

***Lactobacillus acidophilus* Derived Biosurfactant as a Biofilm Inhibitor: A Promising Investigation Using Microfluidic Approaches**

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Abstract:

Background: Biomedical devices and implants are adversely affected by biofilm-associated infections that pose serious public health issues. Biosurfactants (BSs) can combat pathogenic biofilms through their antimicrobial, antibiofilm and antiadhesive capabilities. The objective of our research was to produce biosurfactant (BS) from *Lactobacillus acidophilus* NCIM 2903 and investigate its antibiofilm potential using microfluidics strategies by mimicking the micro-environment of biofilm.

Methods: Antibiofilm and antiadhesive potential was effectively evaluated using different methods like microfluidics assay, catheter assay, polydimethylsiloxane (PDMS) disc assay. Along with this chemical and physical characteristics of BS were also evaluated.

Results: Cell free biosurfactant (CFBS) obtained was found to be effective against biofilm which was validated through the microfluidic or Lab on Chip (LOC) approach. The potency of CFBS was also evaluated on catheter tubing and PDMS surfaces (representative bioimplants). The efficacy of CFBS was also demonstrated through the reduction in surface tension, interfacial tension, contact angle and low critical micelle concentration.

Conclusion: CFBS was found to be a potential antimicrobial and antibiofilm agent. We believe that perhaps this is the first report on demonstrating the inhibiting effect of *Lactobacillus* spp. CFBS via LOC approach. These findings can be explored to design various BSs based formulations exhibiting antimicrobial, antibiofilm and antiadhesive potential for biomedical applications.

Key words: Biofilm; bioimplant; biosurfactant; *Lactobacillus*; microfluidics.

1. Introduction

Medical devices and equipment are routinely used by most healthcare professionals to support and treat the patients. Bioimplants are amenable to develop microbial biofilms. Biofilms are microbial communities with abilities to attach to surfaces, exhibiting high resistance to many antimicrobial agents [1-6]. Pathogenic microbial biofilms exert harmful effects on human health. Increased resistance of bacteria to antibiotic therapy is a major concern for medical professionals worldwide. The inherent resistance of biofilms and their pervasive involvement in implant-related infections has prompted research towards the development of antibiofilm, antiadhesive agents. Biosurfactant (BS) producing microorganisms can disrupt biofilm on medical implants due to their antimicrobial, antibiofilm and antiadhesive potential. Lactic acid bacteria (LAB), such as *Lactobacilli* constitute an important part of the natural microbiota and are recognized as potentially useful bacteria through production of BSs [1-2,6]. Reports by Gomaa [7], Gudiña and co-workers [8-10] suggested that *Lactobacilli* originated BS is highly effective against several pathogenic microorganisms with distinct antimicrobial and anti-adhesive activities. Based on structural details, different types of BSs viz. Glycolipid [5,6], glycoprotein [8,11], glycolipoprotein/peptide [12,13] have been documented from *Lactobacillus* spp. and are therefore considered to be promising agents in tackling biofilms associated infections [7,8,11]. *Lactobacilli* spp. are solely known to produce cell bound/cell associated BS (CABS) and cell free BS (CFBS) [14]. BS mediated synthesis of nanoparticles (NPs) is becoming an interesting approach for many researchers [15]. Currently, several researchers are synthesizing metal NPs from microbial origin and exploiting them for biomedical purposes [16,17]. Reddy et al., [18, 19] reported synthesis of silver and gold NPs by using fractionated BS (as a stabilizer) with NaBH_4 (as a reducing agent). BS plays a crucial role in the synthesis of gold and silver NPs from

B. subtilis ANR 88 by growing in non-hydrocarbon, agro industrial wastes. Synthesis of uniform size and shapes silver NPs (spherical, 4–18 nm) and gold (hexagonal, 40–60 nm) from BS producing *Bacillus* culture has been achieved [20]. Inspiring discoveries or novel formulations introduced by the engineering sector have improved our modern life. Currently incredible applications in the areas of protein crystallization, biochemical screening [21], DNA amplification, DNA sequencing and enzymatic kinetic assays [22] are driving forces to explore advanced developments of engineering. Microfluidics (MF) is one of the pioneering areas of engineering that deals with pico litre volumes of liquids [23]. MF terminology is popularly known as lab-on-a-chip (LOC) where experiments are carried out on small scale *in-vitro*, mimicking *in-vivo* conditions [24]. MF devices have been reported to characterize cellular association in a flow system. Recently, Khor et al., 2018 [25] have cultured HUVECs on the surface of synthetic micro-vascular network in a MF device (SynVivo, INC). This study provided an opportunity to explore possible interactions of molecules with live cells. MF devices with simple and straight microchannels may not be sufficient to replicate the complex structures of the desired systems. However, to some extent MF system are helpful in exploring interactions between nanoparticles with biological molecules. Implementation of fundamental experiments, applied, analytical and diagnostic are often very challenging to carry out due to the availability of compounds in negligible quantities. MF system offers better prospects to overcome these challenges and assist medicinal and therapeutic perspectives. It is also important to highlight that the monetary inputs along with labour cost are also reduced. Automation in the experimental set up facilitates the different combinations for experimental purposes [26].

The advantage of MF set up is that the experiments can be minimized so that extremely sensitive methods can be amalgamated. The design of this innovative technique promotes elucidating the

unexplored biological and diagnostic challenges. Some BS related investigations are quite challenging due to the limited amount of BS produced for *in-vitro* studies. In this study we report the production, physic-chemical characterization of CFBS from *Lactobacillus* spp., some of the physical aspects of CFBS such as surface tension (SFT), critical micelle concentration (*cmc*), interfacial tension (IFT), contact angle (CA) and ionic character. In addition the properties of BS like antimicrobial, antiadhesive and antibiofilm have been explored against pathogenic bacteria on catheter tubing and PDMS based bioimplant surfaces. To the best of our knowledge, this is the first report that describes the functional properties of *Lactobacillus* derived BS using MF approaches.

2. Experimental

2.1 Microbial cultures

L. acidophilus NCIM 2903 used for BS production was obtained from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (NCL), Pune, Maharashtra, India. To visualize the effect of BS on microbial biofilms, bacterial cultures viz., *Escherichia coli* NCIM 2065, *Staphalococcus aureus* NCIM 2079, *Proteus vulgaris* NCIM 2027 were obtained from NCIM, NCL. Other cultures, *Bacillus subtilis* MTCC 2423, *Pseudomonas putida* MTCC 2467 and *P. aeruginosa* MTCC 2297 were obtained from Microbial type culture collection (MTCC), Chandigarh, India. All cultures were grown and maintained as per supplier's instructions.

2.2 *Biosurfactant production and extraction*

Fermentation medium (FM) used for production of CFBS was described in our recent communications [1]. To extract BS, cell free supernatant (CFS) was obtained by centrifuging the culture broth after 72 h at 15,000 rpm/20 min/4 °C and was acidified using 5 N HCl to bring down the pH (from 8.9 to 2.0). Further CFS was kept overnight at 4 °C for precipitation of BS under acidic condition and further it was extracted with ethyl acetate and methanol (4:1) mixture. The organic layer was collected and anhydrous ammonium sulphate was added to remove the water content. Further, organic layer was evaporated to dryness using rotary evaporator [27], and was purified by column chromatographic technique [5,6].

