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PRODUCTION AND CHARACTERIZATION OF BIOEMULSIFIER FROM PROBIOTIC *LACTOBACILLUS PLANTARUM*

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Abstract– Bioemulsifier production from a Probiotic *Lactobacillus plantarum* isolated from a rice based fermented product was studied. The optimization of production of bio emulsifier as a function of fermentation time in shaker and stationary conditions was carried out. The highest production of bio emulsifier with 38.63% decline in surface tension at 72 hoccurred under stationary conditions. The isolated bio emulsifier is partially purified for characterization and anlaysis of CMC (0.45 g l^{1}), oil displacement area, emulsification index, emulsifying activity and emulsion stability. Results showed that the bio emulsifier had good emulsifying property and FTIR analysis indicates the presence of proteins and polysaccharides fractions. Aqueous solutions of the bioemulsifier displayed antimicrobial activity and antiadhesive activity against the pathogens: *Escherichia coli* ATCC, *Escherichia coli* ETCC, *Yersinia enterocolitica* and *Staphylococcus aureus*. All the results indicate that the bioemulsifier produced from *L. plantarum* can be utilized in food formulation as a multiuse food additive.

INTRODUCTION

Probiotics are "livingmicrobes that, when consumed in an acceptable number as part of food, confer a health benefit on the host" (Anonymous, 2001). Bioemulsifiers or Biosurfactants are a structurally mixed group of molecules with surface-active properties secreted by microorganisms. Important groups of bio emulsifier synthesizing microorganisms are lactic acid bacteria from the genus *Lactobacillus*. Many studies on the production of bio emulsifiers using lactic acid bacteria (LAB) have been carried out (Moldes *et al*., 2007). The use of bioemulsifiers from Lactobacilli species is very promising because these human intestinal microbial communities are well known for their probiotic effect (Singh and Cameotra, 2004).

The emulsifying property of bioemulsifiers finds uses in several food industries (Banat *et al*., 2000). Numerous studies have assessed the stable emulsion formation by bioemulsifiers with edible fats and oils. An emulsifying agent with extracellular carbohydrate compound produced from *Candida utilis* has been effectively used for preparation of salad dressing (Shepherd *et al*., 1995).

Along with emulsification, bioemulsifiers show antiadhesive and antimicrobial properties which can be used in regulating the adherence of microorganisms to food contact surfaces providing safe and quality products to consumers (Singh and Cameotra, 2004; Hood and Zottola, 1995). In view of all the properties revealed by bioemulsifiers, they can be proposed as multifaceted food additives for future, displaying emulsifier, antiadhesive, and antimicrobial activities concurrently. They are bound to have a significant influence and might open up novel avenues for their application in food industries (Shepherd *et al*., 1995).

This study is an attempt to produce bioemulsifier from *Lactobacillus plantarum*. So far, they have shown promising characteristics like: potency for producing vitamin B12, β-galactosidase activity for lactose intolerance sufferers, production of short chain fatty acids like lactate and butyrate, and silver

nano-particles production. Here the production, optimization, purification, and characterization of the isolated bioemulsifier is studied. Additionally, the potential of the selected potent probiotic isolate *L. plantarum* for bioemulsifier production as a supplementary property is also evaluated.

MATERIALS AND METHODS

Production of Bioemulsifier

L. plantarum CFR 2194, isolate from 'kanjika' an ayurvedic rice based fermented product was used for the bioemulsifier production. The inoculum was prepared from *L. plantarum* preserved at -60 °C in glycerol (40% v/v) supplemented MRS broth for cryoprotection. Two tubes containing 9 ml of MRS broth was inoculated with 1 ml of bacterial suspension and incubated at 37 °C for 18 h, which was inoculated to 180 ml of MRS media and incubated at 37 °C for 18 h. 20 ml of the bacterial culture was used to inoculate nine 500 ml flasks, each containing 180 ml of MRS media. The samples were removed at 24 h, 48 h and 72 h from flasks incubated at 37°C. This procedure was performed in two batches one set was kept in stationary conditions and the other in a shaker at 150 RPM.

