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ANTIBACTERIAL AND ANTIBIOFILM EFFECT OF BIOSURFACTANT PRODUCED FROM *LEUCONOSTOC MESENTEROIDES* SSP. *CREMORIS* AGAINST BACTERIA FROM CATHETERS

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ABSTRACT

In this study, production of biosurfactant by *Leuconostoc mesenteroides* ssp. *cremoris* isolated from raw cow's milk was studied. Extraction of extracellular and cell-bound biosufactant was completed and partially purified by cold acetone precipitation. The weight of them was determined and the surface activity of each of them was studied. The results showed that extracellular biosurfactant reached to 10.8 gram /1.2 dry cells/ liter and 0.34 gram /1.2 dry cells /liter for cell- bound biosurfactant. Partial purified extracellular and cell-bound biosurfactant showed surface activity with oil displacement diameter 10mm. Antibacterial and antibiofilm effect of partial purified biosurfactant were evaluated against bacterial isolates including *Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus epidermidis* and *Staphylococcus aureus* isolated from catheters. Partial purified biosurfactant had inhibitory effect with MIC at concentration (256) mg\ml against *P. aeruginosa* and *S. epidermidis,* (64) mg\ml against *S.aureus* and (32) mg\ml against each of *P. mirabilis* and *Escherichia coli* and showed inhibitory effect on biofilm formation of these bacteria in Co - incubation and Pre – coated methods with inhibition percentage ranged between (20-92)%. Antibiofilm effect of partial purified biosurfactant on coated catheters was observed against pathogenic bacteria, partial purified biosurfactant recorded maximum biofilm inhibition 54 % against *E.coli* followed by 43% occurred against *P. aeruginosa*. While inhibition on biofilm formation of *p. mirabilis, S. aureus* and *S. epidermidis* was observed with inhibition percentage (3, 38, 35) % respectively.

KEYWORDS: Biosurfactant, *Leuconostoc*, Antibacterial, Antibiofilm, Catheters**.**

INTRODUCTION

Biosurfactants are amphiphilic compounds produced by microbes, either on the cell surface or secreted extracellularly, they have antimicrobial and anti-adhesive properties (Sambanthamoorthy *et al*., 2014) also they have antifungal and antiviral activity (Zhoa *et al*., 2010). They are structurally diverse group of surface active molecules which contained hydrophobic and hydrophilic moieties in the same molecules that accumulate at the interface between fluid phases that show different degrees of polarity and hydrogen bonding, such as oil and water or air (Salleh *et al*., 2011), and thus reducing the surface and interfacial tension (Pereira *et al*., 2013). Biosurfactants can be classified according to their chemical structure and microbial origin. They are classified into two categories by Rosenberg and Ron (1999) include high molecular weight and low molecular weight molecules. High molecular weight molecules include polymeric and particulate surfactants, whereas low molecular weight molecules include glycolipids, lipopeptides and phospholipids (Shoeb *et al*., 2013). They have become an important product of biotechnology for industrial, agricultural, pharmaceutical, cosmetics, detergent, petrochemical, bioremediation and biomedical application (Abdel-Mawgoud *et al*., 2010; Kalyani *et al*., 2011; Henkel *et al*., 2012).

Biosurfactant in pharmaceutical fields used as an agents for stimulating stem fibroblast metabolism (Shoeb *et al*., 2013) and can be a viewed as potential cancer therapeutics (Gudiña *et al.*, 2013). Biosurfactant have the potential to be used as anti-adhesive biological coatings for medical insertional materials, also they can be used as a preventive strategy to delay the onset of pathogenic biofilm growth on catheters and other medical insertional materials, thus reducing hospital infections and use of synthetic drugs and chemicals (Rodrigues *et al.*, 2006a; Gudina *et al.*, 2010a).

The objective of this study was to determine the antibacterial and antibiofilm effect of a biosurfactant produced from *Leuconostoc mesenteroides ssp. Cremoris* against some bacteria isolated from catheters and coating catheters by biosurfactant as a preventive agent.

MATERIALS AND METHODS

Microorganisims

Isolate of *Leuconostoc mesenteroides ssp. cremoris* was isolated from raw cow's milk, then identified through out cultural, microscopical and biochemical test according to Garvie (1986) and Forbes *et al*. (2002) and Vitek 2 system.

