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Environmentally benign antifouling potentials of Triterpenes-Glycosides from *Streptomyces fradiae*: a mangrove isolate

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Running Title: **Antifouling properties of *Streptomyces fradiae***

Abstract

In the present study, an attempt has been made to screen and evaluate the antifouling potentials of actinobacterial strain *Streptomyces fradiae* RMS-MSU, isolated from manakkudy mangroves of Tamilnadu. Screening results showed that ethyl acetate extract of *S. fradiae* RMS-MSU displayed the wide spectrum antagonistic activity (10 to 21mm) against marine biofilm bacterial

strains with least Minimal Inhibitory Concentrations (MIC) and Maximum Bactericidal Concentrations (MBC). It showed promising antimicroalgal activity with the MIC values ranged between 100 and 400µg ml^{-1}. Anticrustacean activity for 50% mortality (LC_{50}) of *Artemia salina* was recorded as 273.77µg ml^{-1}. It revealed the EC_{50} value of 77.03µg ml^{-1} for 50% inhibition of byssus production and attachment of mussel *Perna indica*. Crude extract of *S. fradiae* RMS-MSU showed the LC_{50} value of 718.79µg ml^{-1} for 50% mortality of mussel. The therapeutic ratio (LC_{50}/EC_{50}) of 9.33 indicated the nontoxic nature of the extract. Mollusc foot adherence assay using limpet *Patella vulgata* showed 6.66% fouling and 92.96% regaining at 7mg ml^{-1} after transfer to fresh seawater.

Keywords: *Streptomyces fradiae*, Antifouling, Mussel bioassay, Mollusk foot adherence, Triterpene-Glycosides.

Introduction

Marine biofouling is being defined as the growth of unwanted micro and macro organisms on the surface of natural and artificial structures immersed in seawater and it's created huge material and economic losses towards controlling and maintenance of mariculture, naval vessels and seawater pipelines, etc.. [1, 2] For instance, Bhadury and Wright[3] reported that, government and industry spend nearly 6.5 billion US $ annually to control marine biofouling. Moreover, ecological implications related to biofouling include increased carbon emission and potential dispersion of invasive alien species[4, 5]. There are two major environmental pollutions formed by fouling of hulls which are the more emissions of gas (CO_2, CO and SO_2) into atmospheric region and the dissemination of potentially rare fouling species. [6] On the other hands, the decrease in NOx emissions forced by air quality concerns will have a tendency to shrink the net warming effect due to the tropospheric ozone and CH_4 concentrations. If these

NOx decreases are greater than the parallel augment in CO_2 emissions, then the mutual effect of NOx control could be shrink the global warming impact of the international shipping. [7]

Antifouling paints are mixtures of toxic materials / biocides (copper, lead, mercury and arsenic) were used to control fouling organisms. However, Organotin and Tributylin (TBT) were most effective antifoulants. Studies pertained to toxic biocides indicated that, high TBT concentration was detected in many marine organisms such as invertebrates, vertebrates and marine plants. [8] This led the International Maritime Organization (IMO) to prohibit the toxic organotin based antifoulants and their application to ships on 2008 onwards. [9] Therefore, it is highly essential to develop eco-friendly antifouling compounds from marine organisms. Natural antifoulants have been proposed as one of the best possible alternative approaches to replace TBT coatings. [10] Recent studies propagated that, bioactive compounds derived from marine organisms are much effective and eco friendly in nature.[11, 12] Raveendran and Limna Mol[13] reported that, the majority of natural product antifouling compounds isolated from various marine organisms like sponges, soft corals, tunicates, bryozoans, marine crustaceans, seaweeds, seagrasses and mangroves are terpenoids, steroids, furanones, alkaloids, etc., In addition, several researchers have also been reported that the antifouling performance of marine microbes likes i.e. bacteria (*Pseudoalteromonas tunicata*, *Alteromonas* sp., *Shewanella* sp.), fungi (*Cladosporium* sp.) and cyanobacteria isolated from marine resources and were found to be good source of antifouling compounds.[14-18]

Actinomycetes are distributed extensively in soil, which has provided much information regarding bioactive compounds with potent pharmaceutical values and also inferred that, actinomycetes isolated from marine and mangrove sediments were found to excellent source for discovering new types of halotolerant bioactive metabolites.[19] But, studies related to antifouling

metabolites from mangrove associate actinomycetes are too scanty and few authors reported that the antifouling properties of marine actinomycetes and its isolated from deep sea sediments. More recently, Xu et al.[20] and Cho et al.[21] isolated 2-furanone and Diketopiperazines from deep sea marine actinobacterium *Streptomyces* sp., and proved its promising antifouling activity *viz.* antilarval, antidiatom and seaweed spore settlement activities. Similarly, Xu et al.[22] isolated a branched-chain fatty acid, 12- methyltetradecanoid acid (12-MTA) from a marine sediment isolates, *Streptomyces* sp. UST040711-290 and evidenced that it had strongly inhibited the settlement of larval of polychaete *Hydroides elegans*. Considering the importance of actinomycetes, in the present study an attempt has been undertaken to explore the antifouling and toxic properties of crude extract of mangrove associated actinobacterium *Streptomyces fradiae*.

Results and Discussion

Most of the actinomycetes are identified by the classical method, i.e. based on the colour of mycelium, sporophore arrangement, pigment diffusion and sugar utilization patterns. However, species level strain identification was done by 16S rRNA gene sequencing. In the present study, the candidate strain *S. fradiae* RMS-MSU was subjected to identification through polyphasic characters. The result was inferred that, the strain was Gram positive with filamentous mycelium. The substrate as well as aerial mycelium colour was noticed as yellow and pink. Spiral spore morphology was observed in the aerial mycelium under the light microscope (400×), also it did not produce any pigments in yeast malt extract glucose agar medium. The optimum temperature and pH required for its growth was recorded as 35ºC and pH8, respectively. The candidate strain had NaCl tolerance, up to 5% with the optimum level at 2%. Biochemical characters of the candidate strain such as urease, nitrate and citrate

production showed positive reaction, whereas, H_2S, MR, VP and indole showed negative reaction. In consonance with the present results, Kathiresan et al.[35] and Masilamani Selvam et al.[36] identified as *S. fradiae* Rh1 through the classical method by using various phenotypic characters.

The sugar utilization is an important tool for identifying the unknown actinomycetes up to genus level. Considering the sugar utilization pattern of the tested strain, it was found to be highly positive to lactose, mannitol and maltose and moderately positive in utilizing fructose, xylose and galactose. But, it recorded negative result towards utilizing glucose, sucrose and inositol as carbon source. Besides, it showed positive influence on hydrolyzing protein, lipid, starch, gelatin and pectin. The candidate strain showed production of protease, lipase, amylase, gelatinase and pectinase enzyme. Nevertheless, it showed negative result towards hydrolyzing cellulose (Table 1). Result of BLAST analysis showed 100 % similarity with the reference strain *S. fradiae* (accession number FJ972686). Subsequently, the candidate strain was deposited in NCBI Genbank as *S. fradiae* RMS-MSU with the following accession number HQ267533 (Fig.1).

Deterrence of marine fouling is linked to the control of attachment and development of the fouling assemblages of microfouling and macrofouling communities. [37] In the present study, the antimicrofouling activity of crude extract of *S. fradiae* showed maximum zone of inhibition of 19 to 21 mm against *E.coli, H. aquamarina, Pseudomonas* sp1., *A. hydrophila, Vibrio* sp., *C. freundii, S. sonaii* and *S. fonticola*, followed by 18 mm each against *M. morganii* and *S. liquefaciens*. However, it recorded lesser inhibitory zone ranged between 10 and 12 mm against the biofilm bacterial strains such as *Enterobacter* sp., *Micrococcus* sp., *Salmonella* sp. and *V. parahaemolyticus* (Fig. 2). In accordance to the present study, Bavya et al.[38] investigated

the antimicrofouling activity of crude extract of *S. filamentosus* R1 against the marine fouling bacterial strains such as *Bacillus* sp., *Serratia* sp. and *Alteromonas* sp., and evidenced its promising growth inhibitory activity.

