



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.

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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) Arunachal Pradesh, North East Indian state) in pre-sterilized 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 cm² 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 µl 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'-GGTACCTTGTTAC-GACTT-3). Each PCR reaction mixture contained approximately 10 ng of DNA; 2.5 mM MgCl₂; 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. 16S 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 16S 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 pre-culture and incubated for 72 h at 37 °C at 120 rpm min⁻¹. After 72 h, cells were harvested by centrifugation (5000g, 10 min, 4 °C), washed twice in phosphate buffer saline (PBS: 0.01 mol l⁻¹ KH₂PO₄/K₂HPO₄ 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 (5000g, 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:

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

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

2.10. Pre-adhesion treatment on polystyrene surface

The antiadhesive activity of the purified biosurfactant isolated from *L. helveticus* MRTL91 against several microbial strains was quantified according to the procedure described by Heinemann [17]. Briefly, the wells of a sterile 96 well flat bottomed plastic tissue culture plate (Himedia, India) were filled with 200 μl of the biosurfactant concentration ranging 25 mg ml^{-1} to 1.56 mg ml^{-1} . The plate was incubated for 18 h at 4 °C and subsequently washed twice with PBS. Control wells contained PBS buffer only. An aliquot of 200 μl of a washed bacterial and yeast suspension was added and incubated in the wells for 4 h at 4 °C. Unattached microbial cells were removed by washing the wells three times with PBS. The adherent microorganisms were fixed with 200 μl of methanol (Himedia, India) per well. Then the plates were stained for 5 min with 200 μl of 2% crystal violet used for Gram staining. Excess stain was washed out by placing the plate under running tap water. Consequently, the plates were air dried, the dye bound to the adherent micro-organisms was re-solubilized with 200 μl of 33% (v/v) glacial acetic acid (Himedia, India) per well, and the absorbance of each well was measured at 600 nm. The inhibition percentages at different biosurfactant concentrations for each microorganism were calculated as:

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

Where A_c represents the absorbance of the well with a biosurfactant concentration c and A_0 the absorbance of the control well.

2.11. Biofilm formation on silicon tubes (Co-incubation assays)

The biofilm forming strains of *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus cereus* and *Candida albicans* were used in the present experiment. 10 μl volumes of overnight cultures were added into 1000 μl of fresh LB medium, and the same volume of *C. albicans* was added into 1000 μl of fresh YPD medium. 1000 μl partially purified biosurfactant (final concentration of 25 mg ml^{-1}) solution in LB medium (for bacterial pathogenic strains) and YPD medium for *C. albicans* and 4 cm long pieces of sterile silicone tubes (Himedia, India) also incubated with medium prepared above. Tubes were incubated overnight at 37 °C. After incubation, silicone tubes were removed and washed with distilled water. The silicone tubes were stained with 3000 μl of crystal violet (0.1%) for 20 min. The stained biofilms were rinsed twice with distilled water and allowed to dry at room temperature for 30 min [41].

2.12. Study of biosurfactant stability

Stability studies were performed using the cell free broth obtained by centrifugation at 8000g for 20 min 10 ml of BS suspension (25 mg ml^{-1}) was kept at 0, 5, 15, 25, 50, 75, 100, and 125 °C for 30 min, cooled to room temperature; the surface tension and emulsification index were determined. To elucidate the pH stability of BS, the sample was adjusted to different values (5.0–12.0) with 1 M NaOH and 1 M HCl, and the same aforesaid measurements were performed. All the assays were carried out in triplicates [2].

3. Results and discussion

3.1. Isolation and screening

Initially 10 isolates were isolated from soft and hard variety of Chhurpi cheese after 24 h of incubation at 37 °C. Further all the isolates were screened for biosurfactant production using various qualitative and quantitative methods. When the isolates were spot inoculated over the blood agar plates, resulted in lysis of red blood cells around the colonies which confirmed the production of biosurfactants. In case of drop collapsing test, the flattened drop of supernatant placed over the oil surface indicated the presence of biosurfactant. Screening techniques are essential to identify diverse types of biosurfactants from potential biosurfactant producers. The drop collapse, lysis of red blood cells and oil displacement methods are the most effective tools to prove the biosurfactant production in many of the bacterial strains [42]. On the basis of screening results, isolate MRTL 91 was selected for further study (Table 1). The emulsification activity was found maximum 65% with kerosene oil. Two way ANOVA showed that, the values significantly differed from each other's $P < 0.001$. Biosurfactant produced by *Lactobacilli delbruckii* using peanut cakes showed significant emulsification but comparatively less than the emulsification activity recorded with the standard chemical surfactant Triton X-100 [8]. However, advantages of biosurfactants over chemical surfactants such as lower toxicity, biodegradability and ecological acceptability becomes more favorable. A progressive decrease in surface tension was observed with increase in concentration of biosurfactant up to 2.5 mg ml^{-1} (Fig. 1). The minimum surface tension value of 39.5 mNm^{-1} was observed after 72 h of culture due to excreted biosurfactant, varied markedly from 53.0 (MRS-Lac broth) to 42.3 mNm^{-1} (53.0 mNm^{-1}) and 72.0–39.5 mNm^{-1} by cell bound biosurfactant. The insignificant reductions detected in the surface tension of the culture broth supernatants during the fermentation, it can be concluded that the

