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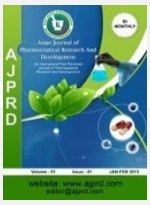

Open Access Research Article

BIOSURFACTANT AND ANTIMICROBIAL ACTIVITY OF LACTIC ACID BACTERIA ISOLATED FROM DIFFERENT SOURCES OF FERMENTED FOODS

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ABSTRACT

Biosurfactants or surface-active compounds are biodegradable, non-toxic and ecofriendly compounds released by microorganisms. The purpose of this work was toevaluate the potential of lactic acid bacteria (LAB) present in fermented foods as biosurfactants producers and their antimicrobial activities against pathogenic bacteria. The results revealed that a total of twenty LAB were isolated from different sources of fermented foods and were screened for biosurfactant production and antimicrobial activity.Six isolates namely Fm1 isolated from fermented milk, Y1 isolated from Arabic yogurt, Gr isolated from fermented grape, So isolated from soil, Fm3 isolated from fermented milk and Y2 from Arabic yogurt produced biosurfactants with antimicrobial activities. Addition of cell free supernatant (CFS) from the six isolates reduced the surface tension of water from 72.22mN/m to an average of 37.21mN/m. The CFS of the six isolates showed emulsion index (EI_{24}) of 90% for diesel and motor oil while 80% for crude oil. Additionally, the CFS of the six isolates showed strong inhibition activity against the target pathogens (P. florescence, P. aeroginosaATCC2785, P. aeruginosa14T28, E. coli and S. Typhimurium) with inhibition zone between 15 and 33.4 mm in diameter. Identification using 16s rDNA identified the isolates as L. acidophilus for Fm1, L.pentosusfor Y1, L.fermentum for Gr, L.plantarum forSo, L.lactis for Fm3 and L.casei for Y2.This study concluded that CFS of LAB isolated from fermented foods possess both biosurfactant and antimicrobial activities that could be further exploited for food and pharmaceutical applications.

Keyword:*Biosurfactant, antimicrobial activity, Lactobacillus strains, emulsification index, surface tension*

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INTRODUCTION

The interest in microbial surfactants has been progressively increasing in the recent years due to their diversity, environmental friendly nature, can be readily biodegraded compared to the recalcitrant chemical surfactants, and their capability to resist high temperature ranges makes them interesting molecules for many industrial formulations. Additionally, biosurfactants are reported to have antimicrobial, anti-adhesive, anti-fungal, anti-viral activities(1). These remarkable characteristics have led biosurfactants to be an asset in a wide range of potential applications, such as therapeutics, biomedical, enhanced oil recovery and food processing (2).The surface-active properties of the biosurfactants are facilitated by the amphiphilicbehaviour of the biosurfactant molecule, which contains hydrophilic and hydrophobic moieties, allowing them to act at the interfaces flanked by aqueous and non-aqueous constituents.Biosurfactants help in reducing surface tension of two immiscible solutions (3). Biosurfactants are molecules with effective emulsifying and surface tension reduction properties at oil–water interfaces.Their surface tension reducing ability, detergency, foaming, and wettability make biosurfactants appropriate for various applications (4). A good biosurfactant can lower the surface tension of water from 72 to 35 mN/m.Biosurfactantsproducing microorganisms also have significant roles in the dissolution of oil molecules (5). Biosurfactants can be used as food additives directly to improve the texture of

certain bakery products or can be used as detergent ingredients indirectly to uncontaminated food surfaces to avoid food contamination (6).

Microorganisms that are reported to produce biosurfanctants include *Bacillussubtilis*(7), *Pseudomonas aeruginosa* S2. (8) and fungi for example *Candida bombicola* (9). Similarly, lactic acid bacteria (LAB) are also reported to be biosurfactant producers with good potential as antibiofilmon various inanimate surfaces (food processing areas, biomedical surfaces, food equipment (3) and in therapeutics and food formulations (10, 3). Sharma et al (3) concluded a significant antiadhesive property by biosurfactant from LAB strains which can potentially be used as a cleaning/coating material for bio-medical equipment's.

LAB such as *L. acidophilus*, *L. casei*, *L.rhamnosus*,&*L. fermentum* were reported to produce biosurfactants (11,12). The ability of *Lactobacillus* to synthesize surfactants makes the bacteria competitive with pathogenic microflora of the gastrointestinal tract (13).These biosurfactants can be cell-bound or excreted by the microorganisms(13). The evaluation of the antimicrobial activity of LAB biosurfactants revealed that the *L.animalis* ATCC35046, *L. paracasei* A20 and *L. agilis* CCUG31450, exhibit a greater antimicrobial activity against *S. aureus* (2).

Petroleum hydrocarbons are important energy resources. However, petroleum is also a major pollutant of the environment (14).

Contamination by oil and oil products has caused serious harm and increasing attention has been paid to the development new technologies for the removal of these contaminants(14). The microbial biosurfactants is an alternative cleaning procedure to decrease the viscosity of sludge and oil deposits by reducing surface tension and formation of an oil-in-water emulsion that facilitates the oil mobility and pumping of wasteDue to the high number of reactive groups in the molecule, biosurfactant bind tightly to oil droplets and form an effective barrier that prevents drop coalescence (15).

The purpose of this work was toevaluate the potential of LAB present in fermented foods as biosurfactantsproducers and their antimicrobial activities against selected bacteria normally present in foods.

MATERIALS AND METHODS

Isolation of LAB

LAB was isolated from different sources of food which are dairy products, breast milk,and fermented shrimp and fruits samples in Malaysia. Ten gram of sample was added to 90 ml 0.1% peptonewater and appropriate dilution was spread plated on de Man,Rogosa and Sharpe (MRS) agar (Oxoid CM0361) plates containing0.8% calcium carbonate.Plates were incubated anaerobically in anaerobic jar at 37 ± 1°Cfor 48 h. Each of the isolates was tested for catalase activity byplacing a drop of 4% hydrogen peroxide solution on the cells.Immediate formation of bubbles indicated the presence of catalasein the cells. Only those isolates which was catalase-negative wasGram-stained and the morphology was observed using Nikonmicroscope (Nikon Eclipse 80i) and streaked on de Man, Rogosaand Sharpe (MRS) agar to obtain pure isolates. All bacterial strainsused in this study were maintained in 15% glycerol stock and storedat -20°C. They were re-cultured in MRS broth (Oxoid CM0359) at37 ± 1°C under anaerobic condition. Prior to beginning theexperiments, each bacterial strain was sub-cultured at least threetimes (1%, v/v) at 24 h intervals (16).

Preparation of cell free supernatant (CFS)

Seventy bacterial isolates were Pre-cultures and grown in MRS broth medium containing 5% crude incubated in shaker at 37°C, 120rpm for 72 h. CFSs from LAB strains were obtained to screenof biosurfactant producing LABand to evaluate their inhibitory activity against theindicator pathogens strains by the agar well-diffusion test .CFS were obtained from MRS broth cultures by centrifugation (Jouan Br4i, France) at 10,000 g for 10 min at 4ºC. To rule out inhibition due to pH reduction caused by organic acids, the pH of the CFSs was adjusted to 6.2 using 1 N NaOH. Inhibitory activity from hydrogen peroxide was also eliminated by addition of catalase. The CFSs were filter-sterilized through 0.22 µm pore-size filters (12).

Screening of BiosurfactantProducing LAB

CFS were tested with drop collapse test, oil spreading technique, blood agar plates, emulsification index(*EI* 24), bacterial adhesion to hydrocarbons (BATH assay) and surface tension measurement to detect biosurfactant producing bacteria.All the experimentswere carried out in triplicates and the average values were calculated.

Drop collapse test

The qualitative Drop collapse test was performed following the protocol of Bodour&Maier (17).To perform this test, 2 µl of hydrocarbons (crude, motor or diesel) was added to the surface of a glass plate and 5 µl of the cultureCFSs wasadded and observed for 1 min. The CFS that made the oil drop collapse were consider positive; those drops that remained beaded were scored as negative compared SDS and distilled water as a positive and negative control respectively.

