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Crude fatty acid extracts of *Streptomyces sps* inhibits the biofilm forming *Streptococcus pyogenes* ATCC 19615

Manickam Rajalakshmi*, Ponnuraj Srinivasan, Maria Francis Poffe, Rajendran Suresh, Venkatesan Brindha Priyadarisini

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Abstract

Crude fatty acid extract of soil *Streptomyces sps* on the biofilm formation by *Streptococcus pyogenes* ATCC 19615 was investigated. Totally, 25 *Streptomyces sps* were isolated identified from the soil samples collected from Nilgiris hill station. All the isolates were subjected to hydrogen peroxide assay, fatty acid extraction and antibiofilm assay. The fatty acid extracts of S8, S9, and S15 inhibited *S. pyogenes* at MIC 10 µg/ml. The BIC was observed as 84.6% , 96.41%, 80.5% at 50 µg/ml concentration. Streptolysin S assay showed that the crude lipid extracts have the capability of inhibiting the Streptolysin S activity. There were changes in extracellular protein of the pathogen exposed to the S8, S9 and S15 crude fatty acid extracts (50 µg/ml) at the range of 100-120 kDa which elucidates that the fatty acid extracts have a significant role in altering the extracellular protein which might be responsible for virulence of the pathogen.

Key words: *Streptococcus pyogenes* ATCC 19615, *Streptomyces sps*, Fatty acid, CLSM, Biofilm inhibition.

Introduction

Streptococcus pyogenes is one of the most important human pathogens associated with extensive human morbidity worldwide. It causes primary infections of skin, throat and mucosal surfaces. Even though the infections are normally self-limited, antibiotic treatment is usually employed to relief discomfort, minimize transmission and reduce complications, (Hoyle 1999; Bisno 2002; Baldassarri 2006). *S. pyogenes* is classified as Group A streptococcus (GAS), Gram-positive, facultative anaerobic bacterium. It is non motile, and does not produce spores. It occurs as long chains of cocci, and occasionally in pairs. GAS typically beta-haemolytic and has a capsule composed of hyaluronic acid (Todar and Ken 2005). The beta-haemolytic streptococci produce a toxin that forms a clear zone of hemolysis on blood agar, demonstrating its ability to destroy red blood cells. This hemolysis is attributed to toxins formed by GAS called streptolysins. Streptolysins can destroy not only red blood cells, but also the white blood cells responsible for fighting off bacteria and disease, as well as other body cells (Tortora 2006).

The cell wall of *S. pyogenes* contains M proteins, which are a major factor behind its virulence. M protein is heat and acid resistant, it aids in the attachment to host tissues, and helps the cell to resist phagocytosis. Host immunity to *S. pyogenes* results the development of antibodies specific to M protein (Jevitz and Maria 2004). Biofilms are formed by a spectrum of microorganisms, including pathogens, and provide a means for these organisms to protect themselves against antimicrobial agents. *S. pyogenes*, biofilm formation has been demonstrated as a potentially significant mechanism conferring to antibiotic treatment failure. The biofilm communities of *S. pyogenes* communities tend to exhibit significant tolerance to antimicrobial challenge during infections. The strains use the biofilm to evade the antibiotic effect, thereby becoming resistant to the treatment. Penicillin remains the antibiotic of choice for *S. pyogenes* infections based of its narrow spectrum of effect, efficacy, good safety profile and low cost (Fux 2003). As there is an increase in the resistance mechanisms of *S. pyogenes* to antibiotics, much effort is being exerted to identify novel compounds with effective antibacterial properties.

A recent study demonstrated three major n-3 PUFA, EPA, DHA, α-linolenic acid (ALA), and their ester derivatives exhibited strong antimicrobial activity against various oral pathogens (Huang and Ebersole 2010). Similar findings were also reported for n-6, n-7, and n-9 fatty acids related to antimicrobial activities against oral microorganisms (Huang 2010; Sylvian 2009). Fatty acids have been known to have antibacterial (Okuyama 2008) and antifungal properties and

Manickam Rajalakshmi*, Maria Francis Poffe, Rajendran Suresh, Venkatachalam Brindha Priyadarsini

Department of Microbial Biotechnology, Bharathiar University, Coimbatore – 641046, Tamilnadu, India.

