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RESEARCH ARTICLE

Inhibition of *Staphylococcus aureus* Producing Biofilm by Rhamnolipid Biosurfactant

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Abstract

Biofilm formation by pathogenic bacteria is associated with reduced efficacy in action of antimicrobial agents against them. Rhamnolipids are a group of bacterial metabolites which exhibit antibiofilm efficacy. The antibiofilm efficacy of rhamnolipid of *Pseudomonas aeruginosa* JS29, produced in glucose rich culture medium, was tested against the human pathogen *Staphylococcus aureus*. Different concentration of rhamnolipid in the range of 0.12 to 4 mg/ml was tested for inhibition of biofilm production and growth of the bacteria *in-vitro*. Results revealed that the biofilm production by the pathogen was inhibited by 90% in presence of rhamnolipid at 2% mg/ml concentration. At 4 mg/ml concentration, the rhamnolipid also caused 100% inhibition of bacterial growth and the effect was at per with that of the standard drug Clindamycin (Clid 300) at 0.5% mg/ml concentration. The inhibition of biofilm formation could be the combined effect of reduced growth and production of Exopolysaccharide (ESP) by the pathogen under the influence of the rhamnolipid.

Keywords: *Biofilm inhibition, Rhamnolipid biosurfactant, Staphylococcus aureus, Pseudomonas aeruginosa JS29*

1. Introduction

The human body harbours a diverse array of microorganisms which are known to play diverse physiological roles, outcome of which is both beneficial and harmful impact on the host. *Staphylococcus aureus* is a bacterial pathogen known to cause mild infections such as pimple and boil as well as life-threatening diseases such as bactericimia and sepsis in human(1; 2; 3). Open wounds are often infected with *Staphylococcus aureus* resulting in wound severity as well as delayed healing(4; 5; 6). The ability of the pathogen to produce biofilm renders strong protection to the individual cells against antimicrobial agents, thereby assisting them to indulge in pathogenicity(6). Biofilm remains the most intense portion of a non-healing wound environment as it continues to be recalcitrant. This leads to difficulty in treatment of biofilm-related infection. Colonization of biofilm in various medical devices such as catheters, pacemakers etc. can also cause exposure of internal organs to the pathogen with consequent deleterious impacts on patients(7). Recently, the emergence of several drug-resistant strains of bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), Vancomycin-resistant *Staphylococcus aureus* (VRSA) has become a new threat to the health of human(8; 9; 10). Currently, various antibiotic such as flucloxacillin, dicloxacillin, cefazolin, cephalothin, cephalexin, clindamycin, lincomycin and erythromycin are prescribed by physician to treat various infections caused by *Staphylococcus aureus*. Although, these antibiotics are efficacious against the pathogen, they also exhibit toxicity on patient's health, which cannot be flouted(11; 12; 13; 14; 15). Therefore, there is a need for finding potentially safe therapeutic alternative for the treatment of Staphylococcus infection. Biosurfactants are surface-active agents produced by different types of microorganisms and are reported to elicit pharmaceutical and agricultural benefits(16; 17). Biosurfactants have grabbed commercial attention as an alternative to various pesticides, chemical substitutes, food additives, and emulsifiers, owing to its eco-friendly nature and low toxicity in environment(18; 19). Antimicrobial activity of rhamnolipid has been enhanced by using it in formulations and producing its derivatives(20; 21). For example, rhamnolipids in formulation with essential oil has been reported to be ef-

fective against MRSA and Candida albicans in *in-vitro* antimicrobial assay(22). Several derivatives of Di-rhamnolipid have been reported to exhibit superior anti-biofilm activity against *Staphylococcus aureus*(23). The *Pseudomonas aeruginosa* strain JS29 has been reported to be producer of rhamnolipid and earlier studies have shown potential of the rhamnolipid in various applications(24; 22). However, there has not been any report on the effect of rhamnolipid in inhibition the growth of the biofilm produced by the human pathogenic bacteria *Staphylococcus aureus*. Therefore, this study was conducted to determine the effect of the rhamnolipid on production of biofilm and the growth of *Staphylococcus aureusin-vitro*.

