Determination of some Optimal Conditions for the Production of Biosurfactant from *Lactobacillus acidophilus* ZA25 Isolated from the Vagina.

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ABSTRACT

Background: Biosurfactants are amphiphilic surface-active molecules produced by microorganisms that have distinctive properties such as emulsifying, anti-adhesive, and antimicrobial behavior. **Objective:** The current study investigates the optimal conditions and identifies the best isolate of *Lactobacillus acidophilus*, a vaginal bacterium,that produces biosurfactant. This isolate was obtained from healthy women. **Methodology:**All isolates were identified by morphological, microscopical, and biochemical tests, and then they were submitted to primary and secondary screening procedures to identify the most efficient *Lactobacillus* isolate for biosurfactant production. **Results:** Among all isolates screened, *L. acidophilus* ZA25 was found to have the highest productivity of the biosurfactant. Optimization findings revealed that the optimal conditions for producing the biosurfactant from this isolate were: MRS broth with 5% olive oil as the culture medium, a temperature of 37°C, anaerobic conditions, a pH of 6, and a 72-hour incubation period. The biosurfactant showed high antifungal activity when tested against *Candida albicans*. **Conclusion**: The chemical composition of the producing media and the physical conditions of incubation influence biosurfactant production by *L. acidophilus*. The produced biosurfactant has antifungal effect.

Keywords: Biosurfactant, *Lactobacillus acidophilus*, Optimization, Emulsifyingactivity.
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INTRODUCTION

Biosurfactants are amphiphilic molecules with various applications in the food, pharmaceutical, and cosmetics industries (1). They include hydrophobic as well as hydrophilic moieties, both of which can lower liquid surface tension. In contrast to synthetic surfactants, biosurfactants are diverse, biodegradable, and capable of performing highly specialized activities (2, 3). They are produced by different microorganisms, including Lactobacilli, which are generally recognized as safe (GRAS) bacteria associated with human health.(4)

Lactobacilli can produce various types of biosurfactants, including glycolipids, lipopeptides, phospholipids, and exopolysaccharides (5). These biosurfactants have different properties, such as reducing surface tension, emulsifying oils, displacing hydrocarbons, and exhibiting antimicrobial activity (6). The production of biosurfactants by Lactobacilli depends on several factors, such as the strain, the substrate, the culture conditions, and the assessment methods (7). Olive oil is a substrate that can be used as a carbon source to produce biosurfactants by Lactobacilli (8). However, the selection of the most promising strains should be based on the final fermentation substrate to avoid misinterpretation of results. Biosurfactants produced by Lactobacilli have potential applications in the biomedical, food, and nutraceutical industries. They can prevent or treat infections caused by *Candida spp*., which are opportunistic fungi that can cause vulvovaginal candidiasis (VVC) in women (9). Biosurfactants can inhibit the adhesion and biofilm formation of *Candida spp*. on the vaginal epithelium and enhance the activity of antifungal drugs (10). Biosurfactants produced by Lactobacilli can also be used as natural preservatives, emulsifiers, stabilizers, and flavor enhancers in food products (11). They can modulate the gut microbiota and exert beneficial effects on human health. To increase the amounts of biosurfactants produced from *Lactobacillus spp*., it is necessary to optimize

the bioprocess, the environmental conditions influence both the yield and the qualities of the biosurfactant that is produced, as the product may be affected by some of the factors including carbon source, temperature, pH, aeration, and incubation time (12). The optimization procedure was discovered to increase biosurfactant production and opened up new possibilities for the application of Lactobacillus spp. as a promising biosurfactant producer.(13)

This study aimed to determine the optimum conditions for producing biosurfactants from the best isolate and tested their antimicrobial activity against *Candida albicans*.

