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المجلة العراقية لبحوث السوق
وحماية المستهلك

USING LOCALLY ISOLATED *BACILLUS SUBTILIS* ZE2013 IN THE PRODUCTION OF SURFACTIN AND STUDYING SOME OF THE OPTIMAL CONDITIONS FOR ITS PRODUCTION

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ABSTRACT

This study was aimed to using the locally isolated *Bacillus subtilis* ZE2013 and molecularly identified *B. subtilis* ZE2013 to produce surfactin,and studying of the optimal it's conditions, the crude bacteria filtrate have been extracted from culture growth for *B. subtilis* ZE2013, surfactin was detected by using high performance liquid chromatography (HPLC) technology, the results of HPLC analysis revealed that retention time of the produced surfactin from *B. subtilis* ZE2013 reached 6.02 min compared to the retention time of the stander surfactin which was 6.09 min, as the concentration of surfactin reached 1588 mg/kg, then detected on the active groups and comparing with the standard surfactin by use the Fourier Transform Infrared Spectroscope(FT-IR),than study the optimal condition for surfactin production, and effectiveness assay determination by use percentage of inhibition of antagonistic activity against selected indicator pathogenic bacteria *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhoi*, *Bacillus cereus*. The results showed that Trypticase Soy Broth (TSB), was the optimal culture medium for producing surfactin, inhibitory and the percentage of inhibition against pathogenic bacteria reached 59%, 70%, 65%, and 57% respectively, the glucose was the best carbon source, and tryptophan was the optimal sources of nitrogen. For surfactin production, the percentage of inhibition reached 51%,69%,62%,and 58% ; 59.5%,69.5%,65% and 58%, respectively, and the optimal temperature, was 35°C and the optimal number of ph=7, the percentage of inhibition was 60.5%, 70%, 65.5%, and 58.5%; 59.5%, 69.7%, 65%, and57.5% respectively, and incubation duration 48 h was 60.1%, 70%, 65.2%, and 55% for surfactin production.

Keywords: *B. subtilis* ZE2013, surfactin, HPLC, Trypticase Soy Broth

[*]**This article is taken from the doctoral dissertation of the first researcher.**

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أستعمال بكتريا *Bacillus subtilis* ZE2013 المعزولة محليًا في أنتاج السرفكتين ودراسة بعض الظروف المثلى لانتاجه

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الخلاصة

هدفت الدراسة الى أستعمال بكتريا *bacillus subtilis* ZE2013 المعزولة محليًا والمشخصة جزيئياً في أنتاج السرفكتين،من خلال فصل الراشح البكتيري من الوسط الزرعي لنمو*B.subtilis* ZE2013، والكشف عن وجود السرفكتين في الراشح البكتيري بأستعمال تقنية الكروموتوكرافي السائل عالي الأداء ،إذ بلغ وقت الأحتجازللسرفكتين المنتج 6.02 دقيقة بالمقارنة مع وقت إحتجازالسرفكتين القياس والبالغ6.09 دقيقة، بتركيز بلغ 1588ملغم/كغم،وبأستعمال مطياف الأشعة تحت الحمراء تم الكشف عن المجاميع الفعالة في السرفكتين المنتج وقورنت مع السرفكتين القياسي،ثم دراسة بعض الظروف المثلى لأنتاج السرفكتين من خلال قياس الفعالية التثبيطية له بأستعمال النسبة المئوية للتثبيط ضد البكتريا المرضية *Escherichia coli* و*Staphylococcus aureus* و*Salmonella typhoi* و *Bacillus cereus* ، وأوضحت النتائج إن وسط Trypticase Soy Broth (TSB) هو أفضل وسط لأنتاج السرفكتين ،إذ بلغت النسبة المئوية للتثبيط 59% و70% و65% و57% على التوالي ،ووجد أن الكلوكوز أفضل مصدر للكاربون ، والتربتوفان أفضل مصدر للنتروجين،إذ بلغت النسبة المئوية للتثبيط 51%و69% و62% و58% ; 59.5% و69.5% و65% و58%على التوالي، وأفضل درجة حرارة 35 مئوي°، وافضل رقم هيدروجيني 7،أذ بلغت النسبة المئوية للتثبيط لهما 60.5% و70%و65.5% و58.5% ;59.5% و69.7% و65% و57.5% على التوالي ، ومدة الحضن 48 ساعة أذ بلغت 60.1% و70% و 65.2% و55%.

الكلمات المفتاحية: *B. subtilis* ZE2013، سرفكتين، الكروموتوكرافي السائل عالي الأداء، تريبتيكاز صويا.

INTRODUCTION

The increasing resistance to traditional antimicrobials has revealed many problems that pose a threat to public health, so many researchers began studying the synthesis of new antibiotics to control the problem of resistance to infectious pathogens and at the same time provide safe and environmentally friendly solutions to their harmful effects **(Hoda *et al.*, 2018),** Antimicrobial Peptides (AMPs) were identified in prokaryotic cells in 1939 **(Marina *et al.*, 2018)**. Explained that peptides are originally small protein molecules produced by living organisms, which play an important role in the living same environment **(Vivian *et al.*, 2021)**, By attacking microorganisms present in their environment **(Jialuo *et al.*, 2019)**, they received great attention again as new generation antibiotics (**Abdel *et al.*,2018**; **Shamary *et al.*,2023**). Research and studies in this direction have continued and crystallized in recent years, as a total of 3240 have been documented in the Antimicrobial Peptide Database (APD), which was updated in August 24, 2020, while in 2021 on December 18, the data documentation reached a total of 3283 AMPs, and at the end of December 2022, the AMP database contained 3569 antimicrobial peptides and proteins in January 2023 (2023 https://aps. unmc.edu) Many studies have been conducted on its production from microscopic organisms **(Luong *et al.*, 2020),** including eukaryotic and prokaryotic, they have good potential for applications in various fields of life. In 1956, Purothionin and Phagocytin were considered the first antimicrobial peptides discovered in plants (wheat) and animals (they were isolated from leukocytes in Rabbits, followed by horses and guinea pigs, respectively **(Mahlapuu *et al.*, 2020)**. Microorganisms are among the largest and most diverse sources for the production of antimicrobial peptides, in addition to being the most widespread in nature. The goal of producing AMPs in microorganism's lies in a kind of competitive strategy used with the

