & Vasconcelos, 2015)**. The source of compounds from these organisms is attributed to the symbiotic microorganisms living in/on them as the actual producers **(Sigwart *et al*., 2021)**. Natural compounds from symbiotic bacteria are reliable and sustainable, and they

form an effective source of bioactive compounds **(Liu *et al*., 2019; Sang *et al*., 2019)**. This is due to the low cost and mass production of bacterial natural sources and easy manipulation under different conditions, which makes them more reliable in the search for AF compounds **(McCauley *et al*., 2020; Muras *et al*., 2021)**. Several symbiotic bacteria with anti-larval and anti-settlement activity against the larval stage of barnacles were reported in previous studies **(Qian *et al*., 2015; Satheesh *et al*., 2016; Liu *et al*., 2020).** The compounds are new, unique, and effective making them target in sourcing for new bioactive compounds with antifouling potentials. Among marine invertebrates, soft corals and its associated bacteria are well known source of bioactive compounds with antifouling potential **(Rocha *et al*., 2011; Hou *et al*., 2019; Liu *et al*., 2019; Sang *et al*., 2019).** Soft corals are known sources of natural products with ability to inhibit the settlement of barnacles **(Standing *et al*., 1984).** Soft coral being sessile have a novel strategy of combating biofouling from their surface and chemically defend themselves against colonization **(Tian *et al*., 2020).** This can be exploited in designing effective novel AF compounds. Since the bacteria associated with the soft corals are mostly responsible for the chemical defense of the organism through their metabolites, there is need to evaluate the compounds produced by these organisms and screened them for antifouling activities in an attempt to find new solution to biofouling problem.

This study was carried out to find an environment-friendly way of preventing biofouling using the bacteria associated with the soft corals as potential new sources of AF compounds. Specifically, the objective of this study was to understand the ability of the bacteria associated with the Red Sea soft corals to produce compounds that will target the larvae of barnacle, a major fouling organism and prevent settlement.

MATERIALS AND METHODS

1. Extraction of secondary metabolites

Bacillus species isolated from soft corals collected from the Red Sea were subjected to intracellular and extracellular solvent extraction process. The *Bacillus* species used in this study and their accession number was provided in Table 1. The extraction process was carried out as previously described with some modifications **(Viju *et al*., 2017; Viju *et al*., 2020).** Briefly, 5ml aliquot of an overnight culture at an optical density of 0.2 at 600 nm (OD_{600}) was inoculated in a 500 ml flask containing marine nutrient broth (MNB) at half its capacity. It was incubated for a week at 28 ^{o}C with shaking and then centrifuged. Extracellular extracts from cell free supernatant were obtained using ethyl acetate as solvent. Equal volume of cell free supernatant and ethyl acetate were mixed in a flask followed by shaking at room temperature for 24 h after which the mixture was separated, and the solvent phase was filtered and then evaporated to dryness. Cold methanol was used as solvent to obtain the intracellular extract from the residue containing the bacterial lysates. To each lysate, 5-fold volume cold methanol was added followed by shaking for 24h in tubes and then filtered to remove cell debris. The extracts

were concentrated by removing the solvents using a rotary evaporator (Buchi, Switzerland). Crude extracts obtained were weighed and then solubilized in dimethyl sulphoxide (DMSO) to a concentration of 125 mg ml^{-1} and then stored in a refrigerator until required for further use.

Table 1. list of *Bacillus* species isolated from Red Sea soft corals and evaluated for anti-larval settlement activity.

Strain Name	Accession no
Bacillus subtilis IAB1^T	*MW774339*
Bacillus subtilis IAB2^T	*MW774340*
Bacillus sp. IAB5^T	*MW774341*
Bacillus sp. IAC1^T	*MW774342*
Bacillus subtilis IAC2^T	*MW774343*
Oceanobacillus sp. IAC4^T	*MW774344*

