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Production of anti-biofouling metabolites by *Streptomyces* sp. strain RD4 for its application in mitigating the bacterial biofilms

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ABSTRACT

This study aims for combating the biofilm formation using four different potential marine actinomycetes strains, isolated from Alang-Sosiya Ship Breaking Yard, Bhavnagar. *Streptomyces* sp. strain RD4 was found to be very effective in producing the anti-biofouling metabolites. The results were analyzed using phenotypic, Congo red agar test and microtiter plate technique. The highest anti-biofilm activity was possessed by the extract of *Streptomyces* sp. strain RD4 with 91.07%, 89.54%, 64.91%, and 79.17% inhibition against biofilm-forming bacteria isolated from the same site, which are identified as *Bacillus sonorensis* strain RD10, *Bacillus licheniformis* strain RD11, *Bacillus subtilis* strain RD14, and *Staphylococcus epidermis* strain RD12, respectively, at 2.5 mg/mL. However, 100% inhibition of all the bio-fouling bacteria was observed when 5.0 mg/mL of the anti-biofoulant was used. Anti-biofouling activity was confirmed using live/dead cell assay, and the anti-biofoulant metabolite was characterized.

Keywords: Anti-biofouling; *Bacillus sonorensis*; Biofilm; Congo red agar; *Streptomyces* sp.

1. Introduction

Marine biofouling is the result of the growth of microorganisms on the surfaces of immersed marine structures in the natural environment [1]. The organisms involved in the marine biofouling are primarily attached or they exist as sessile forms occurring in shallow waters along the coastline. The marine biofilms are much more serious threat than the macro-fouling to the marine systems [2]. The adherence, growth, and colonization of microorganisms on the marine structures result in the formation of a slimy layer called as biofilms [3]. Biofouling is an ongoing problem for water immersed man-made structures resulting in severe material and economic losses by reducing the performance of the marine equipment [4].

In efforts to avoid marine biofouling, many anti-fouling paints are used which are mostly composed of organotin like tri-n-butylin (TBT) binders such as copper and organo-nitrogen [5]. The use of TBT-based paints is banned because they cause enormous pollution and biomagnifies globally in the environment. Currently, there is a major challenge for the production of coatings and in the development of alternative technologies to nullify the effects of biofouling [6]. Owing to the inclined regulations on the restrictive use of TBT and other polluting anti-foulants, there is a need to develop economical and eco-friendly anti-biofouling compounds for marine applications [7–10].

The anti-biofouling metabolites from the microorganisms are mainly through the production of secondary metabolites by many marine organisms, those exhibiting inhibitory effects against the biofoulants [11,12]. The compounds exerting the anti-biofouling activities are usually terpenoids, steroids, carotenoids, phenolics, furanones, alkaloids, peptides,

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and lactones. The aforementioned traits would be ideal for the development of natural anti-foulants [13,14]. Among the diverse marine organisms, antifouling activities were largely reported from sponges and corals as they share close relationships with other organisms and they are known to have viable bioactives [15–17]. Ascidians are also known to possess antagonistic properties against biofouling [18,19]. Several marine organisms especially microalgae and marine invertebrates are known to produce biogenic agents, usually secondary metabolites possessing antagonistic properties to defend themselves and thus, they are rarely fouled by other organisms [14,20]. Till date, many reports are found for the production of antifouling compounds from bacteria and algae [21,22], while the anti-biofouling agents from marine actinomycetes are scanty.

This research is focused on the possible exploration of marine actinomycetes, in particular, which are least exploited for the production of anti-biofouling compounds. Initially, to screen marine bacterial strains those are capable of forming biofilms from the marine structures and to isolate marine actinomycetes that resist the biofouling. Finally, anti-biofouling metabolites from the potential marine actinomycetes were extracted and characterized.

2. Materials and methods

2.1. Screening of biofilm-forming marine bacteria

The samples were scraped marine structures around Alang Ship Scraping Yard, Bhavnagar (India) in a sterile polythene bag. The marine bacteria possessing the ability to form biofilms from the samples were isolated using Zobell marine agar. The flasks were incubated at 37°C for 24–48 h and morphologically distinct colonies were selected, purified and preserved on Zobell marine agar slants. The isolated cultures were streaked on modified Congo red agar (CRA) containing (g/100 mL) sucrose, 2.0; peptone, 1.0; sodium chloride, 0.5; beef extract, 0.25; Congo red dye, 0.2; and agar, 3.0. The plates were inoculated with the test organisms and incubated at 37°C for 24–48 h. The marine bacteria having the biofouling activity were observed as black pigmented colonies on the surface of agar [23].