2.3 *Determination of physical and chemical properties*

Physical properties including SFT, IFT, CA, *cmc*, emulsification, stability at different pH, temperature and ionic character were explored for *L. acidophilus* NCIM 2903 derived CFBS. SFT, IFT, *cmc* measurements were carried out by pendant drop technique, while CA using sessile drop technique with the help of optical contact angle (OCA) Goniometer [28]. In addition; ionic character (IC), emulsification activity (%) was also determined. To find out the emulsification activity of CFBS; 2 ml of hydrocarbon (kerosene, n-decane, n-hexane, xylene, benzene and n-heptane) was vigorously mixed with an equal volume CFBS solution (*cmc* solution). For positive control the *cmc* solutions of four synthetic surfactants (SDS, CTAB, Tween 80, AOT) were included. The mixture of each hydrocarbons and surfactants were mixed thoroughly by vortexing 2 min/RT (30 °C)/1 h [15]. Immediate after mixing and settling down at RT, both the relative emulsion volume (REV, %) and the emulsion stability (ES, %) were measured at zero hour and after 24 h [29]. The stability of CFBS at different temperatures (4, 30,

60 to 121 °C) and pH (ranging between 2 to 14) were examined. Agar double diffusion technique was carried out to determine the ionic character of CFBS [30]. Two regularly-spaced rows of wells were punched in a soft agar (1% w/v). The upper row was filled with reference surfactants (with known ionic character) in around 20 mM concentration (SDS, AOT, CTAB, barium chloride) and lower row was filled with BS (10 mg/ml) and allowed to diffuse at RT. The set up was monitored on 4 h intervals for 48 h to detect formation of a line of precipitation between cationic and anionic pair.

Chemical characterization of CFBS was carried out via thin layer chromatography (TLC) and Fourier-transform infrared (FTIR) spectroscopy to demonstrate the presence of functional groups [1]. Rhamnolipid (RHL) BS (Agae, Technologies, USA) was considered as a reference compound for both TLC and FTIR analysis. Different solvent systems and developers were used to detect presence of sugar and lipid moieties on pre-coated silica gel plates (Merck, India). The presence of UV active spots of CFBS was confirmed under UV light [5,6,11]. For FTIR analysis of CFBS, ~ 1 mg of CFBS paste was grounded with 100 mg of KBr. The translucent pellet was analysed by FTIR device (Jasco FT/IR-6100, Japan). The spectrum ranging between 400–4000 cm^{-1} at a resolution of 4 cm^{-1} was analyzed.

2.4 *Determination of antimicrobial, antiadhesive properties*

Micro-dilution technique in 96-well flat-bottom plastic tissue culture plates (Greiner Bio-One GmbH, Frickenhausen, Germany) was carried out to determine the antimicrobial and antiadhesive activity of CFBS compound (625 $\mu\text{g/ml}$) against six test bacterial cultures as described by Gudiña et al., [8-10] and Satpute et al., [1].

2.5 Preparation of microfluidics (MF) assembly

Polydimethylsiloxane (PDMS) based MF channels were designed by mixing elastomer and curing agent (Elastomer solution kit: 184 Sylgard, Dow Corning, USA) in a ratio 10:1 (w:w). This solution was mixed thoroughly and then bubbles were removed by placing this solution in vacuum desiccators until a clear solution was achieved. This bubble free and clear solution was poured on Si wafer containing impression of rectangular micro-channels with width 200 μm and height 100 μm . While pouring the PDMS liquid, care was taken to prevent the formation of any cracks and minimize air entrapment. It was allowed to solidify at a temperature of 80-100 $^{\circ}\text{C}$ for 6 h in a vacuum oven at a pressure of 100 Pa (10^{-3} to 1 Torr). Mixing process commences the curing reaction that is evident from the gradual increase in viscosity of the solution resulting in gelation and finally altering as a solid elastomer.

After completion of the solidification process, the MF chambers were cut in the shape of typical glass slide size (7.5 cm length and 2.5 cm wide). PDMS liquid was layered on a clean, grease free glass slide and MF chamber (previously cut) were placed on the glass slide. In this way the MF chamber channels were sealed at the bottom. The MF assembly preparation was allowed to solidify at 50-60 $^{\circ}\text{C}$ overnight after which, the MF channels were ready to use. Loading of the medium, sample and reagents was carried out using a Hamilton precision syringe (500 μl). In this case the flow rate of the reagents was not crucial as the method involved CFBS coating on the inner side of the MF channel. As shown in the Figure 1, the sample was loaded from the loading point (A) until it overflowed from the sample exit (B) point to ensure the complete filling/coating of MF channels. At this stage the 'LOC' device was ready for further experimental purpose to visualize the *in-vivo* condition in the laboratory. Around 2 μl sample is loaded in the MF channels which takes 3 seconds to coat the channels completely. The total

length of the MF channel was 2.0 cm with a PDMS thickness of 0.2 cm and a diameter of hole (sample loading and exit points) is 1 mm. The channel width was 200 μm with a height of 100 μm . Figure 1. illustrates the actual construct of MF system used to conduct the experiment.

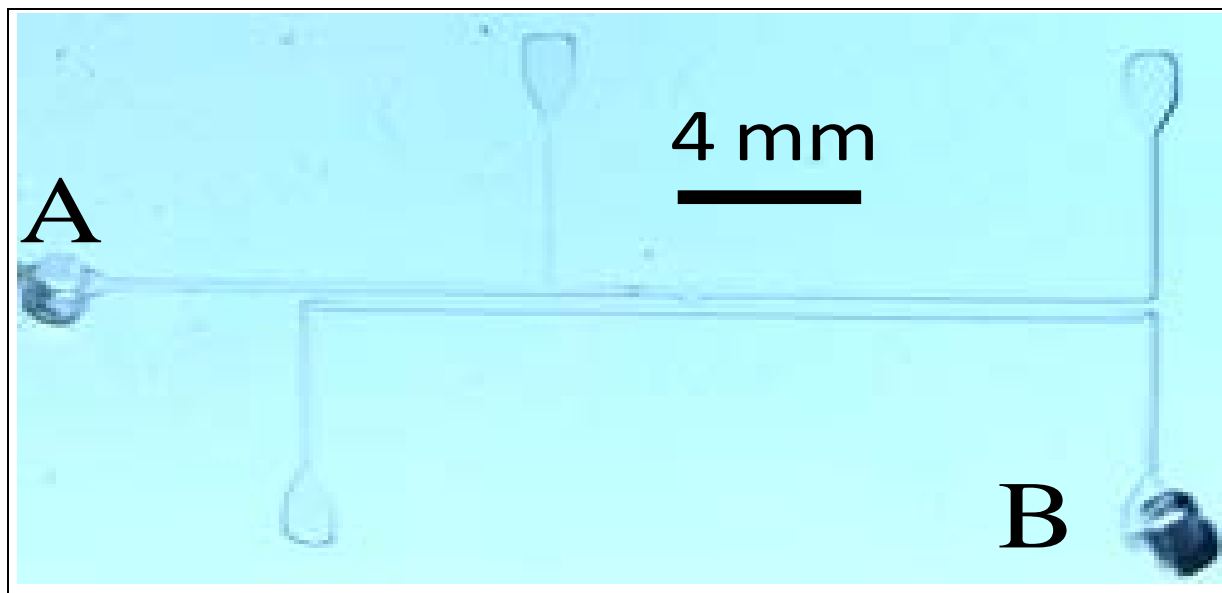


Figure 1. Microfluidics model used to conduct Lab on Chip assay. A: Sample loading point; B: Sample exit point after filling the MF channels completely.