2. Materials and Methods

2.1 Chemicals and others

All the chemicals were procured from Merck and HiMedia, India. All the plastic wares and glasswares were obtained from Tarson, India, and Borosil, India, respectively. Clindamycin drug purchased and used in the study as a comparative control [Clindac A 1% 25ml topical solution (Alkem Laboratories Ltd)].

2.2 Microorganisms, growth conditions

The test organism *Staphylococcus aureus* (MTCC 96) was procured from CSIR- Institute of Microbial Technology (CSIR-IMTECH), Chandigarh, India and was used as a pathogenic strain for antibio film study. The strain was cultured in Mueller Hinton broth (MHB) and Mueller Hinton agar slants at 35˚C for routine use and maintained in 15% glycerol stock at -80˚C for future use. *Pseudomonas aeruginosa* JS29 (Genebank accession no. KC862289), which was reported to produce rhamnolipid(25), was obtained from germplasm of the Institute of Advanced Study in Science and Technology (IASST), Guwahati, Assam, India and was cultured in nutrient agar slants and Nutrient broth at 35˚C. The efficacy of the strain JS29 to produce biosurfactant was observed in a Mineral salt medium (MSM) containing $NaNO_3$ (4 g/l), KCl (0.1 g/l), KH_2PO_4 (0.5 g/l), K_2HPO_4 (1 g/l), CaCl2 (0.01 g/l), $MgSO_4.7H_2O$ (0.5 g/l), $FeSO_4.7H_2O$ (0.01 g/l), Yeast extract (0.1 g/l) and 1% of trace element solution containing (g/l): H_3BO_3 (0.26), $CuSO_4.5H_2O$ (0.50), $MnSO_4.H_2O$ (0.50), $(NH_4)6Mo7O_{24}.4H_2O$ (0.06) and $ZnSO_4.7H_2O$ (0.70). The carbon source used was 2% (w/v) Glucose. The pH of the medium was adjusted to 7.0 ± 0.2. One cfu of the strain JS29 was inoculated into the nutrient broth (NB) and incubated at 35˚C for 24 hrs at 180 rpm. 5% of this overnight incubated NB with the inoculum was added to MSM to observe the growth and surface tension reduction capability of the strain. To observe the growth pattern of the strain, absorbance of the media was observed at 600 nm in UV-Visible Spectrophotometer (Shimadzu, UV-1800) at an interval of 12 hrs up to 72 hrs. The surface tension reduction efficacy of the strain was observed by measuring the surface tension of the media in Kruss 11 Tensiometer (Germany) at an interval of 12 hrs up to 72 hrs.

2.3 Production and Extraction of Biosurfactant

For the production of rhamnolipid, 5% of the overnight incubated NB with the inoculum of *P. aeruginosa* JS29 was added to 200 ml of MSM containing 2% w/v glucose. After 48 h of culture, the cell-free supernatant was acidified to pH 2 with 6N HCl and kept at 4˚C overnight to allow the precipitation of the biosurfactant. The precipitated biosurfactant was then extracted using ethyl acetate as solvent. The solvent was then evaporated under reduced pressure at 40˚C and the crude rhamnolipid was collected. The crude rhamnolipid was then column purified according to the method described by Lahkar et al., 2015(25) for purification.

