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# Inhibition of *Streptococcus pyogenes* Biofilm Formation by Coral-Associated Actinomycetes

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**Abstract** *Streptococcus pyogenes* biofilms tend to exhibit significant tolerance to antimicrobials during infections. We screened coral-associated actinomycetes (CAA) for antibiofilm activity against different biofilm forming M serotype of *Streptococcus pyogenes*. Actinomycetes isolated from the mucus of the coral *Acropora digitifera* were screened for antibiofilm activity against *S. pyogenes* biofilms wherein several isolates clearly demonstrated antibiofilm activity. The biofilm inhibitory concentrations (BICs) and the sub-BICs (1/2 and 1/4 BIC) of the extracts significantly prevented biofilm formation up to 60–80%. The extract of *Streptomyces akiyoshinensis* (A3) displayed efficient antibiofilm activity against all the biofilm forming M serotypes. All the five extracts efficiently reduced the cell surface hydrophobicity (a crucial factor for biofilm formation in *S. pyogenes*) of three M types and thus may inhibit biofilm formation. CAA represent an interesting source of marine invertebrates-derived antibiofilm agents in the development of new strategies to combat Streptococcal biofilms.

## Introduction

Biofilm formation is recognized as an important virulence factor in *Streptococcus pyogenes*, and biofilms of *S. pyogenes* have been reported to be involved in patients with atopic dermatitis and impetigo [1]. Bacterial biofilms have a structural complex and dynamic architecture and develop on many abiotic surfaces (plastic, glass, metal, and minerals) and biotic (plants, animals, and humans) surfaces [7, 18]. The biofilm-associated diseases caused by Gram-positive bacteria are caries, gingivitis, periodontitis, endocarditis, and prostatitis [7]. Many forms of streptococcal infections, especially recurrent and chronic ones, are associated with the formation of bacterial biofilms [13]. Biofilm formation by *S. pyogenes* has been reported in patients with atopic dermatitis and impetigo [1]. Antibiotic treatment failure of *S. pyogenes* infections has been demonstrated to be associated with biofilm formation. Biofilm formation significantly impairs antimicrobial therapy even in those cases caused by strains that are not resistant to the relevant antibiotics [10, 15]. The strains may use biofilms as a barrier to survive in the host against the action of antimicrobials [2, 4]. This newly emerging virulence trait (biofilm formation) of *S. pyogenes* potentially renders these pathogens to become more resistant to antibiotic therapy as well as toward immune responses [13].

To date, only a few antibiofilm agents have been reported against *S. pyogenes* biofilms. The essential oils of certain plants like *Mentha piperita* and *Rosmarinus officinalis* [17] and extracts of certain Thai plant species [14] are shown to possess antibiofilm activity against *S. pyogenes* biofilms. Marine resources have seldom been targeted for antibiofilm agents against *S. pyogenes*.

Marine actinomycetes have been a potential reservoir of pharmaceutical compounds [8, 9] and marine actinomycetes have shown to inhibit biofilm formation by pathogenic *Vibrio* sp. [21]. Only recently it has been shown that corals also harbor diverse species of actinomycetes [11, 12]. To date, actinomycetes associated with corals and their produced metabolites have not yet been explored.

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Recently, we reported for the first time that actinobacteria associated with *Acropora digitifera* possess antibacterial properties [16]. To further unravel the potential of coral-associated actinomycetes (CAA) we screened extracts of 15 CAA for antibacterial and antibiofilm activity against different biofilm forming M serotypes of *S. pyogenes*. Additionally, since the hydrophobic property of bacterial cell surfaces are a major determinant in the adhesion of bacteria and the formation of biofilms on animate and inanimate surfaces [6], the CAA were further screened for properties of reduction of cell surface hydrophobicity.