Five isolates were used to test the antibacterial and antibiofilm properties of the biosurfactant produced by *L. mesenteroides ssp.cremoris,* including *Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, Staphylococcus epidermidis* and *Staphylococcus aureus,* these isolates were isolated from catheter samples of men suffering from Urinary tract infection, then identified through out cultural, microscopical, biochemical test according to the criteria established by Forbes *et al*.(2002) and Vitek 2 system. Morover, these isolates were tested for susceptibility to antibiotics and for ability to biofilm formation .

Biosurfactant production by *L. mesenteroides ssp. cremoris*

Detection of biosurfactant production

Blood haemolysis test

Briefly, *Leuconostoc mesenteroides ssp. cremoris* isolate was streaked on toblood agar plates and incubated for 24h at 30◦C. The plates were visually inspected for zones of clearing around colonies. This clear zone indicates the presence of biosurfactant (Rodrigues *et al*., 2006b and Ali *et al*., 2013).

Surface activity test

Leuconostoc mesenteroides ssp. Cremoris isolate was cultivated in De-Man Regosa Sharpe (MRS) broth at 30°C for 24h, the cultures broth were centrifuged at 6,000×g for 20 min at 4°C, the supernatant was collected. To exclude that the biosurfactant was adherent to the bacterial cell wall, bacteria separated from the supernatant were washed three times, re-suspended in 500 µL of saline and tested by means of the oil spreading assay by using 20 µl of motor oil previously deposited on to the surface of 20 mL of distilled water in a Petri dish (90 mm in diameter) to form a thin membrane. Twenty microliters of bacterial supernatant was gently put on to the center of the oil membrane. Diameters of clearly formed oil displaced circle were measured (Fracchia *et al*., 2010).

Extraction of extracellular biosurfactant

For crude biosurfactant production by *Leuconostoc mesenteroides ssp. cremoris*, 600 ml of culture broth were inoculated with 12ml of an overnight subculture and incubated for 24 h at 30◦C in aerobic condition. Briefly, culture broth was centrifuged at 10000rpm for 10 min at 4◦C, the supernatant was filtered through a Millipore filter (Rodrigues *et al*. 2006a).

Extraction of cell bound biosurfactant

For cell- bound biosurfactant determination, cells were harvested by centrifugation at 10000 rpm for 10 min at 4◦C, washed twice in demineralized water and resuspended in 100 ml of phosphate-buffered saline (PBS). Crude cell-bound biosurfactant extract was produced by gently stirring this suspension for 2 h at room temperature, then the bacteria were removed by centrifugation and the remaining supernatant was filtered through a Millipore filter. The supernatant was dialyzed against demineralized water for 24h at 4◦C using a Cellu-Sep©membrane (molecular weight cut-off 6000–8000 Da) (Rodrigues *et al*., 2006c).

Biomass estimation

The cell pellet which obtained after centrifugation in biosurfactant extraction step was washed, resuspended in pre-sterilized distilled water and centrifuged again. The cell pellet was then desiccated in an electric oven at 105◦**C** until a constant weight was achieved (Raza *et al*., 2006).

Partial purification of biosurfactant

Extracellular and cell-bound biosurfactant was partially purified by cold acetone precipitation. Three volumes of chilled acetone was added to the crude biosurfactant solution and allowed to stand for (15-20) h at 4°C. The precipitate was collected by centrifugation at 10,000rpm for 30 min and the resulting pellet served as partially purified biosurfactant it was further allowed to be evaporate to dryness to remove residual acetone after which it was dissolved in sterile water (Ilori *et al.*, 2005 with some modification).

Surface activity of partial purified extracellular and cell- bound biosurfactant

Surface activity of partial purified extracellular and cell- bound biosurfactant at a concentration of 100 mg/ml was measured by the oil spreading assay.

Antibacterial activity of biosurfactant

Antbacterial activity of partial purified extracellular biosurfactant was determined on the basis of minimum inhibitory concentration (MIC) values, defined as the lowest concentrationn of biosurfactants at which no visible growth could be observed after incubation for the required time . MIC was determined for bacteria isolated from catheters which include *E. coli, P. mirabilis, P. aeruginosa, S. epidermidis* and *S. aureus* by broth dilution method as described by Morello *et al*., (2003).