2.6 Preparation of polydimethylsiloxane (PDMS) surface

The procedure used for preparation of PDMS based surface is described in the section 2.5. The clear solution of PDMS was poured in sterile disposable petri dish and after solidification at 50-60 $^{\circ}\text{C}$ for 1 h, circular PDMS disc (diameter 0.8 cm and thickness of 0.1 cm) were cut. Sterile discs were coated (4 $^{\circ}\text{C}$ /overnight) with sterile CFBS (625 $\mu\text{g}/\text{ml}$ - *cmc* solution) to analyze its inhibiting effect on bacterial biofilm. Other set of disc coated only with PBS (without any CFBS) were considered as control. All discs were immersed in sterile culture medium inoculated with

test cultures and incubated at 37 °C for 48 h. Further discs were removed and rinsed with sterile distilled water and scanning electron microscopic (SEM) images were taken.

2.7 *Foley catheters assay*

Biofilm formed in catheters were visualized as per assay described by Mireles et al., [31]. In brief about 10 µl of overnight bacterial culture (0.5 McFarland standard) was inoculated into 500 µl of medium and injected into 2 cm long clear sterile silicone based urethral catheters (Poly Medicure suction catheter, India). The catheter tubes were capped at both ends and incubated at 37 °C for 48 h. Media and growth conditions were followed as per instructions provided by suppliers. This experimental set up were considered as control and other parallel set was first coated with glycolipid CFBS (625 µg/ml) and left at 4 °C for overnight and then the above procedure was continued. After 48 h cultures were removed from the catheter (control and test) and OD₆₃₀ nm was determined followed by rinsing the catheters with distilled water and further dried at RT for 15 to 20 min. After completing the air drying procedure, ~500 µl of crystal violet (CV) (1% w/v) were added in all catheter tubing and left at RT for 20 min. All biofilms developed on catheters appeared purple due to the CV staining. Further, excess stain of CV was removed by washing with distilled water for several times and catheter tubes were examined for the presence of biofilms.

3. Results

The recurrence of infective diseases and the endless advancement in antibiotic resistance among disease-causing microbes has become one of the greatest threats to human health [32]. This kind of resistance has developed due to the ability of these microorganisms to form a biofilm on

various surfaces where microorganism can shelter. BSs do possess some properties of significant therapeutic potential reflected through anti-microbial, anti-biofilm and anti-adhesive potentials. LAB has been recognized for BS production and beneficiary impact on human system [14]. Among LAB, Lactobacilli spp. have a highly competitive nature in gastrointestinal tract (GIT) by preventing the growth and adherence of pathogens. Consequently, in the current study we explored antibacterial anti-biofilm and anti-adhesion property of BSs derived from *L. acidophilus*.

3.1 *Production and extraction of biosurfactant*

In this paper, we used a comparatively simpler medium for synthesis of CFBS. Reduction in the SFT of CFBS with change in pH indicated the production of CFBS by strain NCIM 2903. In addition, gradual increase in the diameter of the drop of CFS on parafilm surface demonstrated the production of CFBS. MRS medium was found to be more appropriate for growth of NCIM 2903 culture but not for reducing the SFT of CFS. Therefore, FM was considered as a better medium for the production of BS throughout the experiment. CFS collected after 72 h of incubation found to be suitable for extraction of CFBS. Further purification through column chromatography using chloroform:methanol (60:40) yielded 1.5 g/L of BS.

3.2 *Analysis of physical and chemical properties of biosurfactant*

Analysis of physical properties like SFT, IFT, *cmc*, CA, emulsification properties and ionic character proved the effectiveness of CFBS. The SFT of PBS was reduced from 72 to 27 mN/m with a *cmc* 625 µg/ml (Figure 2A). Figure 2B represents the semi-logarithmic reflection of the same *cmc* value from SFT *versus* CFBS concentration.