2.4 Determination of Growth Inhibition

Growth inhibition was determined by broth microdilution in 96 well polystyrene microtiter plates(26). To evaluate the antimicrobial activity of the rhamnolipid, a series of concentrations were prepared. For that, 4 mg column purified rhamnolipid was taken in a 1.5 ml Eppendorf tube and added 1 ml distilled water in it. Then 21 mg of Mueller Hinton Broth was added in it to make the concentration of rhamnolipid 4 mg/ml which was used as stock. The stock was diluted with Mueller Hinton Broth to obtain the concentration in decreasing order of 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, and 0.12 mg/ml. For comparative analysis, the standard antibiotic drug clindamycin was prepared in similar concentrations. 200 µl of the rhamnolipid containing media (concentration 0.12mg/ml to 4 mg/ml) were pipetted in each well of the 96 well plate. 10 µl of the mother inoculum of the pathogen was added in each well, containing media. Mueller Hinton Broth containing no rhamnolipid was considered as growth control for microbial growth. The media without inoculum was treated as sterile control. The plate was incubated at 35˚C for 24 hours. The growth of the pathogen was observed spectrophotometrically at 600nm after 24 hrs of incubation, using Varioskan Flash Multimode Reader (Thermo Scientific). The growth inhibition was calculated as:

$$Percent \quad growth \quad Inhibition = (1 - \frac{Asample}{Acontrol}) \times 100$$

Where A sample and A control are the optical density of treated and growth control cells at 600 nm, respectively.

2.5 Biofilm Inhibition

The pathogen was inoculated in Mueller Hinton Broth and incubated overnight at 35˚C at 150 rpm to prepare the mother inoculum to evaluate biofilm inhibition. 10 µl of the overnight culture of *S. aureus* was inoculated in 200 µl of Mueller Hinton Broth containing rhamnolipid concentrations ranging from 0.12 mg/ml to 4 mg/ml as mentioned previously in 96 well polystyrene microtiter plates. *S. aureus* inoculum in MHB media without rhamnolipid treatment served as the control treatment for biofilm formation, whereas the broth without the pathogen served as sterility control. The plates were incubated at 35˚C for 48 hrs. After 48 hrs of incubation, the planktonic cells were discarded, and the formed biofilm was gently washed thrice with 1X Phosphate Buffered Saline (PBS) and allowed to dry adequately. The wells were then stained with 200µl of 0.1% Crystal Violet for 15 mins. The extra strain was washed off, and the wells were allowed to air-dry adequately. 200µl of 33% Glacial acetic acid was added to the wells to dissolve the biofilm, and the absorbance was measured in 595nm using Varioskan Flash Multimode Reader (Thermo Scientific)(27).

$$Percent \quad growth \quad biofilm = (1 - \frac{Asample}{Acontrol}) \times 100$$

Where Asample and Acontrol are the absorbances of treated and control cells at 595 nm

2.6 Exopolysaccharide Production

Exopolysaccharide (EPS) is an essential factor of biofilm that efficiently stabilizes biofilm and provides resistance

towards various drugs(27). To evaluate the effect of rhamnolipid on EPS production, S.aureus was allowed to grow in 5ml of MHB media in sterile test tubes to obtain an O.D. of 0.1. Then MHB containing rhamnolipidwas added into the media to make a final concentration of 0.5 mg/ml. A control with no treatment was also used in the experiment. The production of EPS by the bacterial cell was allowed by incubating the test tubes for 48 hrs at 37˚C and 150 rpm. After 48 hrs of incubation, the cells were centrifuged at 8000 rpm for 10 minutes, and the supernatant was filtered through a 0.2µ membrane filter. Chilled ethanol was added at a ratio of 3:1 to the filtered supernatant and incubated overnight at 4C for precipitation of EPS. The precipitate was then collected by centrifuging at 10000 rpm and was dried at 50˚C. The production of EPS was quantified gravimetrically, and was expressed in mg/ml(28).

2.7 Statistical Analysis

All the experiments were performed in triplicate and repeated thrice for statistical analysis. Analysis of mean and standard error of triplicate experiments was calculated in Origin Pro 8.5 software (Origin Lab Corporation, Northampton, MA, USA). The values of mean and standard error are reported in graphical plots of the respective experiments. Percent growth inhibition and biofilm inhibition was validated statistically by one-way ANOVA followed by pair-wise least significant difference (LSD) test. EPS production was validated by a one-way ANOVA to determine the significant difference of the treatments.

