PRODUCTION AND CHARACTERIZATION OF BIOSURFACTANT (GLYCOLIPID) FROM LACTOBACILLUS HELVITICUS M5 AND EVALUATE ITS ANTIMICROBIAL AND ANTIADHESIVE ACTIVITY

M. KH. Kadhum N. H. Haydar
Researcher Prof .
Dept. of Biotechnology. College of Science University of Baghdad-Iraq
Email:maha95khalid@gmail.com

ABSTRACT

The current study was aimed to optimize, characterize and evaluate the antimicrobial and antiadhesive activity of biosurfactant(glycolipid) produce from local isolate*Lactobacillus helvetius*M5. The obtained results indicate that the highest emulsifying activity occurred at pH 7, Lactose (5%) the best production medium, 1% peptone as nitrogen source, maximum growth and production of biosurfactant was observed at C: N ratio (5:1) and after 120 h of incubation. Partial purified biosurfactant was characterized byfourier transform infrared spectroscopy and gas chromatography mass. FTIR results indicated aliphatic hydrocarbon chains along with a polysaccharide moiety that confirmed the glycolipid nature of the biosurfactant produced . GC analysis of glycolipid indicated the cycle aliphatic lipid nature of the structures in the biosurfactant.Antibacterial and antiadhesion activities of biosurfactant were evaluated against some pathogenic bacteria. The biosurfactant showed inhibition zones diameter ranged from (12 to 29 mm) and (15 to 31 mm) against *P. aeruginosa and S. aureus* respectively at concentration of glycolipid ranged from 20 to 100 mg/ml.The highest antiadhesive property was observed against *S. aureus* (78%) and *P. aeruginosa* (74.5%) at concentration 50 mg/ml of glycolipid respectively.

Key words:*Lactobacillus*, optimization, characterization,glycolipid, antimicrobial, antiadhesive.

مجلة العلوم الزراعية العراقية -2020 :51 (6):1543-1558 كاظم وحيدر

انتاج وتوصيف المستحلب الحياتي (الدهون السكرية) من بكتريا *Lactobacillus helveticus* M5 وتقيم الفعالية ضد المايكروبية والالتصاق.

مها خالد كاظم ناظم حسن حيدر
باحث استاذ
قسم التقنيات الاحيائية/ كلية العلوم / جامعة بغداد / العراق

المستخلص

هدفت الدراسة الحالية الى انتاج المستحلب الحياتي (دهن سكري) من بكتريا الحليب *Lactobacillus helviticus* وتحديد الظروف المثلى للانتاج وتوصيفه وتقيم فعاليته الضد مايكروبية وضد الالتصاق .بينت النتائج ان اعلى فعالية استحلاب للدهون السكرية المنتجة كانت عند الدالة الحامضية 7 باستخدام اللاكتوز بتركيز 5% كأفضل مصدر كاربوني والببتون بتركيز 1%كأفضل مصدر نتروجيني وبنسبة الكاربون الى نتروجين 5:1بعد 120 ساعة من الحضن .وصف المستحلب الخام الناتج باستخدام كروموتوغرافيا الغاز السائل GC وتنقية FTIR حيث تبينت نتائج FTIR وجود سلسلة كاربوهيدراتية (اليفاتية) مع جزيئات متعدد السكريد والتي تؤكد طبيعة الدهون السكرية للمستحلب الناتج .كما بينت نتائج التحليل بأستحدام الغاز السائل للدهون السكرية بأنها عبارة عن دهون ذات تركيب اليفاتي حلقي في معظم مكوناتها.تم تقيم الفعالية الضد مايكروبية والفعالية ضد الالتصاق للمستحلب الحياتي المنتج ضد بعض الاحياء الممرضة. اظهر المستحلب فعالية مضادة ومثبطة ضد هذه الممرضات حيث تراوحت اقطار تثبيط النمو بين (12–29) و (15–31) ضد بكتريا (*Staphylococcus aureus* و *Psedomonas aerogenosa*) بالتعاقب عند تركيز (20–100)ملغم/مل من المستحلب الناتج. كما كانت اعلى فعالية للالتصاق (78%و 74%)ضد بكتريا (*Psedomonas aerogenosa* و*Staphylococcus aureus*) بالتعاقب عند تركيز 50ملغم/مل من المستحلب الناتج.

كلمات مفتاحية: بكتريا اللبن, الظروف المثلى, توصي ف,الدهون السكرية, ضد المايكروبية, ضد الالتصاق.

*Received:12/1/2020, Accepted:26/4/2020

INTRODUCTION

Amphiphilic molecules with proven surface properties and emulsifying properties are biosurfactants. Biosurfactants are typically amphiphilic molecules, where hydrophobic molecules are either long-chain fatty acid, hydroxyl fatty acid or a-alkyl-b-hydroxy fatty acid, and hydrophilic molecules may be glucose, amino acid, cyclic peptide, phosphate, carboxylic acid, or alcohol (17) . Microbial surfactants play an important role in the solubility of water-insoluble heavy metal compound binding, bacterial pathogenesis, cell adhesion and aggregation, quorum sensing, antimicrobial and anti-biofilm growth (7,11).Biosurfactants were documented for their properties as antibacterial, antifungal, and antiviral. Which make them an alternative to traditional antibiotics against various food-borne pathogens (29). Bacteria are the main group of microorganisms producing biosurfactants although they are also produced by some yeasts and filamentous fungi. Microorganisms that grow on water-inmiscible hydrocarbons may synthesize these compounds; as well as water-soluble compounds such as Glucose, saccharose, glycerol or ethanol that can be excreted or retained in the cell wall (19). The use of cheap substrates such as agro-industrial waste, the optimisation of medium and crop conditions, the development of efficient recovery processes and the production of microorganisms will contribute to making their development more economically attractive by developing cheaper and more effective processes (25).A number of studies have documented the ability of lactobacilli as producers of biosurfactants (29).Biosurfactants formed on silicone rubber and other biomedical instruments by LAB(Lactic acid bacteria) damaged biofilm formations (11). The chemical composition of the biosurfactants developed by lactobacilli was studied from different bacterial species: the *L.helveticus* derived biosurfactant consists mainly of fractions of lipids and sugar; the biosurfactants*L.pentosus, L.lactis and L.paracasei* are glycoproteins or glycolipopeptides, whereas the biosurfactants*L.plantarum* are of glycolipid or glycoprotein nature (10) .The process economics is currently the key factor preventing the widespread use of biosurfactants, and several techniques have been developed to reduce its production costs and make fermentation competitive with chemical synthesis (25). Biosurfactants isolated from several lactobacilli were classified as multi-component mixtures consisting of protein and polysaccharides, in other cases glycolipids were known as surface active compounds (37). Microbial surfactants are called secondary metabolites, play an important role in the survival of microorganism generating biosurfactants by promoting the transport of nutrients or microbe-host interactions, or by acting as biocide agents(14,15), bacterial pathogenesis, and biofilm formation (5). The current study was aimed to production, and characterization of glycolipid produced by *Lactobacillus* sp.and evaluated its antibacterial activity.

MATERIALS AND METHODS

Sample collection and bacterial isolation

One hundred nineteenSamples from various sources were collected from(humans anddairy product). One gm or ml of dairy samples was added to 9 ml of MRS broth and incubation for 48hrs. at 37 ^{o}C. in the presence of 3-5% CO_2 by using Candle Jar, then in test tubes and dilution measures, one ml of sample was applied to 9 ml of 0.1 per cent peptone water. when carried out until 10^{-6} were done. For human, samples were taken from vaginal of healthy women's then the samples were grown on MRS agar medium and incubated at 37 ^{o}C for 48 hr. using Candle Jar (10). antifungal (Nystatin) was applied to crops to prevent fungal growth in crops. The isolates were purified in selective medium by subculturing on MRS-agar (8), then the purified colonies were maintains on the same media until using in the remaining studies.

Screening of *Lactobacillus spp.*isolates for biosurfactant production: The bacterial isolates were cultured in 100 ml MRS broth and grown for 120 h, in anaerobic condition at 37 ^{o}C. for intracellular biosurfactant production, at the end of the experiments (120h), 10 ml of culture were centrifuged for dry biomass estimation. Additionally, cells were harvested by centrifugation (10000, 15min), Washed twice in demineralised water and resuspended in 20 ml phosphate buffered saline (PBS: 10Mm KH2PO4 and 150 Mm NaCl with pH set to 7.0). The bacteria were left upfor 24 hours at medium room temperature with gentle stirring.

Centrifugation was used to remove bacteria and the remaining supernatant was tested for surface tension, Emulsification activity(E24%), and Biuret test(29).

