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

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ABSTRACT

The current study was aimed to optimize, characterize and evaluate the antimicrobial and antiadhesive activity of biosurfactant (glycolipid) produce from local isolate Lactobacillus helveticus 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 by fourier transform infrared spectroscopy and gas chromatography mass. FTIR results indicated a lipha tic 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.

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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 nineteen samples from various sources were collected from (humans and dairy product). One gm or ml of dairy samples was added to 9 ml of MRS broth and incubation for 48hrs. at 37 °C, in the presence of 3-5% CO2 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 °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 °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±1°C 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 Wilhelm plate 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°C before mixing with two milliliters of 1M NaOH solution. Then, drop of two milliliters of 1% of CuSO₄ 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 primarily 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 *Lactobacillus* spp. 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 Gp card 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% (1x10⁶CFU/ml, OD = 0.5) of *Lactobacillus* isolate and incubated in shaker incubator (120 rpm) at 37°C for 120 hrs. for biosurfactant production. Then the samples were taken from each flask for the determination of biomass and biosurfactant production.

**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 biomass and 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 MSM with 0.5% lactose as carbon source liquid medium and 0.1% peptone 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 liquid was 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 centrifugation 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) °C 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 wavenumber (1) was plotted.

Analysis of biosurfactant with 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 °C for 24 hrs. The cultures were stored at 4°C 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 (1x10^8cfu/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 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 °C 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°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 100μl 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).

\[
\text{Microbial antiadhesion (\%) = } \left[1 - \left(\frac{\text{ODc}}{\text{OD0}}\right)\right] \times 100
\]

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 identification bacterial isolates**

One hundred nineteen samples were collected from human 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 %) CO2 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 tests are compared with identification scheme 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 were selected for 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 (Table 1). Therefore the isolate M5 was selected for remaining studies (29).

**Table 1. Screening of Lactobacillus spp. for biosurfactant 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

**Identification of Lactobacillus sp. Isolate**

VITIK 2 compact system was carried out as confirmatory test for the identification of Lactobacillus sp. M5 isolate. The GP card was used for gram positive bacterial isolate, which consists of 43 biochemical tests. The results indicated from the 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](image1.png)

**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 into the production medium with concentration of 0.5% (w/v). Results indicated in (Figure 3) that the E24% (76.5%), surface tension (33.5 mN/m) and dry biomass (6g/l) were achieved when lactose was used as the source of carbon and energy respectively. While the lowest activity was obtained when fructose and glycerol (40%, 32.14 mN/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 72 mN/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 72 mN/m to 49.2 ± 2.43 and lower emulsification index reached 41.85 ± 2.56 when glycerol was used as carbon source (39).

![Figure 3](image2.png)

**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.helviticus*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 peptone was used as nitrogen source. While the lowest emulsification activity and higher surface tension observed with malt extract and urea (36.14%, 45.7 mN/m and 56%, 40.7 mN/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 have 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 *Rhodotorula glutinis* 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 liquid Medium 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% of biosurfactant obtained in the culture was 78.16% 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. helveticus*M5

Effect of incubation period

Different incubation periods (0-126 h) were examined to detect the best period of bacterial growth and biosurfactant production by *L. helveticus*M5. Result in (figure 6) showed that the maximum E24% (77.4%) and the lowest surface tension (27.6 mN/m) and higher dry biomass (6.3 g/l) were obtained during 120 hrs of incubation. Whereas after 126 h 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 conditions along 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 (72 h) and reached its maximum values at 96 to 120 h.

Figure 6. Effect of incubation period on production of biosurfactant from *L. helveticus* 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. Rodrigueṣet 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 *Acenitobacter calcoacti-


cusRAG-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°C, with shak- ing (120 rpm) for 120 h. After that, biosurfactant was extracted using solvent system (chlo- roform: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 moieties 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 report- ed by sharma et al. (29) *Lactobacillus casei* for other glycolipid biosurfactant products. In the previous 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.
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 $C_{9}H_{10}$ 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 | $C_{8}H_{18}O$ | 128 | 8.08 | 42 |
| 3   | 4.343                | Ethanol -2-[-(phenyl methyl)amino] | $C_{9}H_{13}NO$ | 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 | $C_{9}H_{12}$ | 120 | 7.19 | 38 |
| 6   | 7.356                | 2-Methyl decane | $C_{11}H_{24}$ | 156 | 10.01 | 52 |
| 7   | 7.982                | Benzcyclopentane | $C_{9}H_{10}$ | 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 xylo-lipid. 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 to 29 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%), *Shigella flexneri* (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 non-pathogenic 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 anti-biofilm 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 anti-adhesive property S. aureus (78%) and P. aeruginosa (74.5%) respectively at 25 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 antibiofilm properties L. helveticus MRTL91 to various pathogenic microorganisms including L. Monocytogenes, with L. innocua, and B. cereuses, S. aureus, S. epidermidis, that is. Biosurfactant made, however, by L. helveticus MRTL91 displayed low antiadhesive activity to E. coli, P. aeruginosa, S. albi cansTyphi, (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 antiadhesives were 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. aureus, E. faecalis, E. coli and S. epidermidis. In another study, L. fermentum BS4 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 microorganisms.

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            |

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