Table 1
Screening of different *Lactobacilli* for biosurfactant production.

LAB Isolates	Screening methods				
	Drop Collapse	Oil displacement	Hemolytic activity	Surface tension	Emulsification index
MRTL1	+	++	+	43.4 ± 0.23	42.5 ± 0.34
MRTL2	++	++	++	44.1 ± 0.15	41.2 ± 0.25
MRTL3	++	++	++	40.8 ± 0.17	58 ± 0.29
MRTL4	+	+	+	46 ± 0.32	32 ± 0.43
MRTL5	++	++	++	45.8 ± 0.21	34.2 ± 0.41
MRTL6	++	++	++	45.2 ± 0.28	34 ± 0.47
MRTL7	++	++	++	43 ± 0.39	45 ± 0.42
MRTL8	+++	+++	++	40.5 ± 0.19	64 ± 0.51
MRTL9	+++	+++	++	41 ± 0.12	64 ± 0.32
MRTL91	++++	++++	++	39.5 ± 0.5	65 ± 0.34

+++ , higher activity; ++: medium activity; +, less activity; –, no activity $P \leq 0.001$.

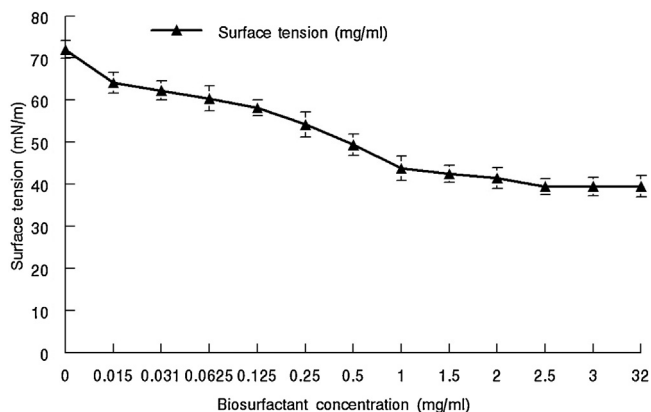


Fig. 1. Progressive decrease in surface tension with increase in concentration of biosurfactant up to 2.5 mg ml⁻¹.

amount of biosurfactant excreted was very low as compared to the cell bound biosurfactant. Velreads et al. [43] also reported a significant reduction in surface tension (72.0–39 mNm⁻¹) while working with *Lactobacillus fermentum* RC-14. In another study Rodrigues et al. [15] has reported similar findings with other LAB. *Streptococcus thermophilus* A and *Lactococcus lactis* 53 reduce surface tension around 36.0–37.0 mNm⁻¹.

A significant property of a biosurfactant is the formation of micelles which are aggregates of amphipathic molecules [3,44,45]. For applied purposes, it is important to differentiate between an effective and an efficient biosurfactant. Effectiveness is measured by the least value to which the surface tension can be reduced, while efficiency is measured by the CMC of the biosurfactant [46]. Surface tension decreases as the surfactant concentration in medium increases and micelles are formed. Critical micelle concentration of biosurfactant was found 2.5 mg ml⁻¹ (Fig. 1) which is quite close to the CMC of commonly used chemical surfactant Sodium dodecyl sulfate (SDS) i.e. 1.8 mg ml⁻¹ which reduced surface tension from 72.0 to 37 mNm⁻¹ [39–41,47]. The CMC determined for biosurfactant produced from peanut oil cake by *Lactobacillus delbrueckii* was found to be 2 mg ml⁻¹ [8].