Biosurfactant analysis using Emulsification Index (E24%)

Two ml of cell free supernatant was added to 2 ml of Toluene,Play in the vortex for 2 minutes, then leave for 24 hours. The height of the emulsifier layer was measured at room temperature. As a percentage of the height of the emulsified layer (mm) the emulsification index is given to the total height of the liquid column (mm) multiplying by 100 (2).

Emulsion Index (E24)% = Height of emulsion layer /Total height of broth× 100

Surface tension assay

The surface tension (ST) of an aqueous solution was measured by the Wilhelm platinum plate with a QBZY-2 Tensiometer (China). Fifteen ml of supernatant was placed on the tensiometer platform and poured into 50 ml glass beaker . The measurement was conducted at 25±1ºC after dipping the plate in the solution, until monitoring the value of supernatant ST following the procedure of measurement written in the manual of the instrument. Between each measurement, the Wilhelmplate was rinsed with acetone and burned by alcohol burner to ensure no contaminant affect the recorded results. In addition to the standard weight of the instrument, distilled-water (72 mN/m), and ethanol (22 mN/m) were used for calibration. For more accurate value, the average of three records was used in the study (27).

Biuret test

The biuret test was used to detect the presence of lipopeptide and glycolipid bio surfactant. Two milliliters of crude extract solution of biosurfactant were first heated at 70 ºC before mixing with two milliliters of 1M NaOH solution. Then, drop of two milliliters of 1% of $CuSO_4$ were slowly added to observe any color change (green color for glycolipid and violet color for lipopeptide) (22).

Dry-weight cell determination

At the end of incubation period, 10 ml of culture was centrifuged at 8,500 g for 20 mins to remove bacterial cells. The collected bacterial cells were washed with phosphate buffer and allowed to dryness in oven (80°C) to obtain a constant dry weight, which is reported in terms of g/L (4).

Identification of *Lactobacillus* sp.

Morphological and biochemical tests: In the current study, *Lactobacillus spp*. Were primary identified according to the morphological tests includes, shape of colonies, size, texture of colonies, production of pigment. Biochemical tests include oxidase, catalase and indole tests.

VITEK 2 system

Pure night rising community of selected*Lactbacillus*sp. on MRS agar plate was used to be identified using VITEK 2 system. Gram negative (Gp) card of this system is used for the automated identification of 135 taxa of the most significant fermenting and non – fermenting Gram –positive bacilli. The Gpcard is based on established biochemical methods and newly developed substrates measuring carbon source utilization, enzymatic activities, and resistance (6), there are 47 biochemical tests and one negative control well.

Optimization of medium composition and culture conditions for biosurfactant production: Carbon sources: Erlenmeyer flasks (250 ml) contains fifty ml of MSM(Ammonium citrate 2g/l , sodium acetate 5g/l , Magnesium sulfate 0.1 g/l , Manganese sulphate 0.05 g/l , dipotassium phosphate 2g/l) were prepared and supplemented with (0.5% v/v) of different carbon sources (whey, glucose, glycerol, Fructose and Lactose). After autoclaving, for 10 min the flasks were inoculated with 2% ($1x10^8$CFU/ml, OD = 0.5on McFarland)of *Lactobacillus* isolate and incubated in shaker incubator (120 rpm) at 37˚Cfor 120 hrs. for biosurfactant production. Then the samples were taken from each flask for the determination ofbiomassandbiosurfactantproduction.

Effect of nitrogen sources

Fifty ml of the defined liquid medium supplemented with (0.5% v/v) lactose asoptimal carbon sources was prepared in 250 ml Erlenmeyer flasks, each contained (0. 1% w/v) of different nitrogen sources (peptone, urea, malt extract and yeast extract). After autoclaving, the flasks were inoculated with 2% (v/v) of *Lactobacillus* inoculum and incubated in shaker incubator (120 rpm) at 37˚C for 120hrs. After the incubation, samples were taken from

each flask for the determination of biomassand biosurfactant production.

Effect of carbon: nitrogen ratios

To determine the best concentration ratio between the carbon and nitrogen sources that support the maximum production of biosurfactant, eight different ratios of the optimized carbon and nitrogen sources were investigated. Fifty ml of the MSM liquid medium was prepared in 250 ml Erlenmeyer flasks each contained a different ratios of carbon (lactose) and nitrogen (peptone) includes (1:1, 2:1, 3:1 , 4:1, 5:1, 6:1 ,5:2, 5:3). After autoclaving, the flasks were inoculated with 2% ($1x10^8$ cell/ml) of *Lactobacillus* inoculum and incubated in shaker (120 rpm) at 37°C for 120hrs then the samples were taken from each flask for the determination of biomass and biosurfactant production.

Effect of pH on biosurfactant production

In order to optimize the effect of pH on biosurfactant production, fifty ml of the MSMwith 0.5% lactose as carbn source liquid medium and 0.1% pepton as nitrogen source was prepared in 250 ml Erlenmeyer flasks. Different values of pH(4, 5, 6, 7, 8,) were applied at medium for finding the best pH value for production. After autoclaving, the flasks were inoculated with 2%($1x10^8$ CFU/ml, OD = 0.5) of *Lactobacillus* inoculum and incubated in shaker 120 rpm at 37˚C for 120hrs for biosurfactant production. After the incubation, samples were taken from each flask for the determination of biomass and biosurfactant production.

Effect of incubation period

In order to optimize the incubation time, fifty ml of the MSM liquidwas prepared in 250 ml Erlenmeyer flasks contained lactose (0.5%) as a source of carbon and peptone (0.1 %) as nitrogen sources at C: N ratio (5:1) at pH 7. After autoclaving, the flasks were inoculated with 2%*Lactobacillus* and incubated in different incubation periods include (24, 48, 54, 72, 78, 96, 102, 120 ,126) using shaker incubator , Then the sample were taken from each flask for estimation of biomass and biosurfactant production.

Biosurfactant extraction

The culture of *Lactobacillus sp.* collected after 120 h of incubation.for intracellular biosurfactant production, at the end of the experiments (120h), 10 ml of culture were centrifuged for dry biomass estimation. Additionally, cells were harvested by centrifugation (10000, 15min), Washed twice in demineralised water and resuspended in 20 ml phosphate buffered saline (PBS: 10Mm KH2PO4 and 150 Mm NaCl with pH set to 7.0). The bacteria were left upFor 24 hours at medium room temperature with gentle stirring. The supernatant contained biosurfactant was transferred to separation funnel and extracted by using different solvent systems: mixture of chloroform – methanol (2:1) (29), chloroform, and methanol. The aqueous layer at the bottom of the separation funnel was removed and the emulsion layer was collected in a glass Petri dish and dried at (40–45) ºC until converting to powder. The resulting powder was weighted and calculated to find the right extraction process and the powder that is contained in a clean vial (30,38).

Characterizations of biosurfactant

FTIR analysis: Biosurfactant FTIR spectra was analyzed using Potassium bromide (KBr) after sample homogenisation. KBr (AR grade)was vacuum-dried at 100 ° C for 48 h and 100 mg KBr was combined separately with 1 mg of biosurfactant to prepare KBr pellets. Data were collected in the range of 500-4000 waves per cm.UV spectra had been recorded in the spectrophotometer Shimadzu- affinity-1. The amplitude of the spectra versus the waven number (1) was ploted.

Analysis of biosurfactantwith Gas chromatography (GC) technique: Biosurfactant was analyzed to their fatty acids components using gas chromatography (GC) according to method described by(44). Fatty acids composition was investigated as follows. Acid methyl ester was prepared by dissolving 10 mg of partial and purified biosurfactant with 1 ml of sulfuric acid – methanol at 90 ˚C for 15 h and 1ml 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 a fused silica capillary column (30 m x 0.25 mm, 0.25 µm film thickness).

Pathogenic microorganisms used in the

antibacterial activity test: The pathogenic bacteria used in the current were isolated from clinical cases obtained from College of Science, Department of Biotechnology. The indicator bacteria used were *Pseudomonas aeruginosa* (isolated from burn) and *Staphylococcus aureus* (isolated from skin). Maintenance of pathogenic bacterial isolates were achieved by streaking on nutrient agar and incubated at 37 ºC for 24 hrs. The cultures were stored at 4ºC and then recultured every three weeks interval time.=

Determination of antibacterial activity of Biosurfactant

The antibacterial activity of biosurfactant was determined against *P.aeruginosa*and *S. aureus*, using paper disc diffusion method (24,32). Overnight growth (24 h) culture of the test bacterium were adjusted to ($1x10^8$cfu/ml) equivalent to (OD= 0.5 on McFarland)Were streaked on sterile Muller Hinton agar surface . Six millimeters diameter of Whatman filter paper discs (GF/C) were prepared by scissors and sterilized in a Petri dish at 121˚C for 15 min. After sterilization, each disc was impregnated with 100µl of different concentration of biosurfactant (10, 20 40, 60, 80 and 100 mg/l), Then the discs were put on the surface of cultured plate with pathogenic bacteria separately. DMSO has been used as a control, due to the fact that it has no antimicrobial activity. Then plates incubated at 37˚C for 24h. Following the incubation, the diameter of inhibition zone was measured using electronic ruler in mm.

