

THE SYNERGISTIC EFFECTS OF LIPOPEPTIDES AND BACTERIOCINS PRODUCED BY *Lactobacillus* sp. OF HUMAN SAMPLES AGAINST CLINICAL PATHOGENS OF WOUND INFECTION

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ABSTRACT

This study was performed for production, purification, characterization and evaluation the antibacterial activity of biosurfactant BS which were produced from local isolate *Lactobacillus plantarum* Z9 with bacteriocin and studying the synergistic effect. A total 100 different samples 85(%) isolates of *Lactobacillus* sp. were isolated from different sources and identified by biochemical tests then subjected to the primary and secondary screening processes to select the active isolate for bacteriocin and biosurfactant production. Among the vagina isolates screened, the results found that *Lactobacillus* sp. Z9 isolated from the clinical samples of Iraqi healthy women had the highest productivity of the bacteriocin and biosurfactant. Partial purification of biosurfactant that produced from *L. plantarum* Z9 was conducted using solvent system (chloroform: methanol, 2:1). A 1:1 ratio of n-butanol was used to extract of partially purified bacteriocin, where the bacteriocin activity up to 320 AU/ml against both *Staphylococcus aureus* and *Pseudomonas aeruginosa* respectively compared to 20 AU/ml before purification.. Antibacterial activity of Bacteriocin displayed a significant activity with inhibition zones diameter ranged from (11 to 26 mm) and (10 to 25 mm) against *P. aeruginosa* and *S. aureus* at concentration ranged from 50 to 200 mg/ml. Maximum antibacterial activity of bacteriocin against *P. aeruginosa* and *S. aureus* showed at concentration 200 mg/ml of bacteriocin. Partial purified lipopeptide of *L. plantarum* Z9 was examined against pathogenic bacteria using well diffusion method. The lipopeptide had inhibition zones diameter ranged from (14 to 28 mm) and (13 to 29 mm) against *P. aeruginosa* and *S. aureus* respectively at concentration ranged from 25 to 200 mg/ml. The combination of lipopeptide with bacteriocin increased the range of inhibition zones and made the pathogens sensitive.

Key words: Purification , characterization, biosurfactant, antibacterial, inhibition.

خليل وحيدر

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دراسة التأثيرات التآزرية للبتيدات الدهنية والبكتريوسينات المنتجة من قبل *Lactobacillus* sp. لعينات بشرية ضد الممرضات

السريية المسببة لالتهاب الجروح

حسن حيدر

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باحث

قسم التقنيات الاحيائية/ كلية العلوم/جامعة بغداد

المستخلص

أجريت هذه الدراسة من أجل إنتاج وتنقية وتوصيف وتقييم النشاط المضاد للبكتيريا للمستحلب الحيوي الذي تم إنتاجه من العزلة المحلية *Lactobacillus plantarum* Z9 مع البكتريوسين ودراسة التأثير التآزري. مجموع 100 عينة مختلفة 85% عزلة من *Lactobacillus* sp. تم عزلها من مصادر مختلفة وتم تحديدها عن طريق الاختبارات البيوكيميائية ثم خضعت لعمليات الفرز الأولية والثانوية لاختبار العزلة النشطة لإنتاج البكتيريا والفاكهة الحيوية. من بين عزلات الفم أو المهبل التي تم فحصها ، وجدت النتائج أن *Lactobacillus* sp. Z9 المعزول من العينات السريية للنساء العراقيات الأصحاء كان له أعلى إنتاجية للبكتيريا والفاكهة الحيوية. تم إجراء التنقية الجزئية للمادة الحيوية الناتجة من *L. plantarum* Z9 باستخدام نظام المذيبات (الكلوروفورم: الميثانول ، 2: 1). تم استخدام نسبة 1: 1 من n- بيوتانول لاستخراج البكتريوسين المنقى جزئياً ، حيث يصل نشاط البكتريوسين إلى 320 AU / ml ضد كل من *Staphylococcus aureus* و *Pseudomonas aeruginosa* على التوالي مقارنة بـ 20 AU / ml قبل التنقية. أظهر نشاطاً معنوياً مع قطر مناطق التثبيط تتراوح من (11 إلى 26 مم) و (10 إلى 25 مم) ضد *S. aureus* و الزنجارية و *S. aureus* بتركيز يتراوح من 50 إلى 200 مجم / مل. أظهر أقصى نشاط مضاد للجراثيم للبكتريوسين ضد *P. aeruginosa* و *S. aureus* بتركيز 200 مل/مجم من البكتريوسين. تم فحص الببتيد الدهني المنقى جزئياً لـ *L. plantarum* Z9 ضد البكتيريا المسببة للأمراض باستخدام طريقة الانتشار الجيد. يتراوح قطر الببتيد الشحمي من (14 إلى 28 مم) و (13 إلى 29 مم) ضد *S. aureus* و الزنجارية و *S. aureus* على التوالي بتركيز يتراوح من 25 إلى 200 مجم/مل. أدى الجمع بين الببتيد الدهني والبكتريوسين إلى زيادة نطاق مناطق التثبيط وجعل العوامل الممرضة حساسة.

