

STUDY THE ANTIMICROBIAL AND ANTIADHESIVE ACTIVITY OF PURIFIED BIOSURFACTANT PRODUCED FROM *LACTOBACILLUS PLANTARUM* AGAINST PATHOGENIC BACTERIA

A. A. Jameel
Researcher

N. H. Haider
Prof.

Depart. of Biology, College of Al-Rasheed, Baghdad - Iraq

Email: alyaa.abd@alrasheedcol.edu.iq.

ABSTRACT

This study was aimed to purification the biosurfactant that produced from *Lactobacillus plantarum* isolated from clinical samples of iraqi healthy women by column chromatography through silica gel column (3.5 × 30 cm) using solvent system (chloroform : methanol, 2:1) and characterization the purified product by Thin layer chromatography (TLC), Fourier Transform Infrared Red spectroscopy (FTIR) and Gas chromatography (GC) mass then evaluated its antibacterial and anti-adhesive activity. The results shown the higher emulsification activity (E₂₄% = 63) and lower the surface tension to 23 mN/m in synthetic MSM, while the natural media gave (E₂₄% = 71) and lower surface tension to 19 mN/m. The using TLC technique indicated presence of lipopeptide in the biosurfactant with R_f value = 0.82. Antibacterial and antiadhesion activities were evaluated against some pathogenic bacteria, including *Pseudomonas aureginosa* and *Staphylococcus aureus*. The results showed a higher inhibitory effect of biosurfactant at concentration 200 mg/ml on *S. aureus* and *P. aeruginosa* with the inhibition zone 27 mm and 33 mm respectively in BCDFTM media. While purified biosurfactant with concentration 200 mg/ml produced in MSM media had an effect on *S. aureus* and *P. aeruginosa* with the inhibition zone 21 mm, and 18 mm respectively. The anti-adhesion activity of purified biosurfactant against *p.aeruginosa* ranging from (34.70 ± 5.256) to (11.7 ± 9.7) produced from natural media BCDFTM and was higher than the anti-adhesion activity of purified biosurfactant against the same pathogen ranging from (46.95 ± 7.37) to (20.15 ± 2.805) produced in MSM.

Key words: chromatography, antimicrobial, antiadhesive.

جميل وحيدر

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دراسة الفعالية المايكروبية والفعالية ضد الالتصاق للمستحلب الحيوي المنتج من بكتريا المعززات الحيوية

LACTOBACILLUS PLANTARUM ضد البكتريا المرضية

ناظم حسن حيدر

علياء عبد الحسين جميل

استاذ

باحث

قسم علوم الحياة - كلية الرشيد الاهلية - بغداد

المستخلص

هدفت الدراسة الحاليه الى تنقيه المستحلب الحيوي المنتج من بكتريا *Lactobacillus plantarum* المعزولة من مصادر سريرية لنساء عراقيات اصحاء. نقي المستحلب الحيوي المنتج جزئيا بواسطة كروماتوغرافيا عمود هلام السليكا (30 x 3.5 cm) باستخدام نظام المذيب (الكلوروفورم : ميثانول 2:1) اظهرت النتائج اعلى فعالية استحلاب (E₂₄ = 63%) واقل شد سطحي 23 m/Nm في الوسط الصناعي بينما اعطت الوسائط الطبيعية اعلى فعالية استحلاب E₂₄ = 71% واقل شد سطحي الى 19 m/Nm. كما اكدت نتائج استخدام تقنية كروماتوغرافيا الطبقة الرقيقة وجود الدهون البروتينية في المستحلب الحيوي مع قيمة R_f مساويا الى 0.82. وبينت النتائج ان اعلى فعالية تثبيطية للمستحلب الحيوي المنتج من الوسط الطبيعي عند التركيز 200 ملغم/مل بلغت 27 و 33 ملم ضد بكتريا *S. aureus* و *P. aeruginosa* بالترتيب. في حين كانت اعلى فعالية تثبيطية للمستحلب الحيوي المنتج من الوسط المصنع عند التركيز 200 ملغم/مل بلغت 21 و 18 ملم ضد البكتريا نفسها بالترتيب. كذلك بلغت معدل الفعالية ضد الالتصاق للمستحلب الحيوي المنتج من الوسط الطبيعي من (34.70 ± 5.256) الى (11.7 ± 9.7) ضد بكتريا *P. aeruginosa* بينما بلغت معدل الفعالية ضد الالتصاق للمستحلب الحيوي المنتج من الوسط المصنع من (46.95 ± 7.37) الى (20.15 ± 2.805) ضد بكتريا *P. aeruginosa*.

المفتاحية: كروماتوغرافيا، الفعالية المايكروبية، الفعالية ضد الالتصاق.

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INTRODUCTION

Biosurfactants is amphipathic molecules and mainly excretions by micro-organisms outside the cells, and sometimes attached to the cells, mostly during growth on water immiscible substrates. They have both hydrophilic and hydrophobic parts. Hydrophilic parts can comprise amino acids or peptides, phosphate, alcohol and mono- di- or poly-saccharides. Hydrophobic parts comprise unsaturated or saturated fatty acids, Biosurfactants (BS) prefer to proliferate at the point where fluid phases interface with various polarity. They are capable of reducing surface and interfacial tension (9). BS can potentially be utilized as therapeutic agents because they are safe and have antibacterial, antifungal, and antiviral functions. They disturb the membranes which results in an increase of the membrane permeability, followed by cell lysis and loss of metabolites. These compounds are able to affect adhesion properties of microorganisms, by partitioning at the interfaces (21,22). Probiotics have been known to alter the adhesive ability of other bacteria through the production of BS able to modify hydrophobic interactions (23). The biosurfactant containing collagen binding proteins derived from *Lactobacillus fermentum* RC-14 was able to inhibit staphylococcal binding to surgical implants (11). It is generally recognised that biosurfactants prevent pathogenic organisms from adhering to solid surfaces and infection sites. BS are also released by probiotic lactic acid bacteria (LAB), including *Lactobacillus helveticus* (26), *Lactobacillus plantarum* (16), and *Lactococcus lactis* (20). BS produced by probiotic LAB possess effective biological activities such as anti-adhesive (13), and antimicrobial (10) effects according to previous research. In addition, the lipopeptide fraction is more effective against pathogenic bacteria and fungus than the glycolipid fraction. Although the cell walls of gram negative bacteria are usually resistant to lipophilic solutes because they consist of a peptidoglycan layer and an additional outer membrane (narrow outer wall) rather than gram-positive bacteria cell walls, which contain peptidoglycan (loose outer wall), which makes gram positive more sensitive (12). This may be because lipopeptide