2.2. Biofouling assay and quantification

The biofouling activity of primarily screened marine bacteria was assayed by the method described by O'Toole and Kolter, [24] with some modifications. A total of 100 μL of Zobell marine broth was loaded in sterile 96-well microtiter plate (Sarstedt, Germany) and 50 μL of each fresh bacterial suspension were added. The microtiter plate was then incubated at 37°C for 48 h under static condition. After incubation, the content of each well was gently removed by tapping the plates. The free-floating debris was removed by flushing with 200 μL of sterile 0.85% saline solution, and the adhering cells in the plate forming biofilm were stained with 0.1% crystal violet and incubated at the room temperature for 20 min. The excess stain was rinsed off using distilled water, and the plates were fixed with 200 μL of 96% ethanol. The absorbance of stained adhered bacteria was measured at 630 nm using an enzyme-linked immunosorbent assay microtiter plate reader (Epoch, BioTek, USA). All the tests were performed in triplicate and the cutoff absorbance was defined as three standard deviations above the mean absorbance values of the negative control (culture medium). The biofilm producing strains such as weak, moderate, strong, and non-biofilm producer were classified as described by Stepanović et al. [25].

2.3. Characterization and identification of biofouling bacteria

The phenotypic characterization such as colony characteristics and microscopy of selected isolates were studied by standard procedures. The biochemical characterization of the bacterial isolates was done using Biolog (BIOLOG MicroStation™ System, Hayward, California, USA) and fatty acid methyl ester (FAME) profiling. The selected biofouling bacteria were identified using 16S rRNA gene sequencing. The sequences have been deposited in the National Center for Biotechnology Information (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov), and accession numbers were obtained.

2.4. Screening of anti-biofouling metabolite producing marine actinomycetes

Actinomycetes from the marine sediments were isolated using Gause's synthetic medium containing (g/L) starch, 20.0; KNO_3, 1.0; NaCl, 0.5; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4$, 0.01; and agar, 3.0, and 0.01% of potassium dichromate was added into the medium to prevent bacterial and fungal growth [26]. After suitable dilution, the samples were inoculated in Luria-Bertani broth containing (g/L) casein hydrolysate, 10.0; yeast extract, 5.0; and sodium chloride, 10.0 (pH 7.5 ± 0.2) and incubated at 30°C under shaking condition (120 rpm). The antifouling activities of the isolated marine actinomycetes were determined using cross-streak method against the isolated biofouling marine bacteria. The extracts of potential actinomycetes were prepared and were subjected to secondary screening, and the zone of inhibition was measured accordingly. The actinomycetes culture those resisting the growth of biofouling bacteria were subcultured, and the glycerol stocks were stored at –80°C. The functional groups in the anti-biofouling metabolite were analyzed by recording the Fourier transform infrared (FT-IR) spectra using a GX-FT-IR system (PerkinElmer, USA).

2.5. Quantification of anti-biofouling activity of marine actinomycetes

A total of 5.0 mg/mL individual extracts of the promising actinomycetes were loaded in a sterile 96-well microtiter plate containing 50 μL of Mueller Hinton broth, and 50 μL of fresh bacterial suspension were added to each well and were incubated at 37°C for 48 h. The biotic control (cells in broth), abiotic control, (only broth) and blank control (broth with extract) were included. The biofilm formation was assayed using crystal violet staining method, and the percentage of biofilm inhibition was calculated using the following relation:

$$\text{Biofilm inhibition}(\%) = \frac{\left(\text{Absorbance}_{\text{Biotic control}} - \text{Absorbance}_{\text{Sample}}\right)}{\text{Absorbance}_{\text{Biotic control}}} \times 100 \quad (1)$$

The biofilm inhibiting concentration is defined as the lowest concentration of the extract that exerts 50% inhibition on the biofilm formation [27].

2.6. Evaluation of anti-biofouling activity by microscopic imaging

Live/dead BacLight™ viability kit (Molecular Probes, Inc., Eugene) was employed to assay the anti-biofouling activity for 20–30 min using two components such as SYTO 9 that stains all bacterial cells with a green fluorescence and propidium iodide, a red fluorescent stain that only penetrates cells with damaged membranes or dead cells [28]. For staining, 200 solution of fluorescent stain was freshly prepared by adding 3 μL of SYTO® 9 stain and 3 μL of propidium iodide stain to 1.0 mL of filter-sterilized water. A total of 5.0 mL of culture broth of individual marine bacterial isolates were tested for live/dead cell assay by adding 2.5 and 5.0 mg/mL extract of potential actinomycetes, respectively, and the tubes were incubated for 72 h under static condition. The samples were fixed on glass slide, stained immediately with 200 μL of staining solution prior drying and incubated in dark for 20–30 min at room temperature. The sample was rinsed with filter-sterilized water and excess stain was removed followed by observation under the fluorescence microscope (Olympus, BX53, Europe).