3. Results and Discussion

3.1 Bacterial Growth and Surface Tension Measurement in the Biosurfactant Producing Strain Culture

The efficiency of the strain in production of biosurfactants in 2% glucose (w/v) was entiched MAM was evident from the value of surface tension measured in the bacterial suspension. The reduction in the surface tension of the media was observed within 12 hrs of incubation and it continued till 72 hrs (Figure 1). Although, the exponential growth phase was not sharp, the stationary phase of the strain in the media was observed to initiate from about 52 hrs and the decline phase of the population began after about 62 hrs after incubation (Figure 1). Subsequently, for extraction of rhamnolipid, the strain was grown in MSM upto 48 hrs.

3.2 Growth Inhibition

Although the minimum inhibitory concentration of an antibiotic can be obtained by visual observation, yet it is necessary to figure out the population of pathogen inhibited by an agent at that concentration. Infact, determination of antimicrobial efficacy is best accomplished through growth inhibition in terms of cell population. The growth of the pathogenic bacteria was inhibited gradually with increase in the concentration of rhamnolipid ($F_{6,14} = 5910.578$, $p<0.001$). At a concentration of 0.5 mg/ml rhamnolipid, growth inhibition was 90%. At 4 mg/ml concentration, rhamnolipid inhibited the growth completely and this was recognized as bactericidal concentration. Thus, the minimum inhibitory concentration (MIC) of the rhamnolipid was considered 0.5mg/ml which killed 90% of the pathogen. The growth inhibition of the pathogen at various concentration of rhamnolipid is shown in Figure 2. The efficacy of the positive treatment i.e. 0.5 mg/ml of clindamycin against the pathogen was found to statistically similar with that of rhamnolipid at 0.5 mg/ml. This study shows that the rhamnolipid as effective as the pharmaceutical control against the pathogenat the concentration of 0.5 mg/ml.

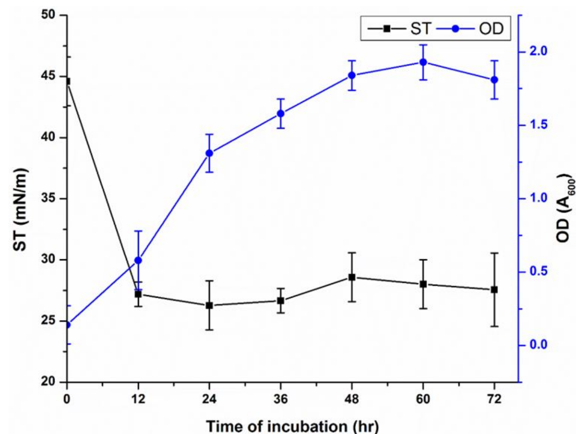

Figure 1: Growth and Surface tension of culture medium inoculated with *P. aeruginosa* JS29 at different time intervals. Error bars represent the standard error of the mean, calculated based on the data from three independent experiments each with three replicates flasks for each time interval

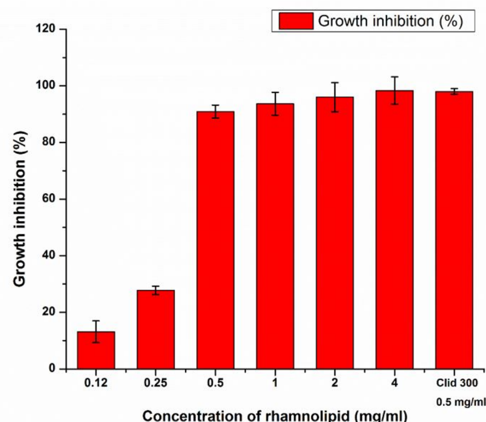

Figure 2: Growth inhibition exhibited against *Staphylococcus aureus* MTCC 96 in culture medium by different concentrations of rhamnolipid extract of *P. aeruginosa*strain JS29. Error bars indicate the standard error of the mean, calculated from three independent experiments with each concentration tested in triplicates