Minimal Inhibitory (MIC) and Minimal Bactericidal (MBC) concentrations of crude extract were tested at different concentrations against the biofilm bacterial strains through 96 multi well plates. Here, the lowest MIC of 25 µg ml^{-1} was recorded against *E. coli*, *Vibrio* sp., *V. parahaemolyticus*, *Micrococcus* sp., *Pseudomonas* sp. 1., *S. sonaii* and *Pseudomonas* sp. 2. But it recorded a considerable increase in MIC ranged between 50 and 200 µg ml^{-1} against *A. hydrophila*, *M. morganii*, *C. freundii*, *P. pudita, H. aquamarina, S. liquefaciens, S. mercescens* and *Enterobacter* sp., respectively. Thus the result of the present study clearly manifested the promising bacteriostatic activity of the crude extract. On the other hand, MBC values of the crude extract exhibited relatively higher concentration against the test biofilm bacterial strains and it varied from 100 to 400 µg ml^{-1}. The positive control (Copper sulphate) evidenced 100% bacteriostatic (MIC) and bactericidal activity (MBC) at the least concentration and it ranged from 5 to 15 µg ml^{-1} and 10 to 20 µg ml^{-1}, respectively (Fig. 3).

Though antifouling compounds were effective against many fouling organisms, but it poorly inhibits fouling microalgal strains like diatoms and cyanobacteria[39]. A recent study showed excellent antimicroalgal activity by crude extracts of several marine bacterial strains *viz. Pseudoaletromonas* sp.[40], *Bacillus* sp.[41], *P. aeruginosa* [42], *Aquimonas* sp.[43], *Vibrio* sp.[44], *Shewanella* sp.[45] and *Streptomyces* sp. L74 [46]. In the present study, crude extract of *S. fradiae* RMS-MSU effectively inhibited the growth of microalgal strains. However, there was significant variation in inhibitory effect. It was found that, 100 - 200 µg ml^{-1} of crude extract had significantly inhibited the growth of *Chlorella* sp., *Nannochloropsis* sp. and *Dunaliella* sp.

Further increase in concentration showed no growth of *Chlorella* sp., *Nannochloropsis* sp., and *Dunaliella* sp. The other microalgal strains such as *Chaetoceros* sp. and *Tetraselmis* sp. showed growth inhibition at 200 µg ml^{-1} extract. The results of copper sulphate (positive control) showed complete growth inhibition against all the tested microalgal strains at a much lower concentrations of 10 µg ml^{-1} (Table 2). In accordance with the result of the present study, Yamamoto et al.[47] studied the antimicroalgal property of crude extract of *S. phaeofaciens* S-9 and observed the growth inhibitory activity of cyanobacterial strains such as *Microcystis aeruginosa* NIES 298, *M. aeruginosa* NIES 112, *Anabaena ucrainica* and *Chlorella* sp. at 1000 µg ml^{-1} concentration. Thus the results of the present study, corroborates with the previous findings and emphasize that the active principle localized within the extract has effectively inhibited the growth of marine microfoulers.

The marine crustacean *A. salina* is ideally suited as a bioassay organism for detecting the toxicity of crude organic extracts or other active compounds [48]. Moreover, toxicity against *A. salina* may be an indication of potential for marine crustacean families [49]. From the present result, it was observed that the *S. fradiae* extract had a good anticrustacean activity against *A. salina* with the LC_{50} value of 273.768 ± 2.76 µg ml^{-1}. Comparatively, copper sulphate (positive control) recorded least LC_{50} value of 9.35 ± 0.76µg ml^{-1}. It was noticed that, after 24h, the percentage mortality of *A. salina* larvae was significantly ($P< 0.05$) increased with respect to increase in concentrations of crude extract as well as copper sulphate (positive control) treated groups (Fig. 4). This result was in agreement with the observation of Anibuo et al.[50] and Manivasagam et al.[51] who investigated the lethality of active metabolites and crude extract of *Streptomyces* spp. by using *A. salina* nauplii with the least toxicity levels.

The mussel is one of the bivalve groups of major fouling organisms in both animate and inanimate surface immersed in the marine environment. Often, they cause serious economic problems. Few authors performed the laboratory assay using different mussel species as a model organism to study the antifouling properties of the crude extracts. [52, 53] In the present study, crude extract of *S. fradiae* and copper sulphate was tested against brown mussel *P. indica* to determine its byssal thread inhibiting activity. The results showed that, the 50% effective concentration (EC_{50}) of crude extract and copper sulphate for inhibition of byssal thread of *P. indica* after 24h was 77.03 ± 1.96 µg ml^{-1} and 5.47 ± 0.06 µg L^{-1} respectively. The same mussels subjected to toxic criterion study inferred that, after 72h of exposure, 50% lethal concentration (LC_{50}) was found to be 718 ± 1.55 µg ml^{-1} for *S. fradiae* extract and 6.08 ± 0.03 µg L^{-1} for Copper sulphate. It is to be noticed that, increasing the test concentration of crude extract and positive control show a gradual reduction in the number of byssal threads formation and its subsequent attachment over the surface of the substrate (Table 3 and Fig. 5a & b).

Li et al.[31] suggested that the LC_{50}/EC_{50} values of active compounds/metabolites depend on properties of antifouling substances and its relative toxicity. Here, the LC_{50}/EC_{50} ratio of crude extract of *S. fradiae* was found to be 9.33; whereas, copper sulphate had recorded LC_{50}/EC_{50} ration of 1.11 which indicated its potential toxicity over the mussel *P. indica*. The present results fell against with the observation of Qian et al.[11] who reported that, natural antifouling compounds with a LC_{50}/EC_{50} ratio >50 µg/ml were considered as a non-toxic and higher LC_{50}/EC_{50} ratio is highly recommended when selecting a candidate compound. However, the higher LC_{50} value of *S. fradiae* against *A. salina* evidenced its non toxic nature.

The mollusc foot adherence assay is a reliable method for evaluating antifouling properties of marine natural products. This assay is based on adherence of the foot to the

substrata through spreading and shrinking with respect to antifouling compounds. In this study, an attempt was taken to determine the fouling (%) and regaining (%) capacity of foot of common limpet *P. vulgata* against crude extract of *S. fradiae* RMS-MSU (Fig. 6). The results showed, 66.66 to 93.33% fouling of *P. vulgata* in 2, 3 and 4 mg ml^{-1} concentrations of the extract coated plates. However, after 10 min exposure to fresh seawater, 33.33% to 72.22% regaining of foot spreading and adherence was noticed in 2, 3 and 4 mg ml^{-1} of concentrations, respectively. Further, increase in concentration of the extract to 5 and 6 mg ml^{-1} concentration inferred a gradual decrease in fouling of *P. vulgata* to 40.0% and 13.33% was noticed with the regaining of 78.25 ± 5.02 and 84.72 ± 4.94%. *P. vulgata* exposed at 7 mg ml^{-1} concentration of crude extract coated plate showed only 6.66% fouling due to immediate reflex in shrinkage of the foot and loss of mobility of the foot over the substrate; also after the transfer to fresh seawater, only 92.96 % regaining and spreading of foot was recorded. At last *P. vulgata* exposed to 8 mg ml^{-1} concentration showed a complete inhibition (0% fouling) of foot adherence (spreading) /fouling over the extract coated plate. The result of copper sulphate showed that at 1 mg/ml^{-1} concentration it displayed 23% fouling of *P. vulgata*, but after transfer to fresh water 10% regaining was recorded. Further, progressive increase in concentration, i.e. at 2 mg ml^{-1} foot of limpets shrunken well and did not show any positive regaining. The result of present study was in consonance with Selvin and Lipton,[32] wherein they reported that adherence (100% fouling) of *P. vulagata* was completely inhibited at 4.02 mg ml^{-1} concentration of methanolic extract of Holothuria, *Holothuria scabra*. Similarly, Aseer et al.[54] in their study pointed out that, methanolic extract of mangrove *A. marina* has shown 100% molluscicidal activity at 6 mg ml^{-1} concentration and at the same concentration, it had also

shown a 80 % regaining of foot spreading and adherence. The results of the present study indicated that this *S. fradiae* RMS-MSU extract posses promising antimolluscan property.