Oil spreading technique

The experiment included measuring the diameters of lightened zones, formed due to the contact of the oil-water interface with the solution containing biosurfactants synthesized by LAB strains.The experiment was conducted on 15.0-cm Petri dishes filled with distilled water (50 mL). Next, 20µl oil (diesel, crud or motor 40 W) was dropped onto the surface of the water, followed by the addition of 10 µl of cell culture CFS.SDS and distilled water was used as a positive and negative control respectively.The diameter and the clear halo visualized under visible light were measured after 30 second. Each experiment was repeated three times to determine an average diameter and the clear zone (18).

Emulsification index

The emulsifying activity of CFS towards hydrocarbons (diesel, motor or crude oil) was carried out as described by Cooper and Goldenberg (19). A 4mL hydrocarbons and 4 mL CFS was added to a test tube, mixed and

homogenized using a vortex for 2 minutes. The emulsion activity was determined after 24 hours and the %EI*24* is given as percentage yielded by dividing the height of the emulsified layer (mm) by the total height of the liquid in the glass test tube (mm), then multiplying by 100. The results were compared with 1% 1%SDS as positive control and distilled water and MRS broth as negative control.

Surface tension measurements

Surface tension measurement: Culture samples were centrifuged (Jouan Br4i, France) at 12,500 rpm for 15 minutes to remove cells and the resultant CFS was submitted to surface activity measurements. Surface tension was measured by using a du Nouy ring-type tensiometer model (KSV-sigma 703D Finland) (20). The measurement of the ST of each sample was conducted three times along with a control (water,MRS and 1% SDS). The presence of biosurfactants in the solution was confirmed based on a decrease in the value of surface tension of the isolated sample against the control sample.

Bacterial adhesion to hydrocarbon (BATH) assay

Cell surface hydrophobicity was determined by bacterial adherence to the hydrocarbon as described by Rosenberg et al. (21). Pure culture of LAB was grown in 250 ml Erlenmeyer flask with 100 ml of MRS broth containing crude 1.0% (v/v). Flasks containing sterilized MRS medium were inoculated with a loopful of LAB culture and the culture flasks were maintained in a shaker at 36 °C for 24 h at 200 rpm. After 24h of incubation, the culture broth was centrifuged at 8000 rpm (Jouan Br4i, France) for 10 minutes, the CFS was removed and the cells were washed twice in normal saline. The washed cells were diluted with saline water to give a final $OD_{550\ nm}$ = 0.5. Next 2ml of the cell suspension was added to 100µl of crude oil in test tube, vortexed for 3 minutes and the aqueous phase were allowed to separate for 15 minutes. The OD was read from the aqueous phase at 550 nm using a micro Elisa auto reader (Model 680, BioRad). Hydrophobicity is expressed as the percentage of cell adherence to hydrocarbon calculated as follows:

Percentage adherence (%) = 100 *(1- OD of the aqueous phase /OD of the initial cell suspension).

For a given sample, three independent determinations were made and the mean value and standard deviation was accounted.

Blood hemolysis test

Blood hemolysis test is a primary method for screening microorganisms capable of producing biosurfactants. The hemolytic activity was determined on blood agar plates. 50 µL CFS were inoculated onto blood agar plates and incubated at 37°C for 48 h. The plates were visually investigated for zone of clearing (haemolysis) around CFS (22).

Antibacterial activity of LAB producing biosurfactant

An agar well diffusion method described by Barefoot and Klaenhammer (23)was used to screen for LAB producing biosurfactant against target bacteria. LAB was grown in MRS broth at 37°C for 24 h and the cells were harvested by centrifugation at 4,000 g for 5 min (Jouan Br4i, France), the CFSs were used to test the antimicrobial activity. In order to exclude the possibility of inhibition caused by organic acids and hydrogen peroxide, the CFSs were adjusted to pH 6.5-7.0 with 5 N NaOH and treated with catalase enzyme (24), respectively. The treated CFSs were filter-sterilized using 0.22 µm membrane filter, Millipore before the antimicrobial assay. The target bacteria (*P. florescence*, *P. aeroginosa*ATCC2785, *P. aeruginosa* 14T28, *E. coli* and *S.Typhimurium*) were obtained from the microbiology laboratory, University Sains Islam Malaysia. They were cultured in nutrient broth (Oxoid, UK) at 37°C for 24 h, and spread on nutrient agar (Oxoid, UK) plates. After agar solidified, wells were made using sterile cork borer of size 6.0 mm then cover the base of the well with agar to avoid leaking. A 80 µl of CFSs were added to each well and the plates were incubated at 37°C for 24 h. Growth inhibition zones (mm) around the wells were measured after diminution the well size. All the experiments were carried out in duplicate.

Phenotypic identification of LAB isolates

Two isolates were identified by API 50 CHL (API system, BioMérieux, France) assay. Purified LAB were cultivated in 20 ml MRS broth incubated at 30°C over night, after which the culture was washed and resuspended into API50 CHL medium (Bio- Merieux SA 69280, France) The turbidity of the suspension was determined by the McFarland method according to the instructions provided by the manufacturer. Cell suspension was applied into API 50 CHL strip wells and paraffin oil was pipetted to each well to create anaerobic condition.The strips were incubated at 30°C. The results were read after 24h and verified after 48 h. Fermentation of carbohydrates in the carbohydrate medium was indicated by a yellow colour except for esculine (dark brown). Colour reactions were score against a chart provided by the manufacture (25). The results were analyzed with API WEB (Bio-Merieux).

Genotypic identification of LAB isolates using 16s rDNA

Genomic identification of the six strains of LAB was determined following the method described by Jarvis & Hoffman (26).Total genomic DNA was extracted from an overnight culture in 20 ml MRS broth at 30 ºC using Master Pure™ Gram positive DNA Purification Kit (USA). One ml of overnight culture was centrifuged 11500 rpm for 10 min at 25°C (Eppendorf centrifuge 5804 R) and the pallet was collected. To the pallet 150 µl of TE buffer was added and incubated at 37 ºC overnight.1 µl of proteinase K (50 µg/µl, Sigma) was mixed to 150 µl of gram positive lysis solution and then added to TE buffered mixture and mixed thoroughly. The sample was incubated at 65-70 ºC for 15 min and vortexed every 5 min, followed by placing in ice for 5 min. 175 µl of MPC protein precipitation reagent were added to each sample, vortexed and centrifuged at 13000 rpm for 10 min at 4 °C (Eppendorf centrifuge 5804 R). The CFSs were transferred to new tubes and the pellets were discarded. 1 µl of RNase II (5 µg/µl) was added to each sample and mixed thoroughly. The samples were

incubated at 37 ºC for 30 min; 500 µl of isopropanol was added to the CFS, centrifuged at 4ºC for 10 min at 13000 rpm (Eppendorf centrifuge 5804 R).Isopropanol was removed using an eppendorf pipette without dislodging the DNA pellet. The pellets were rinsed with 200 µl ethanol 70% and centrifuged at 5000 rpm for 2 min at room temperature.The ethanol was removed carefully and the DNA was resuspended with 35 µl of deionized water and kept at -20 ºC for further study.

Gel electrophoresis

The PCR products amplification from universal bacterial primer were analyzed for expected size. Two µl of each amplification mixture were subjected to electrophoresis in 1.5% (w/v) agarose gels in 0.5 x TEA buffer for 45 min and 110 V. DNA molecular mass marker (250 to 10000 bp) molecular ladders from 1st Base, Malaysia was used as standard. After electrophoresis the gels were stained in ethidium bromide and after washing the gels were visualized and photographed with UV transilluminator (Bio-Rad Laboratories, Segrate, Italy**).** The partial 16S rDNA, Lbp11 and LMM primers sequences were determined by 1st Base, Malaysia and sequences were compare d with databases (Gen- Bank).