كلمات مفتاحية: تنقية، توصيف ، خافض للتوتر الحيوي، مضاد للجراثيم، مثبط.

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INTRODUCTION

Lactobacillus is Gram-positive, non-spore forming, rod or coccobacilli ranging from (0.5-1.2) to (1-10) μm in size are observed in rich habitats containing carbohydrate such as food ; raw milk, fermented foods, habitats of heavy microbial host like the normal microbiota of mouth, female genital tract and gastrointestinal tract (GIT) of animals and humans. Mostly *Lactobacillus* used in bacteriocins production. Bacteriocins are multifunctional proteinaceous substances synthesized by bacteria's ribosomes that have antibacterial activity at specific doses which are big proteins with a molecular weight of more than 20 kDa (18, 29). Bacteriocins are a good helpful tool for several industrial and medical uses due to their harmless for eukaryotes and significantly wider inhibitory ranges; the most common antimicrobial producing bacteria are lactic acid bacteria (49, 48, 50). Broadly agreed theory for the mode of action of bacteriocins including bacteriocin adsorption to specific or non-specific cell surface receptors leading to cell death (41). The type and amount of carbon, nitrogen, and phosphate sources, as well as cations, surfactants, and inhibitors, have a significant impact on bacteriocin synthesis. In addition to biosurfactant production *Lactobacillus* are used to provide a number of benefits to chemical surfactants, including reduced toxicity and greater biodegradability (24). Biosurfactants are surface active agents of microbial origin (bacteria and fungi) with hydrophobic and hydrophilic moieties which are divided into four categories based on their structure: glycolipids, lipopeptides, fatty acids phospholipids and polymeric biosurfactants. Biosurfactants separate between surface active compounds that cling to the cell surface and those that are extracellularly in the growth medium. A large number of cyclic lipopeptides including decapeptide antibiotics (gramicidins) and lipopeptide antibiotics (polymyxins), produced by *B. brevis* and *B. polymyxa*, possess remarkable surface-active properties. Biosurfactants, for example, may be important in reducing pathogenic adhesion capacity, which is an important stage in biofilm formation and bacterial growth (34). The current study was aimed to production, and characterization of produced bacteriocin

and Biosurfactants (lipopeptide) by *Lactobacillus* sp., evaluated its antibacterial activity and study. the synergistic effect of Bacteriocin with Biosurfactant

MATERIALS AND METHODS

Sample collection and bacterial isolation

A total number of 100 human samples were gathered from the mouth cavity and vaginal region of apparently healthy women. The samples were then grown on MRS agar medium for 48 hours at 37 °C with 3-5 percent CO₂ by utilization of Candle Jars at a temperature of 37 degrees Celsius (11). A 0.1 percent antifungal (Nystatin) has been added to the cultures to prevent fungal growth. The isolates were sub- cultured on MRS agar (7), and the pure colonies were kept on the same media when they were employed in the subsequent research. All bacterial isolates were kept in glycerol. *Lactobacillus* sp. was identified using the VITEK 2 system utilizing a pure culture with overnight growth on MRS agar plate

Bacteriocin production

MRS liquid medium was prepared in 250 ml flasks. After autoclaving, these flasks were inoculated with 2% (1×10^8 CFU/ml) of activated *Lactobacillus* inoculum and incubated in shaker (120 rpm) in anaerobic conditions by utilization of N₂ gas at 37°C for 48 hrs. for the extracellular biosurfactant production, at experiment's end (48h), cells have been harvested through centrifugation at 10000 rpm for 15minutes, and the cell-free supernatant (CFS) was collected. and filtered with 0.22 μm Millipore filter paper under sterile conditions (32). NaOH (1M) was used for pH adjustment to (7.0). and catalase solution were added to neutralize the effect of organic acid and H₂O₂ activity.

Detection of bacteriocin activity

The critical dilution assay, which is analogous to the minimum inhibitory concentration technique (MIC) for antibiotic assessment (25) This procedure required the following preparation of a multiple dilution series of the isolate culture to be tested: Each one of the 500 μl sterile normal saline Eppendorf tubes was used. 500 μl of cell-free supernatant (CFS) was transferred to the first Eppendorf

tube using a sterile micropipette, and then vortex mixed. This is the first multiple dilution in the mixer. The 500µl from the first multiple dilution then was transferred to the second tube for a second two-fold dilution (2-1). The bacteriocin activity was determined by the maximum dilution that produced an inhibitory zone. As a result, the bacteriocin activity was calculated as the reciprocal of the highest dilution factor (DF), yielding inhibition zone that can be detected. The following equation (1) was used to compute the bacteriocin activity, which is measured in arbitrary units (AU) (4):

$$\text{AU/ml} = \frac{1}{DF} \times \frac{1000}{\text{volume spotted in } \mu\text{l}}$$