## Materials and Methods

### Bacterial Strains Used in This Study

CAA was isolated from the mucus of the coral *Acropora digitifera* from Gulf of Mannar. The coral surface mucus layer was swabbed using sterile cotton swabs. Mucus samples of ca. 1 $cm^2$ coral surface area were taken with these swabs. The mucus swab samples were transferred to sterile tubes with 1 ml autoclave-sterilized seawater, in a sterile hood. The bacteria from the cotton swabs were suspended in autoclave-sterilized sea water by vigorous vortexing. CAA was isolated using standard serial dilution and plating techniques on Starch casein agar (HiMedia Laboratories, India) [16]. A total of 15 CAA were screened for antibiofilm activity against different biofilm forming M serotype of *S. pyogenes* isolated from throat swabs. All the six *S. pyogenes* isolates were identified at the species level by amplifying the 16S rRNA gene [forward primer 5′-AGAGTTTGATCCTGGCTCAG-3′ (*E. coli* positions 8–27) and reverse primer 5′-ACGGCTACCTTGTTACG-ACTT-3′ (*E. coli* positions 1494–1513)] and sequencing the 16S rRNA gene The M serotypes (M56, st38, M89, M65, M100, and M74) of *S. pyogenes* isolates were determined by *emm* gene sequencing, and their accession numbers are given in Table 2. For all the assays the strains were routinely cultured in Todd Hewitt Broth (THB).

### Preparation of CAA Extracts

The CAA were cultured in ISP-2 broth (HiMedia Laboratories, India) and incubated for 7 days at 28°C. The cells were pelleted at 9,300×*g* for 10 min and the culture supernatant was filtered through a 0.2-µm filter. The CAA extracts were prepared as previously described [16]. Briefly, the cell free supernatant was extracted twice with equal volume of ethyl acetate. The solvent extracts were combined and evaporated to dryness under reduced pressure at room temperature to yield crude extracts and each crude extract obtained was weighed as previously described [16]. The crude extracts were dissolved in double distilled water (MilliQ, Millipore) and used for antibiofilm screening.

### Antibacterial Activity Tests

Antimicrobial activity of the extracts against *S. pyogenes* was assayed by the disc diffusion susceptibility test according to the recommendations of the CLSI 2006 [3]. The disk diffusion test was performed in Muller Hinton Agar (MHA) (Himedia Laboratories, India) supplemented with 5% sheep blood. Overnight cultures of the *S. pyogenes* were sub-cultured in THB until a turbidity of 0.5 McFarland ($1 \times 10^8$ CFU/ml) was reached. Using a sterile cotton swab the culture was uniformly spread over the surface of the agar plate. Absorption of excess moisture was allowed to occur for 10 min. Then sterile discs with a diameter of 10 mm were placed over the swabbed plates and 50 µl (2000 µg/ml) of the extracts were loaded onto the disc. Further, the MHA plates were incubated at 37°C and the zone of inhibition were measured after 24 h. The MIC of the extracts was performed as per CLSI 2006 [3] guidelines. The bacterial suspension $10^8$ CFU/ml were added to THB supplemented with the CAA extracts serially diluted twofold to give final concentrations ranging from 5 to 2000 µg/ml and incubated at 37°C for 24 h. The MIC was recorded as the lowest concentration that produced inhibition of visible growth after overnight incubation.

### Growth Curve Analysis

The effect of the CAA extracts was also checked by growth curve analysis against a strain of M56 alone. The BIC (the lowest concentration which produces visible disruption in the biofilm formation) of the extracts was added to a conical flask containing 50 ml THB, to which a 1% inoculum from the overnight culture was introduced into the media. The flask was incubated at 37°C. Growth medium with the addition of bacteria (*S. pyogenes* M56 serotype) and without the addition of the extract was used as a control. The results were recorded at 600 nm up to 24 h with every 1 h interval.

### Effect of CAA Extracts on Biofilm Formation and Microscopic Visualization

The effect of the CAA extracts against the biofilm forming *S. pyogenes* was tested on 24-well polystyrene plates (Greiner Bio-one, Frickenhausen, Germany) with glass pieces (1 × 1 cm) [14]. The CAA extracts at concentrations ranging from 5 to 2000 µg/ml were added in THB broth containing the bacterial suspension of $10^6$ CFU/ml. The plates were incubated for 24 h at 37°C. After

incubation, biofilm was stained with 0.4% crystal violet. The BIC was determined as the lowest concentration which produces visible disruption in the biofilm formation and significant reduction in the readings when compared with the control wells when read at OD 570 nm [2]. The wells containing the media and with extracts were used as blank. Thus, the BIC was determined by both spectrophotometric quantification and also by microscopic visualization. Further the sub-inhibitory concentrations (1/2 and 1/4 BIC) of the extracts were also tested against the biofilm formation by performing the same protocol. Simultaneously, unstained biofilm and planktonic bacteria were mixed by vigorous vortexing, and bacterial growth was quantified spectrophotometrically at 600 nm. The biofilms grown on glass pieces were stained with crystal violet and were visualized by light microscopy (NIKON ECLIPSE T*i* 100) at magnifications of ×40 and scanning electron microscopy (S-3000H, Hitachi (Japan) [13].