Statistical Analysis

Results are presented as the mean ± standard deviation of three replicates. Statistically significant differences of the experiments achieved in the different assays were evaluated by a one-way ANOVA ($P < 0.05$) and Tukey's test. Statistical analyses were performed using SPSS software (version 22.0) and a significant difference was considered if $P < 0.05$.

RESULTS

Screen of Biosurfactant Producing LAB

A total of 160 bacteria isolated from different sources that showed clear zone on modified MRS-$CaCO_3$ agar, catalase negative and Gram positive and were considered as LAB. Randomly,CFS of 70 isolates were screened for biosurfactantproperties by drop collapse test, oil spreading test, emulsification index (EI_{24}), surface tension and BATH. Results indicated that 20 of the isolates showed variable reactions to the test as described below.

Drop collapse test

Eleven isolates (11/20) namely So, Fm3, Y2, Fm4, Bu2, Fm1, Y1, Gr, Fs4, Da andAp showed positive reaction for the drop collapse test using the three hydrocarbons (crude, motor and diesel) which indicates the production of biosurfactant similar to 1% SDS which is a known a surface active agent. There was no activity observedfor distilled water and MRS broth (Table 1, Figure 1).

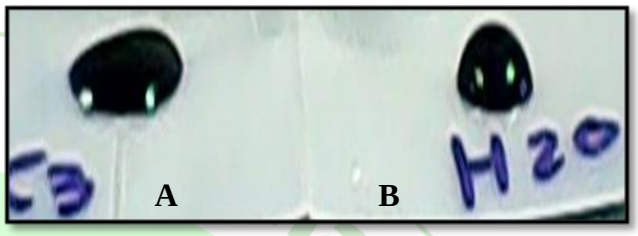
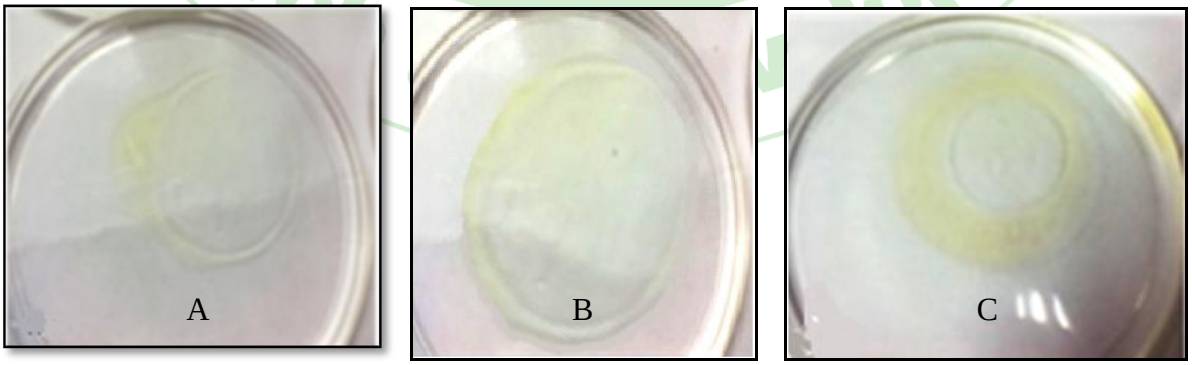


Fig. 1: Drop collapse assay. Collapsed droplet (A) is the motor oil after addition CFS of LAB and (B) H_2O

Oil spreading

Dropping 1% SDS to the hydrocarbons resulted in greater oil spreading diameter (76 to 81 mm) then the diameter of oil spreading by CFS. The oil spreading diameter varied with LAB and hydrocarbon; ten of the CFS caused the oil to spread to a diameter between 12.7 to 55 mm depending on the oil tested (Figure 2, Table 1). Six of the isolates Fm3, Fm1, Y1, Y2, So and Gr showed no significant differences ($p>0.05$) in oil spreading values for the three types of namely, oil, crude, motor and diesel but seems to spread more for motor and diesel. The highest oil spreading diameter (mm) was observed for Fm1 which was 55.2 mm (Diesel), 53.0mm (motor) and 50.0 mm (crude) respectively compared to other LAB isolates. The oil spreading diameter for diesel and motor shown by Y1 was similar to Fm1 with values 51.4 and 50.0 mm, respectively. A slightly lower oil spreading values were observed for Gr (48.0±0.3for diesel, 46.1±0.2 for motor and 42.2±0.4 mm for crude), while oil spreading values for Fm1was 37 to 40 mm for the three types of oil evaluated.



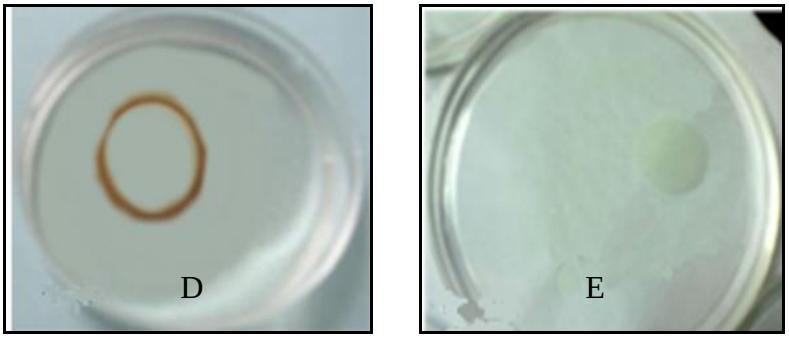


Fig. 2: Oil spreading assay of CFSof LAB on hydrocarbons. (A)1% SDS, (B) diesel, (C) motor oil, (D) crude oil and (E) H_2O after 24 h

Emulsification Index (EI_{24})

Significant differences ($p<0.05$) in $EI_{24}$was observed among the 20 CFS of LAB isolates. Forty percent (8/20) of LAB isolates did not show activity towards diesel, 50% (10/20) of LAB isolates showed no activity towards motor oil, and 60% (12/20) showed no activity against crude (Table 1,Figure 4). The EI_{24}values shown by 1% SDS for diesel, motor and crude were 93.15%, 95.32%, 81.04%, respectively. Among the LAB isolates, six LAB namely, Fm1, Y1, Gr, So, Y2 and Fm3 showed comparable or higher $EI_{24}$with 1% SDS towards the three oil evaluated. There was no significant difference ($p>0.05$) of EI_{24}values for Fm1, Y1, Gr, So and Yfor diesel (ranging from 93.03 to 99.98%) and for motor oil (ranging from 91.54 to 99.55). However, EI_{24} values for crude were significantly lower ($p<0.05$) (ranging 78.32 to 89.44%). The CFS of Fm3 showed a lower EI_{24}values for the oil evaluated compared to 1%SDS which were 86.85, 81.66and 75.10%for diesel, motor and crud, respectively.

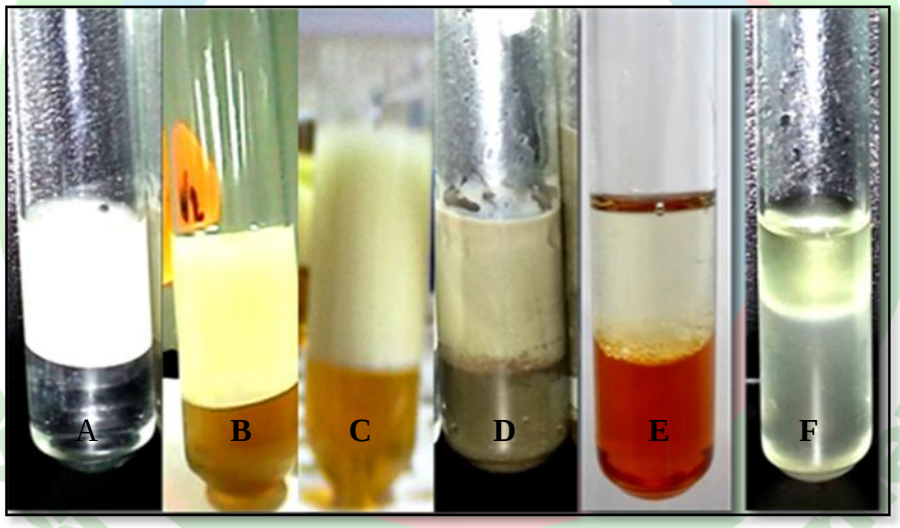


Fig. 4: Emulsion formation.(A) 1% SDS, (B, C and D) diesel, motor and crude oil respectively mixed with CFS of LAB, (E) MRS broth and (F) H_2O after 24 h

