THE ANTIBACTERIAL ACTIVITY OF GLYCOLIPOPEPTIDE PRODUCED FROM *LACTOCOCCUS LACTIS* HN21 AGAINST SOME CLINICAL PATHOGENS IN COMBINED WITH SOME STANDARD ANTIBIOTICS

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ABSTRACT

This study was aimed to produce a biosurfactant from *Lactococcus* sp. and study its synergistic effects with some standard antibiotics. *Lactococcus* sp. HN21 isolate was selected for its highly biosurfactant production and antibacterial activity. It was identified by 16s r RNA as *Lactococcus lactis* HN21. Optimum conditions for production were studied and it was: Modified M17 media (M17 media with some modifications in its composition) at ph 6.5, for 96 hrs incubation time. The E24% at these conditions was 77%. FTIR and GC-MS results identified the produced biosurfactant as glycolipopeptide with a major fatty acid octadecenoic acid. *P. aeruginosa* was successfully inhibited by the glycolipopeptide alone, with overall inhibition ranging from (15 to 19 mm). The combined use of antibiotics and glycolipopeptide, however, resulted in an increase in the total inhibition zones to (17 to 28 mm), while glycolipopeptide alone was effective against *S. aureus* and showed total inhibition zones ranging from (15 to 22) mm. glycolipopeptide in combination with antibiotics, the total inhibition zones were increased (26 to 40) mm. The results observed a great pharmaceutical application in increasing the bactericidal effect.

Keywords: *Lactococcus*, glycolipopeptide, 16s rRNA, antibiotics, synergistic effect

الفعالية ضد البكتيرية لسكريد البروتين الدهني المنتج من بكتريا *Lactococcus lactis* HN21 ضد بعض الممرضات السريرية بالاقتران مع بعض المضادات الحيوية القياسية

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المستخلص

هدفت هذه الدراسة الى انتاج المستحلب الحياتي من بكتريا Lactococcus sp. و دراسة التأثير التآزري لهذا المستحلب مع بعض المضادات الحيوية. العزلة *Lactococcus* HN21 اعطت اعلى فاعلية لانتاج المستحلب الحياتي (67%) و فاعلية ضد بكتيرية. شخصت العزلة بأنها تعود الى جنس *Lactococcus lactis* HN21 من خلال تقنية 16s rRNA. اعلى انتاجية للمستحلب كانت عند الوسط المحور M17 و رقم هيدروجيني 6.5 لمدة 96 ساعة و بلغت فاعلية الاستحلاب (E24%=77) . نتائج تحليل الاشعة تحت الحمراء و كذلك التحليل الطيفي الكتلي للكروماتوغرافيا الغازية. اكدت بأن المستحلب الحياتي المنتج ذو طبيعة دهون سكرية بروتينية و ان معظم الاحماض الدهنية كانت Octadecenoic acid . تم تثبيط بكتريا الزائفة الزنجارية بواسطه المستحلب المنتج بواقع حجم تثبيط (15–19) ملم. التأثير المشترك بين المستحلب و المضادات الحيوية زادت من حجم التثبيط بواقع (17–28) ملم. بينما كان حجم التثبيط للمستحلب ضد بكتريا المكورات العنقودية (15–22) ملم و بالشتراك مع بعض المضادات الحيوية ارتفتعت الى (26–40) ملم . في الدراسة الحالية اظهرت النتائج امكانية استخدام المستحلب الحياتي المنتج في المواد الصيدلانية و زيادة التأثير القاتل للبكتريا.

الكلمات المفتاحية: Lactococcus، سكريد البروتين الدهني، مضادات حيوية، 16s rRNA ، تأثير تآزري

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INTRODUCTION

Lactic acid bacteria (LAB), mainly *Lactococcus, Lactobacillus, Leuconostoc,* Streptococcus *and Pediococcus,* are frequently employed in the food industry. *Lactococcus* sp. considers as an essential group and gaining more focus in the food industry because of its role in biotechnology. As well as prevent pathogenic organisms from cultivation in the ecosystem by producing various antimicrobial agents (2). For obtaining a natural surfactant, micro-organisms are a natural factory for producing biosurfactants. They are multifunctional biomolecules with low toxicity and bio-degradable ability. In contrast, not all biosurfactant-producing microorganisms are ready to use in food products because they have not generally rcognized as safe (GRAS) status (29). LAB are interesting microorganisms producing biosurfactants and have GRAS status, their biosurfactant exert a great role in the biomedical field as they have antimicrobial activity, emulsifier properties and nonstick capability (32). Biosurfactants consider amphiphilic compounds manufactured from micro-organisms on their cell surfaces or excreted outside the cell and can reduce the surface tension in liquids (31). Some LABs, including *Lactobacillus sp*. and *Lactococcus lactis*, simultaneously create cell-bound and extracellular biosurfactants which have various characteristics (6). LAB-derived biosurfactants exhibit good surface, emulsification, antibacterial, and anti-adhesive properties (26). biosurfactants from LABs were divided into a number of categories, including glycolipopeptide (41), glycolipid (16), glycoprotein (19), and lipoprotein (11). LAB biosurfactant yields typically vary between milligrams per liter and higher (28). There are many uses for biosurfactants in a number of fields such as food, cosmetics and pharmaceuticals. They are crucial biotechnological products (17). Biosurfactants have antiviral, antifungal, and antibacterial functions. Because of this, they might be utilized in place of conventional antibiotics to combat several food-borne diseases. (27). Secondary metabolites, commonly referred to as microbial surfactants, are essential for the survival of bacteria that create biosurfactants because they improve nutrient delivery, interact with hosts, or function as biocides (15). This study aimed to produce a biosurfactant from Lactococcus sp. and study the synergistic effects of biosurfactant with some standard antibiotics.