The efficiency of a surfactant is determined by its ability to reduce the surface tension and interfacial tension of production

medium. For example, an effective surfactant can reduce the surface tension of water from 72.0 to 35.0 mNm⁻¹ [48]. Therefore, our results are in conformity with those obtained for biosurfactants isolated from other LAB.

3.2. Contact angle measurement

The surface conditioning with Lactobacilli derived biosurfactant leads to deviations on the water contact angle, a decrease from 86° to around 76° was observed, demonstrating that the surface became less hydrophobic in nature (The values for the contact angles were significantly different, P < 0.05). *Lactobacilli* derived biosurfactant reduce contact angle of the polystyrene surface and consequently microbial adhesion. Hydrophobic surfaces have shown to be colonized by pathogens. Hydrophobic surfaces facilitates the colonization of microorganism, supporting the removal of interfacial water [13,49]. Biosurfactant application on any solid surface turned a hydrophobic surfaces to hydrophilic surfaces, with an anticipated decrease of microbial colonization. The results of present study indicated decrease in hydrophobicity on polystyrene surfaces treated with biosurfactant and also a substantial decrease of bacterial colonization.

3.3. Phenotypic and taxonomic identification

The biochemical and physiological tests revealed that isolates was LAB. 16S rRNA gene of about 1.5 kb were amplified and sequenced using universal gene primers. Taxonomic affiliation of the isolate was retrieved from the blast algorithm (<http://blast.ncbi.nlm.nih.gov/Blast>) to determine the most related sequence relatives in the NCBI nucleotide sequence database. The phylogenetic analysis also confirmed that all the isolates were from Lactobacillus genus. The isolate MRTL91 showed maximum homology (100%) with *L. helveticus* (Fig. 2). Phylogenetic and evolutionary analysis of the 16S rRNA sequence revealed that, *Lactobacillus helveticus* MRTL91 shared high similarity *Lactobacillus helveticus* G61-5 (HM217962) and *Lactobacillus helveticus* NM53-1 (HM218233). Sequence data of the strain MRTL91 was submitted to the gene bank database (BankIt, NCBI) with accession number JX020702. Similar species of *L. helveticus* were also reported from cheese and other variety of Chhurpi cheese from different geographical reasons [50].

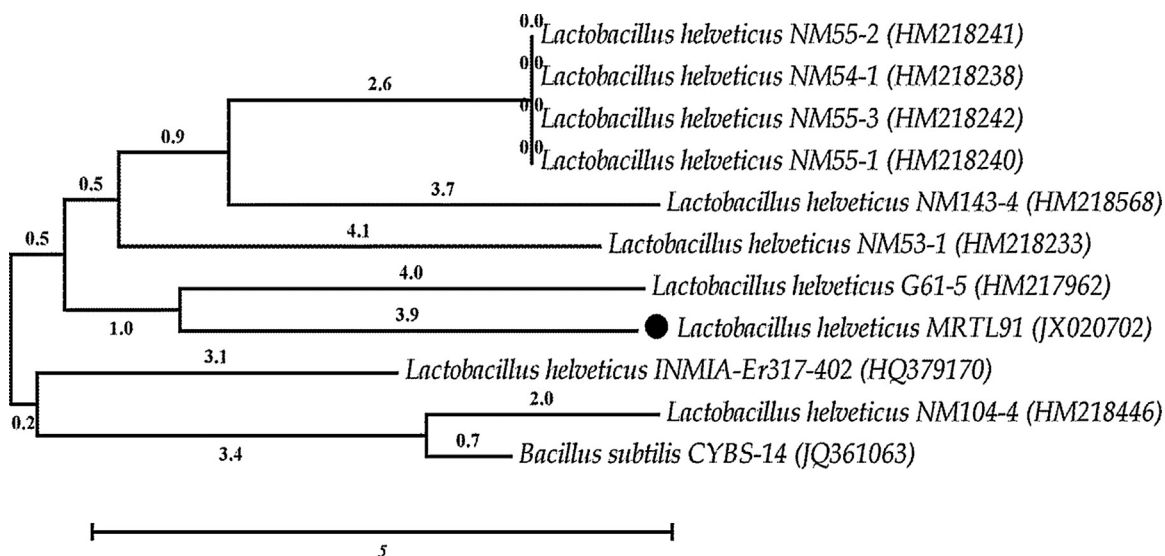


Fig. 2. Phylogenetic tree of biosurfactant producers strain *L. helveticus* MRTL 91.

Table 2Percentages of growth inhibition observed with the biosurfactant isolated from *L. helveticus* MRTL91 at different range of concentration (mg ml⁻¹).