### Microbial Adhesion to Hydrocarbon Assay

The effect of the extracts on cell surface hydrophobicity (CSH) of *S. pyogenes* was measured by Microbial Adhesion to Hydrocarbon (MATH) assay as earlier described [4]. Briefly, 1 ml of bacteria ($OD_{530} = 1.0$) were placed into glass tubes and 100 µl of toluene along with the CAA extracts at their BICs was added. The percent hydrophobicity was calculated by the formula: % Hydrophobicity = [1 − ($OD_{530}$ after vortexing/$OD_{530nm}$ before vortexing)] × 100.

## Results

### Evaluation of *S. pyogenes* Biofilm Formation

A total of 33 *S. pyogenes* strains were isolated from throat swabs of pharyngitis patients, from which 11 different M serotypes were obtained. Out of eleven M serotypes, six M serotypes (M56, st38, M89, M65, M100, and M74) formed biofilms which were confirmed using Confocal Laser Scanning Microscopy (Leica TCS SP2 AOBS equipped with a Leica DMIRE2 inverted microscope, Leica Microsystems, Germany) (unpublished data). Among the six biofilm formers, *S. pyogenes* with the M serotype 56 (M56) was found to be a potent biofilm formation enhancer while the isolates with serotypes M89 and M65 were found to be the least biofilm formers. The *S. pyogenes* isolates were also screened for biofilm formation quantitatively by spectrophotometric analysis [2]. *Streptococcus mutans* UA159 (ATCC 700610) was used a positive control.

### Determination of Biofilm Inhibitory Concentration and Its Sub-Inhibitory Concentrations

All the 15 CAA were identified to the species level by 16S rRNA gene sequencing. The 16S rRNA gene was amplified using the universal eubacterial 16S rRNA forward primer 5′-AGAGTTTGATCCTGGCTCAG-3′ (*E. coli* positions 8–27) and the actinomycetes specific reverse primer 5′-CCGTACTCCCCAGGCGGGG-3′ (ACT878r). The amplified product (approximately 870 bp) was cloned and sequenced. Among the 15 CAA isolates, five isolates (A3, A5, A7, A10, and A13) showed positive results by inhibiting the biofilm formation (Table 1). To determine the MIC of the CAA extracts on *S. pyogenes*, the extracts with concentrations ranging from 5 to 2000 µg/ml has been assessed. Surprisingly, none of the extracts showed antibacterial activity against the *S. pyogenes* strains. Table 2 shown here represents the BIC of the CAA extracts. All the five CAA extracts inhibited biofilm formation against the different serotypes of *S. pyogenes* at the concentrations ranging from 10 to 200 µg/ml (BIC). Interestingly, the CAA extracts A3 (*Streptomyces akiyoshinensis*) and A10 (*Actinobacterium* sp.) showed pronounced effect on the inhibition of the biofilm formation at very low concentrations. The CAA extracts efficiently disintegrated the biofilm formation of M56 (Fig. 1a–f) and also against other five M serotypes (data not shown). The efficiency of the extracts were tested at their sub-inhibitory concentrations by diluting the extracts to yield 1/2, 1/4, and 1/8 BICs where 1/2 and 1/4 BIC showed appreciable antibiofilm activity (Fig. 2a–c). Almost all the CAA extracts reduced the biofilm formation up to 70–80% in BIC. With the increase in dilution, the extracts displayed significant reduction ($P < 0.05$) in the biofilm inhibition by 55–70% in 1/2 BIC and 25–60% in 1/4 BIC. To determine if the molecules responsible for antibiofilm activity were proteins, proteolytic digestions of the extracts were carried out with proteinase K (100 mg/ml). All the extracts from culture supernatants displayed significant antibiofilm activity even after proteinase K treatment which confirmed that the active molecules were not proteins.