METHODOLGY

1. Collection of Samples and Bacterial Isolation

A total of thirty-five clinical samples from the vagina of healthy women aged between nineteen and thirty-six years were collected at Alzafraniah Hospital in Baghdad between January 15, 2021, and November 15, 2022, to isolate *Lactobacillus spp*. One mL of each sample was added to 9 mL of de Man Rogosa Sharpe broth tube and then incubated at 37˚C for 24 hours and 3-5% CO_2 available by utilizing Candle Jar, after that the samples were cultured on MRS agar medium which allows selective growth of *Lactobacillus species* then incubated at 37 °C for 48 hours with CO_2 by using a candle jar (14). In order to inhibit the growth of fungi, 0.1% antifungal (Nystatin) was added to the cultures. The isolates were examined morphologically, microscopically, and by biochemical tests to verify their identification. Only one isolate was subjected to API 50 CH.

Ethical statement: All participants agreed to provide the investigator with the specimens. The ethics committee of college of Science, University of Baghdad approved this work (Ref .CSEC/1122/0139, Appendix I). Informed consent according to the Declaration of Helsinki was obtained from all participants.

2. Screening the *Lactobacillus spp*. isolates for biosurfactant production

2.1. Primary screening (semi-quantitative screening)

2.1.1. Drop collapse assay

25 microliters of each bacterial culture (cultivated in MRS broth at 37 ºC for 48 hours in anaerobic conditions) were dropped on a strip of parafilm as a hydrophobic surface, and the drop's form on the parafilm surface was observed after a minute. If the shape of the drop remains dome-shaped, there is no biosurfactant present. If the form of the drop flattens, a biosurfactant is present. Distilled water is used as a negative control (15).

2.1.2. Oil spreading test

20 µL of engine oil was added to 20 mL of distilled water in a Petri dish. Twenty microliters of the cultivated bacterial broth culture was added to the center of the oil layer. The appearance of a clear zone at 10 seconds was considered a positive result, and the diameter of clear oil-displaced circles was measured (16).

2.1.3. Biosurfactant production from the selected isolates

The selected isolates were inoculated into MRS broth and cultured at 37°C for 48 hours. After the incubation time, the culture broth was centrifuged at 4°C for 10 minutes at 10,000 rpm. A 0.22 µm Millipore filter was used to filter the supernatant, which included extracellular biosurfactant. The remaining cells in the pellet were washed twice with demineralized water, resuspended in 9 mL of phosphate buffer saline (PBS) for intracellular biosurfactant extraction, and then left at room temperature for 2 hours with gentle shaking (17). The bacterial cell components were removed by centrifugation (18).

2.2. Secondary Screening (Quantitative Screening)

2.2.1. Emulsification Index (E24%) measurement of biosurfactant

2 mL of cell-free supernatant was mixed with 2 mL of Toluene, vortexing for two minutes, and left to stand for a day. At 25±1 ºC, the height of the emulsifier layer was measured. The emulsification index was determined by calculating the height of the emulsified layer (mm) by the total height of the liquid column (mm) and multiplying by 100 (19):

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

2.2.2. Surface tension (S.T) measurement

A QBZY-2 tensiometer (China) was used to measure the surface tension of aqueous solutions. The tensiometer platform has been loaded with a glass beaker measuring 50 milliliters and containing 20 milliliters of supernatant. After immersing the plate in the solution and measuring the supernatant surface tension at 25±1 ºC, a standard solution of Triton X-100 at 1 mg/mL concentration was utilized. In this work, the average of three recordings has been used to provide a more accurate value (20).

3. Optimum conditions of biosurfactant production

3.1. Effect of culture media

By cultivating the chosen isolate *L. acidophilus* AZ25 in a variety of culture media, including natural media (wheat flour medium 1g/1.5mL of distil water), synthetic media (MRS medium), and semi-synthetic media (MRS broth with 5% olive oil), the effect of culture media on the production of biosurfactant was investigated. Three flasks containing 50 mL of each used medium were sterilized and inoculated with 1% (1×10^8 cells/mL) of the overnight culture, then incubated for 48 hours at 37 ºC under anaerobic conditions in an anaerobic jar. After an incubation period, the cultures were centrifuged for 10 min at 8000 rpm, and the supernatant was taken (extracellular biosurfactant). The cells for each flask were rinsed twice in distilled water and resuspended in 10 mL of phosphate-buffered saline. Cells were kept at 25 ºC for 4 hours with slight stirring for intracellular biosurfactant production. Emulsification activity and surface tension have been evaluated using the supernatant.