Briefly, a stock solution of partial purified extracellular biosurfactant from *L. mesenteroides ssp cremoris* in Muller Hinton broth were diluted to concentrations ranging 4 to 256 mg/ml.

Antibiofilm activity of biosurfactant

Co-incubation Method

The antibiofilm activity of partial purified extracellular biosurfactant isolated from *L. mesenteroides ssp. cremoris* against *E. coli, P. mirabilis, P. aeruginosa, S. epidermidis* and *S. aureus* were quantified by co-incubation experiments according to the procedure described by Ali (2012). Each of bacterial suspensions in brain heart infusion broth with 2% sucrose (100 µL) were added to 96- well flat bottomed microtiter plates together with (100 µL) subMIC of partial purified extracellular biosurfactant, control wells contained 180 µL of brain heart infusion broth with 2% sucrose and 20 µL of bacterial suspensions, The covered microtiter plate was sealed with Parafilm during incubation at 37°C for 24h. Unattached bacterial cells were removed by washing the wells three times with distilled water. After drying at room temperature for 15 min, 200 µL of crystal violet was added to the wells for 20 min. The stained attached bacterial cells were rinsed three times with distilled water, allowed to dry at room temperature for 15 min, then added 200 µL of 95% ethanol, and the absorbance of each well was measured at 630 nm using ELISA Reader, The inhibition of biofilm percentages of the partial purified extracellular biosurfactant for each pathogenic bacteria were calculated as equation described by Gudina *et al.* (2010a).

$$\% \text{ Inhibition of biofilm} = [1- (\frac{A}{Ao} 100 \times])$$

A. represents the absorbance of the well with a biosurfactant and A_0 the absorbance of the control well.

Pre – Coated Method

In pre-coating experiments 96-well microtiter plates were filled with 200 µL of sub MIC of partial purified extracellular biosurfactant. The covered microtiter plate was sealed with Parafilm during incubation at 37°C for 24h. Biosurfactant solutions were then removed and the plates carefully washed with distilled water and left drying at room temperature for 15 min, 200 µL of bacterial suspensions was added to the wells, microtiter plate covered with Parafilm and incubated at 37°C for 24h. Unattached bacterial cells were removed by washing the wells three times with distilled water, after drying at room temperature for 15 min, the same procedure that was used in the co-incubation method was used by precoating experiment.

Antibiofilm activity of biosurfactant on catheter

Coating of biosurfactant on catheter

The collected catheter was cut in to 1.5cm pieces and sterilized. The cut pieces of the catheter completely immersed in sub MIC of partial purified extracellular biosurfactant suspension and kept in 37ºCfor 24 h. Placed on blotting paper to remove excess suspension and allowed to dry at 40ºc.

Biofilm inhibition assay

The biofilm inhibition activity of partial purified extracellular biosurfactant was evaluated by coating catheter according to the procedure described by Namasivayam *et al.* (2012) with modification. The biosurfactant coated catheter pieces and control (non coated catheter pieces) were immersed in 10ml of 24h bacterial culture, incubated at 37ºc for 24h. After incubation period all catheter pieces (treated and control catheter) was stained with 0.1%weight by volume of crystal violet solution for 30min at room temperature, after staining the catheter was washed with 95% of ethanol for 3 times at room temperature, the washed solution was collected and read spectrophotometrically at 630nm. The percentage of biofilm inhibition was calculated as equation described above.

RESULT AND DISCUSSION

Biosurfactant production by *L. mesenteroides ssp. cremoris*

The tested *Leuconostoc mesenteroides ssp. cremoris* showed zones of clearing in the blood agar with a diameter 18mm, this result indicated *L. mesenteroides ssp cremoris* had ability to produce biosurfactants.

Hemolytic activity has been used for the determination and isolation of strains that produce biosurfactant and the size of the clear zone developed is in proportion to the amount of the produced biosurfactant. (Ghribi *et al.*, 2012), Anadaraj and Thivakaran, (2010) showed that the culture producing beta haemolysis was able to produce biosurfactants.