Extraction of Bioemulsifier

Cells were harvested by centrifugation (10000×g, 15 min, 10°C) and the cell free supernatant (CFS) is collected for surface tension measurement. The gathered cells were washed in demineralized water and dispersed in 100 ml of pH 7.0 phosphate-buffered saline (PBS). The suspension was kept at room temperature for 2h with mild shaking to facilitate the release of bioemulsifier (Rodrigues *et al.*, 2006). The suspension was centrifuged (10000×g, 15 min, 10°C) to separate the bacterial cells. The supernatant was filtered through a 0.22 μ membrane filter (Millipore, USA) to get a clear liquid. The production of bioemulsifier was confirmed by surface tension measurement of the clear supernatant.

Surface tension measurement

The production of Bioemulsifier was confirmed by determining the surface tension using the Kruss-K9 platinum ring type Tensiometer (Singh and Cameotra, 2004). The surface tension was measured at 20 °C after the platinum ring precisely comes in contact with the top layer of the test solution after equilibrium conditions were attained. The surface tension of distilled water was initially tested for calibration. All the measurement were taken in triplicate, and mean value was used to denote the surface tension. Sterile PBS with surface tension of 72.2 mN m^{-1} at 20°C was used as control.

Isolation, Purification and Structural Characterization of Bioemulsifier

Isolation of Bioemulsifier

The filtered supernatant (PBS extract) was dialyzed against demineralised water at 4°C using dialysis tube (Sigma, USA) with molecular weight cut off of 6.0 - 8.0 kDa. The dialysate was freeze dried (Coolsafe 55-4Pro; SCANVAC, India) and stored at -20 °C for further studies.

Purification of Bioemulsifier

Crude bioemulsifier was produced by gentle stirring of the cells in PBS suspension for 2h at room temperature. The cells and the released Bioemulsifier were separated by centrifugation at 10,000 g for 15 min. To ensure complete removal of all bacterial remnants, the supernatant was centrifuged again at 10,000×g for 10 min. At this stage, the crude Bioemulsifier was freeze-dried and stored at -20 °C. The collected freeze-dried crude Bioemulsifier samples were pooled and re suspended in demineralised water. The next step of purification was carried out by acid precipitation using concentrated HCl down to pH 2.0. After removing the supernatant, the precipitate was washed with acidic water (pH 2.0) twice and separated by centrifugation (4000 g). The acid precipitate was further resuspended in distilled water, freeze-dried and stored at -20 °C.

Fourier Transform Infrared Spectroscopy (FTIR)

The sample for FTIR analysis was prepared by dispersing the freeze dried bioemulsifier homogeneously in a matrix of KBr. The bioemulsifier (6 mg) and KBr (300 mg) were ground into fine powder using a small mortar and pestle. The finely ground powder was filled in the pellet holder and screw on top was stiffened softly to form the pellet.

The FTIR, IFS 25 model from Bruker, Germany was equipped with a Michelson interferometer and Deuterated TriGlycerine Sulfate (DTGS) detector with KBr window. The measurement of the spectrum within the range of 4000 – 400 cm^{-1} was performed by placing the sample pellet in the

holder. Sample was scanned at the resolution of 2 cm^{-1} at 0.1%T accuracy. KBr pellet was used as background reference.

Properties of Bioemulsifier

Oil Displacement Test (ODT)

Surface-active property of the freeze dried bioemulsifier was tested using oil spreading technique. The petriplate was filled with 50 ml of distilled water and 20 µl Kerosene was added on the surface. The crude bioemulsifier solution of varying concentrations 4 mg ml^{-1}, 10 mg ml^{-1} and 15 mg ml^{-1} was gradually added on the centre of the oil layer on water. The experiment included

i). a negative control or blank using 20 µl of distilled water and

ii). a positive control using 20 µl of standard surfactant (1 % Tween 80)

Efficiency of bioemulsifier produced was judged by the clear halo zone on oil which was visible, the larger the zone of spreading of oil, higher is the surface-active property of the sample. The diameter of clear zone was measured and the Oil Dispersion Area (ODA) was calculated using the equation 1 (Morikawa *et al.,* 2000).

$$ODA = (\pi/4) \times (Diameter)^2\ cm^2 \quad (1)$$

Determination of Critical micelle concentration (CMC)

A higher bioemulsifier concentration in the test sample provides a lower surface tension until the CMC point is reached. Surface tension was measured at different bioemulsifier concentration (Desai and Banat, 1997). The bioemulsifier of different concentration range (0.05 – 0.65 g l^{-1}) was tested for surface tension until the surface tension reduced no more. CMC was determined using a plot of surface tension as a function of bioemulsifier concentration. The point below which the surface tension does not decrease further even on addition of bioemulsifier is designated as CMC.