In the present study, the crude extract of *S. fradiae* was purified by normal phase silica gel column chromatography. Altogether 32 fractions were collected, among this 25th fraction had showed the excellent antifouling properties against the marine fouling bacteria and microalgal strains (Table S1 and S2). The bioassay guided column fraction (25th fraction) was further purified and characterized by TLC. The TLC results showed the existence of three spots *viz*. 0.54, 0.63 and 0.76 under the UV. Among these spots with Rf value of 0.63 rendered the maximal inhibitory activity against fouling bacterial strains when tested through TLC bioautography (Fig. S1). Besides, the chemical constituents of the bioassay guided spot in TLC plate sprayed with Libermann- Burchards reagent showed fluorescent dark violet (Rf-0.63) color under long wavelength (365nm) of UV light which indicated the presence of triterpene-glycosides. In agreement with the results of the present study, Manivasagan et al.[55,56] reported that chemical constituents such as sesuiqterpenes, terpenoids, polyketides, peptides, carprolactones, butenolides, quinones, alkaloids, macrolides, esters, methyl pyridine, lactams and chinikomycins isolated from marine actinomycetes act as many pharmacological properties. For instance, Kubanek et al.[57] showed that the triterpene-glycoside isolated from marine sponges *Erylus formosus* and *Ectyplasia ferox* had showed promising antifouling activity against marine invertebrate and algal species with the least concentration in field trial up to 90 days.

Infra Red (IR) spectral stretches showed the presence of possible functional groups ranged between the waves lengths numbers ranged from 721.33 to 3394.48 cm^{-1}. The broadband at 3394.48 cm^{-1} (s) may be due to the presence of the phenolic-OH group. The sharp

peak (s) observed in 3029.96, 2923.88 and 2854.45 cm^{-1} indicates the presence of alkene group [CH_2, CH_3]. Presence of aldehydes (H-C=O:C-H) was noticed by registering the peak at 2700.15 and 2628.79 cm^{-1}. A single peak was recorded at 2401.85 cm^{-1} which infers the presence of a nitrile group. A sharp peak at 1741.60 cm^{-1} (s) marks the presence of esters with saturated aliphatics. Likewise, medium peak recorded at 1649.02 cm^{-1} (m) indicates presence of primary amine (N-H). Two sharp (s) stretches observed at 1548.73 and 1529.44 cm^{-1} represents Nitro group (N-O-). Another two peaks exhibited a medium (1460.01 (m) stretch rocks in CH_3 cm^{-1}) and sharp (1375.15 (m) stretch bends in CH_2 cm^{-1}) which was identified as an alkane (C-H-) group. Four medium peaks recorded in 1240.14, 1163.00, 1097.42 and 1053.06 cm^{-1} (m) revealed the presence of the aliphatic amine (C-N-). Finally, two separate peaks found in 975.91 (=C-H bend) and 721.33 cm^{-1} (C-H - out plane rocks) imply the presence of alkenes groups (Fig. S2). The presence of all the above mentioned functional groups indicates the triterpene - glycosides in the bioassay guided column of *S. fradiae* RMS-MSU may be attributed to the bioactivity. Perusal of literatures showed the pharmaceutical importance of triterpene-glycosides in the crude/purified extract of several marine and terrestrial organisms through FT-IR and other spectral analysis. [58-61]

Conclusion

The overall results of the present study clearly emphasized the antifouling potentials of *S. fradiae* RMS-MSU was active against both micro and macrofouling organisms and thus substantiate the less toxic nature. Further research on purification and identification of active antifouling compounds and field trial experiments is being directed now for the development of novel eco-friendly antifouling coatings.

Experimental Section

Chemicals and Solvents

In the present study, the chemicals used are having 95% purity, and the solvents like Hexane, Benzene, Ethyl acetate and Methanol (HPLC grade), microbiological media and other chemicals were purchased from Himedia, India. The genomic DNA isolation kit and PCR kit were purchased from Genei, Bangalore, India.

Candidate actinomycetes strain RMS-MSU and its Taxonomical characteristics

The antifouling metabolite producing strain *S. fradiae* RMS-MSU was isolated from the rhizosphere soil of mangrove plant *Rhizophora mucronata* collected from the Manakudy mangrove ecosystem (77º-7- 77º35' E and 8º-8-35' N) off the southwest coast of Tamilnadu, India. The candidate strain was maintained on Yeast, Malt extract glucose medium (ISP-2; Himedia, Mumbai, India) (Yeast extract: 4g; Malt extract: 10g; Glucose: 4g; 50 % filter sterilized seawater and pH 7.3 ± 2) and preserved in 20 % glycerol at 4 ºC for prolonged usage. The colony morphology (mycelium color) of RMS-MSU was noticed on Yeast, Malt extract (ISP-2) medium at 37 ºC for 7 days. The structure of spores was examined by slide culture method described by Nonomura[23] and the spore structure was observed under light microscope with camera attached on 400 × magnification (Coslab, Model No; HL23, Haryana, India). Physiological, biochemical and utilization of carbon sources by the candidate strain was carried out by following Shrilling and Gottlieb.[24]

Molecular characterization of candidate strain RMS-MSU

Extraction of genomic DNA of candidate strain RMS-MSU was carried out by following Kumar et al. [25] In brief, the genomic DNA was separated and purified by DNA isolation and purification kit (Genei, Bangalore). The 16S rRNA gene was amplified by using universal primers (16S F 5′ AGAGTTTGATCCCTGGCTCAG 3′ and 16S R 5'

GTACGGCTACCTTGTTACGAC 3'). The amplification was performed (Eppendorf gradient thermocycler 96, California, US) by following steps involved, an initial denaturation step for 2 min at 94 °C, thereafter 30 amplification cycles consisting of denaturation at 94 °C for 1 min, followed by annealing at 55 °C for 1 min and then a final extension step consisting of 2 min at 72 °C. The total PCR amplicon was analyzed with 1 % agarose gel electrophoresis and sequenced by genetic analyzer (Applied Bio systems, USA). The comparison of 16S rRNA gene sequence of the candidate strain RMS- MSU with other actinobacterial sequences was matched by NCBI- BLAST database program and identified as *Streptomyces fradiae* RMS-MSU. The 16S rRNA sequence was deposited in NCBI and its accession number (HQ267533) was obtained. The phylogenetic tree was constructed by the Neighbor- Joining (NJ) and Kimura two pair method and topologies were evaluated by performing bootstrap analysis of 1000 sets by using MEGA 4.0 software (The Biodesign Institute, Tempe, AZ, USA). [26]

Fermentation and extraction of secondary metabolites of S. fradiae RMS-MSU

In the present study, the seed culture was prepared by inoculating a loopful of mature sporulating *S. fradiae* RMS-MSU in 100 ml of Yeast-Malt extract glucose broth medium at 150 rpm for 4 days at room temperature. Then the *S. fradiae* RMS-MSU was mass cultivated in optimized broth medium (Mannitol- 10g, Yeast extract - 10g, NaCl- 10g, pH: 7.5, 1000 ml distilled water). About 20 L of autoclaved optimized broth medium was prepared in 10 × 2.5 L of Erlenmeyer conical flasks, cooled and 5% seed culture was inoculated and incubated at 150 rpm for 10 days. After the incubation period, the culture broth was centrifuged at 10,000 rpm for 20 min at 4ºC. Finally, the spent culture free broth was filtered through 0.45 and 0.22 µ cellulose membrane filter paper and the filtrate was extracted by (1:1 ratio) equal volume of ethyl acetate (HPLC grade, Himedia) at 120 rpm for 3 days. Then the organic fraction was

collected by using the separating funnel and concentrated by rotary vacuum evaporator (GOEL, GRFE-2, Gujarat, India) at 35ºC. Finally, the crude extract obtained was weighed and stored in preweighed clean glass vials at 4ºC.

