Available online at www.ijpcr.com
International Journal of Pharmaceutical and Clinical Research 2017; 9(11): 665-670

ISSN- 0975 1556

Research Article

In Vitro Antibiofilm, Antiquroum Sensing Activity of Gamma Tolerant Streptomyces Against Gram Negative Pathogens

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Received: 12th Nov, 17; Revised 15th Nov, 17, Accepted: 20th Nov, 17; Available Online:25th Nov, 17

ABSTRACT

Objective: The objective of this research was to study of antibiofilm, antibacterial, antiquroum sensing activity of gamma tolerant actinomycete isolated from soil. Methods: Secondary metabolites from *Streptomyces albogriseolus* GIS39Ama (genbank Accession number: KX694268) was obtained. The metabolites were tested for antiquroum sensing activity by performing violacein inhibition assay. The minimum biofilm inhibitory concentrations (MBIC) of metabolite against *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Vibrio cholerae* were estimated. The effect of MBIC was further tested for reduction in exopolysaccharide content, viability of bacterial cells of biofilm, bacterial density of biofilm, extracellular DNA. Results The MBIC of *Streptomyces albogriseolus* GIS39Ama metabolite extract was found to be 625ppm against *K. pneumoniae*, *V. cholerae*, 1250ppm against *P. aeruginosa* and 312 ppm against *E. coli.* Upto 95% reduction was achieved in exopolysaccharide content, 82% reduction in Viability, 89% reduction in bacterial density of biofilm was obtained. Inhibition of extracellular DNA was observed. The secondary metabolite extract exhibited antifungal activity. Conclusion: The actinomycete isolated from soil was found to exhibit antibiofilm, antiquroum sensing and antifungal properties.

Keywords: Biofilm, Quorum sensing inhibition, Actinomycetes, extracellular DNA.

INTRODUCTION

Actinomycetes are Gram-positive bacteria which are differentiated from other bacteria due to their distinct morphology and DNA configuration. Their omnipresent nature provided the researchers an opportunity to study them on larger extent.

In past few decades, Actinomycetes are recognized as the diverse group of microorganisms which produces multiple bioactive compounds or secondary metabolites. These bioactive molecules were found to exhibit commercial significance. More than 70% of naturally occurring antibiotics are isolated from various genera of actinomycetes[1].

The genus *Streptomyces* has introduced maximum bioactive secondary metabolites, which have proved to be efficient anticancer, antiparasitic, antifungal, antibacterial, antioxidant and immunosuppressive agents[2-4].

But many antibiotics are now becoming ineffective due to increase in bacterial antibiotic resistance and persistence. The biofilm formation ability of bacterial pathogens is also recognized as one of the major cause of such increasing resistance. There have been many studies suggesting the recurrence of infections caused due to *Pseudomonas* spp, *Klebsiella* spp, *Vibrio* spp, *E.coli* and other Gram negative bacteria forming a biofilm[5,6].

To overcome this problem, there is a requirement of alternative antibacterial substances which can mainly affect the biofilm forming capabilities microorganisms and associated virulence properties.

The previously isolated actinomycete strain which was isolated from soil exposed to 10 Kgy dosage of Gamma radiation. The objective of the present study was to evaluate the antiquorum sensing, antibiofilm activity of the gamma tolerant *Streptomyces albogriseolus* GIS39Ama. The outcome of this study may prove essential with respect to the treatment biofilm forming Gram negative pathogens

MATERIALS AND METHODS

Microbial test cultures used in the study and their growth conditions

The selected bacterial strains were obtained from Microbial type culture collection centre, IMTECH, Haryana, India. The selected strains of pathogens were *Klebsiella pneumoniae* MTCC 3384, *Vibrio cholerae* MTCC 3906, *Escherichia coli* MTCC 687 and *Pseudomonas aeruginosa* MTCC 2453. The antiquorum sensing experimentation was performed with the violacein producing *Chromobacterium violaceum* MTCC 2656. All medical strains were maintained in Brain heart infusion (BHI) broth medium at 37°C, except *C. violaceum* MTCC 2656 which was incubated at 30°C. For all the assays test culture suspension was prepared by using overnight growth with an optical density adjusted to 0.1($OD_{610\,nm}$).