Table 1: Drop collapse test, oil spreading assay and emulsification index of CFS of LAB isolates

Bacteria isolates	Source	Drop collapse test			Oil spreading assay (mm)			Emulsification index (EI_{24}%)		
		Diesel	Motor	Crude	Diesel	Motor	Crude	Diesel	Motor	Crude
So	Soil	+	+	+	40.3 ± 0.1^{eA}	40.6 ± 0.2^{eA}	37.2 ± 0.1^{fA}	99.96 ± 0.05^{aA}	92.98 ± 0.76^{cB}	69.12 ± 04^{fC}
Fm3	Fermented milk	+	+	+	40.0 ± 0.4^{eA}	39.0 ± 0.6^{eA}	35.0 ± 0.5^{eA}	86.85 ± 1.12^{eA}	81.66 ± 0.05^{dAB}	75.10 ± 0.10^{eB}
Fs1	Shrimp	-	-	-	26.1 ± 0.2^{gA}	25.2 ± 0.1^{fA}	15.5 ± 0.1^{iB}	0^{kA}	0^{hA}	0^{iA}
Fm2	Fermented milk	-	-	-	0^{jA}	0^{hA}	0^{lA}	0^{kA}	0^{hA}	0^{iA}
Y4	Yoghurt	-	-	-	0^{jA}	0^{hA}	0^{lA}	8.26 ± 0.00^{jA}	12.78 ± 0.11^{fA}	0^{iB}
Y2	Yoghurt	+	+	+	38.0 ± 0.1^{fA}	40.1 ± 0.1^{eA}	32.1 ± 0.1^{gB}	93.03 ± 0.25^{dA}	91.54 ± 0.05^{cA}	78.32 ± 0.23^{dB}
Fm5	Fermented milk	-	-	-	0^{jA}	0^{hA}	0^{lA}	23.99 ± 0.29^{hB}	45.78 ± 0.01^{eA}	0^{iC}
Fm4	Fermented milk	-	-	-	0^{jA}	0^{hA}	0^{lA}	0^{kA}	0^{hA}	0^{iA}
Fs2	Shrimp	-	-	-	23.0 ± 0.1^{hA}	25.0 ± 0.2^{fA}	20.4 ± 0.3^{hA}	0^{kA}	0^{hA}	0^{iA}
Bu1	Budu	-	-	-	0^{jA}	0^{hA}	0^{lA}	13.85 ± 0.74^{iA}	10.54 ± 0.09^{fA}	0^{iB}
Bu2	Budu	+	+	+	0^{jA}	0^{hA}	0^{lA}	0^{kA}	0^{hA}	0^{iA}
Fm1	Fermented milk	+	+	+	55.2 ± 0.2^{bA}	53.0 ± 0.3^{bA}	50.0 ± 0.1^{bB}	99.98 ± 0.36^{aA}	99.55 ± 0.25^{aA}	89.44 ± 0.04^{bB}
Y1	Yoghurt	+	+	+	51.4 ± 0.1^{cA}	50.0 ± 0.1^{cA}	44.5 ± 0.2^{cB}	97.85 ± 0.21^{bA}	98.90 ± 0.46^{aA}	83.77 ± 0.21^{cB}
Gr	Grape	+	+	+	48.0 ± 0.3^{dA}	46.1 ± 0.2^{dA}	42.2 ± 0.4^{dA}	95.03 ± 0.05^{cA}	98.68 ± 0.19^{aA}	82.72 ± 0.42^{cB}
Bu3	Budu	-	-	-	13.0 ± 0.9^{iA}	17.1 ± 0.2^{gA}	12.7 ± 0.2^{kA}	22.07 ± 0.06^{hA}	0^{hB}	0^{iB}
Y3	Yoghurt	-	-	-	22.0 ± 0.1^{hA}	16.0 ± 0.5^{gA}	18.2 ± 0.1^{jA}	0^{kA}	0^{hA}	0^{iA}
Fs3	Shrimp	-	-	-	0^{jA}	0^{hA}	0^{lA}	12.81 ± 0.01^{iB}	0^{hC}	24.78 ± 51^{gA}
Fs4	Shrimp	+	+	+	0^{jA}	0^{hA}	0^{lA}	29.97 ± 1.24^{gA}	7.43 ± 0.32^{gC}	15.54 ± 83^{hB}
Da	Date	+	+	+	0^{j}	0^{h}	0^{l}	0^{k}	0^{h}	0^{i}
Ap	Apple	+	+	+	0^{j}	0^{h}	0^{l}	0^{k}	0^{h}	0^{i}
Dist water	-	-	-	-	0^{j}	0^{h}	0^{l}	0^{k}	0^{h}	0^{i}
MRS	-	-	-	-	0^{j}	0^{h}	0^{l}	0^{k}	0^{h}	0^{i}
1%SDS	-	+	+	+	77.0 ± 0.2^{aA}	76.0 ± 0.1^{aA}	81.1 ± 0.1^{aA}	81.04 ± 0.28^{fB}	95.32 ± 2.53^{bA}	93.15 ± 0.05^{aA}

Different letters in the same column (lower case)and in same row (upper case) represent significant differences at $p<0.05$

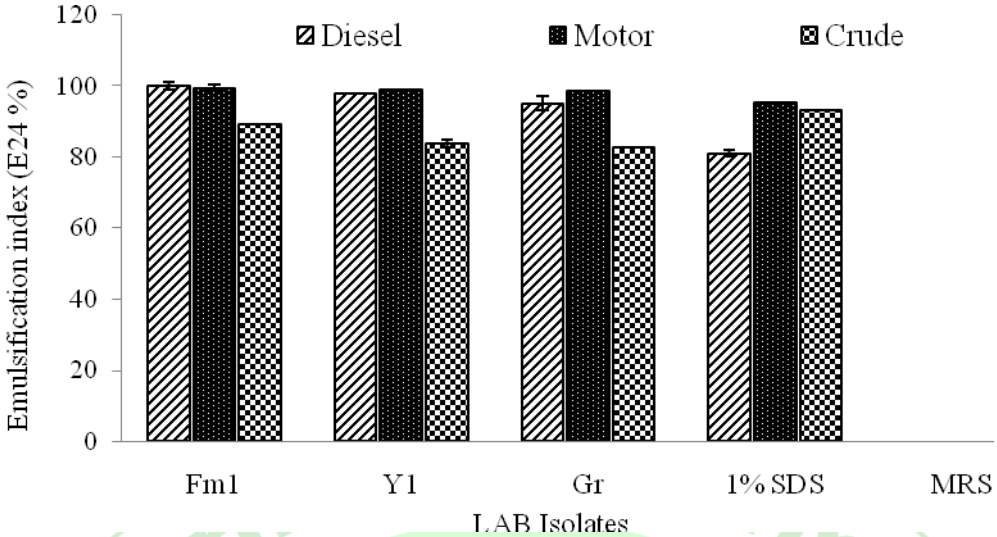


Fig. 5: Emulsion formationof biosurfactant producing LAB

Surface tension measurement

The surface tensions of distilled water and MRS broth were 72.12±2.22and 60.97±2.95 (mN/m), respectively (Figure 6, Table 2).Six (6/20) LAB namely, So, Y2, Fm1, Y1, Fm3 and Gr cultured in MRS broth significantly ($p<0.05$) reduced the surface tension of the broth to 37.14±0.49, 38.81±0.64, 35.5±0.78, 36.76±1.09, 39.22±0.63 and 37.02±1.08(mN/m), respectively. These values arecomparable to the surface tension measurement of 1%SDS which was 32.04±0.02(mN/m). The other LAB (14/20) showed surface tension measurement of greater than 40 mN/m. The results from this experiment indicate that six LAB isolates produced bioactive surface active agent in MRS broth.

