Anti-oxidative property of xylolipid produced by Lactococcus lactis LNH70 and its potential use as fruit juice preservative

Anti-oxidative Property of Xylolipid Produced by *Lactococcus lactis* LNH70 and its Potential use as Fruit Juice Preservative

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Abstract

In the present study, 20 Lactic acid bacteria (LAB) were isolated from different fruit juices, milk and milk products. Based on preliminary screening methods like emulsification index, oil displacement method, hemolysis and reduction in surface tension strain LNH70 was selected for further studies. It was evaluated for probiotic characteristics and identified by 16s rRNA sequencing as *Lactococcus lactis*, submitted to NCBI and accession number was obtained (MH174454). In addition, LNH70 was found to be stable over wide range of temperatures (10 - 45 °C), pH (3 - 10), NaCl (up to 9%), bile (0.7%) and phenol (0.1%) concentrations. Chemical composition of purified biosurfactant obtained from LNH70 was characterized by various physico-chemical analytical techniques and identified as xylolipid. Further, optimization studies at flask level revealed that lactose as carbon source, peptone as organic nitrogen, and inorganic nitrogen (ammonium sulphate) enhanced its production. Xylolipid biosurfactant exhibited anti-adhesion activity against food borne pathogens in *invitro* conditions. Its anti –oxidative property by 1, 1-diphenyl-2-picrylhydrazyl (DPPH), 2, 2'-azino-bis (3- Ethylbenzothiazoline-6-sulphonic acid (ABTS) and ferric reducing antioxidant power (FRAP) radical scavenging activity was found in range of 60.76 ± 0.5 to 83.5 ± 0.73%. Furthermore, xylolipid (0.05, 0.1, 0.3 mg/ml) when used for its potential as orange and pineapple juices preservation revealed miniature changes in the physio-chemical parameters evaluated in this study. However, the microbial population was slightly lowered when xylolipid was used at 0.3mg/ml after 5th day. Hence, this study supports the use of biosurfactant from *L. lactis* for its use as food preservative.

Key words: Emulsification index, surface tension, biosurfactant, antioxidant, biofilm, preservation.

1. Introduction

Biosurfactants are surface active-amphiphilic biomolecules with hydrophilic head (carbohydrate, cyclic peptide, phosphate) and hydrophobic tail (long-chain fatty acid or hydroxyl fatty acid) [1]. Bacteria, yeast and fungi mainly produce biosurfactants and bioemulsifiers [2], which are categorized into four main groups, namely glycolipids (xylolipid, rhamnolipids, sophorolipids and trehaloselipids), lipopeptides, phospholipids and polymeric surfactants [3]. Biosurfactants are advantageous when compared to synthetic surfactants, in terms of low toxicity, high biodegradability, compatibility with human skin, tolerance to temperature, pH and salinity [4]. Based on these advantages and ecofriendly nature, biosurfactants have gained potential use in food, pharmaceutical, agriculture, oil recovery, bioremediation and cosmetic industries [5]. Biosurfactants aid in solubility of water immiscible compounds, cell adhesion and aggregation, binding to heavy metals, as antiadhesive and antibiofilm agents [6]. Even though interest in biosurfactants is increasing, its production is limited because of process cost which depends on media components and other growth parameters (temperature, pH, NaCl and inoculum size) [7].

Probiotic bacteria like *Streptococcus thermophilus* A, *Lactococcus lactis* 53 and *Pediococcus dextrinicus* SHU1593 have been reported to produce various types of biosurfactants [8]. *L. lactis* a probiotic bacteria which is generally recognizedas safe (GRAS) can produce different secondary metabolites like biosurfactants, bacteriocins, acetic acid, lactic acid and diacetyl [9–11]. In addition, biosurfactants show anti-adhesive, anti-oxidative activity and disruption of preformed biofilm formed by pathogenic bacteria [12]**.** However, its characterization and use as food preservative is in preliminary stage.

Hence, the present study aims to isolate LAB from fruit juices, milk and milk products, screen for biosurfactant products and was assess for its ability to preserve orange and pineapple juices for a period of 10 days at 25 °C.

2. Materials and Methods

2.1. Isolation of LAB

Various commercially and locally available raw milk, milk products and fruit juice samples were collected in pre-sterilized screw cap tubes from local markets, Hyderabad and nearby villages of Sangareddy district (Telangana State, India). LAB were isolated from the above sources by serial dilution, plating on deMan, Rogosa, and Sharpe (MRS) agar medium (Himedia, India) amended with 1% calcium carbonate and incubated aerobically for 48 h at 37 °C to categorize lactic acid producers [13]. Further, selected bacterial isolates were streaked on bromo cresol purple plate (BCP) and incubated for 48 h at 37 °C for preliminary characterization [14]. Selected isolates were sub-cultured on MRS agar slants, stored at 4 °C and also preserved at -20 °C in 50% glycerol for further investigations.

2.2 Screening for Biosurfactant Activity by the Isolated Bacteria

For preliminary screening of biosurfactant activity, all 20 bacterial isolates were inoculated separately in 100 ml MRS broth in 250 ml flask and incubated at 37 °C for 72 h. Cell free supernatant was collected after centrifugation at 10,000 x g for 10 min. This supernatant was used for emulsification index (E24), oil displacement (OD) and to measure reduction in surface tension [15].