3.2. The effect of different physiological factors

The effect of pH on biosurfactant production was achieved by using 0.1N HCL or 0.1N NaOH for obtaining various pH values (4, 5, 6), 50 mL of sterilized medium (which was chosen as the best) inoculated with 1% compared with McFarland ($1x10^8$ cells/mL).

The effect of temperature on Biosurfactant production was achieved by incubating at different temperatures (25, 30, 37, and 42°C). Sterilize the medium, and then inoculate it with 1% of the bacterial isolate culture (1×10^8 cells/mL) to determine the optimal incubation period for biosurfactant production. 50 mL of the medium was inoculated with 1% (1×10^8 cells/mL) of the bacterial culture and incubated for different times (24, 48, 72, 96) hours. For incubation conditions, fifty mL of sterilized medium was inoculated with one mL of activated culture (1 × 10^8 cells/mL) of the selected isolate and incubated in both aerobic and anaerobic conditions.

3.3. Antimicrobial activity of a crude biosurfactant

The Petri plates of Muller Hinton agar (MHA) were swabbed with *Candida albicans* isolates, and wells were made with a cork borer (6mm). Fifty microliters of supernatant were loaded into each well and then incubated at 37°C for 24 hr. After incubation, the plates were examined for an inhibition zone. An electronic ruler was used to measure the radius of the inhibitory zone (21).

RESULTS

1. Bacterial isolation and identification

The results showed that only fifteen isolates belonged to the genus *Lactobacillus*. It's recognized as the genus *Lactobacillus* because its colonies appeared tiny (2-5 mm), convex, smooth, and glistening, without pigments, on MRS agar, with a creamy to white color. Under an oil immersion lens (100x), the bacteria have been shown as Gram-positive bacilli organized singly, in pairs, or short chains. In contrast, biochemical testing showed that all isolates have negative results for oxidase, catalase, and indole tests; only the best biosurfactant production isolate was subjected to api 50 CH to identify the *lactobacillus* strain.

2. Screening of *Lactobacillus spp*. isolates for biosurfactant production:

2.1. Primary screening (semi-quantitative screening)

The results for the drops collapse assay showed that from all fifteen tested *Lactobacillus* isolates, only seven isolates gave positive results and became flattened in shape. The instability of liquid droplets by biosurfactants is the basis for this test. The other eight isolates remain stable and gave negative results. For the oil spreading test, only four isolates showed a clear zone (which was considered a positive result) from all fifteen isolates. A clear zone forms in

the middle of the plate where the biosurfactant-producing isolates slide the oil, indicating the presence of the biosurfactant. The diameters of the clear zones were (3,6,7,20) mm. The L. acidophilus ZA25 showed high surface activity by forming a 20 mm diameter oil-clear zone. No clear zone observed with negative control (distilled water).

2.2. Secondary screening (quantitative screening).

For secondary screening, two *Lactobacillus* isolates with the best results were selected from the primary screening methods.

2.3. Determination of surface tension

Tensiometer measurements of the surface tension revealed significant biosurfactant activity of the two chosen isolates (23.26, 32.42 mN/m under anaerobic conditions), while (27.17-36.0 mN/m under aerobic conditions). The isolate ZA25 showed a high reduction in surface tension (23.27 mN/M) in anaerobic conditions.

2.4. Determination of emulsification activity (E 24%)

The two selected isolates showed variable emulsification activity, ranging from 43% to 62% under anaerobic conditions. While (39%-50%) under aerobic conditions.