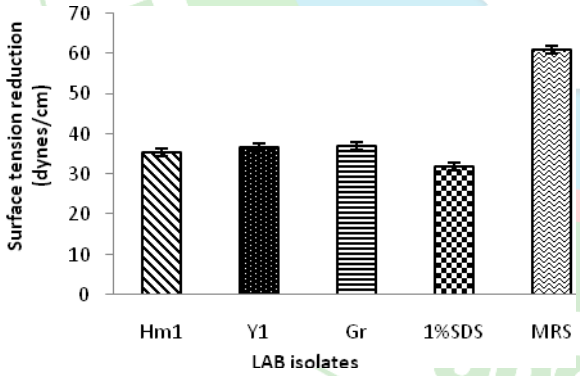


Fig. 6: Surface tension reduction by biosurfactant producing LAB

Bacterial adhesion to hydrocarbons (BATH) assay

The results of BATH assay on crude showed no activity for distilled water, MRS broth and 1%SDS (Table 2). Although, all the LAB strains showed positive reaction for the BATH assay, six (6/20) of the isolates (Fs1, Fm4, Bu2, Bu3, Y3 and Ap) had values below 50%. The isolates Fm1, Y1 and Gr showed BATH assay values of 99.92, 98.99 and 98.23%, respectively. Additionally, the isolates So, Fm3 and Y2 have comparatively high BATH values of 92.08, 89.64 and 86.66, respectively (Figure 7). This indicates that these LAB cells have affinity towards the crude oil and attached to the hydrocarbon.

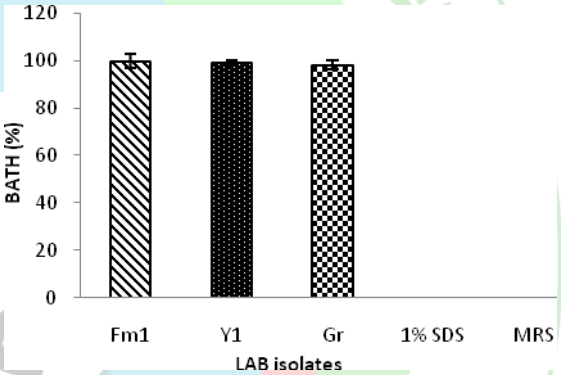


Figure 7: BATH assay of LAB Fm1, Y1 and Gr cells using crude oil

Hemolytic activity

Hemolytic activity was observed in CFS of eight (8/20) LAB isolates (Table 2). The CFS of Fm1, Y1 and Gr showed significantly greater ($p<0.05$) hemolysis activity with diameter 5.0, 4.9 and 4.7 cm, respectively than the hemolysis activity of commercial surfactant 1% SDS with 2.4 cm (Table 2, Figure 8 and 9).

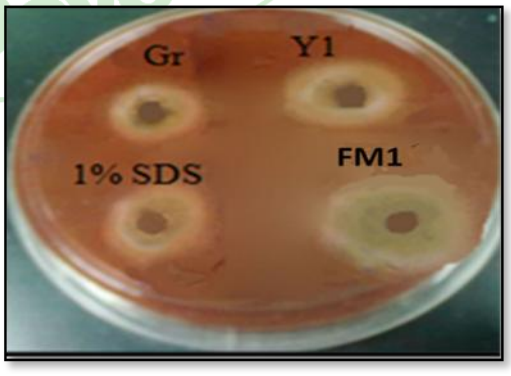


Fig. 8: Hemolytic activity on blood agar plated with CFS of LAB

Table 2: Surface tension, BATH and Hemolytic Activity of CFS of LAB isolates

Isolates	Source	ST(mN/m)	BATH (%)	Hemolytic Activity (cm)
So	Soil	37.14±0.49 [b]	92.08±0.72 [c]	4.5±0.2 [a]
Fm3	Fermented milk	39.22±0.63 [d]	89.64±0.97 [d]	4.5±0.2 [a]
Fs1	shrimp	51.43±0.84 [f]	55.17±2.03 [j]	0 [e]
Fm2	Fermented milk	53.28±2.59 [h]	72.73±1.02 [g]	2.0±0.2 [d]
Fs1	Yoghurt	57.72±0.96 [j]	44.16±0.40 [l]	0 [e]
Y2	Yoghurt	38.81±0.64 [c]	86.66±0.58 [e]	4.4±0.2 [b]
Fm5	Fermented milk	59.98±4.81 [k]	53.59±1.15 [k]	0 [e]
Fm4	Fermented milk	51.72±0.62 [g]	45.4±0.71 [l]	0 [e]
Fs2	shrimp	46.08±1.36 [e]	64.64±1.38 [h]	3.0±0.l [c]
Bu1	Budu	58.71±0.40 [j]	77.11±2.04 [f]	0 [e]
Bu2	Budu	53.22±1.00 [h]	25.42±1.36 [o]	0 [e]
Fm1	Fermented milk	35.5±0.78 [b]	99.92±0.89 [a]	5.0±0.4 [a]
Y1	Yoghurt	36.76±1.09 [b]	98.99±0.9 [ab]	4.9±0.2 [a]
Gr	Grape	37.02±1.08 [b]	98.23±0.87 [b]	4.7±0.1 [a]
Bu3	Budu	52.55±2.13 [g]	37.74±0.77 [n]	0 [e]
Y3	Yoghurt	51.63±0.54 [g]	46.96±1.39 [m]	0 [e]
Fs3	shrimp	54.90±1.01 [i]	59.18±0.78 [i]	0 [e]
Fs4	shrimp	50.19±0.46 [f]	61.31±2.49 [i]	0 [e]
Da	Date	48.97±0.94 [f]	76.1±0.83 [f]	0 [e]
Ap	Apple	49.01±0.63 [fg]	48.25±0.56 [m]	0 [e]
Dis.water	-	72.12±2.22 [l]	0 [p]	0 [e]
MRS	-	60.97±2.95 [k]	0 [p]	0 [e]
1%SDS	-	32.04±0.02 [a]	0 [p]	2.5±0.2 [d]

Different letters in the same column represent significant differences (p< 0.05)

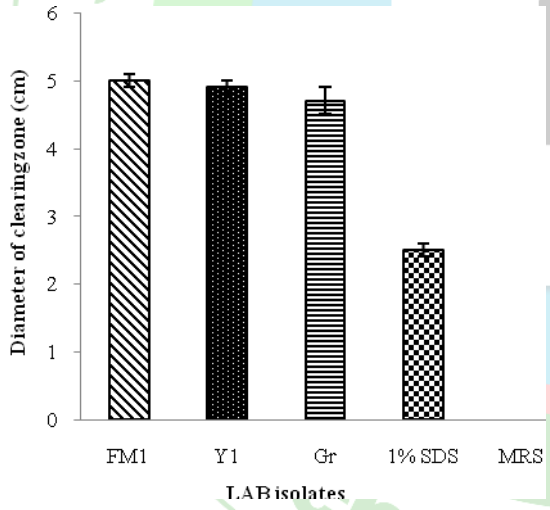


Fig. 9: Hemolytic activity of CFS of LAB isolates Fm1, Y1 and Gr

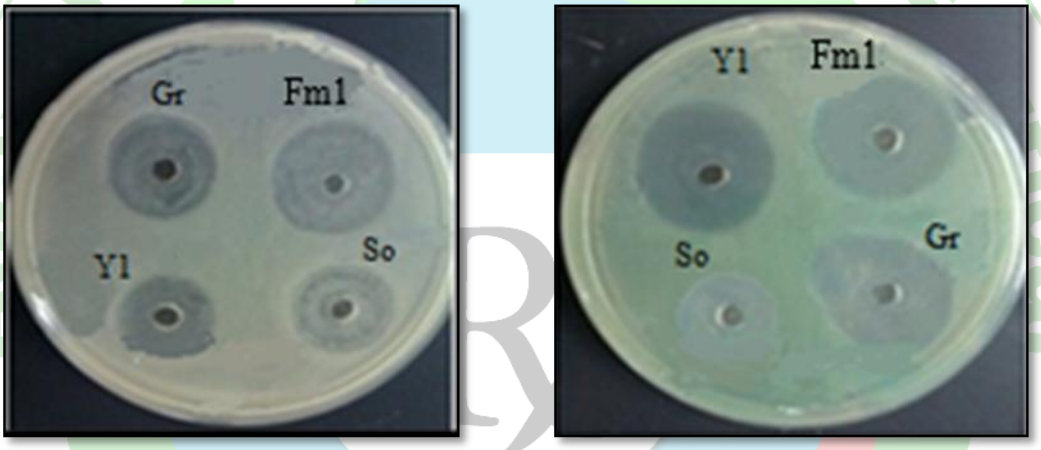
Antibacterial activity of CFS of LABby Agar well diffusion agar plates