Test organisms	Biosurfactant (mg ml ⁻¹)					Control PBS
	25	12.5	6.25	3.12	1.56	
<i>E. coli</i> ATCC 25922	90.4 ± 0.65	82.1 ± 0.39	74.1 ± 0.15	51 ± 0.11	40.9 ± 0.36	0
<i>P. aeruginosa</i> ATCC 15442	75.6 ± 0.52	69.2 ± 0.25	55.1 ± 0.11	44.1 ± 0.16	39.2 ± 0.46	0
<i>S. typhi</i> MTCC 733	78.6 ± 0.60	71.6 ± 0.85	62.4 ± 0.07	53.1 ± 0.10	34.1 ± 0.28	0
<i>S. flexneri</i> ATCC9199	70.2 ± 0.53	65.4 ± 0.45	51.3 ± 0.47	40.8 ± 0.28	29.1 ± 0.10	0
<i>S. aureus</i> ATCC 6538P	92.5 ± 0.47	83.3 ± 0.38	69.3 ± 0.05	55.3 ± 0.30	39. ± 0.14	0
<i>S. epidermidis</i> ATCC12228	98.4 ± 0.37	86.6 ± 0.37	66.3 ± 0.35	58.2 ± 0.40	41.2 ± 0.20	0
<i>L. monocytogenes</i> MTCC 657	99.5 ± 0.38	84.3 ± 0.38	63.1 ± 0.17	49.3 ± 0.32	32.4 ± 0.51	0
<i>L. innocua</i> ATCC 33090	97.7 ± 0.42	84.1 ± 0.95	72.1 ± 0.23	46.3 ± 0.32	33.1 ± 0.23	0
<i>B. cereus</i> ATCC 11770	99 ± 0.16	79.3 ± 0.28	61.1 ± 0.28	39.2 ± 0.20	28.1 ± 0.20	0

ATCC-American type culture collection, USA MTCC-Microbial type culture collection, INDIA P ≤ 0.001.

3.4. Antimicrobial activity

The increasing demand for novel antimicrobial agents due to the increased antimicrobial resistance of clinical which drawn attention of many researcher to use biosurfactant as a novel antimicrobial compound against antibiotic resistance pathogens [51–56]. Biosurfactants disrupt cytoplasmic membranes leading to cell lysis and metabolite leakage and disrupting protein conformations which ultimately altering important membrane functions [57,58]. Biosurfactant was found effective against various pathogenic and nonpathogenic microorganisms at different degrees (Table 2). The purified biosurfactant showed antimicrobial activity against a broad range of pathogenic and non-pathogenic strains, including Gram-positive and Gram-negative bacteria, as well as yeasts. Nearly complete inhibition was observed for different biosurfactant concentrations ranging between 1.56 and 25 mg ml⁻¹, except for *Pseudomonas aeruginosa*, *Salmonella typhi*, *Shigella flexneri* and *Candida albicans*. The highest concentration of biosurfactant tested i.e. 25 mg ml⁻¹ showed highest percentage of inhibition for *Escherichia coli* (90.4%), *Pseudomonas aeruginosa* (75.6%), *Salmonella typhi* (78.6%), *Shigella flexneri* (70.2%), *Staphylococcus aureus* (92.5%), *Staphylococcus epidermidis* (98.4%), *Listeria monocytogenes* (99.5%), *Listeria innocua* (99.7%) and *Bacillus cereus* (99%). Furthermore it is remarkable that, a high degree of growth inhibition was observed (from 73.2 to 100%) with the biosurfactant concentration assayed (25 mg ml⁻¹). Biosurfactants derived from different genera of microorganism that exhibit antimicrobial activity have been previously described [61,62]. But, there are only few reports about the antimicrobial activity of biosurfactants isolated from LAB. Biosurfactants derived from *S. thermophilus* A, *L. lactis* 53, *Lactobacillus paracasei* ssp. *paracasei* A20 and *Lactobacillus paracasei* also have been documented for

efficient antimicrobial properties in opposition to various bacterial human pathogens and yeast strains [9,13,16].