Bacteriocin partial purification of n-Butanol extraction technique: The bacterial isolate was inoculated into MRS, centrifugation at 6000 rpm for 15 min, cells were neglected, after that the CFS was heated for 10 min at 80°C, cooled, and centrifuged for 15 minutes at 6000 rpm (31). The supernatant was thoroughly mixed with n-butanol in a ratio 1:1. To achieve phase separation, the mixture was centrifuged at 4000 rpm for 10 minutes. At 55°C, the organic phase was evaporated by utilization of oven, and then the sediment was resuspended in sodium phosphate buffer (pH 6.5, 1.0mM) and dubbed partly purified bacteriocin (PPB). The agar well diffusion test was used to measure bacteriocin's antibacterial activity (2).

Characterization of bacteriocin amino acid analyzation: Amino acid analyzer is offers the digestion and abstraction of amino acids, briefly 0.2 g of purified bacteriocin was taken and 12 ml of (6M HCl) was added then placed in the oven at 110°C for 1h. The mixture was filtrated through a filter paper type 0.8 µm. Then the filtrate was washed twice with distilled water. Rotary evaporator at 50 °C was used for drying the filtrate .A volume of 10 ml of D.W was add, then the component was returned again to the rotary until it completly dryness. Then dissolved in 3.5 ml of HCl (0.02 M). the pH of solution was adjusted by adding NaOH (1m). The product was injecting to the

amino acid analyzer after addition the reagent OPA (27).

Production of biosurfactant

MRS broth medium was prepared in 250 ml flasks. After autoclaving, these flasks were inoculated with 2% (1x 10⁸ CFU/ml) of activated *Lactobacillus* inoculum and incubated in shaker (120 rpm) in anaerobic conditions by utilization of N₂ gas at 37°C for 120 hrs. for the intracellular biosurfactant production (35).

Extraction of biosurfactant

After 120 hours of incubation, the *Lactobacillus* sp. culture was harvested by centrifugation (10000, 15min). Ten ml of culture were centrifuged for dry biomass measurement. Furthermore, , washed twice in de-mineralized water, and re- suspended in PBS (20 ml) (PBS: KH₂PO₄ (10Mm), NaCl (150Mm). The cells were sonicated at 600Hz for 2 min and centrifuged at 10000 rpm for 15minute. The biosurfactant- containing supernatant was transferred to a separating funnel and extracted with various solvent systems, including a 2:1 mixture of chloroform and methanol (8). The aqueous layer at the bottom of the separating funnel was removed, and the emulsion layer was collected in a glass Petri dish and dried at (40–45) °C until it converted to powder. The resulting product was weighed and stored in a clean vial at 5°C for further use. (42,38).

Characterizations of *Lactobacillus* biosurfactant

The spectrum of the Fourier transforms infrared (FTIR): Following the homogenization of the material with KBr, FTIR spectra associated to biosurfactant were examined at wavelength ranged (500-4000) wave number/cm of data was obtained . In a Shimadzu-I Raffinity-1 spectrophotometer, UV spectra were acquired. The spectra have also been displayed as intensity vs. wave number (3).

Fatty acid analysis (GC-Mass):

Biosurfactants were evaluated using gas chromatography (GC) to determine their composition of fatty acid. The composition of fatty acids was examined in the following way. Following 10mg of partially

purified biosurfactant was dissolved in 1 ml of sulfuric acid – methanol at 90 °C for 15 hours, then 1 ml of hexane was added with mixing, and the hexane layer was extracted after that the sulfuric acid was evaporated. A volume 1 ml D.W was added to the hexane layer and mixed thoroughly. The fatty acid methyl ester was recovered with hexane and analyzed by GC using helium as the carrier gas on a Shimadzu 17-A GC equipped with a fused silica capillary column (30 m x 0.25 mm, 0.25 m film thickness) (51).

Collection, Isolation and identification of the pathogenic isolates: A total 150 specimens pathogenic isolates from infected wounds and burns that were used as indicators in the current study, were collected from AL-Yarmouk Teaching hospital in Baghdad were collected between 15/9/2020 to 15/1/2021 for isolation. All isolates were diagnosed by using VITEK system after a preliminary identification on blood agar, Mannitol salt agar and MaCconkey agar (43).

Antibiotic Susceptibility

Antibiotic susceptibility was conducted using the disc diffusion method on a Mueller Hinton agar which was previously seeded with the selected indicators isolates (40) and use 9 Antibiotics Discs (Azithromycin, Erythromycin, Gentamicin, Vancomycin, Oxacillin, Aztreonam, Ceftazidim, Levofloxacin and Piperacillin). The aim of antibiotic susceptibility to select amore antibiotic resistant bacteria.

