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Screening and Characterisation of Biosurfactant Producing Lactic Acid Bacteria

Mitali H. Tillawala[1], Dr. Manisha N. Shah[2]

Shree Ramkrishna Institute of Computer Education & Applied Science, Surat- 395001, India

Abstract: ***Biosurfactant is a surface active compound having amphiphilic nature that reduce surface tension and interfacial tension between two insoluble phases. This study is intended to isolate lactic acid bacteria (LAB) and their capabilities to produce biosurfactants. LAB is widely found in milk and milk products, oil contaminated soil, and fruits etc., and produce biosurfactant during their late exponential and stationary phase of growth. Total 10 samples were collected from various milk and milk products and studied for lactic acid bacteria for production of biosurfactant. LAB are isolated using sterile MRS agar supplemented with 1% calcium carbonate. Oxidase and catalase negative isolates showing hemolytic activity are screened for biosurfactant production. Modified MRS broth without tween 80 is used for the production of biosurfactant, under static Condition up to 72 hours and centrifuged the broth for obtaining cell free supernatant (CFS) and checked it for biosurfactant production by oil spreading method. Among 10 samples collected, 18 isolates were identified as lactic acid bacteria. 10 isolates among were screened and further tested for biosurfactant activity. Anti-adhesive activity of biosurfactant producing LAB was checked against Pseudomonas aeruginosa, Staphylococcus aureus. Thin layer chromatography was carried out to characterized the biosurfactant produced by LAB.***

Keywords: Lactic acid bacteria (LAB), Biosurfactant, Amphiphilic, Hemolytic activity, Anti-adhesive activity

1. Introduction

Various different microorganisms are capable of producing such kind of surface active agents having both 'hydrophilic' means water loving and 'hydrophobic' means water repellent moieties within the same molecule that show action at the interfaces of two immiscible phases[1]. Hydrophilic moieties contain acid, peptide cations, or a Biosurfactant is used as an alternative of chemically synthesized surfactants. Biosurfactant are surface active amphiphilic compound having both hydrophilic moieties contain cations or anions, mono-, di-, or polysaccharides and hydrophobic moieties contain unsaturated or saturated hydrocarbon chains or fatty acids. Because of this structure of biosurfactant, it able to reduce surface tension and interfacial tension between two phases [2].

Lactic acid bacteria belongs to the family *Lactobacillaceae* are concern for biosurfactant production. Because of their probiotic nature, it can be easily deal with any industrial processes and are widely used in fermentation of various dairy products. LAB are the normal flora of animal and human gastrointestinal tract and women vagina [3]. LAB are known for the production of organic acid, carbon peroxide, hydrogen peroxide and bacteriocin and now it can also be used for the production of adhesion inhibitor, Biosurfactant to protect environment from harmful effect of chemically synthesized surfactant [1].

Human pathogenic microorganisms like *Pseudomonas aeruginosa, Staphylococcus aureus* are causative agents for biofilm production. They are found to produce biofilm which is responsible for various diseases such as nosocomial infections like urinary, respiratory, peritonitis and septic arthritis etc. Biosurfactant producing LAB are now concerned to decrease such infections due to it's anti-adhesive activity. ([4] ;[5] ;[6]).

The aim of this study is to screening out biosurfactant producing lactic acid bacteria from milk and related Products and check it's anti-adhesive activity against different biofilm forming human pathogens.

2. Materials and Methodology

Collection of samples
Different milk and milk products, dairy waste effluent were collected for isolation of lactic acid bacteria ([7] ;[8] ; [9]).

Sample enrichment and Isolation of LAB
In case of raw milk samples, enrichment is carried out using MRS broth incubated at 37°c for 24 hours. Serially diluted samples from 10^{-1} to 10^{-6} and spread 0.1 ml of aliquot on sterile MRS (De man, Rogosa, Sharpe) agar plates supplemented with 1% $CaCo_3$. and in case of enriched sample directly streak it on sterile MRS agar plates. All plates were incubated at 37°c for 24 hours under aerobic and anaerobic conditions. Various colonies were picked up for further study. ([9] ; [10]).