3. Results and discussion

3.1. Isolation and identification of biofouling marine bacteria

Presently, marine microorganisms are of greater attention to overcome the exploitation of marine organisms such as corals and sponges for the production of bioactive molecules [29]. The anti-biofouling mechanism usually involves the inhibition of ion channel and obstructs the sensing and transmission of chemical signals affecting the release and/or production of adhering adhesives [14,29]. In this investigation, 35 bacterial isolates were selected on the basis of their diverse morphology and color variations, and upon purification the isolates were screened for the biofouling activity by CRA method. It was found that only 10 among the 35 isolates could form black-colored colonies on CRA plates (Fig. 1(a)) indicating the biofilm formation [23,30]. Further screening of all the 10 isolates was done using microtiter plate assay and 4 isolates were found to exhibit higher biofouling activity on Zobell marine agar (Fig. 1(b)). The four isolates were cultured further to yield a consistent colony and the 16S rRNA gene sequence data having 1,300 bp were analyzed according to BLASTn to identify the isolates, and the sequences were submitted to NCBI for obtaining the accession numbers. The isolates were identified as *Bacillus sonorensis* strain RD10, *Bacillus licheniformis* strain RD11, *Staphylococcus epidermidis* strain RD12, and *Bacillus subtilis* strain RD14 as shown in Fig. 2.

Several reports by the other researchers suggest that the biofilm forming bacteria were isolated from the marine environment such as harbors and coastal waters [31–36]. The frequently identified isolates were *Bacillus megaterium*, *Bacillus thuringiensis*, *Bacillus atrophaeus*, *Staphylococcus saprophyticus*, *Agrobacterium vitis*, *Serratia* sp., and *Alteromonas* sp. [32,37]. It was noted that *Bacillus* sp. was found to be predominant and commonly prevalent biofouling bacteria [31].

3.2. Screening of anti-biofouling marine actinomycetes

The cross-streak method was done for evaluating the anti-bacterial activity as shown in Fig. 3, and it was found that 10 isolates exhibited antibacterial activity against biofouling bacteria and out of which only 4 isolates of actinomycetes were selected as they possess maximum anti-biofouling activity. The four isolates were cultured further to yield a consistent colony, and it was found that the isolates were Gram positive with mycelial hyphae. The metabolic profiles of these isolates from Biolog GENE III micro-plates and FAME analyses revealed that the isolates closely matched with *Streptomyces* [38]. 16S rRNA gene sequence data were analyzed to identify the isolates and

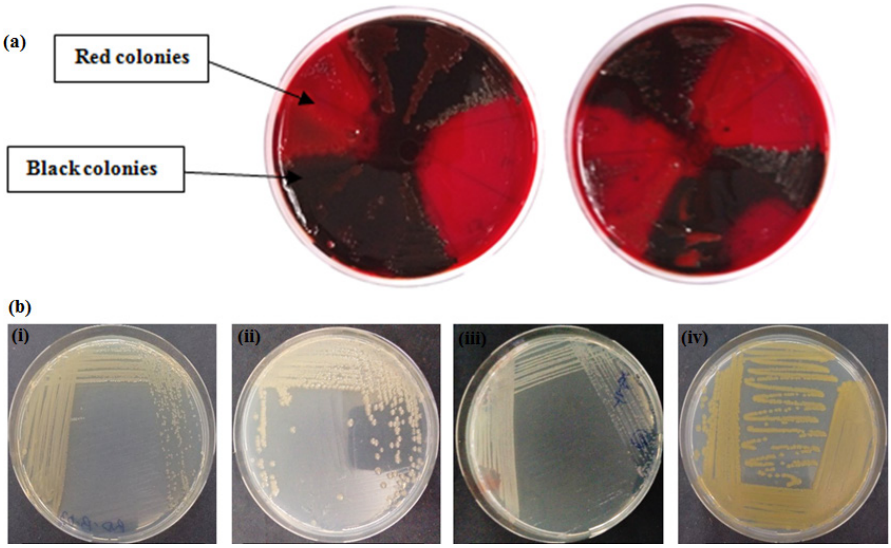

Fig. 1. (a) Bio-fouling bacteria on CRA plate and (b)colony characteristics of four different marine biofouling bacteria on Zobell marine agar.