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The antiquorum sensing and antibiofilm studies were conducted using *Streptomyces albogriseolus* GIS39Ama (genbank Accession number: KX694268) isolate.

Estimation of Antiquorum sensing activity of the Streptomyces albogriseolus GIS39Ama using violacein inhibition assay

The violacein inhibition assay was performed using the method prescribed by McLean et al[7]. *Streptomyces albogriseolus* GIS39Ama (genbank Accession number: KX694268) was grown in Actinomyces broth media, and incubated for 3 weeks. After incubation broth was centrifuged at 15000 rpm for 20minutes to obtain cell-free supernatant (CFS).

10 µl of cell free supernatant was inoculated into a microtiter plate well containing 180µl of Luria broth and 10 µl of the culture suspension of *C. violaceum* MTCC 2656 (0.1 OD $_{610nm}$) The plates were incubated overnight at 37°C and observed for the reduction in violacein pigment production. Wells were observed for violet colour pigmentation. The percentage of violacein inhibition was calculated by following the formula:

percentage of violacein inhibition= (control OD_{580nm}−test OD_{580nm} / control OD_{580nm}) ×100.

Extraction of secondary metabolites for Streptomyces albogriseolus GIS39Ama

The secondary metabolite extracts (SME) were prepared using method previously described by Usup et al[8]. The cell-free supernatant of *Streptomyces albogriseolus* GIS39Ama was prepared as per the process described above. The cell fee supernatant was mixed with equal volume of ethyl acetate. The mixture was kept under shaking conditions (200 RPM) for 1 hour. The secondary metabolite was obtained using separating funnel (middle layer). The middle layer obtained from separating funnel was dried to in a petri dish. Antibiofilm properties of secondary metabolite were studied after dissolving the metabolite in dimethyl sulfoxide.

Antibiofilm study

To study the biofilm inhibition of standard strains of pathogens by Streptomyces albogriseolus GIS39Ama

A quantitative microtiter plate based assay was conducted to evaluate the minimum biofilm inhibitory concentration (MBIC) of secondary metabolite extracts (SME)[9].

Concentrations of 5000, 2500,1250, 625, 312, 156, 78 and 39ppm of secondary metabolite extract were made by performing serial dilution technique. Dilutions were made using 2% Glucose containing BHI medium. 50µl of test cultures were inoculated in 200µl BHI broth supplemented with 2% glucose and secondary metabolite extracts. The plate was incubated at 37°C for 24 hours. After incubation, medium was removed aseptically. Each well was washed with sterile phosphate buffered saline (PBS) (pH 7.0). Such three washings were provided to each well. After washing, plate was allowed to dry and the biofilm formation was checked by staining wells with 0.2% (aq.) Crystal violet (200µl) for 45 minutes. After staining, wells were washed five times gently with sterile PBS. Wells were destained using 200 µl of absolute ethanol. 100ul from each well was transferred to a new microtiter plate and OD was measured at 595 nm. Sodium hypochlorite 0.4%(v/v) was used as antibiofilm control.

The control for each test culture was also maintained (i.e. untreated 48h old biofilm). The percentage reduction in biofilm was calculated by using following formula:

Percent reduction in biofilm = (Control $OD_{595\ nm}$ –Test $OD_{595\ nm}$)/Control $OD_{595\ nm}$ x 100

To evaluate the effect of Minimum biofilm inhibitory concentration on of Streptomyces albogriseolus GIS39Ama exopolysaccharide (EPS) content of biofilm

The Exopolysaccharide content was estimated by total carbohydrate assay[10]. The biofilm of test cultures was developed in a Brain heart infusion broth supplemented with 2% glucose for 48 hours in a test tube. The minimum biofilm inhibitory concentration of secondary metabolite extract was added over biofilm of each test culture, for 6 hours. The secondary metabolite was then removed aseptically, and biofilm was washed gently using sterile PBS (pH 7.2). The 5% phenol and H_2SO_4 containing 1% hydrazine sulphate was added to each of the tube. The tubes were incubated for 1 h in dark conditions. The optical density was measured at 490 nm. The untreated biofilm of each test culture was maintained as a positive control. The percentage reduction in EPS content of biofilm was calculated by using following formula:

Percent reduction in EPS = (Control $OD_{490\ nm}$ –Test OD490 $_{nm}$)/Control $OD_{490\ nm}$ x 100

To evaluate the effect of Minimum biofilm inhibitory concentration on biofilm bacterial density

To quantify the effect of SME on biofilm density method described by Lokegaonkar and Nabar[9]. The biofilms of test cultures were grown on 1x1 cm PVC slides for 48 hours. These biofilms were exposed to the minimum biofilm inhibitory concentration of secondary metabolite extracts and incubated for 6hours.

After incubation, the biofilm was removed from the slide by rigorous shaking. The treated biofilm was suspended in the 5ml of phosphate buffer (pH 7.6). The 0.1ml of fluorescein diacetate (FDA) in acetone, was added to 3ml of the suspension. Tubes were incubated for 1h at 37ºC in dark. The reaction was stopped using 3ml Acetone and absorbance was read at 490nm. The 0.3ml volume of biofilm suspension was used to determine the bacterial titer by spread plate technique on LB agar. The results were compared with the untreated biofilm of each test culture respectively. The biofilm density was calculated by following formula:

Surface density of biofilm= (Bacterial concentration x Buffer Volume)/ Area of slide

To evaluate the effect of Minimum biofilm inhibitory concentration on the viability of bacterial cells of biofilm

The biofilms of test culture were developed in a microtiter plate by inoculating 20µl of test culture in 180µl of 2% Glucose containing BHI medium. Plates were incubated for 48h, and then medium was decanted. The cell debris was removed by washing the biofilm gently with sterile PBS (pH 7.2). The biofilm was treated with minimum biofilm inhibitory concentration of secondary metabolite extract for 6 hours. After the treatment biofilm washed to remove residual secondary metabolite extract components.

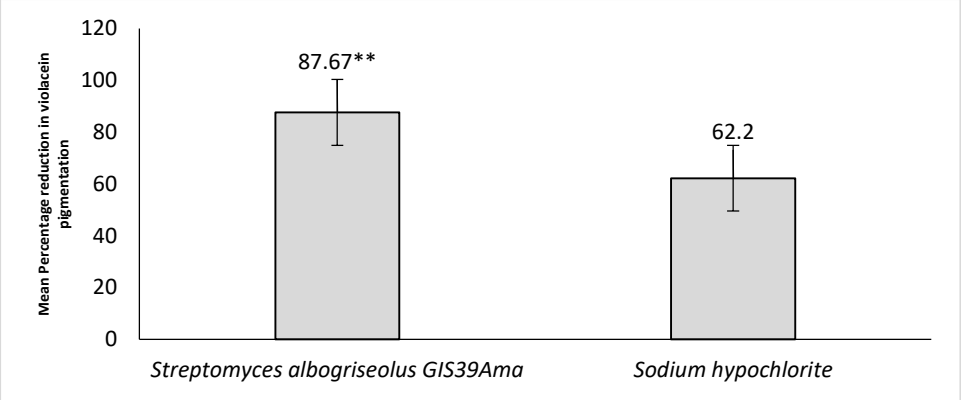

Figure 1: Percentage reduction in Violacein. Graph represents mean percent reduction in violacein pigmentation. The one way ANOVA was performed to compare mean values. **: significance value less than 0.01