Emulsification index (EI)

Emulsification index was calculated based on the increase in height of emulsion. A mixture of freeze dried bioemulsifier (1 ml, 1 mg ml^{-1}), water (4 ml) and hydrocarbon / oil (6ml, kerosene, xylene, hexane, heptane, coconut oil and sunflower oil) were taken in same sized graduated glass test tubes with stopper. The mixture was vigorously shaken for 2 min. to achieve maximum emulsification. After 24 h period, the emulsion layer height and wholemixture height was noted to calculate Emulsification index (EI) using equation 2. Water and Tween 80 (1%) served as negative controland positive controlrespectively (Cooper and Goldenberg, 1987).

$$EI = \frac{\text{Height of emulsion layer}}{\text{Total height}} \times 100 \quad (2)$$

Emulsification Activity and Emulsion Stability (EA & ES)

Freeze dried bioemulsifier (1 mg/ml^{-1}) prepared in PBS (pH 7.0) was added to 7.5 ml of PBS along with 0.1 ml of substrate (kerosene, xylene, hexane, heptane, coconut oil and sunflower oil) in a screw capped tube. After vigorous shaking for 2min, absorbance was measured at 540nm. Emulsification Activity (EA) was defined as the measured optical density. After 24 h, absorbance was taken to calculate Emulsion Stability (ES) of the formed emulsion using equation 3 (Kim *et al.,* 2000).

$$\text{Emulsion Stability} = \frac{\text{Absorbance at 0h}}{\text{Absorbance after 1h}} \quad (3)$$

Antimicrobial Activity

The antimicrobial activity of 1.0, 2.0 or 4.0% (wt/v) aqueous solution of freeze dried bioemulsifier was assayed with the diffusion method using a well technique against indicatory bacteria: *Escherichia coli* ATCC, *Escherichia coli* ETCC, *Salmonella typhii*, *Yersinia enterocolitica* and *Staphylococcus aureus*. The cultures of the different bacteria (mentioned above) were prepared by adding the 5.0 ml of bacterial suspension to 100 ml of sterile Nutrient agar (HiMedia Pvt Ltd., India) and cooled to a temperature of 45 °C. After mixing, 20 ml portions of the culture medium were poured into sterile petri dishes solidified. Subsequently, 5 mm in diameter wells were cut under sterile conditions by means of a sterile cork-boring device and filled with 60 µL of the analyzed aqueous solutions of bioemulsifier preparation. The petri dishes were incubated at a temperature of 37 °C for 16 h. The diameters (mm) of bacterial growth inhibition zones around the wells were determined with respect to redistilled water for control (Golek *et al.,* 2009).

Antiadhesive Activity

The antiadhesive activity of the crude freeze dried bioemulsifier isolated from *L. plantarum* against a

few microbial strains were measured according to the procedure described by Reid *et al.* (1992). Crude freeze dried bioemulsifier (200 µl) were filled in sterile wells of 96-well flat-bottomed ELISA plate (HiMedia Pvt Ltd., India). Numerous bioemulsifier concentrations ranging from 3 to 50 mg ml^{-1}were subjected to testing.

The plate was incubated for a period of 18h at 4°C and later washed twice with PBS. Wells containing PBS buffer was taken as control. An aliquot of 200 µl of washed bacterial cells was added and incubated in the wells for a period of 4h at 4 °C. Unattached microbes were removed by washing the wells thrice with PBS. The adherent microbes were fixed with 200 µl of methanol (99% purity) per well, and after 15 min. Now the plates were emptied and left to dry. Then the plates were stained for 5 min with 200 µl of 2% crystal violet. Excess stain was rinsed out by placing the plate under running tap water. Then, the plates were air dried, the dye bound to the adherent micro-organisms was resolubilized with 200 µl of 33% (v D v) glacial acetic acid per well, and the absorbance of each well was measured at 595 nm. The microbial inhibition percentages at different bioemulsifier concentrations for each micro-organism were calculated as:

$$\% \text{ Microbial inhibition}_c = [\, 1 - (A_c/A_0)] \times 100 \quad (4)$$

where, A_c represents the absorbance of the well with a bioemulsifier concentration c and A_0 represents the absorbance of the control well. The antiadhesion assay evaluates the percentage of microbial adhesion reduction in relation to the control wells, which were set at 0% to indicate the absence of bioemulsifier and therefore of its anti-adhesion properties. In contrast, results with a negative percentage indicates the percentage increase in microbial adhesion at a given surfactant concentration in relation to the control. The anti-adhesion assay allows the determination of the crude bioemulsifier concentrations that are effective in decreasing adhesion of the microbes studied (*Escherichia coli* ATCC, *Escherichia coli* ETCC, *Staphylococcus aureus, Salmonella typhii and Yersinia enterocolitica*).

RESULTS AND DISCUSSION

Optimization of Conditions for Bioemulsifer Production

The reduction in surface tension of the PBS cell extract confirms to the ability of *L. plantarum* to produce bioemulsifier. The average surface tension readings of PBS cell extract after 24, 48 and 72 h of fermentation in MRS medium in shaking and at stationary conditions are presented in Table 1.

Table 1. The surface tension of Bioemulsifier solution from *L. plantarum* grown under stationary and shaking conditions

Fermentation	Surface tension of PBS cell extract, mN m^{-1}	
Time	Stationary	Shaking
Control PBS	72.2 ± 0.00	72.2 ± 0.00
24 h	64.46 ± 0.25	67.2 ±0.15
48 h	53.56 ± 0.35	56.6 ±0.26
72 h	44.30 ± 0.20	50.3 ±0.15

There was higher reduction in the surface tension of PBS cell extract obtained from cells grown under stationary condition (50.3 mN m^{-1}) when compared to control PBS (72.0 mN m^{-1}). The bioemulsifier production was highest at 72 h with a 38.63% decrease in surface tension under stationary conditions (Figure 1).

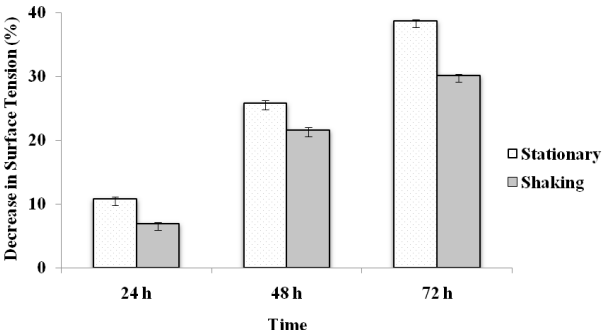

Fig. 1. Decrease in surface tension against control PBS at different time intervals.

CFS obtained after 24, 48 and 72 h yielded surface tension values in the range of 43 48 mN m^{-1}. This indicates only a negligible change in surface tension when compared to MRS control (50.8 mN m^{-1}). Hence it can be concluded that the production of bioemulsifier was mostly cell bound.

Isolation, Purification and Structural Characterization of Bioemulsifier

Isolation of Bioemulsifier

The PBS extract after dialysis was freeze dried to get the crude bioemulsifier powder. The bioemulsifier obtained has to be purified further to analyse its constituents and evaluation of the properties.

Purification of Bioemulsifier

The crude bioemulsifier was purified by acid precipitation and the purified bioemulsifier was used for characterization studies.

Fourier Transform Infrared Spectroscopy

The FTIR spectra obtained (Figure 2) established that the bioemulsifier is proteinaceous in nature. The recorded absorbance maxima at wavelengths of 3408.9, 1661.2, 1556.0 cm^{-1} typical of stretching bonds α N-H, stretching bonds CO-N and N=O indicates the presence of proteins in the sample analyzed. The presence of absorption peak at 2945cm^{-1} indicates presence of bonds occurring in aliphatic chains (-CH_3, -CH_2-).

The occurrence of a spectrum between wavelength range of 1300–900 cm^{-1} specifies the presence of the polysaccharide of bioemulsifiers. Since, strong absorption at a wavelength of 1087.2 cm^{-1} is characteristic of oscillations of C–O–C bonds.