microorganisms found in their environment **(Xiaoyun *et al.*, 2024**), in contrast to the rest of the organisms (**Moretta *et al.*, 2020**), which is the goal of The production of peptides is protection against infection **(Magana *et al.*, 2020).** Fungi and yeasts are one of the important organisms in the synthesis of antimicrobial peptides **(Buda *et al.*, 2020).** During the examination of natural products of fermentation by the yeast Papularia sphaerosperma in the 1970s, the peptide Echinocandins were discovered, which is an 2020important class of peptides. Non-ribosomal antifungal lipids **(Ana *et al.*, 2021).** Antimicrobial peptides (AMPs) are a class of small peptides that widely exist in nature and they are an important part of the innate immune system of different organisms **(Rodrigues *et al.*, 2022).** The AMPs produced by Bacillus are of great importance in the pharmaceutical industry, and in the food and agricultural sectors. Bacillus species can produce 154 secondary metabolites with different compositions, and a large percentage of these substances possess a broad spectrum of antimicrobial activity **(Serwecińska, 2020)**. Studies show that at least 4-5% of the genetic material of the bacterial genome is dedicated to the production of antimicrobial compounds (AMCs), Members of the *Bacillus subtilis* family produce a variety of antibacterial and antifungal antibiotics **(Fa'iza *et al.*,2011; Adil & Elham,2021)**. Some of them are of ribosomal origin, such as subtilin, subtilosin A, Sublancin, and of non-ribosomal origin, such as Bacilysin, Chlorotetain, Mycobacillin**, (Zhao *et al.*,2021)** Rhizocticins, Bacillaene, Difficidin, Lipopeptides of the Surfaceactin family, Iturin, Fengycin. Surfactin is the first peptide indicated in research, produced by *Bacillus subtilis*, with antimicrobial and anticancer **(Luo *et al.*, 2022)** activities. Surfactin is an amphipathic cyclic lipopeptide produced by many types of bacilli and was identified for the first time. Once in 1968, from a culture of the *B. subtilis*, a long chain of (12-16) carbon atoms is formed to form the ring structure. The heptapeptide structure is formed ((L-Glu-L-Leu-D-Leu-L-Val-L-Asp). Surfactin consists of two D-amino acids (Leu, Leu) and five L-amino acids (Val, Asp, Leu, Glu, Leu) **(Al-Badran& Elham, 2019)** indicates Its composition is due to its amphipathic nature, because it has in its structure a lipophilic and a hydrophilic part **(Vivian *et al.*, 2021)**. As the lipophilic group consists of a hydrocarbon chain of a fatty acid or a sterol ring **(Yuchen *et al.*, 2022**), the hydrophilic part contains a carboxyl group**,(Dobler, 2022; Dijksteel *et al.*, 2021)** forming Surfactin production is controlled by several factors, including the producing bacterial strain, cultural conditions, temperature and pH, duration of incubation, and type of carbon and nitrogen sources **(Zhou *et al.*, 2023; Alyaa *et al.*, 2018).** The current study aims to reveal the ability of local bacterial isolates to produce the natural antimicrobial agent Surfactin and to increase their ability to produce it by identifying the best nutritional and environmental conditions affecting production.

MATERIALS AND METHODS

Isolation sources of *Bacillus subtilis* ZE2013

Soil samples were collected from 10 different locations, It was included 21 soil samples in November 2021, the samples were taken at a deepness of 10 cm from the surface of the ground, transferred directly to the laboratory for the isolation.

Culture media

Nutrient Broth (N.B)

The N.B media was prepared as described by the manufacturing company, pH was adjusted to 7, media was autoclaved at 121℃ and 15 pound/inch² for 15 min.

Nutrient agar (NA)

The NA media was prepared as described by the manufacturing company Himedia by dissolving 28 g and it in a liter of distilled water.

Production using Trypticase Soy Broth (TSB)

The TSB media was prepared as described by the manufacturing company, adjusting the pH 7, it was autoclaved at 121℃ and 15 pound/inch² for 15 min, and use production of antimicrobial peptides from bacterial isolates.