MATERIALS AND METHODS

Collecting and isolation of bacterial samples: One hundred fifteen (115) different dairy samples were collected from local markets to isolate *Lactococcus sp*. A small amount from a dairy sample was taken into a sterile container then sterile distilled water was added to the sample to make a suspension by homogenization. A volume of (1 ml) dairy sample was put on (9 ml) MRS broth and then incubated for 24hr at 37 Celsius. After the incubation, 1 ml of broth culture was added to 9 ml of normal saline in the test tubes then dilution until 10^{-4} was done. Samples from each dilution have been cultured on MRS and M17 agar medium for 48 hours at 37°C, an anaerobic condition was applied to the plates via candle jar. After incubation, Subculturing the isolates on M17 agar media allowed for their purification to obtain single colonies. Colonies were tested by microscopic examination. The isolates which showed *Lactococcus* cells morphology were further subjected to biochemical identification with catalase and oxidase tests (18).

Screening of *Lactococcus sp*. Isolates for biosurfactant production and antibacterial activity :In 250 ml Erlenmeyer flasks, a 50ml of Modified M17 broth media (di sodium β-glycerophosphate replaced with Na2HPO4 15g/l and glycerol 1ml/l) was prepared, autoclaved and inoculated with 2% ($1x10^8$ CFU/ml, OD_{600} = 0.5 on McFarland) of *Lactococcus* isolates were then incubated in a shaker at 120 rpm with N2 gas for making anaerobic condition at 37°C for 96hrs for biosurfactant production. Cells were collected from 20 ml of broth culture by centrifugation for 8000 rpm, for 15 minutes. The cells were then rinsed with distilled water, 4 ml of PBS was added, and the mixture was vortexed. The bacterial suspension with phosphate buffer saline (PBS) was then sonicated for 2 minutes at a power of 600 Hz (one minute on, one minute off) (42) with ice surrounding the container, for cell bounded biosurfactant extraction. After sonication, bacterial debris

was discarded by centrifuge while the supernatant was put to test for emulsification index (E24%) and antibacterial activity (29).

Emulsification Index (E24%) for Biosurfactant analysis: Cell-free supernatant of (2ml) has been added to Toluene of (2ml), mixed for 2 minutes by vortex, and then left to stand for a day. The emulsifier layer's height was measured and analyzed. The emulsification index is expressed as a proportion of the height of the emulsified layer (in mm) to the height of the entire liquid column (mm) multiplied by 100 (1).

Indicator Microorganism *Pseudomonas aeruginosa* was previously identified by the Dept. of Biotechnology, College of Science, University Of Baghdad and used as an indicator pathogen for testing the antibacterial activity of *Lactococcus*-biosurfactant.

The antibacterial effectiveness of crude *Lactococcus* biosurfactant: By using paper disc diffusion assay, the antibacterial effect of crude biosurfactant was assessed against *P. aeroginosa* On Muller Hinton agar plate (22).

Molecular identification by 16s r RNA: *Lc* isolate with higher biosurfactant production and antibacterial activity was identified by 16s rRNA technique. The 16s r RNA from the genome was amplified using the universal bacterial primer set. 27F: 5-AGAGTTTGATCCTGGCTCAG 3 and 1492R: CGGTTACCTTGTTACGACTT3 -, were used to amplify the PCR-amplified 16S rRNA fragments. Ethidium bromide (10 mg/ml), loading dye, DNA ladder marker, and X-TAE buffer are the solutions that are employed. The PCR reaction mixture was made. The set's PCR cycling parameters were a touchdown approach with 30 cycles. as follows: Genomic DNA underwent an initial denature phase of 1 cycle at 95 °C for 5 min, 30 cycles at 95 °C for 30 sec, 40 °C for 30 sec, and 72 °C for 1 min, and a final extension step I cycle at 72 °C for 7 min. The end of the PCR program was extended by 10 minutes of incubation at 10°C. PCR products were sent to Macrogen Corporation in Korea for Sanger sequencing utilizing an automated DNA sequencer called the ABI3730XL.The results were received by email and then analyzed using genious software'.

Optimization of culture conditions for biosurfactant production

The effect of medium composition: Different media were used for biosurfactant from local isolate *Lc.* HN 21 includes: MRS, modified M17, Turner (39) without TTC, Elliker (8), and Mineral salt medium (MSM) consisting of g/l (K2HPO4 1g, KH2PO4 1g, MgSO4.7H2O 6g, NaCl 0.005g, CaCl2 0.02g, yeast extract 0.5g and lactose 5g) these media were used to estimate more effective media for lipid production. The PH value of all media was fixed at 6.5 by using NaOH and NaCl solutions 1M, then Autoclaved at 121 °C for 15 min. After autoclaving, each media was inoculated with (1ml) of overnight pre-cultured broth of *Lc.* isolate HN21 ($1x10^8$ CFU/ml, OD600 = 0.5 on McFarland) to 50 ml broth from each media in 250 ml Erlenmeyer flask supplemented with N2 gas by flashing system to make anaerobic condition and incubated on shaker incubator 120 rpm, 37˚C for 96 hrs. for biosurfactant production (35). Following incubation, a volume of 20 ml of broth culture was taken, and centrifuged to obtain the cells for (8000, 15 minutes), after that, washed the cells with distilled water and in 4 ml PBS, they will be re-suspended, then mixed with vortex. The bacterial suspension with phosphate buffer saline (PBS) was then sonicated for 2 minutes at a power of 600 Hz (one minute on, one minute off) (42) with ice surrounding the container, for cell bounded biosurfactant extraction. After sonication, bacterial debris was discarded by centrifuge while the supernatant was tested for emulsification index (E24%) and biosurfactant yield (mg/l) (29).