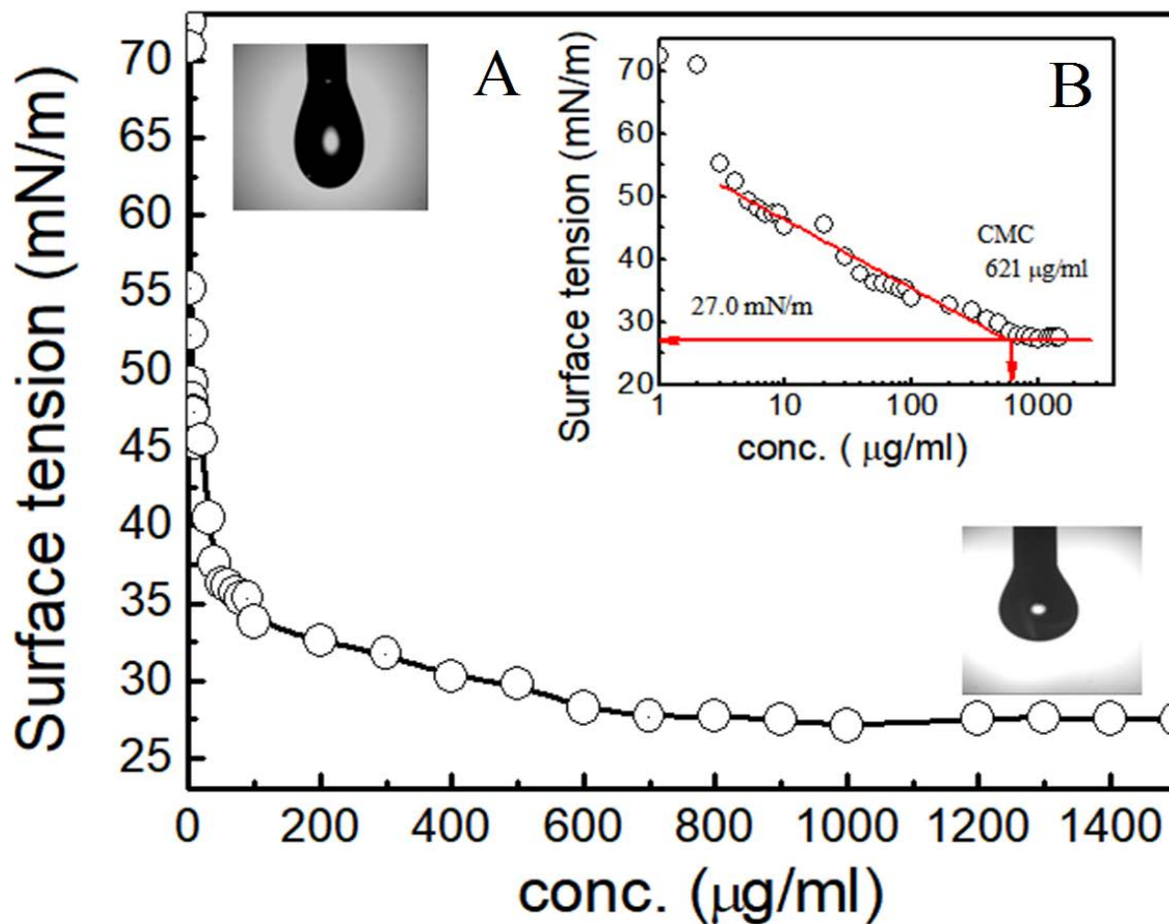


Figure 2. (A) Variation in surface tension and (B) semi-logarithmic reflection of critical micelle concentration (*cmc*) value from SFT (mN/m) versus CFBS concentration for derived from NCIM 2903.

Other physical properties like IFT, CA, EA, ES also confirmed the efficacy of CFBS produced by NCIM 2903 with great certainty. The IFT measurements with immiscible liquids (hydrocarbons), showed lowest IFT against benzene (1.47 mN/m) followed by kerosene, o-

xylene, n-hexane (Figure 3). There was good reduction in IFT values by CFBS against all hydrocarbons tested in comparison to other synthetic surfactants.

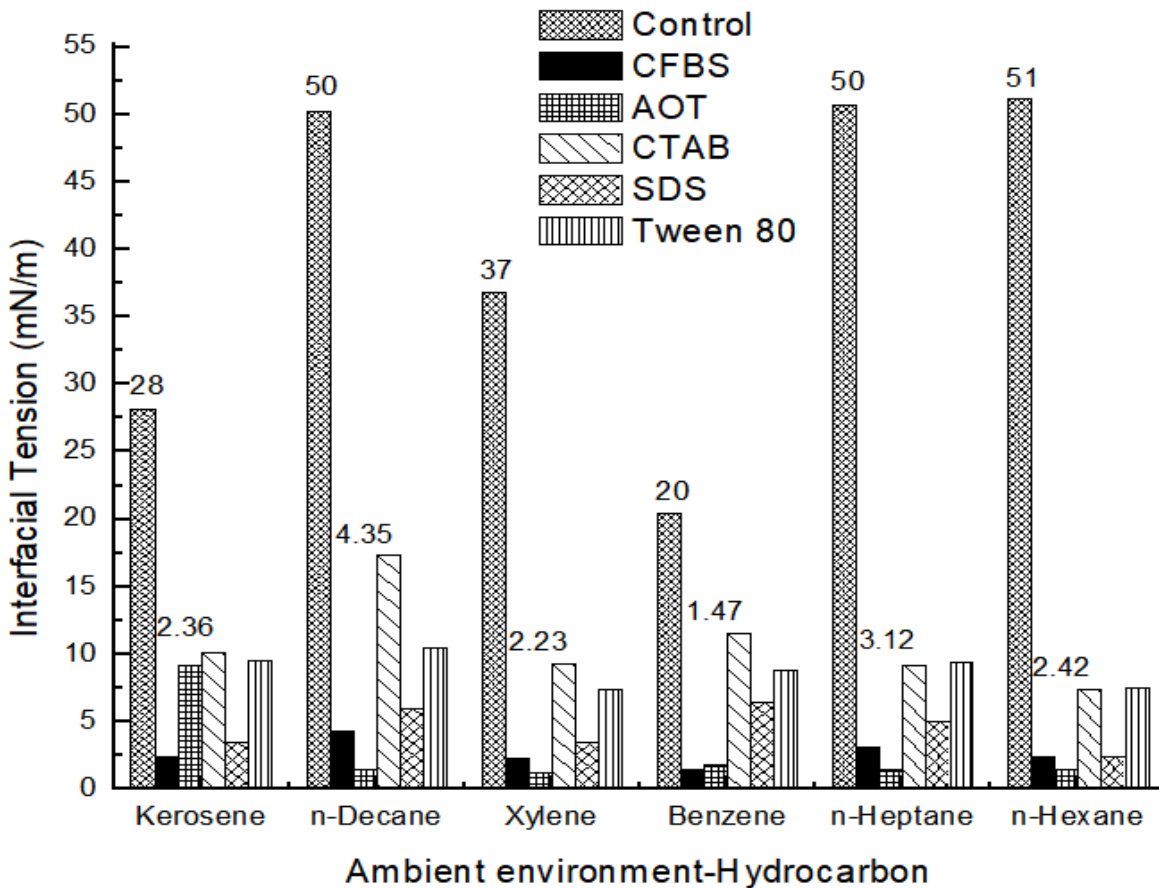


Figure 3. Measurement of interfacial tension (IFT) mN/m for CFBS obtained from *L. acidophilus* NCIM 2903 and synthetic surfactants against water insoluble liquids. Control: Phosphate buffer saline; CFBS: Cell free biosurfactant; AOT: Aerosol OT; CTAB: cetyl trimethyl ammonium bromide; SDS: sodium dodecyl sulphate.

Effective spreading abilities for CFBS were tested through the measurement of CA. The highest reduction in CA was observed on polystyrene (From $\theta = 90^\circ$ to 49°) and PDMS (from $\theta = 115^\circ$ to

74°) followed by Teflon (from $\theta = 115^\circ$ to 75°). Polystyrene and PDMS surfaces are used regularly in biomedical industries. CA on OHP transparent sheet was reduced from $\theta = 69^\circ$ to 44° . However, on the highly hydrophilic glass surface; there was no significant reduction in CA values (from $\theta = 39^\circ$ to 25°) (Figure 4).