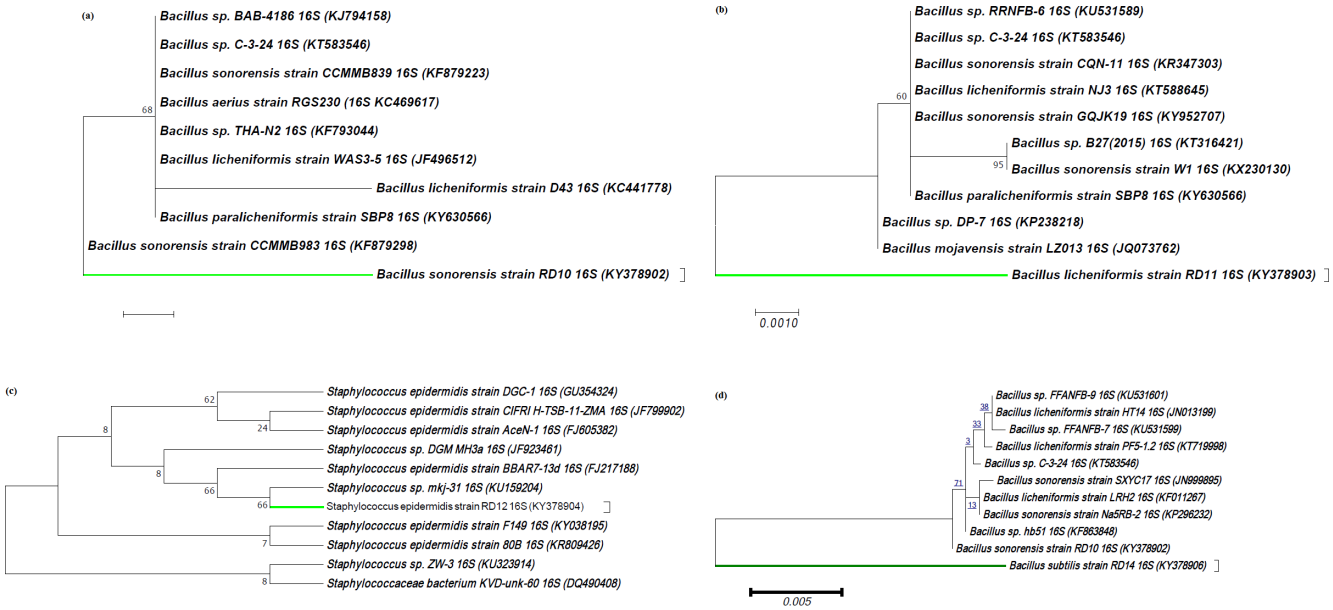

Fig. 2. Phylogenetic relationships of (a) *Bacillus sonorensis* strain RD10, (b) *Bacillus licheniformis* strain RD11, (c) *Staphylococcus epidermidis* strain RD12, and (d) *Bacillus subtilis* strain RD14with other strains.

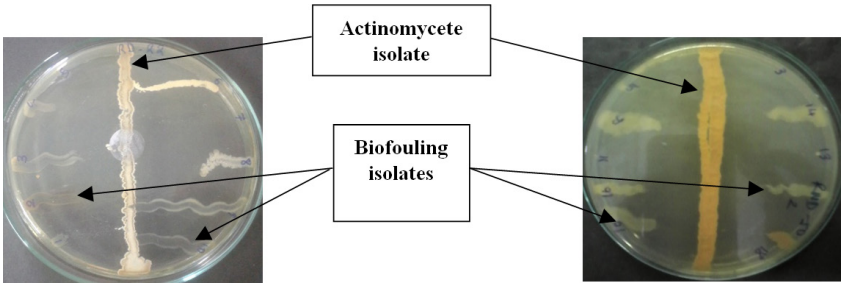

Fig. 3. Cross streak method for primary screening of anti-biofouling activity.

found as species belonging to genera *Streptomyces*. They were identified as *Streptomyces cyaneus* strain RD1; *Streptomyces pseudogriseolus* strain RD2, *Streptomyces* sp. strain RD4, and *Streptomyces variabilis* strain RD5, and their phylogenetic relationship is illustrated in Fig. 4. The identified actinomycetes were inoculated in Gause's synthetic broth individually and incubated at 30°C for 7–9 d. The effect of incubation time on growth and anti-biofouling activity was analyzed every 24 h interval for 9 d.