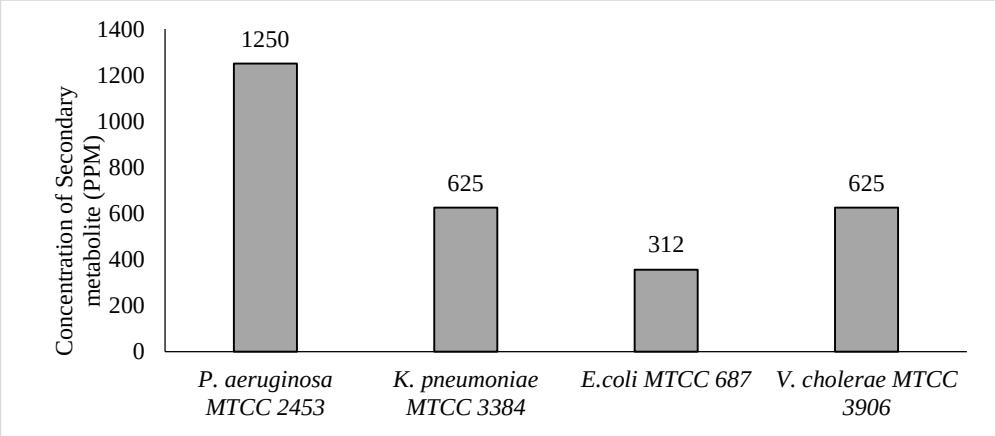

Figure 2: Minimum biofilm inhibitory concentration of *Streptomyces albogriseolus GIS39Ama* metabolite against the standard strains of microbial pathogens.

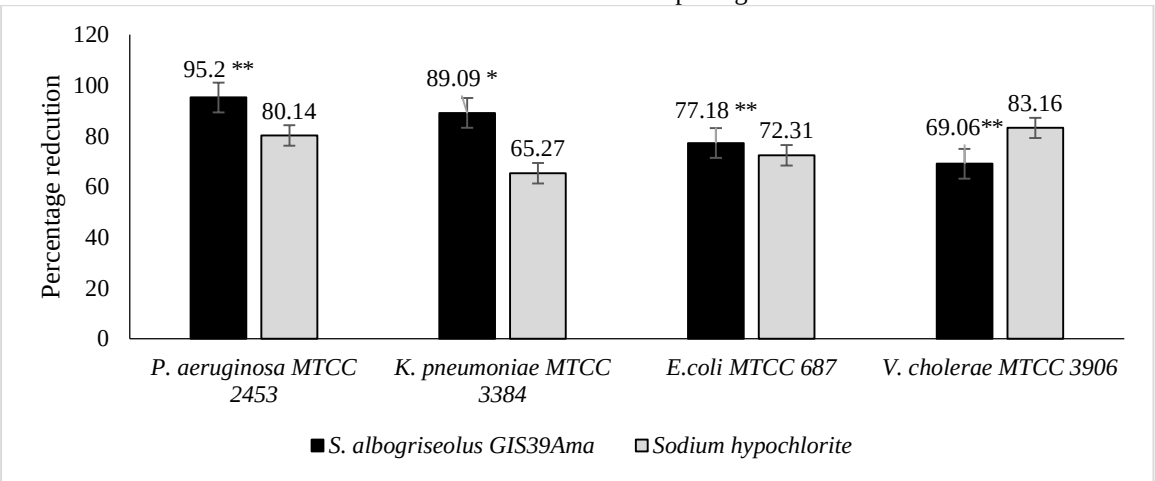

Figure 3: Effect of *Streptomyces albogriseolus GIS39Ama* metabolite on exopolysaccharide content of biofilm. Graph represents mean percent reduction in exopolysaccharide content. The one way ANOVA was performed to compare mean values. *: significance value less than 0.05, **: significance value less than 0.01.

The biofilm was then disrupted by applying mechanical shear force (vigorous pipetting) and suspended in a sterile saline. A load of viable cells was determined by serial dilution technique using spread plate method. The positive control of untreated biofilm of test pathogens was also maintained individually. Reduction in viability was estimated by comparing the load of bacterial viable cells of secondary metabolite treated biofilm with control biofilm.

Reduction in Viability= (Control CFU/ml – Treated CFU/ml)/Control CFU/ml x 100

To evaluate the effect of Minimum biofilm inhibitory concentration on extracellular DNA

The effect of secondary metabolite extract on the presence of extracellular DNA was studied using sub-MBIC levels of the extract. In a microcentrifuge tube, 200µl of test pathogens were inoculated in 1800µl 2% glucose containing BHI medium along with sub MBIC