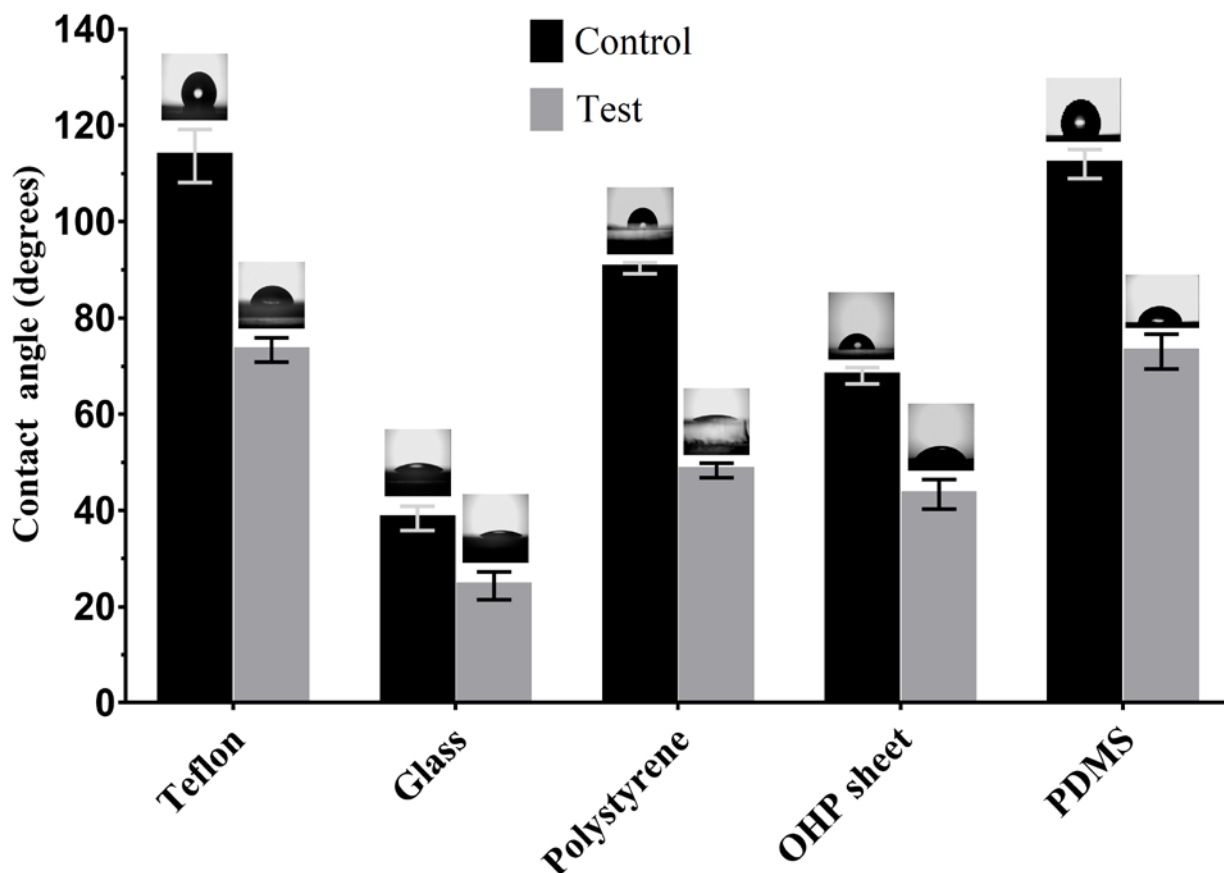


Figure 4. Measurement of contact angle (CA) (degrees) for CFBS obtained from *L. acidophilus* NCIM 2903 on different surfaces, OHP: Overhead Projector Transparent sheet; PDMS: polydimethylsiloxane.

The CA measurements for CFBS derived from *Lactobacillus* sp. have seldom been reported in literature. The emulsification capacity and stabilization activity of CFBS against different water

insoluble liquids analysis showed that CFBS has relative EV between 25-65% and ES between 45-87% after 24 h. The highest EV (65%) was seen against n-decane with 87% of ES. CFBS demonstrated good EV (46%) and ES (87%) with xylene. Treatment of CFBS (*cmc* solution) at different pH (2.0 to 12.0) indicated higher stability between the pH of 6-10 and lowest SFT value at pH 7.0 indicating the potency of CFBS at neutral to alkaline conditions rather than acidic conditions. However, treatment of acidic pH result the precipitation of CFBS. Regarding the temperature effects, CFBS worked well at different temperatures (4, 30, 70 and 121 °C). Determination of ionic character by agar double diffusion technique indicated passive diffusion between CTAB (cationic compounds) and CFBS proving the presence of anionic charge on CFBS (Figure 5). A prominent line of precipitation formed on soft gel helped rapid determination of ionic character of CFBS.

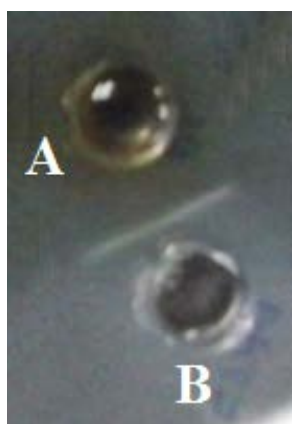


Figure 5. Double diffusion of cationic surfactant (B) (positively charged) CTAB against CFBS (A) on agar plate indicating a line of precipitation due to formation of ionic pair.

Sugar and lipid moieties were detected using TLC confirming the presence of glycolipid typed CFBS. The chemical composition was confirmed by FTIR analysis (Figure 6).

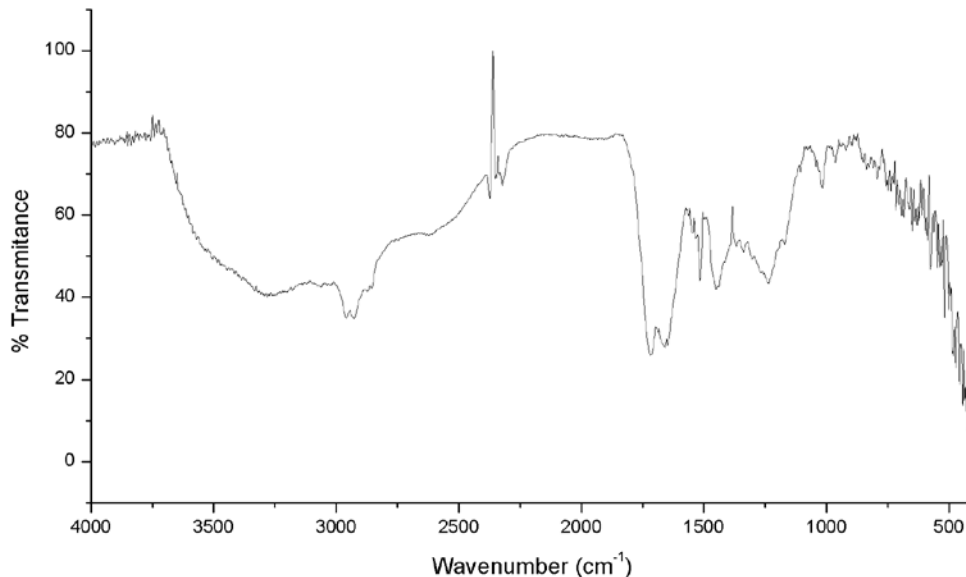


Figure 6. FTIR spectrum for CFBS obtained from *L. acidophilus* NCIM 2903.

The peak at 3320 cm^{-1} depicts the presence of OH stretching. The presence of hydrocarbons is confirmed by the peak at 2900 cm^{-1} . The peaks at 1730 cm^{-1} significantly denote the presence C=O stretching in ester bond. The presence of ether moiety was confirmed by the presence of peak at 1230 while the presence of sugar moiety was clearly indicated by the peak at 1000 cm^{-1} (C-O stretching in sugars). The results of our study strongly suggest a glycolipid nature of CFBS.