Isolation of bacterial from soil sources

The samples were taken at a deepness of 10 cm, the samples were mixed, and all tubes were placed in a water bath at 80°C for 30 min, from which a series of decimal dilutions were made **(Adil& Elham, 2021).** The pour plate method was used in the isolation process by transferring 1mL of prepared dilutions using N.A incubated at 37°C for 48 h, after the end of the incubation period **(Ting *et al*., 2020),** The purification process of bacteria isolated was carried out by sequential transferring in to N.A by streaking method the plates were incubated at 37°C for 48h (**Serwecińska, 2020),** colonies appeared with different sizes and shapes were selected for identification **(Zahraa& Elham, 2018).**

Activated Bacteria

Bacteria have been activated (*E.coli, Staphylococcus aureus, Salmonella typhoi,* and *Bacillus cereus*). Using medium N.B were inoculated with pathogenic bacteria (1ml contains 10^5 cell) CFU/mL, according to McFarland each individually and incubated at a of 37°C for 24 h (**Mahmud& Alkhafaji,2023).**

Anti-microbial peptide production from locally isolated *B.subtilis* ZE2013

The (TSB) was inoculated with 2% *B. subtilis* ZE2013 (1ml contains 10^8 cell), incubated at 35°C for 48 h. After the end of the incubation period,the cultuer was a centrifugation process was performed 10000 xg for 20 min at 4°C in order to obtain a the bacterial filtrate,(**Buda *et al*. 2020; Hiba *et al*.,2020).** The Bacterial filtrate was separated sterilely using millipore filter 0.22 µm and preserved in the refrigerator. added was 45% of the bacterial filtrate to the sterile N.B inoculated with 1 mL (1mL contains 10^5 cell) of pathogenic bacteria individually then incubated at 37°C for 48 hours, in addition to the control treatment to which the jest pathogenic bacteria were added at 1 mL to the nutrient broth without bacteria filtrate, The absorbance was measured at 600 nm; the law was used to measure the percentage of inhibition (**Ghanbari& Ebrahimpour**, **2017**).

% inhibition = $[(A_o - A_1)/A_o] \times 100$

A_o = absorbance of control; A1 = absorbance of the sample

High-Performance Liquid Chromatography

The bacterial filtration produced from *B.subtilis* ZE2013 was lyophilized by CHRIST in Ministry of Industry and Minerals, Ibn Al-Bitar Center, Industrial Research and Development Authority, This analysis was carried out using the Sykam HPLC American, in Ministry of Science and Technology/Department of Environment and Water, Detection the lyophilized, using the mobile phase. Phase Acetonitrile and ammonium acetate (ACN) (10mM) in a ratio of 40:60 (v/v) at a flow rate of 2 mL/min, where a C18-ODS type separation column

with dimensions (4.6cm,25cm) was used to separate the compound. The lyophilized filtrate was injected with 5 microliters at a wavelength of 254 nm and compared with standard surfactin with a purity of 98%, prepared from Solarbio/China, and the concentration of surfactin in the lyophilized filtrate was estimated.

Fourier Transform Infrared Spectroscope

An infrared spectroscopy (FT-IR) examination of the lyophilized filtrate was producte form *B.subtilis* ZE2013 and standard surfactin. The sample was prepared by mixing1mg of standard surfactin powder with potassium bromide (KBr) after grinding them well with an agate mortar to obtain a fine powder. Press it into a tablet. By applying 400 MPa pressure on it by the device, it was transferred to the spectrometer to measure the infrared spectrum in a range of wavenumbers (4000- 400) cm^{-1}, and all the visible bands were fixed with their wavenumbers, and most of the main bands were identified, and the main functional groups of surfactin were noted. Between 400 and 4000 wave lengths **(Huan *et al*., 2020; Janek *et al*., 2021).**

Study of the Impact optimal conditions for the production of surfactin from locally isolated *B. subtilis* ZE2013

The effect of the cultural medium on the production of surfactin

The effect of different types of culture media from (Hi media) company was used to determine the optimal culture medium for the production of surfactin from the local isolate *B. subtilis* ZE2013, including (Tryptone Soyabean Casein Digest Broth, Nutrient Broth, Landy medium, Luria Bertani Broth) individually.

Effect of carbon source

The effect of different carbon sources, on surfactin production by the isolate *B.subtilis*ZE2013, including (Glucose, Sucrose, Galactose, and Fructose), and compared them with Dextrose, which is the original carbon source in the optimal culture medium for the production of surfactin, The tested carbon source was added at a concentration equivalent in its carbon content to that of Dextrose (2.50) g/L, the medium was inoculated after sterilizing it with 12 mL of activated *B.subtilis* ZE2013(1mL contained10^{8} cells/mL),and it incubated at 35ºC for 48 h.

Effect of nitrogen source

The different nitrogen sources were included Organic nitrogen (yeast extract, meat extract), and inorganic nitrogen including (ammonium nitrate, ammonium sulfate), compared them with Tryptone, which is the original nitrogen source in the optimal culture medium for the production of surfactin, The tested nitrogen source was added at a concentration equivalent in its nitrogen content to that of Tryptone (17. 00) g/L, taking into account the optimal conditions obtained from previous experiments.

Effect of temperature

The effect of temperature on the production of surfactin from the locally isolated ***B. subtilis*** ZE2013, the flasks containing the production medium inoculated with the bacteria were

incubated at different temperatures, including (20, 25, 30, 35,and 40) ºC, separately, with the previous optimal conditions remaining constant.

Effect of pH

The effect of temperature on the production of surfactin from the locally isolated *B.subtilis* ZE2013,The optimal production medium was prepared with different pH numbers (5, 6, 7, 8, and 9) using a solution of 1M of hydrochloric acid and 1M of sodium hydroxide to adjust the pH, taking into account the results obtained in previous experiments.

Effect of incubation duration

Different incubation times were tested, including (24, 48, 72, and 96) h for the medium inoculated with *B.subtilis*, to obtain the best fermentation duration for the isolate, taking into account the optimal conditions obtained from previous experiments.

High-Performance Liquid Chromatography

Testing the concentration of the produced surfactin after testing the optimal production conditions by using the same device and conditions used previously.