The results showed that the CFS of 9/20 LAB isolates significantly ($p<0.05$) inhibited the growth of tested strains of Gram positive and Gram negative bacteria with variable diameter of inhibition zone (Table 3, Figure 10).The inhibition zone was rated strong (>13mm), moderate 13 to 9 mm) and weak (<9 mm) (24).The results showed that six isolates (So, Fm1, Y1, Gr, Fm3 and Y2) had strong inhibition activity against the target bacteria (*P. florescence*, *P. aeroginosa*ATCC2785, *P. aeruginosa*14T28, *E. coli* and *S. Typhimurium*) with inhibition zone between 15 and 33.4 mm in diameter (Table 3). All the target bacteria were strongly inhibited by isolate Fm1: *P. florescence*(33.4 mm), *S.* Typhimurium (30.4 mm), *P. aeroginosa*ATCC2785 (29.7 mm), *P. aeruginosa*14T28 (25.5 mm) and *E.coli* (20.2 mm). Moderate to weak inhibition against the target bacteria was observed for isolate Da, Ap and Fs2.

Table 3: Growth inhibition zone (mm) of CFS of selected LAB against target bacteria

LAB	Target bacteria				
	P. florescence	***P. aeroginosa*ATCC2785**	***P. aeruginosa* 14T28**	***E. coli***	***S.Typhimurium***
So	15.2±2.49^{d}	17.3±0.2^{d}	14.2±0.10^{de}	15.5±0.52^{b}	16.0±^{cd}
Fm3	15.0±0.97^{d}	13.2±0.97^{e}	15.1±0.63^{d}	16.1±0.4^{b}	13.0±0.11^{e}
Y2	15.7±0.1^{d}	17.0±0.4^{d}	13.2±0.12^{e}	13.1±0.31^{c}	15.3±1.08^{c}
Ap	5.1±0.01^{f}	7.3±0.1^{f}	11.1±0.20^{f}	5.2±1.36^{e}	9.1±0.52^{f}
Fm1	33.4±0.1^{a}	29.7±0.72^{a}	25.5±1.36^{a}	20.2±0.1^{a}	30.4±0.60^{a}
Y1	18.3±0.84^{c}	21.1±1.08^{b}	22.3±2.22^{b}	15.8±0.71^{b}	16.7±1.07^{d}
Gr	23.8±0.90^{b}	18.4±0.56^{c}	17.0±0.54^{c}	20.1±0.4^{a}	24.1±0.32^{b}
Da	6.1±1.08^{f}	4.0±0.5^{g}	7.0±0.94^{h}	4.3±1.10^{e}	10.1±1.01^{f}
Fs2	10.0±0.11^{e}	9.0±2.03^{g}	9.0±0.62^{g}	10.2±0.12^{d}	10.0±0.16^{f}

Different letters in the same row represent significant differences ($p < 0.05$).



S. Typhimurium *P. aeroginosa*ATCC2785

Fig. 10: Growth inhibition zone of LAB CFS against pathogenic bacteria by well diffusion method after 24 h incubated at 30 °C

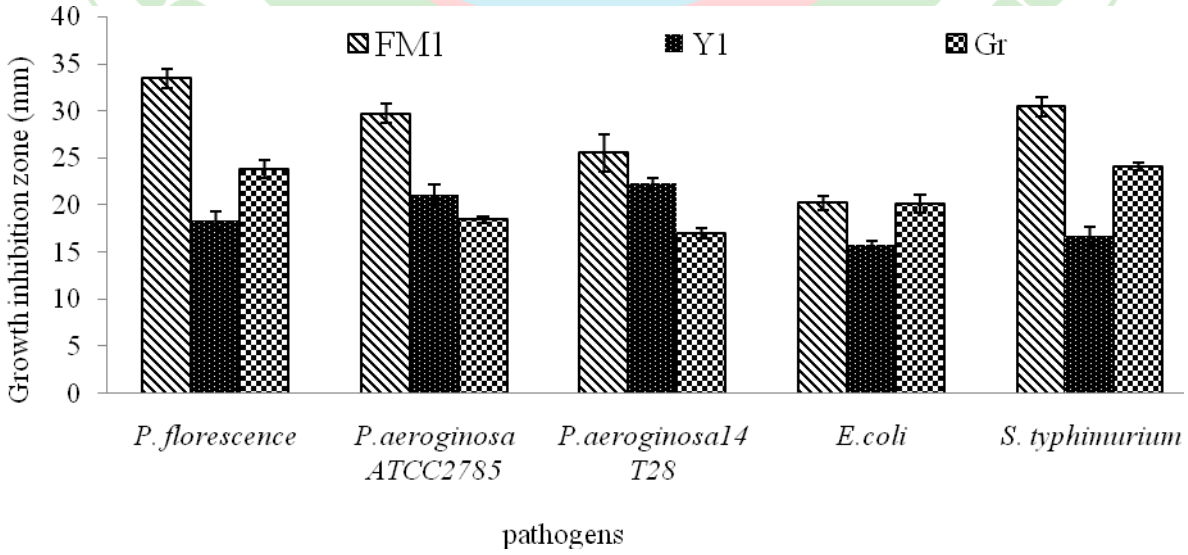


Fig. 11: Growth inhibition zone of biosurfactant producing LAB against target bacteria

Phenotypicidentification of LAB Isolates

Results from API 50 CH test kits and API web identified the six LAB isolates (Fm1, Y1, Gr,So, Fm3 and Y2) as *L. acidophilus* , *L. pentoususL .fermentum, L.Plantarum, L.lactis*and *L.casei*. with similarity 99.2, 82.9, 99.0, 97.4, 98.7 and 98.5 %, respectively (Table 4).

Genotypic identification

Genotype identification of DNA using universal primer showed clear bands of isolates (Figure 12) with approximate molecular weight 1500 bp and similarity 99.9% for (LAB-Fm1) *L. acidophilus* , 100% for (LAB-Y1) *L. pentosus*, 99.6% for (LAB-Gr) *L.fermentum*, 99.0% for (LAB-So) *L.plantarum*, 98.0% for (LAB-Fm3) *L.lactis* and 99.3% for (LAB-Y2) *L.casei*. The sequences of these isolates were determined and deposited in the Gene Bank database under accession number GU138532.1, GU451063.1, GU451063.1, JN560879.1, JX129203.1 and GU253892.1, respectively (Table 4).