The effect of initial medium pH: mM17 of (50 ml) liquid medium was prepared in a 250 ml Erlenmeyer flask. Different (5, 6, 6.5, 7, 8) pH values were applied to the medium for determining the optimal pH value for biosurfactant production. Following autoclaving, an inoculum of 2% ($1x10^8$ CFU/ml, OD600 = 0.5) of *Lc.* HN21 were inoculated to the flasks and incubated in shaker incubator (120 rpm) and with N2 gas (flashing system) to make the anaerobic condition at 37˚C for 96 hrs. Samples have been taken from each flask at the end of

incubation time, for the estimation of E24% and biomass in g/l (29).

Determination of Dry-weight: To estimate bacterial cells after the incubation period, 10 ml of the culture broth was centrifuged at (8000 rpm for 15 min), then discharged the supernatant. Biomass were washed gently with PBS and placed in the oven at 80 °C to dry. After drying, biomass was measured in terms of g/l (3).

The effect of incubation time: The incubation period for *Lc.* HN21 isolate was estimated for biosurfactant production. Erlenmeyer flasks (250 ml) containing 50 ml of m M17 broth media with a pH of 6.5 were autoclaved. After that, 2% of inoculum was transferred to the flasks with anaerobic conditions by N2 gas (flashing system) and incubated in a shaker incubator at 120 rpm, 37˚C for (24, 48, 54, 72, 78, 96 and 120 hrs.). The samples have been taken at different times throughout the incubation period for the estimation of biomass and E24 %.

Purification of biosurfactant by column chromatography: In a column with (1.5 x 60 cm), the partial purified biosurfactant obtained from solvent extraction was purified using filled with silica gel (60 -120 mesh). Silica gel powder was activated for 18hr in the oven at 100 °C before using. It was packed tightly by a continuous flow of equal volume of methanol: chloroform and washed with the same solvent mixture. The biosurfactant was then loaded into the column until the overall solution was absorbed. Chloroform and methanol were then used to elute the column (1:1 v/v). three ml from each fraction of the eluted extract, which flowed at a rate of 20 ml per hour, were collected. All eluted fractions were gathered, and their emulsification activity was assessed. Furthermore, the effective fractions have been collected for extraction with a solvent system mixture of methanol and chloroform (1:2) and dried at (40–45°C). The purified powder was stored in a clean vial at 4 ˚C for the remaining experiment (23).

Characterization of partial purified biosurfactant

Fourier Transform-Infrared spectroscopy (FTIR): The process of determining the biosurfactant nature by FTIR was done as follow: 100 mg of KBr (AR grade) have been mixed separately with one mg of biosurfactant after being dried under vacuum for 48 hours at a temperature of 100 °C to create the KBr pellet. Data was gathered in a wave number/cm range of 500 to 4000. In the Shimadzu-IR affinity-1 spectrophotometer, FTIR spectra were captured. Additionally, the spectra have been shown as wave number vs intensity (3).

Analysis of biosurfactant by Gas Chromatography-Mass Spectrum (GC-MS): the fatty acid composition was determined by GCMS. Based on the method described by Zheng *et a*l., (43), 10 mg of biosurfactant were dissolved in 1 ml of sulfuric acid – methanol at 90 °C for 15 h to create Acid methyl ester. 1ml of hexane was added and mixed, and then removing the hexane phase once the sulfuric acid had evaporated. Then 1 ml of distilled water was added to the hexane phase. Hexane was used to extract the fatty acid methyl ester, and the sample was then subjected to a GC analysis using helium as the carrier gas on a Shimadzu 17-A GC with a fused silica capillary column (30 m x 0.25 mm, 0.25 m film thickness).

Application studies of purified biosurfactant

Synergistic effect of biosurfactant with some standard antibiotics: *P. aeruginosa* and *S. aureus* isolated from wounds and burns of Iraqi patients were tested its antibiotic susceptibility with 10 types of antibiotic discs for each genus and choose the moderate resistant isolates. Antibiotic discs were used in combination with *Lc. lactis* HN21 biosurfactant to evaluate its antibacterial activity. On Muller Hinton agar, overnight growth of *P. aeruginosa* and *S. aureus* adjusted to ($1x10^8$ CFU/ml) equal to (OD600 = 0.5) are swapped and left to dry for a while. Then antibiotic discs are placed and fixed by sterile forceps on the surface of agar plates (24). Antibiotics selected in this test include (Meropenem 10 µg, Gentamicin 10 µg, Ceftazidime 30 µg, Aztreonam 30 µg, Piperacillin 100 µg)for *P. aeruginosa* and (Levofloxacin 5 µg, Vancomycin 5 µg, Doxycycline 30 µg, Oxacillin 5 µg, Azithromycin 15 µg) for *S. aureus*. Then gel filtration purified biosurfactant of 100 mg/ml was prepared by dissolving in DMSO and

filtered by a filter unit (0.22 µm in diameter). Sterilized Whatman filter paper discs and antibiotic discs were socked in the stock solution of biosurfactant and placed on plates of *P. aeruginosa* and *S. aureus* and incubated at 37°C for 24 hr. The inhibition zone was measured by the electronic ruler (13).

RESULTS AND DISCUSSION

Isolation and identification of *Lactococcus* Isolates: A number of 115 samples have been gathered from various dairy sources (yogurt, cheese, milk, butter, labneh, whey, and cream) from Iraqi local markets. As a selective media for isolation, M17 agar plates were used to grow the samples predominantly. According to the results observed Only 38 isolates (33%) that belonged to the genus *Lactococcus* were found. according to the findings, these isolates were then identified using morphological, microscopy, and biochemical testing. The isolates' morphological characteristics showed that they belonged to the *Lactococcus* genus by their small (2-3mm), Creamy; little sticks smooth round colonies and opaque without pigment on M17 agar, gram-positive cocci organized singly, in pairs or short chains Fig.1A and B, and were negative for catalase and oxidase tests.

