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The inhibitory effect of a *Lactobacillus acidophilus* derived biosurfactant on biofilm producer *Serratia marcescens*

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ABSTRACT

Objective(s): *Serratia marcescens* is one of the nosocomial pathogen with the ability to form biofilm which is an important feature in the pathogenesis of *S. marcescens.* The aim of this study was to determine the anti-adhesive properties of a biosurfactant isolated from *Lactobacillus acidophilus* ATCC 4356, on *S. marcescens* strains.
Materials and Methods: *Lactobacillus acidophilus* ATCC 4356 was selected as a probiotic strain for biosurfactant production. Anti-adhesive activities was determined by pre-coating and co- incubating methods in 96-well culture plates.
Results: The FTIR analysis of derived biosurfactant revealed the composition as protein component. Due to the release of such biosurfactants, *L. acidophilus* was able to interfere with the adhesion and biofilm formation of the *S. marcescens* strains. In co-incubation method, this biosurfactant in 2.5 mg/ml concentration showed anti-adhesive activity against all tested strains of *S. marcescens* (P<0.05).
Conclusion: Our results show that the anti-adhesive properties of *L. acidophilus* biosurfactant has the potential to be used against microorganisms responsible for infections in the urinary, vaginal and gastrointestinal tracts, as well as skin, making it a suitable alternative to conventional antibiotics.

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Introduction

Serratia marcescens is a hospital-acquired pathogen commonly causing secondary infections like urinary, respiratory, sinusitis, wound, peritonitis and septic arthritis in the hospitalized patients (1, 2). The ability to adhere to medical devices and host epithelial surfaces to form biofilm is an important feature in the pathogenesis of *S. marcescens*. Biofilms are surface-attached microbial communications and the basis of resistance to biocides and antibiotics as compared to planktonic cells and hence commonly involved in medical device-associated infections (3-5).

Probiotic bacteria, such as lactobacilli, are known to have a helpful effect on the maintenance of human health (6, 7). They constitute an important part of usual microbiota, which are also known as potential interfering bacteria by producing numerous antimicrobial agents such as organic acids, H_2O_2, diacetyl, bacteriocins, low molecular weight antimicrobial substances and adhesion inhibitors, such as biosurfactants (8). In particular, lactobacilli have been identified for their antimicrobial activity and capability to delay pathogens attached to the epithelial cells of urogenital and gastrointestinal tracts (9-11), and for their anti-biofilm production on catheter devices (12) as well as hearing-aids (13, 14). Biosurfactants are a structurally various group of surface active molecules which are synthesized by microorganisms and have recently become an important product of biotechnology for medical applications (15-17). They have several advantages over artificial surfactants including low toxicity, intrinsic superior biodegradability, and ecological acceptability (17). Adsorption of biosurfactants to a substratum surface changes its hydrophobicity and thereby interferes in the microbial adsorption and desorption processes (18); for this reason, the release of biosurfactants by probiotic bacteria within a living organism can be considered as a defence mechanism against other colonizing strains especially in the urogenital and intestinal tracts (19) and on medical devices. Therefore, pretreatments by biosurfactants can be used as a preventive strategy

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to interrupt the onset of pathogenic biofilm formation on catheters and other medical insertional materials (18, 20, 21).

The aim of this study was to determine the antiadhesive capability of the biosurfactant produced by *Lactobacillus acidophilus* ATCC 4356, against pathogenic, biofilm- producing strains of *Serratia marcescens.*

Materials and Methods

Bacterial strains and culture conditions

Lactobacillus acidophilus ATCC 4356, *Serratia marcescens* ATCC 13880 (biofilm producer) and *S. marcescens* ATCC 19180 (biofilm producer) were purchased from the collection center of bacteria and fungi in Iranian Research Organization for Science and Technology (IROST). Other strains of *S. marcescens* were isolated from patients in Namazi Hospital (Tehran, Iran) with high ability of biofilm formation (biofilm formation of *S. marcescens* strains was quantified by the crystal violet method) (22). *S. marcescens* strains were grown in Nutrient agar (NA, Darmstadt, Merck, Germany) and incubated at 37 ˚C for 24 hr. The identification of strains was done with the usual biochemical tests (SIM, TSI, Gellatinase test) (23). *L. acidophilus* ATCC 4356 as a probiotic source was cultured in de Man, Rogosa, Sharpe Broth or agar (MRSB or MRSA, Darmstadt, Merck, Germany) and incubated at 37 ˚C in an anaerobic jar for 24 hr.