biosurfactant causes loss or damage of the peptidoglycan layer may also inhibit the biochemical reactions in the cell wall and prevent peptidoglycan growth. The lipopeptide fraction showed high antimicrobial activity against *Candida albicans* which may reflect its effect on the fungal membrane by disorganising the fungal membrane or preventing the cell wall from synthesising. The current study focused on characterization of biosurfactant produced by *L.plantarum* and evaluated its antibacterial and antiadhesion activity.

MATERIALS AND METHODS

Production and extraction of Biosurfactant

Production of biosurfactant was carried out in optimum condition. The fermentation medium containing 50 ml of (BCDFT (banana, corn, date, fig and tomato media with pH 3) and MSM with pH 5) media were inoculated with 1 ml of selected isolate *L. plantarum* 1.5×10^8 bacteria / ml (O.D₆₀₀ about 0.5). The cultures were incubated at 30°C for 72 and 96 hr, respectively with anaerobic conditions. Then the cells were harvested by centrifugation at 8000 rpm for 20 minutes, thereafter were washed twice in distilled water and resuspended in 10 ml of phosphate buffer saline. The cells were then incubated at room temperature for 4 hrs. with gentle stirring, after 4 hrs. the broth was centrifuged at 8000g for 20 min. The supernatant was taken for extraction. Equal volumes of chloroform: methanol was added in the ratio of (2:1 v/v). These mixtures were shaken well to ensure proper mixing and were left overnight for evaporation. White coloured precipitate if seen at the interface between the two liquids proved the presence of biosurfactant, The yields were determined gravimetrically in terms of g/L. (4).

Purification of Biosurfactant

A portion of crude extract was dissolved in methanol to obtained (1 gm/ 10 ml) that used for purification by silica column chromatography with following dimension (3.5 x 30 cm) using filled with silica gel (60 mesh). It was packed tightly by a continuous flow of methanol, then the column was washed with methanol. One gram of crude exerted biosurfactants were dissolved in 10 ml of methanol and loaded in column until majority

of the solvent is absorbed. Then the column was eluted with gradient of chloroform and methanol ranging from 50 : 3 (250 ml) , 50:5 (200 ml), 50:50 (100 ml), and methanol alone (100 ml). The eluted extract was flow rate 20 ml / hr. and 3 ml from each fraction were collected. All eluted fractions were collected and tested for their surface tension and emulsification activity, then each fraction which contain the (biosurfactant) tested for antimicrobial and antiadhesion activity (17).

Characterization of partial purified biosurfactant

Analysis of biosurfactant by thin layer chromatography (TLC): The identification of the bioactive compound present in the biosurfactant was screened by Thin Layer Chromatography (TLC). Silica coated plates (20 x 20 cm) were prepared and the pelleted partial purified biosurfactant obtained was spotted onto the plate. The solvent used for separation was chloroform: water: methanol (65:24:4). Ninhydrin reagent was sprayed over it and the appearance of red spots indicates the presence of lipopeptide in the biosurfactant (5).

Fourier Transform Infrared Red spectroscopy (FTIR) spectral analysis

The functional groups and chemical bonds (post purification) were detected using FTIR spectrometry (shimadzu 8400, Japan). The spectrum was limited at the range of 4000-650 cm^{-1} with resolution of 4 cm^{-1} .

Analysis with Gas chromatography (GC)

Lipids were analyzed to their fatty acids components using gas chromatography (GC) (32). Fatty acids composition was investigated as Acid methyl ester by dissolving 10 mg of partial and purified biosurfactant with 1 ml of sulphuric acid - methanol at 90 °C for 15 min., and 1 ml of hexane was added with mixing, then hexane phase was taken after evaporated the sulfuric acid. To the hexane phase, 1 ml of D.W was added with mixing. The fatty acid methyl ester was extracted with hexane and subjected to an analysis with GC, by using helium as carrier gas on a Shimadzu 17-A GC equipped with an fused silica capillary column (30 m × 0.25 mm, 0.25 μm film thickness).

Critical Micelle Concentration (CMC) of purified biosurfactant; Critical micelle concentration (CMC) is a concentration at

which the surface tension of a solution reached a point that any further addition of surface active agents has little effect on reducing that value, and will aggregate as micelles. The CMC can be calculated by plotting the surface tension as a function of biosurfactant concentration as the curve slope abruptly changes at the point of CMC, which called the intersection point (25,5). For measuring CMC of partial purified biosurfactant, the serial concentrations (100, 110, 120, 130, 140, 150, 160, 170, 180 and 200 mg/ml) from stock solution (200 mg/ml) at room temperature, the surface tension of these solutions were recorded (17). The negative control is the distilled water. The CMC as well as the surface tension at the point of CMC were specified from the cut point in the surface tension of these solutions and recorded (25).

Application studying of purified biosurfactant

Antibacterial activity of produced

Biosurfactant: The antimicrobial activity of purified biosurfactant was examined against some bacteria (*P.aeruginosa* and *s.aureus*). It was evaluated by an agar disc diffusion method (19). The pathogenic bacteria were cultured in Muller Hinton broth and incubated over night at 37°C. Volume (0.1 ml) from each bacterial strain(O.D₆₀₀ about 0.5) were swabbed on the plates of Muller Hinton agar, and wells were made with cork borer on the surface layer. Different concentrations (100, 110, 120, 130, 140,150, 160, 170, 180 and 200 mg/ml) of purified biosurfactant were prepared, and one hundred microliter of each concentration was added into of the wells. Then the plates were incubated at 37 °C for 24 hrs. The clear zone marked as antimicrobial activity of biosurfactant. Thereafter calculated to determine the actual zone diameter (33).