Selection of marine biofilm bacterial and Microalgal strains

Totally, 17 different marine biofilm bacterial strains such as *Aeromonas hydrophila* (JN561697); *Halomonas aquamarina* (JN561698); *E. coli* (JN585664); *Vibrio* sp. (JN585665); *Morganella morganii* (JN596112); *Citrobacter freundii* (JN585667); *Vibrio paraheamolyticus* (JN585666); *Enterobacter* sp. (JF970207); *Salmonella* sp. (JN596113); *Micrococcus* sp. (JN596114); *Serratia liqufacieans* (JN596115); *Pseudomonas* sp. 1 (JN596116); *Shigella sonnai* (JN596117); *Serratia marcesences* (JN596118); *Pseudomonas* sp. 2 (JN596119); *Pseudomonas putida* (JN596120); *Serratia fonticola* (JN596121) were isolated from marine substrata immersed in Thondi Coasal Water, Palk Bay, Tamilnadu, India and identified by 16S rRNA gene sequencing. Marine fouling microalgal strains such as *Chaetoceros* sp., *Chlorella* sp., *Nannochloropsis* sp., *Dunaliella* sp. and *Tetraselmis* sp., were obtained from the marine algal culture unit, CMFRI (Central Marine Fisheries Research Institute), Tuticorin, Tamilnadu, and the individual seed culture was maintained in Conway medium at 20 ºC in 12h light period for 7 days.

Antimicrofouling assays

Antibacterial activity against marine biofilm bacterial strains

Crude extract of *S. fradiae* RMS-MSU was tested against the marine biofilm bacterial strains by agar well diffusion method.[27] In brief, biofilm bacterial strains at logarithmic phase were seeded individually on Muller Hinton Agar (MHA) with 50% sterile seawater and the wells were made by using sterile cork borer. Finally, each well was filled with

100 µl DMSO (Dimethyl Sulphoxide) containing 400 µg ml^{-1} of crude extract. DMSO (100 µl) was used as negative control, whereas copper sulphate (20 µg/ ml^{-1}) was used as positive control. The agar plates were incubated at 32ºC for 24h. After the specified incubation period of zone of inhibition was measured in mm level from the edge of the well. Each assay was carried out in triplicate. Furthermore, MIC and MBC concentrations were assessed through a 96 well microplate assays (Flat Bottom; Polystyrene, Eppendorf, India) by following the method of Sharma and Kumar.[28] Briefly, the different concentration (6.25 to 400 µg ml^{-1}) of crude extract and positive control (copper sulphate) ranged from 5 to 20 µg ml^{-1} were prepared and loaded in a 96 clear polystyrene plate using Methanol: Ethyl acetate at 3:1 as carrier solvent and evaporated under sterile condition. Thereafter, 1×10^{8} CFU ml^{-1} cell densities (Amsterdam, 1996) of 100 µl of each biofilm bacterial inoculum were added into each well. Well plate containing only biofilm bacterial strains suspension was used as negative control and incubated at 30ºC for 24h, each wells were examined for microbial growth by turbidity observation. To determine the MIC and MBC, a loopful of inoculum was streaked onto Zobell marine agar plates and incubated at 37°C for 24 h. [27] The concentration which inhibits the bacterial growth was recorded as MIC value and in which there is no visible bacterial growth on ZMA plates as MBC.

Antimicroalgal assay against marine fouling microalgae

Antimicroalgal assay was also conducted with different concentrations (25, 50, 100, 200, 400 and 800 µg ml^{-1}) of crude extract *S. fradiae* RMS-MSU and positive control as copper sulphate (10 ug ml^{-1}) separately using methanol: ethyl acetate (3:1) as carrier solvent loaded in a 96 clear polystyrene plate (polymeric materials). The assay was started with an initial cell density of 1×10^{5} cells ml^{-1} of each marine microalgal strains. Then the plates were incubated at

20ºC under 12h light periods for five days. The least concentration of crude extract, where no algal growth was observed in 4 to 6 wells was recorded as the minimum inhibitory concentration. [29]

Anticrustacean assay

In the present study, the anticrustacean assay was conducted using brine shrimp *Artemia salina* larvae (II in star) as a model organism. Anticrustacean assay was performed by 24 well clear polystyrene plate (polymeric materials) method. [30] For the toxicity test, 500 µl of different concentrations (25 to 500 µg ml^{-1}) of crude extract of *S. fradiae* RMS-MSU was coated onto 24 well microplates and the solvent was evaporated at room temperature with sterile condition. Then, ten live and healthy (most active swimming) larvae of *A. salina* was introduced into the coated micro plate with 500 µl of filter sterile seawater. After 24 h of incubation at 25ºC, the mortality was counted in each concentration loaded wells. Copper sulphate (2.5 to 80 µg ml^{-1}) was used as positive control, whereas wells free of extract and copper sulphate served as negative control was recorded as the percentage of mortality noticed after 24h and LC_{50} value was calculated by probit analysis. The assay was carried on triplicates.

Antimacrofouling assay

Byssal thread inhibitory activity of crude extract

The method described by Iyapparaj et al.[5] was followed for the mussel bioassay. For the present study, the brown mussel, *Perna indica* with a uniform size range (1.5 to 2 cm length) was used. Before the start up of the experiment, the byssal threads of all the mussels were scrapped off completely and ten numbers of young and healthy mussels were introduced into individual beakers containing 100 ml of filtered and sterilized seawater. Then the different concentrations of crude extract (50 to 800 µg ml^{-1}) were dissolved individually in DMSO and

introduced into experimental beakers. Beakers containing, only seawater was used as negative control; whereas seawater with copper sulphate ($CuSO_4$) was used as positive control (1-124 µg ml^{-1}). The experiment was carried out in triplicates. The beakers were then incubated at room temperature with mild aeration (dissolved oxygen ranged from 4.2 to 5.1 ml/L). The settlement of the mussels at each concentration was recorded after 24 h. The EC_{50} (Effective concentration at which 50% of mussel showed inhibition of byssal attachment) was recorded based on the minimum concentration, which prevented the byssal thread production and the subsequent attachment. The LC_{50} value was estimated by probit analysis, after exposing the *P. indica* in test concentrations of the extract for 96 h. In the toxicity assay, the same mussels used in LC_{50} estimation which failed to develop byssal thread in seawater containing the crude extract were transferred to the extract free sea water and observed for 72 h for byssal production and attachment. The therapeutic ratio (LC_{50}/EC_{50}) value was calculated for the active extract to test its non-toxic nature. [11, 31]

Mollusc foot adherence assay on *Patella vulgata*

The mollusc foot adherence assay is a more rapid and reliable assay methodology which requires a minimum quantity of the test compound to determine its effect on the settlement of mollusc by spreading their foot. [32] In the present study common limpet, *Patella vulgata,* a rocky common fouler was used as a model organism to describe the antifouling property of a crude ethyl acetate extract of *S. fradiae* RMS-MSU. Collected limpets were transported to the laboratory in a plastic container with aerated seawater. Before the start up of the experiment, they were acclimatized in the laboratory condition for four days in seawater filled, sand bedded FRP tank. During the course of acclimatization, they were fed *ad libitum* with algae *Ulva fasciata*. The assay was performed in a series of assay plates (100 mm petri plates). Briefly,

assay plates were spread evenly with different concentrations (1 to 7 mg ml^{-1}) of crude extract of *S. fradiae* RMS-MSU and subsequently evaporated to dryness in a hot air oven at 35°C to obtain uniform film of the extract. The plates containing concentration 1 and 2 mg ml^{-1} copper sulphate as positive control also maintained separately. Then one third of all the coated plates were filled with filtered seawater, control plate without extract was also maintained separately. The whole setup was carried on the transparent glass surface to observe the foot reflex. The assay was performed in triplicates at the rate of 10 animals per plate. The immediate foot reflex and mobility was monitored continuously until the foot was completely shrunken. Similarly, fouling and the regaining percentage (only unsettled organisms) on the coated plates were also recorded.