***Tel: 00**91 422-242 8100; **Fax:** 0091 422 242 2387;
Email: raji09mb@gmail.com

Ponnuraj Srinivasan

Department of Biotechnology, Karpagam University, Coimbatore – 641021, Tamilnadu, India.

especially capric acid (10:0) and lauric acid (12:0) are known to have anti-*Candida* effects by inhibiting growth of planktonic cells (Bergussonn 2001) and butyric acid (4:0) have been shown to inhibit hyphal formation by *C. albicans* (Noverr 2004). Actinomycetes represent a high proportion of the soil microbial biomass and have the capacity to produce a wide variety of antibiotics and extracellular enzymes. Most of the known natural antibiotics are produced from actinomycetes (Kavitha 2011; Lee 1993). As they are the novel source of the antibiotics, they have a high pharmacological and commercial interest including control of infectious diseases. Compounds produced by the actinomycetes are diverse in their clinical effects and are active against many pathogens (Aghamirian 2009). The ability of Actinobacteria to grow in aerated fermentation vessels and the vast amount of knowledge obtained from antibiotic production is useful in formulating commercially viable and more valuable products on large scale.

Despite the presence of a variety of gene clusters, these groups of bacteria are underexplored in the area of lipids, which indicates that many types of potentially valuable compounds are yet to be discovered from this Phylum that could have tremendous commercial potential in the medical sector (Hyoung-pyo 1994). Actinomycetes are reported for lipid biosynthesis and are relevant, not only for the generation of cell membrane phospholipids but also for the synthesis of poly ketide compounds with useful pharmaceutical properties. The objective of this study is to extend the research to lipids, in order to find an alternative source of biofilm inhibiting compound; using soil isolates of actinomycetes.

Materials and Methods

Bacterial strain

Streptococcus pyogenes ATCC 19615, obtained from Karpagam medical college, Karpagam University, Coimbatore, Tamilnadu, was used for antibiofilm activity. The bacterium was routinely cultured in Todd Hewitt Broth (THB) for all assays. For the present study crude fatty acid extracts of *Streptomyces sps*. were evaluated for their role on bacterial biofilm.

Screening of PUFA producing actinomycetes

Screening of the Streptomyces sps isolate for fatty acid production was performed by following the method of Ashwini Tilay and Uday Annapure (2012). Briefly, Starch casein agar (SCA) medium plates, supplemented with 1 mM Sodium azide (NaN_3) (catalase inhibitor), were freshly prepared. 48 hrs culture of *Streptomyces sps* (OD = 1.0 at 600 nm) isolated and identified using conventional methods; from rhizosphere soil obtained from, The Nilgiris district, Tamilnadu, was swabbed on the agar surface uniformly. Hydrogen peroxide solution was prepared at different concentrations of 0.1 %, 0.5 % and 1.0 % from a stock of 30 % solution. Whatmann no.1 filter paper was cut into small pieces of 5 mm sized disc and was placed on the surface of the agar plates. Then the filter paper discs were incorporated with different concentration of hydrogen peroxide solution and the plates were incubated at 37° C for 48 h. After incubation, the plates were observed for the zone of inhibition around the filter paper discs. Absence of inhibition zone correlates to the resistance exhibited by Streptomyces sps against hydrogen peroxide due to fatty acid synthesis.