RESULTS AND **DISCUSSION**

Isolation sources

The process of choosing the most appropriate source, addition to the process of isolation and selection of the microorganism, constitute two major details in the success of carrying out the study correctly, Isolation samples were process at a temperature of 80°C for 30 min in a water bath in order to remove the vegetative cells and maintain the spores and their growth into vegetative cells when appropriate environmental and nutritional environmental conditions were provided.

Anti-microbial peptide production from local isolate *B.subtilis* ZE2013

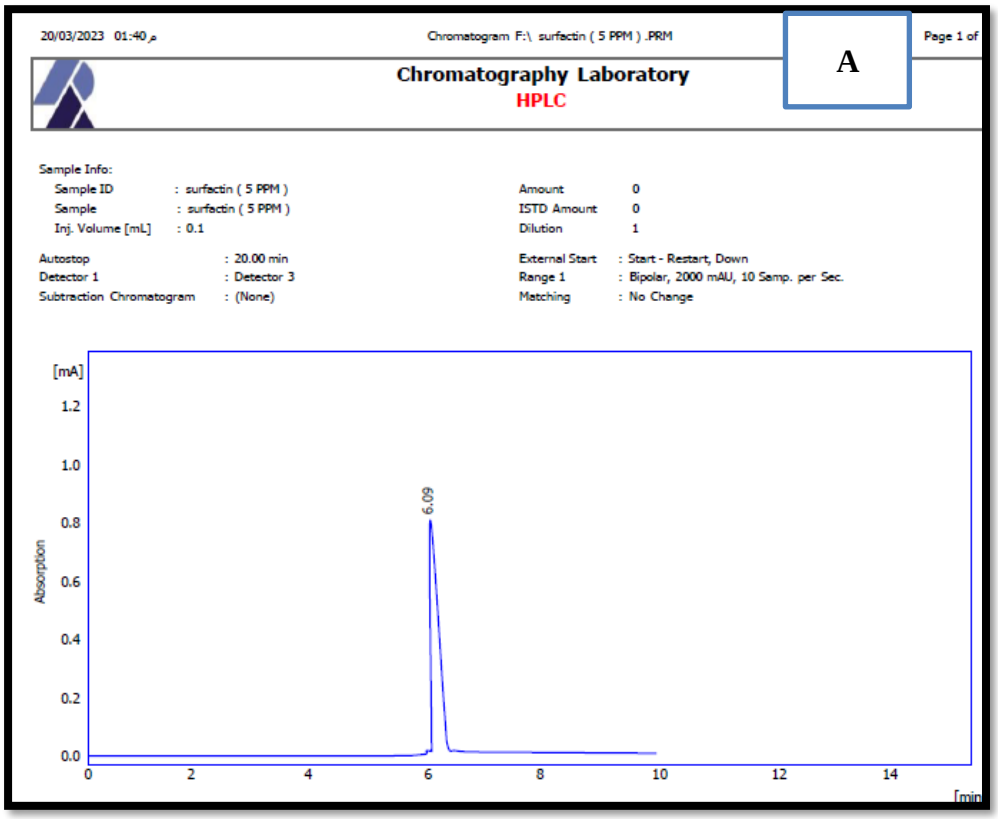
According to the results Percentage inhibition of *B.subtilis* ZE2013 (Table 1), which amounted to 58.6%, 68.8%, 65%, and 57.6% against (*E.coli, S.aureus, Salmonella typhoi* , and *B.cereus*), respectively, where the highest percentage was observed. *S.aureus*, followed by *Sal. typhi*, then *E.coli*, and finally *B.cereus* , surfactin structure consists of a peptide ring that has a horse saddle and a fatty acyl chain on the opposite end. So the fatty acyl chain is able to extend into lipid bilayers and this leads to interaction with biological membranes through interactions with hydrophobic groups **(Sahar *et al.*, 2024)**. It also explains the results maximum percent inhibition against Gram-positive bacteria, this is compatible with has been reported that surfactin exhibits greater antimicrobial activity against Gram-positive bacteria than Gram-negative bacteria **(Marina *et al*., 2018; Ghofran *et al*., 2016).**

Table (1): Inhibition of percentage (%) of *B. subtilis* ZE2013 against pathogenic bacteria

Pathogenic bacteria inhibition %	*Escherichia coli* 58.6%	*Staphylococcus aureus* 68.8%	*Salmonella typhoi* 65%	*Bacillus cereus* 57.6%

Detection of Ant microbial peptide using HPLC technology

The lyophilized filtrate of the *B.subtilis* ZE2013 was analysis using the Sykam HPLC system, the results showed the retention time for the standard surfactin and the lyophilized from the filtrate of the *B.subtilis* ZE2013 (6.09,6.02) min respectively, noted that the retention time for the samples with surfactin is identical to as in (Figure1). The concentration of surfactin (1040) mg/kg in the lyophilized filtrate, and this was consistent with a study conducted by **(Yang *et al.*, 2015).** Using HPLC to diagnose surfactin produced from *B.subtilis* under the same conditions **(Dan *et al.*, 2020)**. This test depends on the flow of the different components of the sample mixture through column C18 at different flow rates due to their different interaction with the adsorbent. Which leads to the separation of components as they flow out of the column, conducted the purification and analysis of lipopeptide homologues from the *B.velezensis* SK isolate using a high-performance liquid chromatography system **(Sagar *et al.*, 2022).**