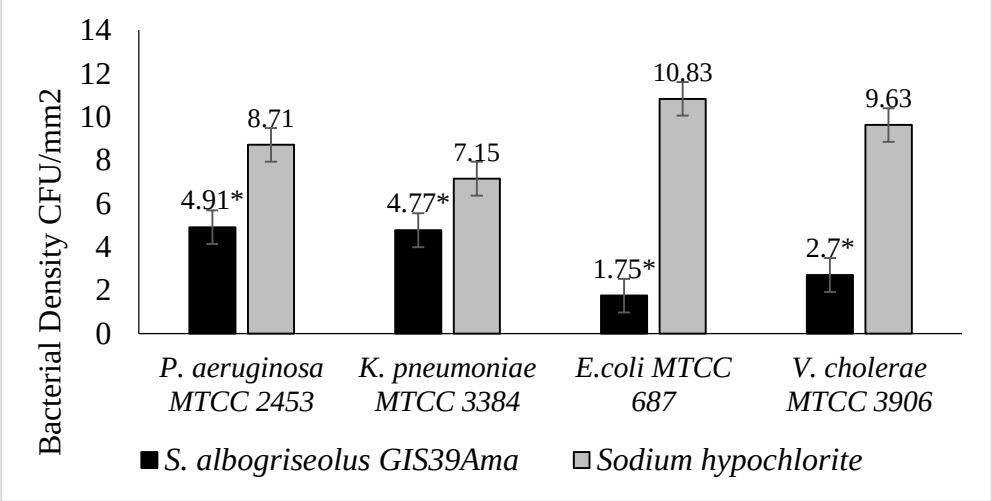

Figure 4: Effect of *Streptomyces albogriseolus GIS39Ama* metabolite on bacterial density of biofilm. Graph represents mean bacterial density of biofilm. The one way ANOVA was performed to compare mean values. *: significance value less than 0.05.

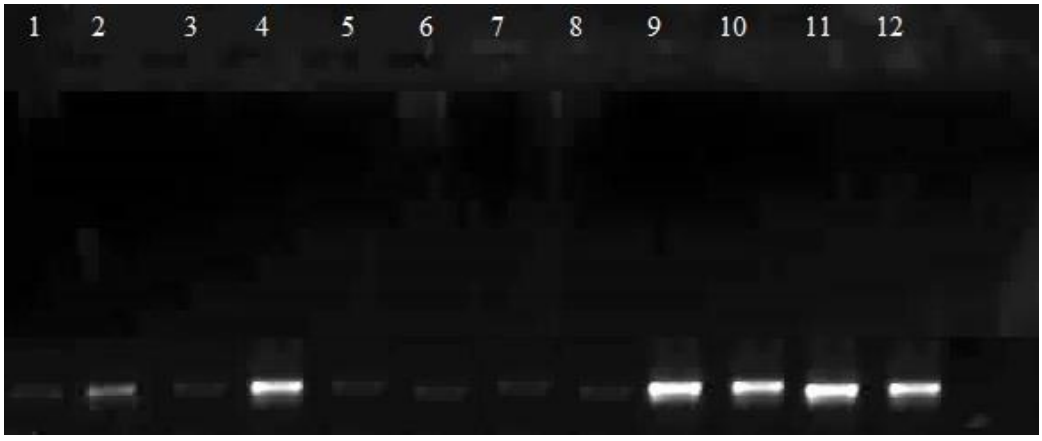

Figure 5: Effect of *Streptomyces albogriseolus GIS39Ama* metabolite on extracellular DNA of biofilm Well no. 1-4 represents secondary metabolite treated supernatants of *P. aeruginosa* MTCC 2453, *K. pneumoniae* MTCC 3384, *E. coli* MTCC 687, and *V. cholerae* MTCC 3906 respectively. Well 5-8 represents supernatants of dnase enzyme treated biofilms of *P. aeruginosa* MTCC 2453, *K. pneumoniae* MTCC 3384, *E. coli* MTCC 687, and *V. cholerae* MTCC 3906 respectively. Well no 8-12 represents supernatants of untreated biofilms of *P. aeruginosa* MTCC 2453, *K. pneumoniae* MTCC 3384, *E. coli* MTCC 687, and *V. cholerae* MTCC 3906 respectively.

concentration. Tubes were incubated for 24h, at 37°C. After incubation tubes were centrifuged at 5000 rpm for 10 minutes. Supernatants were subjected to agarose gel electrophoresis to check the presence of DNA. Electrophoresis was carried out with 1% Agarose gel in Tris-Borate buffer with ethidium bromide as a visualizing agent. Agarose gel was observed under UV transilluminator to check the presence of DNA band. The untreated biofilm was considered as positive control. The negative control was also maintained, where the biofilm formation of each test culture was carried out in present of 10 µg of dnase enzyme.