Morphological and biochemical identification
Morphological identification of LAB isolates were done by gram staining, catalase test and oxidase test was carried out for biochemical identification [1].

Storage of LAB isolates
Isolates were stored on sterile MRS agar slant at 4°c and also store in MRS broth having 20% glycerol at 4°c ([3] ; [8]).

Screening of isolates for biosurfactant production
Blood agar was used for screening of biosurfactant producing LAB. Blood agar plate was inoculated and incubated at 37°c for 24 hours and observed for hemolysis. Isolates showing hemolysis were used for biosurfactant production [3].

Growth curve determination
1ml of 24 hours old culture of selected isolates was inoculated in 100 ml of sterile modified MRS broth without

tween 80 and incubated at 37°c up to 72 hours in static Condition. Observed bacterial growth at specific period of time by taking optical density at 600 nm [1].

Biosurfactant production

1 ml of overnight grown culture in modified MRS broth without tween 80 was used as inoculum to inoculate 100 ml of sterile modified MRS broth without tween 80 and incubated it at 30°c overnight in static Condition. Centrifuged the inoculated broth at 10000 rpm for 30 minutes to obtain cell free supernatant (CFS). In case of cell wall adherent biosurfactant, cell pallet was washed twice with Phosphate Buffer Saline (PBS), pH 7.2; then suspended in PBS and allowed it at room temperature for 2 hours with gentle stirring for biosurfactant release. Again centrifuged at 9000 rpm for 10 minutes to remove cell pallets and obtained bottom to collect biosurfactant adhere to cell surface [8].

Biosurfactant extraction

Supernatant and CFS were acidified to pH 2 with 6 N HCl and stored overnight at 4°c. Biosurfactant extracted three time with chloroform/methanol (2:1) and organic fraction was evaporated under vacuum. To recover biosurfactant, acetone was added and allow it to dry. Collected biosurfactant was dissolved in PBS buffer or 10% DMSO (dimethyl sulphoxide) [11].

Oil spreading method

30ml of water was poured in empty petri plate and added 20 microliter of motor oil to form thin layer of oil on the surface of water. 20 microliter of bottom/CFS is then added on oil layer to observe clear zone due to the biosurfactant present in CFS/bottom [12].

Emulsification index

1 ml of supernatant/bottom was added in a blank test tube.. 3 ml of distilled water and 1 ml of kerosene were also added. And mixture was vortexed for 2 minutes and placed overnight in refrigerator. Next day observed and calculated the height of emulsion and total height of mixture to calculate emulsification index [12].

Surface tension measurement

By using balance beam method, reduction in surface tension of cell free supernatant (CFS)/ bottom was calculated by using mathematical formulas.

Anti-adhesion activity

Precoating and co-incubation experiment was used to check anti-adhesive activity of LAB supernatant/bottom against different biofilm forming human pathogenic microorganisms. 96 Wells microtiter plates was used. 200 µL of CFS/bottom was added in each well and incubated it at 37°c for 24 hours. Next day decanted non adherent CFS/bottom and washed twice with 100 µL PBS pH 7.2. Add 150 µL of 24 hours grown test culture ($1.5 * 10^{-7}$ cfu/ml) and incubated at 37°c for 24 hours. After incubation, decanted non-binding microorganisms and washed twice with PBS. To fix the biofilm, 100 µL of 99% methanol was added for 15 min and allowed it to air dried. Then 100 µL 2% Crystal violet was added for 20 min for easy quantification of activity. Additional Crystal violet was removed and washed with tap water. To solubilize the stain particlesadhere to the microorganisms, 100 µL 33% glacial acetic acid was added and take optical density at 595 nm using ELISA autoreader and calculated % microbial adhesion using following equation [6].