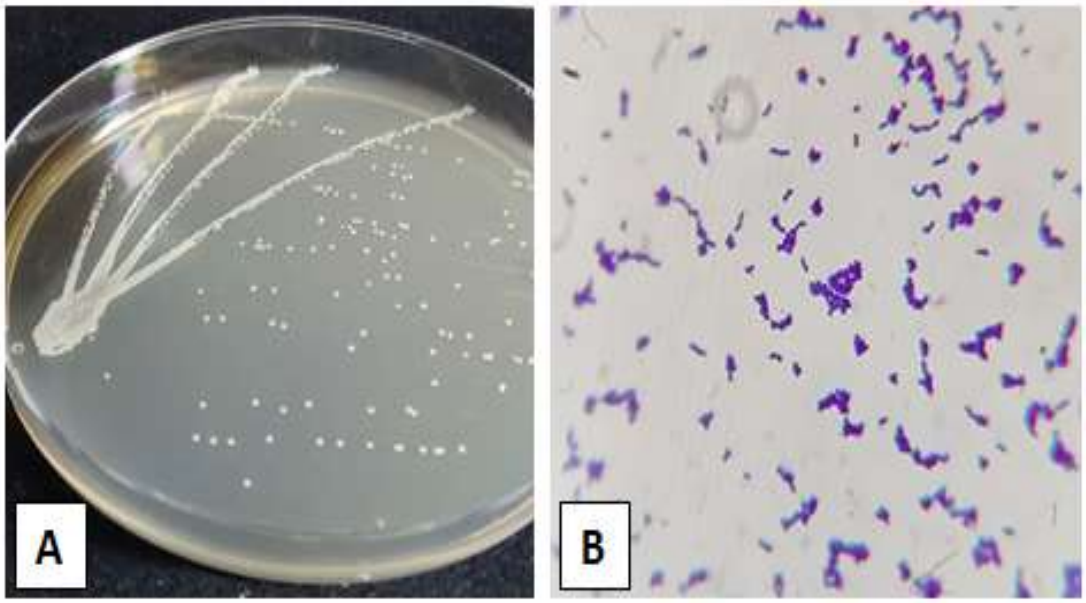

Figure 1.A) *Lactococcus* isolate on M17 agar after 48h of anaerobic incubation at 37°C. B) Colony observation of *Lactococcus sp*. under a microscope (100x) lens after gram staining

Screening of *Lc sp*. isolates for biosurfactant production: The emulsification index (E24%) was used to detect the intracellular products for isolates of *Lc sp*. This screening has been carried out in the mM17 medium. For an additional level of reliability, all isolates have been refined under identical conditions in the term of the size of the inoculum (2% v/v), (pH 7), the incubation period (96 h), and shaking speed (120 rpm) in anaerobic conditions (flashing test). Among 38 isolates of *Lc* bacteria screened for biosurfactant production, only 24 isolates can produce biosurfactants with different emulsifications ranging from (50 - 67) %. The isolates (HN21, HN33, HN70, and HN79) gave the highest and stronger emulsification activity (67.85%, 61.53%, 62.96%, and 67.85%) respectively. These isolates have been chosen to complete the remaining screening tests.

Antibacterial activity of selected isolates: To assess the antimicrobial activity of crude biosurfactants in PBS extracted from *Lc* species, the isolates (HN21, HN70, HN79, and HN33) that showed higher E24% activity were tested for bacterial pathogen-inhibitory action. Figure 2 demonstrated that the biosurfactants had a suppressive impact on indicator *P. aeruginosa* by the formation of an inhibition zone around the discs. The isolate *Lc* HN21 showed higher antibacterial activity with an inhibition zone of 18 mm. Therefore, isolate *Lc. lactis* HN21 was selected for the remaining experiments in the current study.

Figure 2.Antibacterial activity of *Lactococcus* isolates against indicator *P. aeruginosa*

Molecular identification of *Lactococcus sp.* by 16s rRNA: DNA has been extracted from the presumed *Lc sp*. HN21 isolate to conduct molecular analysis. Following DNA extraction and subsequent PCR amplification, *Lc*-derived DNA, which quickly amplifies with common bacterial primer sets, can be easily found in investigations of bacterial populations. Here, bands were found using such universal primers (27F/1492R), confirming the putative isolate's identity as a member of the genus *Lc*. The isolate (HN21) was consequently identified as *Lc. lactis* by identity 100% as shown in figure 3, and chosen for the subsequent research. After Bacterial ribosomal RNA result amplification, analysis of sequences and validation of microorganism's homogeneous data using the rRNA database (NCBI) suggests *Lc. lactis* strain CAU937 16S ribosomal RNA gene, partial sequence, accession number MF582953.1.

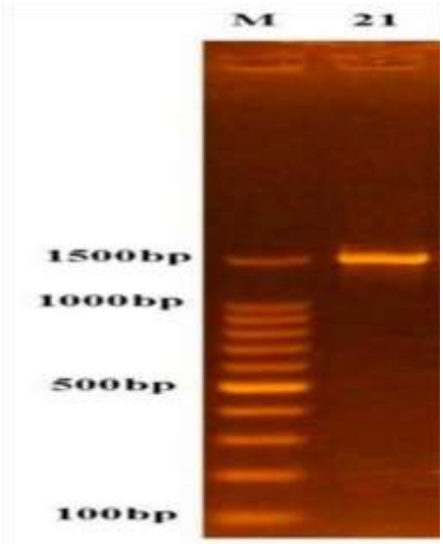

Figure 3.Agarose gel electrophoresis: The first line is a positive *Lc. lactis* HN21 isolate (1500bp), M: marker, and was visualized under UV light under staining with Ethidium bromide (agarose Con. 1% and ran at 5V/cm)

The optimum conditions for biosurfactant production

Optimum media composition: To enhance biosurfactant production from *Lc. lactis* HN21 isolate, a number of different culture media have been tested. The results in Figure 4 showed that modified M17 media (which contains 5g/l sucrose as a carbon source) were the highest media for biosurfactant production and biomass formation by emulsifying index equal to (67%) and the yield of products (685 mg/l), followed by MRS (62.5%, 660 mg/l), Turner (60%, 640 mg/l), Elliker (59%, 313 mg/l) and MSM (54.54%, 255 mg/l) respectively.