Extraction of total lipid from actinomycetes

Fatty acid extraction was done by using the method of Bligh and Dyer (1959). Briefly, the *Streptomyces* isolates were grown on 250 ml Starch casein broth for 1 to 7 days at 37 °C. After their growth, the cultures were centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the pellet was re-suspended with half the volume 1% NaCl and centrifuged at 10,000 rpm for 10 min. The resulting pellets were lyophilized under vacuum drier and stored at -20 °C. 1 g of the lyophilized cells were weighed and dissolved in the 50 ml solvent mixture (Chloroform: Methanol: Distilled water/ 2:1:1 v/v) and 15 mg of Butylated hydroxy toluene (BHT) was added and mixed thoroughly. Then, the flasks were sealed and kept in orbital shaker for 18 h at 37 °C on continuous agitation. After incubation the solution was separated by using the separating funnel for 6 hours until both the phases got separated. The aqueous phase subjected to the separation step twice for better extraction. The aqueous phase was then subjected to esterification.

Esterification of fatty acid into fatty acid methyl esters

Esterification was done according to Hoshi et al. (1973). Thus, 1 ml of 20 mM cupric acetate monohydrate in methanol was added to the lipid extract. Then, 1 ml of 0.5 N HCl in methanol was added and mixed. This solution was kept at room temperature for 2 to 3 h. The reaction was stopped by addition of 5 ml distilled water. Then the lower chloroform layer was pooled and allowed to evaporate slowly in hot water bath at 35 °C until the solvent was fully evaporated. The final fatty acid methyl ester was dissolved in 1ml of concentrated hexane and filtered using Whattman no.1 filter paper and dried using vacuum drier. The esterified fatty acid was weighed. Then the samples were stored at -20 °C.

Minimum inhibitory concentration assay

Anti-biofilm activity was tested by using various concentrations of crude fatty acid extract (5, 10, 15, 20,25,30,35,40,45,50 µg/ml). 1 µl of the overnight culture of *S. pyogenes* (ATCC 19615) with the optical density of 0.1 at 600 nm was added to each wells of 96-well titre plate. Different concentration of the lipid extract was added to each wells of the titre plate. The 96-well plate was then incubated at 37 °C for 16 h. The incubated plate was later observed for the growth of *S. pyogenes* (Nithya 2010). Growth of the pathogen was also observed against various concentration of the crude fatty acid. Different concentration of crude lipid was added to 1 ml of the test culture and incubated at 37 °C overnight. After incubation the growth was recorded spectrophotometrically at 600nm.

Biofilm inhibition assay

Overnight culture of *S. pyogenes* was incubated on 24 well microtitre plate containing 1 ml of Todd-Hewitt Broth (THB) with and without lipid extracts (Nithya 2010). Plate was incubated without agitation at 37 °C for 18 h. After incubation, planktonic cells and spent media were discarded; the adherent cells on the slide were gently rinsed twice with deionized water and air dried. The biofilm was stained with 0.4% crystal violet solution for 5 min and then rinsed twice with deionized water. Finally it was resuspended in 1 ml absolute ethanol and the absorbance was observed at 570 nm.

Microscopic observation of biofilm

For visualization of biofilm by light microscopy (Nithya 2010), the biofilms were allowed to grow on glass pieces (1x1 cm) placed in 24-well polystyrene plates supplemented with crude fatty acid methyl esters (10, 20, 30, 40, 50 µg/ml) and incubated for 24 h at 28 ºC. The slides were stained using

crystal violet and were placed on slides with biofilm pointing upwards. The slides were observed under light microscopy at magnification of ×40. Visible biofilms were documented with an attached digital camera (Kozo Optics Model: XJS900T).

Confocal laser scanning microscopy (CLSM) was used to determine the three-dimensional architecture, thickness and morphology of biofilms formed by *S. pyogenes* (ATCC 19615) treated with the crude fatty acid methyl esters. The biofilms formed on glass slides were transferred to a 24 well plate and incubated for 45 min at 37°C in 2 ml of PBS containing the fluorescein isothiocynate-concanavalin A (FITC-ConA; 50 µg ml^{-1}) in 10 $mmol^{-1}$ hydroxyl ethyl piperazine ethane sulfonic acid (HEPES). The stained slides were subjected to visualization under CLSM (Zeiss LSM710 meta, Germany). The objective used was a water immersion C-apochromat lens (×20). The 488 nm-line of an Ar laser was used for excitation and the fluorescence was detected through emission filter 590/70 (high-quality band pass), centered at 590 nm with 70-nm bandwidth. Images were captured and processed by using Zeiss LSM Image Examiner Version 4.2.0.121.