Antibacterial activity of bacteriocin

The antibacterial activity of partially, purified bacteriocin was tested against two selected pathogenic isolates (*S. aureus* and *P. aeruginosa*). It was performed by disc diffusion method Pathogens were grown in Muller Hinton broth and then placed in incubator at 37 °C overnight. Each bacterial strain's volume (0.1 ml) was swabbed onto Muller Hinton ager dishes. A 200 mg of partial purified bacteriocin was dissolved in 1 ml phosphate buffer (1M) for preparation of the stock solution of bacteriocin then serial . dilution A 100 µl of each concentration of bacteriocin 50,

100, 150, 200 mg/ml were prepared and added to each well. The plates were then incubated for 24 hours at 37 °C. The antibacterial activity of bacteriocin was indicated by measurement the diameter of growth inhibition zone around each well (4).

Antibacterial activity of biosurfactant

As before the antibacterial activity of partially purified biosurfactant was tested against the same indicator (two pathogens) the same indicator pathogens (*S. aureus* and *P. aeruginosa*). For each Petri plate, 20 mL Muller Hinton Agar was produced. A 0.1 ml of pathogens indicators culture was adjusted to (1×10^8 cell/ml) and streaked across all dishes as described previously. The wells were prepared on plates using a cork borer, 100 µ l of biosurfactant at concentrations (25, 50, 100, and 200 mg/ml) was poured into each well created in MHA followed by a 24-hour incubation period at 37°C. These plates were examined after incubation to find a definite zone of inhibition had appeared. A digital ruler was used to measure the radius of the inhibitory zone in millimeters (47).

The synergistic effect of bacteriocin with biosurfactant:

To test the synergistic effect of bio-surfactant with bacteriocin against pathogenic bacteria. The solution from partial purified bacteriocin has been freshly made through dissolving partial purified bacteriocin in phosphate buffer with CMC of biosurfactant .A 0.1 overnight growth of *S. aureus* and *P. aeruginosa* after adjusting to (1×10^8 UFC/ml) was spread on surface of Muller Hinton agar the diffusible disks were impregnated with 50µl of known concentration of bacteriocin and 50µl of biosurfactant. Other disks containing only bacteriocin and biosurfactant were used as control. The plates were incubated at 37 °C for 24 h. The inhibition zone was measured. All the experiments were carried out in duplicate.

RESULTS AND DISCUSSION

Isolation and identification of *Lactobacillus*

A total 100 samples were collected from several sources includes eighty vaginal swabs, twenty swabs from mouth cavity.

81(81% of *Lactobacillus*), 69(85%) from vaginal swab and 12(14.8%) from mouth cavity appeared white color, soft, large or small, convex, creamy and smooth, circular colonies when the samples were primarily grown MRS agar plates (7), on the other hand Gram stain showed as the gram positive purple rods that occurred singly, in pairs or in short chains and non-spore forming (20). All bacterial isolates were catalase, oxidase and gelatinase negative. Catalase and oxidase testes were usefulness tests to identify the genus of *Lactobacillus* and therefore, the above tests were performed for all isolates (46). Hemolysis test for all *Lactobacillus* isolates examined shown gray colonies with alpha hemolysis in blood agar plate medium. Finally, the best isolate *Lactobacillus* Z9 for bacteriocin and biosurfactant producers was chosen in this study for further experiments.

Identification of *Lactobacillus* sp. isolate

VITIK 2 compact system was carried out as confirmatory test for the identification of *Lactobacillus* sp. Z9 isolate. The GP card was used for gram positive bacterial isolate, which consists of 43 biochemical tests. The results indicated that the isolate *Lactobacillus* sp. belong to the species *Lactobacillus plantarum*.

Detection of bacteriocin activity

The partial purified bacteriocin produced by *L. plantarum* Z9 was tested against two clinical isolates that more resistance to all antibiotics *P. aeruginosa* (P4) and *S. aureus* (S7) isolated from wound infections. Bacteriocin displayed a significant activity with diameter of inhibition zones ranged between (11-26 mm) and (10 -25 mm) against *P. aeruginosa* and *S. aureus* respectively at concentration ranged from 50 to 200 mg/ml. Maximum antibacterial activity of bacteriocin against *P. aeruginosa* and *S. aureus* showed at concentration 200 mg/ml (36).

Partial purification of bacteriocin

Bacteriocin formed by *Lactobacillus plantarum* Z9 was partially purified by using n-butanol extraction method for select the best procedure for partial purification of this bacteriocin, primarily heating step did not effect on crude bacteriocin activity, since the

activity remaining 20 AU/ml against *S. aureus* and *P. aeruginosa* isolates was detected. Bacteriocin was partially purified by extraction with n-butanol in a ratio 1:1. Bacteriocin was removed from the aqueous phase and could be recovered from the organic phase. By using this method, the activity of bacteriocin reached to 320 AU/ml against both pathogens. Butanol extraction exhibited complete recovery of bacteriocin activity, suggesting that at least part of the bacteriocin molecule has a hydrophobic character and shares this property with other bacteriocins (6,10). Extraction of bacteriocins using n-butanol in a1:1 ratio was reported for plantaricin 35d (26), plantaricin AA135(2) and plantaricin JY22 (22). It was found that the activity of the crude extract prepared by extraction with n- butanol was significantly higher (30).