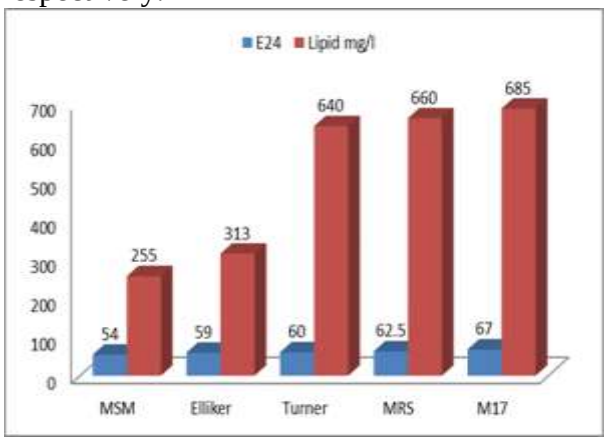

Figure 4.The effect of different media composition on *Lc. lactis* HN21, on biosurfactant production and the yield of the product at pH 6.5 after 72 h

Approximately similar results were obtained by Zainab and Nadhim (42), They pointed out that E24% 100 % were achieved when sucrose was utilized as energy and carbon source for biosurfactant (glycoprotein) production from local isolate *Lactobacillus plantarum*.

The effect of pH value: Choosing the proper medium pH and maintaining it are crucial steps in reducing intracellular stress and maximizing growth (34). To find out how pH

affects the formation of biosurfactants, mM17 medium was chosen as the ideal media according to the previous test. The media were adjusted to variable values of pH, based on the result in Figure 5 the highest value of E24% and biomass was at pH 6.5 reaching 66.66% and 5 g/l respectively, while the lowest activity was obtained at pH 5 (52.6%) and biomass of (2 g/l). Also, the same results found by Guera *et al*., (12) noticed that the optimal pH ranges for maximum biosurfactant production were 6-6.8.

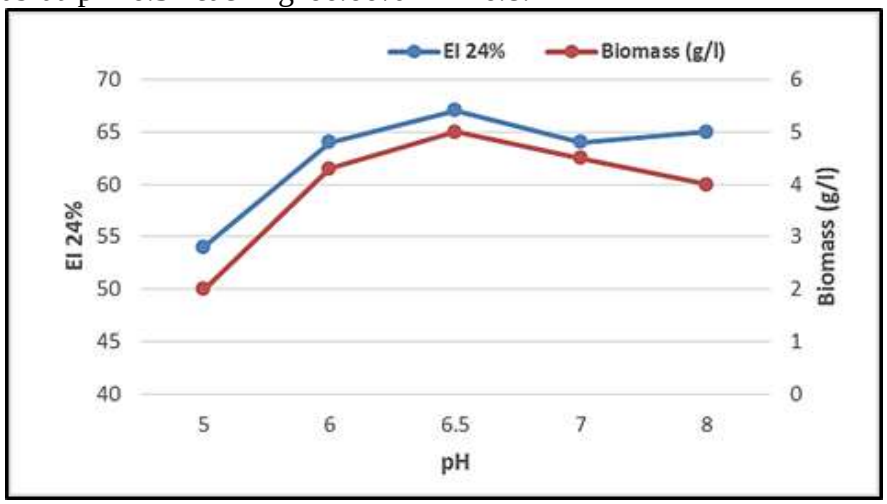

Figure 5.The effect of pH value on biomass and E24% formation in shaker incubator (120 rpm) after 96hr

The effect of incubation time: The result in Figure 6 showed that the maximum E24% (77%), higher dry biomass (6.6 g/l) was obtained during 96 hrs. of incubation. However, the emulsification activity started to decline after 96 hours of incubation. Since this is a batch culture type, which inhibits bacterial growth, the cause may be a shift in the culture conditions along with periods of diminishing nutrients and accumulation of harmful metabolites. The results of this test demonstrated that the biosurfactant produced by *Lc. lactis* HN21 is growth-related, with production beginning at an early exponential phase (48 h) and reaching its highest values at 78 to 96 h. Persson *et al*., (21) demonstrated that biosurfactants synthesized during the exponential phase as a primary metabolite and so have a relationship with cellular biomass creation.

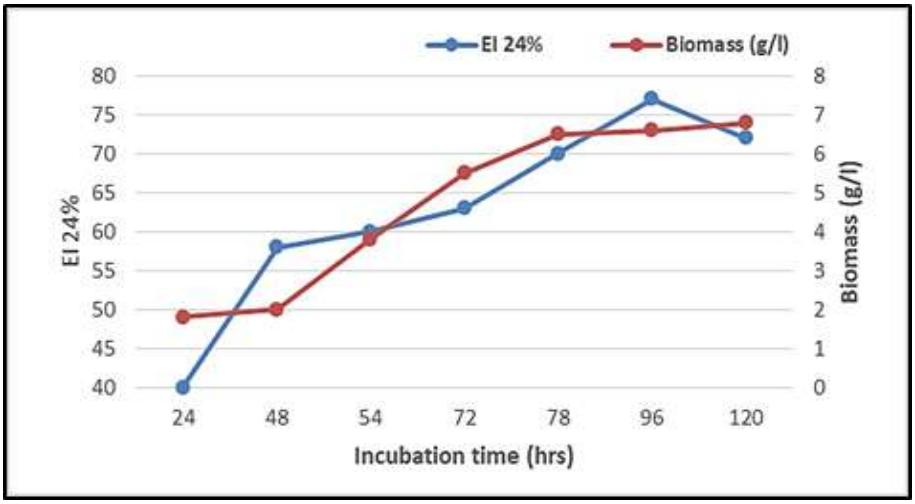

Figure 6.Optimum incubation time for biosurfactant production from *Lc. lactis* HN21 at pH 6.5, in shaker incubator (120 rpm) for 120 hr