Table4: Phenotypic and genotypic identification of LABisolates

Code of LAB	Source	Phenotype identification		Genotype identification		
		ID[a]	SI[b] %	ID	SI %	Accession No.
Fm1	Breast milk	*L. plantarum*	99.2	*L. acidophilus*	99.9	GU138532.1
Y1	Yogurt	*L. pentosus*	82.9	*L.pentosus*	100	GU451063.1
Gr	Grape	*L.plantarum*	99.0	*L.fermentum*	99.6	GU451063.1
So	Soil	*L.plantarum*	97.4	*L.plantarum.*	99.0	JN560879.1
Fm3	Breast milk	*L.fermentum*	98.7	*L.lactis*	98.0	JX129203.1
Y2	Yogurt	*L.plantarum*	98.5	*L.casei*	99.3	GU253892.1

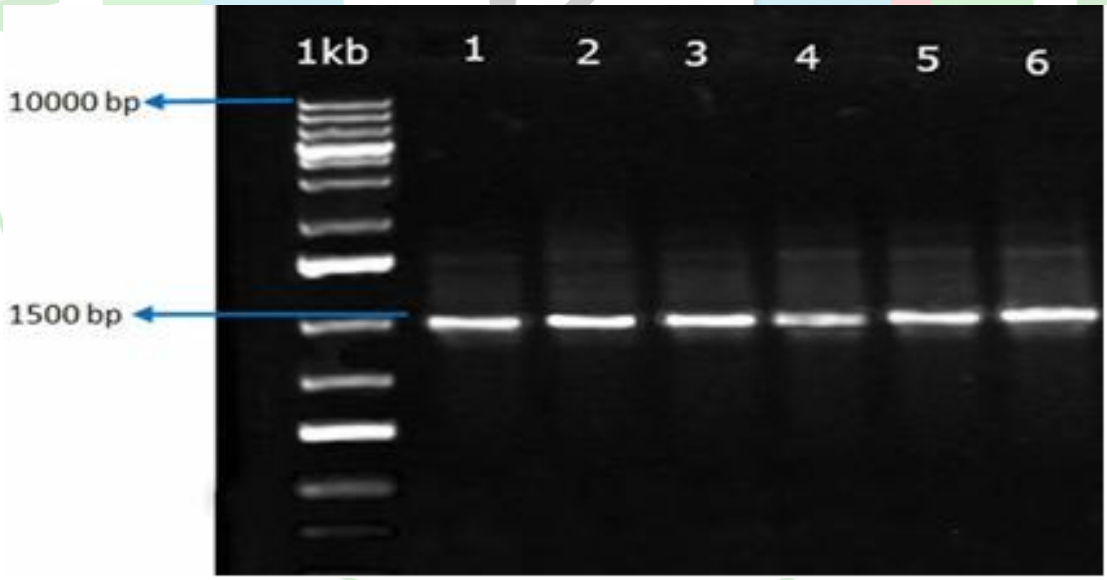


Figure 12: The DNA bands of LABs on the 1.5 % agarose gel.

Lane 1: Fm1, 2. Y1, 3. Gr, 4. So, 5. Fm3 and 6. Y2.

DISCUSSION

Drop collapse test and oil spreading assay

Preliminary detection for the presence of biosurfactant in a bacterial CFS are normally carried out by using drop collapse test and oil spreading assay. The CFS will cause a drop of oil to collapse and spread when it is added to the oil by which the force between the liquid drop and hydrophobic surface of oil is reduced (27). Strains of LAB were reported to produce biosurfactant (10,3). Preliminary screening of 70 LAB isolates obtained from different food sources (9 samples), fermented milk (5 sample), fermented shrimp (2 sample and soil (5 sample) showed that eleven of the isolates showed positive drop collapse test and oil spreading assay (Table 1). Augustin &Hippolyta (28) reported the CFS of eight *Lactobacillus*sps. isolated form fermented milk obtained in Cameroon market showed positive drop collapse assay of palm oil. Youssef et al. (29) compared three methods (drop collapse, oil spreading

and haemolytic activity) to detect biosurfactant production by *Bacillus* spp. from soil obtained in Mexico and USA using crude oil. They recommended that both drop collapse and oil spreading assay methods should be carried out as reliable techniques for testing biosurfactant production. Youssef et al. (29) reported a 5.0 mm diameter of oil displacement in the oil spreading technique is an indication of the presence of biosurfactant. The oil spreading test is used for the rapid detection of biosurfactant produces because of probable lipase activity in the biosurfactant isolated from microorganisms. The oil may be degraded into free fatty acids, i.e. di- and monocylglycerols, since these compounds have surfactant properties which can result in a positive oil spreading test (30). The biggest clear zone for Fm1 isolate 55.2mm, 53.0 mm and 50.0 mm diesel, motor and crude, respectively, followed by Y1 51.4 mm, 50.0mm and 44.5mm, Gr 48.0mm, 46.1mm and 42.2 mm, So 40.3 mm, 40.6mm and 37.2 mm, Fm3 40 mm, 39 mm and 35 mm and Y2 38 mm, 40.1 mm and 35 mm(Table 1, Figure 2). The clear zone diameter for oil spreading obtained in this study was bigger than clear zone obtained by Thavasi1 et al. (31). He reported that, out of one hundred five strains isolated from sea water samples in India and screened, eighty one strains were positive for the oil spreading assay using crude oil with biggest clear zone diameter of 35 mm. Five strains namely *B. megaterium*, *B. subtilis*, *C. kutscheri*, *L. delbrueckii*and *P. aeruginosa*showed the highest clear zone.

Emulsification index ($\%EI_{24}$)

Quantitative analysis including emulsification index ($\%EI_{24}$)and surface tension (ST) measurement was found to be a more reliable method for quantification of the soluble biosurfactant in the medium. A criterion cited for emulsion stabilizing capacity is the ability to maintain at least 50% of original emulsion volume 24 hours after formation (32). It was observed that the isolate Fm1, Y1, Gr, So, Fm3 and Y2 was able to maintain the emulsion stability after 24 h with $\%EI_{24}$ range between86.9-100, 81.7-99.6 and 69-89% for diesel, motor and crude, respectively. In addition CFSs of these isolates were stable after 48 h(Table 1, Figure3).These EI_{24}values were higher than the value of 56.80 for palm oil obtained from *Lactobacillus* spp TM1 isolated from fermented milk in Cameroon (28). In another study, the MRS CFS of *L. lactis*isolated from fermented milk showed EI_{24} of 72% evaluated using paraffin oil (33). Similarly,*Bacillus* spp. and *Lactobacillus* spp. isolated from butter milk showed EI_{24} of 66.8% and 85% on paraffin oil, respectively (10). Augustin et al. (34) evaluated potential biosurfactant properties of *Lactobacillus* spp. on palm oil and reported EI_{24} of 61.11±1.37%.Most data published in the literature reported that bacteria with high potential of emulsifying activity of 50 to 60% are promising microbial candidates for biosurfactant production (35). In this study, however, the six LAB isolatesFm1, Y1, Gr, So, Fm3 and Y2 showed high EI_{24} values ranging from 62 to 100% for the three oil evaluated, and therefore, these strains of *Lactobacillus* spp. can be considered promising biosurfactant production on diesel, motor and crude.