Characterization of bacteriocin by amino acid analyzer system :

The results in Table (1) of amino acid analysis revealed the existence of 5 peaks in bacteriocin produced from *L. plantarum* Z9. the results were discovered with the highest mole fraction expressed through comparing the retention times of the results to those of the authentic standards amino percent of Glycine, Threonine, and Alanine were 28.7, 32.8, and 63.3, respectively, with Alanine having a higher relative abundance of 100% compared to other amino acids as show in (Table 1). Approximately similar results (27), from bacteriocin Produced by *Lactobacillus* sp L45. The amino acid composition of the purified bacteriocin, lactocin contained approximately 33 amino acid residues, of which about 50% were the non-polar amino acids alanine, valine and leucine. While (50) observed two fractions of purified bacteriocin from *Lactobacillus acidophilus* IBB 801. the amino acid composition revealed the presence of many hydrophobic amino acids, averaging 50% of the total amino acid content (21). noticed that the amino acid composition of bacteriocin produced by *Pediococcus* caacidilactici contained higher nmol of alanine, Aspartine, glycine, and lowest nmol of glucine, serine and valine.

Table 1. Amino acid analysis profile of the *L. plantarum* Z9 of bacteriocin extract

No.	Retention time (min)	Amino acid	Area %	Relative Abundance %
1	8.85	Alanine	633.6	100
2	8.06	Threonine	328.2	51.80
3	7.93	Glycine	287.0	45.29
4	9.44	Tyrosine	197.5	31.17
5	11.40	Cysteine	176.3	27.82

Extraction of biosurfactant

The isolate *Lactobacillus plantarum* Z9 was grown in optimal culture conditions for production of biosurfactant including MRS medium, pH 6.5 at 37 °C, with shaking (150 rpm) for and 120 hr . Then extraction was done by using Chloroform: Methanol 2:1 through separation funnel. The extraction of BS produced up to 1.5 g/L in MRS medium, this system used to obtain partially purified viscous milky colored product. 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. The remaining aqueous phase after extraction was measured by Tensiometer to insure that all the biosurfactant was collected by solvents and no remnants were left without extraction (9).

Characterization of *L. plantarum* Z9 biosurfactant

FTIR spectrum: This technique was performed by using MRS medium for partial purified biosurfactant production the result in (Figure 1) was showed band at 3288 cm⁻¹ and broad band at 3431 cm⁻¹ where that may be due to the hydroxyl groups (OH) of biosurfactant. These results were agreed with (39) who confirmed the broad stretching intense peak is existed at around 3428 cm⁻¹ which is characteristic of OH and

(CH) model between 2854 and 2925 cm⁻¹. In addition, there is a strong band at 1639cm⁻¹ and 1119 cm⁻¹, those may be due to the NH and CN groups, respectively. In most effective biosurfactants, alkene and alkyl benzene components are significant. The novel anionic surfactants are derived from aromatic or substituted aromatic molecules and alkenes. Peaks at 1500-1650 cm⁻¹ indicates the presence of C=C alkene component. This technique indicates the existence of groups of alkene, alkyne and conjugated diene in the biosurfactant which lead to higher activity of surface. According to FTIR spectrum obtained, the molecular composition of BS is composed of carbohydrates and lipopeptide. The hydrophobic chain of BS is composed of lipid and hydrophilic part is mainly composed of sugar. Comparison of the spectra obtained revealed that the BS is closely similar to Xylolipid (glycolipid) reported earlier from different LAB strains (44,33). Several reports have been published on BS produced by LAB but inadequate information about their chemical composition is known. LAB derived BS were initially characterized as multicomponent mixtures consisting of protein, lipid and polysaccharide fractions, polysaccharides and phosphate groups (45, 44,13, 37).