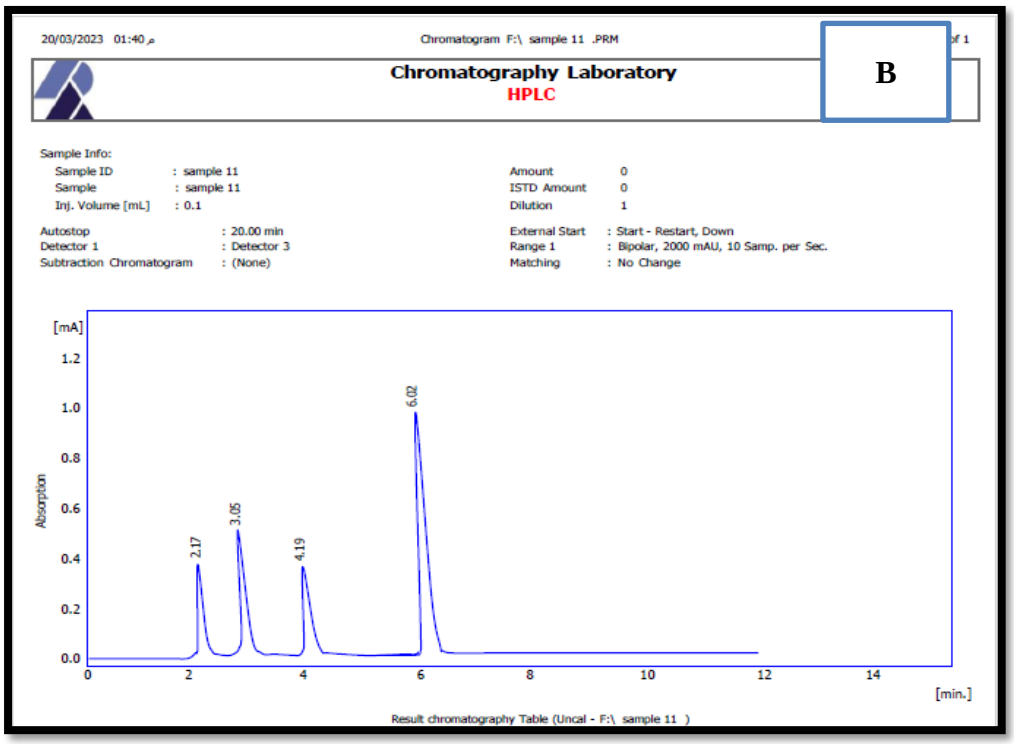


Figure (1):A/ HPLC standard surfactine and B/ (HPLC) of the dried filtrate of *B. subtilis* ZE2013.

Detection of antimicrobial peptide production by Fourier Transform Infrared Spectroscope

The sample was measured within a wavelength range of (400 and 4000) cm^{-1}as the specific FTIR spectrum of surfactin depends primarily on the fine structure of the compound, including the specific amino acid residues and fatty acid chains present, the results of the analysis of the red ray spectrum for each of the standard surfactins and Surfactin product, (A) spectrophotometer diagnosis of the standard surfactin, (B) lyophilized filtrate of *B. subtilis* ZE2013 (Figure 2), and their comparison with the results of the analysis of the functional groups that are supposed to be present in the lyophilized bacterial filtrate of the isolate *B.subtilis* ZE2013, notice that the functional groups present are largely identical to the functional groups found in the standard surfactin. The results showed the presence of deformation of the hydroxyl group (H-O) in the standard surfactin at wave numbers (3304)cm^{-1} and in the lyophilized filtrate of isolate *B.subtilis* ZE2013 at wave numbers (3224) cm^{-1}, also notice the presence of bond expansion between carbon and nitrogen (C-N) are at wave number 1629 cm^{-1} in the standard surfactin. As for the isolated bacteria filtrate, they appear at (1552) cm^{-1}. This indicates the presence of an amide group in the model, as surfactin contains a cyclic peptide, which means the presence of an amide group. Bands, We also notice that there is an extension of the covalent bond between carbon and hydrogen (C-H) in the standard surfactin at wave numbers 2958 cm^{-1}, while in the lyophilized leachate of isolation at

wave numbers (2989) cm^{-1}, and this indicates the presence of a saturated aliphatic chain, which is usually present. In the series of amino acids, there is also the presence of a sharp and strong peak in the dried filtrate of the isolate at the wavenumber (1629) cm^{-1}, and this is close to the standard surfactin 1651 cm^{-1}, and this indicates the presence of a carbonyl group (C=O), compared to the (C-O) group. notice the expansion of the ester bond, which indicates the attachment of carbon to amino acid residues In the standard surfactin, it is present at wavenumbers 1548 cm^{-1}, but as for the lyophilized filtrate of the isolate, it appears at wave numbers 1552 cm^{-1} a region the fingerprint in the lyophilized filtrate *B.subtilis* ZE2013 is at (466,597, 661, 906, 1043, and 1134), and notice a similarity in the fingerprint area to the standard compound Surfactin, the examination revealed the presence of surfactin in the filtrate of the local isolate *B.subtilis* ZE2013, and this is consistent with **Xiaoyun *et al.*** (**2024**) pointed out in diagnosing *B.subtilis* LSFM-05,also pointed out the use of FTIR technology in detecting the surfactin produced by *Bacillus velezensis* SK(**Sagar *et al.*** ,**2022**).