Antiadhesive activity (14)

preparation of epithelial cells: Epithelial cells were obtained from mouth cavity of healthy people using sterile swabs and placed in sterile tubes containing phosphate buffer saline (PBS), mixed well, centrifuged at (2000 rpm) for 10 minutes, then washed three times with PBS and resuspended in PBS again, the number of epithelial cell should be balanced to give approximately 1 x 10⁵ cells / ml by using hemocytometer.

preparation of bacterial suspension

Bacterial cells were inoculated in BHI broth and incubated overnight at 37°C. The culture should be balanced to give approximately 1.5×10^8 bacteria / ml (O.D₆₀₀ about 0.5). Then the bacteria were washed twice with PBS after centrifugation 20 minutes at (2000 rpm) and resuspended in PBS again.

***In vitro* adhesion test**

Test tubes containing a mixture of 0.2 ml of bacterial suspension, 0.2 ml of epithelial cells suspension, and 0.1 ml of PBS were incubated at 37°C for one hr. Unattached bacteria were removed by centrifugation for 10 minutes at (2000 rpm). The final pellet was resuspended in a drop of PBS, dropped onto a glass slide microscope then, air dried and fixed with methanol, and finally stained with methylene blue. The number of attached bacteria was counted on 20 epithelial cells by light microscope. A control of epithelial cells was performed by fixing and staining the epithelial cells alone with methanol and methylene blue stain respectively.

Anti-adhesive test by purified biosurfactant produced by *Lactobacillus plantarum* (31)

Equal amount of different concentrations (100, 110, 120, 130, 140, 150, 160, 170, 180, and 200 mg/ml) of purified biosurfactant were mixed with equal amount of the cultures of *P.aeruginosa* isolate containing approximately 1.5×10^8 bacteria / ml (O.D₆₀₀ about 0.5), and incubated at 37 °C for 18hr. Then 0.1 ml of the mixtures were then spread onto brain heart infusion agar plates and incubated at 37 °C for 18 hr. The colonies grown on the brain heart infusion agar were tested for adhesion test as previously mentioned as mention above.

RESULTS AND DISCUSSION**Extraction and partial purification of produced Biosurfactant**

L. plantarum ADK2 was growing in optimum culture condition for biosurfactant production include (in mineral salt medium (pH5) and BCDFTM (pH3) at 30 °C, with shaking (120 rpm) for 96 and 72hr respectively. Then extraction was done by using Ch:M 2:1 through separation fanyl. The BS extraction produced reached to 4.2 and 10.8 g/L in MSM and natural medium respectively. Previous studies showed the highest crude biosurfactant

yield was 0.32 g/L by using chloroform extraction, and when used Ethyl acetate extraction the biosurfactant yield was reached to 0.24 g/l, while the used acid precipitation coupled with chloroform-methanol (2:1) extraction the yield was 0.27 g/l (7).

Purification of produced biosurfactants using chromatography technique

In order to obtain a purified biosurfactant, silica gel column chromatography (3.5 × 30 cm) was used, by loading the column with crude biosurfactant which was dissolved in chloroform. All eluted fractions were collected, then the emulsification activity and surface tension for each one was measured. The results of relation between emulsification activity, surface tension and fraction number are illustrated in Figure 1. The results were revealed that the presence of 2 peak of BS produce in synthetic MSM media in which the first one appeared between (77- 82) fraction number in elution3 using Ch:M (50 : 50), while the second one between (83-88) result in elution4 using methanol. In natural BCDFTM three peaks appeared, one of them appeared with elution 2 using (chloroform : methanol, 50:50) in which the first peak appeared in fraction number (48- 65), second one at fraction number (66-82) in elution 3 (chloroform : methanol, 50:50), and the third one appeared with elution 4 methanol with fraction number (83-89). Results also indicated that the first peak gave the higher emulsification activity (E_{24%}= 63) and lower the surface tension to 23 mN/m in synthetic MSM , while the third peak, with eluted4 using methanol gave (E_{24%} =71) and lower surface tension to 19 mN/m. Partially purified cell bound BS was appeared as crystalline dirty white powder. The BS formed white precipitation line between sample well and cationic compounds CTAB with barium chloride. The BS derived from *E. faecium* was confirmed as an anionic BS. Generally BS produced from other lactic acid bacteria were found as anionic surfactants. Xylolipid produced by *Lactococcus* 11Q2AWSZX ERactis was also reported anionic in nature (23). Silica gel column chromatography was used in several studies to purify biosurfactant compounds, (34) used silica gel column chromatography to purify rhamnolid produced

by *P. aeruginosa*. culture of *L. acidophilus* and *L. pentosus* was purified after solvent extraction, *L. acidophilus* biosurfactant was eluted with chloroform: methanol: water (60:20:1, v/v) gradient ranging as the mobile phase, while *L. pentosus* biosurfactant was

eluted with chloroform: methanol (95:5, v/v) gradient ranging as the mobile phase) by adsorption chromatography 60 silica gel. The biosurfactant compositions of the derived from *L. acidophilus* (lipopeptide) and *L. pentosus* (glycolipid) (1).