2.2.1 Emulsification Index (E24)

The emulsifying ability of culture supernatant was evaluated by E24 test. E24 was performed by mixing equal volume (2 ml) of culture supernatant and different hydrocarbons (engine oil and toluene) in test tubes, vortexed at high speed for 2 min and allowed to stand for 24 h [16]. Percentage E24 was calculated using the equation.

$$E24 = \frac{\text{Emulsified layer height}}{\text{Total height of the liquid column}} * 100$$

2.2.2 Oil Displacement Method

Oil displacement method was carried out by taking 30 ml distilled water in Petri plate, to this 20 µl of engine oil (synthetic diesel engine oil) was added to form a thin oil layer. Then, 10 µl of culture supernatant was gently placed on the center of the oil layer. The surfactant activity correlates with diameter of clearing zone of engine oil layer [17].

2.2.3 Hemolysis Test

Hemolysis test was carried out by spot inoculation of overnight grown cultures on sheep blood agar plates and incubated at 37 °C for 48 h. Colonies which showed clear zone of lysis were positive for biosurfactant production [18].

2.2.4 Surface Tension Measurement

Biosurfactant production was monitored by measuring reduction in surface tension of culture supernatant using a du Nouy ring type tensiometer (Surface Electro Optics, Republic of Korea). Surface tension of distilled water 72 mN/m was taken as standard value. Distilled water was used to calibrate tensiometer, then surface tension of supernatant was measured, by dipping Platinum ring in solution until equilibrium was attained [19].

2.3 Identification of the strain LNH70 based on 16S rRNA Gene Sequence

Molecular identification of the bacterial isolate LNH70, was determined by 16S rRNA sequencing (MACROGEN, Seoul, Korea). The16S rRNA sequence obtained was compared using BLAST (basic local alignment search tool), submitted to National Center for Biotechnology (NCBI) and accession number was obtained.

2.4. Evaluation of Probiotic Characteristics of LNH70

Transit tolerance to upper gastrointestinal tract (GIT) was evaluated by *in vitro* model simulating gastric and pancreatic conditions as described by Pisano et al. [20]. Briefly, overnight grown culture (LNH70) was harvested by centrifugation, inoculated to 10 ml gastric juice (pH 3) and incubated at 37 °C in shaker incubator to simulate peristalsis movement. After 90 min, 17.5 ml of synthetic duodenum juice with pH 7.4 and 4 ml of 10% oxgall were added to simulate passage into the upper intestinal tract. After 0, 90 and 180 min, 0.1 ml of gastric and pancreatic treated samples were plated on MRS agar plates to enumerate the bacterial count. Auto-aggregation and co-aggregation properties of LNH70 was determined according to method described by Sui et al. [21]. For aggregation studies, strain LNH70 cell suspension absorption was adjusted to 0.3 ± 0.05 at OD_{600} nm (A_o) with sterile distilled water in order to standardize the cell number to $10^7 – 10^8$ cells/ml. For auto-aggregation, optical density of aqueous phase was recorded after 24 h of incubation without vortexing (A_T). For co-aggregation study, equal volumes (2ml) of strain LNH70 and (2ml) pathogen cultures ($10^7 – 10^8$ cells/ml) were mixed, vortexed, absorbance was monitored at 0 h (A_o), 24 h of incubation at 37 °C (A_T) and percentage was recorded. Strain LNH70 was evaluated for its tolerance to different temperatures from 4 to 50°C, pH 2 to 12, osmotic stress tolerance 1 to 9%, phenol 0.1 to 0.5% and bile 0.1 to 1%. To assess the viability of the isolate optical density (OD) was recorded at 600 nm [22].

2.5 Production of Biosurfactant under Aerobic and Anaerobic Conditions and its Purification

Production of biosurfactant under aerobic conditions was carried out in 100 ml MRS broth medium prepared in 250-ml flask. Under anaerobic conditions, biosurfactant production was carried out in 100 ml serum vials with 90 ml MRS broth medium (nitrogen gas purged to replace oxygen). For aerobic and anaerobic biosurfactant production, the flasks/serum vials were inoculated with 1% (v/v) overnight grown culture (16 h) of strain LNH70 and incubated at 37 °C in orbital shaker (150 rpm) and static conditions respectively. After 96 h incubation, cell pellet was segregated from both aerobic and anaerobic conditions by centrifugation at 10,000 x g for 10 min at 4 °C [23]. Cell biomass of strain LNH70 was recorded using gravimetric measurement using 2 ml eppendorfs at 24, 48, 72 and 96 h. Supernatant was collected separately and acid precipitated to pH 2 using 6 N hydrochloric acid (HCl) and kept at 4 °C for 16 h. Then supernatant was mixed in 1:1 ratio with ethyl acetate in separating funnel, shaken vigorously for 10 min and allowed to separate two layers, upper organic layer that contained biosurfactant was collected and concentrated using rotary evaporator. The obtained crude biosurfactant was used for oil displacement and E24 (%) using engine oil and data was recorded for 24, 48, 72 and 96h. Column purification of the obtained biosurfactant was carried out using silica (G 60-120, SRL India) and separated by chloroform and methanol in 20:1 to 2:1 ratio.

2.6 Production of Biosurfactant using Different Carbon Sources

Production of biosurfactant was investigated using different carbon sources such as glucose, sucrose, maltose, lactose, fructose, galactose and xylose (Himedia, India). All the carbon sources were amended separately as 2% (w/v) in MRS broth medium (90 ml) taken in 100 ml serum vials. Overnight culture (strain LNH70) was used as inoculum [(1% (v/v)] and the vials were incubated at 37 °C for 96 h in static conditions. After 96 h cell pellet was separated by centrifugation (at 10,000 x g for 10 min at 4 °C) and supernatant was evaluated for reduction in surface tension and E24 was calculated using engine oil [24].