Biosurfactant production

15 ml of *L. acidophilus* cultured overnight was inoculated into 600-ml of MRS broth and incubated for 24 hr at 37 ˚C. The cells were harvested by centrifugation at 10,000×g for 5 min at 10 ˚C, washed twice in demineralized water, and resuspended in 100 ml of PBS. The lactobacilli were incubated at room temperature for 2 hr with gentle stirring for biosurfactant production. Subsequently, the bacteria were removed by centrifugation, and the remaining supernatant liquid was filtered through a 0.22 mm-pore-size filter (Millipore). Aliquots (10 ml) of the supernatant were used immediately in the adhesion assay. The remainder was dialyzed against demineralized water using 6,000 kDa dialysis tubing (Sigma, St. Louis, Missouri, USA) for 48 hr at 4 ˚C, and was freeze- dried as described by Velraed *et al* (24).

Drop-collapse method

In order to test whether produced biosurfactant was able to decrease the surface tension between water and hydrophobic surfaces, the ability to collapse a droplet of water was tested as follows: 25 µl of extracted biosurfactant was pipetted as a droplet onto parafilm; the flattening of the droplet and the spreading of the droplet on the parafilm surface was followed over seconds or minutes. Then, methylene blue (with no influence on the shape of the droplets) was added to the water spot for photographic purposes. The droplet was allowed to dry and the diameter of the dried droplet was recorded by ruler (25, 26).

Fourier transform infrared spectroscopy

Freeze-dried biosurfactants (2 mg) were ground with 100 mg KBr and compressed by 7,500 kg for 3 min to obtain translucent pellets. Infrared absorption spectra were recorded by Bruker Tensor 27 instrument. KBr pellet was used for background correction. The quantity of a spectral region of interest was determined by normalization of the area under the absorption bands relative to the area of the CH absorption band around 2,930 cm^{-1} (24, 27).

Molecular weight determination by SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). Gel electrophoresis of biosurfactant was carried out using 12% (w/v) resolving gel and 4% stacking gel and run at a constant 150 V for about 240 min. The molecular weight was determined by comparison with the protein ladder (Prestained protein ladder, Tris-Glysine 4-20%, CinnaGen, Iran) after staining with Coomassie blue G250 (Merck, Germany) (28).

Biofilm formation assay

In order to generate *S. marcescens* biofilms on glass slide, 10 µl of *S. marcescens* (ATCC 13880) overnight culture (10^8 CFU/ml) was inoculated into microtiter wells containing 1 ml of sterile MBD medium and two slides with and without *L. acidophilus*–derived biosurfactant (2.5 mg/ml). The glass slides were washed in detergent solution, rinsed in distilled water twice, then air dried and autoclaved before use. The microtiter plate was incubated in an orbital incubator (100 rpm) at 37 ˚C for 18-20 hr. The glass slides were removed and rinsed twice with 1 ml of the PBS solution in order to remove unattached cells (29).Removed glass slides were fixed in 2% (w/v) glutaraldehyde for 2 hr at 4 ˚C, washed with saline solution, and dehydrated for 5 min in increasing ethanol concentrations (30%, 50%, 70%, and 90% [v/v]) followed by 15 min incubation in absolute ethanol. Samples then were coated with gold in argon atmosphere. The scanning electron microscopy (SEM) observations were carried out using a scanning device (Vega3 Tescan, USA) (30).