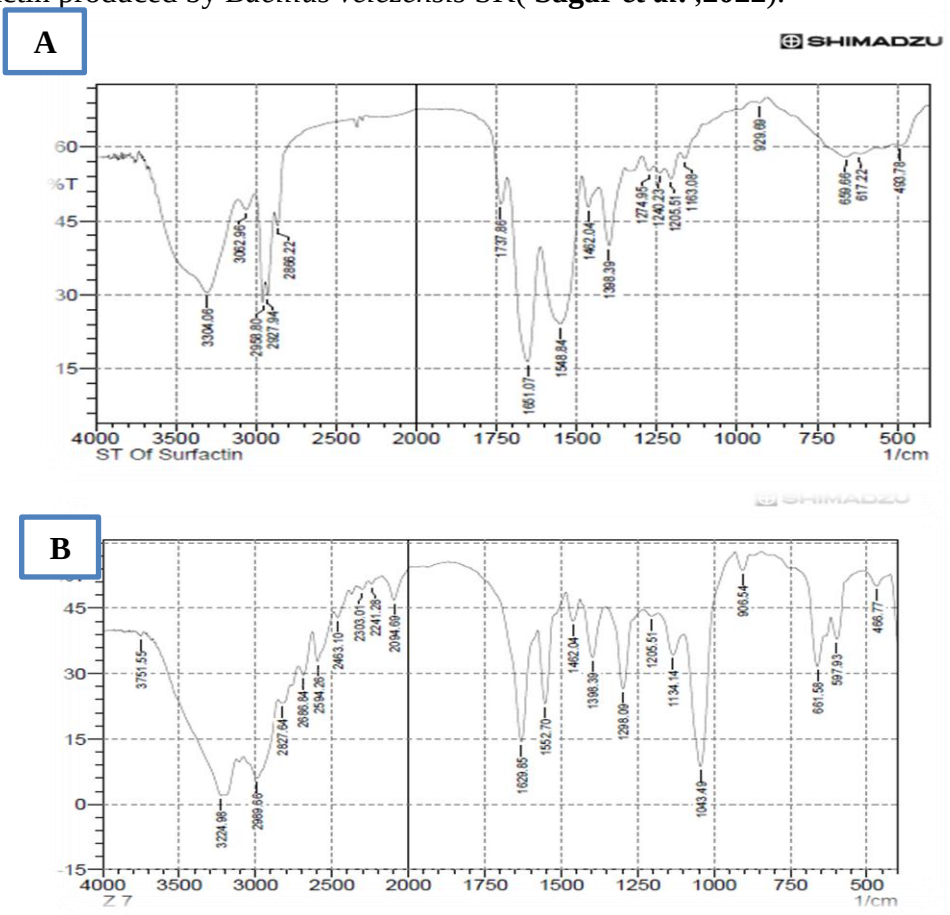


Figure (2): FITR (A) spectrophotometer diagnosis of the standard surfactin and (B) lyophilized filtrate of *B. subtilis* ZE2013.

Study of the optimal conditions for production surfactine by *B. subtilis* ZE2013

This study was conducted for the purpose of determining the optimal conditions for the production surfactin in two replicates and using the percentage of inhibition as a measure of the amount surfactin produced by the local isolate *B. subtilis* ZE2013. The effect culture medium on the production of surfactin, The effect of different types of culture media (ready-made) was used to determine the optimal culture medium for the production of surfactin from the local isolate *B. subtilis* ZE2013, which included (Tryptone Soyabean Casein Digest Broth, Nutrient Broth, Landy medium, and Luria Bertani Broth) individually, as the percentage inhibition of *S.arues* reached 70%, 59%, 60%, and 66%, respectively, than *E.coli* 59%, 54%, 55%, and 56% respectively, *Sal.typhi* 65%, 57%, 60%,and 54% , respectively and *B.cerus* 57%,50%, 51%,and 50% respectively (Table2), the results indicate that all media encouraged production of surfactin, with the observation that (TSB) medium, which represents the medium adopted for production since a beginning of the study, outperformed the rest of the media, and by estimating the percentage (**Meena *et al*.,2020;Mahlapuu *et al*.,2020).**

Table (2): The effect different media on the production of surfactin from *B.subtilis* ZE2013.

Culture media	**Percentage inhibition(%) of filtrate *B.subtilis* ZE2013 against of pathogenic bacterial**			
	Escherichia coli	*Staphylococcus aureus*	*Salmonella typhoi*	*Bacillus cereus*
Tryptone soyabean Broth	59%	70%	65%	57%
Nutrient Broth	44%	59%	57%	50%
Landy medium	55%	60%	60%	51%
Luria Bertani Broth	56%	66%	54%	50%

Effect of carbon source

The effect of various carbon sources, including, Sucrose, Galactose and Fructose replacement of Dextrose (glucose), which is the original carbon source in the optimal culture media TSB for the production of surfactin, at the same concentration reported in the media, which is (2.50 mg/L). The results show that the original carbon source is the best (Table3). In the production of surfactin by estimating the percentage inhibition, its reached using glucose, Sucrose, Galactose, and Fructose (69, 65, 52, and42) % against *S.arues*; (62, 55, 48, and 40) % against *Sal.typhi*; (58, 50, 45, and 37)% against *B.cereus* and (51, 48, 42, and 31)% *E.coli* (Table 3), the production of similar levels of surfactin from *B.subtilis* BS5 used glucose, sucrose, maltose, D-sorbitol, and malt extract as a source of carbon, while a clear decrease in the production of secretin was observed when lactose and galactose were used as a carbon source(**Zhou *et al*., 2023; AL-Shamary *et al*., 2023).**