2.7 Effect of Nitrogen Sources on Biosurfactant Production

Based on the above study, lactose was selected as carbon source and amended with different organic and inorganic nitrogen sources for biosurfactant production using strain LNH70. Yeast extract, protease peptone, casein acid hydrolysis, meat extract, beef extract and soya bean meal (0.5 g/L, Himedia, India) were used as organic nitrogen source. Ammonium citrate, ammonium sulphate, sodium nitrate, potassium nitrate, ammonium oxalate and ammonium chloride (0.5 g/L, Himedia, India) were all tested as inorganic nitrogen source [25].

2.8 Optimization of Physical Parameters for Biosurfactant Production

Optimization of biosurfactant production was carried out at different temperature (25, 30, 37 and 45 °C), pH (3, 5, 7, 9 and 12) and salt [NaCl (w/v, 0, 1, 3, 5, 7 %)]. All the parameters were carried out in MRS broth medium supplemented with (2%) lactose as carbon source, (0.5 g/L) protease peptone as nitrogen source. Overnight culture (strain LNH70) was used as inoculum [(1% (v/v)] and the vials were incubated at 37 °C for 96 h in static conditions. After 96 h cell pellet was separated by centrifugation (at 10,000 x g for 10 min at 4 °C) and supernatant was evaluated for reduction in surface tension and E24 was calculated using engine oil [26].

2.9 Chemical Characterization of Biosurfactant

Preliminary characterization of column purified biosurfactant was carried out by TLC analysis. Biosurfactant was dissolved in methanol and spotted on silica plate (Merck DC, Silica gel 60 F_{254}). The mobile phase consisted of chloroform, methanol and water in (65:15:2 v/v) ratio. Once dried, the plate was developed with anthrone reagent

for detection of glycolipids, chamber saturated with iodine vapors was used for lipids detection and subsequently potassium dichromate reagent for xylose sugar[27]. For Fourier-transform infrared spectroscopy (FTIR) characterization, ten milligrams of biosurfactant were mixed with 100 mg of potassium bromide (KBr) and pressed to get translucent pellet. Infrared absorption spectra were scanned and recorded on FTIR spectroscopy (Bruker Tensor 27) in the range of 4000-10 cm^{-1}. Spectral resolution and wave number was recorded with accuracy of 4 and 0.01 cm^{-1} respectively [15]. Fatty acid analysis of biosurfactant was determined by GC-MS (Shimadzu, QP2020 NX), Helium gas was used as carrier at 0.8 ml/min with 20 and 50 Kilo Pascal pressure. Sample injection volume is 20 µl, initial column temperature was maintained at 100 °C and increased to 300 °C for 2 min [28]. Mass of the biosurfactant was analyzed with liquid chromatography–mass spectrometry (LC-MS, Agilent Technologies, USA) using HPLC equipped with C18 column (Phenomenex, 250 X 4.60 mm). The mobile phase contained (A) of water spiked with 0.05% formic acid (v/v) and acetonitrile (B). Flow rate of the mobile phase was 0.5 ml/min and gradient method was employed: 5% B ramped to 80% in 12 min (liner). Fragmentor voltage calibrated at 135 volts and data was obtained by scanning from m/z 50-1000 [29].

2.10 Applications of Biosurfactant

2.10.1 Anti-adhesive Assay

Anti-adhesive assay of biosurfactant was studied as methodology described by Sharma et al. [30]. Pathogenic indicator strains used in this study were *Klebsiella pneumonia (*ATTC 70603), *Enterococcus faecalis* (ATTC 29212), *Bacillus cere*us (ATTC 11770), *Salmonella arizonae* (ATTC 13314), *Salmonella enterica* Subsp. *Enterica* serotype *Abony* (29.56%), *Salmmonella enterica* Subsp. *Enterica* serovar *poona* (26.78%), *Salmonella enteritidis* (ATTC 13076), *Salmonella enterica* (MTCC 733), *Escherichia coli* (ATTC 25922) and yeast, *Candida albicans* (ATTC 14053). The Wells of 96 well polystyrene plate were loaded with 200 µl of different concentrations of Biosurfactant (ranged from 5, 10, 15 and 20 mg/ml). The plate was incubated at 4 °C for 16 h and subsequently washed with phosphate buffer saline (PBS) pH 7. Control wells (without biosurfactant) were maintained and treated with PBS only. Later on, an aliquot of 200 µl individual pathogen cell suspension [(10^7 cells/ml, (OD 0.2)] prepared using active culture of pathogenic strain was inoculated in 3 ml Luria Bertaini broth)] were added to each well separately and incubated at 4 °C for 4 h. Adherent cells were fixed by addition of 200 µl methanol per well, stained with 200 µl of 2% crystal violet. Plate was air dried and then 200 µl of 33% (v/v) acetic acid was added per well to solubilize the dye bound to microbial cells. Optical density (OD) of each well was measured at 595 nm (Epoch Microplate Spectrophotometer, BioTek Instruments).

Percentage anti-adhesion activity is calculated by.

$$\%\ \text{anti} - \text{adhesion activity} = 1 - \left(\frac{\text{Asample}}{\text{Acontrol}}\right) * 100$$

Where, A_{sample} represents absorbance of biosurfactant treated well, $A_{control}$ represents absorbance of untreated well.

