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ANTI-ADHESIVE ACTIVITY OF THE BIOSURFACTANT RHAMNOLIPID ISOLATED FROM *STREPTOMYCES* SP

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ABSTRACT

Surfactants produced by microorganisms are known as biosurfactants. Biosurfactants are becoming important biotechnology products for industrial and medical applications due to their specific modes of action, low toxicity, relative ease of preparation and widespread applicability. The present study is to determine the anti-adhesive properties of a biosurfactant rhamnolipid isolated from *Streptomyces matensis* (PLS-1) and *Streptomyces coelicoflavus* (NDYS-4) against several micro-organisms, including Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi. This biosurfactant producing actinomycetes was isolated from soil samples collected near poultry litter and naval dockyard in Visakhapatnam. The anti-adhesive properties of this biosurfactant were tested on several pathogenic strains that colonize animal's gastrointestinal tract, namely, *Staphylococcus aureus* (NCIM 2122), *Bacillus subtilis* (NCIM 2045), *Bacillus pumilus* (NCIM 2108), *Escherichia coli* (NNICM 2137), *Proteus vulgaris* (NCIM 2027), *Pseudomonas aeruginosa* (NCIM 2053), *Candida albicans* (NCIM 3102), *Aspergillus niger* (NCIM 652), *Aspergillus oryzae* (NCIM 1212), *Pectinotricum lanenceae* (NCIM 2118). Results of anti-adhesive activities of biosurfactants obtained from *Streptomyces matensis* (PLS-1) and *Streptomyces coelicoflavus* (NDYS-4) against such a broad group of microorganisms was compiled. Although the anti-adhesive activity of biosurfactants isolated from lactic acid bacteria has been widely reported, their antimicrobial activity is quite unusual and has been described only for a few strains. Further, anti-adhesive properties of this biosurfactant opens up future prospects for its use against micro-organisms responsible for diseases and infections in the urinary, vaginal and gastrointestinal tracts, as well as on the skin, making it a suitable alternative to conventional antibiotics.

INTRODUCTION

Surfactants are amphiphilic compounds that reduce the free energy of the system by replacing the bulk molecules of higher energy at an interface. It contains a hydrophobic moiety with little affinity for the bulk medium and a hydrophilic portion that is attracted to the bulk medium. As a result, surfactants reduce the forces of repulsion between unlike phases at interfaces or surfaces and allow the two phases to mix more easily. Surfactants have been used industrially as flocculating, wetting, foaming agents, adhesives and demulsifies, lubricants and penetrants. Another interesting characteristic of surfactants in aqueous solution is their tendency to aggregate into larger, oriented groups called micelles. At very low