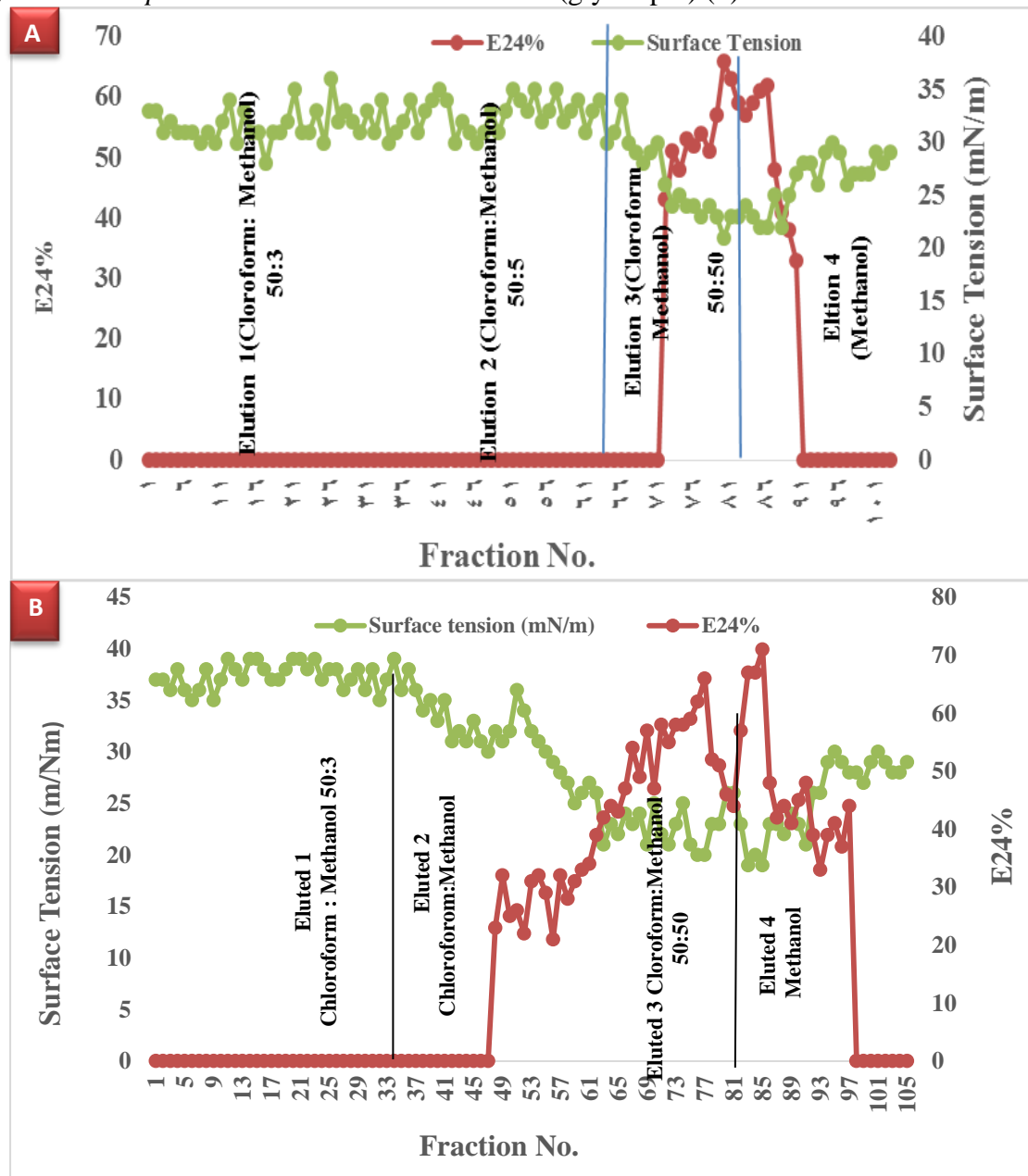


Figure 1. Purification of produced biosurfactant by *L.plantarum* using Silica Column chromatography, A: MSM, B: Natural media

Characterization of *L.plantarum* biosurfactant
TLC

The identification of the bioactive compound present in the biosurfactant was done by Thin Layer Chromatography. The Rf (Relative flow) value was 0.82 visualized under UV-illuminator as Figure 2 .The appearances of red colored spots on the TLC plate indicate the

presence of lipopetide in the biosurfactant. According to the Rf value, The Rf value is used to quantify the movement of the materials along the plate and determined the type of compound, Rf value is equal to the distance traveled by the substance divided by the distance traveled by the solvent. Its value is always between zero and one. The molecular composition of the partial purified

biosurfactant was evaluated by TLC that disclosed the presence of (Lipopeptide). Similar peaks for functional groups were additionally allotted to the biosurfactant obtained from *L. lactis*. Ninhydrin chemical agent disclosed the presence of lipopeptide compound within the biosurfactant extracted from eubacterium as red spots, this can be quite contrastive to earlier reports on antimicrobial actions of the biosurfactants wherever the lipopeptide biosurfactants are reported to move principally against most of the microorganism (28). After purification of crude RL by column chromatography, the separated fractions were analyzed using TLC. The spots appeared on the TLC plates were corresponding to rhamnsoe domains (Rf value 0.41 and 0.17) which represent mono- and di-RLs respectively, this indicates that this isolate (*P. aeruginosa* A3) produce a mixture of mono- and di-RL. The preliminary characterization of this type of biosurfactant was a glycolipid (3).