3.3. Anti-biofouling activity of marine actinomycetes

The anti-biofouling activity of *S. cyaneus* strain RD1, *S. pseudogriseolus* strain RD2, *Streptomyces* sp. strain RD4, and *S. variabilis* strain RD5 were tested, and the percentage inhibition of biofilm formation against the biofouling bacteria is given in Fig. 5. The results from other researchers were compared against our findings and it was reported that *Streptomyces filamentosus* (RI) showed 13–15 mm inhibition against three biofouling bacteria such as *Bacillus* sp. (BB11), *Serratia* sp. (BB12), and *Alteromonas* sp. (BB14) [39]. *Streptomyces fradiae* RMS-MSU displayed antagonistic activity (10–21 mm) against marine biofilm bacterial strains [8].

In this study, the highest anti-biofouling activity was observed by the extract of *Streptomyces* sp. RD4 with 91.07%, 89.54%, 79.17% and 64.91% inhibition of *B. sonorensis* strain RD10, *B. licheniformis* strain RD11, *S. epidermidis* strain RD12, and *B. subtilis* strain RD14 at concentration of 2.5 mg/mL, whereas complete inhibition of all the biofouling bacteria was observed at 5.0 mg/mL concentration. *S. pseudogriseolus* strain RD2 exerted minimum anti-biofouling activity. The results from this study were similar to the antifouling activity expressed by *Streptomyces* sp. reported by other researchers [6,7,21].

3.4. Growth and production of anti-biofouling metabolite

The production of anti-biofouling metabolite by *Streptomyces* sp. RD4 was tested by growing the cells in different medium such as starch casein agar, yeast malt extract agar (ISP2), inorganic salt agar (ISP-4), glycerol asparagine agar (ISP5), tyrosine agar (ISP-7), and Gause' synthetic agar (GSA). It was found that *Streptomyces* sp. RD4 grew luxuriantly on GSA medium with higher cell mass as shown in Table 1, and growth trend along with the production of anti-biofouling metabolite is shown in Fig. 6. From the growth curve, it was learnt that the lag phase extended up to 48 h followed by the