Table (3): The effect of carbon source on the production surfactin by *B.subtilis* ZE2013

carbon source	Percentage inhibition(%) of filtrate *B.subtilis* ZE2013 against of pathogenic bacterial			
	Escherichia coli	*Staphylococcus aureus*	*Salmonella typhoi*	*Bacillus cereus*
Glucose	51%	69%	62%	58%
Sucrose	48%	65%	55%	50%
Galactose	42%	52%	48%	45%
Fructose	31%	42%	40%	37%

Effect of Nitrogen source

The source of nitrogen is one of the basic sources for the growth of living cells, as it is involved in the construction of organic compounds **(Moretta *et al*.,2021)**, especially amino acids that represent the basic units of proteins, as well as nuclear acids **(Hoda, 2018; Paulo *et al*., 2018)**. In this study test Effect of Nitrogen source (organic and inorganic), which included Yeast extract, Meat extract meat, ammonium nitrate, and ammonium sulphate, in addition to using the nitrogen source tryptophan and soybeans for the optimal medium for production. The results showed, the nitrogen source in the optimal medium, its preservation of approximately the same percentages of inhibition, amounting to (59.5, 69.5, 65, and 58) % against (*E.coli ,S.aureus, Sal.typhi,* and *B.cereus*) while it decreased to (40, 50, 48, and 44) %; (50, 64, 62, and 48.3) %; (46, 62, 58, and 55) % and (36, 45, 41, and 48)%, when using Yeast extract, Meat extract, ammonium nitrate, and ammonium sulfate, respectively (Table 4). Mentioned that the source of nitrogen is important for growth and production of surfactin of *B. subtilis*, and a medium poor in a nitrogen source leads to low cell growth and little production of surfactin **(Neela *et al*., 2022)**. In a study in which two strains, DM-04 and *B.subtilis* DM-03, were used, they were grown on a medium poor in nitrogen and produced secretin, but a clear decrease in its properties was observed (**Katiany *et al*., 2023**).

Table (4): Effect of nitrogen source on surfactin production by *B.subtilis* ZE2013.

Nitrogen source	Percentage inhibition (%) of filtrate *B.subtilis* ZE2013 against of pathogenic bacterial			
	Escherichia coli	*Staphylococcus aureus*	*Salmonella typhoi*	*Bacillus cereus*
Yeast extract	40%	50%	48%	44%
Meat extract	50%	64%	62%	48.3%
Ammonium nitrate	46%	62%	58%	55%
Ammoniumsulphate	36%	45%	41%	48%
Tryptophan	59.5%	69.5%	65%	58%

Effect of temperature

Temperature plays a major role in the growth of microscopic organisms and in the production of secondary metabolic materials in general **(Koim *et al.*, 2021).** From reviewing the literature, there is no consensus on what is the optimal temperature that will lead to the best production of surfactin (**Thérien**, **2019; Neeloffer, 2020**). Shows (Table 5) the results the effect of temperature on the production of surfactin from the most efficient local isolate *B. subtilis* ZE2013, as it is noted that the temperature 35°C gave percentage of inhibition (60.5, 70, 65.5, and 58.5)% against (*E.coli ,S.aureus, Sal.typhi, and B.cereus*) respectively, while the percentage of inhibition at two 30 and 40 ºC (58, 67, 63, and 55)% ; (53, 62, 57, and 50) % respectively, with the observation of decrease in the inhibitory activity of surfactin produced at temperatures of 20 and 25°C, some studies indicated that 37.4 ºC increased the production of surfactin and it is necessary to emphasize that the ideal conditions of temperature and pH for production of surfactin can vary according to other cultivation conditions and also according to the strain used **(Hofer,2019;Dobler *et al.*,2022**) Reported a study conducted on *B.subtilis* and *B.mojavensis*, The optimum temperature for surfactin production at higher temperatures was 30°C **(Meena *et al.* ,2020 ;Magana *et al.*,2020**).

Table (5): Effect temperature on the surfactin produced from *B.subtilis* ZE2013.

Temperature^{o}C	**percentage of inhibition (%) of filtrate *B.subtilis* ZE2013 against of pathogenic bacterial**			
	Escherichia coli	*Staphylococcus aureus*	*Salmonella typhoi*	*Bacillus cereus*
20	23%	30%	26%	20%
25	35%	44%	41%	31%
30	58%	67%	63%	55%
35	60.5%	70%	65.5%	58.8%
40	53%	62%	57%	50%

Effect of pH

Studied the effect of optimal pH for producing surfactin. The values included (5, 6, 7, 8, and 9), according to the results obtained from (Table 6), the highest percentage of inhibition against the bacteria *E.coli*, *S.aureus, Sal.typhi*, and *B.cereus* under study was obtained, For the surfactin produced at pH 6 and 7, with pH exceeding 7 which represents the pH of the optimal production medium, the percentage of inhibition reached (56, 68.5, 63, and 55.5)% ; (59.5, 69.7, 65, and 57.5)%respectively, changes pH of the culture medium greatly affects the metabolism of the microscopic organism (including the transport of compounds across the cell membrane), and effects on the ability organism to produce surfactin (**Katiany *et al.* ,2023**). The optimum pH for surfactin production its (7) for the growth of *B.subtilis* isolates ATCC 21332, while reported in study pH is between (6.5-7) **(Koim *et al.*, 2021)**. Found that it is optimal for producing surfactin from *B. subtilis*, when growing *B.subtilis* BS5 bacteria, it occurs at pH numbers ranging from (6 - 9.0) **(Hamdia *et al.*,2022)**