Determination of anti-adhesive activity of produced biosurfactant

The anti-adhesive activity of the biosurfactant s fractions against target pathogens was performed in co-incubation as described by (12) .The 96-well microtiter plates were coated with 200 µl of biosurfactant fraction solutions prepared in PBS at different concentrations (5, 7.5, 10, 15, 20, 25, 40, 50 mg/ml).And they incubated the microtiter plates at 37 ^oC for 24 hours. The biosurfactant solution was subsequently drained and the plate rinsed twice at 100 µl PBS pH 7.2 to reduce biosurfactant that was not adhesive . The next step was the addition of 150 µl of a washed bacterial suspension in PBS, adjusting it to 0.5 McFarland standard turbidity (a final density of 10^8 CFU ml-1) to individual wells after which the microtiter plate was again subjected to 24 hour incubation at 37^{o}C. By gently rinsing the wells twice with PBS pH 7.2 no adhering cells were removed. Quantification was carried out using violet crystal assay(23). After that 100 µl of 99 per cent methanol was applied to each well, the biofilm was mixed for 15 min and the plate was then air-dried. In the next step, 100 µl of crystal violet 2 percent was added and retained before removing the superfluous crystal violet by pipetting for 20 min, and the residue in the wells was rinsed with tap water. The stain associated with the adherent pathogens was solubilized with 100 µl of 33% glacial acetic acid for each well and the optical density readings of individual wells were recorded at 595 nm using micro Elisa auto reader (Model 680, Bio-Rad). This was followed by the preparation of Bacterial suspension with no biosurfactant, as control. The percentage of adherence reduction was computed with the formula of (12).

$$\textbf{Microbial antiadhesion (\%)} = [1 - (ODc)/OD0] \times 100$$

Where: ODc, is the optical density of the well with a biosurfactant concentration and pathogen, and OD0 is the optical density of the pathogen suspension with no biosurfactant (control). Triplicate assays were conducted and the mean of optical density was taken.

RESULTS AND DISCUSSION

Isolation and identificationbacterial isolates

One hundred nineteen samples were collected fromhuman and dairy product. The samples were primarily grown onto MRS agar plates as selective media for isolation and incubated at 37 °C for 48 hr. with the presence of (3-5 %) CO_2 by using Candle Jar**.** The results were showed that only eighty two isolates were found belongs to genus *Lactobacillus* which subjected to morphological, microscopy, and biochemical tests in order to confirm their identification. The isolates were identified as related to the genus *Lactobacillus* by their small (2-5 mm), convex, smooth, glistening colonies, and opaque without pigment on MRS, (Figure 1).Microscopically, the bacteria appeared under oil immersion lens (100x) as gram positive bacilli, arranged singly, pairs or short chains as shown in (Figure 1-A**)**. While biochemical test results are revealed that all isolates were negative for oxidase and catalase

tests and for indole test. The biochemical testes are compared with identification schematic diagram of (16).

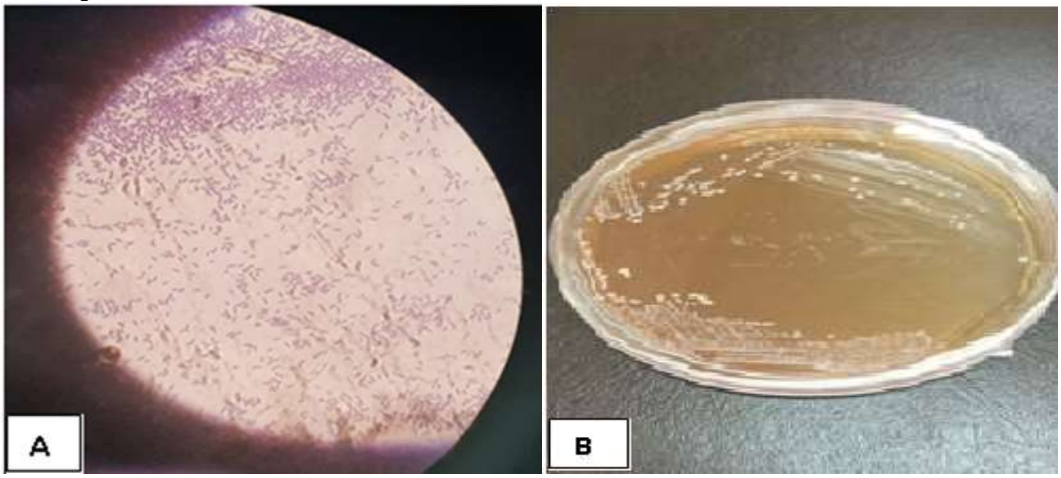

Figure 1. A. Microscopic field of *Lactobacillus* cells, bacilli in shape and pairs in chain. B.The growth of *Lactobacillus* on MRS agar after 48h of anaerobic incubation at 37°C.

Screening of *Lactobacillus* isolates for biosurfactant production: The ability of eighty two isolates of *Lactobacillus*sp.wereselecetedfor screening a higher biosurfactant development isolates which may be used for further experiments in this analysis, the screening process was undertaken. Among eighty two isolates were screened for biosurfactant production, eight isolates were exhibited glycolipid biosurfactant production according to biuret test (formation of green ring over the surface of the supernatant) as well as the isolates revealed higher biosurfactant production. The isolates *Lactobacillus*(M5) has demonstrated maximum development of biosurfactants compared with other isolates . The result showed higher Emulsification activity E24% (75.3%) and reduction in surface tension (33.2mN/m) and biomass(5.5 g/l) after five days of incubation (Table1). Therefore the isolate M5 was selected for remaining studies (29).

Table 1. Screening of *Lactobacillus* spp. for biosurfctant production in MRS media after five days in shaker incubator(120 rpm) at 37° C after 120 hrs.

No.of isolate	sources	Surface tension mN/m	E24%	Biomass g/l	Biuret test
M4	yogurt	34.4	63.45	4	+
M5	yogurt	33.2	75.37	5.5	+
M6	yogurt	37.6	73.12	5.2	+
M11	yogurt	39.1	51.65	4.5	+
M14	yogurt	38.0	53.5	3	+
M22	vagina	37.8	57.32	3.7	+
M24	vagina	39.9	52.80	4.8	+

+ = Green ring indicate for glycolipid biosurfactant

Identificationof*Lactobacillus* sp. Isolate

VITIK 2 compact system was carried out as confirmatory test for the identification of *Lactobacillus* sp.M5isolate. The GP card was used for gram positive bacterial isolate, which consists of 43 biochemical tests. The results indicated fromthe figure below that the isolate Lactobacillus sp. Belong to the genus *Lactobacillus helveticus*

Optimization of media composition and culture Conditions Effect of Ph

To investigate the effect of initial pH medium on biosurfactant production by *L. helveticus* (M5) ,MSM media which selected in previous study was adjusted to different pH values. The obtained results in(Figure 2)indicate that the highest emulsifying activity (75%),lowest surface tension (32.9mN/m) and dry biomass (5.5g/l) occurred at pH 7.The synthesis of the biosurfactant decreased without the pH control, indicating the importance of maintaining it throughout the fermentation process (33)**.** On the other hand, lower biosurfactant observed at PH inferior to 5 and greater than 7. Environmental factors and conditions of development, such as the pH effect on the production of biosurfactants through their cell growth or activity effect. Development of

rhamnolipids by the *Pseudomonas* spp. Goal achieved at pH 7 (34).