Purification and partial characterization of active metabolites

Purification of bioassay guided fraction by chromatography techniques

The crude extract (1.5 gm) of *S. fradiae* was purified by using normal phase silica gel column chromatography (60 - 200 µm mesh size) by using the stepwise gradient solvents likes hexane, benzene, ethyl acetate, methanol and end with warmed methanol. Totally, 32 fractions (each 20 ml) were collected, dried under gloomy condition and then all the fractions (100 µg/ml) were screened individually against both biofilm bacterial and microalgal strains through microplates assay. Amongst the fractions, 25^{th} fraction showed the best antifouling properties over the other fractions. Furthermore, the 25^{th} fraction was purified by Thin Layer Chromatogram (TLC) method. [27] In brief, the 25^{th} fraction was redissolved in ethyl acetate: methanol (6:4) and made up to a volume of 100 mg/ml. From this, 100 µl was taken and spotted on TLC plates (TLC aluminum sheets, 20 x 20 cm, silica gel $60F_{254}$, Merck, USA) using the same solvent systems as mobile phase. Thereafter, the TLC plates were dried at room

temperature and observed under UV/Vis absorption (Bio-Rad; AlphaImager^{TM} 3300) for detection and marking different spots at different wavelengths of 254 and 365 mm. The Rf values of the TLC plate were then calculated and recorded. There after the TLC plate were tested against fouling bacterial strains through TLC bioautography. [27]

Chemical constituents and FT-IR analysis of bioassay guided fraction

The chemical constituent of the bioassay guided fraction was identified through conventional TLC plate spray method. [33] In brief, the TLC plate was sprayed with Liebermann-Burchard reagent (A mixture of 5 ml acetic anhydride with 5 ml of 97% sulfuric acid was prepared in chilled 50 ml of ethanol) was sprayed TLC plate kept in hot air oven for 10 min at 110°C, after that the plate visualized under the UV light with short and long wavelength 365 nm for the detection of Triterpene glycosides. Furthermore, the presence of possible functional groups in the bioassay guided fraction of *S. fradiae* was also determined by Fourier Transmission - Infra Red spectroscopic analysis (Shimadzu FTIR-820 IPC, Japan), the frequency of the spectral analysis was set between 4000 and 400 cm^{-1}. [34]

Statistical analysis

The results obtained in the present study were subjected to relevant statistical analysis by one-way ANOVA and further *post hoc* multiple comparison using the Dunnett test with varying significant level ($P< 0.05$; $P< 0.01$; $P< 0.001$; $P< 0.0001$) using the SPSS -16 version (SPSS Inc, Chicago, USA) and the EC_{50} and LC_{50} values for *A. salina* and *P. indica* were calculated in probit analysis by using the EPA software (Environmental Protection Agency, Cincinnati, Ohia, USA).

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Table 1 Morphology, biochemical and physiological characteristics of strain RMS-MSU

Characters	Results
Cell Shape	Mycelium Round & irregular
Colony Morphology	Margin: Concave & irregular
Aerial mycelium Colour	Pink
Substrate mycelium Colour	Yellow
Spore Colour	Grey
Spore Shape	Spiral
Diffusion Colour	-
Melanoid pigment	-
Growth	Aerobic
Grams Reaction	++
Range of temperature	20 to 40ºC
Optimum temperature	35ºC
Range of pH for growth	5 to 9
Optimum pH	7.5 to 8
NaCl tolerance (%)	0.5 to 5 %
Optimum NaCl	2 %
Urease	+
H_2S Production	-
Nitrate Reduction	+
MR Reaction	-
VP Reaction	-
Indole Production	-
Citrate Utilization	+
Glucose	-
Mannitol	++
Lactose	++
Fructose	+
Sucrose	-
Xylose	+
Inositol	-
Maltose	+++
Galactose	+
Protein	++
Lipids	++
Starch	+
Cellulose	-

Gelatine	+
Pectin	+

+++: High positive; ++: Moderate positive; +: Positive; - : Negative

Table 2 Antimicroalgal activity of crude extract of *S. fradiae* RMS-MSU against marine micro algal strains

Microalgal strains	Control (Only microalgal strains)	Different concentration of crude extract (µg/ml)						Positive control $CuSo_4$ (10 ug/ml)
		25	50	100	200	400	800	
Chaetoceros sp.	+++	+++	+++	++	+	-	-	-
Chorella sp.	+++	++	+	-	-	-	-	-
Nannochloropsis sp.	+++	++	++	+	-	-	-	-
Dunaliella sp.	+++	++	+	+	-	-	-	-
Tetraselmis sp.	+++	+++	+++	++	+	-	-	-

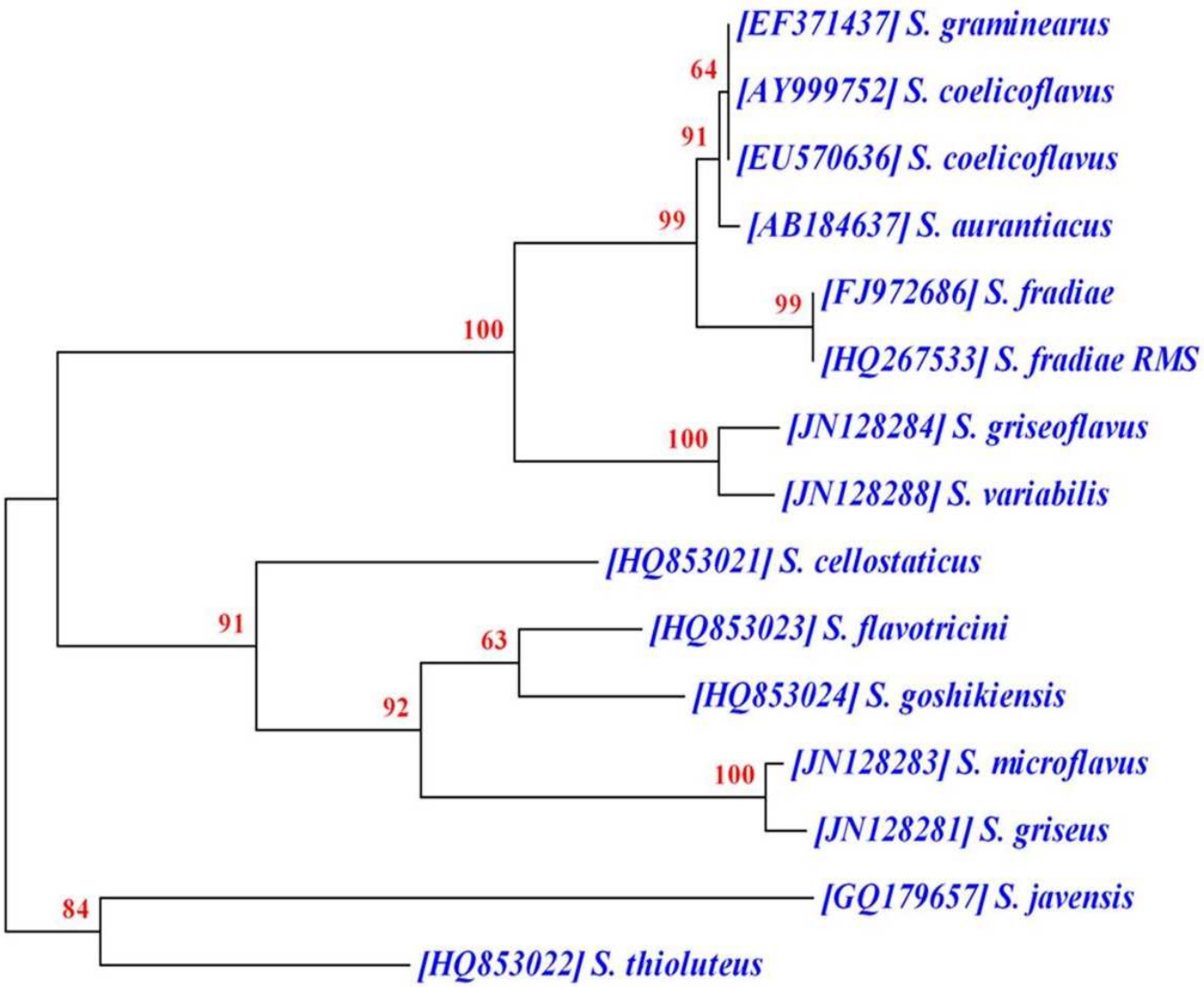
+++: Excellent growth; ++: good growth; +: moderate growth; - : No growth.