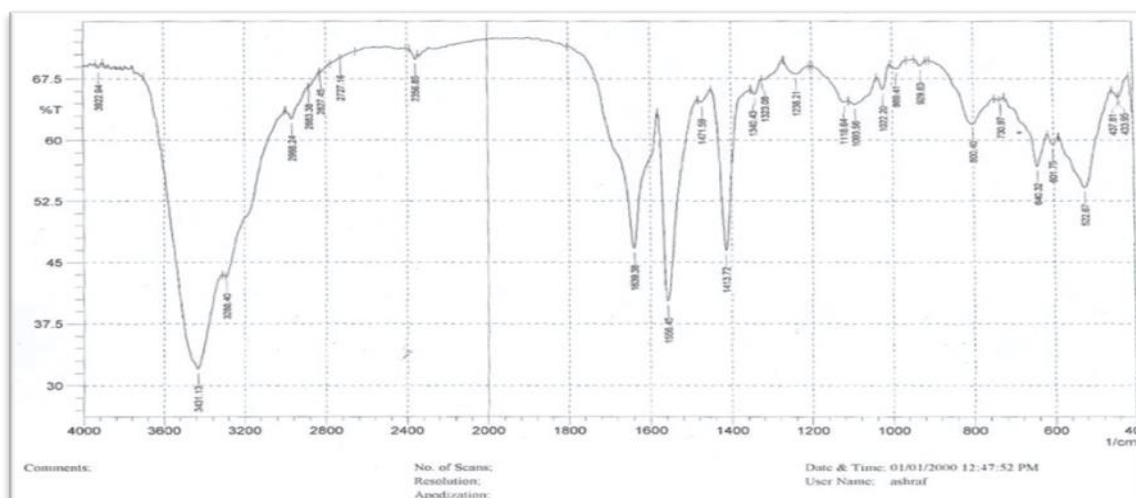


Figure 1. FTIR spectrum analysis lipopeptide produced by *L. plantarum* Z9

GC-MS

Based on the results, the purified biosurfactant composed of 100% of Trisobutyl (3-phenylpropoxy) silane carbazol-1-one,1,2,3,4-tetrahydro-6-phenoxy-, in addition to other components with a lesser percentage. the results also revealed that the biosurfactant was consist of 55.5% of Anthracenol pyridino phane-1,13-diene, Pyrido (2,3) pyrimidine-dion, 6-amino-5-hydroxy-1,3-dimethyl-7- (2-oxo-1-pyrrolidiny), (30.1%) of oleic Acid,cis- vaccenic acid,cis-13-Octadecenoic and- other components in different time as (Figure 2). These results agreed with (3) showed that the purified biosurfactant produced by *L. plantarum* consists of 94% of Benzene, 1, 2, 4- trimethyl. psi. -Cumene As-Trim ethylbenzene Pseudocumene

Pseudocumol 1, 2, 4- Trim ethylbenzene 1,2. In addition, there are other components with a lesser percentage. In comparison, the results of the purified biosurfactant also showed that it mainly consist of 93% of Benzene, 1-ethyl-3-methyl- Toluene, m-ethyl- m-Ethyl methylbenzene m- Ethyl toluene m- Methyl ethylbenzene 1- Ethyl-3 and other components in different time. Saravanakumari and Mani, (33) have isolated biosurfactant from *Lactobacillus lactis* which consists octadecanoic acid as a chain of fatty acid related with moiety of sugar. Rhamnolipids which are consist of molecules of β -hydroxydecanoic acid as branched fatty acids. Stearic acid and palmitic acid were major type of fatty acid in cell bound biosurfactant produced by *Lactobacillus pentosus* (17).

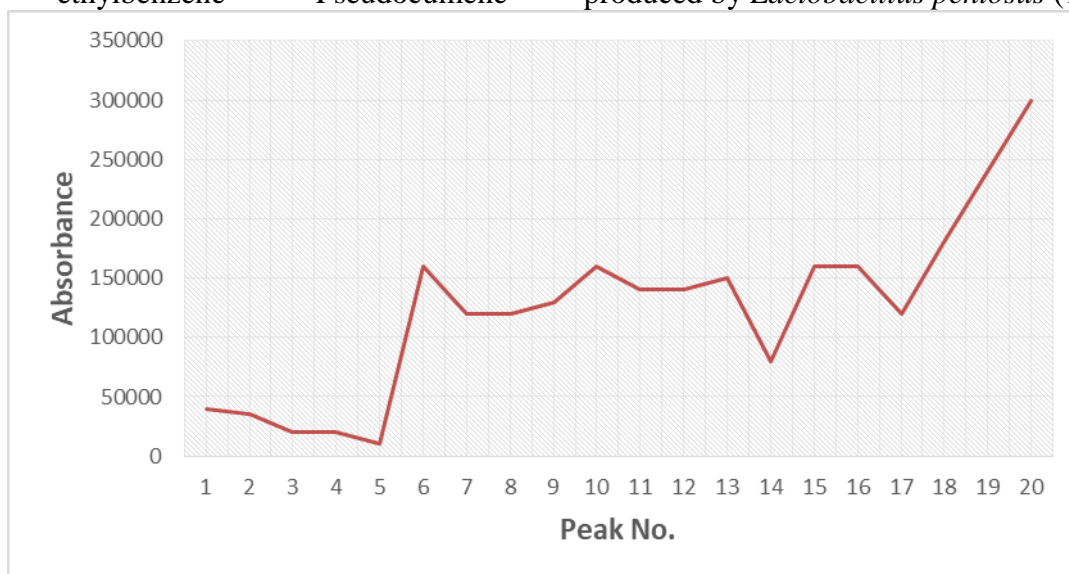


Figure 2. GC mass analysis of partial purified biosurfactant produced by *L. plantarum* Z9