Fourier transform infrared spectroscopy demonstrated that the chemical composition of bioemulsifier synthesized by *L. plantarum* was non-homogeneous. Bioemulsifier was composed of protein and polysaccharide fraction. Literature data indicate that only a few microorganisms synthesize the surface-active compounds as chemically pure ones.

Properties of Bioemulsifier

Oil Displacement Test (ODT)

The oil displacement method indirectly measures surface activity of anemulsifier sample when tested alongside oil, a large diameter is an indication of higher surface activity (Rodrigues *et al.*, 2006). In this study surface activity of the crude freeze dried bioemulsifier in different concentrations was measured in comparison with that of tween 80 and water.

The area of clearly formed zone is measured as the activity of bioemulsifier (Table 2). The diameter of the circle is 1.2 cm and 1.9 cm for 4 mg ml^{-1} and 10mg ml^{-1} concentration of crude bioemulsifier, their ODA equal to 1.13 cm^2 and 2.83 cm^2. The bioemulsifier solution with 15 mg ml^{-1} concentration dispersed the oil layer completely similar to that of the positive control Tween 80 (1%) indicating high surface active properties in higher concentration of bioemulsifier. Although the mechanism of oil displacement by bioemulsifiers has not been explained on the molecular level, this is a sensitive and easy method.

Determination of CMC

The value of surface tension decreases after the CMC is reached. When the bioemulsifier concentration is greater than the CMC, the surface tension remains constant at some minimum value. In order to determine CMC the surface tension at different bioemulsifier concentration was measured and a graph of surface tension and bioemulsifier concentration is plotted (Desai and Banat, 1997).

From Figure 3, it is clear that the surface tension does not reduce below 44 mN m^{-1} even when the concentration is increased; the corresponding

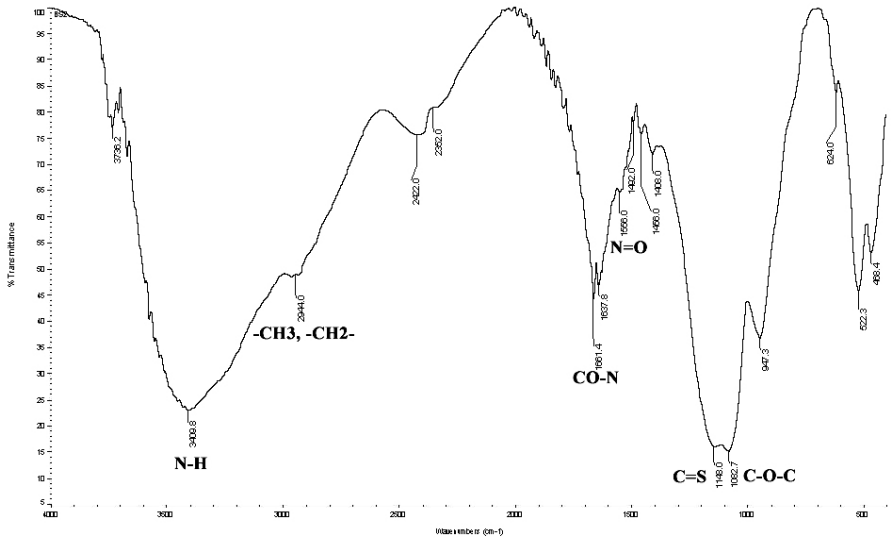

Fig. 2. FTIR spectrum of bioemulsifier

concentration of 0.45 g l^{-1}, beyond which there is no drastic change in the surface tension even on increasing the bioemulsifier concentration, is the CMC value of the bioemulsifier.