Streptolysin-S Assay

Sheep blood was collected and centrifuged at 3000 rpm for 3min. The serum was collected and then washed with PBS buffer trice by centrifugation at 3000 rpm for 3 minutes. 900 µl of 10 mM Tris buffer was added and 100 µl of *S. pyogenes* ATCC 19615 culture supernatant (control & lipid extract treated) was added with 100 µl of sheep RBC and incubated for 20 min at - 4 ºC. It was centrifuged at 3000 rpm for 5 min. The absorbance of resulting supernatant was measured at 540 nm (Betschel 1998).

Preparation of extracellular protein of S. pyogenes and SDS PAGE

Overnight culture of *Streptococcus pyogenes* (ATCC 19615) were grown on 4 separate conical flasks containing THB at 37°C. Each of the three flasks was incorporated with 50 µg/ml crude lipid extract of S8, S9 and S15 respectively. The fourth flask was maintained as control. After incubation, cells were removed by centrifugation followed by filtration through a 0.2 µm pore size filter. Total proteins were obtained by precipitation with trichloro acetic acid (TCA; 20% w/v final concentration) on ice for 4 h, washed with acetone, and resuspended in 1x gel loading buffer to 1/40 volume. Equal amounts of proteins (as measured by Lowry method) were loaded in each lane. Proteins were separated using 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Betschel 1998).

Results and Discussion

Screening of fatty acid synthesizing Streptomyces sps

In H_2O_2 - plate assay (Fig 1), the cells which were susceptible to externally-added H_2O_2 were not able to grow suitably and thus showed a zone of inhibition, which was dependant on the added concentration of H_2O_2 on Whattman filter paper disc. Growth was observed on the agar plate without the zone of inhibition, which indicated that the actinobacterial cells produced fatty acid and were able to grow in presence exogenous H_2O_2, due to the membrane-shielding effect of fatty acids (Okuyama 2008). The concentration of NaN_3 was opted from report studied by Teixeira and Mota (1992). If microorganism produced catalase enzyme, NaN_3 inhibited catalase enzyme (Lichstein 1944) which helped to infer and promote the actual interpretation of plate assay. Out of 25 *Streptomyces sps* isolates used in this study, 22 isolates were found to be non producer of fatty acid under primary screening. In this assay, 3 isolates did not show, zone of inhibition and these were selected for further studies (Table 1) (Fig 1).

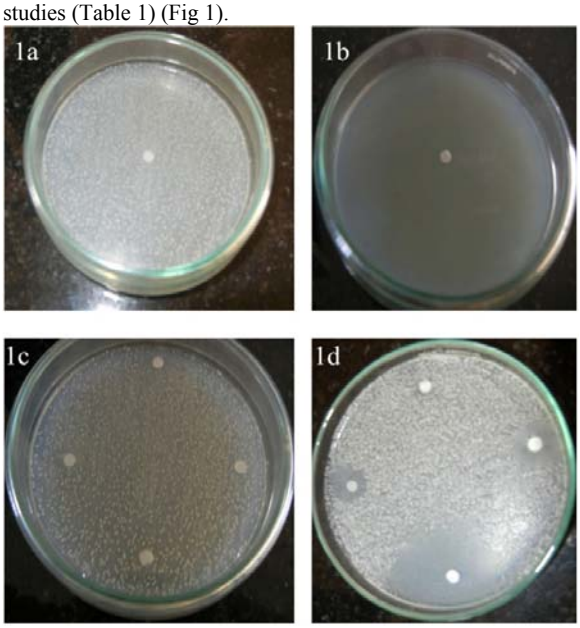

Figure 1: Screening of PUFA producing Streptomyces sps using Hydrogen peroxide assay. 1(a) Positive control – shows the growth of actinobacteria, 1(b) Negative Control – shows the absence of microbial growth, 1(c) PUFA producer – the isolate S8 was not inhibited by H_2O_2 on all concentrations, 1(d) PUFA non producer – the S1 isolate neither produced catalase nor PUFA and so it is inhibited by H_2O_2 at all concentration which showed that the zone of inhibition was proportional to the H_2O_2 concentration.