2. Larval rearing of the barnacle

The adults of the barnacle, *Balanus amphitrite* attached to a hard substrate submerged in the Obhur Creek area of the Red Sea, North of Jeddah (N21°42.562′ E039°05.764′) were collected and reared under laboratory conditions. The adult organisms were maintained in aerated aquarium (20L capacity) containing 18L fresh and 0.45µ filtered seawater (FSW) at an ambient temperature (25 °C) after being exposed to air for 2 h. The adult barnacles were fed with the microalga *Chaetoceros calcitrans*. The nauplii released by the adults (stage III) were collected after a light source was placed in a side of the aquarium below the water level. The nauplii collected were either used for nauplius toxicity assay or reared to cyprid stage by transferring them to a new smaller aquarium (5L capacity) containing 4L FSW and fed with the microalga, *C. calcitrans* at a density of 2.5 x 10^5 cells/ml. The larval density in the aquarium was maintained at 2.0 larvae/ml and the cyprid culture was carried out in a walk-in environmental chamber at 28 ^oC under an alternating cycle of light and dark period of 12 hours each as reported previously **(Siddik and Satheesh, 2019).** Waste and other metabolic byproducts are removed daily by replacing the used FSW with fresh FSW and the larvae are fed with new algal diet as explained above. The cyprids were collected after 6 -8 days in batches just before metamorphosis to the adult stage for anti-settlement assay.

3. Activity of the extracts against barnacle settlement

The anti-larval settlement activity of all the extracts was carried out using the competent cyprid larval stage of the barnacle, *B. Amphitrite* using the procedure described by **Rittschof *et al*. (1986)** with brief modifications. The assay was conducted in glass Petri dishes (120 mm X 20 mm, Saudiplast, KSA). The concentration of the extracts was adjusted to 1000 μgl^{-1} and 500 μgl^{-1} in sterile FSW. Each of the extracts at a concentration of 1000 μgl^{-1} and 500 μgl^{-1} (n = 4) was added to the glass Petri dish containing 10 ml of sterilized FSW and 10 competent cyprids that were reared from the nauplii stage. The control used contained FSW only alongside the cyprids without extract (n=4). All plates were incubated in a walk-in environmental chamber at 28 ^{o}C in a light and dark period of 12 hours each for 48 hours with observation after every 24 hours. A dissection microscope (Leica, M80) microscope was used to count the number of settled larvae (attached and metamorphosed) and the rate of settlement was obtained by comparing the number of settled and unsettled larvae with the control. The number of larvae that settled for each extract was averaged (mean of the four replicates) and expressed as a percentage of the total number of cyprid larvae in the plates.

4. Nauplius toxicity assay

For this assay which aimed to determine the toxicity of the extracts, 15 newly hatched stage III nauplii were transferred into a 6-well gamma sterilized polystyrene plates (SPL, USA). This was followed by the addition of 10 ml of FSW. The concentration of the extracts was adjusted to 1000 μgl^{-1} and 500 μgl^{-1} in sterile FSW and added to the well (n=4). Two types of controls were used with one containing the nauplii with DMSO (10% and 5%) while the other containing only the larvae. Results were recorded after 24 h, 48 h and 72 h by counting the dead nauplii under a microscope (Leica, M80). The toxicity was expressed as a percentage of the mortality recorded from the total nauplii in the well.

RESULTS

1. Cyprid settlement inhibition

Twelve extracts (intracellular and extracellular) were obtained from the six bacterial strains, 2 from each strain. All the 12 crude extracts obtained from the six strains were evaluated for their ability to target and inhibit the settlement and metamorphosis of the cyprid of *B. Amphitrite.* The results indicated that the extracts exhibit different inhibitory activity. Based on the activity, they are divided into three groups. The first group comprises of those extracts that completely inhibit the settlement of barnacle larvae. There was 100% settlement inhibition at the concentration of 500 μgl^{-1} and 1000 μgl^{-1} from the extracellular extract of *Bacillus* sp. IAB5 after 24 h. The second group of extracts demonstrate moderate to high settlement inhibition activity. This comprises of 6 extracts out of the 12 extracts obtained from the bacteria. Moderate to high activity was