2.11 Evaluation of Antioxidant Activity of Biosurfactant

Different concentrations of biosurfactant (0.5, 1, 3 and 5 mg/ml) was dissolved in methanol and used to carry out 1, 1-diphenyl-2-picrylhydrazyl (DPPH), 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and ferric reducing antioxidant power (FRAP) assays. In DPPH assay, 500 µl of different concentrations of biosurfactant were mixed with 125 µl freshly prepared 0.3 mM DPPH solution. To the above mixture, 375 µl pure ethanol was added,

incubated in dark for 30 min and absorbance was recorded at 520 nm. For ABTS assay, 5 µl different concentrations of biosurfactant were mixed with 198 µl ABTS solution, incubated for 10 min and absorbance was recorded at 734 nm. In FRAP assay, reaction mixture was prepared by mixing (2 µl) different concentrations of biosurfactant and 198 µl FRAP reagent. Absorbance was recorded at 595 nm after 10 min of incubation. Ascorbic acid is used as positive control (1 mg/ml) [31].

Percentage scavenging activity of biosurfactant was calculated by formula.

$$\text{Scavenging activity \%} = \frac{\text{A control} - \text{A test sample}}{\text{A control}} * 100$$

2.12 Preservation of Orange and Pineapple Juice with Biosurfactant

Fresh orange and pineapple juices were purchased from the local market of Hyderabad, Telangana state, India. 50 ml of juice samples were dispensed into the sterile serum vials and closed with cotton plugs. Biosurfactant was dissolved in 0.5 ml ethanol and then mixed with juice samples, resulting in three different concentrations of 0.05, 0.1 and 0.3 mg/ml respectively. Chemical preservative benzoic acid (sodium benzoate) was added to fruit juice (0.1 mg/ml) as positive controls. A total of six treatments (T) were carried out namely, T_1 blank control (juice plus sterile distilled water), T_2 positive control (juice plus 0.1 mg/ml benzoic acid), T_3 (juice plus 0.05 mg/ml biosurfactant), T_4 (juice plus 0.1 mg/ml biosurfactant), T_5 (juice plus 0.3 mg/ml biosurfactant) and T_6 (juice plus 10^6 *L. lactis* cells/ml). Treated juice samples were incubated at 25 °C for 10 days and the quality of the samples were examined on 1st and 10th day respectively [32].

2.12.1 Effect of Biosurfactant on Bacterial and Yeast Growth

One milliliter of juice sample was serially diluted and plated (0.1 ml) on nutrient agar (NA) for bacteria and yeast extract peptone dextrose (YPD) agar plates for yeast count. Inoculated plates were incubated at 37 °C for bacterial growth, 30 °C for yeast growth (24 h) and CFU/ml was counted. The effect of different concentrations of biosurfactant and benzoic acid on bacterial and yeast growth were studied on 1st and 10th day [32].

2.12.2 Physical-chemical Analysis of the Stored Juice Samples

Treated juice samples were further, studied for pH, total acidity, total soluble solids (TSS), fiber, fat, vitamin C, total sugars, protein, mineral content like Magnesium, Potassium, phosphates as (PO_4) and calcium were estimated on 1st and 10th day. Total acidity was assessed by phenolphthalein titration method. Each (5 ml) juice sample was diluted with sterile distilled water to 25 ml and added with two drops of phenolphthalein indicator and titrated against 0.1 N NaOH solution, until pink color was formed. Titratable acidity conveys the amount of malic acid (grams) present in 25 ml juice samples. 2, 6-dichloroindophenol titrimetric method was used to measure vitamin C present in juice samples. Diluted (25 ml) juice sample was titrated with (0.1%) 2, 6-dichloroindophenol until pink color was formed. Vitamin C was expressed as milligrams of ascorbic acid per 25 ml juice samples [33]. Digital refractometer (MA 871 Digital Brix, Milwaukee Electronics, Europe) was used to measure total soluble solids (TSS) and values were reported as $^{\circ}$Brix. Total sugars present in juice samples were measured by phenol-sulfuric acid method and protein content was estimated by Lowry method. Fiber, fat and mineral content were measured according to Food Safety and Standards Authority of India (FSSAI) methods [34].

2.13. Statistical Analysis

All the experiments were conducted in triplicates and the results showed are mean of three independent experiments showing steady results. The experimental data was statically analyzed using ANOVA at ($p < 0.05$) significance, followed by comparative analysis by Duncan multiple range test (DMRT) using SAS 9.3. The graphs were created by OriginPro 2015.

3. Results

3.1 Sample Collection and Isolation of LAB

A total of 20 bacterial isolates showed zone of clearance due to acid production on MRS agar medium. All 20 bacterial isolates showed yellow coloration on BCP agar plates with in 48 h which conformed acid production (Fig. S1). All the 20 bacterial isolates were used for further studies.

3.2 Screening for Biosurfactant Activity for the Isolated Bacteria

The highest E24 was shown by one of the 20 bacterial isolates LNH70 with toluene (74%) followed by engine oil (70%). Bacterial isolate LNH70 showed maximum oil displacement activity (4.2 cm) in 5 seconds followed by LNH34 and LNH52 with 3.5 and 3.0 cm respectively (Fig. S2). No hemolysis (γ-hemolysis) was observed on blood agar with LNH70 whereas, LNH34 and LNH52 showed α-hemolysis (Fig. S2). γ-hemolysis could be due to inability of biosurfactant to diffuse into surrounding media, which prevented hemolytic activity. Isolate LNH70 showed Surface tension reduction up to 33 ± 0.5 mN/m followed by LNH34 and LNH52 with 37.41 ± 0.4 and 39 ± 0.9 mN/m respectively (Table S1). Based on the above screening methods, LNH70 showed maximum biosurfactant activity and was selected for further studies.