concentrations, the individual single molecules are present or their ions. As the surfactant concentration increase a point is reached called critical micelle concentration (CMC). At this point there is an abrupt change in the solution properties, such as, surface tension, osmotic pressure, viscosity, density and electrical conductivity. Commercially available surfactants that are chemically synthesized, seem to have certain limitations because many of them are toxic and either slowly or non-biodegradable (Swisher, 1970). Most of them are produced from hydrocarbons, which are a non-replenishable resource, and their manufacture involves a series of processing steps where several chemical reactions are followed by a number of costly purification steps. In this process corrosive chemicals are used and quite frequently corrosion problems are encountered in the process equipment. Some of the limitations encountered in synthetic surfactants have stimulated research to find and produce new surfactants from microbes. Recently there has been great deal of interest in biosurfactants, i.e., surface-active compounds produced by microorganisms (Haferburg *et al*., 1986). These biosurfactants are extracellular products excreted by microbial cells grown on certain hydrocarbons, although it may possible to produce them from other substrates such as carbohydrates. The main characteristic of these microbial cultures is their ability to excrete relatively large amount of surface-active substances that emulsify or wet the hydrocarbon phase thus making it available for absorption. There are many reports of extracellular microbial products capable of stabilizing emulsions and most of them originate from bacteria growing on hydrocarbons or related substrates (Akit *et al*., 1981). There are few exceptions such as the lipopeptide surfactin from *Bacillus subtilis* produced on water-soluble substrates (Cooper *et al*., 1981). Biological surfactants possess a number of potential advantages over their chemically manufactured counterparts, including lower toxicity, biodegradability (Zajic *et al*., 1977) and effectiveness at extreme temperatures, pH and salinity (Kretschmer *et al*., 1982). An increased interest for potential application of microbial surface active compounds is based on their range of functional properties which mainly comprise emulsification, phase separation, wetting, foaming, surface activity and viscosity reduction of heavy crude oils (Finnerty *et al*., 1983). Potential applications can be envisaged in several industries such as agriculture, food, textiles, cosmetics, pharmaceutical preparations, petrochemical and petroleum production (Thanomsub *et al*., 2004). Their capability to reduce surface and interfacial tension with low toxicity, high specificity and biodegradability, lead to an increasing interest on these microbial products as alternatives to chemical surfactants (Banat *et al*., 2000; Abouseoud *et al*., 2007). Almost all surfactants currently used are petroleum derivatives, which are toxic and potential source of pollution. These hazards associated with synthetic emulsifiers have, in recent years, drawn much attention to the biosurfactants. Biosurfactants are promising compounds often showing anti-microbial and anti-adhesive properties and sometimes penetrating and removing mature bio-films. Microbial surfactants can interact with interfaces and inhibit the adhesion of microorganisms to different surfaces. They are an alternative to synthetic surface-active agents because of their low toxicity and biodegradability. The present study is to determine the anti-adhesive properties of a biosurfactant rhamnolipid isolated from *Streptomyces matensis* (PLS-1) and *Streptomyces coelicoflavus* (NDYS-4) against several microorganisms, including Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi.

MATERIALS AND METHODS

Microorganisms and culture conditions:

Streptomyces matensis and *Streptomyces coelicoflavus* isolates were obtained from terrestrial soils and maintained on Starch casein agar medium for *Streptomyces matensis* and Bennett's agar medium for *Streptomyces coelicoflavus*. These are subjected to fermentation for biosurfactant production. The composition of the production medium (g/l): olive oil 30 ml, $NaNO_3$ 1.0, KH_2PO_4 0.1, $MgSO_4.7H_2O$ 0.1, $CaCl_2$ 0.1, Yeast extract 0.2 and distilled water up to 1000 ml, pH 6.0 (Kalyani *et al*., 2014) in 250 ml EM flask and incubated at 28ºC on rotary shaker for 7 days at 150 rpm. The anti-adhesive property of purified biosurfactants NDYS-4 E and PLS-1 I was tested on several pathogenic strains that colonize animal's gastrointestinal tract or

medical devices. Six bacterial strains *Staphylococcus aureus* (NCIM 2122), *Bacillus subtilis* (NCIM 2045), *Bacillus pumilus* (NCIM 2108), *Escherichia coli* (NNICM 2137), *Proteus vulgaris* (NCIM 2027), *Pseudomonas aeruginosa* (NCIM 2053) were grown at 28ºC in LB medium (10 g/l bacto-tryptone, 5 g/l bacto-yeast ectract, 10 g/l NaCl). Four fungal strains, *Candida albicans* (NCIM 3102), *Aspergillus niger* (NCIM 652), *Aspergillus oryzae* (NCIM 1212), *Pectinotricum lanenceae* (NCIM 2118) were grown in a 6.7 g/l yeast nitrogen base broth with pH 5.5 containing 2% D-glucose for adhesion tests.