### Effect of Extracts on *S. pyogenes* Growth

All the CAA extracts (2000 µg/ml) when evaluated for their antibacterial activity against *S. pyogenes* (M56) by disc diffusion assay and spectrophotometric analysis did not show any antibacterial activity.

### Scanning Electron Microscopy

The SEM picture revealed the primary adhesion of the bacterium leads to dense biofilm formation on the surface

**Table 1** Strains of the coral associated actinobacterial extracts exhibiting antibiofilm activity

| S.No. | Extract No. | CAA isolates | GenBank accession number |
|---|---|---|---|
| 1 | A3 | *Streptomyces akiyoshinensis* | FJ662855 |
| 2 | A5 | *Streptomyces rochei* | FJ662857 |
| 3 | A7 | *Propionibacterium* sp. | FJ662859 |
| 4 | A10 | *Actinobacterium* sp. | FJ662870 |
| 5 | A13 | *Micrococcus luteus* | FJ662862 |

**Table 2** Biofilm inhibitory concentration (BIC) of CAA extracts against the different biofilm forming M serotypes of *S. pyogenes* strains after 24 h

| M serotypes | CAA Extracts (μg/ml) | | | | |
|---|---|---|---|---|---|
| | A3 | A5 | A7 | A10 | A13 |
| M56 (EU636227) | 50 | 200 | 50 | 100 | 100 |
| st38 (EU636229) | 50 | 150 | 50 | 100 | 80 |
| M89 (EU660375) | 10 | 100 | 20 | 25 | 50 |
| M65 (EU660377) | 10 | 100 | 20 | 25 | 50 |
| M74 (EU660380) | 50 | 100 | 50 | 50 | 80 |
| M100 (EU660379) | 50 | 100 | 50 | 50 | 80 |
| *S. mutans* UA159 (positive control) | 50 | 200 | 50 | 50 | 150 |

The GenBank accession numbers of the various M serotypes are given in parentheses

of the glass piece by the strain M56 (Fig. 3a) whereas Fig. 3b depicts the antibiofilm activity of the CAA extract A3 at 1/2 BIC against M56 biofilm formation.

### Effect of Various CAA Extracts on Cell Surface Hydrophobicity

The adhesion of streptococci to toluene was used to measure the hydrophobicity of *S. pyogenes*. The degree of the effect of biofilm inhibitory and its sub-inhibitory concentrations of the CAA extracts on cell surface hydrophobicity of *S. pyogenes* was investigated. All the five extracts showed reduction in the cell surface hydrophobicity against three M types namely M56, st38, and M89 (Supplementary Table S1). The mean values of triplicate independent experiments and standard deviations are shown. Dunnett test demonstrated significant difference between the tests and the control ($^*P < 0.05$).

## Discussion

To date, only medicinal plants [14] have been reported to inhibit the biofilm formation of *S. pyogenes*. The bioactive capabilities of CAA have not yet been explored and the present study shows that CAA may be promising candidates with potential antibiofilm activity against *S. pyogenes.* The metabolites of CAA extracts inhibited biofilm formation by reducing the formation of microcolonies by *S. pyogenes*. The bioactive compounds present in the extracts, like berberine sulfate [19] might have interfered with the adherence of *S. pyogenes* by releasing the adhesin, lipoteichoic acid (LTA) from the streptococcal cell surface.

An important step in biofilm development is the formation of the characteristic biofilm architecture [21]. Figure 1 shows that the architecture of the *S. pyogenes* microcolonies that were treated with CAA extracts were found to be looser than the biofilms of the control. It is envisaged that the natural products of CAA possibly interfered at any step of the *S. pyogenes* biofilms but evidently did not inhibit the growth of *S. pyogenes* at all biofilm inhibitory and its sub-inhibitory concentrations tested [14]. When compared with the control (Fig. 3a), extract A3 showed good antibiofilm activity at their sub-inhibitory concentration against M56 serotype (Fig. 3b) which was further confirmed by scanning electron microscopy. On investigating the effect of extract A3 on preformed biofilms, it was found out that it disintegrated the microcolony formation of biofilms which shows that the extract acted on the early stage of biofilm formation.