% microbial adhesion = $1 - (OD_t/OD_0)*100$

OD_t = optical density of test

OD_0 = optical density of blank

Characterization of biosurfactant

Biosurfactants were identified by Thin Layer Chromatography. Silica plates were prepared with silica gel and the crude biosurfactant was spotted on the plate. Chloroform: water: methanol in ratio (65: 24: 4) was used as a solvent system for separating biosurfactants. Ninhydrin reagent and iodine vapours were sprayed to detect lipopeptide biosurfactant as a red spots and glycolipid biosurfactant as a yellow spot respectively [13].

3. Results & Discussion

Isolation and partial identification of LAB

LAB were successfully isolated from the different samples using MRS agar supplemented with 1% calcium carbonate. As shown in figure, After incubation clear zone surrounding colonies was observed due to the lactic acid production so it was confirmed that the selected eighteen isolates were LAB.

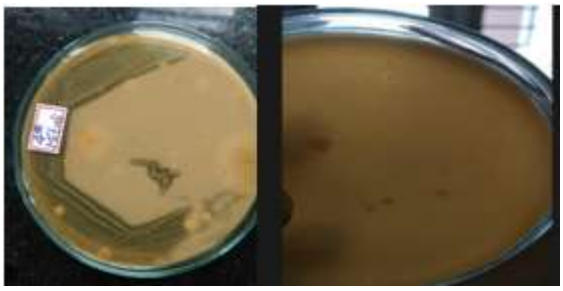

Figure 1: clear zone surrounding colonies on MRS agar + 1% $CaCo_3$

As per morphological identification, eight isolates are gram positive rods shaped organisms and other ten isolates are gram positive cocci (Fig: 1). All eighteen isolates were found catalase negative and fourteen isolates were observed oxidase negative during biochemical identification (Table: 1).

According to P. Kale, dairy products are essential sources of LAB. In accordance to K. Puphan, Isolation of LAB was carried out using MRS agar supplemented with calcium carbonate. In contrast to L. Fracchia, yeast were also isolated from various fruits samples, but according to L. Fracchia, isolates are partially confirmed by absence of oxidase and catalase enzymes & by morphological characteristics.

Screening of LAB for biosurfactant production

Microorganisms derived biosurfactant are known to haemolysed red blood cells. Thus hemolytic activity of isolates were targeted for screening. From 18 LAB isolates, total 10 isolates were screened out for further biosurfactant production (Fig: 2; Table:1).

Figure 2: hemolytic activity by biosurfactant producing LAB.

According to A. soni, hemolysis test was used to confirm that the isolates produce biosurfactant. Furthermore he also carried out Foaming activity, oil spreading method for the same. C. Cornea used drop collapse method for screening of biosurfactant producing isolates.

Table 1: Morphological and Biochemical identification & screening for Biosurfactant

Isolate no	Morphology	Gram reaction	Catalase test	Oxidase test	Biosurfactant production
1	Cocci	+	-	-	+
3	Cocci	+	-	-	
4	Cocci	+	-	-	
5	Rod	+	-	+	
6	Cocci	+	-	-	+
7	Rod	+	-	-	+
S-1 (2)	Cocci	+	-	-	+
S-1 (3)	Rod	+	-	+	
S2	Cocci	+	-	-	+
S3	Cocci	+		-	+
S-4 (1)	Rod	+	-	-	+
S-4 (2)	Rod	+		+	
BM1	Rod	+	-	-	+
BM2	Rod	+	-	+	+
DW1	Rod	+	-	-	+
DW2	Cocci	+	-	-	+

Growth curve & Biosurfactant production

LAB derived biosurfactant was produced during late exponential and stationary phase of growth. So it is necessary to linked growth curve of isolates with production of biosurfactant. As shown in figure upto 12 hours, there was lag phase of growth after that from 12 hours to 48 hours, it was an exponantional phase and then after decline of growth. According to growth
curve from 12 to 48 hours, there was highest production of biosurfactant observed that was confirmed by further tests.