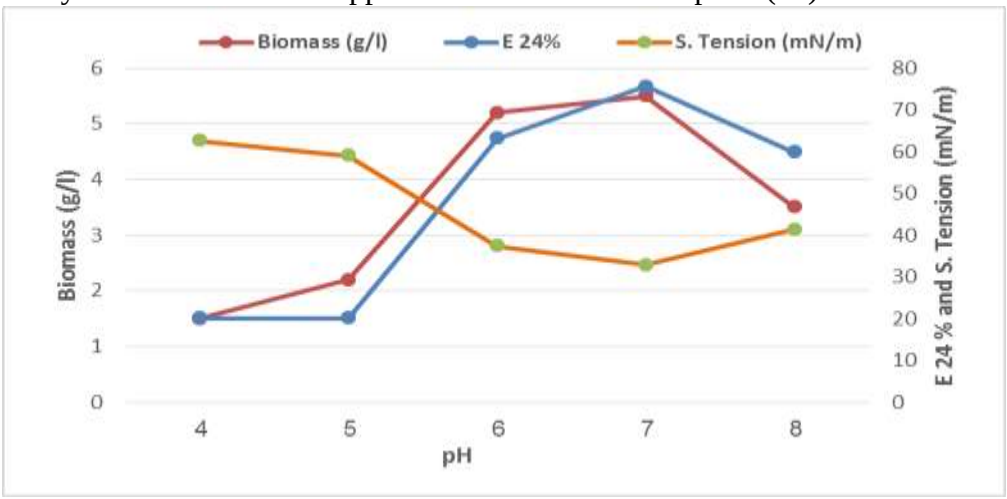

Figure 2. Effect of pH on production of biosurfactant from *L.helviticus* M 5

The effect of carbon sources

Biosurfactant production was tested in the presence of different carbon sources incorporated in to the production medium with concentration of 0.5% (w/v).Results indicated in (Figure 3)that the E24% (76.5 %), surface tension (33.5mN/m) and dry biomass (6g/l) were achieved when lactose was used as the source of carbon and energy respectively. While the lowestactivity was obtained when fructose and glycerol (40%, 32.14mN/m and 44%, 38.4 mN/m) were used respectively. These results demonstrated the ability of this bacterium to degrade a wide range of carbon sources and biosurfactant production. The biosurfactants produced *L. plantarum* utilized molasses as substrate exhibited high surface tension reduction from 72mN/m to values ranged from 47.50 ± 1.78 and high emulsification index reached 49.89 ± 5.28. While, the isolate exhibited lower surface tension reduction from 72mN/m to to 49 2 ± 2.43 and lower emulsification index reached 41.85 ± 2.56 when glycerol was used as carbon source(39).

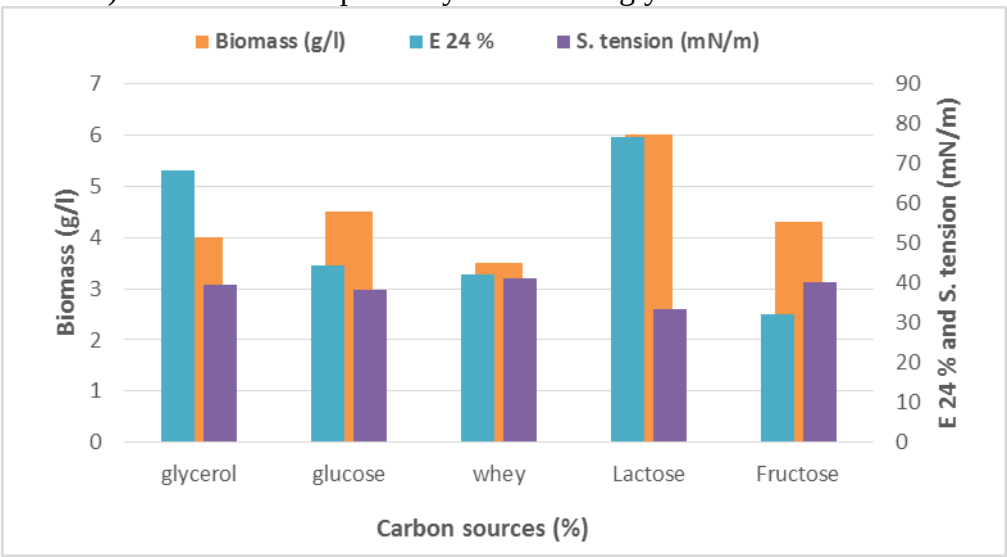

Figure 3. Effect of carbon source on production of biosurfactant from *L.helviticus*M 5.

Effect of nitrogen sources

In order to determine the effect of different types of nitrogen sources on biosurfactant production by *L.helveticus*M5, different nitrogen sources were tested. Results (Figure 4) showed that the production of biosurfactant varies with different nitrogen sources. The highest E24% (77.84%) with lowering the surface tension of (32.1 mN/m) and dry biomass (6.5g/l) were obtained when peptonewas used as nitrogen source. While the lowest emulsification activity and higher surface tension observed with malt extract and urea (36.14%,45.7mN/m and 56%,40.7mN/m) respectively, compared with other nitrogen sources. The bacteria require nitrogen to complete its metabolic pathways and it is essential for the microbial growth as protein and enzyme syntheses depend on it(2).

Previous studies has found comparable results, they noticed that the best source of nitrogen for biosurfactant (bacteriocin) production is yeast extract, and glucose as the best carbon source by *L. plantarum*(38). The ammonium salts and urea were preferred nitrogen sources for biosurfactant production by *Arthrobacter paraffineus*, whereas nitrate supported the maximum surfactant production by *P. aeruginosa* and *Rhodococcus*sp (14). However, the potassium nitrate support the maximum production of biosurfactant by the yeast *Rhodotorulaglutinis*IIP30(9).

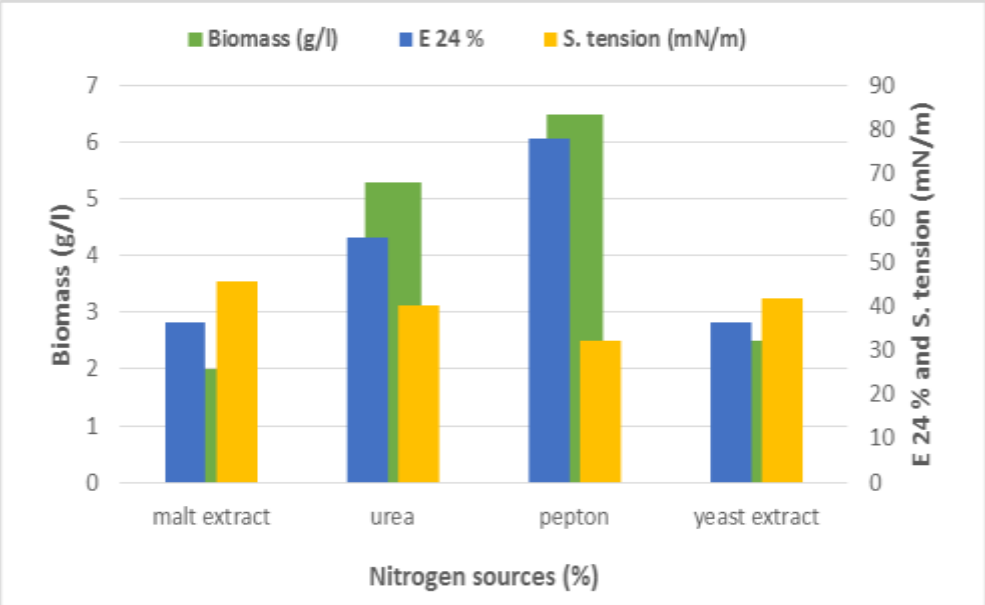

Figure 4. Effect of nitrogen source on production of biosurfactant from *L. helviticus*M5

Effect of C: N ratio

The requirements of carbon in living organisms are usually larger than nitrogen and therefore the balance between the concentrations of them in the culture medium is a crucial aspect as it can determine how microorganisms use these sources (28,43).Thus,To boost the production, of biosurfactant, C:N ratios of (1:01, 2:01, 3:01, 4:01, 5;01 ,6:01, 5:02, 5:03) in the MSM liquidMedium used to identify appropriate proportions . It was found that microbial growth and production of biosurfactant was maximum affected at C: N ratio (5:1) that was used in the previous experiments, (figure 5).Since the bacterial cell require carbon source in large amounts, while the production of biosurfactant is induced by the depletion of nitrogen (21). The results in figure 6 indicated that maximum E24% ofbiosurfactant obtained in the culture was78.16% with reduction in the surface tension to 28.7mN/m and dry biomass was reached 6.2 g/l.