3.3 *Antimicrobial, antiadhesive and antibiofilm potential of biosurfactant*

Our studies showed good antimicrobial, antiadhesive and antibiofilm potential of CFBS. It could be clearly seen in Figure 7 that CFBS exhibited antimicrobial activity ranging between 65 to 87% against all bacterial strains used in the study. At the concentration of $625\text{ }\mu\text{g/mL}$, it

inhibited the growth of *S. aureus* (87%), *P. aeruginosa* (85%), *B. subtilis* (82%) and followed by *E. coli* (80%) and *Ps. putida* and *P. vulgaris* (70%).

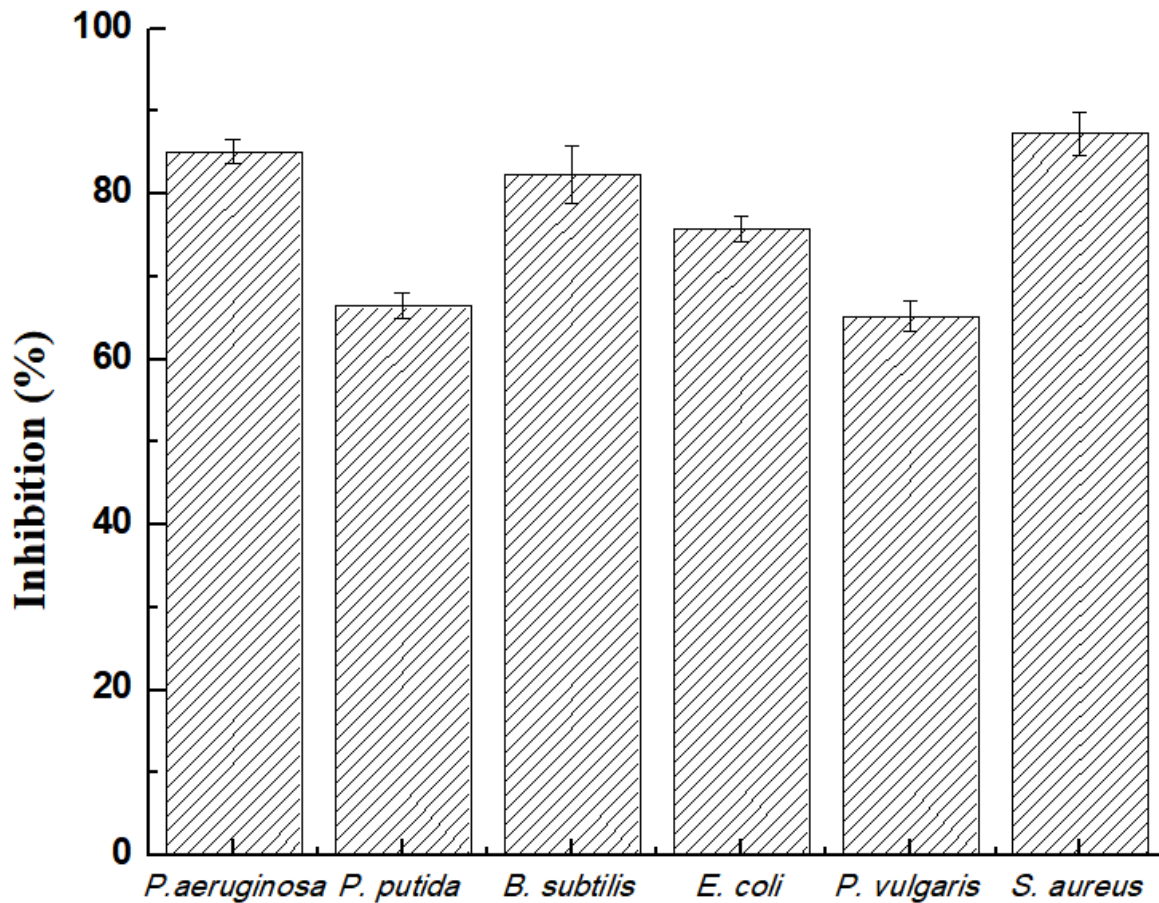


Figure 7. Antimicrobial potential of CFBS (625 µg/ml) derived from NCIM 2903 against *P. aeruginosa* MTCC 2297, *P. putida* MTCC 2467, *B. subtilis* MTCC 2423, *E. coli* NCIM 2065, *P. vulgaris* NCIM 2027 and *S. aureus* NCIM 2079.

3.4 Investigation of antiadhesive and antibiofilm potential of CFBS via microfluidic approaches

The MF assembly shown in Figure 1 was used to investigate the antiadhesive and antibiofilm effect of CFBS against *B. subtilis*. Biofilms were observed at different locations of the MF channels without any coating of CFBS. No noticeable effects were seen in the control treatments of PBS which was evident by the high growth and viability of the test culture (Figures. 8A, B, C). The MF assembly coated with CFBS showed clear channels after staining with CV indicating that coating with CFBS inhibited the adhesion and biofilm formation on the surface (Figure. 8D, E, F).