The absence of DNA in supernatant indicated the DNAse activity of crude extracts.

Statistics

Each assay was performed in triplicate and the values were expressed as the means ± SD. Linear regression analysis and One-way analysis of variance (ANOVA) was performed using IBM SPSS 23.

RESULTS AND DISCUSSION

Estimation of Antiquorum sensing activity of Streptomyces albogriseolus GIS39Ama using violacein inhibition assay

The antiquorum sensing activity of secondary metabolite was estimated on the basis of violacein inhibition assay. Figure 1 represents the results of quantitative violacein inhibition assay. As per the results, it was observed that *Streptomyces albogriseolus* GIS39Ama metabolite reduced violacein pigmentation by 87.67%. The metabolite was found to be more efficient than the Antibiofilm control with respect to violacein pigment inhibition. Hence it was concluded that the microbial metabolite exhibits antiquorum sensing activity. In next phases, secondary metabolite extract was made by solvent extraction method and used to study antibiofilm properties. Teasdale et al reported that the *Halobacillus salinus* isolated from a seagrass sample tends to exhibit violacein inhibitory properties[11]. The decanoyl homoserine

Table 1: Effect of *Streptomyces albogriseolus GIS39Ama* metabolite on Viablility of bacterial cells of a biofilm formed by standard strains of microbial pathogens.

Pathogens	Positive control	*Streptomyces albogriseolus GIS39Ama*	Sodium hypochlorite
P. aeruginosa MTCC 2453	165×10^{12}	128×10^{3}	84×10^{3}
K. pneumoniae MTCC 3384	190×10^{12}	86×10^{2}	115×10^{2}
E.coli MTCC 687	154×10^{12}	105×10^{2}	64×10^{2}
V. cholerae MTCC 3906	140×10^{12}	108×10^{3}	118×10^{2}

lactone, the native autoinducer for *Burkholderia pseudomallei* was also reported to inhibit the HHL quorum sensing system of *C. violaceum* by McClean et al[7].

Determination of minimum biofilm inhibitory concentration of Streptomyces albogriseolus GIS39Ama metabolite against standard strains of pathogens

The compounds exhibiting anti quorum sensing activity, have proven their efficiency as a good antibiofilm agents. The antiquorum sensing component extracted from *Delisea pulchra* (*5Z*)-4-Bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone (furanone) found to inhibit biofilm formation of *Escherichia coli* 100 µg/ml[12]. Similar activity is reported by Younis, Usup and Ahmad, who explained the biofilm inhibitory activity of crude secondary metabolite extract of *Streptomyces* spp against uropathogenic *Proteus mirabilis* isolate[8]. In our present study we examined the antibiofilm activity of secondary metabolite extract of *Streptomyces albogriseolus GIS39Ama*. Figure 2 depicts the MBIC values of *Streptomyces albogriseolus GIS39Ama* metabolite towards the bacterial pathogens. The concentration giving more than 99% reduction in biofilm formation was considered as MBIC. Among the four tested pathogens, *P. aeruginosa* MTCC 2453 exhibited MBIC value of 1250ppm. The biofilm formation of *V.cholerae* MTCC 3906 and *K. pneumoniae* MTCC 3384 were inhibited with MBIC of 625.

Effect of MBIC of Streptomyces albogriseolus GIS39Ama on exopolysaccharide content of biofilm

Exopolysaccharides are a crucial part of the biofilm that enables mechanical stability and compact physical structure. Exopolysaccharides play a dual role in nature with respect to biofilm formation. Even though in most of the cases exopolysaccharide has been reported for its adhesive properties, many studies suggest otherwise. Guzman-Murillo et al and Guezennec et al described antiadhesive and biofilm inhibitory properties of microalagae and *Alteromonas, Pseudomonas,* and *Vibrio* spp respectively[13,14]. In another study, Nasu and coworkers examined several antibiotics, of which clindamycin and macrolides were found to be efficient in reducing exopolysaccharide content of Gram negative pathogen[15].