Table 3 Toxicity Profile (EC_{50} and LC_{50} values) of *S. fradiae* RMS-MSU against *P. indica* and *A. salina*

Fouling Organisms	Test animal	*EC_{50}	*LC_{50}	LC_{50}/ EC_{50}	Mode of action
Bivalve	*P. indica* (µg ml^{-1})	77.03 ± 1.96	718.79 ± 1.55	9.33**	Less / Non toxic
Positive Control - $CuSo_4$ (µg L^{-1})		5.47 ± 0.006	6.08 ± 0.003	1.1**	High Toxic
Crustacean	*A. salina* (µg ml^{-1})	ND	273.77 ± 2.76	ND	Less / Non toxic
Positive Control - $CuSo_4$ (µg L^{-1})		ND	9.35 ± 0.76	ND	High toxic

*Mean ± SD of three observations; ND: Not Determent and** referring to Qian et al., 2010

1 **Fig. 1.** Phylogentic relationship of *S. fradiae* RMS-MSU with closely related BLAST
2 sequences



3
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Fig. 2. Antimicrofouling activity (zone of inhibition - mm) of crude ethyl acetate extract of *S. fradiae* RMS-MSU against biofilm bacterial strains

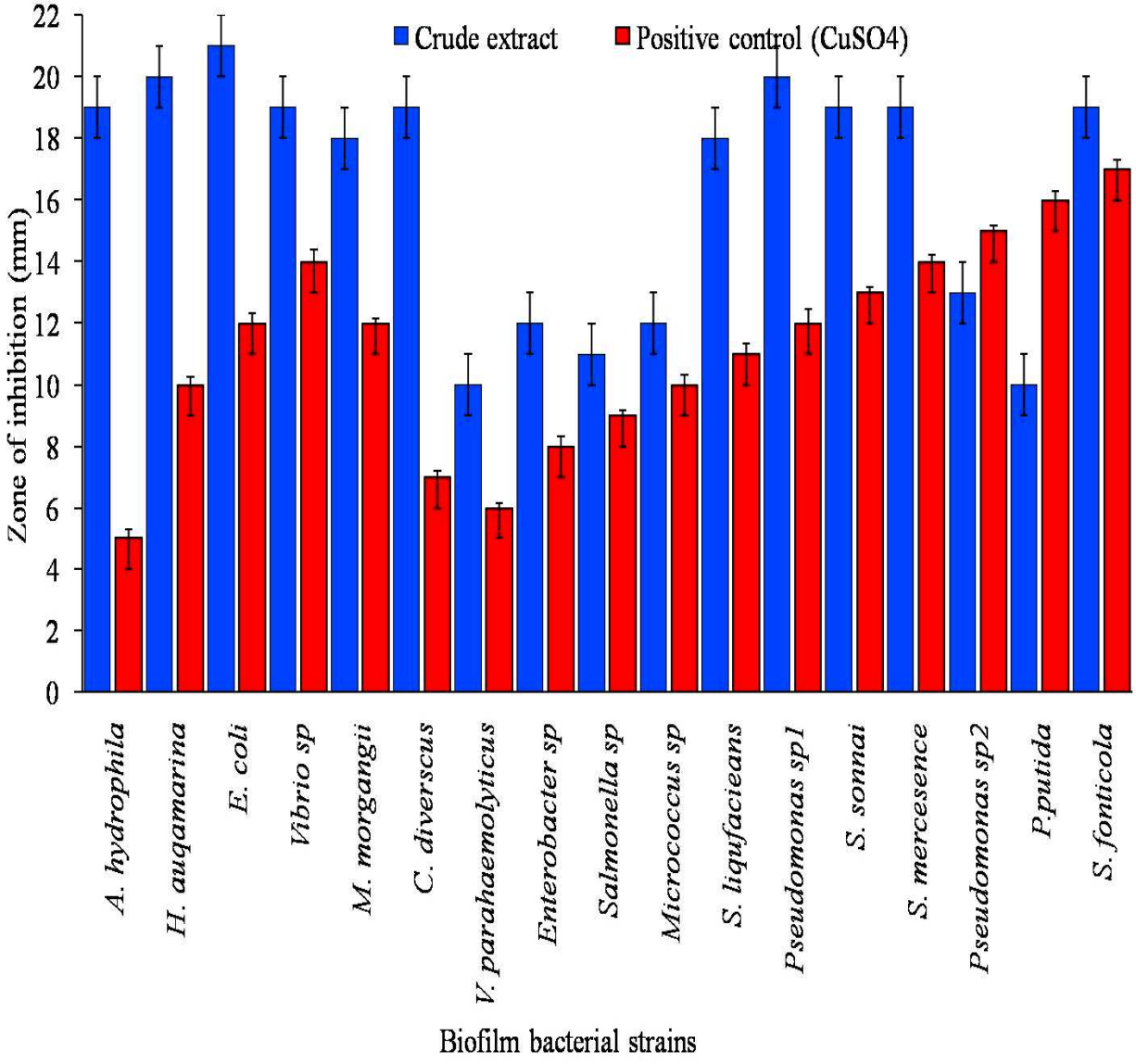


Fig. 3. Minimal Inhibitory (MIC) and Minimal Bactericidal (MBC) concentrations tested on (A) crude extract of *S. fradiae* (B) Copper Sulphate ($CuSO_4$) against biofilm bacterial strains