Anti-adhesion assays:

Inhibition of microbial adhesion by purified biosurfactants NDYS-4 E and PLS-1 I was tested in 96-well plates (Sarstedt, Numbrecht, Germany). Briefly, the wells of a sterile 96-well flat-bottom plate were filled with 100 µl of 0.035-0.5 mg/ml purified biosurfactant dissolved in PBS (phosphate buffer saline). The plates were incubated for 2 h at 37ºC on a rotary shaker (MixMate, Eppendrof, Hamburg, Germany) at 300 rpm and subsequently washed twice with PBS. Negative control (blank) wells contained purified biosurfactant at the highest concentration tested (0.5 mg/ml) while positive control wells contained PBS buffer only. The overnight cultures of microbial strains were centrifuged, washed twice with PBS (pH = 7.4) and re-suspended in PBS to an optical density OD_{600} = 1.0 for bacterial and OD_{600} = 0.6 for fungal strains. The highest adhesion without purified biosurfactant was observed at these optical densities. A 100 µl aliquot of washed microbial suspension was added and incubated in the wells. After 2 h incubation at 37ºC in a rotary shaker at 300 rpm nonadherent cells were removed by three washes with PBS. Then the plates were stained with 0.1% crystal-violet for 5 min and again washed three times with PBS. The adherent microorganisms were permeabilized and the dye was resolubilized with 100 µl of 33% (v/v) glacial acetic acid per well, and the absorbance of each well was measured at 595 nm. Purified biosurfactant did not affect the absorption of negative control (crystal violet in blank wells). The microbial inhibition percentages at different biosurfactant concentrations for each microorganism were calculated as

$$\% \text{ Microbial inhibition}_c = [1-(\frac{Ac}{Ao})] \times 100$$

Where A_c represents the absorbance of the well with a biosurfactant concentration c and A_o the absorbance of the control well. The micro titre-plate anti-adhesion assay estimates the percentage of microbial adhesion reduction in relation to the control wells, which were set at 0% to indicate the absence of biosurfactant and therefore of its anti-adhesion properties. The micro titre-plate anti-adhesion assay allows the estimation of the purified biosurfactant concentrations that are effective in decreasing adhesion of the microorganism's studied. Assay was carried out three times in triplicates.

***Preparation of Phosphate buffer saline (PBS)*:**

Phosphate buffered saline (PBS) is composed of 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.42 g KH_2PO_4. These salts were dissolved in distilled water and volume made up to 1000 ml. The pH adjusted to 7.4 and sterilized by autoclaving.

Preparation of Biosurfactant stock solution:

A solution of purified biosurfactant 2000 µg/ml (2 mg/ml) was made in PBS and pH adjusted to 7.0. This solution was sterilized and stored at 4ºC.

Preparation of Crystal violet solution:

Solution A: Crystal violet- 2.0 g, Ethyl alcohol 95% - 20 ml

Solution B: Ammonium oxalate - 0.8 g, Distilled water - 80 ml and Solutions A and B were mixed and stored for 24 h before use. Then the resulting stain is stable.

RESULTS AND DISCUSSION

Antiadhesive activity of purified biosurfactant NDYS-4E:

Adhesion of pathogenic microorganisms to solid surfaces or to infection sites has to be inhibited by biosurfactants capable of modifying the Physico-chemical properties of the surface to reduce adhesion and bio film formation on a given biomaterial.

Table. 1: Microbial adhesion inhibition in the micro titter plate by purified biosurfactant NDYS-4E

Microorganisms	Microbial adhesion inhibition (%)						Control (PBS)
	Biosurfactant concentration (mg/ml)						
	0.5	0.25	0.20	0.150	0.075	0.035	0
S. aureus	55 ± 3	48 ± 3	47 ± 3	19 ± 3	15 ± 3	13 ± 3	0
B. subtilis	69 ± 2	63 ± 2	62 ± 1	56 ± 2	50 ± 3	47 ± 3	0
B. pumilus	41 ± 2	37 ± 2	32 ± 1	7 ± 2	5 ± 4	5 ± 4	0
E. coli	51 ± 2	47 ± 2	46 ± 2	46 ± 2	23 ± 3	15 ± 3	0
P. vulgaris	72 ± 1	72 ± 1	71 ± 1	70 ± 1	65 ± 2	47 ± 0	0
P. aeruginosa	72 ± 1	69 ± 1	66 ± 0	62 ± 1	58 ± 1	53 ± 0	0
C. albicans	77 ± 1	70 ± 1	69 ± 1	68 ± 2	66 ± 1	57 ± 2	0
A. niger	41 ± 2	39 ± 2	33 ± 3	18 ± 2	13 ± 1	12 ± 3	0
A. oryzae	52 ± 1	40 ± 1	38 ± 1	38 ± 1	31 ± 1	27 ± 2	0
P. leuteum	65 ± 1	57 ± 2	57 ± 1	54 ± 3	51 ± 3	43 ± 2	0

PBS was used as control and set as 0% as no microbial inhibition occurs.

