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Antibacterial Activities of Soil Bacteria Isolated from Hashemite University Area in Jordan

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Abstract

Soil is an important source of antibiotics-producing microorganisms. This study aims at investigating the isolation of antibiotics-producing bateria from soil samples collected from the Hashemite University area in Jordan. A large number of bacteria was isolated from soil samples collected from the Hashemite University Campus and assessed for their antimicrobial activity. Five of these isolates showed an inhibition activity against six pathogenic bacteria. The isolates were identified as *Bacillus firmus*, *B. circulans* and two *B. stearothermophilus*, while the last isolate was unidentified. To achieve the maximum antimicrobial activity of each isolate, growth optimization was investigated. The isolates showed high efficiency in inhibiting the growth of strong gram-positive and gram-negative pathogenic bacteria using the agar-well diffusion method and liquid cultures of the pathogens. The results revealed that the most suitable nitrogen source, pH value, and temperature needed for maximum antibiotic production were different among the bacterial isolates. Among the different carbon sources studied, glycerol was the most effective in enhancing the antimicrobial activity of *B. firmus* and *B. circulans* followed by starch, sucrose and glucose, whilst, starch was the most effective in increasing the antimicrobial activity of the two strains of *B. stearothermophilus* and the unknown strain. The highest antimicrobial activity was achieved from the ethyl acetate extract of *B. circulans* against *K. oxytoca* with an inhibition zone of 33.67 ± 0.88 mm, while the lowest antimicrobial activity was obtained from the ethyl acetate extract of *B. stearothermophilus* isolate 2 against *Staphylococcus aureus* with an inhibition zone of 17.67 ± 1.45 mm.

Keywords: Antibacterial activity, Soil bacteria, Ethyl acetate extract, Hashemite University, Jordan

1. Introduction

Antibiotics are among the most important commercially exploited secondary metabolites produced by bacteria and employed on a large scale. Most of the antibiotic producers used today were soil microbes. *Bacillus* species is predominant soil bacteria, and their resistant endospore formation and production of vital antibiotics such as bacitracin etc. are always found inhibiting the growth of other organisms (Gupta *et al.*, 2017). The emergence of pathogenic bacteria, which are resistant to multiple antibiotics, represent a growing threat to human health and has given additional impetus to scientists to search for new drugs. In fact, novel approaches for the development of new antibiotics have been pursued, such as the combinatory chemistry tools, but only a few new antibiotics are produced by the pharmaceutical industry nowadays (Coates and Hu, 2007). In this context, unusual sources, such as microorganisms from extreme environments have begun to capture the attention of scientists (Lo Giudice *et al.*, 2007). Polypeptide antibiotics which constitute the *Bacillus* bacteria have been gaining importance as a result of studies. Yilmaz *et al.* (2006) maintain that *B. subtilis*, *B. polymyxa*, *B. brevis*, *B. licheniformis*, *B. Circulans*, and *B. Cereus* are the most widley studied *Bacillus* species for the production of antibiotics. Polypeptide antibiotics produced by *Bacillus* that are used in medical treatments are bacitracin, gramycidin S, polymyxin, and tyrotricidin (Morikawa *et al*., 1992; Perez *et al*., 1992; 1993; Drablos *et al*., 1999).

A great number of microorganisms that inhabit soils are in a constant interaction that partially determines the physical, chemical, and biological properties of this habitat. In the soil, microorganisms are highly important for the biogeochemical cycle since they carry on most of the biological changes in this environment. Thus, reports on antibiotics-producing soil microorganisms were of high interest to enlarge the list of target microorganisms (Thakur *et al*., 2007). In their work of screening 110 actinomycete strains isolated from the soil of Indian protected areas, a high level of active isolates were selected for the isolation of more compounds.