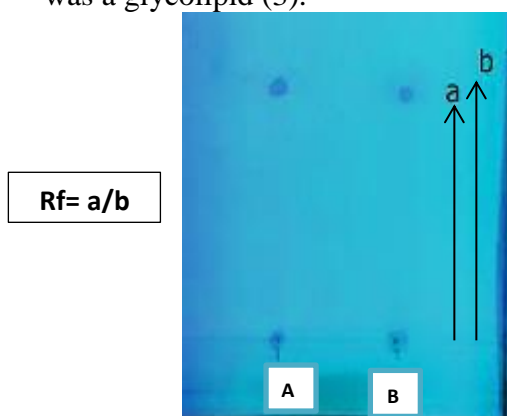
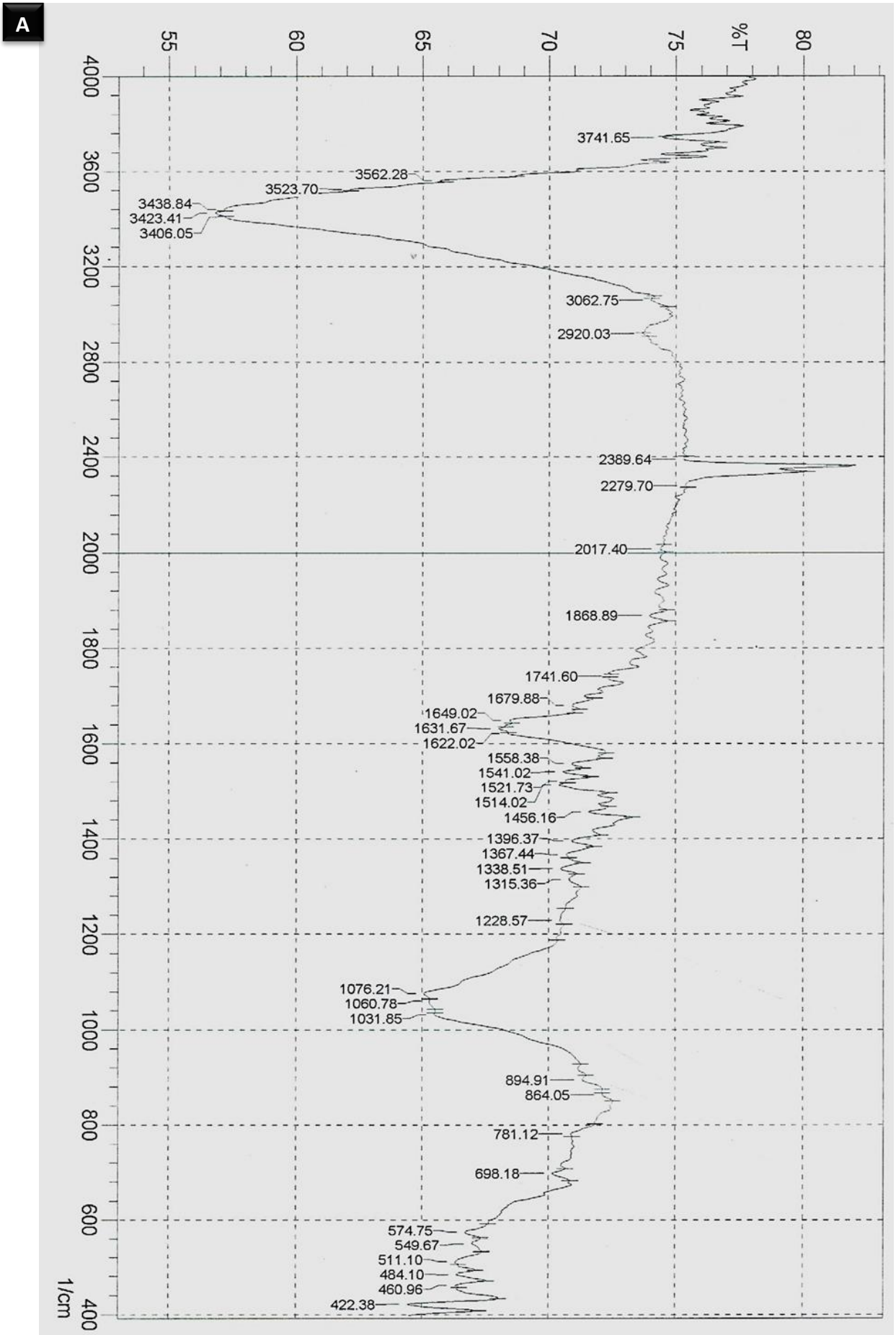


Figure 2. Analyzing of Biosurfactant produced by *L.plantarum* using TLC. A: from MSM , B: from BCDFTM

FTIR

The FTIR spectrum of partial purified biosurfactant produced by the *L.plantarum* using MSM media Figure 3 showed a broad

band at 3440 cm^{-1} and another band at 3394 cm^{-1} this may be attributed to the (O-H) groups of polysaccharide. This result was in accordance with (28) who demonstrated the presence of a broadly stretching intense peak at around 3428 cm^{-1} which is characteristic of hydroxyl groups (O-H) and (C-H) model between 2854 and 2925 cm^{-1} . The broad peak at 1745 cm^{-1} comprises both the carbonyl(C=O) of the ester group, and the carboxylic-acid part. Figure 3 also showed a strong band at 1650 cm^{-1} and 1161 cm^{-1} , those may be attributed to the N-H and C-N respectively. The FTIR indicates the presence of alkene, alkyne group and conjugated di-ene in the surfactant which indicates higher surface activity Figure 4 . Alkene and alkyl benzene components of surfactant are being significantly most effective surfactants. New anionic surfactants are derived from aromatic or substituted aromatic molecules and alkenes. Peaks at $1500\text{-}1650\text{ cm}^{-1}$ indicates the presence of C=C alkene component. Also the result revealed in Figure 3 B that the partial purified biosurfactant produced by the *L.plantarum* from BCDFTM media have a broad band at 3438 cm^{-1} and 3423 cm^{-1} and another band at 3406 cm^{-1} this may be attributed to the (O-H) groups of polysaccharide. Peaks at $1500\text{-}1650\text{ cm}^{-1}$ indicates the presence of C=C alkene component. According to FTIR spectrum obtained, the molecular composition of BS is composed of carbohydrates and lipopeptide. The hydrophobic chain of BS is composed of lipid and hydrophilic part is mainly composed of sugar. Comparison of the spectra obtained revealed that the BS is closely similar to Xylolipid (glycolipid) reported earlier from different LAB strains (30,25).