recorded by extracellular extracts from *Bacillus subtilis* IAB2, *Bacillus* sp. IAC1 and *Oceanobacillus*sp. IAC4 as presented in **Fig. 1**. The same applies to intracellular extracts of *Bacillus subtilis* IAB2, *Bacillus* sp. IAB5 and *Oceanobacillus* sp IAC4. The last category (group) comprises of extracts that produced no or little effect on the settlement with some having more settlement rate than the control. The extracts that have no effect on the larval settlement are the extracellular extracts of the *Bacillus subtilis* IAB1 and *Bacillus subtilis* IAC2 that did not show any settlement effect against the barnacle larvae after 48 hours at both concentrations (500 μgl^{-1} and 1000 μgl^{-1}) with number of settled larvae higher than the control. It was observed that the extracellular extract of *Bacillus subtilis* IAB2 treatment at 1000 μgl^{-1} and intracellular extracts from *Bacillus subtilis* IAC2 and *Bacillus* sp. IAC1 also recorded low settlement activity than the control at both concentrations but with little anti-settlement activity. None of the intracellular extracts shows 100% settlement inhibition compared with the extracellular extract after 48 hours. A total of 7 extracts out of 12 (58%) showed some kind of antifouling activity by inhibiting the settlement of barnacle cyprids larvae and its metamorphosis.

2. Larval toxicity of the extracts

All the 12 different extracts were tested against the nauplii (stage III) of *B. amphitrite* to determine their environmental toxicity and bioactivity for 72 h at 1000 μgl^{-1} and 500 μgl^{-1}. **Fig. 2.** shows the mortality/survival of nauplii treated with the extracts. The survival in the control ranged from the maximum of 100% after 24 hours to 46 % maximum after 72 hours. After 24 hours, only extracellular extract from *B. subtilis* IAB2 has 0 % survival or 100 % mortality at 500 μgl^{-1} but the survival of the remaining 11 extracts ranged from 0 to 92% after 24 hours which reduced to 40% after 48 hours and 19 % maximum after 72 hours. At a concentration of 500 μgl^{-1}, extracellular extract and intracellular extract of *Bacillus* IAC2 and extracellular extract from *B. subtilis* IAB2 and *Bacillus* sp. IAC1 recorded 100 % mortality after 72 hours. But after 48 hours, only extracellular extract from *B. subtilis* IAB2 recorded 100 % mortality at 500 μgl^{-1} (it recorded same after 24 h). At 1000 μgl^{-1}, the survival rate at 24 h ranged from 0 % (100% mortality) to a maximum of 54 % which was reduced to 16.5 % after 48 hours. After 48 hours at 1000 μgl^{-1}, intracellular extract from *Bacillus sp.* IAC1 recorded survival of 16.5% and extracellular extracts from *B. subtilis* IAB1 and *Bacillus* sp. IAB5 recorded 2.5 % survival each. None of the larvae survive after 72 hours at the concentration of 1000 μgl^{-1} for all the extracts (100% mortality). Also, the toxicity becomes higher as the incubation period progresses from 24h to 72h.