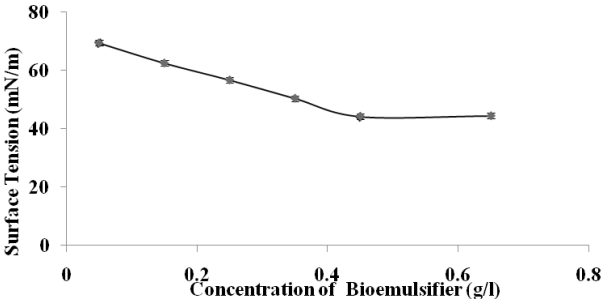

Fig. 3. CMC – Surface Tension at various concentration of bioemulsifier

Emulsification index (EI)

The emulsification index of the produced bioemulsifier (1 mg ml^{-1}) was tested against different substrates like kerosene, xylene, coconut oil, sunflower oil, hexane and heptane. Tween 80 was used as a standard. Among the hydrocarbons heptane gave the highest EI value of 38.2, followed by xylene (16.22) kerosene (15.62) and hexane (13.6). In vegetable oils, coconut oil and sunflower oil gave an EI value of 37.95 and 19.43. Hence the bioemulsifier has good emulsification with vegetable oils and can be used in food formulations (Figure 4).

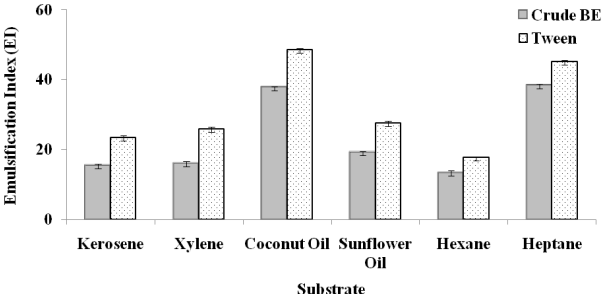

Fig. 4. Emulsification Index for different substrates

Emulsification Activity and Emulsion Stability (EA and ES)

The emulsification activity of the produced bioemulsifier was tested with different substrates (kerosene, xylene, coconut oil, sunflower oil, hexane and heptane). Emulsion stability was calculated based on the decrease in absorbance. From the graph, we observe that coconut oil and sunflower oil were efficiently emulsified. Furthermore, kerosene and xylene were considerably emulsified. Interestingly, EA with n-alkanes increased with the increase of the number of carbon atoms. Emulsions formed with vegetable oils, sunflower oil and coconut oil were more stable than emulsions formed with hydrocarbons (Figure 5 and 6).

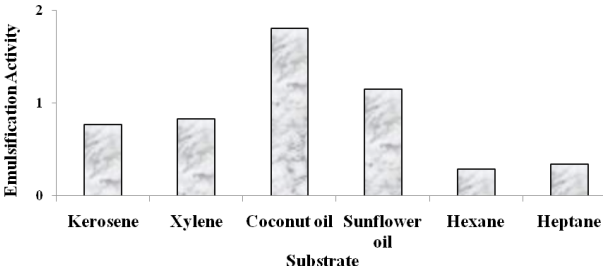

Fig. 5. Emulsification Activity for different substrates

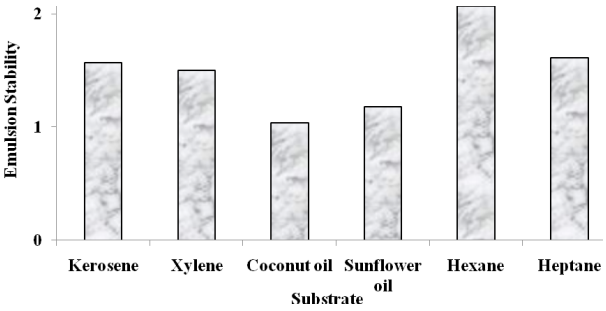

Fig. 6. Emulsification Stability for different substrates

Antimicrobial Activity

Bioemulsifier obtained from the lactic acid bacteria have shown antibiotic properties, which inclines

Table 2. Comparison of oil spreading efficiency of standard surface active agents and crude bioemulsifier

Agents	Zone of oil Spreading (cm)	ODA (cm^2)
Distilled water	0	0
Tween 80 (0.1%)	[a]	[N.F]
Crude Bioemulsifier (4 mg ml^{-1})	1.2	1.13
Crude Bioemulsifier (10 mg ml^{-1})	1.9	2.83
Crude Bioemulsifier (15 mg ml^{-1})	[a]	[N.F]