The antimicrobial properties of biosurfactant derived from *L. helveticus* MRTL91 against *L. monocytogenes*, *L. innocua*, *B. cereus*, *S. aureus*, *S. epidermidis*, *P. aeruginosa*, *S. typhi*, *S. flexneri* were found equivalent to that obtained with the crude biosurfactants produced by *L. lactis* 53 and *S. thermophilus* A, among various concentrations between 25 and 100 mg ml⁻¹ [13,16]. Biosurfactant produced by *S. thermophilus* A reported for antimicrobial properties against *C. tropicalis* at even very low biosurfactant concentration of 2.5 mg l⁻¹. Biosurfactants of *Lactobacillus paracasei* were also reported for antimicrobial activity against *S. aureus*, *S. pyogenes* and *S. agalactiae* with a biosurfactant concentration of 25 mg ml⁻¹. In another study antimicrobial activities of biosurfactant produced by the *Lactobacillus paracasei* ssp. *paracasei* A20 showed maximal growth inhibition at a concentration ranging between 25 and 50 mg ml⁻¹. The biosurfactant showed antimicrobial properties against all the pathogenic and nonpathogenic strains assayed, and result showed significant antimicrobial activities against pathogenic *Candida albicans*, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus agalactiae*. Minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were attained for biosurfactant concentrations ranging between 25 and 50 mg ml⁻¹. Additionally, the biosurfactant was found to be a significant antibiofilm molecule against most of the pathogens studied [4].

3.5. Antiadhesive activity on polystyrene surface

Biosurfactant form a film that changes wettability of the original surface affecting the adhesion properties of pathogens [56]. It has been hypothetical that biosurfactant manipulate

Table 3Antiadhesive properties of partially purified biosurfactant isolated from *L. helveticus* MRTL91 at different range of concentration (mg ml⁻¹).

Test organisms	Biosurfactant (mg ml ⁻¹)					Control PBS
	25	12.5	6.25	3.12	1.56	
<i>E. coli</i> ATCC 25922	50.1 ± 0.15	29.1 ± 0.17	21.1 ± 0.20	14.1 ± 0.10	6.4 ± 0.40	0
<i>P. aeruginosa</i> ATCC 15442	49.1 ± 0.10	32.1 ± 0.15	26.1 ± 0.15	19.1 ± 0.15	3.4 ± 0.11	0
<i>S. typhi</i> MTCC 733	56.1 ± 0.15	28.1 ± 0.10	19.1 ± 0.10	12.1 ± 0.10	1.7 ± 0.17	0
<i>S. flexneri</i> ATCC9199	40.1 ± 0.23	26.1 ± 0.20	12.1 ± 0.20	8.13 ± 0.15	3.1 ± 0.10	0
<i>S. aureus</i> ATCC 6538P	83.1 ± 0.15	75.1 ± 0.17	58.1 ± 0.11	42.1 ± 0.15	27.1 ± 0.20	0
<i>S. epidermidis</i> ATCC12228	85 ± 0.57	79.2 ± 0.26	61.1 ± 0.20	43.1 ± 0.10	31.1 ± 0.15	0
<i>L. monocytogenes</i> MTCC 657	84.3 ± 0.36	74.2 ± 0.20	60.3 ± 0.26	47.1 ± 0.10	38.1 ± 0.15	0
<i>L. innocua</i> ATCC 33090	82.1 ± 0.10	78.1 ± 0.10	58.1 ± 0.15	40 ± 0.11	21 ± 0.05	0
<i>B. cereus</i> ATCC 11770	87 ± 0.11	81 ± 0.11	63.1 ± 0.10	52.1 ± 0.15	32 ± 0.11	0

ATCC-American type culture collection, USA MTCC-Microbial type culture collection, INDIA P ≤ 0.001.

bacterial-surface interactions [30]. The biosurfactant showed antibiofilm activity against most of the pathogens but the spectrum of activity was varied for different microorganisms and also depends upon the concentration of the biosurfactant (Table 3). The highest antiadhesive property was observed against *B. cereus* (87%), *S. epidermidis* (85%), *L. monocytogenes* (84.3%), *S. aureus* (83.1%) and *L. innocua* (82.1%) on the other side, low activity was observed against *E. coli* (50.1%), *P. aeruginosa* (49.1%), *S. typhi* (56.1%) and *S. flexneri* (40.1%) (Table 3). The antiadhesive activity against yeast *C. albicans* was quite low even at the highest biosurfactant concentration assayed i.e. 25 mg ml⁻¹. In addition it is remarkable that, a high degree of antiadhesive activity was observed (from 69.2 to 87%) with the highest biosurfactant concentration assayed (25 mg ml⁻¹).

A microbial biofilm is a few group of microorganisms in which bacterial cells adhere to a solid surface. The medical equipments or implants including; urinary catheters, orthopedic and surgical implants, and contact lenses are barely available to adhesion by various opportunistic pathogens [41]. Furthermore, along with the antimicrobial activities, the biosurfactant also exhibited considerable antibiofilm properties against most of the microorganisms tested but to different degrees.