Application studies of produced bacteriocin and lipopeptide

In vitro antibacterial activity of bacteriocin

The partial purified bacteriocin produced by *L. plantarum* Z9 was tested against two clinical isolates that more resistance to all antibiotics. *P. aeruginosa* (P4) and *S. aureus* (S7) isolated from wound infections. Bacteriocin displayed a significant activity with inhibition zones diameter ranged from (11 to 26 mm) and (10 to 25 mm) against *P. aeruginosa* and *S. aureus* respectively, at concentration ranged from 50 to 200 mg/ml. Maximum antibacterial activity of bacteriocin against *P. aeruginosa* and *S. aureus* showed at concentration 200 mg/ml of bacteriocin as shown in (table 3) and (figure 2). One of the most significant findings to emerge from this study is that bacteriocin

produced by *Lactobacillus* showed high antibacterial activity against many clinical isolates of *S. aureus*, that is considered as Methicillin-resistant Staphylococcus aureus (MRSA). Another study showed the inhibitory spectrum of *L. plantarum* bacteriocin was assessed by the agar well assay against many stains revealed the bacteriocin could strongly suppress common harmful and spoilage bacteria, such as *S. aureus*, *B. subtilis*, *B. anthracis*, *E. coli*, and *Salmonella*, which had an inhibition zone diameter above 28 mm. At the same time, it also had a certain inhibitory effect on *L. monocytogenes*. The bacteriocin also had the characteristics of most LAB bacteriocin, which has a certain inhibitory effect on the closely related *lactobacilli*. It was indicated the bacteriocin

also had partial inhibition of the yeast. In a word, this bacteriocin could inhibit

Gram-positive, Gram-negative, and yeast, so it was a broad-spectrum bacteriocin (36).

Table 3. Antibacterial activity of bacteriocin against *P. aeruginosa* and *S. aureus*

Bacteriocin (mg/mL)	<i>P. aeruginosa</i> (mm)	<i>S. aureus</i> (mm)
50	11	10
100	16	15
150	20	21
200	26	25

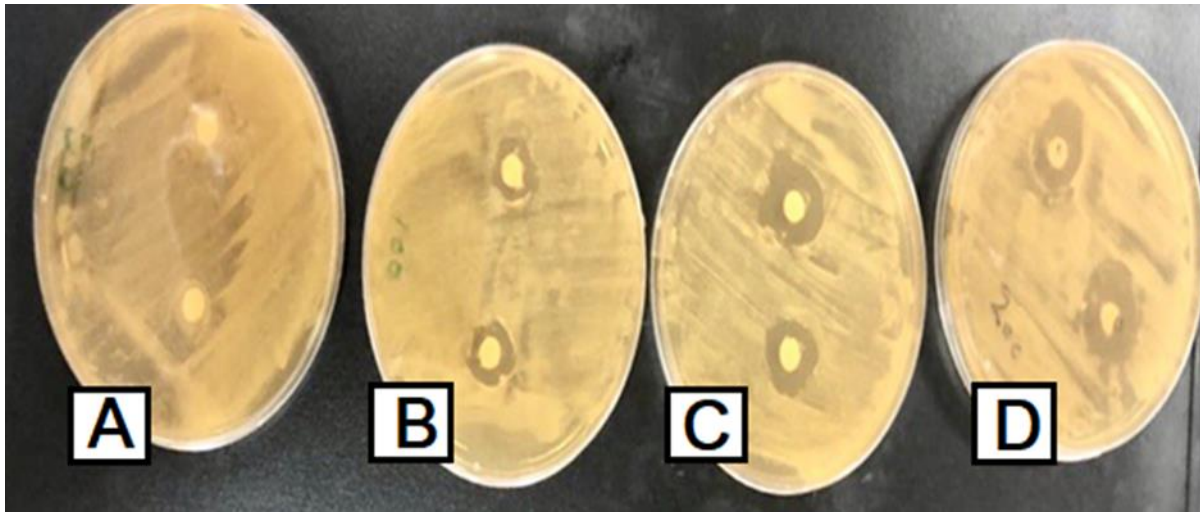


Figure 3. Growth inhibition zone of pathogenic bacteria with concentrations A: 50 mg/ml, B:100 mg/ml C: 150 mg/ml and D: 200 mg/ml of bacteriocin produced from *L. plantarum* in MRS

Antibacterial activity of lipopeptide

Partial purified lipopeptide of *L. plantarum* Z9 was examined against pathogenic bacteria using the approach of the well diffusion method. The results showed several antibacterial effects on the growth of pathogens as in (table 4). The biosurfactant had inhibition zones diameter ranged from (14 to 28 mm) and (13 to 29 mm) against *P. aeruginosa* and *S. aureus* respectively at concentration ranged from 25 to 200 mg/ml of lipopeptide. This result agreed with Madhu and Prapulla, (28) who reported that the biosurfactant which formed by *Lactobacillus plantarum* CFR 2194 have strongly inhibitory effects on *S. aureus* at 25 mg/mL, as well as these result similar (3). where reported partial purified biosurfactant which produced from *L. plantarum* have antimicrobial activity against some pathogens. The concentration 200 mg/ml, showed the best effect of the biosurfactants have effects on both *P. aeruginosa* and *S. aureus* with the inhibition zone (18 mm and 21 mm), respectively. The mechanism of antibacterial activity of biosurfactants result in the disruption of the cytoplasmic membrane, which results in the