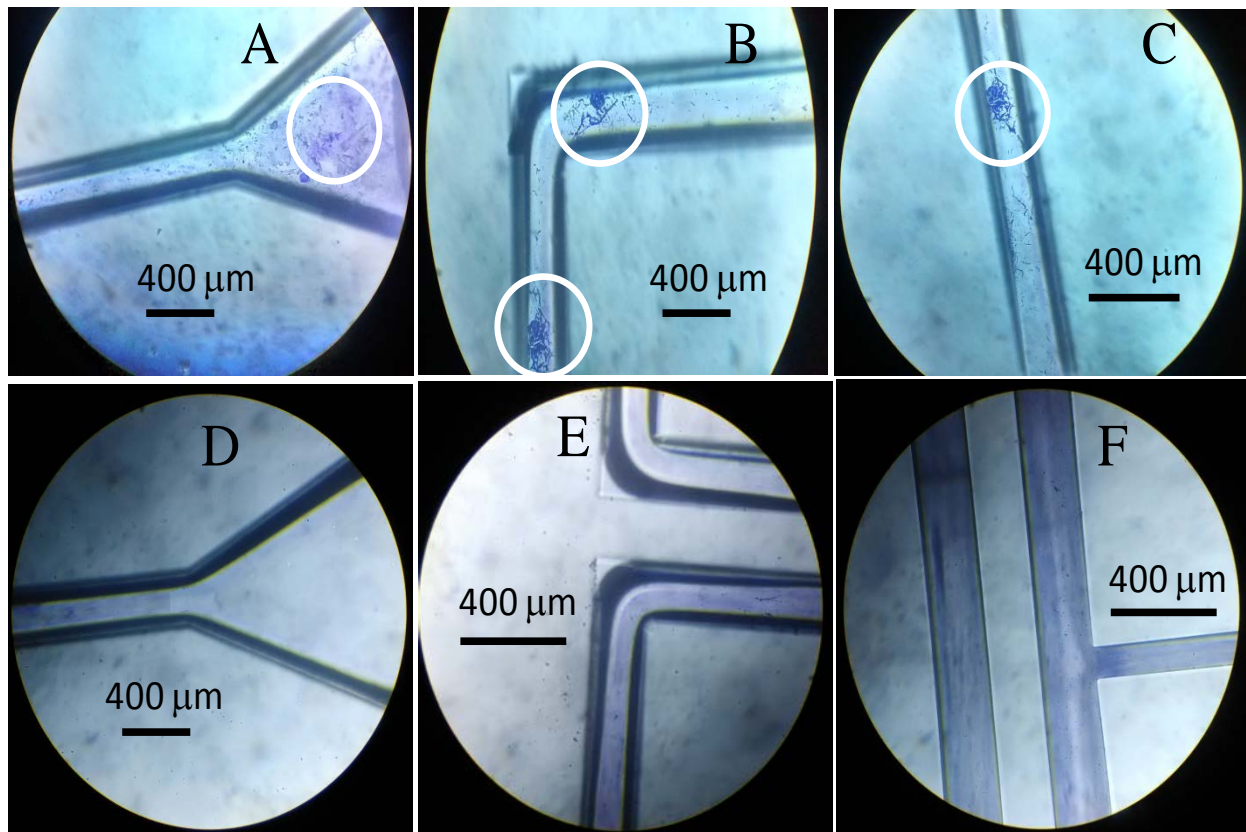


Figure 8. Optical microscopic images of microfluidics (MF) assembly and biofilms on polydimethylsiloxane (PDMS) surfaces with (Test- Lower row D, E, F) and without (Control- Upper row A, B, C) CFBS coatings (625 $\mu\text{g/ml}$). In absence of CFBS coating, no inhibition of adhesion and therefore biofilm formation by *B. subtilis* (A, B, C) occurred. White coloured circles indicates the biofilm stained with crystal violet (1%) solution. In contrast, the test MF channels (D, E, F) are seen clear without biofilms.

3.5 *Biofilm formation on PDMS surface*

In addition to MF based approach, PDMS surfaces were also used to demonstrate the inhibiting effect of CFBS against biofilms formed by *B. subtilis* MTCC 2423, *P. vulgaris* NCIM 2027. We used SEM technique to distinguish the conglomerations of biofilms on PDMS surfaces. Under SEM, Figures. 9: A, C (Control) where confluent biofilms were clearly visualized than in Figures. 9: B, D (Test) representing the CFBS pre-coated PDMS discs. Formation of microbial biofilms was restricted on surfaces of PDMS disc when they were previously coated with CFBS.

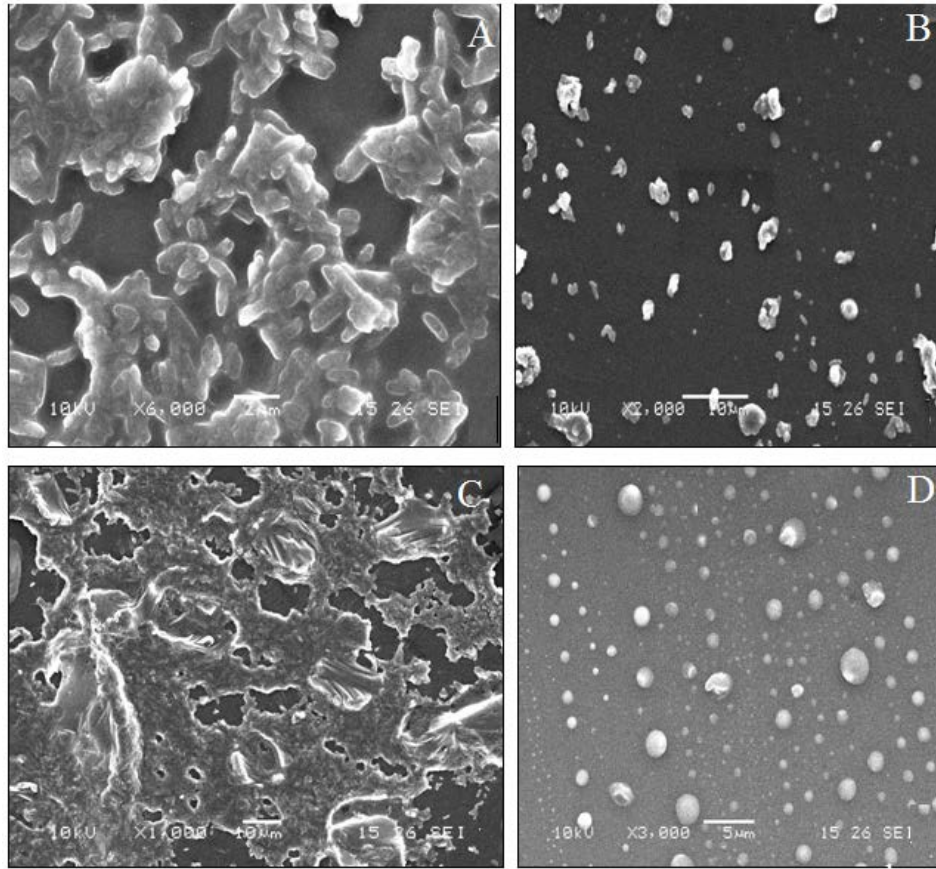


Figure 9. SEM images of polydimethylsiloxane (PDMS) disc with (A, C) and without biofilms (B, D) of two bacterial cultures. The upper row (A and B) represents for *B. subtilis* MTCC 2423 and lower row (C and D) represents for *P. vulgaris* NCIM 2027. Figure A and C represents the control surfaces indicating the PDMS disc without any CFBS coating where confluent growth of biofilms is seen. Whereas, Figure B and D represents the test surfaces indicating pre-coating with CFBS that inhibited the adherence and growth of bacteria proving the antibiofilm and antiadhesion potential of CFBS.

3.6 *Foley catheter assay*

A commercially used medical grade catheter was also tested to explore the effect of CFBS on pathogenic bacterial biofilms. Figure 10A shows dark blue colouration due to the staining of biofilms formed by *P. vulgaris* NCIM 2027. Whereas Figure 10B did not show any blue colour development on catheter surface indicating that glycolipid CFBS inhibited the formation of biofilm of *P. vulgaris* NCIM 2027. The pre-coated of silicone urethral catheters with glycolipid CFBS completely inhibited the adherence and growth of *P. vulgaris* NCIM 2027.

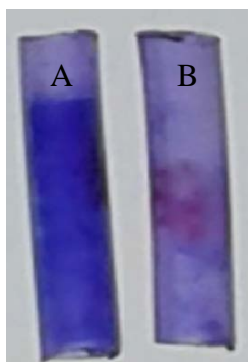


Figure 10. Foley catheters assay: Inhibition of biofilm formed by *P. vulgaris* NCIM 2027 by CFBS on urethral catheters. A: The control where bacterial biofilm appears as a dark blue colour. B: The test, showing anti-biofilm property of CFBS against the bacterial strain. Development of biofilm was diminished absolutely on test catheter tubing.