L. mesenteroides ssp cremoris isolate showed surface activity with oil displacement in diameter 7mm for both extracellular and cell- bound biosurfactant. The diameter of this clearing zone on the oil surface correlates to biosurfactant activity (Walter *et al*., 2010). Youssef *et al*. (2004) and Plaza *et al*. (2006) demonstrated that the oil spreading technique is a reliable method to detect biosurfactant production by diverse microorganisms.

Acetone is a good purifying agent for proteins (Nadrrmullah and Mukhtar, 2013) and for biosurfactant (Satpute *et al.*, 2010). The recovery of biosurfactant was maximum by using acetone as solvent in comparison to other solvent (Ray, 2012).

Extracellular biosurfactant production yield from *L. mesenteroides ssp. Cremoris* was achieved 10.8 g/L of medium and 0.34g/L for cell-bound biosurfactant and biomass concentration of 1.2 g/L of medium.

Lactic acid bacteria have ability to produce biosurfactant as metabolic products and in different concentration (Rodrigues *et al*., 2006d).

Gudina *et al*., (2010b) showed that Lactobacilli are produced biosurfactant in lower amounts (20–100 mg/L). Salman and Alimer (2014) observed biosurfactant production yield from *Lactobacillus rhamnosus* was achieved 48mg/L of medium, and biomass concentration of 1.5 g/L of medium.

Antibacterial activity of biosurfactant

The antibacterial activity of the partial purified extracellular biosurfactant isolated from *L. mesenteroides ssp cremoris* was determined by measuring the growth obtained for some

bacteria isolated from catheter. From those results, the minimum concentration (MIC) of the partial purified extracellular biosurfactant was found to be 256 mg/ml against *S. epidermidis* and *P. aeruginosa*, 64 mg/ml against *S.aureus* and 32 mg /ml against *E.coli* and *P. mirabilis*. The biosurfactants produced by *Streptococcus thermophilus* and *L. lactis* showed significant antimicrobial activity against several bacterial and yeast strains isolated from explanted voice prostheses (Rodrigues *et al.*, 2004, Rodrigues *et al*., 2006b). Salman *et al.*, (2013) observed that the biosurfactant isolated from *S. thermophilus* showed inhibitory effect against *E.coli, Klebsiella* spp., *P. aeruginosa* and *S.aureus*. Another study, biosurfactant produced from *L.rhamnosus* showed inhibitory effect against UTI causative bacteria *S. aureus, K. pneumoniae, Burkholderia cepacia,* and *E.coli* (Salman and Alimer, 2014). The mechanism of antimicrobial action of biosurfactant regards to the fact that biosurfactants may disturb membrane structure through interaction with phospholipids as well as membrane proteins (Lotfabad *et al.,* 2013). Another explanation of the antimicrobial effect of biosurfactants is the adhering property of biosurfactants to cell surfaces caused deterioration in the integrity of cell membrane and also breakdown in the nutrition cycle. Also the biosurfactant prevent the protein synthesis by inhibition of the peptidyltransferase in binding mainly the 23S rRNA in the 50S subunit of the bacterial ribosome (Gomaa *et al*., 2012).

Antibiofilm activity of biosurfactant

Antibiofilm activity of the partial purified extracellular biosurfactant was evaluated against bacteria isolated from catheters, using Co-incubation Method, The extracellular biosurfactant that obtained from *L. mesenteroides ssp cremoris* showed antibiofilm activity against all bacteria, the highest inhibition percentage was observed for *P. aeruginosa* 92%, followed by *E. coli* and *S. aureus* with inhibition percentage (87, 79)% respectively, while (36, 25)% was obtained for *S. epidermidis* and *P. mirabilis* respectively (Table 1).

Table1: Antibiofilm activity of partial purified extracellular biosurfactant isolated from *Leuconostoc mesenteroides ssp cremoris* (Co-incubation method).

Pathogenic bacteria	(O.D)		Inhibition percentage (%)
	Partial purified biosurfactant	Control	
Pseudomonas aeruginosa	**0.091**	**1.136**	**92**
Staphylococcus aureus	**0.121**	**0.560**	**79**
Staphylococcus epidermidis	**0.088**	**0.137**	**36**
Escherichia coli	**0.134**	**0.971**	**87**
Proteus mirabilis	**0.108**	**0.144**	**25**

Pre – coated method showed that biosurfactant had antibifilm effect with inhibition percentage (88, 71, 69, 62, 20) % against *P. aeruginosa, S. aureus, S. epidermidis, E. coli* and *P. mirabilis* respectively (Table 2).