Surface tension

The ability to reduce the surface tension is another criterion used to determine the effectiveness of a surfactant. For instance, a good surfactant can reduce the surface tension of water from 72.2 to 35.0 mN/m (36). Youssef *et al.* (29) reported that bacteria which have potential in producing biosurfactant recorded surface tension measurement below 42 mN/m. Surface tension value above 60mN/m produced by the culture medium is an indication of its inability to produce biosurfactan. In this study it was observed the surface tension values of distilled water were 72.12±2.22 dynes/cm, MRS broth 60.97±2.95 dynes/cm and SDS (32.04±0.02 mN/m). The six LAB isolates (FM1,Y1, Gr, So, Fm3 and Y2) showed high reduction in surface tension which were 35.5±0.78, 36.76, 37.02, 37.14, 39.22 and 38.81±0.63 mN/m, respectively compared to distilled water, MRS and SDS.Bodour and Miller-Maier (17) suggested for LAB to be considered a biosurfanctant producer it must be able to reduce the surface tension below 40 mN/m. In this study it was observed that all the six isolates showed the surface tension below 40 mN/m indicating they are good biosurfactant producers.Studies on CFS of *B. subtilis* observed that the final surface tension of the CFS was 34.5 mN/m and EI_{24} of 76% evaluated using crude oil (37) while another study by Dadrasnia and Ismail (38) reported surface tension of the CFS was 36.5 mN/m. and EI_{24} of 65% using lubricating oil.Viramontes-Ramos et al. (39) indicated that the surface tension reduction by *Pseudomonas* strains was 27.2mN/m and EI_{24} were 100% and 92.8% while the surface reduction by *Bacillus* strain was 33.3mN/m and EI_{24} were 0.8% and 80.3%, using diesel and motor oil respectively. The six LAB isolates (FM1,Y1, Gr, So, Fm3 and Y2) showed high reduction in surface tension of CFS from 60.97 mN/m to 35.5, 36.76, 37.02, 37.14, 39.22 and 38.81mN/m, respectively (Table 2). Rodrigues (40) screened three*Lactobacillus*strains (*L. casei*CECT-5275, *L. rhamnosus*CECT-288 and *L. pentosus*CECT-4023) obtained from the Spanish Collection of Type Cultures (Valencia, Spain) for their potential to produce biosurfactant and reported that the best biosurfactant producer was *L. pentosus* which showed a surface tension reduction of 21.5 mN /m. Similarly, selected strains of *P. aeruginosa*and *B.subtilis* lower the surface tension of water from 70 mN m-1 to 29 mN m-1 and 27 mN m-1, respectively (41).The surface tension reduction in this study is higher than those previously described in theliterature.Saravanakumariand Mani (42) reported an extracellular biosurfactant from *L. lactis* isolated from cheese way in India able to reduce the surface tension of MRS broth from 60 to 40.5 mN/m.

Bacteria adhesion to hydrocarbon (BATH)

BATH is another quantitative method developed by Rosenberg et al. (21) estimate the cell hydrophobicitywith hydrophobic compounds like crude oil.Cells attach themselves to the oil droplets by producing surface active compounds called biosurfactants. Bacterial strains with high cell

hydrophobicity are reported as potential biosurfactant producers (43). Positive cell hydrophobicity was reported as an indication of biosurfactant production (44). Visualization of bacterial cells adhered to crude confirmed the affinity of cells towards crude droplets (31).In the present work, the hydrophobicity of six LAB, Fm1, Y1, Gr, So, Fm3 and Y2 were evaluated on crude oil and the values were 99.92, 98.99, 98.23, 92.08, 89.64 and 86.66%, respectively. The result is in agreement with previous study by Thavasi et al. (31) that reported 91 from 105 bacterial strains isolated from seawater in USA were positive for the BATH assay, which indicated the affinity of the bacterial cells towards hydrophobic substrate. Maximum cell attachment with crude was found with *P. aeruginosa* (95.15±0.21%) followed by *L. delbrueckii* (92.6±0.84%). In this study, Fm1, Y1 and Gr showed BATH value of >98% with crude, suggesting that these isoaltes are good producers of biosurfactant.

The blood agar method

Additionally, the blood agar method was included in this study since it is widely used to screen for biosurfactant production, and in some cases, it is the sole method used (45). Johnson et al. (46), Banat (45) included hemolyticactivity in the screening for the possibility of biosurfactant production by microorganisms. This method is based on the fact that biosufactants are able to haemolyse the red blood cell present in blood (47). Results of this study showed that six LAB namelyFm1, Y1, Gr, So, Fm3 and Y2gave different halo ring diameter which were 5.0 cm, 4.9 cm, 4.7 cm, 4.5 cm, 4.5 cm and 4.4 cm, respectively (Table 2). The results of blood agar method were similar to the work done by Mulligan et al. (48) and Mulligan et al. (49), who have isolated biosurfactant overproducer mutants with blood agar method. They found hemolysis positive results with all bacteria strains isolated from soil. Abbas et al. (50) studied biosurfactant production by *Bacillus*spp. isolated from hydrocarbon using blood agar hemolysis test and observed that eight from 18 strains showed a zone of clearance around the colony, confirming the production of surface active molecules. The hemolytic activity of biosurfactants wasfirst discovered when Bernheimer and Avigad (51) reported that thebiosurfactant produced by *B. subtilis*, surfactin, lysed red blood cells. Carrillo et al. (52) found an association between hemolytic activity andsurfactant production and they recommended the use of blood agarlysis as a primary method to screen biosurfactant production. Biosurfactant interact strongly with cellular membranes, while exotoxins cause lysis of the red blood cells. Biosurfactants are amphiphilic in nature and can partition into the phospholipid membrane of cells (53).

Antibacterial activity

Biosurfactants were recorded to have variable degrees of antimicrobial activity (54). Several biosurfactants that exhibit antimicrobial activity have been previously described(55). Results of this study revealed that from 20 isolates tested, nine isolates showed antibacterial activity against tested Gram positive and Gram negative bacteria and caused growth inhibition with different diameter of clear zone (Table 3, Figure10 and 11). The isolate Fm1 had strong inhibition activity (20.2 to 33.4 mm) against all the tested bacteria, Y1 had strong inhibition activity against *P. aeroginosa*ATCC278 (21.1mm), *P. aeroginosa*14T28 (22.3 mm) and Gr that showed strong inhibition activity against *P. florescence*, *E. coli* and*S. typhimuriu*(23.8, 20.1 and 24.1mmrespectively). The results of this study are in agreement with this work of Rodrigues et al. (12). The biosurfactants produced by *S.thermophilus* and *L. lactis* showed significant ($p>0.05$) antimicrobial activity against several bacterial and yeast strains isolated from explanted voice prostheses. Rodrigues et al. (56) reported that the biosurfactants produced by *S.thermophilus* and *L. lactis*completely inhibited *S. aureus* and *S. epidermidis*at concentrations 100 mg/ ml. Likewise, biosurfactants obtained from *L. paracasei*showed antimicrobial activity against *E. coli*, *S. aureus*, *S. epidermidis* and *S. agalactiae* (55).Salman et al.(57) observed that the crude biosurfactant isolated from *S. thermophilus* showed inhibitory effect against *Klebsiella* spp. and *P. aeruginosa*. Similarly, Salman andAlimer (58) studied the antibacterial activity of the crude and partial purified biosurfactantof *L. rhamnosus*against some bacteria causing urinary tract infection including *K. pneumonia*, *B. cepacia*,*E. coli* and *S. aureus* and found that the crude and partial purified biosurfactanthad good activity against these bacteria.Biosurfactants produced by *lactobacilli* have been shown to reduce adhesion of pathogenic micro-organisms to glass (59) silicone rubber (60) surgical implants and voice prostheses (56). Consequently, previous adsorption of biosurfactants can be used as a preventive strategy to delay the onset of pathogenic biofilm growth on catheters and other medical insertional materials, reducing the use of synthetic drugs and chemicals (61).The suggested mechanism of antimicrobial action of biosurfactant may be by disorganizing the membrane structure through interaction with phospholipids as well as membrane proteins(62). Another explanation of the antimicrobial effect of biosurfactants is the adhering property of biosurfactants to cell surfaces that caused deterioration in the integrity of cell membrane and also breakdown in the nutrition cycle. Also the biosurfactant prevent the protein synthesis by inhibition of the peptidyltransferase in binding mainly the 23S rRNA in the 50S subunit of the bacterial ribosome (63).

CONCLUSION

Six LAB isolated from different sources of fermented foodswere able to produce biosurfactants with antibacterial activities. The biosurfactant produced reduced surface tension of about37.21mN/m with emulsification index (%*EI24*) 90% for diesel and motor oil while 80% for crude oil and therefore, these strains of *Lactobacillus* spp. can be considered promising biosurfactant production on diesel, motor and crude. This study observed that LAB from different sources of fermented foodshas varying antibacterial activity against different pathogens. Growth of all tested pathogens was readily inhibited by CFS of the Fm1 (*L. acidophilus)*, Y1 (*L.pentosus) and*Gr (*L.fermentum*)isolates. Additionally, antimicrobial compounds produced by this naturally present LAB may play important role in

enhancing the antimicrobial properties and the medicinal benefit.

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