Among the several actinobacteria members screened in this study, *Streptomyces akiyoshinensis* (A3) and *Actinobacterium* sp. (A10) were found to be the potent biofilm inhibitor of *S. pyogenes* because they were able to inhibit the biofilms of all the serotypes of *S. pyogenes* at very low concentrations of the extract, i.e., 10–50 μg/ml. Limsuwan and Voravuthikunchai [14] reported that the plant extracts screened, had antibacterial activity at a higher concentrations and only the subinhibitory concentrations of the plant extract inhibit biofilm formation against *S. pyogenes*. The actinomycetes extracts used in this study did not possess antibacterial activity even at high concentrations and inhibited only the biofilm formation of *S. pyogenes*. This property of inhibiting only the biofilm formation and not the bacterial growth of a pathogen is considered as a hallmark of a good antibiofilm compound and from our results we suggest that the coral bacterial extracts used in this study be developed as antibiofilm compounds against *S. pyogenes*.

Cell surface charge and cell surface hydrophobicity play a crucial role in bacterium-host cell interaction [20]. LTA is a major hydrophobin that contributes to the

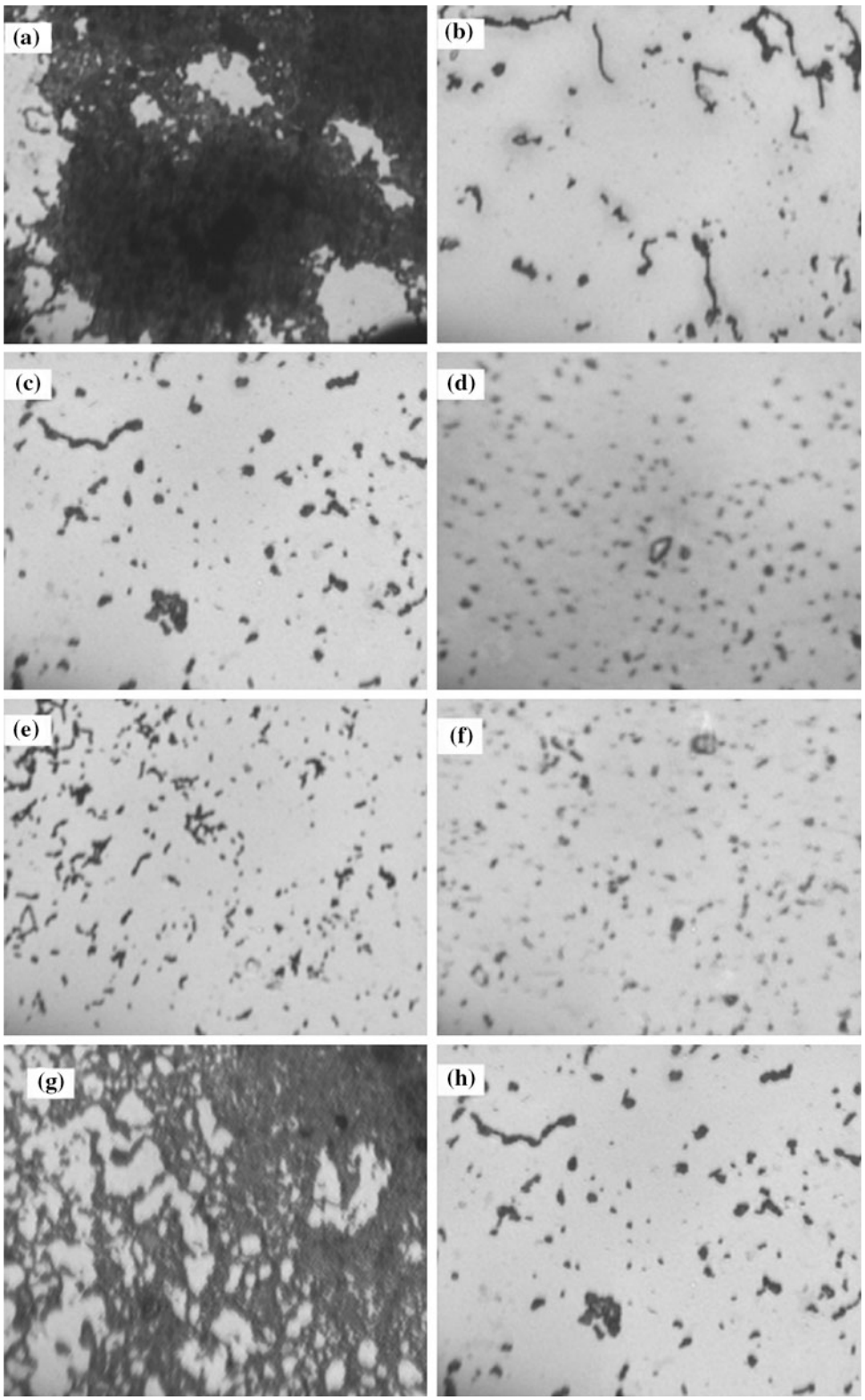


**Fig. 1** Microscopic visualization of antibiofilm activity of CAA extracts against the *S. pyogenes* serotype M56. **a** Control, **b** A3 (50 μg), **c** A5 (200 μg), **d** A7 (50 μg), **e** A10 (100 μg), **f** A13 (100 μg), **g** biofilm formation by *Streptococcus mutans* UA159 (positive control), **h** antibiofilm activity of CAA extract A3 (50 μg) against *Streptococcus mutans* UA159

hydrophobicity of a variety of Gram-positive bacteria [5]. In *S. pyogenes* LTA functions not only as a hydrophobin but also mediates adhesion of the organism to a variety of host cells. Earlier, Limsuwan and Voravuthikunchai [14] have reported that none of the plant extracts showed reduction in the cell surface hydrophobicity against *S. pyogenes* biofilms. In contrast, the CAA extracts showed reduction in hydrophobicity at sub-BICs. All the five CAA extracts reduced the cell surface hydrophobicity of only M56, St38, and M89 serotypes.