Table (6): Effect of pH on the inhibition (%) of surfactin produced by *B.subtilis* ZE2013.

pH	percentage of inhibition (%) of filtrate *B.subtilis* ZE2013 against of pathogenic bacterial			
	Escherichia coli 32.2%	*Staphylococcus aureus*	*Salmonella typhoi*	*Bacillus cereus*
5	56%	42.3%	38.5%	25%
6	59.5%	68.5%	63%	55.5%
7	40.5%	69.7%	65%	57.5%
8	28.2%	55.2%	49.2%	35.2%
9		39.2%	32.5%	20%

Effect of incubation period

A study of an effect different incubation durations included (96, 72, 48, and 24) h for the production medium inoculated with *B. subtilis* ZE2013, The results of (Table 7) showed the highest percentage of inhibition was obtained after 48 hours of incubation reached (60.1, 70, 65.2 , and 55)% while the incubation period of(24,72) hours percentage of production reached (50, 53, 40, and 40)% ; (59, 66.3, 64, and 52)% respectively, While the incubation period of 96 hours reached (9, 11.2, 10, and 6)% against (*E.coli ,S.aureus, Sal.typhi, and B.cereus*) respectively, according of previous studies explained process synthesis of surfactin ,it was a parallel with the formation of biomass as observed a continuous increase in surfactin, Maximum surfactin production during stationary phase **(Fadia *et al*.,2023;Yulu *et al*.,2023)** and then was a decrease amount of surfactin due to decreased metabolism of *B. subtilis* cells (**Hiba *et al*.,2020**; **Majeed *et al*., 2020; Gabrielle *et al*.,2021)**.

Table (7): Effect of incubation period on the inhibition (%) surfactin produced by *B.subtilis* ZE2013.

incubation period	percentage of inhibition (%) of filtrate *B.subtilis* ZE2013			
	Escherichia coli	*Staphylococcus aureus*	*Salmonella typhoi*	*Bacillus cereus*
24	50	53	40	40
48	60.1	70	65.2	55
72	59	66.3	64	52
96	9	11.2	10	6

Estimating the concentration of surfactin produced from the local isolate *B.subtilis* ZE2013 under optimal conditions using HPLC

The concentration surfactin produced under the optimal conditions was estimated, and it was shown that the retention time of the surfactin produced and lyophilized under the optimal conditions was identical with the standard surfactin, at a concentration of 1588 mg/kg (Figure 3).

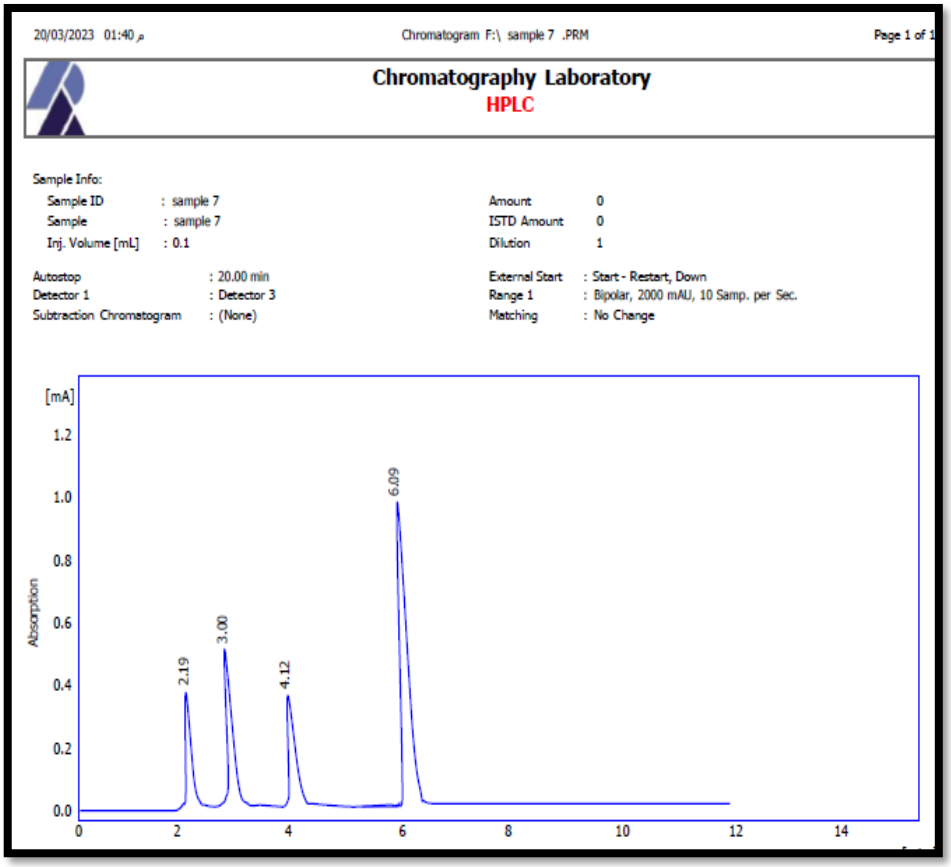


Figure (3): Estimating the concentration of surfactin produced from the local isolate B.subtilis ZE2013 under optimal conditions using HPLC technology.

CONCLUSION

This study found that, the acquired results indicate presence of surfactin in the bacterial filtrate from the local isolate *B.subtili*ZE2013, as indicated, the best results to biosynthesis surfactin by growth *B.subtilis*ZE2013 on the medium production (TSB), by measuring the percentage of inhibition against pathogenic microorganisms and measuring the concentration before and after applying optimal production conditions.

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