Biosurfactant extraction: In every part of the optimization of biosurfactant production, tests have been done to reach a maximum amount of production from *Lc. lactis* HN21 isolate. The final results of the optimization found that using mM17 media at pH 6.5 for cultivation in anaerobic condition at 37C° for 96 hrs in a shaker incubator (120 rpm) and extraction of cell bounded biosurfactant with PBS (ph7) using ultra-sonication 20 kHz for 5 minutes.

Thereafter, separation and extraction of lipids using solvent system methanol: chloroform (1:2 v/v) to yield a higher amount of biosurfactant (lipid) from *Lc. lactis* HN21. The results showed appearance of a white material in the middle or bottom of the separation funnel which is indicates to the biosurfactant product. The organic side (chloroform) is discharged; lipids are collected in a clean glass dish and the superior aqueous phase was once more extracted using the same amounts of the solvents mentioned above. To gain a crude biosurfactant, solvents should be removed by evaporation at 45 C° in the oven to obtain a honey-like powder material which was scraped, and collected in a clean vial and measured in terms of g/l (37).

Purification of biosurfactant Using Gel-Filtration chromatography technique: The yellowish honey precipitate from chloroform: methanol precipitation was further purified using Silica (60-120 mesh) gel filtration chromatography with the dimensions (1.5 x 60 cm) column. 600 mg was dissolved in 10 ml methanol: chloroform (1:1) emulsification activity of each fraction was then measured. Figure 7 illustrates the results of emulsification activity and fraction number. The results indicated that the occurrence of peaks of Biosurfactant appeared between fraction numbers (40 - 90) in elution Chloroform: Methanol (1:1) with higher EI 24% activity at fraction numbers (70 – 77) ranging from 62 to 71%. All fraction numbers from (40-90) were extracted again with solvent system methanol: chloroform (1:2 v/v) and dried as mentioned before to obtain purified biosurfactant and use in remaining experiments. Results of the present study were confirmed with (14) revealed that the gel filtration chromatography techniques using silica gel presence of biosurfactant molecules (lipopeptide) from *Lactobacillus plantarum* in fraction numbers between (77- 89) eluted with Chloroform: Methanol, the E24% values ranged from (63 – 71%).

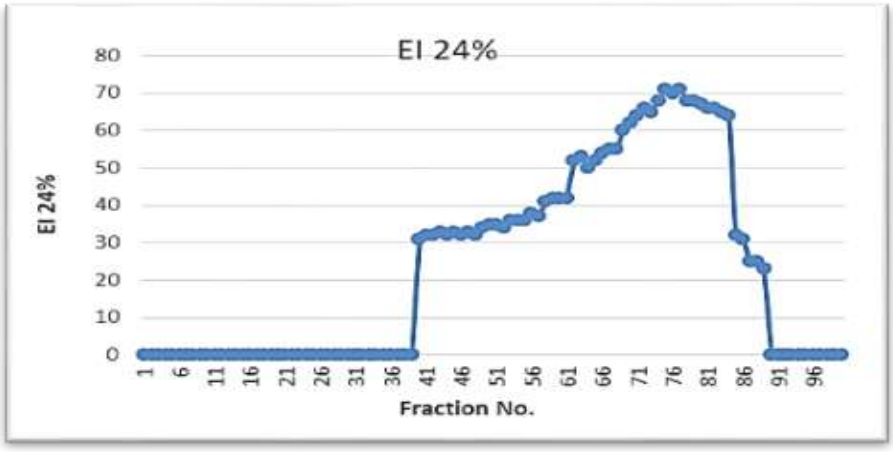

Figure 7.Purification of biosurfactant from *L. lactis* HN21 by silica gel column (1.5×60 cm) eluted with chloroform: methanol (1:1) in 20 ml/hour flow rate, 3 ml per fraction

Characterization of *Lc. lactis* HN21 biosurfactant: FTIR Analysis: Based on the distinctive infrared absorption bands of the chemical groups, FTIR is frequently used to determine the functional groups of organic molecules (20). The results in the current study in Figure 8 show the occurrence of a wide band 3402 and 3427 at 3200–3500 cm−1 in the spectra of the biosurfactant produced by *Lc. lactis* HN21 It demonstrates the existence of OH groups of polysaccharides and NH groups of glycoproteins. These results agreed with Morais *et al.,* (20), the spectra of the biosurfactants made by L. gasseri P65 suggest the existence of OH groups (and, perhaps, NH groups) in the large band between 3500 and 3200 cm-1. Also, ingh *et al*., (33), confirmed the broad peak, which existed at 3428 cm^{-1}, a distinctive OH group of biosurfactant produced by Lactobacillus sp. Another band appeared on a 1645 cm-1 area and was related to the C=O peptide bond stretch. A band at 1404 cm- 1 (AmII band: NOH bending in proteins), band 1244 cm-1 (PI band: phosphates), and the absorption bands between 1200 -1000 cm−1 can be related to ester C–O–C stretching. Alkene and alkyl benzene molecules of biosurfactant are the most effective components. Peaks between 1500-1650 cm-1 revealed the existence of the C=C

alkene part. Peaks between 2250 – 2400 indicate the presence of the C≡C alkyne component. The significant bands were between 2800 - 3000 (C–H stretching bands of CH2 and CH3 groups), 1739 cm−1 (C = O stretching vibrations of the carbonyl groups), and 775 cm−1 (CH2 group). The observed results prominently confirmed the presence of glycolipopeptide type of biosurfactant produced by *Lc. lactis* HN21. Similar results were obtained by Shokouhfard *et al*., (30).