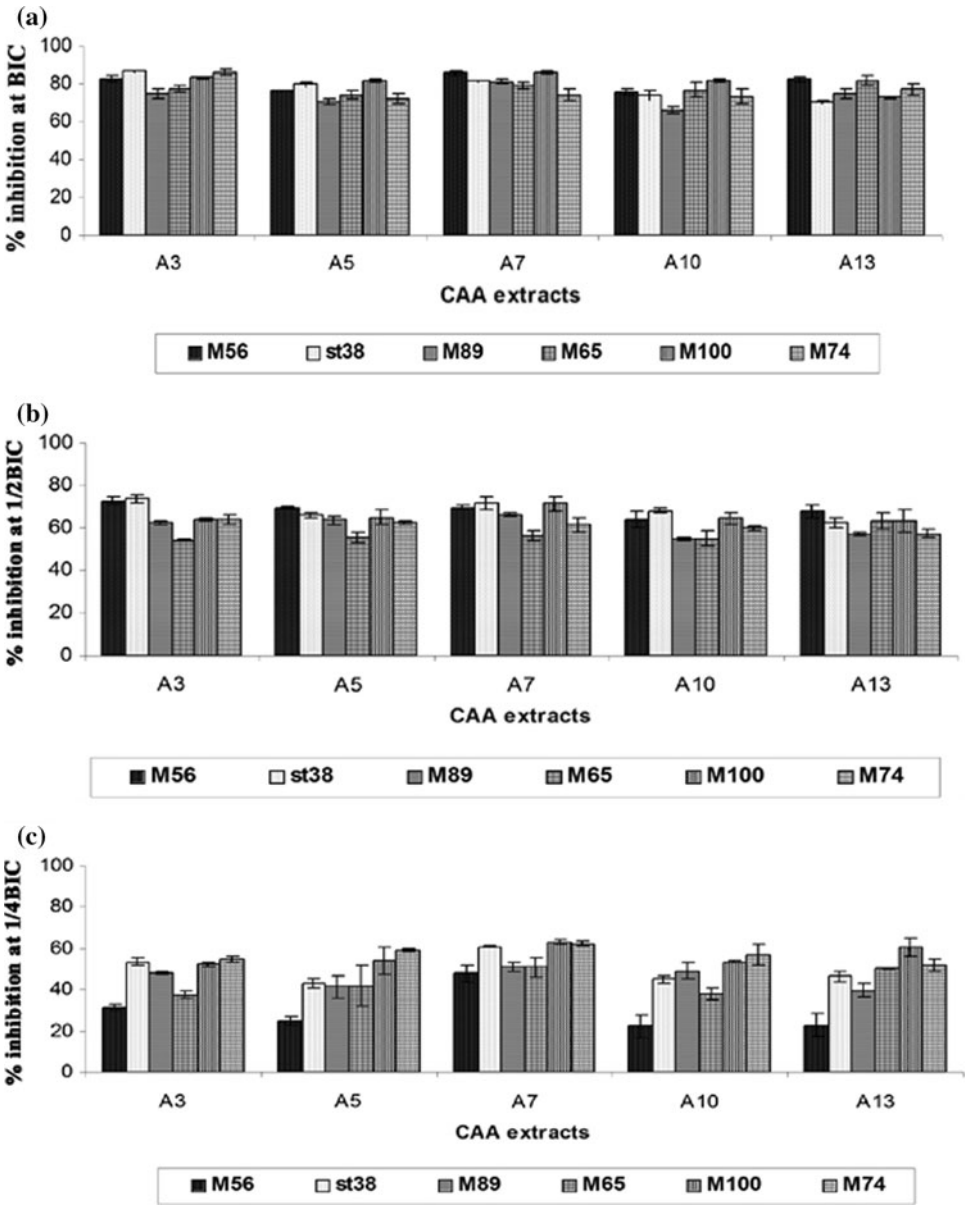


**Fig. 2** Effect of CAA extracts in the biofilm formation of *S. pyogenes* strains quantified by crystal violet staining and measuring absorbance at 570 nm. **a** Percentage inhibition of biofilm formation at Biofilm inhibitory concentration (BIC), **b** percentage inhibition of biofilm formation at 1/2 BIC, **c** percentage inhibition of biofilm formation at 1/4 BIC. The mean values of triplicate independent experiments and SD are shown. Dunnett test demonstrates significant difference between the tests and the control ($P < 0.05$)

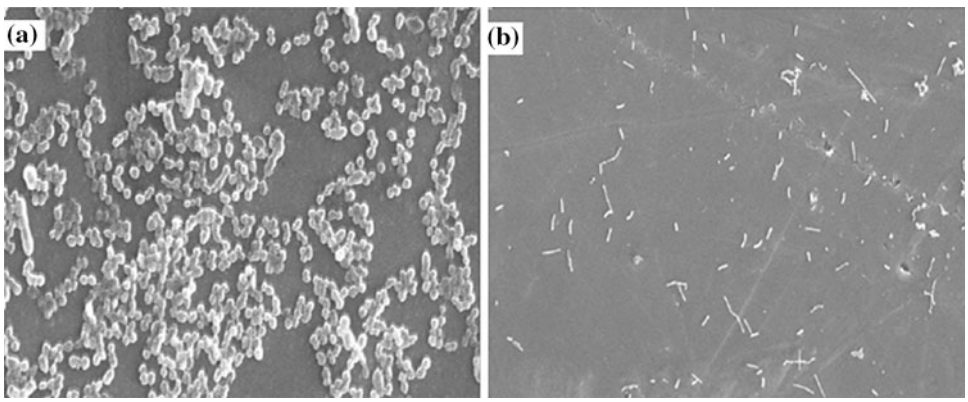


**Fig. 3** Scanning electron micrographs of *S. pyogenes* serotype M56 biofilms formed on glass surface. **a** *S. pyogenes* serotype M56 (biofilm control), *bar* 10 μm; **b** the sample when treated with sub-inhibitory concentration of the extract A3 (25 μg/ml) showed absence of biofilm formation, *bar* 100 μm

A possible mechanism by which the coral-associated bacterial extracts reduce cells surface hydrophobicity might be by affecting the hydrophobins like LTA on the bacterial structures thus affecting the initial step of colonization and most probably the development of infection.

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