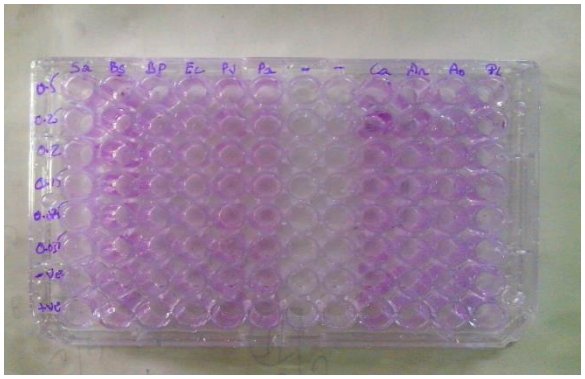

Fig. 1: 96-well micro plate containing purified biosurfactant fractions

Table. 2: Microbial adhesion inhibition in the micro titter plate by purified biosurfactant PLS-1I

Microorganisms	Microbial adhesion inhibition (%)												Control (PBS)
	Biosurfactant concentration (mg/ml)												
	0.5		0.25		0.20		0.150		0.075		0.035		0
S. aureus	55	± 5	53	± 5	45	± 6	32	± 7	26	± 7	21	± 7	0
B. subtilis	67	± 1	62	± 0	61	± 0	60	± 0	53	± 0	51	± 0	0
B. pumilus	47	± 1	44	± 2	43	± 2	12	± 1	13	± 6	3	± 1	0
E. coli	51	± 2	50	± 1	49	± 1	46	± 1	22	± 2	16	± 1	0
P. vulgaris	74	± 1	74	± 1	72	± 1	68	± 1	66	± 1	53	± 0	0
P. aeruginosa	73	± 1	72	± 1	67	± 1	66	± 1	62	± 2	56	± 1	0
C. albicans	73	± 1	69	± 0	67	± 1	65	± 1	59	± 1	56	± 1	0
A. niger	40	± 3	38	± 2	33	± 2	17	± 1	16	± 1	13	± 3	0
A. oryzae	53	± 2	48	± 2	40	± 1	38	± 2	36	± 1	35	± 1	0
P. leuteum	53	± 8	53	± 8	52	± 9	43	± 10	41	± 11	38	± 11	0