The ZA25 isolate showed the highest E24 activity 62 % in anaerobic conditions, and 50% in aerobic conditions, and it was chosen for detecting the optimum parameters in the following experiment.

3. Optimum conditions for biosurfactant production

3.1. Effect of fermentation media

After cultivation *of L. acidophilus ZA25* in three different media, A: synthetic medium (MRS medium), B: natural medium (flour), C: semi-synthetic medium (MRS broth with olive oil), emulsifying activity and surface tension were determined. Among the three media used, semi-synthetic was the perfect medium for producing biosurfactants, as shown in Figure (1).

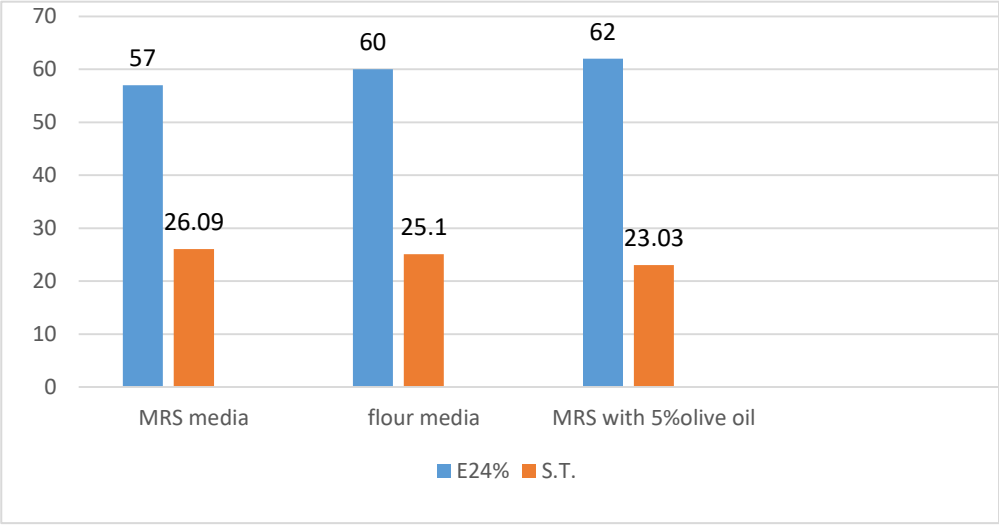

Figure (1): The effects of different media on biosurfactant production by *L. acidophilus* at 37°C, semi-synthetic medium (MRS broth with 5% olive oil) was used as the culture medium.

3.2. Effect of pH

The semi-synthetic media that were chosen in the previous experiment had been adjusted to different pH values to examine the effect of initial pH medium on biosurfactant production by *L. acidophilus* ZA25. The results given in Figure 2 indicate that pH 6 had the highest emulsifying activity (63%) and the lowest surface tension (22.8 mN/m).