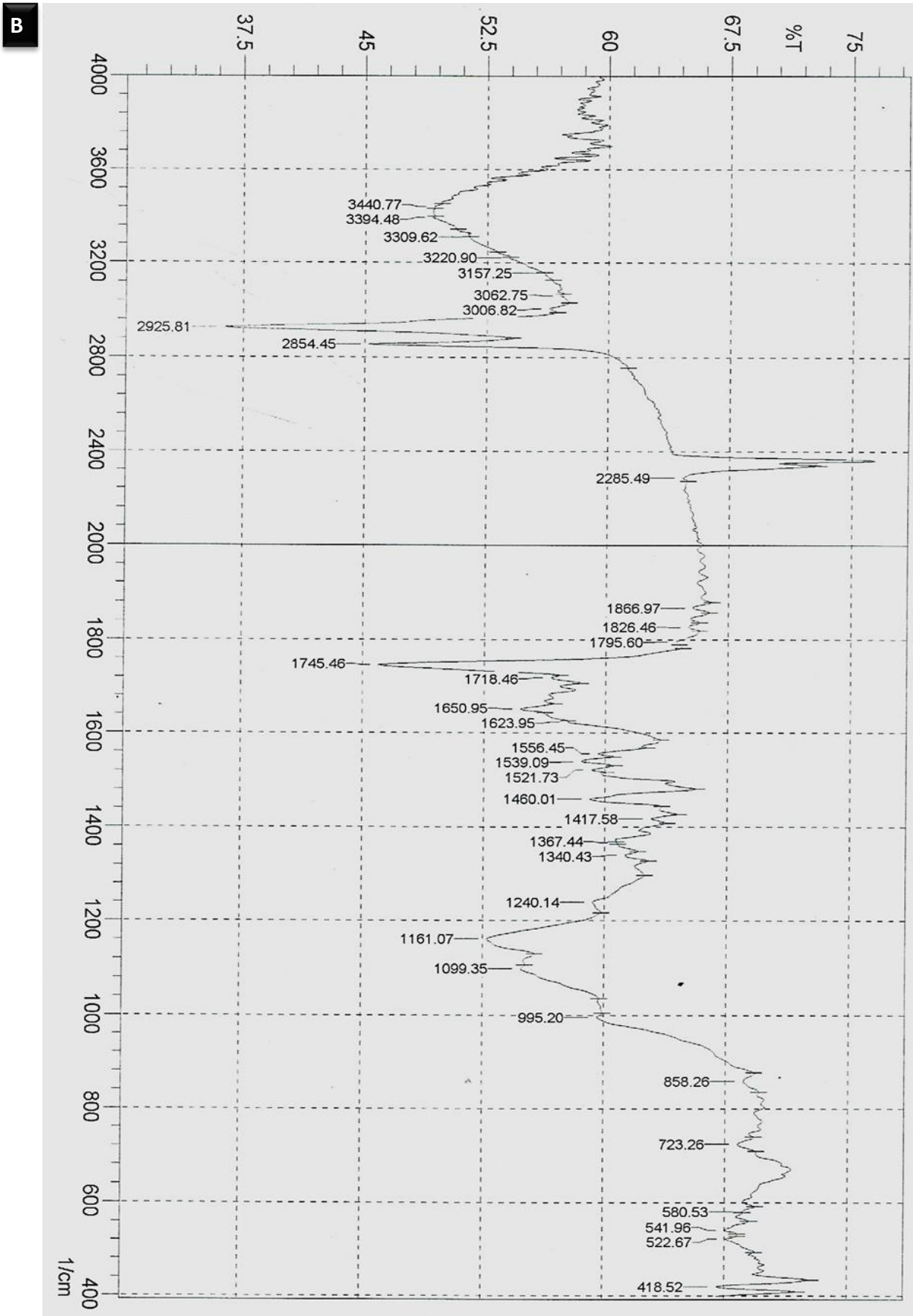


Figure 3. FTIR spectrum of partial purified biosurfactant produced by *L.plantarum* (A): in MSM (B): in BCDFTM media

GC-MS

Results in Figure 4 showed that the purified biosurfactant produced by *L.plantarum* from MSM consists of a high percentage (94%) of Benzene, 1,2,4-trimethyl .psi.-Cumene As-Trimethylbenzene Pseudocumene Pseudocumol 1,2,4- Trimethylbenzene 1,2. Besides there are many other components with a less percentage. In comparison, the results of the purified biosurfactant also showed that it mainly consists high percentage (93%) of Benzene, 1-ethyl-3-methyl- Toluene, m-ethyl-m-Ethylmethylbenzene m-Ethyltoluene m-Methylethylbenzene 1-Ethyl-3. While the purified biosurfactant produced from

BCDFTM media according to the results in Figure 4 B consists of a high percentage (91%) of (1,2-dithiolan-3-yl) pentanoic acid and other components with a less percentage (89%) of 4-Methyl-1-(propan-2-yl)bycyclo [3.0.1] hexane-3-one. (25) have isolated BS from *L. lactis* which also contains octadecanoic acid as a fatty acid chain associated with sugar moiety. Rhamnolipids are the extensively isolated glycolipids which are also composed of β -hydroxydecanoic acid molecules as branched fatty acids (8). Palmitic acid and stearic acid were found major fatty acid type in cell bound biosurfactant produced by *L. pentosus* (32).