Table 1. Hydrogen peroxide plate assay for determination of PUFA synthesizing actinomycetes.

Strain	H_2O_2[@] concentration (%)			PUFA[*] +ve/-ve
	0.1	0.5	1	
S1 to S7	-	-	-	- ve
S8	+	+	+++	**+ve**
S9	+	++	+++	**+ve**
S10 to S14	-	-	-	-ve
S15	+	++	+++	**+ve**
S16 to S25	-	-	-	-ve

[@]describes zone of inhibition due to presence H2O2; +/++ describes growth of microorganisms or no zone of inhibition due to presence of PUFA. [*]PUFA +ve denotes PUFA producer and −ve denotes PUFA non-producer.

Extraction of total lipid and esterification

Total lipid content was extracted from the selected isolates S8, S9 and S15 using Bligh and Dyer protocol and their dry weight were found to be 40, 48, 42 µg/ml respectively. According to Teixeira Mota, (1992), the EPA- and DHA-expressing bacteria were reported to be more resistant to exogenous H_2O_2. The bacterial cell gets protected by the effect of EPA that has reported earlier. The membrane-shielding effects of *n*-3 LC-PUFAs have been shown only for bacterial cells producing EPA (Nishida 2007; Nishida 2006). The study conducted by Ashwini Tilay and Uday Annapure (2010) revealed that the long chain fatty acids of marine bacteria also responsible for the same protecting effect against exogenous H_2O_2 which was also well supported with studied carried out by Okuyama (2008). These results support our hypothesis that fatty acid synthesis by actinobacteria might also be responsible for protection against exogenous H_2O_2.

Minimum inhibitory concentration assay

There was a gradual decline in the turbidity of isolates treated with increasing concentration of crude fatty acid extract. All the three crude fatty acid lipid extract of S8, S9, and S15 isolates inhibited the pathogen at a minimal concentration of 10 µg/ml. Synthetic compounds such as fluoroquinolone failed to inhibit clinical isolates of *Streptococcus pyogenes* at a minimal concentration which proved that biofilms are developing resistance strategies against the metabolic drug (Thenmozhi 2009). The effectiveness of the fatty acid against the biofilm forming *S. pyogenes* has not been assessed previously. Long chain fatty acids from marine sources have been extensively studied for their antibiofilm property against *Candida albicans* and *Candida dubliniensis* (Vuyisile et al. 2010).The present study elucidated that natural crude fatty acid extract from *Streptomyces sps* could inhibit the biofilm forming *Streptococci* at a much lower concentration thus enabling us to confer its effectiveness (Figure 2).

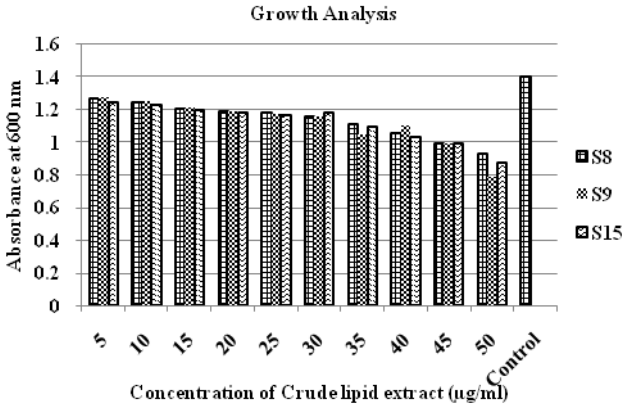

Figure 2: Represents the gradual inhibition of *S. pyogenes* ATCC 19615 exposed to the varying concentrations of the crude lipid extracts after 16 h incubation. The MIC of the S8, S9, and S15 crude lipid extract against *S. pyogenes* was found to be in range of 10 to 50 µg/ml concentrations, above which complete inhibition was observed.