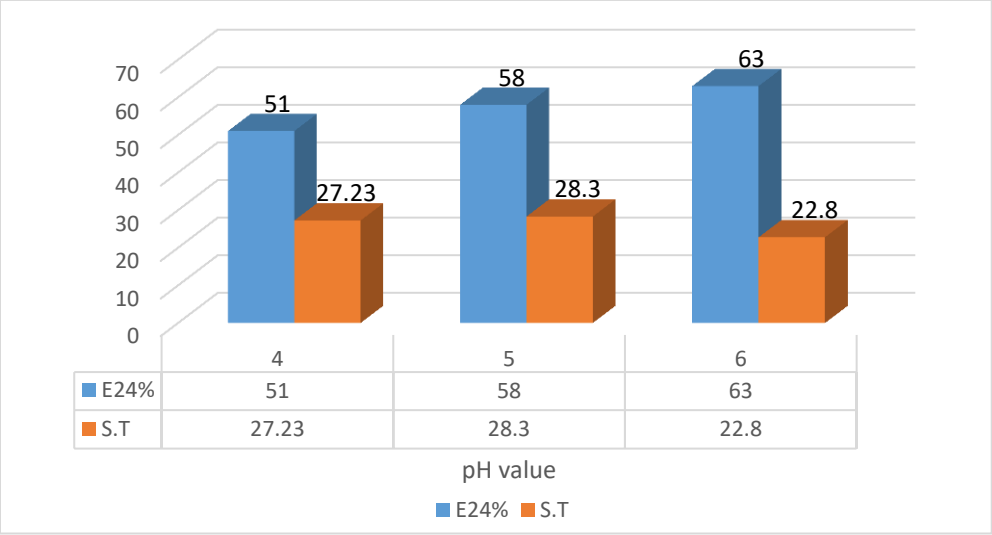

Figure (2): The effect of pH levels on biosurfactant synthesis by *L. acidophilus* at 37°C, semi-synthetic medium (MRS broth with 5% olive oil) was used as the culture medium.

3.3. Effect of temperature

The results showed that isolate *L. acidophilus* ZA25 could grow and produce biosurfactant at a variety of temperatures, including 25, 30, 37, and 42 °C. When semi-synthetic culture medium (MRS broth with 5% olive oil) was utilized, the best temperature for biosurfactant synthesis was 37 °C, with an emulsification activity of 63% and a surface tension of 22.8 mN/m.

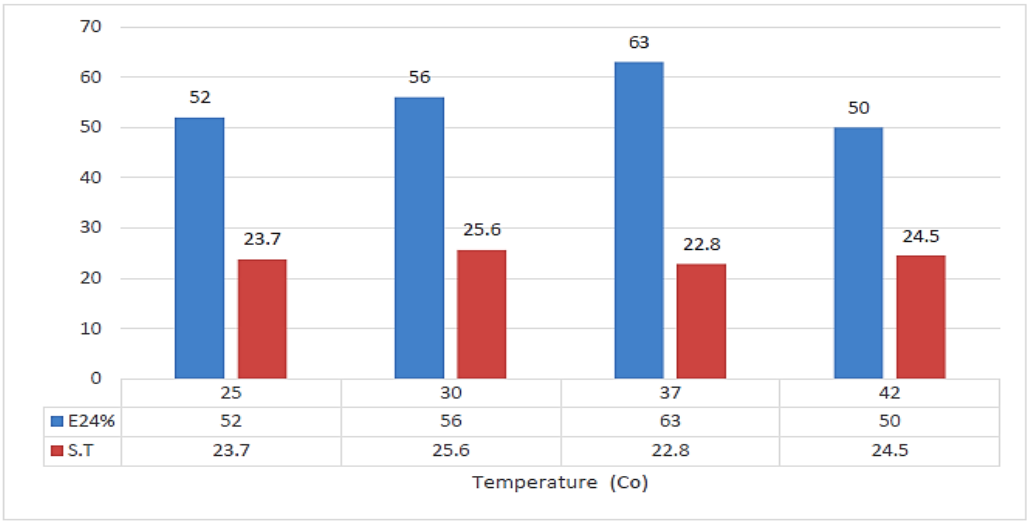

Figure (3): The effect of incubation temperature on biosurfactant production by *L. acidophilus* ZA25 was studied using a semi-synthetic medium (MRS broth with 5% olive oil) as the culture medium.

3.4. Effect of incubation period

The results in figure (4) shown that the high E24% (63%) and low surface tension (22.8 mN/m) had been gotten after 72 hours of incubation by utilize semi synthetic medium (MRS broth with 5% olive oil), while the lowest E24% (49%) and lowest surface tension (27.3mN/m) were obtained after 96 hours.