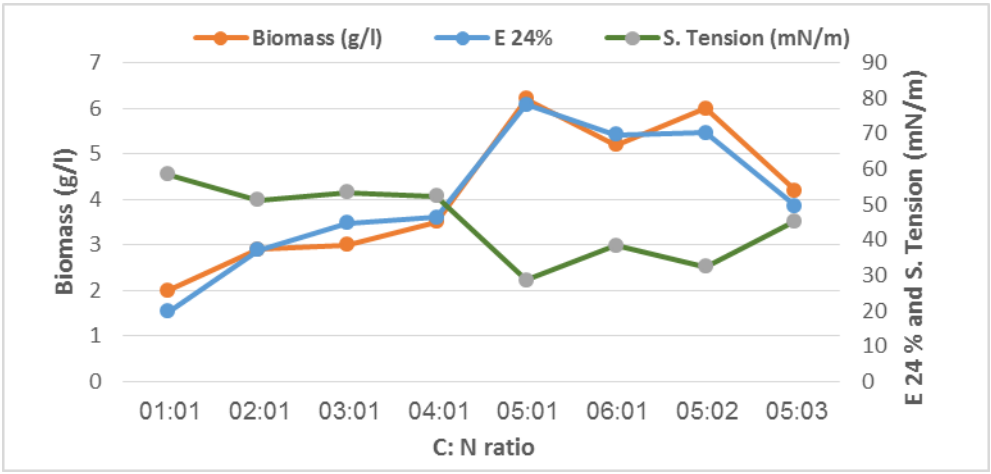

Figure 5. Effect of C: N ratio on production of biosurfactant from *L.helviticus*M5

Effect of incubation period

Different incubation periods (0-126 h) were examinedto detect the best period of bacterial growth and biosurfactantproduction-by*L.helveticus*M5. Result in(figure 6) showed that the maximumE24% (77.4%) and the lowest surface tension (27.6mN/m) and higher dry biomass (6.3g/l) were obtained during 120hrs.ofincubation.Whereas after 126h of incubation, the emulsification activity was decreased and with an increasing the surface tension values with increasing the incubation time. This may be due to the change in the culture conditionsalong with periods such as diminishing of nutrients and accumulating of toxic metabolites which inhibit the bacterial growth**.** The result in the current study pointed out that biosurfactant produced by *L.helveticus* increased with incubation period and the production started at early stationary phase (72h) and reached its maximum values at 96 to120h.

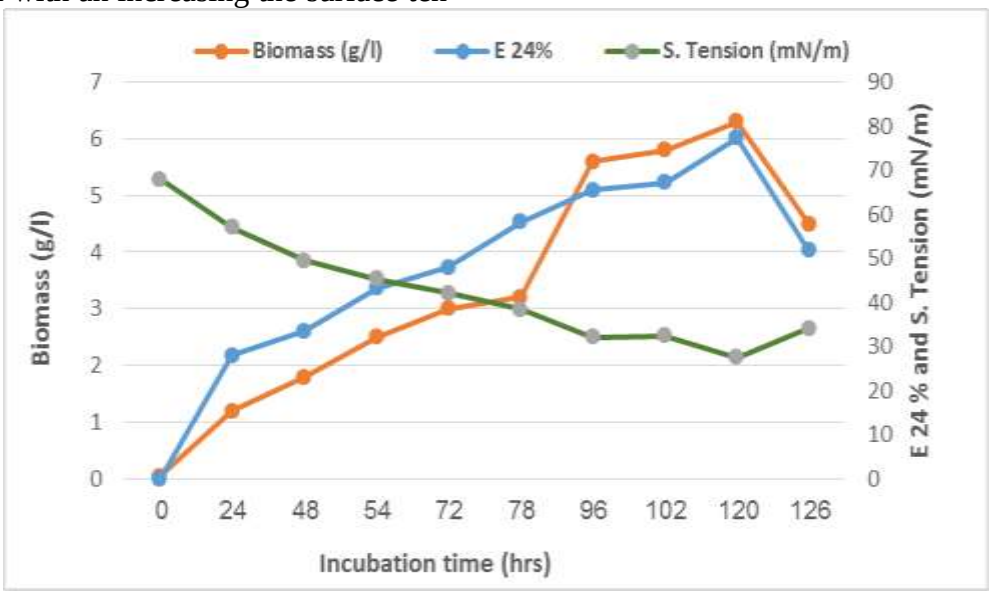

Figure 6. Effect of incubation period on production of biosurfactant from *L. helviticus* M5

As regards the development of biosurfactants in lactose medium during cultivation, at least for biosurfactants linked to cells, it has been shown slight increase of biosurfactant production observed after 48 h (Fig.6). The results in the current study showed that the produced biosurfactant is cell-bound biosurfactant. The production of biosurfactant increased after 72 h of cultivation, with highest values after 120 h of cultivation. These results indicate biosurfactant development via isolate *L. helveticus* begins during the exponential growth cycle, and stays stationary for at least two days. Rodrigues*et al.*(26)observed the same findings for various species of lactobacilli (*L. casei, L. brevis*). Erum *et al.*(10) mentioned that the biosurfactant biosynthesis stopped, probably due to the production of secondary metabolites which could interfere with emulsion formation and the adsorption of surfactant molecules at the oil–water interface. A maximum emulsan production by *Acenitobactercalcoaceti-*

*cus*RAG-1 during the stationary growth phase(37). While peeteres *et al.*(23) were showed that the biosurfactant biosynthesis using olive oil occurred predominantly during the exponential growth phase, suggesting that thebiosurfactant was produced as a primary metabolite accompanying cellular biomass formation (growth-associated kinetics). The RL production was increased with time until it reaches the maximum level after 108 h of incubation where 10.6 g/L was obtained by (2).

Extraction of produced biosurfactant

Optimum conditions for biosurfactant production by isolates *L.helveticus* M5 were utilized for at optimum conditions. The isolate was grown of in mineral salt medium (pH 7) containing 5% lactose as carbon source and 1% peptone as nitrogen source at 37^{o}C, with shaking (120 rpm) for 120 h.After that, biosurfactant was extracted using solvent system (chloroform:methanol, 2:1) this system used to obtain partially purified biosurfactant. It obvious that solvent extraction method has the highest yield, by virtue of the existence hydrophobic end in the biosurfactant, making it soluble in organic-solvents. Equal amount of supernatant of culture and (chloroform:methanol, 2:1) were kept in separating funnel overnight for evaporation and dry weight of biosurfactant obtained were reached to 6.2g/l.

Characterization of produced biosurfactant FTIR spectrum analysis

Biosurfactant FTIR analysis provided by *L. helveticus* M5 in(Figure 7), Indicate the presence of aliphatic hydrocarbon chains along with polysaccharide moiety that confirmed glycolipidity of biosurfactants. The absorption bands at 3444.63 cm -1 and 3429.43 cm -1 indicate the existence of – OH groups, this was similar to the (18) results. At a height of 2929.67 cm -1 the compound showed C-H bond (of sugar moiety). The 2854.45 cm peaks -1, 1452.30 cm -1 represent an aliphatic chain (CH3,-CH2-). 1741.60 cm -1 for C = O stretching in community of esters.Lipid and fatty acids with an elevation of 1643,24 cm -1 say C = O. Asymmetric ester (O-C-O) attributes may have peak at 1315.36 cm -1. In the sugar moiety structure 1047.27cm -1, due to the sugar contacts C-O, the absorption peak of about 1192.01 cm -1 indicates carbon atoms expanding with hydroxyl group. Tops 700.11 cm -1 and 1047.27 cm -1 Associated with the CH2 group (Glycolipid moieties) and the stretching of glyosidic linkage confirming the biosurfactant's glycolipid nature. The sugar residue in the biosurfactant structure displays the hydrophilic characteristics, while the lipid fractions were responsible for the hydrophobic characteristics The biosurfactant glycolipid FTIR spectra was nearly identical to those reported by sharma *et al.* (29) *Lactobacillus casei* for other glycolipid biosurfactant products. In the previus study surekha *et al.*(35) sugar and lipid moieties were identified using TLC confirming the existence of CFBS typed with glycolipids. An study of FTIR has confirmed the chemical composition. The height at 3320 cm−1 depicts OH stretching in presence. Hydrocarbon concentration is confirmed at peak 2900cm The peaks at 1730 cm−1 imply the presence ofC = O stretching in the ester bond is important. The presence of ether moiety was confirmed by peak at 1230 while sugar moiety was clearly indicated by peak at 1000 cm−1 (C-O stretching in sugars). Our study findings strongly suggest that CFBS is of a glycolipid nature.