Biosurfactant obtained from LAB have been reported for their effective antibiofilm properties against various pathogens [4,30,13,33,43,57,28]. Role of biosurfactants in microbial adhesion and desorption has been broadly considered, and implementation of biosurfactants derived from various lactobacilli to surface might be an valuable move toward to diminish adhesion and combating colonization by pathogenic strains in biomedical and food surfaces [7,28]. Antibiofilm properties were observed with biosurfactant produced by *L. helveticus* MRTL91 against various pathogenic microorganisms such as *L. monocytogenes*, *L. innocua*, *B. cereus*,

S. aureus, *S. epidermidis*. Antibiofilm activities by biosurfactant produced by *L. helveticus* MRTL91 are extremely promising for sinking microbial colonization on different surfaces. However, biosurfactant produced by *L. helveticus* MRTL91 showed low degree of antiadhesive activity against *E. coli*, *C. albicans*, *P. aeruginosa*, *S. typhi*, *S. flexneri* in contrast with the antimicrobial activity exhibited against these strains at the same biosurfactant concentrations. Falagas and Makris [11] has projected the utility of biosurfactants produced by probiotic microorganism to patient care equipment's (catheters and other biomedical insertional implants) in hospitals to decline colonization by microorganisms accountable for nosocomial infections. Rodrigues et al. [13] studied inhibition of microbial colonization to silicone rubber exposed to biosurfactant produced by *S. thermophilus* A.

Drop off in the initial deposition rates were observed for *Rothia dentocariosa* and *S. aureus*. The amount of bacterial cells adhering to the silicone rubber with pre adsorbed biosurfactant after 4 h was further declined by 89% and 97% by two Lactobacilli strain, respectively. Gudina et al. [4] reported LAB which possess antiadhesive activity against various pathogens. The maximal antiadhesive percentages were observed for *S. aureus*, *S. epidermidis* and *S. agalactiae* at a concentration of 25 mg ml⁻¹. Antibiofilm potential of biosurfactant derived from *Lactobacillus paracasei* ssp. *paracasei* A20 and *Lactobacillus paracasei* also has been documented for efficient antimicrobial properties in opposition to various bacterial human pathogens and yeast strains and approximately 75% destruction of microbial population was observed against various pathogens.

Antiadhesive property against *Candida albicans* by biosurfactant derived from *Lactobacillus* sp was also reported by Fracchia et al. [29]. In pre-coating assay, biofilm formation of the strain was reduced 82% at concentration of 312.5 µg/ml. Biosurfactant