Biofilm inhibition assay

Biofilm inhibition assays with the extracted *L. acidophilus* ATCC 4356 biosurfactant were carried out in pre-coating and co-incubation experiments. Briefly, in pre-coating experiments (12), flat-bottomed polystyrene 96-well microtiter plates were filled with 200 µl of different concentrations of

L. acidophilus ATCC 4356 biosurfactant (ranging from 2.5 mg/ml to 0.312 mg/ml) and incubated for 24 hr at 37 ˚C at 130 rpm. Control wells containing sterile water only were treated in the same way. Biosurfactant solutions were then removed and the wells carefully washed twice with phosphate buffer saline (PBS) pH 7.2 to remove non-adhering biosurfactant. Aliquots of 150 µl of each *S. marcescens* suspension in the MBD medium at the concentration of 1×10^7 CFU/ml were then added to each well and the plates were incubated at 37 ˚C for 3 hr at 75 rpm. After this time, non-adherent cells were removed by gently washing twice the wells with PBS. 150 µl of fresh MBD medium were added to each well after which plates were incubated again at 37 ˚C for 48 hr at 75 rpm. In co-incubation experiments, aliquots of 150 µl of each *S. marcescens* suspension at the concentration of 1×10^7 CFU/ml were added to microtiter wells together with different concentrations of the extracted biosurfactant, ranging from 2.5 mg/ml to 0.312 mg/ml (from 0.5 mg/well to 0.0624 mg/well) and incubated for 3 hr as previously described. After this stage, procedures were exactly the same as the pre-coating experiments in which each well was filled with fresh MBD medium without different biosurfactant concentrations (31). Finally biofilm production by *S. marcescens* strains was quantified by crystal violet method (22). The microbial inhibition percentages at different biosurfactant concentrations for each micro-organism were calculated as:

$$\% \text{ Microbial inhibition}_c = [1 - (A_c/A_0)] \times 100$$

Where A_c represents the absorbance of the well with a biosurfactant concentration c and A_0 the absorbance of the control well. The microtitre-plate anti-adhesion assay estimates the percentage of microbial adhesion reduction versus the control wells, which were set at 0% to indicate the absence of biosurfactant and therefore its anti-adhesion properties. In contrast, negative percentage results indicate the percentage increase in microbial adhesion at a given surfactant concentration in relation to the control (32). The microtitre-plate anti-adhesion assay allows the estimation of the crude biosurfactant concentrations that are effective in decreasing adhesion of the microorganisms studied.

Statistical analysis

Experiments were conducted in triplicate. The results are presented as means±SD. Statistical analysis was conducted using SPSS version 20. After assumptions of normality and variances of homogeneity were checked one way analysis of variance (ANOVA), Kruskal-Wallis test and paired sample t-test were also performed. The significance level was set at $P<0.05$.

Results

Drop collapse assay

Drop collapse method is a sensitive and easy to perform method which requires a small volume (~5 µl) of broth culture or biosurfactant solution to test the surfactant property. According to the results of this method, no activity was detected in distilled water as predicted. The biosurfactant was able to collapse a droplet of water (Figure 1), representing their effects on reduction of surface tension.

Fourier transform infrared spectroscopy

The molecular composition of the biosurfactant used in this study was evaluated by Fourier transform infrared spectroscopy (Figure 2). The most important bands were located at 2'929 cm^{-1} (CH band: CH2- CH3 stretching), 1'655 cm^{-1} (AmI band: CAO stretching in proteins), 1'402 cm^{-1} (AmII band: NOH bending in proteins), 1'260 cm^{-1} (PI band: phosphates), and 1'056 cm^{-1} (PII band: polysaccharides). Therefore biosurfactant of *L. acidophilus* ATCC 4356 appeared to be mostly protein.

Figure 1. Drop collapse assay. Collapsed droplets (A) is H_2O and (B) is *Lactobacillus acidophilus* ATCC 4356-derived biosurfactant

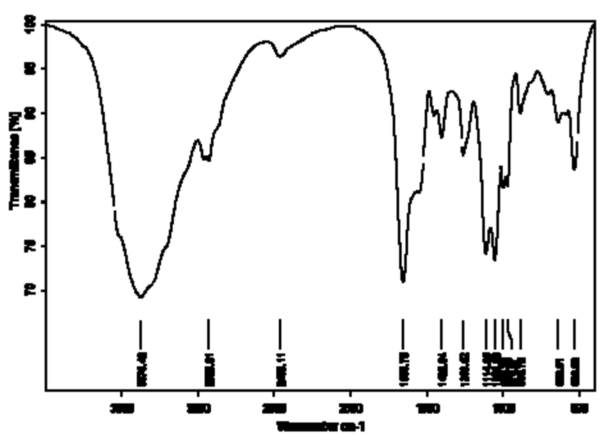

Figure 2. Fourier transform infrared absorption spectra of the freeze-dried biosurfactant released from *Lactobacillus acidophilus* ATCC 4356