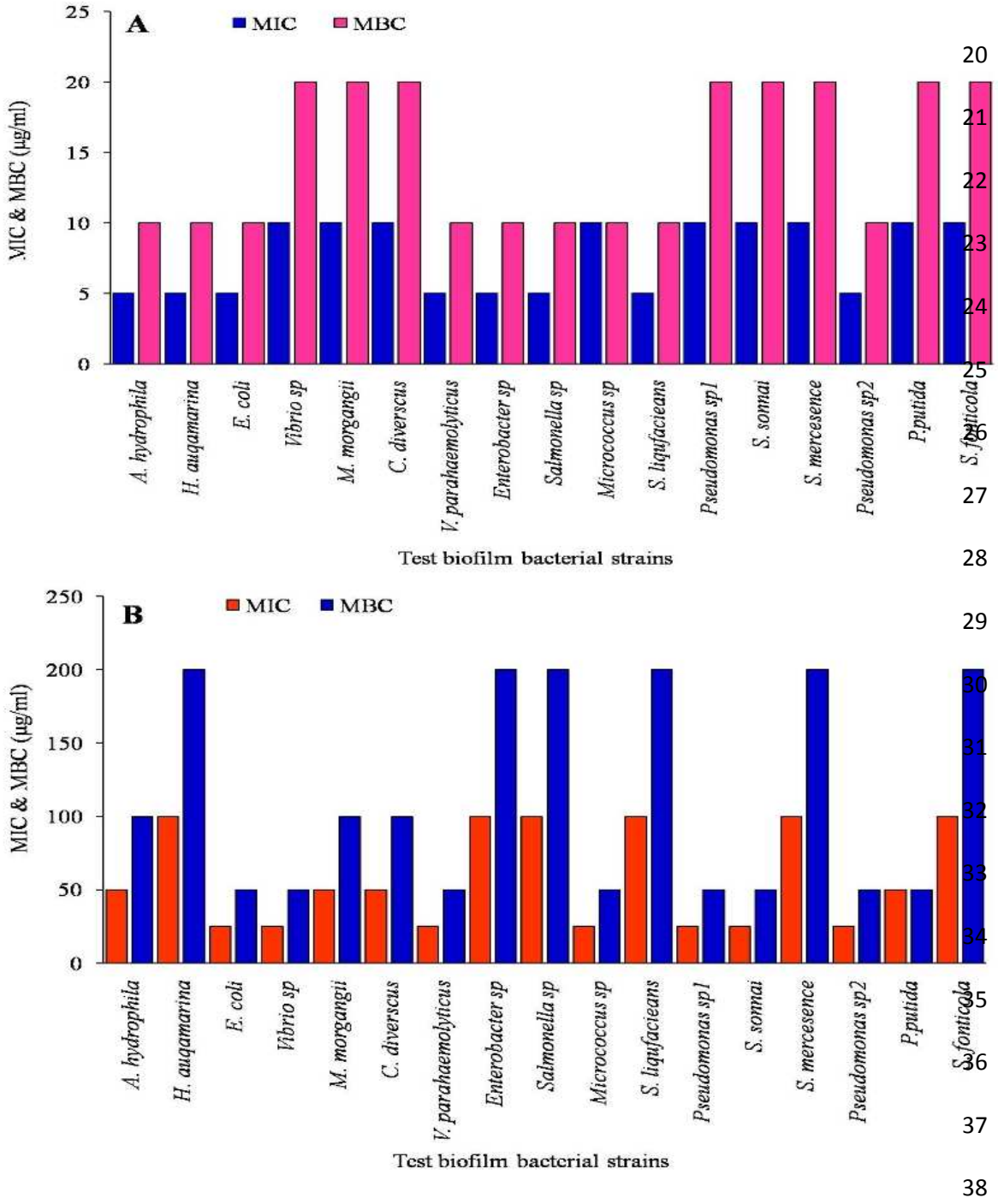
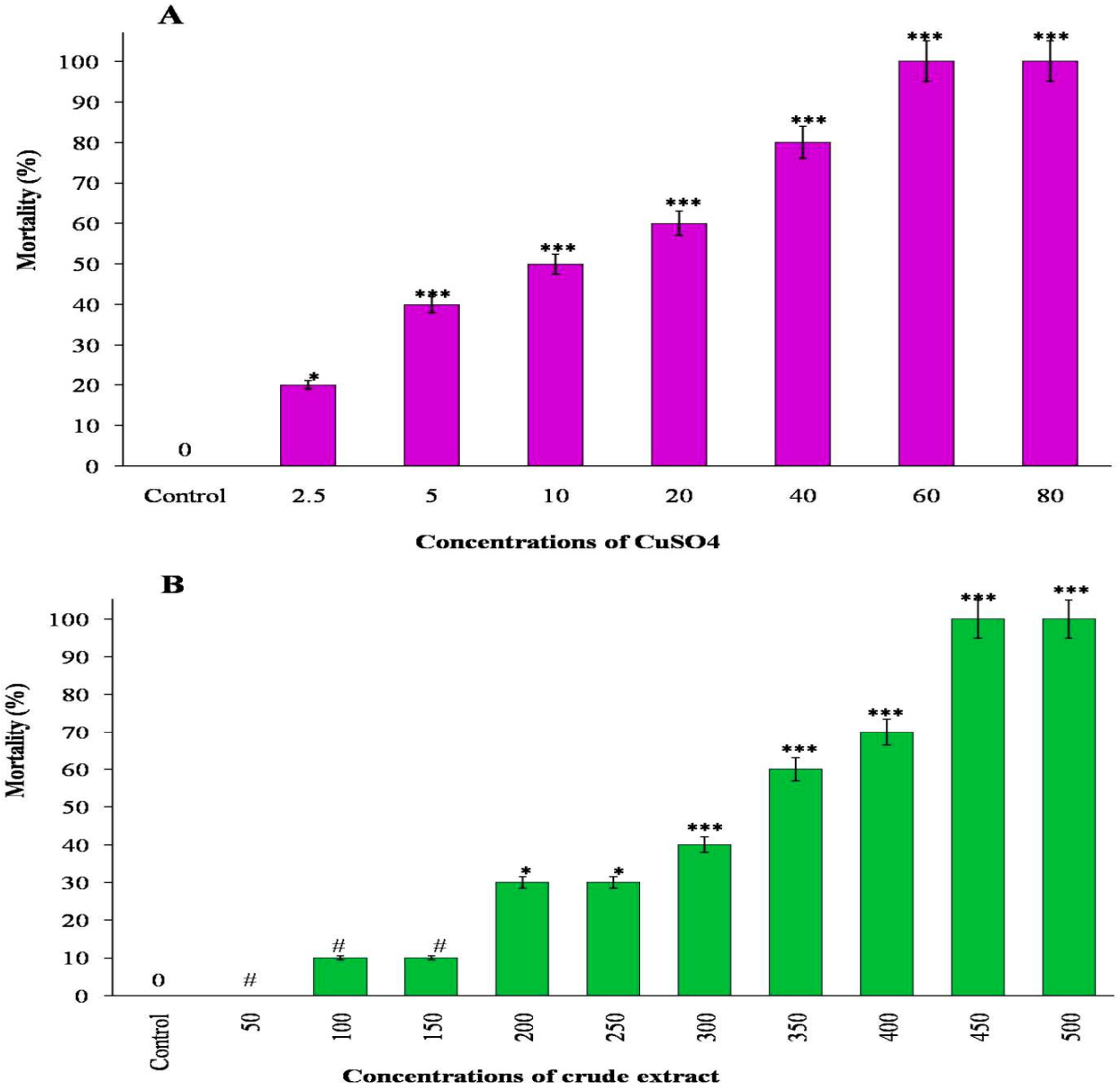


Fig. 4. Anticrustacean assay with different concentrations of (A) Copper Sulphate ($CuSO_4$) and (B) crude extract of *S. fradiae* against *A. salina* larvae



Mean ± SD value indicates significant percentage mortality on *A. salina* in different test concentrations of crude extract and copper sulphate compared with control (one-way ANOVA, Dunnett test, *P< 0.05; ***P< 0.0001; # non significant).