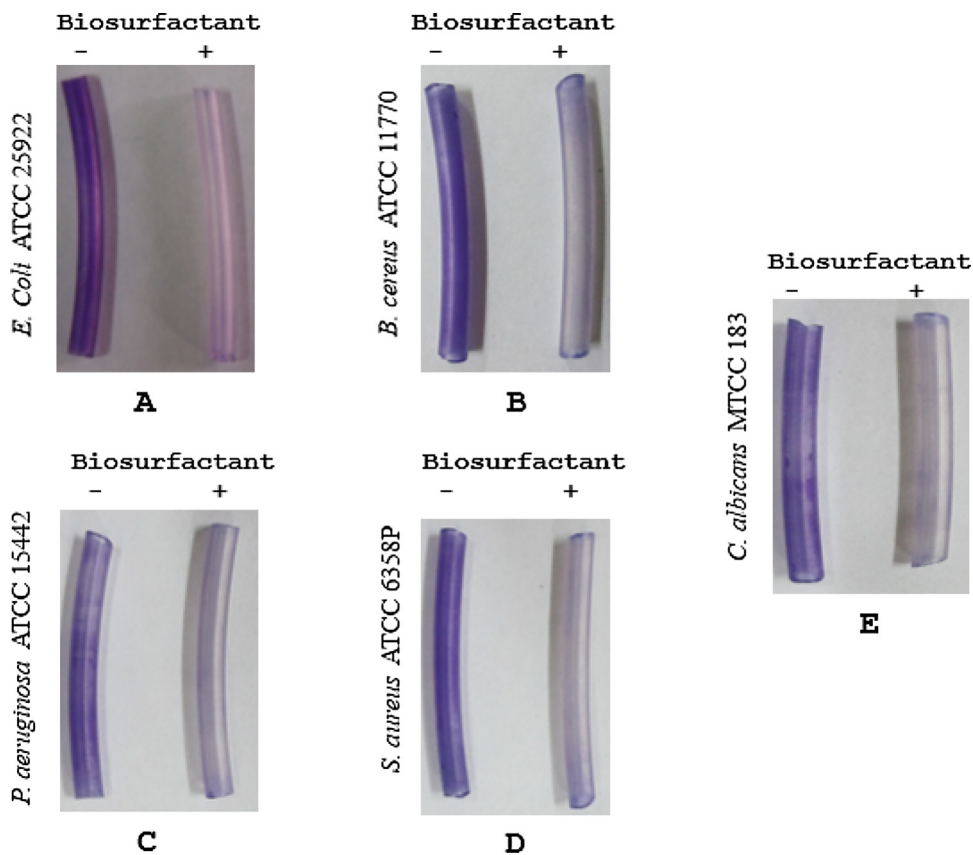


Fig. 3. BS derived from *L. helveticus* inhibits biofilm formation by (A) *E. coli* ATCC 25922 (B) *S. aureus* ATCC 6358 P (C) *P. aeruginosa* ATCC 15442 (D) *B. cereus* ATCC 11770 (E) *C. albicans* MTCC183 on silicone tubes, (-) without biosurfactant treatment (+) with biosurfactant coating.

produced by *L. helveticus* MRTL 91 strain reduced biofilm on silicone tubing near to completely. Biosurfactant showed anti-adhesive properties against several pathogenic microorganism which are potential biofilm formers on surgical implants and silicone tubing of medical equipment's. More than 95% was achieved with 25 mg ml⁻¹ of biosurfactant solution added to the medium. Biosurfactants derived from *Lactobacillus acidophilus* reported for more than 50% of deposition of pathogenic strains of *C. albicans*, *S. aureus*, *E. faecalis*, *E. coli* and *S. epidermidis*. In another study, *Lactobacillus fermentum* B54 strain derived biosurfactant showed antiadhesive activity against uropathogenic microorganisms [31,43]. Various strains of LAB were reported for their biosurfactant producing ability which decreases the rate of deposition (biofilm formation) of potent biofilm pathogens [31,43,15]. *Lactococcus lactis* 53 inhibits microbial growth of *Rothiadent cariosa* and *Candida tropicalis* on silicone tubings in the presence of biosurfactants.

In current experiments all the tested Gram-negative and Gram-positive pathogenic and nonpathogenic bacteria showed decrease of adhesion on both polystyrene and silicone tubes. The results of the present study indicate that LAB derived biosurfactants have potential to be used for competent removal and prevention of biofilms for pathogenic microorganisms. Adsorption of biosurfactants derived from LAB to solid surfaces might establish an effective strategy to reduce microbial adhesion and combating colonization by pathogenic micro-organisms in the biomedical and food industry [13,58,11].

3.6. Biofilm hindrance on silicone tubes

To evaluate biofilm development on medical grade silicone tubes, 4 cm pieces of the silicone tubes formerly treated with *L. helveticus* MRTL91 derived biosurfactants were incubated with biofilm forming pathogenic strains (Fig. 3). Biofilm development was diminished absolutely on silicone tube at exceptionally low concentration of 25 mg ml⁻¹. Reasonable anti-biofilm property was observed against the *C. albicans*. Biosurfactants derived from *S. thermophiles* A reduced the number of pathogenic cells by 89% and 97% after 4 h of incubation [13]. Antibiofilm property of biosurfactant obtained by *Lactobacillus* sp. against *Candida albicans* was reported in pre-coating approach leads to the reduction in microbial population by 82% at concentration of 312.5 µg/ml [29]. Approximately 95% reduction of pathogenic microbial cells was

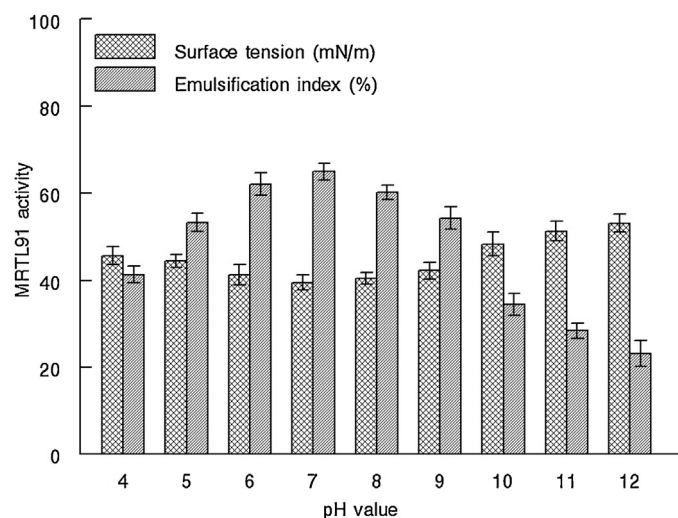


Fig. 4. Stability study of biosurfactant derived from *L. helveticus* at different pH with conviction of surface tension measurement and emulsification index.