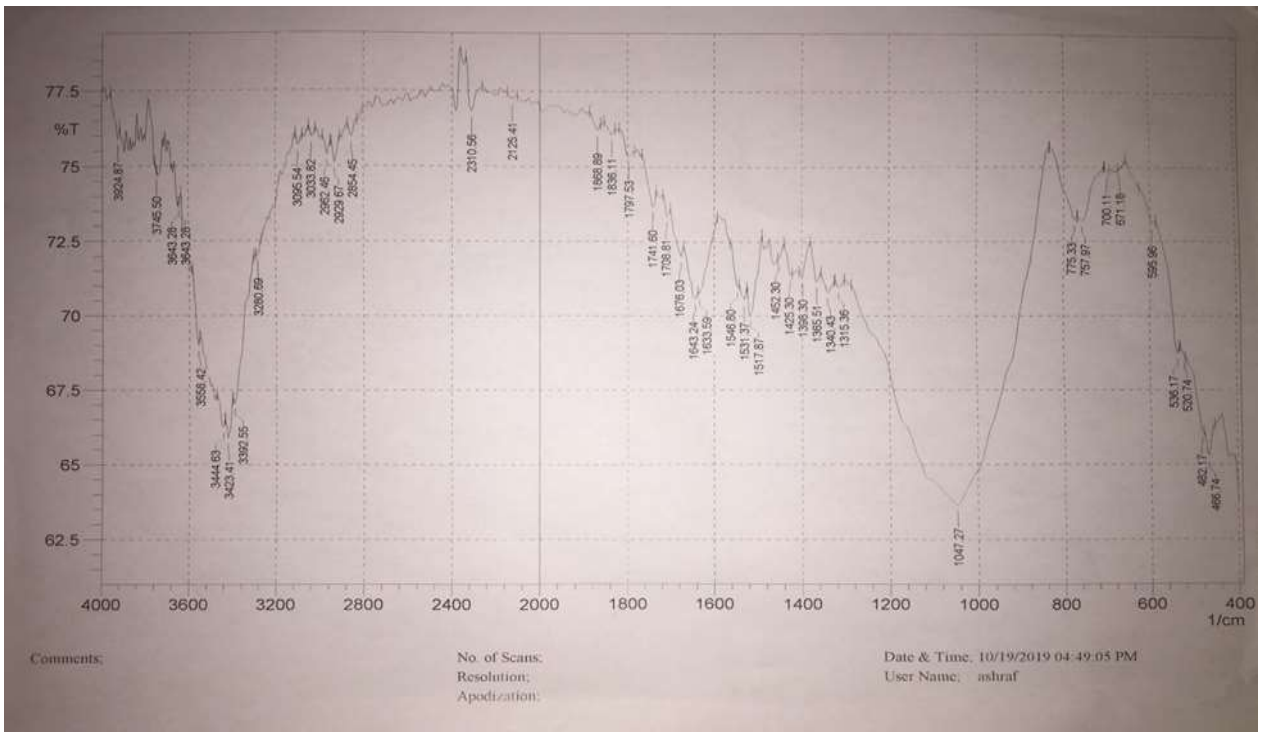

Figure7.FTIR spectrum analysis of biosurfactant produced by *L. helveticus* M5

GC-MS analysis of produced biosurfactant

The chemical composition of semi-purified glycolipid extract were analyzed by GC-MS (figure 8). In comparison, of the constituents with the NIST library, a total of 44 peaks were observed, from that 7 active peak were predicted (Table 2). Of the 7 compounds identified, the data revealed the occurrence of two major separable components with the molecular formulas of $C_{10}H_{14}$ and C_9H_{10} with relative abundance of 100 and 65 % respectively (Table 2). The two major fatty acids were then identified as Benzen,1 methyl – 3- propyl and Benzo cyclopentane with molecular weight 134 and 118 (g/mole) respectively. Both peaks with GC analysis indicated the cycle aliphatic lipid nature of the structures.

Table 2. GC mass profile of the *L. helveticus* M5 glycolipid

No.	Retention time (min)	Compounds	Molecular formula	Molecular weight (g/mole)	Area %	Relative Abundance %
1	2.630	1-Hexane, 3- methyl-6-phenyl-4- (1-phenyl ethoxy)	$C_{21}H_{26}O$	294	4.39	23
2	2.893	Methyl octane	C_9H_2O	128	8.08	42
3	4.343	Ethanol -2-[-(phenyl methyl)amino]	$C_9H_{13}NO$	151	5.33	28
4	5.706	Benzen,1 methyl – 3- propyl	$C_{10}H_{14}$	134	19.14	100
5	6.098	n- propyl benzene	C_9H_{12}	120	7.19	38
6	7.356	2- Methyl decane	$C_{11}H_{24}$	156	10.01	52
7	7.982	Benzocyclopentane	C_9H_{10}	118	12.41	65

The production of glycolipid complex containing carbohydrate (mono or oligo saccharide) and lipid moiety with surface active properties is widely accepted in case of *Pseudomonas* spp. Whereas, in case of *Lactobacillus* spp. the majority of the literature appears to be protein-based biosurfactant. There are few exceptions biosurfactant/s from Lactobacilli species where glycolipid-type biosurfactant obtained from Lactobacilli spp. (29). reported again glycolipid-type biosurfactant from Lactobacilli spp. having mixture of sugar and lipid fractions which was claimed to be similar to xylolipid. Fourier transform infrared spectroscopy and nuclear magnetic resonance analysis confirmed the presence of glycolipid with hexadecanoic fatty acid (C16) chain. Saravanakumari,*et al.* (31) have isolated biosurfac-

tant from *L. lactis* which also contains octadecanoic acid as a fatty acid chain associated with sugar moiety. The compounds in the current study were identified from the GC analysis of the extract (Table 2) might be responsible for the antibacterial activity. Sharma *et al.*(29) used *L. casei* MRTL3 as biosurfactant producing strain and reported glycolipid-type biosurfactant analyzing through thin-layer chromatographic studies. The presence of lipid and sugar moieties in biosurfactant was confirmed using 1H-Nuclear magnetic resonance spectroscopy. The presence of methyl esters glycolipid biosurfactant was correlated to an increased hydrophobicity and, as a result enhancing not only the biosurfactant surface activity but also hemolytic and antifungal activities