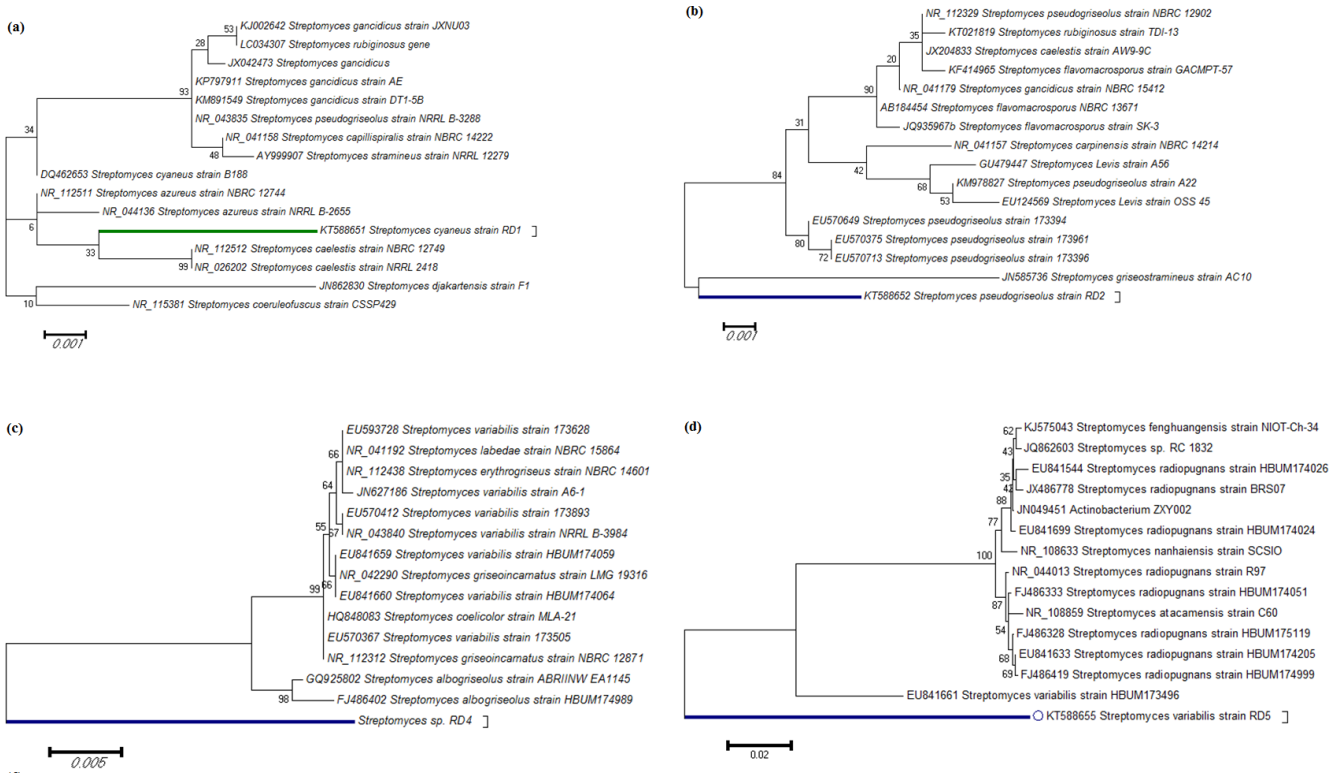

Fig. 4. Phylogenetic relationships of (a) *Streptomyces cyaneus* strain RD1, (b) *Streptomyces pseudogriseolus* strain RD2, (c) *Streptomyces* sp. strain RD4, and (d) *Streptomyces variabilis* strainRD5 with the other strains.

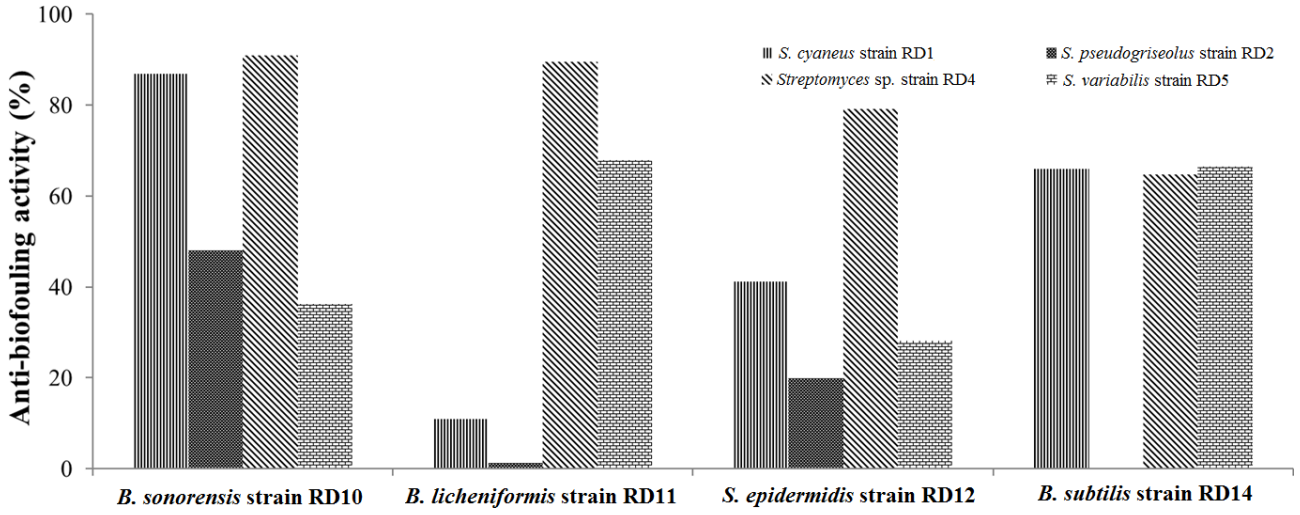

Fig. 5. Anti-biofouling activities of the marine actinomycetes against the biofouling marine bacteria.

exponential phase up to 168 h and stationary phase to 216 h. The production of the anti-biofouling metabolite was observed after 72 h which increased with time till 168 h. At this point of time, *B. sonorensis* strain RD10 was the most sensitive while *B. subtilis* strain RD14 was the least sensitive as shown in Fig. 6. There are few reports on anti-biofouling compounds from *S. filamentosus*, which showed maximum inhibition against the biofouling bacteria [32]. *S. fradiae* RMS-MSU from mangrove and estuarine sediments was reported to show promising activity against various biofouling bacteria [8].

3.5. Spectral characterization of anti-biofouling metabolite

FT-IR spectrum of anti-biofouling metabolite from *Streptomyces* sp. strain RD4 as shown in Fig. 7 revealed a broad absorption band at 3,408 cm^{-1} for the presence of OH stretching of the hydroxyl group [40]. The peak at 2,929 refers to presence of C–H stretching vibration of alkanes and 1,075 cm^{-1} refers to the C–N stretching vibration of aliphatic amines. The presence of C–O group was witnessed by the peaks at 1,277, 1,121, and 1,033 cm^{-1} while the absorption

Table 1
Growth characteristics of *Streptomyces* sp. strain RD4 on different media