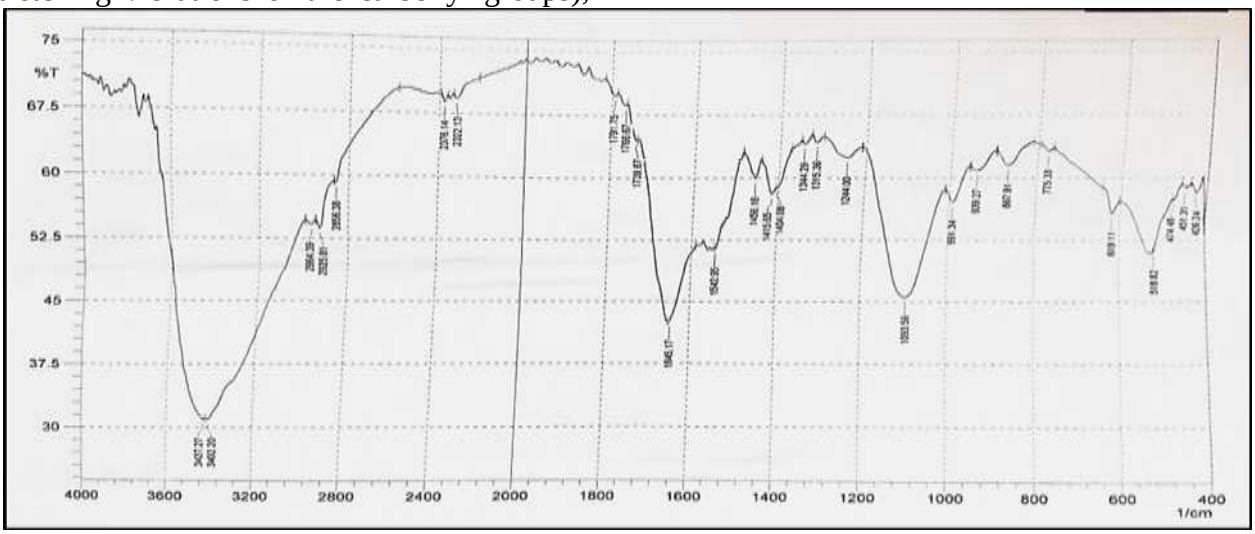

Figure 8. FTIR spectrum analysis of biosurfactant produced by *Lc. lactis* HN21

GC-MS Analysis of Biosurfactant Produced by *Lc .lactis* HN21: GC-MS is a useful technique to determine and understand the molecular structure of any compound. The Biosurfactant produced by the local isolate *Lc. lactis* HN21 was further evaluated by GCMS. Gas chromatography is a very efficient method for quantitative estimation as well as the characterization of biosurfactants. The total ion chromatogram of the partial purified biosurfactant (glycolipopeptide) of *Lc. lactis* HN21 confirmed the presence of a complex containing different biologically active compounds including lipid and peptides moiety with surface active properties with different retention times. Based on the results in Figure 9, the purified glycolipopeptide is composed of (100%) 11- Octadecenoic acid, methyl ester, (E) - peak no. 8, in addition to a lesser percentage of other compositions. The results also revealed that the biosurfactant consisted of (57.20 %) 2-methyl -z, z-3,13-Octadecadienol, (40.76 %) of 9,12-Octadecadienoic acid, methyl ester, and (40.43 %) Hexadecanoic acid, methyl ester in addition to other components at different times. Saravanakumari and Mani (25) reported that the biosurfactant obtained from *Lactobacillus lactis* consists of octadecanoic acid as a fatty acid related to the sugar moiety. Stearic acid and palmitic acid are the main types of fatty acids in cell-bound biosurfactants obtained from *Lactobacillus pentosus* (40).

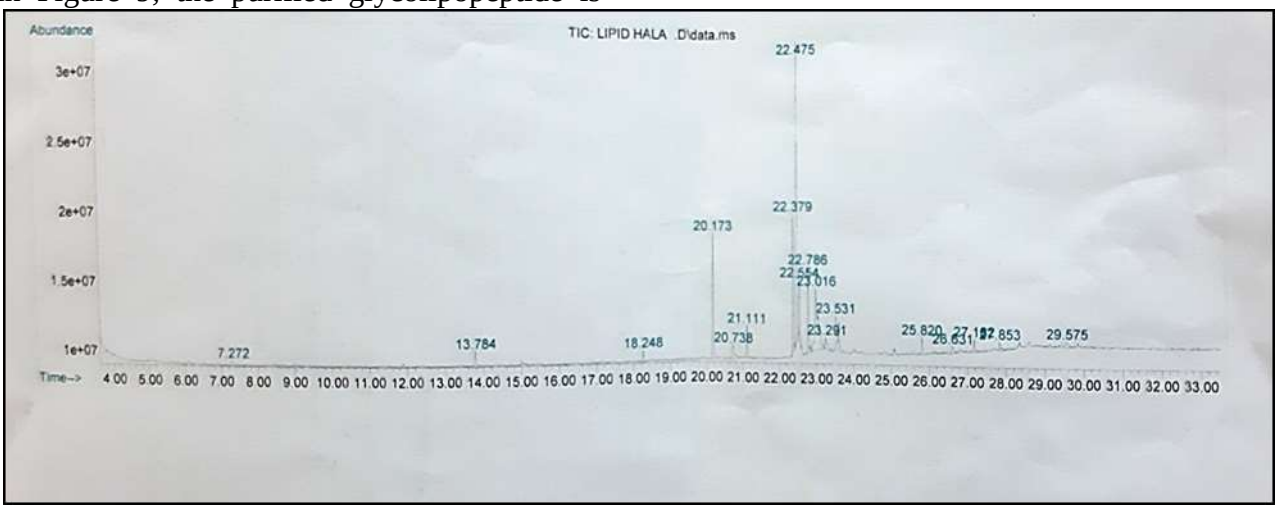

Figure 9.GC mass analysis of partially purified biosurfactant produced by *Lc.* lactis HN21