metabolite leakage and cell lysis, as well as the disruption of the conformations of the protein that eventually alter the significant functions of the membranes (15). The reason of lipopeptide may be attributed to its concentration, where it's supposed to make its effect on the permeability of cell membrane as detergent and that effect similar to the effect that emulsified lipid in bacterial membranes and formation of a pore-bearing channel in membrane, as well as, the lipopeptide fraction is more effective against pathogenic bacteria and fungus than the glycolipid fraction. Although the cell wall of gram-negative bacteria are usually resistant to lipophilic solutes because they consist of lipopolysaccharide layer and an additional outer membrane (narrow outer wall) rather than gram-positive bacteria cell walls, which contain peptidoglycan (loose outer wall), which makes gram positive more sensitive (14). This phenome is due to that the biosurfactant can causes damage or loss of the layer of peptidoglycan which lead to inhibit the biochemical reactions in the wall of cell and prevent growth of peptidoglycan.

Table 3. Inhibition zone (mm) of pathogenic bacteria by lipopeptide produced from *L. plantarum* in MRS

Biosurfactant (mg/mL)	<i>P. aeruginosa</i> (mm)	<i>S. aureus</i> (mm)
25	14	13
50	18	19
100	22	24
200	28	29

Synergistic effect of lipopeptide with bacteriocin: A synergistic association is when two or more agents act to enhance efficacy compared to a single one (12). Results showed the combination of lipopeptide with bacteriocin increased the range of inhibition zones and made the pathogens sensitive. As shown in (figure 3), the inhibition zone of bacteriocin at concentration of 200 mg/ml against pathogenic bacteria *S. aureus* was 20 mm and with lipopeptide at CMC concentration (200 mg/ml) increased the inhibition zones to 24 mm. Together, biosurfactant interfere with bacterial membranes and forming pores that permit the entrance of antibiotics into the cells, which increase the antimicrobial activity, owing to the amphiphilic quality of the surfactants, which destabilize lipid packing of biological membranes, altering integrity and penetrate these coats through hydrophobic interactions, that lead ultimately to an increase of the antibiotic impact (19). Antibiotic compounds are thought to diffuse freely through the cell wall of gram-positive bacteria. However, in

gram negative bacteria the diffusion of a given antibiotic agent depends on the permeability of the outer membrane, the outer membrane of bacteria contains various peochannel called porins, which are involved in the inflow of various compound, including several classes of antibiotics. Bacterial adaptation to reduce influx through porins is an increasing prim worldwide that contributes, together with efflux system to the emergence and dissemination of antibiotic. Bacteriocins are multifunctional proteinaceous substances synthesized by bacteria's ribosomes that have antibacterial activity at specific doses (5). Other combinations contain bacteriocin of *Lactobacillus* was reported, there is strong synergistic effect between bacteriocin produced by *L. acidophilus* and killer toxin produced by *Saccharomyces cerevisiae* against *C. albicans* which was inhibited by 160 AU/ml of bacteriocin alone, and by 80 AU/ml of killer toxin alone, while the activity of bacteriocin and killer toxin mixture was 640 AU/ml, such as antibiotics (16).

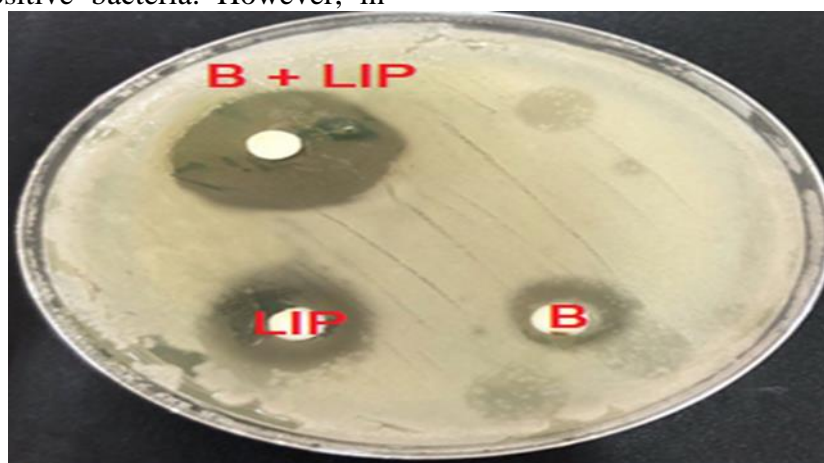


Figure 4. Combine effect of bacteriocin with lipopeptide against *S. aureus* (LIP: Lipopeptide, B: Bacteriocin and B+LIP: Combination of lipopeptide and bacteriocin).

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