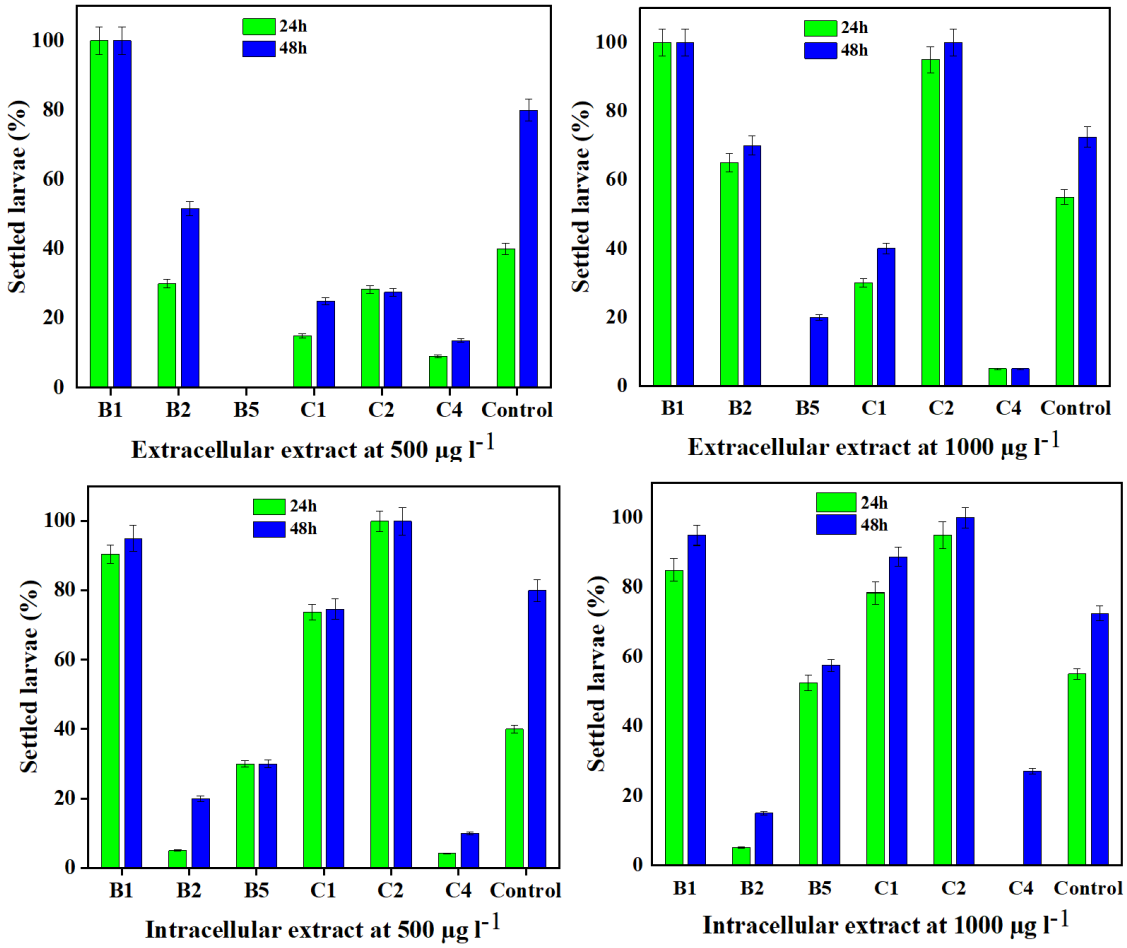

Fig. 1. The anti-settlement activity of extract from soft coral associated bacteria against the cyprid larvae of barnacles. The control comprises of cyprids and filtered seawater only. The higher the settled larvae the lower the activity of the extracts.

Key: B1: *Bacillus subtilis* (IAB1^T)*;* B2: *B. subtilis* (IAB2^T), B5*: Bacillus* sp. (IAB5^T),C1: *Bacillus* sp. (IAC1^T), C2: *B. subtilis* (IAC2^T). C4: *Oceanobacillus* sp. (IAC4^T)