Biofilm inhibition assay

The crude fatty acid extracts of S8, S9 and S15 were able to inhibit the biofilm formation of the *S. pyogenes* ATCC 19615. The concentration of the fatty acid extracts used to assess the biofilm inhibition ranged from 10 µg - 50 µg /ml. From Figure 3, it was observed that all the concentrations of crude fatty acid extracts of

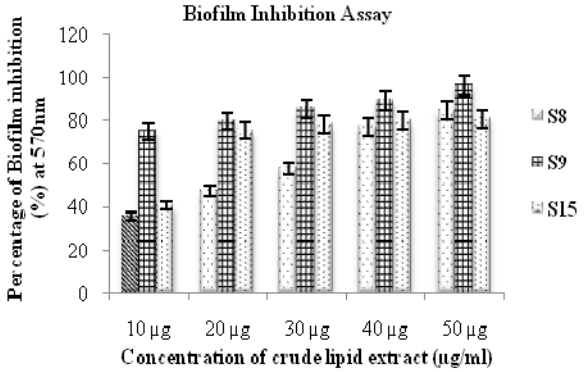

Figure 3: Representation of the percentage inhibition of the lipid extracts of actinobacterial isolates S8, S9, and S15 against the biofilm forming *S. pyogenes* ATCC 19615.

the isolates showed good biofilm inhibition. It is promising from the Figure 4. (2a, 2b and 2c) that S8, S9 and S15 exract showed the highest percentage inhibition of 84.6% , 96.41%, 80.5% at 50 µg/ml and hence it was observed to be the biofilm inhibitiory (BIC) concentration.

Microscopic observation of biofilm assay

Analyses of growth kinetics and the architecture of biofilms are important to understand and interpret their behavior including molecular genomics and proteomics. Therefore evaluation of crude fatty acid against *S. pyogenes* ATCC 19615 biofilm was performed using both conventional and novel quantification techniques. The crude lipid extracts (S8, S9, and S15) showed effective antibiofilm activity against the *S. pyogenes* ATCC 19615biofilm formation even at minimal concentration of 10 µg/ml. Fig 4 depicts the efficient disintegration of *S. pyogenes* ATCC 19615 biofilm by compounds even at a minimal concentration which is visualized using light microscopy, as the concentration of the fatty acid extract increased the biofilm formation decreased. The effect of crude lipid extracts of *Streptomycetes sps* on biofilm formation was also observed using CLSM (Fig 5). The decrease in the biofilm formation and the reduction in the surface area of the biofilm by the treatment of crude lipid extract, were assessed by comparing the control biofilm formed on the matrix material.

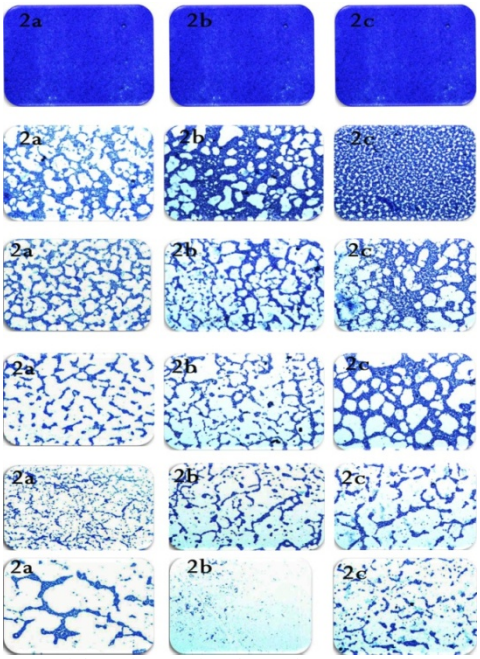

Figure 4: Light Microscopic observation (x40) of *S. pyogenes* ATCC 19615 adhesion phases on the glass surfaces with different concentration (10 µg/ml to 50 µg/ml, top to bottom order) of S8 2(a), S9 2(b), and S15 2(c) crude *Streptomyces sps* lipid extracts against control which is indicative of glass surface with uniformly distributed cells stained with crystal violet.