[a]Complete dispersion of oil layer, [N.F]Not feasible to calculate

that group of chemical compounds for potential application in the production of antibiotics (Rodrigues *et al.,* 2004). Bearing in mind the above findings, experiments were conducted to determine the antimicrobial activity of preparations of bioemulsifiers synthesized by *L. plantarum* against *Escherichia coli* ATCC, *Escherichia coli* ETCC, *Salmonella typhii, Yersinia enterocolitica* and *Staphylococcus aureus.*

The antimicrobial activity of the preparations of bioemulsifier synthesized by *L. plantarum* increased with their concentration in aqueous solution (Table 3). The results obtained shows best antimicrobial activity in 4 mg ml^{-1} and 25 mg ml^{-1} aqueous solutions of bioemulsifier preparations against *Escherichia coli* ETCC and *Staphylococcus aureus.* Positive results were achieved for *Escherichia coli* ATCC and *Yersinia enterocolitica* with the use of 25 mg ml^{-1} aqueous solutions that yielded inhibition zones of the growth of indicatory bacterial strains, thus indicating the antimicrobial activity of the preparations of bioemulsifiers synthesized by *L. plantarum*. Both the concentrations of the bioemulsifier were not able to inhibit the growth of *Salmonella typhii.*

Table 3. Antimicrobial activities of the crude bioemulsifier from *L. plantarum*.

Microorganism	Zone of Inhibition	
	4 mg ml^{-1}	25 mg ml^{-1}
Escherichia coli ATCC	-	+
Escherichia coli ETCC	++	+++
Salmonella typhii	-	-
Yersinia enterocolitica	-	++
Staphylococcus aureus	+	++

Results represent mean of three independent experiments carried out. Positive (+) sign indicates inhibition of microbial growth, whereas negative (–) sign indicates inability to inhibitthe growth of the tested microorganisms; + means Zone of inhibition around 5 mm around the application of bioemulsifier, ++ means zone of inhibition around 5–10 mm around the application of bioemulsifier, +++ means zone of inhibition more than 15 mm around the application of bioemulsifier.

Antiadhesive Activity

The antiadhesive activity of the bioemulsifier was evaluated against a few pathogens listed in Table 4. The bioemulsifier showed antiadhesive activity against all the tested microbes, but the antiadhesive effect depends on the concentration and the microbes tested.

Table 4. Antiadhesive properties of crude bioemulsifier from *L. plantarum*.

Microorganism	Bioemulsifier (mg ml^{-1})	
	4.0	25.0
Escherichia coli ATCC	8.04 ± 0.2	54.43 ± 0.1
Escherichia coli ETCC	10.65 ± 0.1	56.78 ± 0.3
Salmonella typhii	13.92 ± 0.1	61.60 ± 0.2
Yersinia enterocolitica	15.23 ± 0.3	64.72 ± 0.4
Staphylococcus aureus	25.9 ± 0.1	67.18 ± 0.2

The antiadhesive activity was higher against *Staphylococcus aureus* (67.18 ±0.2% inhibition) for a bioemulsifier concentration of 25 mg ml^{-1}. For *Yersinia enterocolitica* the highest antiadhesive percentages were (64.72 ± 0.4%), whereas the lowest were for *Escherichia coli* ATCC (54.43 ± 0.1%). Regarding the other pathogenic bacteria, high antiadhesive percentages were obtained for *Escherichia coli* ETCC (56.78 ± 0.3%) and *Salmonella typhii* (61.60 ± 0.2%).

Negative controls were set at 0% to indicate the absence of bioemulsifier. Positive percentages indicate the reductions in microbial adhesion when compared to the control, and negative percentages indicate increased microbial adhesion. Results are expressed as means ± standard deviation of results from triplicate experiments.

CONCLUSION

Bioemulsifier production from probiotic *L. plantarum*, optimization of the production as a function of fermentation time is carried out. Further, characterization studies and evaluation of different properties were done. The studies indicate that the production of bioemulsifier is more when *L. plantarum* is grown for 72 h at stationary conditions.

Isolation and purification processes yielded a partially purified bioemulsifier. The FTIR studies have shown spectrum bands typical of proteins and polysaccharides. CMC of the bioemulsifier was found to be 0.45 g l^{-1}. The EI, EA and ES values of the bioemulsifier have confirmed their emulsification property. Additionally, they have antimicrobial and antiadhesive properties which enable them to be used as multipurpose food additives.

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