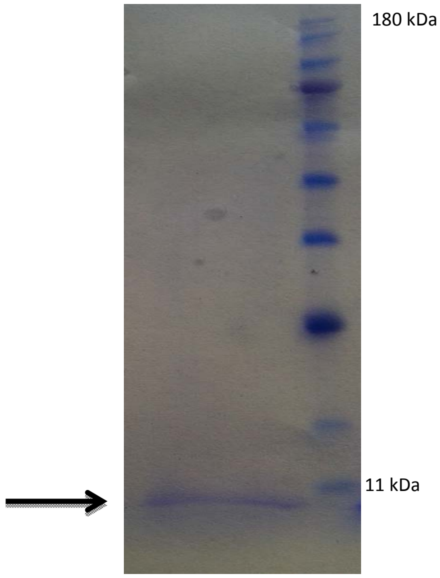

Figure 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profile analysis of *Lactobacillus acidophilus* ATCC 4356 extracted biosurfactant

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The freeze-dried biosurfactant released from *L. acidophilus* ATCC 4356 was analyzed using SDS-PAGE. Protein profile showed one band with approximate size of 10 kDa (Figure 3).

Biofilm formation

As shown in Figure 4, the presence of 2.5mg/ml *L. acidophilus* ATCC 4356- derived biosurfactant, dramatically reduced the process of *S. marcescens* ATCC 13880 attachment and biofilm production.

Effect of L. acidophilus ATCC 4356 biosurfactant on biofilm formation of S. marcescens strains

The effect of pre-coating of *L. acidophilus* biosurfactant on biofilm formation of *S. marcescens* isolates is shown in Table 1. The concentration of 2.5 mg/ml of biosurfactant significantly reduced the percentage of cell adhesion against all of the isolated strains tested (P<0.05) except for *S. marcescens* (1) (P=0.128), *S. marcescens* (2) (P=0.496), and *S. marcescens* (5) (P=0.57), while 1.25 mg/ml biosurfactant significantly reduced *S. marcescens* ATCC 13880 and ATCC 19180 cell adhesion (P=0.00, P=0.002 respectively). Co-incubation results of biosurfactant are shown in Table 2. The concentration of 2.5 mg/ml of biosurfactant significantly reduced the percentage of cell adhesion of all isolated strains tested (P<0.05). While concentration of 1.25 mg/ml of biosurfactant significantly affected cell adhesion of *S. marcescens* ATCC 13880 and ATCC 19180 (P=0.005, P=0.000 respectively). There is no statistically significant difference between the two methods, pre-coating and co-incubation. The anti-adhesive effect depends on the concentration; by decreasing concentration, the anti-adhesive activity is noticeably reduced.

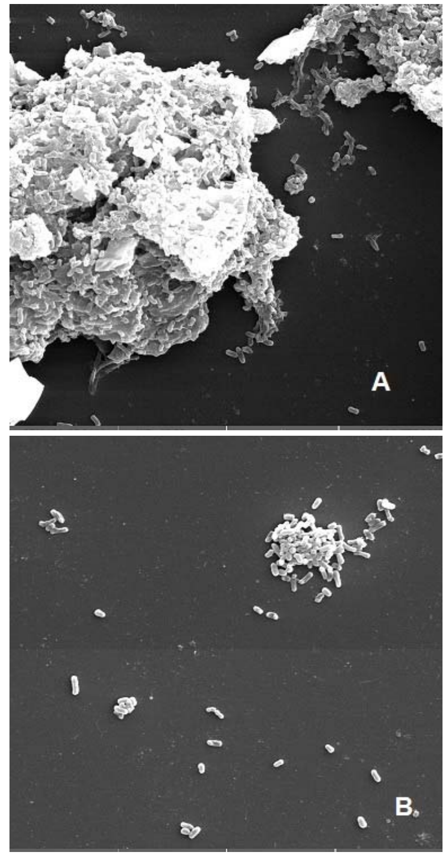

Figure 4. The scanning electron microscopy (SEM) of *Serratia marcescens* ATCC 13880. (A) control group (in the absence of biosurfactant). (B) experimental group (in the presence 2.5 mg/ml of *Lactobacillus acidophilus* ATCC 4356-derived biosurfactant)