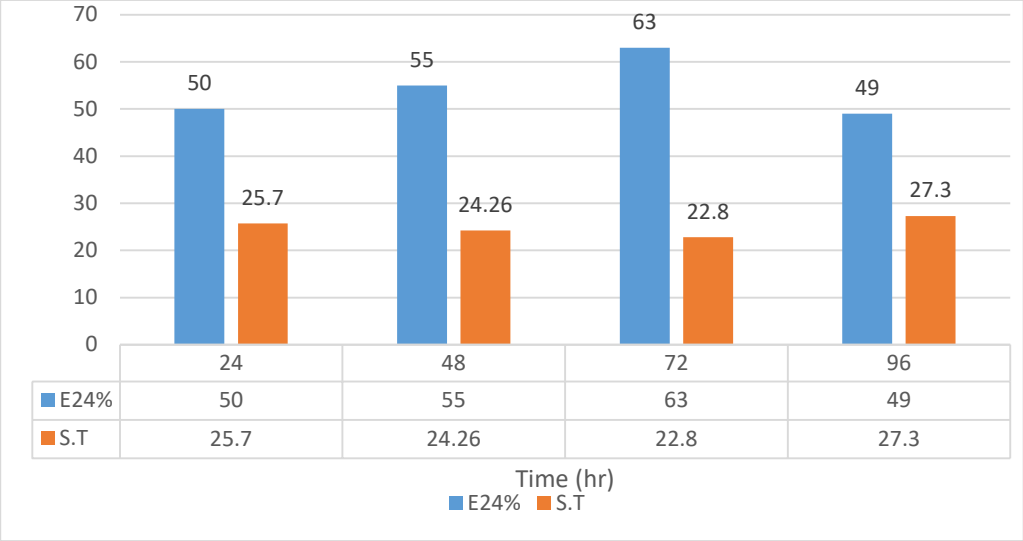

Figure (4): The effect of the incubation period on biosurfactant production by *L. acidophilus* ZA25 was studied using a semi-synthetic medium (MRS broth with 5% olive oil) as the culture medium.

3.5. The effect of incubation (aeration) condition

An additional factor influencing biosurfactant production was an anaerobic environment, which resulted in higher emulsification activity and lower surface tension compared with aerobic conditions (Figure **5)**.

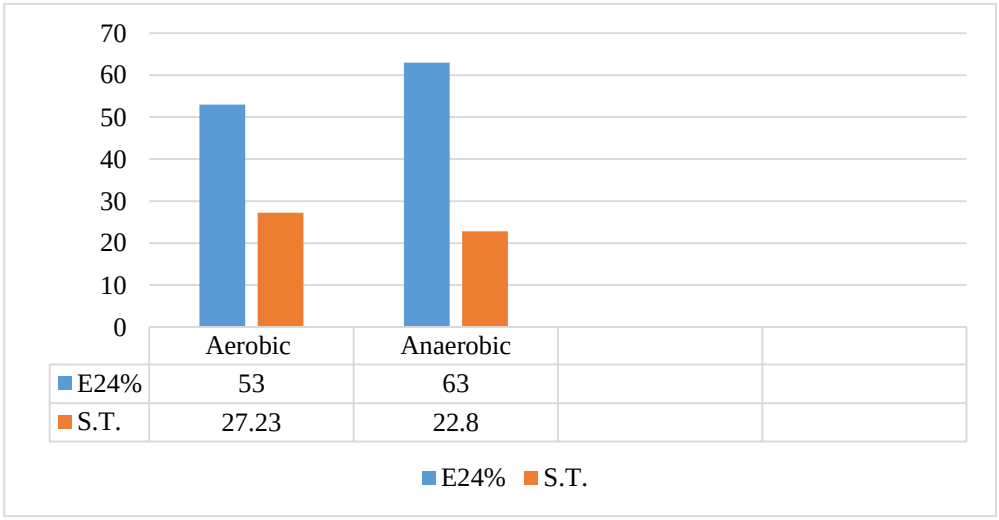

Figure (5): The effect of aerobic and anaerobic conditions on biosurfactant production by *L. acidophilus* ZA25 was studied using a semi-synthetic medium (MRS broth with 5% olive oil) as the culture medium.