Medium	Growth	Mycelium		Pigment
		Aerial	Substrate	
Starch casein agar	Moderate	Brownish white	Brownish white	None
Yeast malt extract agar (ISP-2)	Good	White	Brownish white	Brownish yellow
Inorganic salt agar (ISP-4)	Good	Brownish white	Brownish white	Brownish yellow
Glycerol asparagine agar (ISP-5)	Good	Slight orange	Light brown	Light brown
Tyrosine agar (ISP-7)	Moderate	Light brown	Brownish white	None
Gause's synthetic agar (GSA)	Excellent	Brown	Brownish white	Brown

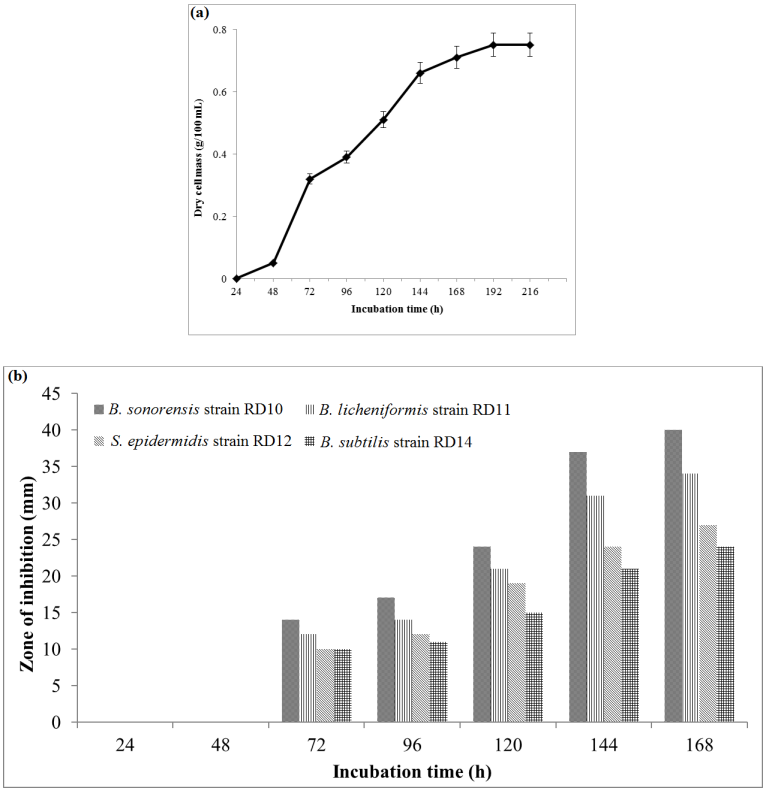

Fig. 6. (a) Growth curve and (b) anti-biofouling activity of *Streptomyces* sp. strain RD4.

at 2,369 cm^{-1} indicates the presence of N–H group [41]. The peaks at 2,159 and 1,722 cm^{-1} refers to the presence of C–N group whereas, the peaks at 850 and 614 cm^{-1} attributes to the C–H stretch of alkanes [42]. The peak at 1,629 cm^{-1} indicates the presence of N–H bend 1°amines [43].

3.6. Evaluation of anti-biofouling activity by microscopic imaging

The viability of *B. sonorensis* strain RD10 against different concentrations of the anti-biofouling metabolite of *Streptomyces* sp. strain RD4 was assessed using live/dead cell assay. It was found that the anti-biofouling metabolite decreased the viability of *B. sonorensis* strain RD10. In the control set, the green-stained cells correspond to live *B. sonorensis* strain RD10 which were higher in number as shown in Fig. 8(a). Whereas Figs. 8(b) and (c) explain the effect of anti-biofouling metabolite concentrations such as 2.5 and 5.0 mg/mL on the growth of *B. sonorensis* strain RD10.

4. Conclusions

This study successfully identified four potential biofilm-producing marine bacteria and meanwhile to combat the problem of biofouling, four actinomycetes those are antagonists to these marine bacteria were also screened and identified. *Streptomyces* sp. strain RD4 was found to be very effective and the anti-biofouling metabolite from *Streptomyces* sp. strain RD4 showed excellent efficacy toward combating the biofilm produced by distinct marine bacterial strains especially *B. sonorensis* strain RD10. Conclusively, this study bridges the gap for understanding the mechanisms of anti-biofouling metabolite against the biofouling organisms and would establish a model for the examination of antifoulant using molecular tools.