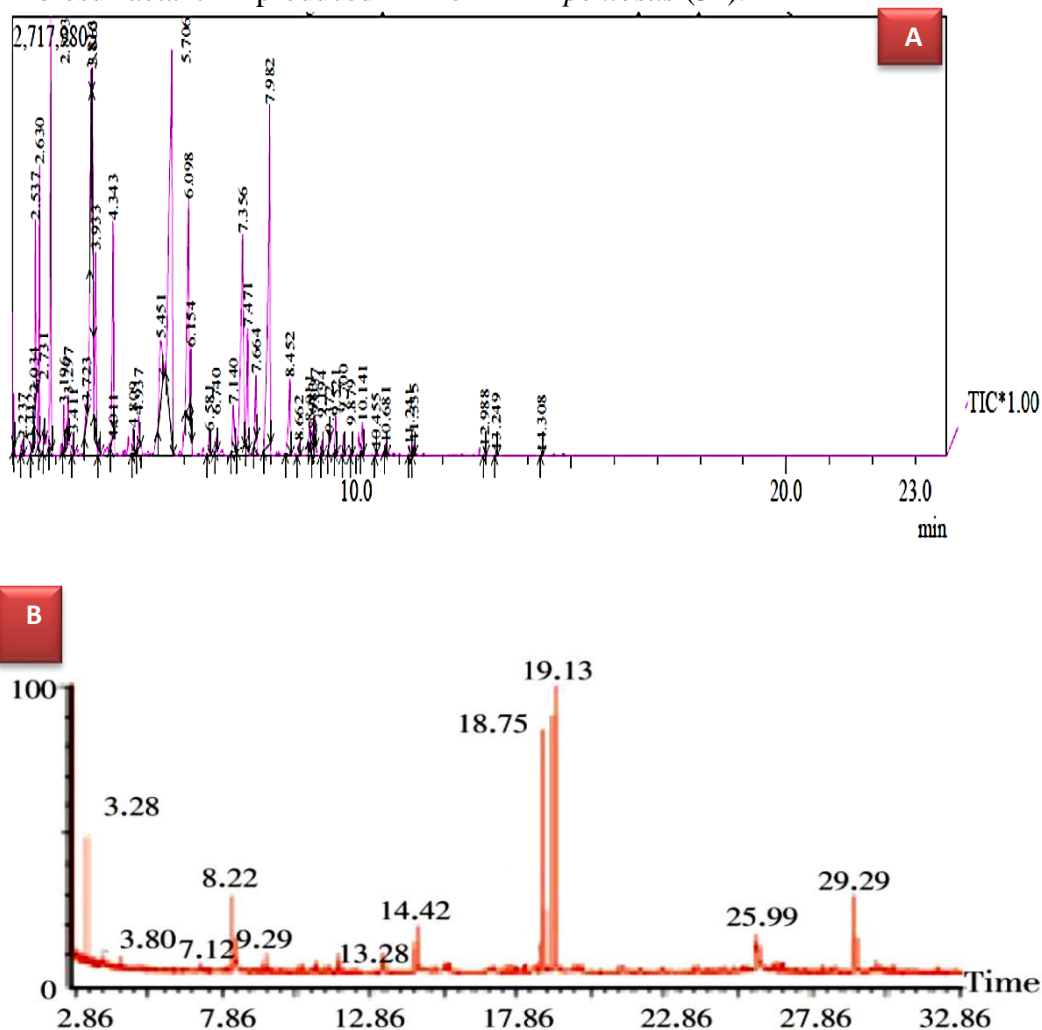


Figure 4. GC analysis of partial purified BS produce from *L.plantarum*. A: MSM, B: Natural BCDFTM

Critical Micelle Concentration (CMC)

The biosurfactant produced by *L.plantarum* ADK2 was able to reduce the surface tension of distilled water to 23 mN/m when the CMC value is 200 mg/2ml. This means that only 200 mg/2ml of biosurfactant is needed to reduce the surface tension to the minimum

level as Figure 5. The ST and CMC are correlated to each other in a way that the most efficient biosurfactant in terms of maximum reduction in ST of solution have low CMC value (17). The CMC of crude RL produced by mutated *P. aeruginosa* was 120 mg/L compared with the 170 mg/L for the non-

mutant isolate crude extract, while the maximum CMD obtained after 100-fold dilution compared to only 10-fold dilution of control (2).

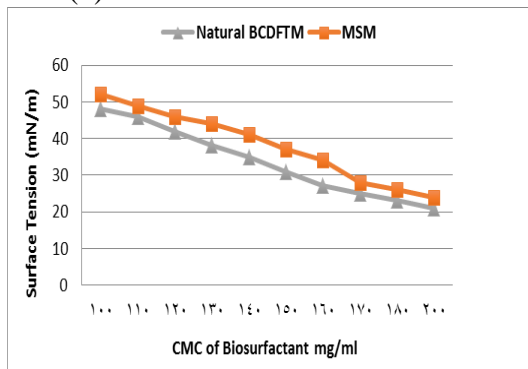


Figure 5. Critical micelle concentration of biosurfactant produced by *L.plantarum* ADK2
Determination of Antibacterial activity

The antimicrobial activity of purified biosurfactant of *L. plantarum* was examined against some microorganisms. The results showed that the biosurfactant had different antibacterial effect on the bacterial growth as shown on (Table 1) and Figure 6 . The biosurfactant with concentration 200 mg/ml had an effect on *S. aureus* and *P. aeruginosa* with the inhibition zone 27 mm, and 33 mm respectively in BCDFTM media. While in MSM The biosurfactant with concentration 200 mg/ml had an effect on *S. aureus* and *P. aeruginosa* with the inhibition zone 21 mm, and 18 mm respectively. This effect may be attributed to the concentration of biosurfactant, it is supposed to exert its toxicity on the cell membrane permeability as detergent like effect that emulsified lipid bacterial membranes and/or form a pore-bearing channel inside a lipid membrane. The antimicrobial activity of purified biosurfactant of *L. plantarum* was examined against some microorganisms such as *S. aureus* and *P.aeruginosa* . In study of (200 mg/ml) showed that MIC values for the two kinds of BS against *S. aureus* are higher than 100 mg/mL. In the time-killing curve test, the two BS showed minimal effects on bacterial growth, with concentrations of BS in the range of 12.5–50 mg/mL. This result similar from those of previous reports by (16) who documented that the BS produced by *L. plantarum* CFR 2194 poses strong inhibitory effects on *Staphylococcus aureus* at a concentration of 25 mg/mL. At present, specific antibacterial mechanism of BS has not been elucidated, as observed from relevant

studies, but it is possibly related to the type of BS and target bacterial strain. Antimicrobial activity of BS has not been observed in all cases (8).

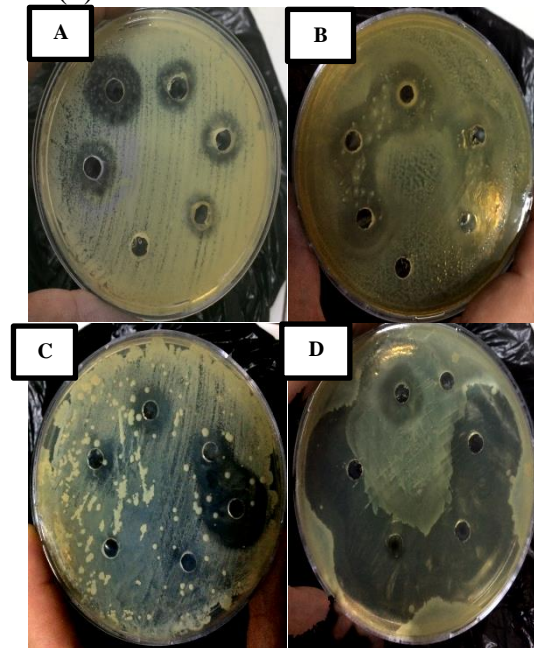


Figure 6. Antimicrobial activity of purified biosurfactant produced by *L. plantarum* (A,B) synthetic media against (A): *S.aureus* , (B): *P.aeruginosa* and (C,D) natural media against (C): *S.aureus* , (D): *P.aeruginosa* respectively

Table 1. Growth inhibition zone (mm) of Pathogenic bacteria by biosurfactant produced from *L. plantarum* in 2 media (MSM and BCDFTM).