3.6. Antimicrobial activity of biosurfactant

With inhibition zones of 22 mm and 26 mm, respectively, the antibacterial activity of supernatant containing biosurfactant from the ZA25 isolate had a substantial inhibitory impact on *C. albicans* isolates.**Figure (6)**

Figure (6): Antimicrobial activity of biosurfactant produced by *L. acidophilus* ZA25 isolate against *Candida* isolate.

DISSCUSION

From thirty-five samples, only fifteen strains were identified as *Lactobacillus*, according to the colony characteristics, biochemical tests, and microscopic observations (22,23).

The Primary screenings for biosurfactant production in *Lactobacillus* isolates revealed that only seven isolates out of 15 were positive in the drop collapse experiment, where the drop became flattened after one minute and spread as a result of the interfacial tension between the liquid drop and the hydrophobic surface. In contrast, the other negative results remained stable. Drop stability is affected by biosurfactant concentration and is related to interfacial tension (24). The drop-collapse method is a sensitive and convenient method. It has advantages, including needing a small volume of samples, being rapid, easy to carry out, and not requiring specialized equipment (25). The oil spreading test is also a primary technique for detecting the presence of biosurfactants. It relies on the displacement of oil to create a clear zone in the middle of the plate. (26), only four of the fifteen isolates formed a clear zone (positive result), ranging from 3 to 20 mm. (27) demonstrated that the diameter of the clear zone is proportional to the amount of biosurfactant produced. Secondary screening (quantitative screening) was performed in two major ways: Firstly, by the determination of surface tension method: the results of the two chosen tested isolates were (23.26, 32.42 mN/m in anaerobic condition) while (27.17-36.0 mN/m in aerobic condition). The ZA25 isolate showed a high reduction of surface tension (23.26 mN/M) in anaerobic conditions. The surface-active features of biosurfactant rely on the composition of the homologue, and this depends on the producing strain, as well as time and culture conditions (28). Sarubbo *et.al*., 2022 show that biosurfactants with low molecular weight can reduce surface tension and form stable emulsions.(3)

Secondly, by measuring the emulsification activity of biosurfactant produced by two chosen lactobacilli isolates, the results were (62%, 43% in anaerobic condition) respectively, while (50%, 39% in aerobic condition) respectively. Emulsification activity, an indirect method used to examine biosurfactant production, is considered a simple, reliable, and rapid assay to measure the amount of biosurfactant (29),(30) demonstrated that microorganisms with high emulsifying activity are good microbial candidates for biosurfactant production;, in addition, there is a relationship between E24% and surface tension such that a higher E24% indicates a lower surface tension and vice versa (24). Secondary screening findings show that the isolate *L. acidophilus* ZA25 was the most productive isolate for biosurfactant synthesis and was chosen to identify the best biosurfactant production conditions. The effect of culture media on biosurfactant production was studied by cultivating the isolate *L. acidophilus* ZA25 in various media Figure 1, and based on the findings; a semi-synthetic (MRS broth with 5% olive oil) medium was chosen to determine other optimal parameters. (31) Find out that olive oil was the best carbon source for biosurfactant synthesis. At the same