3.3 Identification of LNH70 based on 16S rRNA Gene Sequence

Sequence analysis of 16S rRNA gene sequence was carried out and compared with the existing sequences in BLAST, NCBI and the isolate was identified as *Lactococcus lactis*. The sequence was submitted to NCBI under the name *Lactococcus lactis* LNH70 and accession number obtained was MH174454. Phylogenetic tree was constructed in MEGA XI software using neighborhood joining method and shown in Fig. S3.

3.4 Evaluation of Probiotic Characteristics

Strain LNH70 showed resistance to pH 2 of gastric juice with 95% survival rate (8.36 log CFU/ml) after 90 min incubation and exposure to artificial duodenum juice (pH 8) in small intestine showed 60% survival (8.17 log CFU/ml) after 180 min of incubation. Strain LNH70 exhibited 77% auto-aggregation and varied co-aggregation with different food borne pathogens, *Salm. enterica* (34.13%) followed by *Salm. arizonae* (3.14%), *Salm. abony* (29.56%), *Salm. poona* (26.78%), *Salm. enteritidis* (24.85%), *B. cereus* (28.47%) and *Ent. faecalis* (21.69%). Strain LNH70 showed tolerance to temperature (10-45 °C) with maximum growth at 37 °C, pH (3-10) optimal at pH 7, salt (NaCl - 9%), phenol (0.1%) and bile concentration (0.7%).

3.5 Production of Biosurfactant under Aerobic and Anaerobic Conditions and its Purification

Cell biomass recorded was 1.6 – 6.5 mg/ml and 1.2 to 5.1 mg/ml under anaerobic and aerobic conditions respectively. In oil displacement method, supernatant obtained from anaerobically incubated culture (24 to 96 h) exhibited 1.4 to 4.7 cm increase in displacement of engine oil whereas, supernatant from aerobic conditions (24 to

96 h) showed 1.2 to 4.2 cm. Simultaneously, E24 with engine oil was higher under anaerobic (80%), when compared to aerobic conditions (75%) (Table 1).

3.6 Production of Biosurfactant using Different Carbon Sources

Among the seven different carbon sources screened, lactose favored highest production of biosurfactant with 75 ± 0.4% E24 after 96 h incubation. Carbon source ranking for biosurfactant production as follows: glucose (E24 73 ± 1.14%), maltose (70 ± 1.84%), sucrose (67 ± 0.83%), galactose (65 ± 0.74%), fructose (60 ± 0.86%) and xylose (53 ± 0.2 EI %). The highest reduction in surface tension was favored when lactose (32 ± 0.5 mN/m) was used whereas, lowest reduction in surface tension was obtained with xylose (45 ± 0.04 mN/m) (Fig. 1A).

3.7 Effect of Nitrogen Sources on Biosurfactant Production

Among the different organic nitrogen sources screened, protease peptone favored highest biosurfactant production with E24 (78 ± 0.8%) and least was with casein acid hydrolysis (61 ± 0.89%) (Fig. 1B). Among the inorganic nitrogen sources, maximum biosurfactant was obtained with sodium nitrate E24 (75 ± 0.8%) and minimum with ammonium chloride (61 ± 0.79%) (Fig. 1C). Reduction in surface tension among the organic nitrogen sources tested ranged from 31 ± 0.8 mN/m to 39 ± 0.42 mN/m and inorganic nitrogen sources (34 ± 0.47 mN/m to 44 ± 0.33 mN/m).

3.8 Optimization of Physical Parameters for Biosurfactant Activity

Studies on optimization of physical parameters for both biosurfactant activity and reduction in surface tension revealed that 37 °C temperature, pH 7 were optimum. At temperature 37 °C E24 (74 ± 0.9%) and reduction in surface tension was 32 ± 0.86 mN/m (Fig. 2A). Also, initial pH of the culture medium affected biosurfactant production and at pH 7, an E24 (75 ± 0.34%) and reduction in surface tension to 32 ± 0.42 mN/m was observed (Fig. 2B). MRS broth without NaCl showed E24 (71 ± 0.7%) and reduction in surface tension to 32 ± 0.37 mN/m. At >3% NaCl, biosurfactant activity and surface tension were reduced (Fig. 2C).

3.9 Chemical Characterization of Biosurfactant

TLC analysis of biosurfactant showed green color band (retention factor 4.7 cm) with anthrone reagent, development of brown color band with iodine vapors which indicated the presence of lipid content, which inferred positive for glycolipid and further treatment with potassium dichromate reagent showed yellow color spot which specified xylose as the sugar component (Fig. 3A,B,C). FTIR spectra showed chemical composition of biosurfactant as sugar and lipid fractions. Most predominant bands were located at 3431 cm^{-1} (OH stretch of hydroxyl groups attached to ring structure), 2922 cm^{-1} (C-H stretch of CH_2 and CH_3), 1723 cm^{-1} (C=O stretch vibrations of carboxyl groups), 1458 cm^{-1} (C=H stretch of ring structure), 1128 cm^{-1} (C-O stretch between carbon atom of ring and oxygen) and 1047 cm^{-1} (C-H bending) these stretches confirm the glycolipid type of biosurfactant (Fig. 3D). Based on the GC-MS analysis, retention time 9, 12 Octadecanoic acid ($C_{18}H_{32}O_2$) were found as major fatty acid component with retention time 19.66 min and molecular weight 294 g/mol, followed by Octadecanoic acid ($C_{18}H_{36}O_2$) and Hexadecanoic acid ($C_{16}H_{32}O_2$) (Table S2). Mass analysis of glycolipid biosurfactant showed approximate molecular weight of 493 g/mol (Fig. 3E).