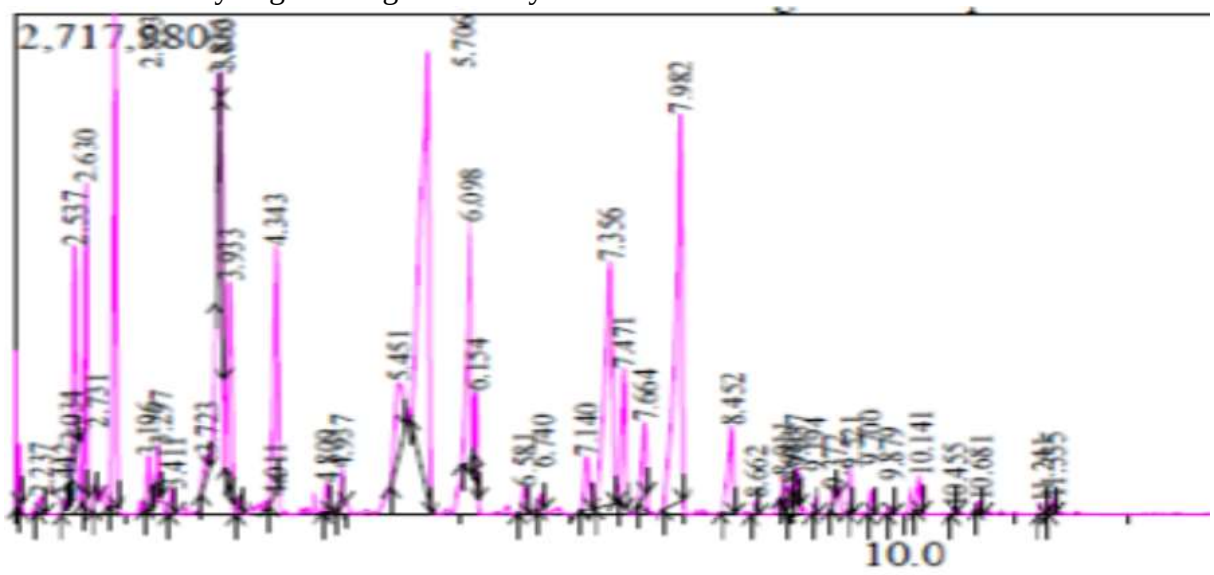

Figure 8. GC-MS analysis spectrum of produced biosurfactant

Determination of Antimicrobial activity

Biosurfactants interact with cytoplasmic membranes leading to cell lysis and metabolite leakage, and disrupt protein conformation that eventually alters essential membrane functions (20). Glycolipid are the best known class of biosurfactant with antimicrobial effects. The biosurfactants, such as rhaminolipid and lipopeptides, showed an inhibitory effect against bacteria and fungi (41)The antibacterial activity of produced biosurfactant by *L. helveticus* M5 was tested using disc diffusion method on Muller-Hinton agar (MHA) plates against pathogenic bacteria *P.aerogenosa and S. aureus*. It can be observed from the results in Table 3 that the biosurfactant showed inhibition zones diameter ranged from (12 to29 mm) and (15 to 31mm) against *P. aerogenosa and S. aureus*respectively at concentration of glycolipid ranged from 20 to 100 mg/ml. The results also indicated that glycolipid fraction is more effective against gram positive bacteria than gram negative. Although the cell wall of gram negative bacteria are usually resistant to glycolipid fractions because they consist of a peptidoglycan layer and an additional outer membrane (outer wall) rather than gram – positive bacteria cell walls, which contain peptidoglycan, which makes gram-negative bacteria more sensitive (31). This may be because glycolipid biosurfactant causes loss or damage of the peptidoglycan layer and inhibit the biochemical reactions in the cell wall.There are very few reports of the antimicrobial activity of biosurfactants isolated from LAB .sharma*et al.*(29) Biosurfactant developed by was found to be*L. helveticus* MRTL91 is successful in different degrees against various pathogenic and nonpathogenic microorganisms including gram-positive and gram-negative bacteria. properties deriving from *L. helveticus* MRTL91 *L. monocytogenes*, The highest concentration of biosurfactant tested, i.e. 25 mg ml-1, showed the highest percentage of inhibition of *Escherichia coli* (90.4%), *Pseudomonas aeruginosa* (75.6%), *Salmonella typhi* (78.6%), *Shigellaflexneri* (70.2%), *Staphylococcus aureus* (92.5%), *Staphylococcus epidermidis* (98.4%), *Listeria monocytogenes* (99.5%), *Listeria innocua* (99). , *S. aureus, P. aeruginosa, S. flexneri* were found to be similar to that obtained from *L.helveticus*produced

crude biosurfactants.The biosurfactant displayed antimicrobial properties against all the pathogenic and nonpathogenic strains studied, and the result showed good antimicrobial activity against pathogenic *Candida albicans, Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis and Streptococcus agalactiae*). Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were obtained for concentrations of 25 to 50 mg /ml of biosurfactant. Furthermore, the biosurfactant was found to be a major antibiofilm agent against most of the pathogens tested (12)

Table 3.Antibacterial activity of glycolipid produced by *L. helveticus* M5 against pathogenic bacteria using disc diffusion method

Surfactant (mg/ml)	*P.aeruginosa*(mm)	*S.aureus* (mm)
10	-	-
20	12	15
40	19	19
60	21	23
80	26	28
100	29	31

Anti-adhesive activity of glycolipid produced by*L. helveticus*M5

One of the essential properties of Biosurfactant is the shaping of a film that affects the wettability of the original surface affecting pathogen adhesion properties (3). Biosurfactant developed out of *L. helveticus* M5 has demonstrated antibiofilm activity against pathogens but the extent of activity has varied and depends also on the concentration of biosurfactants(Table 4).against *S.aureus* we found the highest antiadhesivepropert*yS. aureus* (78%) and *P. aeruginosa* (74.5%) respectively at 50 mg / ml glycolipid concentration (Table 4). A microbial biofilm is any group of microorganisms that bacterial cells bind to a solid surface Medical devices or implants, including; urinary catheters, orthopedic and surgical implants; and contact lenses, various opportunistic pathogens are hardly eligible for adhesion(13). LAB-derived biosurfactant has been documented for its positive antibiofilm properties against various pathogens (42,12)Rodriguez*et al.*(25) studied inhibition of microbial colonization to silicone rubber exposed to biosurfactant developed by *L.helveticus* has observed antibiofilmproperties*L. helveticus* MRTL91 to various pathogenic microorganisms including L. Monocytogenes, with *L. innocua*, and *B. cereuses*, *Saureus*., *S. epidermidis*, that is. Biosurfactant made, however, by *L. helveticus* MRTL91 displayed low antiadhesive activity to *E.coli*, *P. aeruginosa, S. albicansTyphi,* (29) biosurfactant produced by *S. thermophilus* A. Drop off for *Rothiadentocariosa* and was observed in the initial deposition levels*S.aureus*. The quantity of bacterial cells that adhered to silicone rubber with pre-adsorbed biosurfactant after 4 h was further reduced by 89% and two strains of Lactobacilli by 97%, respectively (12)reported LAB that has an anti-adhesive activity against different pathogens.The maximum percentages of antiadhesiveswere observed for *S.aureus, S. epidermi and S. agalactiae*, at 25 mg ml-1 concentration. Antiadhesive property derived from Lactobacillus sp., against *Candida albicans* by biosurfactant. Biosurfactants derived from *L. acidophilus* reported for more than 50% of deposition of pathogenic strains of *C. albicans, S. aurues, E. faecalis, E. coli and S. epidermidis*. In another study, *L. fermentum* B54 strain derived biosurfactant showed antiadhesive activity against uropathogenic microorganisms (40).The findings of this study indicate that glycolipids derived from LAB *L. helveticus* M5 have the ability to remove and prevent pathogenic biofilms from pathogenic microorganisms. The adsorption of LAB-derived biosurfactants to solid surfaces may provide an effective strategy for reducing microbial adhesion and combating colonization by pathogenic biomedical micro-organisms

Table 4.Antiadhesive activity of glycolipid produced *by L. helveticus* M5

Glycolipid mg/ml	*P. aeruginosa* (%)	*S.aureus* (%)
5	15	18
7.5	16	21
10	18.6	30
15	22	37
20	36	52
25	56	68
40	60	72
50	74.5	78

REFERENCES

1.Augustine, S. K., S. P., Bhavsar, M. Baserisalehi, and B.P. Kapadnish, 2005. A nonpolyene antifungal antibiotic from Streptomyces albidoflavus PU 23: Indian J. Exp. Biol., 42, pp: 928-932

2.Alshaikh A. M.; Faqri,. N. H.; Hayder, A. J. Hashim, 2019 . Lab-scale production of rhamnolipid by *pseudomonas aeruginosa* A3 and study its synergistic effect with certain antibiotics against some.Iraqi Journal of Agricultural Sciences. 50(5):1290-1301

3.Arutchelvi, S. Bhaduri, P.V. Uppara, and M. Doble, 2008. Mannosylerythritol lipids: a review, J. Ind. Microbiol. Biotechnol. 35 :1559–1570

4.Bordoloi, N.K. and B.K. Konwar, 2008. Microbial surfactant-enhanced mineral oil recovery under laboratory conditions. Colloids and Surfaces B: Biointerfaces. 63: 73-82.

5. Chen, C., S. C., Baker, R. Darton, 2007. The application of a high throughput analysis method for the screening of potential biosurfactants from natural sources. J. Microbiol Method,; 70: 503-510 .8

6.Chang YH, J, Han, J, Chun K.C., Lee, M. S, Rhee Y. B, Kim, and K.S.Ba.e 2002. Comamonaskoreensissp.nov. a non-motile species from wetland in Woopo, Korea. Int. J. Syst. Evol. Microbiol. 52:377-318

7. Cortes-Sanchez, H. and M.E. Hernandez-Sanchez, 2012. Jaramillo-Flore, Biological activity of glycolipids produced by microorganisms: new trends and possible therapeutic alternatives, Microbiol. Res. 168 : 22–32

8. De Man, J. C. ; M. ; Rogosa, and M. E. Sharpe, 1960. A medium for the cultivation of Lactobacilli. J. Appl. Bacteriol. 23: 130 – 135. Cited By: Boris, S. ; Suarez, J. E. ; and Barbes, C. (1997). Charaterization of the aggregation promoting factor from *Lactobacillus gasseri* a vaginal isolates. J. Appl. Microbiol. 83: 413 – 420.

9.Dastgheib, S. S. M.; M. A.; Amoozegar, E.; Elahi, S. Asad, and I. M. Banat, 2008. Bioemulsifier production by a halothermophilic-*Bacillus* strain with potential applications in microbially enhanced oil recovery. Biotechnol. Lett., 30:263–270

10. Erum, S.; B.; Uzma, A.; Jameela, A. A.; Faiza, W. Maheen, and A. A. Maqsood, 2012. Screening of surfactant producing bacterial strains isolated from soil samples of an Automobile Workshop, Karachi University Journal of Science, 40, 31-36.13

11. Gudina, A. Teixeira, and L.R. Rodrigues, 2011. Biosurfactant-producing lactobacilli: screening, production profiles, and effect of medium composition, Appl. Environ. Soil Sci. 1–9.

