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Functional characterization of biomedical potential of biosurfactant produced by *Lactobacillus helveticus*

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ABSTRACT

Various lactic acid bacteria (LAB) have been isolated and screened for biosurfactant production and their biomedical and food applications. Additionally, various different concentrations of the biosurfactant (0.625–25 mg ml^{-1}) were used to evaluate its antimicrobial and antiadhesive potential against a range of pathogenic microorganisms. Biosurfactant was found to be stable to pH changes over a range of 4.0-12.0, being most effective at pH 7 and showed no apparent loss of surface tension and emulsification efficiency after heat treatment at 125 °C for 15 min. Present study demonstrated that biosurfactant obtained from *Lactobacillus helveticus* has the ability to counteract effectively the initial deposition of biofilm forming pathogens to silicone surfaces and to significantly sluggish biofilm growth.

1. Background

Biosurfactants are amphiphilic molecules with recognized surface and emulsifying properties. In general, biosurfactants are amphiphilic molecules, where the hydrophobic moiety is either a long-chain fatty acid, hydroxyl fatty acid, or α-alkyl-β-hydroxy fatty acid and the hydrophilic moiety can be a carbohydrate, an amino acid, a cyclic peptide, a phosphate, a carboxylic acid, or alcohol, among others [1]. Different groups of microbial surfactants exhibit different properties and displayed a range of physiological functions [2]. Microbial surfactants plays an important role in the solubility of water insoluble compounds, binding of heavy metal, bacterial pathogenesis, cell adhesion and aggregation, quorum sensing, production of antimicrobial and antibiofilm compounds [3,4]. Biosurfactants have been reported for their antibacterial, antifungal, antiviral and antiadhesive properties, which make them an alternative to conventional antibiotics against various food borne pathogens [5–7]. A number of studies have been reported the potential of lactobacilli as biosurfactant producers [5,8–17].

The attachment of bacteria to surfaces and the consequent biofilm formation has serious impacts in food, environmental and biomedical fields. The occurrence of biofilm in food processing environments can lead to spoilage and transmission of diseases representing a health risk to the consumers. There is enough evidence indicating that the biofilm mode of life leads to increased resistance to antimicrobial products [18–25]. Moreover, microbial species can become resistant to disinfectants, hence making difficult the cleaning of surfaces. Thus, controlling the microorganism's adherence is an essential step towards food safety assurance and developing new adhesion control strategies. The biosurfactant from the probiotic LAB has tremendous applications in these areas. Application of biosurfactants to a surface modifies its hydrophobicity, interfering in the microbial adhesion and desorption processes; in that sense, the production of biosurfactants by probiotic bacteria *in vivo* can be considered as a defense against other colonizing [27,28]. Biosurfactants produced by LAB impaired biofilm formations on silicone rubber and other biomedical instruments [9,10,15,26–33]. Evidence on the chemical composition of biosurfactants produced by LAB are inadequate. Biosurfactants produced by LAB have been composed of complex biological mixtures, but their structural composition has not been broadly studied [4,34]. Better information of biosurfactants structure is essential to understand their major components in order to expand their behavior.

The aim of the present study was to isolate, screen and characterize the functional characteristic of the biosurfactant produced by *Lactobacilli* from ethnic fermented food (Chhurpi cheese) for the production of biosurfactants. Characterization included the determination of surface tension reduction, critical micelle concentration and stability at different pH and temperature in conviction of change in surface tension and emulsification

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index. The antimicrobial and anti-adhesive activities of this biosurfactant were assayed against various pathogenic microorganisms. Antiadhesive potential was also assayed with silicon tube using co-incubation of tubes with biosurfactant.

2. Materials and methods

2.1. Sample collection and isolation of LAB

Samples of Yak milk cheese (Chhurpi cheese) were collected from local market and farmers of Bomdila (Coordinates: 27.25°N 92.4°E) Arunanchal Pradesh, North East Indian state) in presterilized containers. Biosurfactant producing LAB isolates were cultured by enrichment in 100 ml of sterile minimal media (MM) with 1% paraffin oil as carbon source. The suspension was incubated at 28 °C for 48 h. Inoculum from culture flask was sub cultured in deMan Rogosa and Sharpe (MRS) agar (Sarvanakumari and Mani, 2010). Isolates was stored at −20 °C in MRS broth (Himedia, India) containing 20% (v/v) glycerol stock until it was used in current study.

2.2. Screening for biosurfactant production

Pure culture of LAB isolates were used to screen biosurfactant production by hemolytic activity, oil displacement test, drop collapsing test, surface tension measurement, critical micelle concentration (CMC) and emulsification index [35,36]. Surface tensions of supernatants were measured by the De Nouy ring method, using a Tensiometer equipped with a 1.9 cm platinum ring at room temperature (Lauda, Germany).