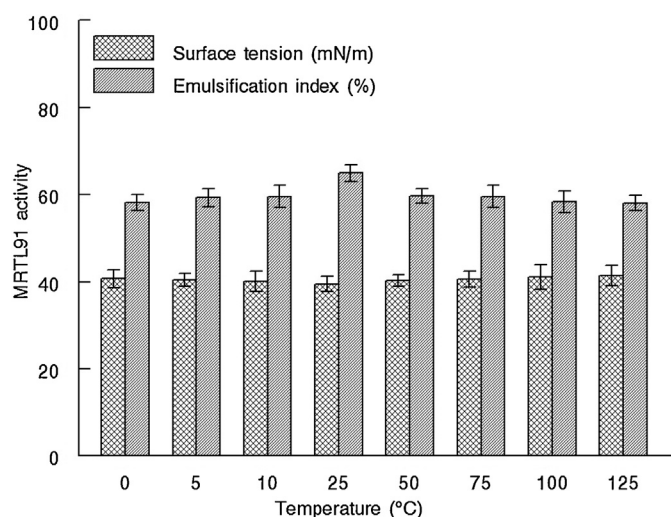


Fig. 5. Stability study of biosurfactant derived from *L. helveticus* at different temperature with conviction of surface tension measurement and emulsification index.

attained with the concentration of 25 mg ml⁻¹ of added to the growth medium. Biosurfactant incorporated surface cleaning strategies could be a newer generation solutions for extremely sophisticated lab and biomedical surfaces. The current study recognized a stage onwards for emergent alternative strategies for the prevention of microbial colonization on surgical equipment's and silicone rubber prostheses.

3.7. Stability

The utility of biosurfactants mainly depends on their behavior at diverse temperatures and pH exposures. In array to study the effect of pH on biosurfactant steadiness, different samples of biosurfactant were exposed with a concentration of 50 mg ml⁻¹ at different pH values (ranging 4–12). As can be seen in Figs. 4 and 5, the minimum surface tension value was observed at pH 7 (41.8 mNm⁻¹) with maximum emulsification value i.e. 65%. Biosurfactant retained activity over a pH range from 4 to 12 with a minimal divergence in surface tension values. The pH values below and above 7 surface tension were found to be stable. The biosurfactants showed persistent activity even after treatment at 0–125 °C for 30 min. The biosurfactant was found to be successfully stable over the different temperature range with constant reduction in surface tension and stable emulsification. The maximum reduction in surface tension was at 25 °C i.e. 65%. Heat treatment (autoclaving at 120 °C for 15 min) on biosurfactant showed no substantial changes in their surface and emulsifying activities [55–60]. Biosurfactant obtained from *Pseudomonas aeruginosa* P4 isolated from petroleum contaminated soil was also found to be stable at different temperature and pH ranges [59]. Lipopeptides of *Bacillus subtilis* also documented with stability at elevated temperature and different range of pH [60–62]. The utility of biosurfactants as coating agents is reliant on their stability at different pH range as saliva can exhibit pH variations according to the patient diet.

4. Conclusion

In current study, we have demonstrated the antimicrobial and antiadhesive properties of the biosurfactant isolated from *L. helveticus* MRTL91 against various pathogenic and nonpathogenic microorganisms. The isolated strain of *L. helveticus* has not been reported for biosurfactant production earlier and the

functional properties of isolated biosurfactant is the key finding of the study. The results obtained recommended the potential use of the biosurfactant from *L. helveticus* MRTL91 as an alternative antimicrobial agent in the biomedical field for applications against pathogenic microorganisms, as well as in the skin care product, making it a suitable alternative to conventional antibiotics for further use in food and cosmetics field.

Conflict of interest

The author(s) declare that they have no competing interests.

Author's contributions

“DS carried out the experiments and drafted the manuscript. BSS carried out design and editing manuscript. All authors read and approved the final manuscript.

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