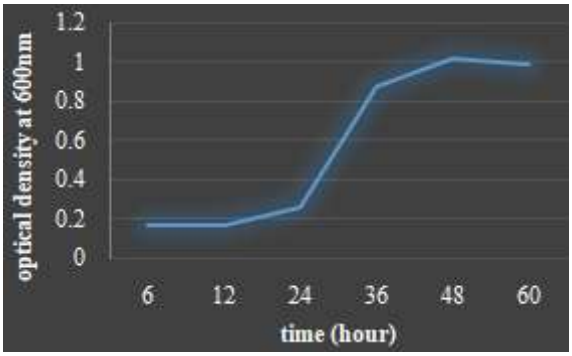

Figure 3: growth curve of LAB.

As per the study of L. Fracchia, during exponential phase and stationary phase, Biosurfactant production was maximum, which was started after 5 hours of incubation. But as per my study, after 24 hours exponential phase was started and after 36 hours there was maximum production of biosurfactant.

Confirmation test for biosurfactant production

1) Oil spreading method

In oil spreading method, supernatant and bottom was collected by centrifuging the broth of 24 hours old grown LAB. When drop of biosurfactant (supernatant/bottom) was added to the oil layer on the water, clear zone was observed (Fig: 4).

Figure 4: zone of clearance by crude biosurfactant

C. Cornea et al used kerosene & olive oil to detect biosurfactant producing isolates for oil spreading method. According to G. Matei et al, engine oil was used for this method to easy detection of clear dispersion zone of oil.

2) Emulsification index

When water and kerosene, two immiscible phases were mixed together with crude biosurfactant and as both hydrophobic and hydrophilic moieties present in the biosurfactant, it emulsify water and kerosene after some hours (Fig: 5 ; Table: 2). According to the C. Cornea et al, kerosene gave little emulsification than that was of olive oil and sunflower oil. He got highest emulsification index with kerosene was about 12.75. As per my study I found highest emulsification index was 14.28.

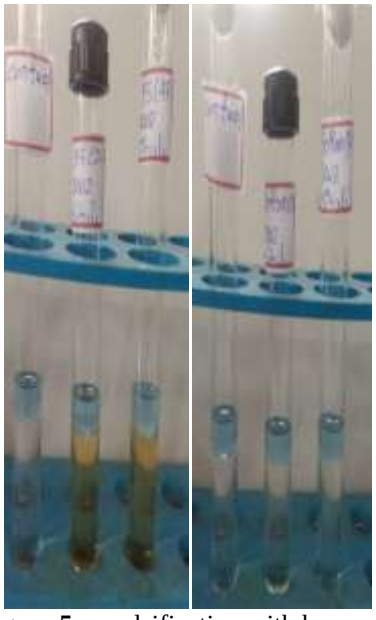

Figure 5: emulsification with kerosene

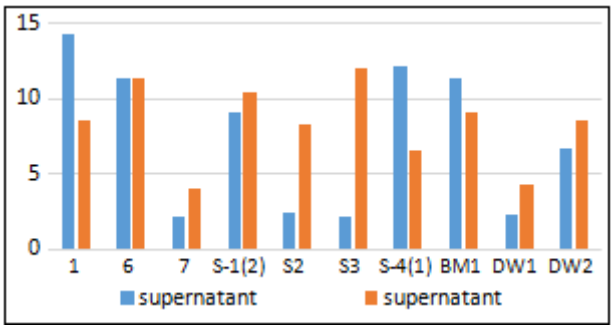

Figure 6: emulsification index of supernatant

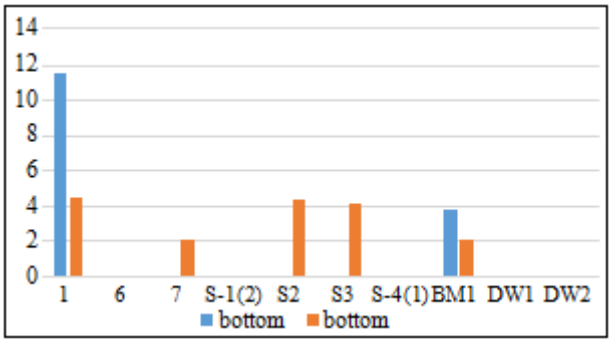

Figure 7: emulsification index of bottom

3) Surface tension measurement

Biosurfactant reduced the surface tension as emulsification of immiscible phases occur. As shown in table:2 , supernatant without biosurfactant have high surface tension than those having biosurfactant.