Surfactant (mg/ml)	<i>P.aeruginosa</i> (mm)		<i>S. aureus</i> (mm)	
	MS M	BCDFT M Media	MS M	BCDFT M Media
100	-	-	-	-
110	-	-	-	-
120	-	-	-	-
130	-	-	-	-
140	-	-	-	-
150	-	14	6	-
160	14	24	10	4
170	15	29	11	7
180	17	31	14	11
200	18	33	21	27

Determination of Anti adhesive activity

First of all adhesion capability was performed for the *p.aeruginosa* isolate using the method described by (12), adhesive ability of *p.aeruginosa* to epithelial cells isolated from human buccal cavity of healthy people was compared by the frequency of distribution of bacteria on epithelial cells and by the mean number of bacteria adhering to (20) epithelial cells as shown in Figure 7 , and considered as

criteria for adhesive capability of cells, as long as adhering bacterial cells visible under light microscope and easy to count, *p.aeruginosa* isolate displayed differences in their adherence to human buccal cavity epithelial cells and showed a mean number of adhering bacteria (60.50 ± 7.101) bacteria / epithelial cell as indicated in (Table 2). The antiadhesion assay estimated for purified biosurfactant with different concentrations that could effectively inhibit adhesion of the microorganism. The lipopeptide fraction showed significant ($p < 0.05$) anti-adhesion activity against *p.aeruginosa*. Results clearly show that the anti-adhesion activity rises as the concentration of biosurfactant increases. In addition, the anti-adhesion activity of purified biosurfactant against *p.aeruginosa* ranging from (34.70 ± 5.256) to (11.7 ± 9.7) produced from natural media BCDFTM was higher than the anti-adhesion activity of purified biosurfactant against the same pathogen ranging from (46.95 ± 7.37) to (20.15 ± 2.805) produced in MSM. The purified biosurfactant reduced the adhesion of *p.aeruginosa* increased the concentration of purified of biosurfactant. as Figure 8 :C and D and (Table 3 and 4).

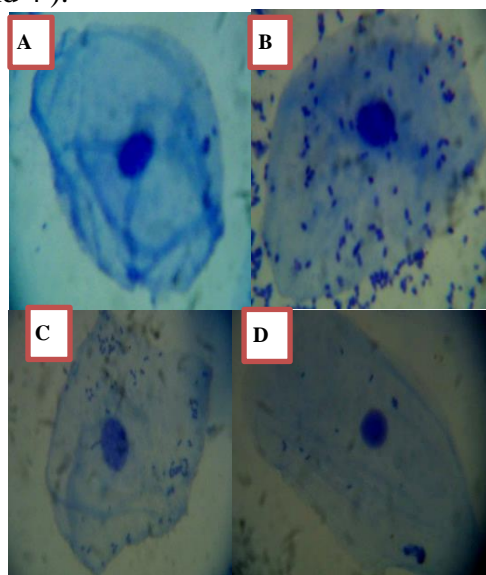


Figure 7. Adhesion capability of *p.aeruginosa* A: Epithelial cell from human buccal cavity as control visualized . B: *p.aeruginosa* isolate adhered to human buccal cavity epithelial cell visualized at (100x). C: Inhibition of adhesion by biosurfactant produced by *L. plantarum* at (200 mg/ml) in MSM. D: Inhibition of adhesion by biosurfactant produced by *L. plantarum* at (200 mg/ml) in BCDFTM

Table 2. Mean of Adhesive capability of *p. aeruginosa* isolate to human buccal epithelial cells

pathogenic isolate	In Phosphate Buffer Saline					Mean No. of Adherent <i>p.aeruginosa</i> / cell \pm SD
	No. of Adherent <i>p.aeruginosa</i> . to No. of epithelial cells					
	0	1-5	6-20	21-50	>50	* Mean \pm SD
<i>P. aeruginosa</i>	0	0	5	4	11	60.50 \pm 7.101

Table 3. Effect of different concentration of biosurfactant produced by *L.plantarum* from MSM on adherence capability of *p.aeruginosa*

Surfactant (mg/ml) (MSM)	In Phosphate Buffer Saline					Mean No. of Adherent <i>p.aeruginosa</i> / cell \pm SD
	No. of Adherent <i>p.aeruginosa</i> . to No. of epithelial cells					
	0	1-5	6-20	21-50	>50	* Mean \pm SD
100	0	3	4	4	9	46.95 \pm 7.379
110	0	2	5	5	8	45.25 \pm 7.427
120	0	2	6	8	4	32.75 \pm 4.620
130	0	3	7	6	4	32.04 \pm 1.262
140	0	4	6	6	4	31.45 \pm 5.211
150	0	4	7	5	4	30.35 \pm 5.349
160	0	5	7	4	4	27.75 \pm 5.571
170	0	4	9	7	0	21.15 \pm 3.771
180	0	4	9	7	0	21.15 \pm 3.771
200	0	2	10	8	0	20.15 \pm 2.805

Table 4. Effect of different concentration of biosurfactant produced by *L.plantarum* from BCDFTM on adherence capability of *p.aeruginosa*

Con. of biosurfactant (mg/ml) (BCDFTM)	In Phosphate Buffer Saline					Mean No. of Adherent <i>p.aeruginosa</i> / cell \pm SD
	No. of Adherent <i>p.aeruginosa</i> . to No. of epithelial cells					
	0	1-5	6-20	21-50	>50	* Mean \pm SD
100	0	3	5	7	5	34.70 \pm 5.256
110	0	4	6	6	4	32.35 \pm 5.164
120	0	2	6	8	4	32.7 \pm 4.6
130	0	3	7	7	3	28.25 \pm 4.762
140	0	4	8	6	2	24.5 \pm 4.7
150	0	2	11	5	2	23.3 \pm 4.1
160	0	7	8	5	0	19.1 \pm 3.6
170	2	4	10	4	0	14.1 \pm 9.9
180	1	4	8	7	0	13.5 \pm 12.2
200	0	5	5	10	0	11.7 \pm 9.7

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