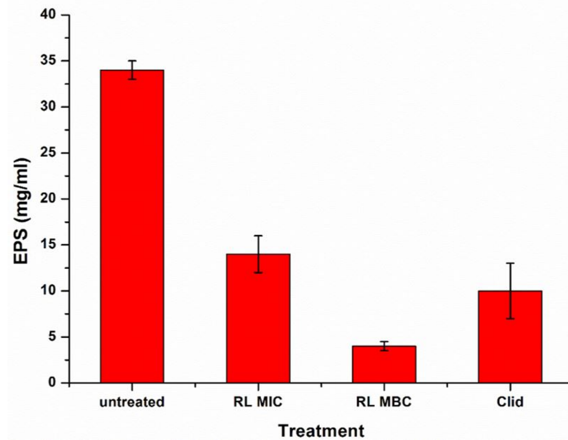

Figure 3: Reduction of EPS production when *Staphylococcus aureus* cells were treated with 0.5 mg/ml of rhamnolipid (RL-MIC), 4mg/ml (RL-MBC) and 0.5 mg/ml of Clindamycin (Clid) in comparison with rhamnolipid untreated control. Error bars indicate the standard error of the mean, calculated from three independent experiments with each concentration of rhamnolipid tested in triplicates

3.3 Biofilm Inhibition

Biofilm produced by pathogens complicatesthe treatment of an infection. Inhibition of the biofilm is an important strategy in treatment of infaction by biofilm producer pathogen. The capability to inhibit biofilm formation is an essential feature of the antimicrobial agent, as it inhibits the reappearance of biofilm-mediated infection. The biofilm inhibition efficacy of the rhamnolipid extracted from *P. aeruginosa* was found to be dose-dependent ($F_{6,14}$=2819.857, p<0.001). Rhamnolipid at a concentration of 0.5 mg/ml and 2 mg/ml was found to inhibit 72% and 91% of biofilm formation respectively. The biofilm inhibition at different concentrations of rhamnolipid is represented in Figure 3. The biofilm inhibition efficacy of an antimicrobial agent is also crucial as planktonic cells often tend to form biofilm on various biotic and abiotic surfaces, resulting in various kinds of infections(29). The inhibition of biofilm formation in this study was accomplished due to the efficacy of the rhamnolipid with which it killed the planktonic cells. It seems that increasing concentration of rhamnolipid killed the planktonic cells in more numbers and therefore, requisite number of cells to initiate cellular aggregation to finally produce biofilm were reduced and consequently biofilm concentration was reduced (Figure 3).

3.4 Exopolysaccharide (EPS) Production

Bacterial biofilms aresurface adherent microbial communities held by self-produced exopolysaccharides (EPS) that serve as the pathogen's adherence matrix enabling the biofilm formation on various substrates(30). Cells embedded in EPS within the biofilm tend to revert the action of various antibiotics. In this study, it was found that rhamnolipid could effectively inhibit EPS production, as shown in Figure 4. The production of EPS by the untreated cells was found to be 34 mg/ml. When the actively growing cells were treated with 0.5 mg/ml and 4 mg/ml concentration of rhamnolipid, it was found that the production of EPS was reduced to 10 mg/ml and 4 mg/ml respectively. These results indicate that EPS may not have been produced in sufficient quantity to allow biofilm formation to the full capacity of the pathogen. Overall, these results suggest that the biofilm inhibition could be reflection of the combined effect of reduced growth of pathogen and production of EPS brought against by the presence of rhamnolipid.

4. Conclusion

This study indicates that the rhamnolipid biosurfactant has a tremendous potential to inhibit in formation of biofilm by the pathogenic bacterial strain of *S. aureus*, as a result of which, the wound caused due to infection of the pathogen would efficiently be controlled. However, to confirm the finding, an in-vivo study would be required to compliment the efficacy of rhamnolipid in wound healing.

Conflict of Interest The authors declare no conflict of interest.

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