Table 2: surface tension measurement

Isolate no	*Surface tension (N/m)*
Blank	0.0772
1(S)	0.0386
1(B)	0.0515
6(S)	0.02575
7(S)	0.0515
7(B)	0.0515
S-1 (2)(S)	0.0386
S2(S)	0.02575
S2(B)	0.0321
S3(S)	0.0321
S3(B)	0.04505
S-4(1)(S)	0.0386
BM1(S)	0.0321
BM1(B)	0.04505
DW1(S)	0.0386
DW2((S)	0.02575

(S) - supernatant
(B) - bottom

Anti-adhesive activity

Crude biosurfactant (CFS/bottom) was added to the 96 well microtiter plate that inhibit the adhesion of biofilm forming *Staphylococcus aureus & Pseudomonas aeruginosa* organisms. After crystal violet assay for quantification, each well reading was compared with control well reading and calculate % adhesion of each test wells (Table: 3)

Table 3: % Antiadhesion of biofilm forming organisms by LAB derived crude biosurfactant

Isolate no	*Test organisms*	
	S. aureus	*P. aeruginosa*
1(S)	8.13	18.58
1(B)	19.15	19.31
6(S)	8.04	3.66
7(S)	12.72	20.09
7(B)	8.98	8.42
S-1 (2)(S)	10.54	13.49
S2(S)	0.82	0.62
S2(B)	9.66	2.63
S3(S)	20.23	10.50
S3(B)	5.44	11.38
S-4(1)(S)	2.28	20.11
BM1(S)	9.11	0.25
BM1(B)	10.41	24.12
DW1(S)	0.75	8.02
DW2((S)	16.54	19.38

B. Bulgasem et al carried out anti-adhesion activity against Candida spp. by crude supernatant treated at specific temperature. While S.Kaur used *E.coli & S.aureus* as a biofim forming test organisms. Same test organisms as I used for my study were taken but different way (in bioflux channel) to detect effect of biosurfactant was used by M. A. Diaz De Rienzo et al.

Thin layer chromatography

By using chromatographic method, we can characterize the nature of biosurfactant. Ninhydrin reagent identified lipopeptide nature and iodine vapours identified glycolipid nature of Biosurfactant (Fig : 8 ; Table: 4)

Figure 8: Thin layer chromatography of extracted biosurfactant

Table 4: Biosurfactant nature according TLC

Isolate no	*Nature of Biosurfactant*
1(S)	Lipopeptide
1(B)	Lipopeptide
6(S)	Lipopeptide
7(S)	Lipopeptide
7(B)	Lipopeptide
S-1 (2)(S)	Glycolipid
S2(S)	Lipopeptide
S2(B)	Lipopeptide
S3(S)	Lipopeptide
S3(B)	Lipopeptide
S-4(1)(S)	Glycolipid
BM1(S)	Glycolipid
BM1(B)	Glycolipid
DW1(S)	Glycolipid
DW2((S)	Lipopeptide

In contrast to L. Fracchia et al, TLC was analysed by UV light at 254 nm or by 5% anisaldehyde solution in ethanol. But as per Anayata sharma et al, lipopeptide was detected by ninhydrin reagent and glycopeptide was detected by anthrone reagent. According to my study, ninhydrin used for lipopeptide and iodine vapours was used for detection of glycolipids.

4. Conclusion

Biosurfactant derived LAB, which is partially identified by morphological and biochemical characteristics; screened by hemolysis; confirmed by various methods; characterized by TLC, are widely showing anti-adhesion of many biofilm forming human pathogenic organisms. So, it has many applications in medical field. Not only that biosurfactant can be used as an alternative of many chemically synthesized detergents. Therefore it protects environment from hazardous chemicals.

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