Discussion

Serratia infections are in general nosocomial, affecting compromised patients who receive broad-spectrum antibiotic therapy and often with indwelling urinary catheters, endotracheal tubes or other foreign bodies. New evidence from ophthalmic infections, however, indicates that even healthy contact-lens wearers may be at risk of serratia keratitis (33). One of the main problems associated with *S. marcescens* infection is increase of resistance against a great number of antibiotics through biofilms formation (34). Increasing problems of resistance to synthetic antimicrobials have encouraged the researchers to focus on alternative natural products such as probiotic bacteria. Some microorganisms such as lactic acid bacteria were found to be biosurfactant- producing strains. One of the major roles known for biosurfactants is their negative effect on other microbial species (13, 22, 25).

Table 1. Percentage of anti-adhesive properties after *Lactobacillus acidophilus* ATCC 4356 biosurfactant pre-coating at different concentrations (mg/ml). Negative controls were set at 0% to indicate the absence of biosurfactant. Percentages indicate the reductions in microbial adhesion when compared to the control. Results are expressed as means±standard deviation of values obtained from triplicate experiments

Microorganism (isolate number)	[Biosurfactant] (mg/ml)			
	2.5	1.25	0.625	0.312
S. marcescens (1)	55.2 ± 0.59	49.1 ± 0.16	42.4 ± 0.51	36.9 ± 0.66
S. marcescens (2)	43.9 ± 0.36	31.1 ± 0.72	29.98 ± 0.82	19.44 ± 1.02
S. marcescens (3)	39.3 ± 1.34	32.3 ± 0.61	31.8 ± 0.74	23.5 ± 1.02
S. marcescens (4)	40.3 ± 0.16	35.8 ± 0.81	29.7 ± 0.39	30.7 ± 0.74
S. marcescens (5)	24.1 ± 0.35	16.8 ± 0.55	18.9 ± 0.79	13.2 ± 0.55
S. marcescens (6)	51.1 ± 0.21	56.0 ± 0.79	56.1 ± 0.28	54.1 ± 0.27
S. marcescens ATCC 13880	49.5 ± 0.21	35.27 ± 0.79	0.01 ± 0.27	0 ± 0.28
S. marcescens ATCC 19180	60.0 ± 1.77	50.4 ± 0.59	17.1 ± 1.29	8.74 ± 0.03

On the basis of the results, we conclude that the biosurfactant from *L. acidophilus* has a relatively high protein content compared to other components such as polysaccharides and phosphates. According to Figures 4-A and 4-B, it is also shown the adhesion of *S. marcescens* to glass slide could reduce by biosurfactants. Velraeds *et al* (24) demonstrated that, biosurfactants from *L. acidophilus* RC14 and *L. fermentum* B54 were richer in protein and also had less polysaccharides than biosurfactants from *L. casei* subsp. *rhamnosus* ATCC 7469.

Inhibitory effect of biosurfactants on bacterial adhesion and biofilm formation has also been previously reported (27). However, the definitive mechanisms of such effects have not yet been described in detail. The mechanism appears to be exceedingly dependent on biosurfactant type and the properties of the target bacteria. The common technique to explain biosurfactant anti-adhesion and anti-biofilm activities would be their direct antimicrobial activity. However, the antimicrobial activity of biosurfactants has not been observed in all cases (27, 35). Walencka *et al* (35) reported that the way in which surfactants influenced bacterial surface interactions appeared to be related to the surface tension changes and bacterial cell-wall charge. These factors are very important in overwhelming the initial electrostatic repulsion barrier between the microorganism cell surface and its substrate. Biosurfactants may also affect both cell-cell and cell-surface interactions. The results indicate that lactobacilli-derived agents have significant effects on these interactions (22, 35).