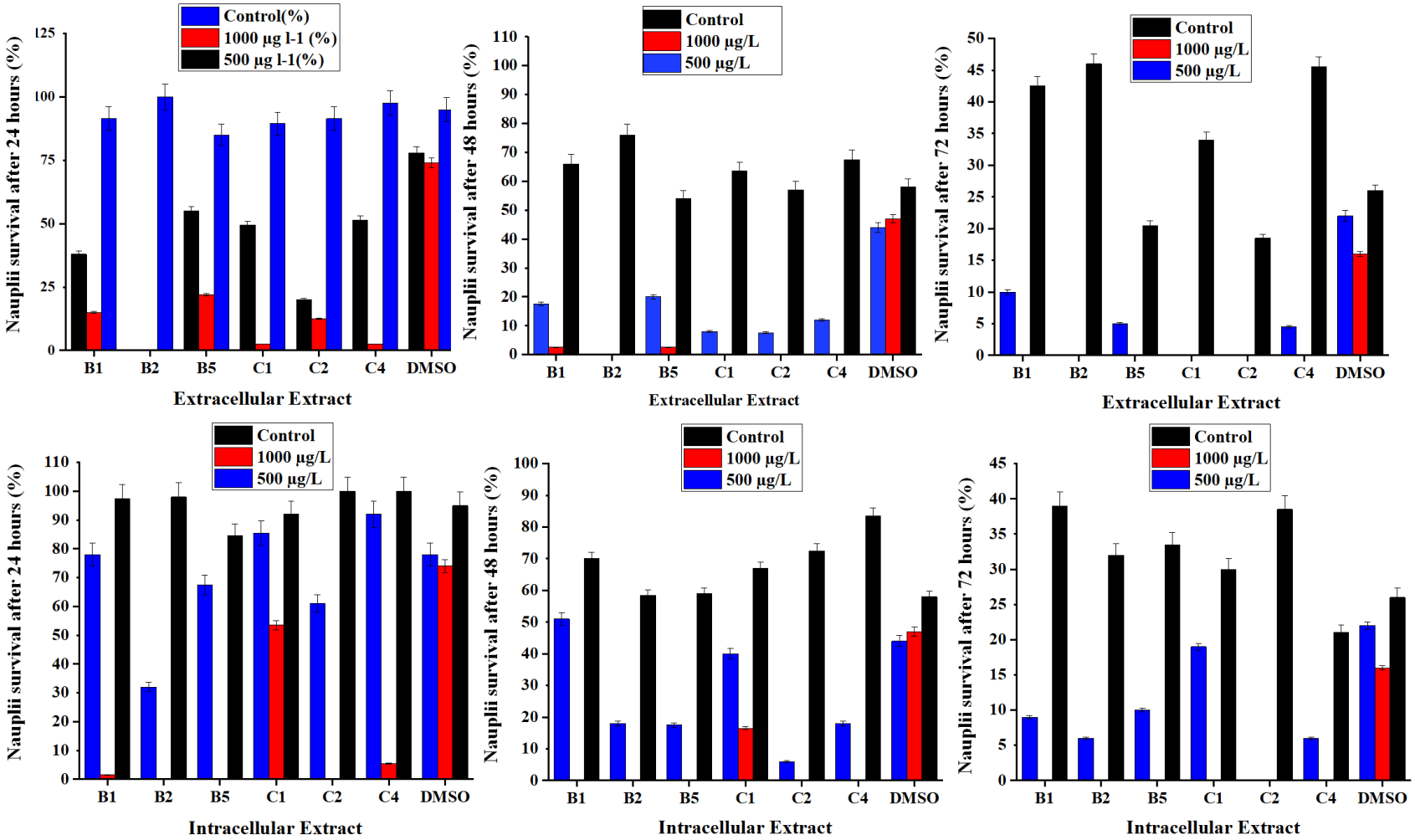

Fig. 2. Toxicity assay of extract from soft corals associated bacteria. The result displays the survival of the nauplii at a concentration of 500 μgl^{-1} and 1000 μgl^{-1} after 24 hours. The higher the survival, the lower the toxicity/mortality.

NB: DMSO (Dimethyl sulphoxide) was evaluated at 5% and 10% respectively.

Key: B1: *Bacillus subtilis* (IAB1^{T}), B2: *B. subtilis* (IAB2^{T}), B5*: Bacillus* sp. (IAB5^{T}), C1: *Bacillus* sp. (IAC1^{T}), C2: *B. subtilis* (IAC2^{T}), C4: *Oceanobacillus* sp. (IAC4^{T}).

DISCUSSION

The extracellular and intracellular crude extracts from the bacteria associated with soft corals demonstrated potential antifouling activity by inhibiting the settlement of cyprid larvae of barnacle *B. amphitrite*. The anti-larval settlement assay against cyprids larvae revealed the potential of compounds released by the bacteria as prospective marine natural products with antifouling activity. A total of 7 crude extracts (4 from extracellular and three from intracellular) from 4 organisms revealed some form of settlement inhibitory effect against the cyprid larvae. Extracellular extracts demonstrated higher anti-settlement activity than the intracellular ones. Usually, extracellular substances from bacteria can inhibit biofouling at a higher rate **(Yu-Mei *et al*., 2013).** Zero settlement was recorded by the extracellular extracts of *Bacillus* sp. IAB5^T proving it to be a prospective source for the isolation of new AF agents. The promising anti-settlement activity demonstrated by *Bacillus* sp. IAB5^T may be attributed to its ability to produce secondary metabolites that are capable of preventing the settlement of barnacles larvae. The organism is closely related with members of the *Bacillus cereus* group based on its 16S rRNA gene sequence similarity and several members of the *B. cereus* group were reported to have AF activity. *Bacillus cereus* QDG-B509 has been reported previously to have significant antifouling activity against barnacle larvae **(Gao *et al*., 2014)** while symbiotic *Bacillus cereus* SS05 from sponge revealed antifouling activity against biofilm bacteria and microalgal settlement **(Satheesh *et al*., 2012).** Members of the *Bacillus* genus from marine environment are well known to produce secondary metabolites with bioactive properties **(Caulier *et al*., 2019; Kaspar *et al*., 2019; Kai, 2020).** Several compounds from marine *Bacillus* with potential antifouling activity have been reported from many studies **(Ortega-Morales *et al*., 2008; Satheesh *et al*., 2012; Gao *et al*., 2014; Ramasubburayan *et al*., 2017; Muras *et al*., 2021).** Members of the *Bacillus* genus are becoming an attractive source of AF compounds **(Caulier *et al*., 2019; Kaspar *et al*., 2019; Liu *et al*., 2019).** The compounds represent different classes of bioactive compounds such as volatile organic compounds, ribosomal peptides, polyketides, non-ribosomal peptides among others **(Modolon *et al*., 2020).** The species of bacteria associated with soft corals are capable of producing bioactive compounds with significant antifouling activity against larval settlement.