Table2: Antibiofilm activity of the partial purified extracellular biosurfactant isolated from *Leuconostoc mesenteroides ssp. cremoris* (Pre – coated method).

Pathogenic bacteria	(O.D)		Inhibition percentage (%)
	Partial purified biosurfactant	Control	
Staphylococcus epidermidis	**0.243**	**0.760**	**69**
Proteus mirabilis	**0.216**	**0.268**	**20**
Staphylococcus aureus	**0.259**	**0.874**	**71**
Escherichia coli	**0.137**	**0.359**	**62**
Pseudomonas aeruginosa	**0.215**	**1.671**	**88**

Rivardo *et al*. (2009) mentioned that both methods Co-incubation and Pre – coated give positive result for inhibition of biofilm formation and also reported that the biosurfactant obtained from *Bacillus* spp. have antibiofilm activity against *S. aureus* and *E.coli*. Biosurfactants synthesized by *Lactobacillus* had inhibition activity on biofilm formation for *E coli*, *S.* aureus, *Salmonella arizonae* and *Listeria monocytogenus* (Fracchia *et al*., 2010). Ali, (2012) was demonstrated that the biosurfactant isolated from *L.acidophilus* inhibit biofilm formation of *Proteus mirabilis*. Salman and Alimer (2014) showed that partial purified biosurfactant isolated from *L.rhamnosus* had antibiofilm activity against UTI causative bacteria.

One mechanism that could explain this global inhibition of pathogenic adherence by biosurfactants, the Biosurfactants are amphipathic molecules that have a variety of purposes, including adsorption to surfaces (Spurbeck and Arvidson, 2010). Rivardo *et al.* (2009) demonstrated that biosurfactant were able to inhibit biofilm formation when used to coat surfaces before treated with the pathogenic bacteria.

Antibiofilm activity of biosurfactant on catheter

Anti biofilm effect of the partial purified extracellular biosurfactant from *L. mesenteroides ssp. cremoris* on coated catheter was observed against all bacterial isolates that isolated from catheters, biosurfactant recorded maximum biofilm inhibition 54% against *E.coli*, followed by 43% occurred against *P. aeruginosa*, While inhibition effect on biofilm formation of *P. mirabilis*, *S. aureus* and *S. epidermidis* was (39, 38, 35) % respectively (Table 3). The pretreatment of polystyrene surfaces and other medical instrument with pseudofactin II

obtained from *P. fluorescens* significantly decreased the adhesion of bacteria and yeast which include *E. coli, E. faecalis, S. epidermidis, P. mirabilis, C. albicans* (Janek *et al*., 2012). Biosurfactants produced by *Streptococcus thermophilus* and *L. lactis* showed inhibition activity on biofilm formation of pathogenic bacteria on voice prostheses (Rodrigues *et al.*, 2004). Salman *et al.* (2014) demonstrated that biosurfactant- PVA mixture had inhibitory effect on biofilm formation of pathogenic bacteria in glass and plastic materials.

Table 3: Antibiofilm activity of the partial purified extracellular biosurfactant isolated from *Leuconostoc mesenteroides ssp cremoris* against pathogenic bacteria on catheter.

Pathogenic bacteria	(O.D)		Inhibition percentages (%)
	Partial purified biosurfactant	Control	
Staphylococcus epidermidis	**0.087**	**0.133**	**35**
Staphylococcus aureus	**0.078**	**0.125**	**38**
Proteus mirabilis	**0.076**	**0.123**	**39**
Escherichia coli	**0.054**	**0.117**	**54**
Pseudomonas aeruginosa	**0.065**	**0.113**	**43**

CONCLUSION

In conclusion, the *Leuconostoc mesenteroides ssp. cremoris* isolated from raw milk had ability to produce extracellular and cell-bound biosurfactant, the biosurfactant had antibacterial, antibiofilm properties against pathogenic bacteria isolated from catheters. Also had potential to be used as anti biofilm coating for catheters.

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