2.3. Contact angle measurements

The polystyrene surfaces of 3 cm^2 were conditioned with the *Lactobacillus helveticus* MRTL91 derived biosurfactants for 24 h at room temperature, washed gently with demineralized water and left to dry for 24 h. Control, polystyrene surfaces were immersed in demineralized water for 24 h [37]. The contact angle of water was assessed by the sessile drop technique at 20 °C using a drop volume of 4 ml on a contact angle system (CAM 200-KSV).The values are an average of at least 20 measurements taken from three independent samples.

2.4. Taxonomic identification

Preliminary identification of putative isolates were carried out by booth microscopic and biochemical experiments based on Bergey's manual of determinative Bacteriology [38]. Genotypic identifications were performed by universal primers 27F, (5′ AGAGTTTGATCCTGGCTCAG-3′) and 1492 R (5′-GGTTACCTTGTTACGACTT-3). Each PCR reaction mixture contained approximately 10 ng of DNA; 2.5 mM $MgCl_2$; 1 × PCR buffer (Bangalore Genei, Bangalore, India); 200 mM each dCTP, dGTP, dTTP and dATP; 2 pmol of each forward and reverse primer; and 1U of Taq polymerase (Bangalore Genei, Bangalore, India). The amplification was performed using the Eppendorf Gradient Master cycler system with a cycle of 94 °C for 5 min; 30 cycles of 94 °C, 60 °C and 72 °C for 1 min each; and final extension at 72 °C for 10 min, and the mixture was held at 4 °C. The PCR product was precipitated using polyethylene glycol, washed thrice using 70% ethanol and dissolved in Tris-HCl buffer (10 mM, pH 8). The amplified DNA products were controlled in 1% Agarose gel electrophoresis. 16 S rRNA universal primers were used to amplify the selective fragment of genomic DNA. Samples were run on an ABI PRISM 3730XL DNA Analyzer (Applied Biosystems, USA). The 16 S rRNA sequences were analyzed using the DNA sequence analyzer computer software (Applied Biosystems, USA).

2.5. Production media and cultivation conditions

500 ml of MRS-Lac (glucose was replaced by lactose) broth (pH 6.2) was inoculated with 1% (v/v) of selected isolate preculture and incubated for 72 h at 37 °C at 120 rpm min^{-1}. After 72 h, cells were harvested by centrifugation (5000*g*, 10 min, 4 °C), washed twice in phosphate buffer saline (PBS: 0.01 mol l^{-1} KH_2Po_4/K_2HPO_4 and 0.15 mol l^{-1} (pH 7.2) and re-suspended in 100 ml of PBS [13]. The suspension was kept at room temperature for overnight with gentle stirring for release of cell-bound biosurfactant. Consequently, bacterial cells were removed by centrifugation (5000*g*, 10 min, 4 °C), and the supernatant liquid was filtered through a 0.22 μm pore size filter (Axiva, India).

2.6. Purification of biosurfactant

The suspension was dialyzed against demineralized water at 4 °C in a dialysis membrane (molecular weight cutoff 6000-8,000 Da, Himedia, India) and freeze dried. Freeze dried biosurfactant was partially purified in silica gel (60–120 mesh) column eluted with gradient of chloroform and methanol ranging from 20:1 to 2:1 (v/v).

2.7. Biosurfactant stock solution and dilutions

The partially purified biosurfactant was dissolved in PBS (pH 7.2) at the final concentration of 50 mg ml^{-1}. These solution was filtered through 0.2 μm PTFE syringe filters and then stored at 4 °C.

2.8. Pathogenic strains preparation

All the pathogenic strains were obtained from American Type Culture Collection (ATCC, USA) and Microbial Type Culture Collection (MTCC, INDIA).The bacterial strains were cultivated in Tryptic soy agar supplemented with 6 g l^{-1} of yeast extract and incubated at 35 °C for 24 h. Yeast strains were cultured in Yeast peptone dextrose agar and broth.

2.9. Antimicrobial assay

The antimicrobial activity of the biosurfactant against various pathogenic and nonpathogenic strains was determined by the 96 well flat bottom plastic tissue culture plates (Himedia, India). Briefly, 125 μl of sterile, 2X culture medium were placed into the first well of the 96 well microplate and 125 μl of sterile, 1X culture broth in the remaining wells. Further, 125 μl of biosurfactant solution in PBS (50 mg ml^{-1}) were added to the first column of the microplate; this results in a biosurfactant concentration of 25 mg ml^{-1}; in sequence, 125 μl were transferred to the successive wells. All the wells (except negative control) were inoculated with 2.5 μl of an overnight pathogenic strain. Plates were incubated for 48 h at 37 °C. After incubation, the absorbance at 600 nm was recorded for each well. The growth inhibition percentages at different biosurfactant concentrations for each pathogenic strain were calculated as:

$$\%\ \text{Growth Inhibition}_c = [1 - (A_C/A_O)] \times 100$$

Where Ac represents the absorbance of the well with a biosurfactant concentration c and A_0 the absorbance of the control well (without biosurfactant).