In this study, the anti-adhesive activity of *L. acidophilus*–derived biosurfactant against strains of *S. marcescens* was investigated. Particularly, in co-incubation experiments, the percentage of cell adhesion of *S. marcescens* 1 was reduced by 73% at

Table 2. Percentage of anti-adhesive properties after *Lactobacillus acidophilus* ATCC 4356 biosurfactant co-incubation at different concentrations (mg/ml). Negative controls were set at 0% to indicate the absence of biosurfactant. Percentages indicate the reductions in microbial adhesion when compared to the control. Results are expressed as means ± standard deviation of values obtained from triplicate experiments

Micro-organism (isolate number)	[Biosurfactant] (mg/ml)			
	2.5	1.25	0.625	0.312
S. marcescens (1)	73.4 ± 0.38	68.3 ± 0.60	66.9 ± 0.60	67.2 ± 0.56
S. marcescens (2)	58.4 ± 0.83	57.6 ± 0.76	49.0 ± 0.86	15.54± 0.94
S. marcescens (3)	45.8± 0.79	48.0± 1.84	33.3 ± 1.58	18.61± 2.09
S. marcescens (4)	59.48± 0.96	54.2± 0.97	55.31 ± 0.61	20.54± 0.85
S. marcescens (5)	60.8± 0.87	54.8 ± 0.71	48.28 ± 0.64	40.69 ± 0.31
S. marcescens (6)	57.0± 0.83	46.3 ± 1.59	60.0 ± 0.627	58.0± 0.37
S. marcescens ATCC 13880	47.46 ± 0.76	33.24 ± 0.87	7.96 ± 0.98	2.24± 1.45
S. marcescens ATCC 19180	52.0 ± 2.13	46.55± 0.12	26.15 ± 0.38	0.66± 0.35

the concentration of 2.5 mg/ml and the percentage of cell adhesion of all other strains was reduced at the concentration of 2.5 mg/ml. These results look very encouraging since to the best of our knowledge, this is the first time that a lactobacilli biosurfactant displays such a high anti-adhesive activity against *S. marcescens* biofilm formation. Anti-adhesive activity of biosurfactant produced by lactobacilli has been also described against biofilm formation of bacterial pathogens by prerequisite materials used in the urogenital tract or the oral cavity, glass or plastic (24, 35). Results obtained from this study also indicates the efficacy of *L. acidophilus* biosurfactant against biofilm formation of *S. marcescens* on polystyrene. These surfactants influence surface interactions of bacteria which appear to be more strictly related to modifications in bacterial cell-wall charge and surface tension (31, 35). In conclusion, the anti-adhesive properties of the biosurfactant against eight *S. marcescens* biofilm producers suggest its potential usage as an anti-adhesive product on medical devices (catheters, prosthesis) to prevent *S. marcescens* infections.

However, the biosurfactant isolated in this study exhibited a considerable anti-adhesive activity against most of the microorganisms tested. Biosurfactant can involvement in microbial adhesion and desorption has been widely described, and adsorption of biosurfactants isolated from lactobacilli to surfaces might constitute an effective strategy to reduce microbial adhesion and conflicting colonization by pathogenic bacteria, in the biomedical field or in the food industry (18, 20, 21, 36).

The anti-adhesive activity observed with this biosurfactant on micro-organisms such as *S. marcescens* is very promising for additional studies and therapeutic applications targeted at reducing microbial colonization on different material. These antimicrobial and anti-adhesive properties make biosurfactants appropriate therapeutic agents in the battle against many infections(18, 32). Falagas and Makris (20) have proposed the application of biosurfactants, isolated from probiotic bacteria, to patient-care equipments in hospitals, to reduce hospital-acquired infections.

Conclusion

In this work we have demonstrated the anti-adhesive properties of the crude biosurfactant isolated from *L. acidophillus* against pathogenic microorganisms, including bacteria. The results obtained suggest the possible use of this biosurfactant as an antimicrobial agent with applications against microorganisms responsible for diseases and infections in the vaginal, urinary and alimentary tract, in addition to the skin, making it a suitable alternative to conventional antibiotics.

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