Synergistic effect of Glycolipopeptide with some standard antibiotics: Modern antimicrobial approaches are sought after as a result of the need for novel antimicrobials to combat bacterial drug resistance. Therefore, combining other antimicrobial chemicals with antibiotics to boost their effectiveness is thought to be a workable solution to the issue. And when two or more agents work together, their combined efficacy increases compared to when they act alone (9). The Combination effect of glycolipopetide (100 mg/ml) (CMC of glycolipopeptide) with different standard antibiotics, was utilized against pathogenic bacteria. The Antibacterial activity of antibiotics and glycolipopeptides against *P. aeruginosa* was shown in Figure 10. The findings indicated that *P. aeruginosa* was successfully inhibited by the glycolipopeptide alone, with overall inhibition ranging from (15 to 19 mm). The combined use of antibiotics and glycolipopeptide, however, resulted in an increase in the total inhibition zones to (17 to 28 mm). The combination effect of glycolipopeptide with antibiotics (Aztreonam, Gentamicin, and Meropenem) enhanced 21.18, 27.32, and 53.12 % of the inhibition zone for *P. aeruginosa* respectively. While the combined effect of glycolipopeptide with antibiotics Ceftazedime and Piperacillin did not exhibit any enhancement in inhibition zones. The best combination effect of glycolipopeptide with antibiotics was found with clinical isolate *S. aureus* as shown in Figure 11. It has been observed from the results that the glycolipopeptide alone also was effective against *S. aureus* and showed total inhibition zones ranging from (15 to 22) mm. However, when glycolipopeptide and antibiotics have been utilized in combination, the total inhibition zones were increased (26 to 40) mm. The combination effect of glycolipopeptide with antibiotics (Vancomycin, Azithromycin, Levofloxacin, Oxacillin, and Doxycycline) enhanced 25.30, 26.37, 32.14, 31.12, and 62.25 % of the inhibition zone respectively. These results were following standards values (CLSI, 2022). The results in the present work were confirmed by previous studies. Faqri *et al*., (10) demonstrated that the Rhamnolipid produced by Pseudomonas aeruginosa A3 has a synergistic impact when paired with antibiotics to fight pathogenic bacteria and enhance the inhibition zone.

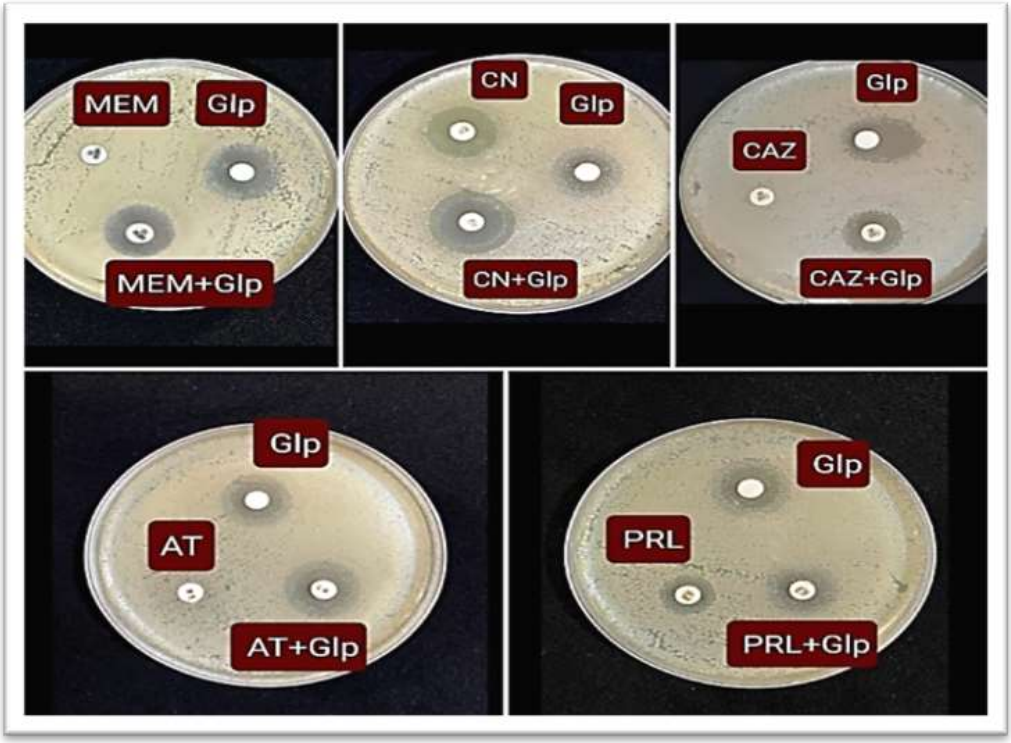

Figure 10 Synergistic effect of Glycolipopeptide (Glp) produced from *Lc. lactis* HN21 with standard antibiotics against *P. aeruginosa*. MEM: Meropenem, CN: Gentamicin, CAZ: Ceftazedime, AT: Aztreonam, PRL: Piperacillin

Figure 11.Synergistic effect of Glycolipopeptide (Glp) produced from *Lc. lactis* HN21 with standard antibiotics against *S. aureus*. LE: Levofloxacin, VA: Vancomycin, DO: Doxycycline, OX: Oxacillin, AZM: Azithromycin

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