12. Gudina EJ1, V, Rocha, J. A, Teixeira, and LR. Rodrigues Antimicrobial and antiadhesive properties of a biosurfactant isolated from Lactobacillus paracasei ssp. paracasei A20. LettApplMicrobiol. 2010; 50(4): 419-24

13. Hua, J. Chen, S. Lun, X. Wang, 2003. Influence of biosurfactants produced by Candida antarctica on surface properties of microorganism and biodegradation of n-alkanes, Water Res. 37 (17) : 4143–4150

14. Joshi, S.J., H., Suthar, A.K., Yadav, K. ,Hingurao, and A. Nerurkar, 2013. Occurrence of biosurfactant producing *Bacillus* sp. in diverse habitats. ISRN Biotechnology; 1-6.23

15. Kuiper, I., Ellen, L., Lagendijk, R.P., Jeremy, P.D., Gerda, E.M.L., Jane, E.T., Ben, J.J.L., Guido, V.B., 2004. Characterization of two *Pseudomonas putida*lipopeptidebiosurfactants, putisolvin I and II, which inhibit biofilm formation and break down existing biofilms. MolMicrobiol,; 51(1): 97-113.26

16.Kotzamanidis,C., A. Kourelis, Litopoulou-E. Tzanetaki, N.,Tzanetakis, and M. Yiangou, 2010. Evaluation of adhesion capacity,cellsurfacetraits and immunomodulatory activity of presumptive probiotic *Lactobacillus* strains. Int. J.FoodMicrobiol. 140, 154–163.doi:10.1016/j.ijfoodmicro.2010. 04.004

17. Luna, L.A. ,and GM. Sarubbo, Campos-Takaki 2009 .A new biosurfactant produced by Candida glabrata UCP1002: characteristics of stability and application in oil recovery, Braz. Arch. Biol. Technol. 52 :785–793.

18.Morais, A. L. Cordeiro, G. S. Teixeira, V. S. Domingues, R. M. D. Nardi, A. S. Monterio, R. J. Alves, E. P. Siqueira and V. L. Santos. 2017. Biological and physiochemical properties if biosurfactant produced by *Lactobacillus jensenii*P6A and *Lactobacillus gasseri*P65. Microb Cell Fact 16:155.

19.Mukherjee, S., P. Das, and R. Sen, 2006. Towards commercial production of microbial surfactants. Trends in Biotechnology, vol. 24, no. 11, pp. 509–515,.31

20. Neu, Significance of bacterial surface-active compounds in interaction of bacteria with interfaces, Microbiol. Rev. 60 (1) (1996) 151

21.NeiraGladys, Astrid Lorely and Fanny Dugarte. 2003. Parameters Examination of a

Biosurfactant Production at laboratory Scale. Futuro vol.2 no.4 Bucaramanga Jan

22.Parveen Jamal1, Shajrat Mir1, Md. Zahangir Alam1 and Wan M. Fazli Wan Nawawi3. 2014. Isolation and Selection of New Biosurfactant Producing Bacteria from Degraded Palm KernelCake under Liquid State Fermentation.Journal of Oleo Science.63, (8) 795-804

23.Peeteres E, HJ,Nelis., and T. Coenye 2008. Cop mparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. Journal of microbiological methods.; 72:157-165

24.Petersdo.Rg, JC.Sherris 1965. Methods and significance of in vitro testing of bacterial sensitivity to drugs. American Journal of Medicine 39(5):766

25.Rodrigues, L. R., and J. A. Teixeira, 2008. Biosurfactants production from cheese whey," in Advances in CheeseWhey Utilization, M. E. Cerd´an, M. Gonz´alez-Siso, and M. Becerra, Eds., pp. 81– 104, Transworld Research Network.36

26.Rodrigues, L., A., Moldes, J.,.Teixeira, R, Oliveira 2006. Kinetic study of fermentative biosurfactant production by *Lactobacillus* strains. J BiochemEng,; 28: 109-116.38

27.Saikia, R.R., S., Deka, M. Deka, and H. Sarma, 2012. Optimization of environmental factors for improved production of rhamnolipidbiosurfactant by *Pseudomonas aeruginosa*RS29 on glycerol. Journal of Basic Microbiology. 52: 446-457

28.SamerThamir Mahmoud and Khalid JaberKadhum and ReemWaleedYounis. 2015. The effect of microbial elicitors on prodigiosin production by local isolate of Serratiamarcescens. Iraqi Journal of Science, vol 56, No. 3A, PP: 1938-1951

29.Sharma D, B.S. Saharan, N, Chauhan S, Procha and S, Lal x 2014. Simultaneous Production of Biosurfactants and Bacteriocinsb Simultaneous Production of Biosurfactants and BacteriocinsbyProbiotic*Lactobacillus casei*MRTL3.International Journal of Microbiology.698713, 7

30.Sifour, M.; M. H. Al-Jilawi, and G. M. Aziz, 2007. Emulsification properties of biosurfactant produced from *Pseudomonas aeruginosa* RB 28. Pakistan Journal of Biological Sciences. 10 (8): 1331 – 1335.

31. Saravanakumari, K. Mani, 2010. Structural characterization of a novel xylolipidbiosurfactant from Lactococcuslactis and analysis of antibacterial activity against multi-drug resistant pathogens, Biores. Technol. 101 (22) : 8851–8854.

32.Skov, R., R., Smyth, A. R., Larsen, A., Bolmstrom, A., Karlsson, and K.Mills, 2006. Phenotypic detection of methicillin resistance in *Staphylococcus aureus* by disk diffusion testing and Etest on Mueller-Hinton agar. Journal of clinical microbiology, 44(12), 4395-4399

33.Sekhon, R. S.; H.; Lin, K. L.; Childs, C. N.; Hansey, C. R.; Buell, N. de Leon, and S. M. Kaeppler, 2011. Genome-wide atlas of transcription during maize development. Plant J., 66(4):553-63

34.Sekhon, K.K., S., Khanna, and S.S. Cameotra, 2012. Biosurfactant Production and Potential Correlationwith Esterase Activity. J. Pet. Environ Biotechnol. 3,133

35.SurekhaK Satputeetal 2016. Biosurfactant/sfrom Lactobacilli species: Properties,challenges and potential biomedical applications Department of Physics, Centre for Advanced Studies in Materials Science and Condensed Matter Physics,SavitribaiPhule Pune University, Pune, Maharashtra, India

36. Tahmourespour, R. Salehi, R.K. Kermanshahi, G. Eslami, 2011. The anti-biofouling effect of Lactobacillus fermentum-derived biosurfactant against Streptococcus mutans, Biofouling 27 (2011) 385–392.

37. Thavasi, S. Jayalakshmi, and I.M. Banat,2011. Effect of biosurfactant and fertilizer on biodegradation of crude oil by marine isolates of Bacillus megaterium: Corynebacteriumkutscheri and Pseudomonas aeruginosa, Biores. Technol. 102 (2) (2011) 772–778.

38.Tahzibi, A.; F. Kamal, and M. M. Assadi, 2004. Improved production of Rhamnolipids by a *Pseudomonas aeruginosa* mutant. Iranian Biomedical Journal. 8 (1): 25 – 31

39.Tene HippolyteMouafo*et al* . 2018. Effect of Different Carbon SourcesonBiosurfactantsProduction by Three Strains of *Lactobacillus* spp.Centre for Research on Food and Nutrition, Institute of Medical Research and Medicinal Plants Studies,P.O. Box 6163, Yaounde, Cameroon,Volume 2018, Article ID 5034783, 15 pages

40. Van der R.H. Mei, Free, G.J. Elving, R. van Weissenbruch, F.W.J. Albers, and H.J. Busscher, 1997. Effect of probiotic bacteria on probiotic organisms for infection control and prevalence of yeasts in oropharyngeal biofilms on silicone rubber voice prostheses in vitro, J. Med. Microbiol. 49: 713–718

41.Varnier AL, L, Sanchez P, Vatsa L, Boudesocque A, Garcia-Brugger F, Rabenoelina and S. Dorey Bacterial 2009. rhamnolipids are novel MAMPs conferring resistance to Botrytis cinerea in grapevine. Plant, Cell & Environment; 32(2): 178–193. 14.

42. Walencka, S. Ro_ zalska, B. Sadowska, B. Ro_ zalska, 2008. The influence of Lactobacillus acidophilus derived surfactants on staphylococcal adhesion and biofilm formation, Folia Microbiologia 53 : 61–66

43.Wei, Y.H., C. L.Chou, and J.S. Chang, 2005. Rhamnolipid production by indigenous *pseudomonas aeruginosa* J4 originating from petrochemical wastewater. Biochemical Engineering Journal. 27:146-154.Vandana, P., Peter, J.K.(2014). Partial purification and characterization of biosurfactant from *Pseudomonas aeruginosa* (C). Int. J. Eng. Sci. Res Technol;3:45-50

44.Zheng, C.; J.; He, Y.; Wang, M. Wang, and Z. Huang, 2011. Hydrocarbon degradation and bioemulsifier production by thermophilic*G. pallidus* strains. Bioresource technology. 102:9155- 9161.