Fig. 3: 96-well micro plate containing purified biosurfactant fractions

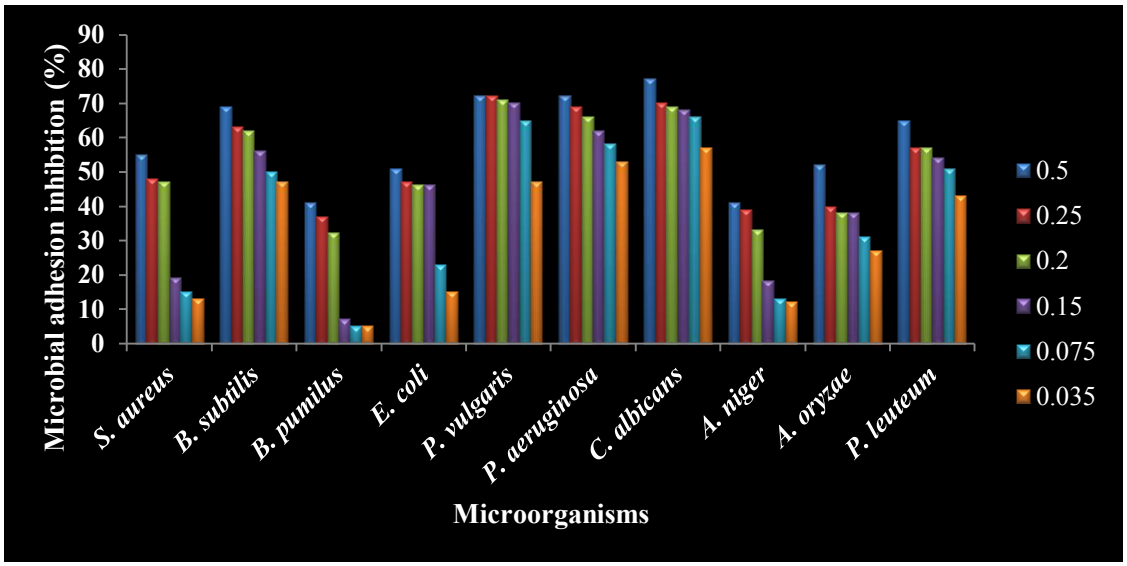

Fig. 2 Microbial adhesion inhibition in the micro titter plate by purified biosurfactant NDYS-4E

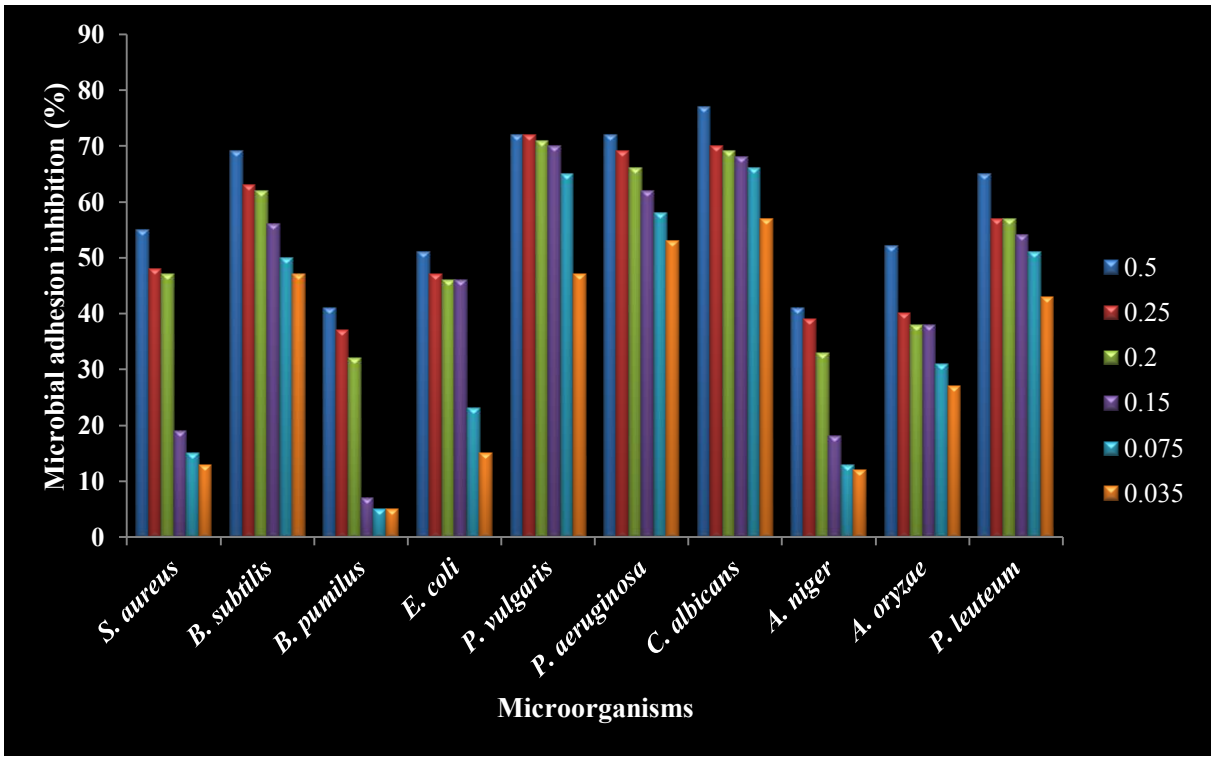

Fig. 4 Microbial adhesion inhibition in the micro titter plate by purified biosurfactant PLS-1I