Streptolysin-S assay

Streptolysin-S (SLS) is a toxic immunogenic protein (oxygen resistant) produced and secreted by most *S. pyogenes* group A strains. It is cytolytic or cytotoxic toward erythrocytes and other types of eukaryotic cells. The erythrocytes remained

intact, when the crude fatty acid extract treated, *S. pyogenes* ATCC 19615 culture supernatant was added. Thus from the spectrophotometric observation it is confirmed that absorbance of the control was low representing that the release of haemoglobin whereas the treated showed the high absorbance values representing the intact erythrocytes (Figure 6). Hence it may be deduced that the actinobacterial lipid extracts might be able to modify the activity of SLS synthesized by *S. pyogenes* ATCC 19615. Serum without culture supernatant was used as negative control.

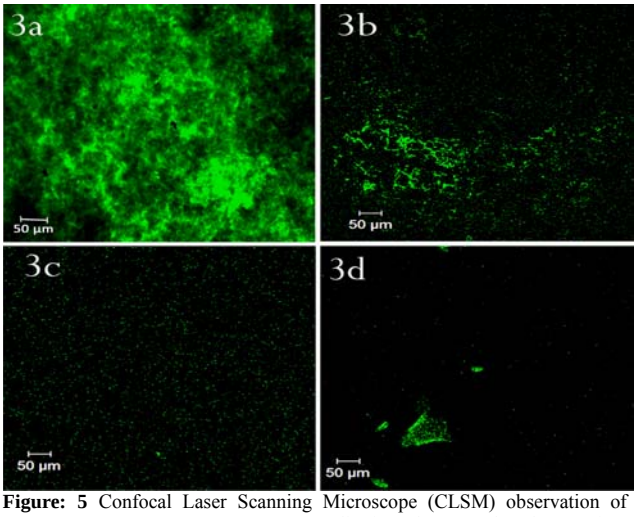

Figure: 5 Confocal Laser Scanning Microscope (CLSM) observation of *Streptococcus pyogenes* ATCC 19615 adhesion phase on the glass surface 3(a) Biofilm Control, 3(b) S8 lipid extract treated glass surface, 3(c) S9 lipid extract treated glass surface, 3d) S15 lipid extract treated glass surface. The lipid extract was incorporated at a concentration of 50 µg/ml. The biofilm cells were stained with fluorescein isothio cyanate-concanavalin A (FITC - con A).

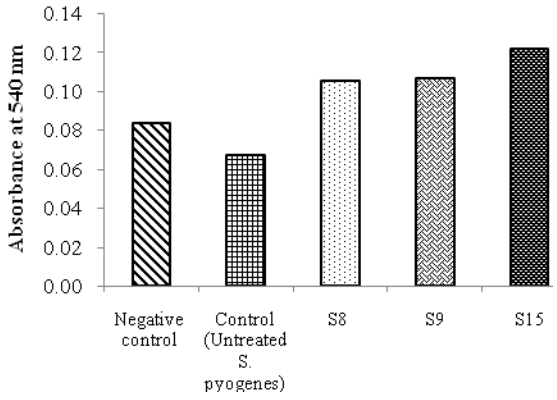

Figure 6: Representation of the lysis of intact erythrocytes with the release of haemoglobin when the *S. pyogenes* ATCC 19615 culture supernatant was added. The erythrocytes remained intact, when the crude fatty acid extract treated, *S. pyogenes* ATCC 19615 culture supernatant was added.