4. Discussion

4.1 *Production and extraction of biosurfactant*

Most of the literature documented the use of de man Rogosa Sharpe (MRS) medium [33] for the synthesis BS from LAB including *Lactobacilli* spp. [1,14,34]. We have documented the lowest SFT value as 26 mN/m for FM observed after the growth of NCIM 2903 [1].

4.2 *Analysis of physical and chemical properties of biosurfactant*

Physical properties viz., SFT, *cmc*, IFT, CA, emulsification clearly proved the potency of CFBS. We observed the maximum reduction in SFT of PBS from 72 to 27 mN/m at a *cmc* value of 625 µg/ml (Figure 2A). In addition, noticeable reduction in CA on various surfaces and IFT values against various hydrocarbons entirely proved the potency of CFBS isolated in the present studies. It is important to note that the CA measurements of CFBS isolated from *Lactobacillus* sp. were rarely reported in literature. The emulsification with n-decane, and xylene was observed to be comparatively better. Our results are comparable with the Moldes et al., [13] for emulsions of octane/water stabilized by BS isolated from *L. pentosus* and SDS. Along with these results; the stability of BS at a wide physiological condition would contribute towards broadening its scope as antibiofilm agent. Our studies revealed the anionic nature of the CFBS. Similar results were reported by Sharma et al., [35] for the BS isolated from *Enterococcus faecium*. Structural examination of CFBS confirmed the glycolipid type. Closer observation in literature, we can say that our results are comparable with that of the work reported by Sharma et al., [5,6] who suggested the Xylolipidic type BS from *Lactobacillus* spp. In the literature, there are some instances, where researchers have claimed the production of glycolipid type BS from *Lactobacillus* sp. [5,6].

4.3 Antimicrobial, antiadhesive and antibiofilm potential of biosurfactant

Several microorganisms have been used for a wide range of BS production some of which with some antimicrobial properties [3-4,7,11]. However, limited number of reports documented antimicrobial activity of BS obtained from Lactobacilli spp. CFBS obtained from few Lactobacilli spp, displayed antimicrobial activity at low concentrations [14]. The CABS are popular for their antiadhesive property rather than antimicrobial potential (concentration ranging between 4 to-50 mg/ml) [11]. In this work we have demonstrated antimicrobial activity of CFBS at a concentration of 625 µg/mL. More than 80% antimicrobial effect of CFBS were observed by the test organisms (*S. aureus*, *P. aeruginosa*, *B. subtilis* and *E. coli*) used in this study. Gudiña et al., [9] showed 76.8% antiadhesive activity at a 50 mg/ml of CABS isolated from *L. paracasei* spp. *paracasei* A20 against *S. aureus*.

The growth of bacteria like *E. coli* ATCC and *Y. enterocolitica* were inhibited at a concentration of 25 mg/ml for CABS isolated from *L. plantarum* CFR 2194. The same surfactant at both concentrations was found to be ineffective against *S. typhi*. The possible mode of action of CFBS needs thorough investigation. BS can act on microbial system through interfering with the membrane functions and energy generating mechanisms. BS can reduce the cell surface hydrophobicity and microbial adherence to surfaces. Thus, microbial colonization can be prevented or reduced. BS can also increase the cell permeability and can cause the leakage of the metabolites. Alteration in physical membrane structure as well as interference of the protein conformations were also usually reported [4].

4.4 Investigation of antiadhesive and antibiofilm potential via microfluidic approaches

To the best of our knowledge, this is perhaps the first report of MF based studies to demonstrate the inhibitory effect of *Lactobacillus* derived glycolipid CFBS against bacterial biofilms. CFBS inhibiting the adherence, growth and biofilm formation of pathogens is significant. Similar observations have been put forward by De Rienzo et al., [36] indicating the effect of sophorolipid (SPL) on *B. subtilis* using BioFlux channel system. The authors also suggested that multidrug-resistant bacterial infections can be reduced with the help of combinations of RHL and SPL.

4.5 Biofilm formation on PDMS surface

The BS strongly inhibited the biofilm formation on PDMS surface. Our results are comparable to studies carried out by Velraeds et al., [37] who showed inhibitory effect of CFBS produced by *Lactobacillus* sp on *E. faecalis* biofilms on glass surfaces. Our work also gave a strong indication towards the high capability of CFBS to act as antiadhesive and antibiofilm agents against pathogens.

4.6 Foley catheter assay

Our results obtained for silicon based urinary catheter are comparable with Rivardo et al., [38], who showed that BSs of *Bacillus* spp. inhibits biofilm formed by *E. coli* and *S. aureus*. Similarly, Irie et al., [39] also displayed biofilm dispersion ability of *P. aeruginosa* originated RHL against *Bordetella bronchiseptica*. BS molecules can work excitingly in synergistic way (against pathogens) in association with antibiotics, NPs etc. Gómez-Graña et al., [40] suggested that the CFBS exhibit deep impact in reducing the metal precursor and also stabilizing the NPs. More

importantly BS stabilized NPs exhibit the antimicrobial properties against various well known pathogens like *E. coli*, *P. aeruginosa*, *S. aureus*. Such evidence reflects the broad spectrum potential of BS mediated NPs for tackling the antimicrobial resistance. Promising experimental procedures are mandatory for synthesis of NPs where Green technology is the major driving force for the researchers. Currently nanotechnology is in an attempt to use clean, non-toxic and eco-friendly to synthesize nanomaterials in order to minimize the disposal of wastes. Microbes are popularly known for synthesis of inorganic molecules which can be deposited intra or outside the cells [15]. Use of nanomaterials amalgamated with extraordinary molecules like BS, antibiotics definitely provides opportunities to explore the innovative applications.

5. Conclusions

Glycolipid BS displayed virtuous antimicrobial, anti-adhesion and anti-biofilm potential against pathogenic bacteria on catheters and PDMS surfaces. MF confirms its ability to restrict the primary adhesion of biofilm forming pathogenic bacteria on surfaces. These studies are noteworthy to be widely used to examine biofilm cohesion under a variety of physiological like temperature and pH. CFBS may have commercial applications for several medical devices in prolonging the life of some biomaterials as well as reduce the possibilities of opportunistic infections. It is possibly the first time that MF system were used to demonstrate the antibiofilm effect CFBS derived from *Lactobacillus* spp. This simple technique can be used for testing various antibiofilm and antiadhesive formulations against various pathogens at laboratory level.

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Compliances with Ethical standards

This research does not involve any studies with human participants or animals performed by any of the authors.

Declaration on conflict of interest

Authors declare that they do not have any conflict of interest.

Authors' Contributions

SKS, IMB and AGB conceived the research project and SKS & AGB achieved financial support from funding agency. SKS, IMB and AGB designed the study. SKS, NSM, PD, AGB performed the experiments and analyzed the results and all authors participated in writing the manuscript and approved the final manuscript for submission.

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