Purified biosurfactant NDYS-4E isolated from NDYS-4 isolate was found to possess anti adhesive activity against all tested microorganisms shown in Table 1 and its 96-well micro plate containing purified biosurfactant concentrations shown in Figure 1 and Figure 2. Purified biosurfactant NDYS-4E was found to possess anti-adhesive activity against all tested microorganisms. The percentage of polystyrene surfaces with purified biosurfactant significantly decreased the adhesion of all bacteria and fungi, and this anti-adhesive effect was concentration dependent. The anti-adhesive property was proportional to the concentration of the biosurfactant. The highest reduction of adhesion (69 and 77%) was observed for *B. subtilis* (NCIM 2045), and *C. albicans* (NCIM 3102). Low inhibitions were observed for *B. Pumilus* (NCIM 2108), *E. coli* (NCIM 2137) and *A. niger* (NCIM 652), with values of 41 and 51%, respectively, at the maximum biosurfactant concentration.

Antiadhesive activity of purified biosurfactant PLS-1I:

Purified biosurfactant PLS-1I isolated from PLS-1 isolate was found to possess anti adhesive activity against all tested microorganisms shown in Table 2 and its 96-well micro plate containing purified biosurfactant concentrations shown in Figure 3 and Figure 4. Purified biosurfactant isolated from PLS-1 isolate was found to possess anti-adhesive activity against all tested microorganisms. The percentage of polystyrene surfaces with purified biosurfactant significantly decreased the adhesion of all bacteria and fungi, and this anti-adhesive effect was concentration dependent. The anti-adhesive property was proportional to the concentration of the biosurfactant. The highest reduction of adhesion (67 and 74%) was observed for *B. subtilis* (NCIM 2045), *P. vulgaris* (NCIM 2027). Low inhibitions were observed for *B. pumilus* (NCIM 2108) and *A. niger* (NCIM 652), with values of 40 and 47%, respectively, at the maximum biosurfactant concentration. Similar studies were observed by Rivardo *et al*., 2009. According to him the adhesion of pathogenic bacteria to polystyrene surfaces was inhibited by two lipopeptide biosurfactants produced by *B. subtilis* and *B. Licheniformis*, and adhesion of Listeria monocytogenes to polystyrene micro-plates was reduced by 84% on pretreating the surface with surfactin (1 mg/ml), and by 82% when it was treated with purified rhamnolipid (7.5 mg/ml) (De Araujo *et al*., 2011). Gudina *et al*., 2010, characterized the anti-adhesive activity of biosurfactants against several microorganisms including Gram-positive and Gram-negative bacteria. This biosurfactant at concentration 25 mg/ml showed high anti-adhesive activity against *Staphylococcus aureus* (72.0%), *S. epidermidis* (62.1%), *Streptococcus agalactiae* (60.0%) and low anti-adhesive activity against *P. aeruginosa* (16.5%) and *E. coli* (11.5%).

Similar studies were carried out by Tomasz Janek *et al*., 2012 on pseudofactin II secreted by the Arctic bacterium *Pseudomonas fluorescens* BD5 and by Ruffino *et al*., 2011 on Rufisan biosurfactant produced by *Candida lipolytica* UCP 0988. According to them as biosurfactant concentration increases the anti-adhesive activities of the microorganisms also increases.

CONCLUSION

The anti-adhesive properties of biosurfactants were tested on several pathogenic strains that colonize animal's gastrointestinal tracts. The results found suggest that when the surface is covered by purified biosurfactant micelles attached to polystyrene by Vander Waals forces, the adhesion is inhibited more strongly than it is with monomers.

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