In present study, secondary metabolites were tested for their exopolysaccharide inhibitory properties using total carbohydrate assay. The exopolysaccharides were inhibited up to 95.2% and 80% by microbial metabolite and Sodium hypochlorite respectively. Figure 3. Represents the plot of mean percentage reduction of exopolysaccharide content of biofilm of microbial pathogens.

Effect of Streptomyces albogriseolus GIS39Ama metabolite on Viability of bacterial cells of a biofilm formed by standard strains of microbial pathogens

Destruction of biofilm by inhibiting exopolysaccharides enables antibiofilm compound to reach inner layers of biofilm. By virtue of this ability, antibiofilm compound tends to exhibit antibacterial activity in a larger extent. Multiple studies reported that the several genera of actinomycetes display antibiofilm as well as antibacterial properties[16,17]. In the present study, we have examined the antibacterial properties of *Streptomyces albogriseolus* GIS39Ama secondary metabolite. Bactericidal properties of MBIC of metabolite and antibiofilm control measured by estimating the viable count of treated biofilm. In 6 hours of exposure biofilm, the viable count of biofilm was reduced to 10^{3}CFU/ml from 10^{12} CFU/ml. Table1 represents the results of the viable count for biofilms upon treatment with metabolite and sodium hypochlorite.

Effect of Streptomyces albogriseolus GIS39Ama metabolite on the bacterial density of biofilm

The efficiency of biofilm eradication is determined by reduction in biofilm biomass and decreased bacterial density. Hence in this study, we estimated the effect of microbial metabolite on the bacterial density of biofilm. The examination of the bacterial density of biofilm provides an idea about the impact of antibiofilm agent on biofilm bacterial population with respect to the area covered by a biofilm. Figure 4 depicts the results bacterial density reduction upon treatment with secondary metabolite. Reduction in bacterial density was obtained upto 1.75 CFU/mm^2 and 8.71 CFU/mm^2 by microbial metabolite and Sodium hypochlorite respectively.

Effect of Streptomyces albogriseolus GIS39Ama metabolite on extracellular DNA of biofilm

Relationship of biofilm exopolysaccharide and involvement of cell free or extracellular DNA is explained in many studies. Multiple hypotheses propose extracellular DNA containing exopolysaccharide as significant defence mechanism system of biofilm forming bacteria[17-20]. Even though role of extracellular DNA in biofilm formation is not fully understood. But it is considered to be significant with respect to adherence of bacterial cells to abiotic surfaces, as well as intercellular attachments. Figure 5 represents the agarose gel electrophoresis image of supernatants of biofilms treated with metabolite, dnase enzymes, and untreated biofilm. Supernatants from biofilm of positive control showed presence of DNA on electrophoresis, thus confirmed presence of extracellular DNA. DNase treated biofilms, and metabolite treated *P. aeruginosa* MTCC 2453, *E.coli* MTCC687 inhibited extracellular DNA. Metabolites proved ineffective against

V. cholerae MTCC 3906, and *K. pneumoniae* MTCC 3384. Tetz and tetz reported reduction of biofilm biomass in presence of Dnase I, explaining the significance of extracellular DNA in biofilm formation[21]. 5µg/ml of DNase I reduced biofilm biomass of *E.coli* and *S. aureus* biofilm by 54% and 51% respectively. Thus the exonuclease activity expressed by *Streptomyces albogriseolus GIS39Ama* indicates its strong antibiofilm nature.

CONCLUSION

Many *Streptomyces* spp have been reported in past decades for their antibacterial, antiquorum sensing, antitumor properties. But this is the first study which report efficiency of *Streptomyces albogriseolus* GIS39Ama as antiquorum, antibiofilm activity against Gram negative biofilm forming pathogens. The isolates not only inhibited the biofilm formation of pathogens but also controlled the other biofilm associated virulence properties included in study.

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