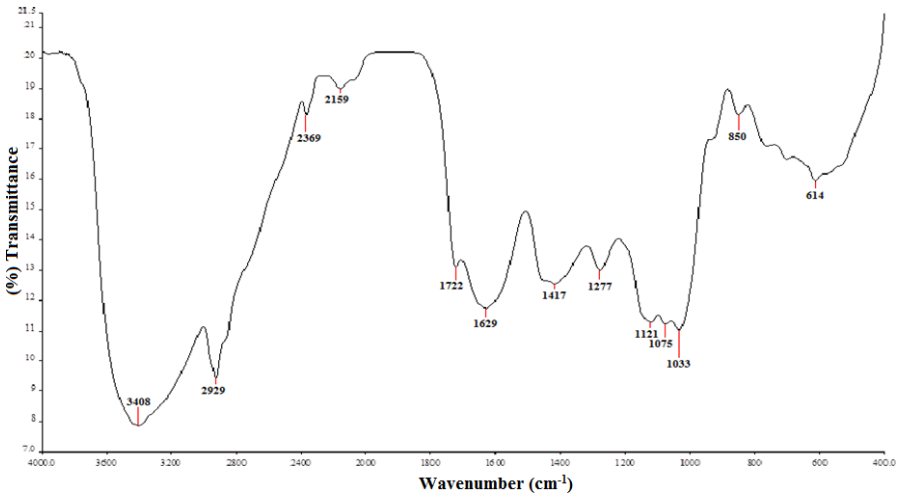

Fig. 7. FT-IR spectra of anti-biofouling metabolite.

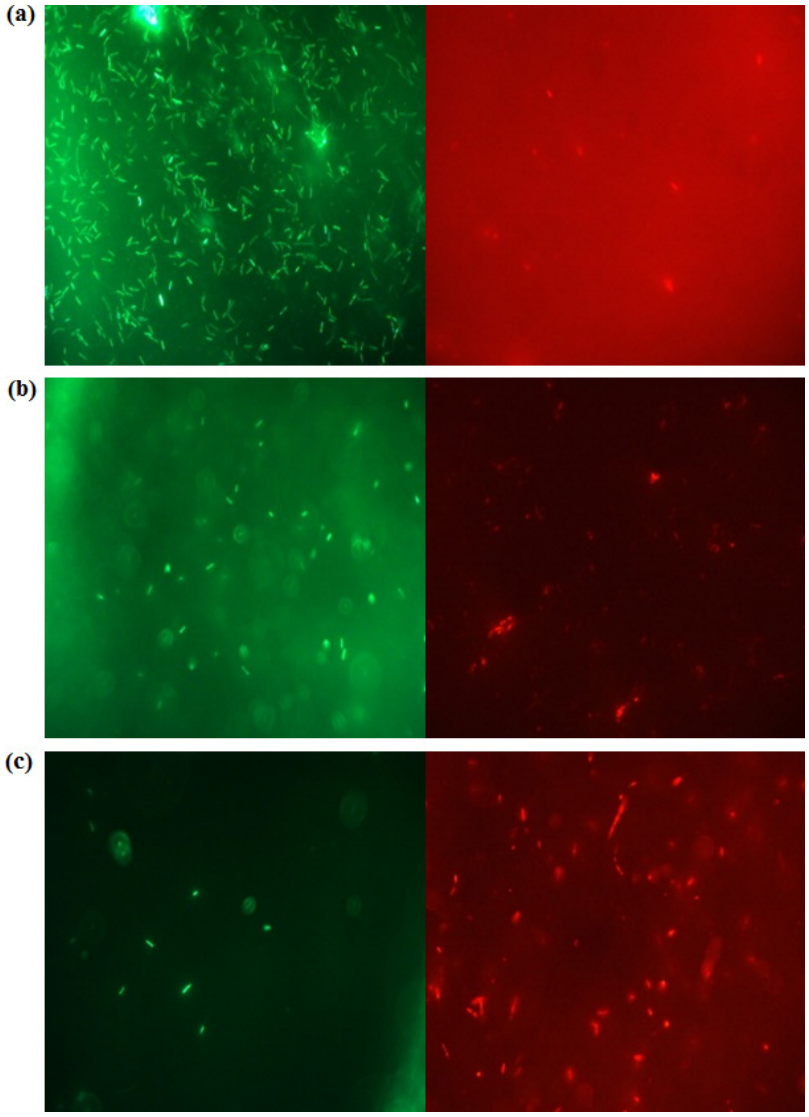

Fig. 8. Microscopic images of live/dead assay of *Bacillus sonorensis* strain RD10 (a) biotic control, (b) with 2.5 mg/mL, and (c) with 5.0 mg/mL of anti-biofouling metabolite of *Streptomyces* sp. strain RD4.

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