Little attention has been paid for the screening and isolation of new antibiotic producers in Jordan. Saadoun *et al*. (2008) were able to isolate 161 different *Streptomyces* isolates from soil samples representing different habitats of north Jordan. These were then characterized and assessed for their antagonistic activity against four clinical multi-drug resistant *Pseudomonas aeruginosa* test pathogens. El-Banna (2004) reported the isolation of *Corynebacterium xerosis* from Jordanian soils in Jerash and studied its antimicrobial activity against some bacteria and fungi.

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To understand microbial diversity in Jordan especially in the eastern desert where the Hashemite University is located, the surrounding environment should be studied first. In Jordan, the Badia region (Eastern Desert) is divided into two broad areas: (i) Hammad Land, which expands from Naqab to the Jordan-Iraqi borders in the northeast; and (ii) the volcanic area of Hurra Land, which is a part of Hurra of the Syrian Badia, and extends from southwest Syria through northeast Jordan (McEachern, 1991). The Badia encompasses seven million hectares and receives an average annual rainfall of less than 100 mm. The prevailing climate is dry and hot during the summer and very cold during winters, with rain in the form of thundershowers. There are two main types of soil in this area, the first type was formed as a result of the desert climate; the second was formed as a result of a humid climate, but now falls under the effect of the desert climate. Soils in the eastern and central areas are affected by lime rocks, and there are soils rich in gypsum, especially in flat plains. This research has focused on studying the area of the Hashemite University attempting to isolate different antibiotic producers from soil samples and evaluate their efficiency against different human pathogens.

2. Materials and Methods

2.1. Sampling Procedure

Twenty-four soil samples were collected from different regions around Hashemite University Campus using sterile labeled conical tubes. The soil samples were collected from the surface, 5 cm below the surface, and 20 cm below the surface. The soil samples were processed immediately.

2.2. Isolation of Antagonistic Bacteria

According to the method used by Chilcott and Wigley (1993), each 1 g of the soil sample was suspended in 9 mL sterile distilled water and shaken vigorously for two minutes. The liquid was serially diluted in sterile distilled water, and a 0.1 mL sample of the dilutions 10^{-4} to 10^{-7} was added into 20 ml of melted TBA agar. After solidification of the medium, the plates were incubated at 30 ˚C for 24-48 hours. The bacterial colonies which showed antagonism to the adjacent bacterial colonies were picked up and subcultured to make pure cultures as explained by Dubey and Maheshwari (2002).

2.3. Identification of Isolates

The isolates that showed antibacterial activity were identified to the species level by observing their morphology and biochemical reactions according to the methods described by Brawn (2004) and Garrity *et al*. (2001).

2.4. Extract Preparation

Each isolate was cultivated in flasks containing nutrient broth (NB). The flasks were incubated at 30˚C in an incubator shaker (Human Lab, Korea) running at 100 rpm for two days. After incubation, the cultures were centrifuged at 6000 rpm for fifteen minutes, and the supernatant was used as a cell-free extract as reported by Yilmaz *et al*. (2006). An ethyl acetate extract for each isolate was prepared by the addition of ethyl acetate to the cell-free extract in a 1:1 ratio. The organic phase was concentrated by evaporating the ethyl acetate at 45 ˚C. The resulting crude extract was stored at 4 ˚C.

2.5. Antimicrobial Activity

The inhibitory effect of each isolate was tested against different pathogens including *Proteus mirabilis* ATCC 12453, *P. vulgaris* ATCC 33420, *Streptococcus pneumonia* ATCC 6303., *Staphylococcus aureus* ATCC 11632, *Klebsiella oxytoca* ATCC 13883, *K. pneumonia* ATCC 10536, *Escherichia coli* ATCC 10145, *Pseudomonas aeruginosa* ACTT 29737, *Enterobacter sp.* ATCC 13047, and *Salmonella* sp. Group A ATCC 9150. For each pathogen, a suspension of 0.1 mL from a culture (OD_{550} = 0.5) was spread on a plate, and wells of 6 mm in diameter were made in the agar. This was followed by the transfer of 100 µL of the cell-free extract representing each isolate into the wells in the agar plates directly. The inoculated plates were incubated for twenty-four hours at 37˚C, and the diameter of the inhibition zone was measured. Streptomycin, amoxicillin, and bacteriocin were used as controls.