3.10 Applications of Biosurfactant

3.10.1 Antiadhesive Activity

Biosurfactant extracted from strain LNH70 exhibited anti-adhesive activity against all the pathogenic microorganisms. Biosurfactant (at 20 mg/ml) exhibited highest anti-adhesive activity against Gram positive bacteria (*B. cereus*) 86 ± 0.32% and least with Gram negative bacteria (*Kl. pneumonia*) 44 ± 0.12%. Anti-adhesive activity against different *Salmonella* spp. showed in the range from 71 ± 0.13 to 76 ± 0.42% and yeast, *C. albicans* exhibited 41 ± 0.26% (Table 2).

3.11 Antioxidant Activity

Radical scavenging activity of biosurfactant from *L. lactis* (at 5 mg/ml) was 81 ± 0.7% by DPPH and 82 ± 0.93% by ABTS assay. Similar results were observed in FRAP assay, as concentration of biosurfactant increased from 0.5 to 5 mg/ml, scavenging activity increased from 61 ± 0.65 to 83 ± 0.73%. In the above three scavenging assays, reduction of free radicals was observed in a concentration dependent manner. Whereas, positive control (ascorbic acid) showed maximum radical scavenging activity of 93.12 ± 0.8% at 5 mg/ml concentration (Fig. 4).

3.12 Preservation of Orange and Pineapple Juice with Biosurfactant

3.12.1 Effect of Biosurfactant on Bacterial and Yeast Growth

Bacterial and yeast count was not uniform in all the treated juice samples. During 10 days of incubation, pineapple juice favored more microbial growth compared to orange juice. Chemical preservatives sodium benzoate (T_2) completely inhibited microbial load on 10^{th} day, when compared with strain LNH70 (T_6) and lower concentrations of biosurfactant (T_3 and T_4). Increase in biosurfactant concentration from 0.05 to 0.3 mg/ml decreased both bacterial and yeast growth in dose dependent manner. Biosurfactant concentration 0.3 mg/ml (T_5) completely inhibited microbial growth in both the juice samples up to 10^{th} day of storage. Control sample (T_1) exhibited increased microbial growth from first day onwards (Table 3).

3.12.2 Physico-Chemical Analysis of the Stored Juice Samples

The physico-chemical properties of juice samples can be used to determine their freshness. Initial pH of orange juice sample was acidic (5.4) compared to pineapple (6.2). The pH of both juice samples decreased with increased storage period except for sodium benzoate (T_2) and 0.3 mg/ml biosurfactant treatment (T_5). Initial total titratable acidity (TTA) of pineapple and orange juices was 0.23 – 0.24%, however, there was moderate variation of TTA in both juice samples, when stored for a period of 10 days, except sodium benzoate (T_2) and 0.3 mg/ml biosurfactant treatment (T_5). Initial total soluble solids (TSS) present in both the juice samples was 15 Brix. To a large extent, the TSS reflects the sugar content in the juice samples. A decrease in TSS was noticed in all the treatments of both juice samples except sodium benzoate (T_2) and 0.3 mg/ml biosurfactant treatment (T_5). In treatments T_2 and T_5 decrease in TSS was not significant ($p > 0.05$) could be due to absence of bacterial growth. TSS was reduced to the lowest level in the sample treated with distilled water (T1) and varied among the different treatments used. Fiber content was found in the range 1.42 to 1.46% in pineapple juice and 0.50 to 0.53% in orange juice. No significant difference was noted in fiber and fat content in both juice samples from first day to 10^{th} day of storage (Table 4). The amount of vitamin C present in fresh pineapple and orange juice was 9.5 and 50 mg/100 ml respectively. Samples treated with different concentrations of biosurfactant (T_3, T_4 and T_5) and sodium benzoate (T_5) reduced vitamin C levels similar to that of control (Figure 5A). The level of total sugars present in fresh pineapple and orange juice samples was 7 and 8.45 g/100 ml respectively. A decrease in total sugars in all the treatments was correlated with microbial count

present in both juice samples. In treatments (T_2) sodium benzoate and (T_5) 0.3 mg/ml biosurfactant, the total sugars reduction was not significant, this could be due to the inhibition of microbial growth (Figure 5B). The average protein content in fresh pineapple and orange juice was 0.5 and 2 g/100 ml respectively. There was no significant change in protein content in both the juice samples during 10 days of storage (Fig. 5C).

3.12.3 Elemental Composition of Orange and Pineapple Juice

Mineral content in orange and pineapple samples were analyzed by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES). The four major elements which are typically found in both juice samples are Magnesium, Potassium, Calcium and Phosphate. There was no significant change ($p > 0.05$) in the elemental composition in both juice samples before and after storage period (Table S3).