Extracellular protein profile of S. pyogenes

The synthesis of SLS is one of the major defining characters of *S. pyogenes*. In addition GAS also secrets a wide range of extracellular enzymes and cell wall associated protease that aid in the virulence of the organism. Biofilm formation by *S. pyogenes* represents a key factor in their survival, and has important clinical repercussions (Baillie and Douglas 2000; Chandra et al. 2001; Ramage 2005). Many extracellular proteins secreted by *S. pyogenes* have the capability to cleave the fibrin clots of the host and help establish infection. Our experiment aimed to study whether the crude actinomycete fatty acid extract influenced the extra cellular protein secreted by the pathogen. From the Fig 7, it is clear that culture supernatant of *S. pyogenes* treated with S8, S9 and S15 crude fatty acid extract of actinomycetes, have a different pattern of protein expression compared to *S. pyogenes* ATCC 19615 untreated control (Lane 2). In Lane 3-5, a predominant band between 100-120 kDa was observed, representing that the particular protein is expressed, when the bacterial culture was treated with the crude fatty acid extract (S8, S9, and S15 respectively). The lipid extracts were accountable for the significant reduction of biofilm formed by *S. pyogenes* ATCC 19615. It was also confirmed using biofilm inhibition assay and microscopic observation. Thus, further characterization of this protein will reveal its potential role in inhibition of biofilm formation.

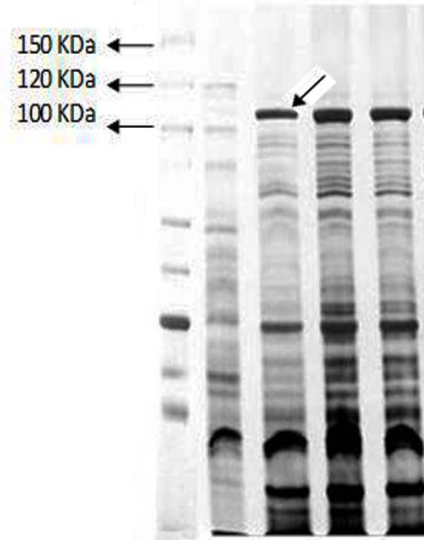

Figure 7: Protein profile of *S. pyogenes* ATCC 19615 culture supernatant, **Lane 1:** Marker (~ 10 µg), **Lane 2:** Control (~ 25 µg), **Lane 3:** *S. pyogenes* ATCC 19615 treated with S8 (~ 25 µg), **Lane 4:** *S. pyogenes* ATCC 19615 treated with S9 (~ 25 µg), **Lane 5:** *S. pyogenes* ATCC 19615 treated with S15 (~ 25 µg). All the cultures were treated with 50 µg/ml concentration of crude lipid extracts for 16 hrs.

Conclusion

The results emphasize the role of *Streptomyces sps* crude fatty acid extract, inhibiting the biofilm formation of *S. pyogenes* ATCC 19615. Among the 25 isolates, the crude lipid extract of the *Streptomyces sps* isolates S8, S9, and S15 isolated from the rhizosphere soil of Nilgiris hills, Tamilnadu have significant property to inhibit the biofilm formation. CLSM images showed the disruption and inhibition of biofilm at a concentration of 50 µg/ml. All the three extracts inhibited the biofilm at a minimal inhibitory concentration of 10 µg/ml. The result of the Streptolysin S assay showed a change in the absorbance level which corresponded to the influence of lipid extract on the enzyme. The protein profile also showed the expression of specific protein at a molecular weight range from 100 – 120 kDa which was evident from the prominent band pattern on the SDS PAGE.

This elucidates that the crude fatty acid extracts have influenced the extracellular protein secretion of the pathogen. Till date, no data has reported lipids to inhibit the production

of SLS, which is very important for virulence. The 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 conclude that these crude fatty acid extract might belong to the long chain fatty acid groups and have the ability to effectively inhibit the biofilms of *S. pyogenes* ATCC 19615. Extending the study on these active compounds with their ability to inhibit quorum sensing and biofilm formation, may provide complimentary medicine for biofilm associated infections.

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