Fig. 5. Inhibition of byssal attachment and mortality of *P. indica* exposed at different concentrations of (A) crude extract of *S. fradiae* RMS-MSU and (B) Copper Sulphate ($CuSO_4$)

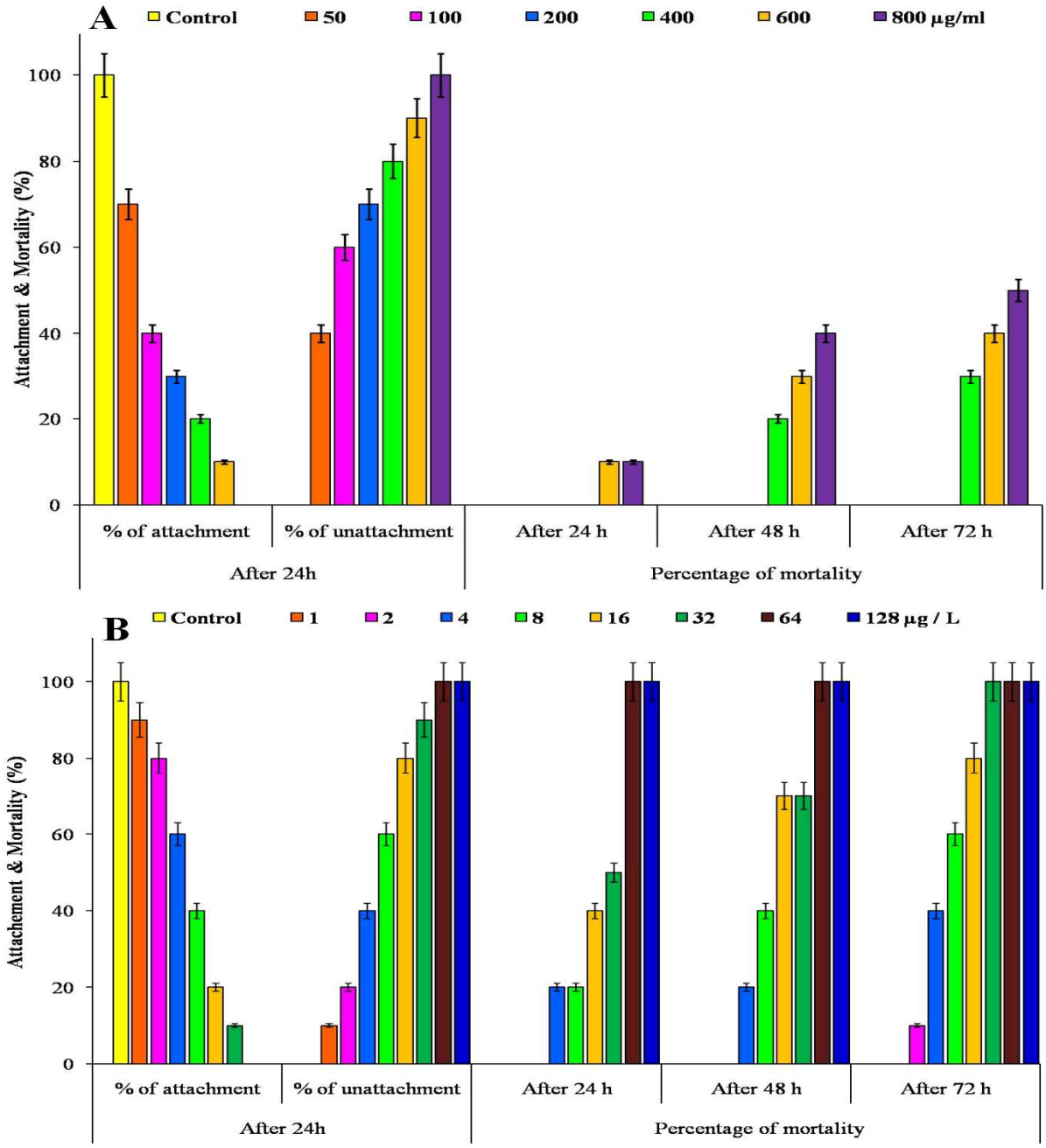
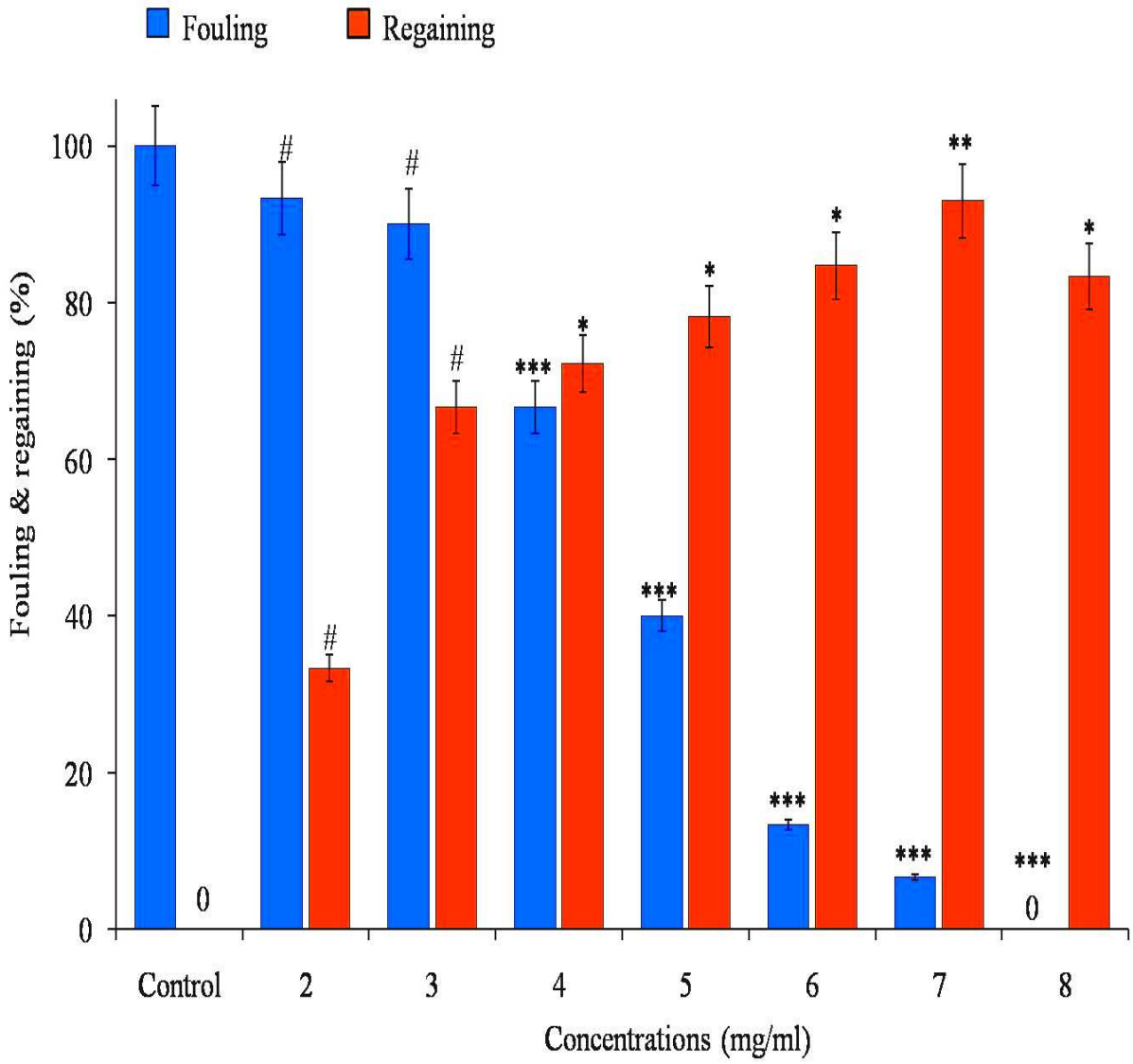


Fig. 6. Antimolluscidal activities of crude extract of *S. fradiae* RMS-MSU and Copper Sulphate ($CuSO_4$) against marine limpet *P. vulgate*



Mean value indicates significant percentage of fouling and regaining of *P. vulgata* in different test concentrations compared with the control (One-way ANOVA, Dunnett test, *P< 0.05; **P< 0.01; ***P< 0.0001 and #non significant).