4. Discussion

A total of 20 LAB were isolated from different source samples on MRS agar medium and BCP agar medium. Lee and Lee [35] stated as it was difficult to differentiate different strains of LAB on MRS medium, further confirmation of acid producers can be done on BCP agar medium. Among the 20 bacterial isolates tested for semi qualitative screening methods of biosurfactant activity, one potential isolate (LNH70) showed E24 74% with toluene, 4.2 cm oil displacement and γ hemolysis on blood agar plate. Similar screening methods like hemolysis on blood agar, E24 with hexadecane and oil displacement using vegetable oil were reported by Ghasemi et al. [8] for biosurfactant producing *Pediococcus dextrinicus* SHU1593. Oil spreading techniques and E24 are recommended as qualitative and quantitative assays to select biosurfactant producers. In addition, these screening methods need small volume of sample, are quick, simple to perform and do not require specialized equipment. Youssef et al. [36] discovered a link between hemolytic activity and biosurfactant production. However, due to sensitivity, non-reliability and the presence of compounds other than biosurfactants that may lyse red blood cells, this method is treated as a complementary method [37]. A reduction in surface tension using culture supernatant (after 96 h) was observed for strain LNH70 to 31 ± 0.42 mN/m. Biosurfactants accumulate at the interface of two immiscible fluids and lower the interfacial tension and permit the two phases to interact and mix easily. In comparison, Sharma et al. [15] reported biosurfactant from *Enterococcus faecium* reduced surface tension (40.02 mN/m) of fermented broth in 72 h.

. Previous studies reveal probiotic strain can tolerate 0.3% bile, which aid in colonization and metabolic activity [38]. In our study, strain LNH70 showed 0.7% bile tolerance which is quite high in comparison with previous reports from other *Lactobacillus* strains (0.3%) [39]. Likewise, phenol tolerance is another characteristic feature of a probiotic bacteria which is assessed to check resistance against phenolic metabolites in the intestine [38]. In this study, LNH70 was found to exhibit survival at 0.3% phenol for 24 h. Gastric juices in stomach (pH 3) and duodenal juice in small intestine (pH 8) are the main stress factors that decrease viability of probiotic bacteria [20]. The present study findings show that strain LNH70 could survive in conditions that simulated human gastro-intestine (*in vitro*). It's well explained that auto-aggregation, co-aggregation properties help probiotic bacteria to inhabit the gut, urogenital tracts and provides resistance to pathogen colonization. In this study, strain LNH70 showed 77% auto-aggregation and 21.69 to 34.13% co-aggregation with *Salmonella* spp. Previous report on *Lact. plantarum* M2 demonstrated 38.46% auto-aggregation and 35.30% co-aggregation with *E. coli* after 24 h incubation [40]. In continuation, strain LNH70 showed temperature, pH and NaCl tolerance similar to that of other LAB reported by Menconi et al. [41].

The production of biosurfactant using various growth conditions is significant since a slight change in the composition of critical nutrients leads to variation in the biosurfactant activity. In the current study, biosurfactant production was measured in terms of emulsification activity (E24) and reduction in surface tension. Among different carbon sources screened, lactose was the most suitable for biosurfactant activity. Nitrogen is the obligate component of MRS medium, that helps bacteria in synthesis of proteins, enzymes and other cellular components [42].Our results revealed that, organic nitrogen source (protease peptone) favored maximum biosurfactant production compared to inorganic nitrogen source. Hu et al. [42] reported similar results regarding carbon (lactose) and nitrogen sources (yeast extract) for biosurfactant production using *Vibrio* sps 3B-2. The metabolism (enzymatic activity) of microorganism is directly related to its optimum growth temperature and initial pH of the culture medium. In this study, temperature 37 °C and pH 7 was determined optimum for biosurfactant activities. Our results are in line with optimum temperature (35 °C) and pH (7) as reported by Najafi et al. [43] for biosurfactant production using *Paenibacillus alvei* ARN63. In the current study, strain LNH70 showed biosurfactant activity upto 3% NaCl concentration and retained its production up to 7%. This result was in agreement with Khopade et al. [26] who reported biosurfactant production from *Nocardiopsis* sp. B4 at 3% NaCl concentration and retained up to 12%.

In the current study, green color band with anthrone reagent and brown band with iodine vapors indicated a glycolipid type of biosurfactant. Further appearance of yellow color with potassium dichromate reagent on TLC confirms it as xylose sugar. Similar result about TLC analysis was reported by Shalini et al. [44] for glycolipid biosurfactant from endophytic *Acinetobacter* sp.ACMS25. The present results of FTIR, GC-MS and mass spectrometry analysis of biosurfactant revealed that it is glycolipid (xylolipid) in nature. Characterization of xylolipid biosurfactant from other LAB disclose similar functional groups, chemical bonds, fatty acid composition and molecular weight [28,45].

Biosurfactants from GRAS bacteria can be used safely as anti-adhesive agents in food, dairy industries and medical fields, where biofilms are formed on the structures, pipes and cause contamination. Biosurfactants can form a thin layer on surfaces and change adherence properties on the nature of the surfaces and inhibit the growth of microbial pathogens [46,47]. In this study, biosurfactant of strain LNH70 showed anti-adhesion activity against various pathogens at lower concentrations (20 mg/ml) used. In comparison, previous report of Gudina et al. [48], reported the use of biosurfactant from *Lactobacillus paracasei* A20 at 25-50 mg/ml concentrations is required to inhibit various medical pathogens. Awareness of safety and environmental friendly credentials led to increased interests in natural preservatives globally for potential use in the food and pharma sector. Giri et al. [49] reported the use of biosurfactant as potential antioxidant molecules. In our study, xylolipid biosurfactant showed radical scavenging activity with slightly varied results based on the method used. Our DPPH, ABTS and FRAP results are in line with antioxidant activity of Zouari et al. [50] who reported biosurfactant from *Bacillus subtilis* SPB1 showed similar outcome by DPPH (70.4%) and FRAP (80.32%) assays. Previous study of Vecino et al. [51] has reported that, fatty acid component of biosurfactant serve as natural antioxidant by preventing the formation of free radicals.