Not all the extracts were found to be effective in preventing the settlement of the cyprid larvae. Some of the extracts demonstrated no activity on the settlement of the larvae allowing 100% settlement which is higher than the control after 48 hours. This is an indication that the bacteria produce compounds in the extracts that might probably enhanced the settlement and metamorphosis of the larvae by recording higher settled larvae than the control. This is not surprising as many bacteria in the marine environment are known to produce compounds that enhance settlement of invertebrate larvae including barnacle larvae **(Maki, 1999; Unabia and Hadfield, 1999; Tait and Havenhand, 2013; Siddik and Satheesh, 2019).** Indeed, several bacteria and other microorganism have

been identified as natural settlement cues for many marine invertebrates **(Dobretsov and Rittschof, 2020).** Most bacteria form biofilm by attaching to surfaces and produced compounds that enhance the settlement and metamorphosis of barnacle larvae **(Tait and Havenhand, 2013; Siddik and Satheesh, 2019; Dobretsov and Rittschof, 2020).**

The toxicity of the extracts evaluated using the nauplii larvae revealed that the higher the concentration of the extracts the higher the toxicity effect on the larvae. This instance is higher in intracellular extracts with 3 extracts and only one in extracellular extract. This is an indication that the intracellular compounds produced within the bacterial cells are more toxic than the extracellular ones produced outside of the cells. This is a reversal in terms of anti-settlement activity where extracellular compounds demonstrate more settlement inhibitory activity. The results also indicate that there was no relationship between toxicity of an extract andits anti-settlement of activity and the activity and toxicity of the extracts is dependent on concentration of the extracts.

The use of crude bacterial extract for screening antifouling activity without necessarily identifying the individual compounds could be more advantageous due to synergistic action of compounds and will surely reduce the cost of production **(Muras *et al*., 2021).** Since biofouling is multifaceted, caused by different organisms at different stages, the use of crude extracts can enhance the development of antifouling agents with multiple activities against the fouling agents. Compounds from marine bacteria can overcome the challenge of toxicity associated with many chemical compounds and can be target specific without altering the natural biodiversity and environmentally friendly **(Prakash *et al*., 2015).** However, antifouling activity evaluation should be tested against multiple targets that contribute to biofouling including antibacterial, antibiofilm, antialgal, antiprotozoal, anti-larval and antisettlement **(Dobretsov *et al*., 2006).** An Antifouling screening program that consists of the combination of antibacterial, antibiofilm with anti-settlement tests is an effective strategy for the evaluation of natural products to determine its potential application biofouling control **(Xu *et al*., 2010).** The strains that produce these extracts with multiple activities show promising potential of being natural sources of antifouling compounds. **Xu *et al*., (2007)** revealed in a study, the potential of deep-sea bacteria to inhibit bacterial growth and settlement of barnacles larvae making them potential antifouling compounds.

The potential activity demonstrated by the extracts produced by the bacteria is an indication of their role in protecting the surface of the soft corals against biofouling. Further, this study showed that *Bacillus* species associated with soft corals couldplay significant role in the development of antifouling compounds. However, to complement the full potential of the prospective antifouling natural product, there is a need to identify the compounds responsible for the anti-settlement activity and further field experiments.

CONCLUSION

The compounds produced by the bacteria have demonstrated strong activity in preventing larval settlement with little toxicity. This is an indication that bacteria associated with soft corals belonging to the genera *Bacillus* and *Oceanobacillus* will play significant role in the development of promising antifouling compounds.

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