time, *Pseudomonas* sp. strain LP1 produces biosurfactant with the greatest emulsification activity (E24%) when grown in a medium containing heavy oil (32). PH control is important for biosurfactant production, as it affects the growth and metabolism of the microorganisms that produce them (33). The results found that pH 6 was the optimum pH, as shown in Figure 2. Similar research found that *Candida antarctica* and *Candida apicola* produce the most glycolipids when the pH is kept at 6.6 (34). Temperature is an essential factor influence on biosurfactant production, multiple incubation temperatures had been used, Figure (3) showed that the optimal temperature for biosurfactant production was 37 °C, with an emulsification activity of 63% and surface tension was 22.8 mN/m, temperature is a key component in the generation of biosurfactants and effect on the rates of biological processes by promoting or suppressing enzyme production and change in the amount of the biosurfactant produced (35).(36) Observed that culture temperature affects *Lactobacillus spp*. growth, with 37 °C giving the maximum bacteria population density and biosurfactant production. Different incubation periods (24,48,72,96) hours were examined of biosurfactant production by *L. acidophilus* ZA25, the results indicated that increasing of incubation time increased emulsification activity and decreased surface tension values until reach to the maximum at 72 hours, after 96 hours the emulsification activity was decreased as showed in Figure 4, this might owing to alterations in culture conditions over this time period, such as a decrease in anaerobic conditions, nutrients, and the accumulation of toxic metabolites that impede bacterial growth. (37) Said that biosurfactant biosynthesis has ceased, most likely due to the generation of secondary metabolites that might interfere with emulsion formation and surfactant adsorption. Also, anaerobic environment effect on biosurfactant production, Figure 5 showed a high emulsification activity under anaerobic conditions, the mechanism which cause that is not fully understood, but it may involve different metabolic pathways, regulatory genes, or environmental signals, pattern of behavior might be explained by low oxygen levels, which could produce a suitable habitat for these microaerophilic bacteria (38). High antimicrobial activity of biosurfactant against *C. albicans* isolates was observed; Its toxicity on cell membrane permeability is ascribable to a detergent-like impact that emulsifies lipid fungi membranes and forms a pore-bearing channel inside the lipid membrane; a resemblance study found that biosurfactants produced by *Bacillus subtilis* SPB1 have significant antimicrobial activity against various bacteria and yeast strains isolated from clinical specimens (39).

CONCLUSION

The outcomes of this research showed that the isolate *L. acidophilus* ZA25 that isolated from clinical sources was the best for biosurfactant production with high quality and quantity among the different isolates, by using MRS with 5% olive oil as culture media, the optimum conditions for biosurfactant production involve temperature 37°C, pH value 6,72 hours of incubation period in anaerobic condition. The antimicrobial properties and stability of biosurfactant indicate to their potential use as alternative antimicrobial agents in the medical field for applications against different pathogens that are responsible for various infections.

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تحديد بعض الظروف المثلى لإنتاج الفاعل بالسطح الحيوي لبكتيريا *Lactobacillus acidophilus* ZA25 المعزولة من المهبل

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الخلاصة

خلفية البحث: المواد الخافضة للتوتر السطحي هي جزيئات ذات نشاط سطحي برمائي تنتجها الكائنات الحية الدقيقة ولها خصائص مميزة مثل الاستحلاب، والسلوك المضاد للالتصاق، والمضاد للميكروبات. **الهدف من البحث:** تبحث الدراسة الحالية في الظروف المثلى وتحديد أفضل العزلات المنتجة للفاعل بالسطح الحيوي من بكتيريا *Lactobacillus acidophilus* المهبلية التي تم عزلها من النساء الأصحاء. **المواد وطرق العمل :** تم التعرف على جميع العزلات عن طريق الاختبارات المورفولوجية والمجهرية والكيميائية الحيوية بعد إخضاعها لإجراءات الفحص الأولية والثانوية لتحديد عزلة *Lactobacillus* الأكثر كفاءة لإنتاج الفاعل بالسطح الحيوي. **النتائج:** وجد من بين جميع العزلات التي تم فحصها أن بكتيريا *L. acidophilus* ZA25 لديها أعلى إنتاجية من الفاعل بالسطح الحيوي. أظهرت نتائج التحسين أن أفضل الظروف لإنتاج الفاعل بالسطح الحيوي من بكتيريا *L. acidophilus* ZA25 هي مرق MRS مع 5% من زيت الزيتون كوسط استزراع، درجة حرارة 37 درجة مئوية، حالة لاهوائية ، درجة حموضة 6 وفترة حضانة 72 ساعة.

الكلمات المفتاحية: المستحلب الحيوي ، الظروف المثلى ، نشاط الاستحلاب ، *Lactobacillus acidophilus* .