Biopreservation of foods using microbial derived products has gained importance in recent days to avoid the use of chemical preservatives and their remnants (sodium nitrate, propionate and sodium benzoate). Various microorganisms and their metabolites, like biosurfactants, have been suggested as preservatives or to extend the

shelf life of food [32]. Preliminary study on fruit juice (pineapple and orange) preservation at 25 °C for a period of 10 days was achieved with the presence of xylolipid of strain LNH70. It was observed that biosurfactant used at 0.3 mg/ml was at par the sodium benzoate (0.1 mg/ml) in certain physico-chemical parameters analyzed.

To our knowledge only few reports were available for *in vivo* preservation of fruit juice samples using biosurfactant from GRAS bacteria. But for searching an alternative to chemical additives, some natural products like lemon grass oil and *Eucalyptus globulus* oil were reported to inhibit yeast growth in mixed fruit juice [52]. From the present study, fruit juice preservation indicated that biosurfactant (0.3 mg/ml) completely inhibited microbial growth in stored juice (10 days at 25°C) which was comparable with that of sodium benzoate (0.1 mg/ml). In this study pH, TTA and TSS did not show any significant change during 10 days storage, after 0.3% biosurfactant addition which could be due to absence of microbial growth. Shi et al. [32] showed comparable results for TTA and TSS in the preservation of orange juice with iturin A (0.76 mg/ml) from *Bacillus amyloliquefaciens*. Vitamin C levels in the samples were found to be lower, which could be attributed to the presence of oxygen and oxidation reactions in the preserved juice sample [53]. Similarly in the present study, we also observed some reduction in vitamin C content in all the treated juice samples during 10 days storage. The use of xylolipid bisourfactant did not have any discernible difference in terms of fiber, fat and protein composition, this might be due to less content of protein or insolubility in juice samples.

5. Conclusion

In the present study, 20 LAB were isolated and screened for biosurfactant production. One potential bacterial strain LNH70 was selected for further study as efficient probiotic and biosurfactant strain. Lactose and protease peptone favored maximum bacterial growth and highest emulsification which indicated biosurfactant production. Chemical characterization studies reveal biosurfactant of *L. lactis* LNH70 as xylolipid biosurfactant. Further, biosurfactant of *L. lactis* LNH70 showed antiadhesive activity and anti-oxidative activity which propose its potential to be exploited in food and medical sector. Further investigations at larger scale and more sampling data may confirm the use of xylolipid as fruit juice preservative.

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Authors' contributions

LN, HB conceived the idea. JP contributed to methodology, resources. The article was written by LN with the inputs of HB, PKSMR, IMB. All the authors have agreed to the final version of manuscript.

Availability of data and materials

The 16s rRNA gene sequence of *Lactococcus lactis* LNH70 is available in NCBI database under accession number MH174454.

Conflicts of interest/competing interests

The authors do not have any conflict of interest

7. References

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Figure captions

1. **Fig. 1** Optimization of biosurfactant production using *Lactococcus lactis* LNH70. (a) Effect of different lab grade carbon sources (b) organic nitrogen and (c) inorganic nitrogen sources on production of biosurfactant with conversion of emulsification of index (E24) and surface tension values. All three experiments are carried out in deMan, Rogosa, and Sharpe (MRS) broth. Results represent average of triplicates and error bars represents ± standard deviation values

2. **Fig. 2** Optimization of xylolipid biosurfactnat production from *Lactococcus lactis* LNH70 Effect of (a) temperature (b) pH and (c) NaCl on biosurfactant production using deMan, Rogosa, and Sharpe (MRS) broth medium, with conversion of emulsification of index (E24) and surface tension values. Results represent average of triplicates and error bars represents ± standard deviation values

3. **Fig. 3** Chemical characterization of column purified biosurfactant extracted from *Lactococcus lactis* LNH70. Thin layer chromatogram (TLC): (a) Anthrone was used for detection of sugars (b) Iodine vapors was used for detection of lipid (c) Potassium dichromate was used for detection of xylose content (d) FTIR (Fourier transform infrared spectroscopy) analysis (e) Mass spectrometry analysis

4. **Fig. 4** Free radical scavenging activity of different concentrations (0.5 to 5 mg/ml) of xylolipid biosurfactant produced by *Lactococcus lactis* LNH70 on 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2, 2'-azino-bis (3- Ethylbenzothiazoline-6-sulphonic acid (ABTS) and Ferric reducing antioxidant power (FRAP) radicals and compared with that of Ascorbic acid as standard. Results represent average of triplicates and ± designates standard deviation values

5. **Fig. 5** Estimation of a) vitamin C b) total sugars c) protein content of pineapple and orange juices stored for 10 days at 25 °C. PAT: Pineapple treatments, OT: Orange treatments. T_1 blank control (juice plus sterile distilled water), T_2 positive control (juice plus 0.1 mg/ml benzoic acid), T_3 (juice plus 0.05 mg/ml biosurfactant), T_4 (juice plus 0.1 mg/ml biosurfactant), T_5 (juice plus 0.3 mg/ml biosurfactant) and T_6 (juice plus 10^6 *Lactococcus lactis* cells/ml), were set in this experiment. Results represent average of triplicates and ± specifies standard deviation values