2.6. Growth Optimization of Bacterial Isolates

The isolates were grown in Erlenmeyer flasks containing a nutrient broth and were incubated at 28 ˚C, 31 ˚C, 34 ˚C, 37 ˚C and 40 ˚C in order to study the effect of temperature on growth and antimicrobial activity. After three days of incubation, the ethyl acetate extract was prepared from each flask, and was tested at suitable concentrations for the antimicrobial activity. To study the time profile of growth and antimicrobial activity, each isolate was grown in a nutrient broth at optimum temperature and the samples were withdrawn every twelve hours. To study the effect of medium pH on growth and antimicrobial activity, each isolate was grown in a sterile NB medium of different pH values (6.5-8). Furthermore, the effect of nitrogen sources on growth and antimicrobial activity was studied. The nitrogen sources used for this purpose included yeast extract, peptone, urea and ammonium sulfate $(NH_4)_2SO_4$ at a conc. of 1 % (w/v). Finally, the effect of the carbon source on growth and antimicrobial activity was studied. The experiment was performed as previously mentioned except for the addition of different carbon sources. The C- sources used were glucose, glycerol, starch, and sucrose and were mixed with the medium before sterilization. To analyze the results of the process of optimization, the dissolved protein content was recorded according to Lowry *et al*. (1951) and the CFU was calculated each time a sample was withdrawn. This was followed by estimating the antimicrobial activity of each isolate after a specific treatment as previously mentioned.

2.7. Effect of Extracts Concentration on Pathogens Growth

The effect of ethyl acetate extract concentration prepared from each isolate on the pathogens was studied through two experiments: (1) elevating the concentration of ethyl acetate extract to cause more inhibitory effects. This was achieved by adding different concentrations of the ethyl acetate extract of each isolate (40, 60, 80 and 100 µL) in the wells of agar plates which were pre-streaked with pathogens. (2) Adding different concentrations of the ethyl acetate extract of each isolate (40, 60, 80, 100, 500 and 1000 µL) to the 100 mL nutrient broth inoculated with

0.1 mL (10^7 CFU) of the bacterial broth culture containing a test pathogen, incubated for twenty-four hours at 37 °C. Thereafter, the optical density (OD_{550}) of the culture was measured.

2.8. Statistical Analysis

Statistical analysis of the data was performed using ANOVA (analysis of variance), and Tukey test was applied to test the significance at ($P \leq 0.05$) (Tukey, 1949). The significant differences among the values were expressed as letters. Standard errors among the replicates and duplicates were represented by bar on the figures and ± in the tables.

3. Results

3.1. Isolation and Identification of Antibiotic –producing Bacteria

Under the objective of the isolation of antimicrobial producers, soil samples from the Hashemite University area were collected. A large number of bacteria were isolated and tested for their antimicrobial activity. Five isolates showed antimicrobial activity against different bacterial pathogens. Four of these isolates were identified as *Bacillus* species: *B. firmus*, *B. circulans* and two *B. stearothermophilus* isolates, while the fifth isolate was undetermined due to variations in the results of the biochemical reactions of this isolate (Table 1).

Table 1. Biochemical activities and characteristics of the isolates.

Tests	Bacterial isolates				
	B. stearothermophilus isolate 1	*B. stearothermophilus* isolate 2	*B. firmus* isolate	*B. circulans* isolate	Unknown isolate
Gram´s reaction	+	+	+	+	+
Cell shape	rod	rod	Rod	rod	rod
Motility	+	+	-	+	-
Catalase production	+	+	+	+	-
Glucose fermantation	+	+	-	+	-
Starch hydrolysis	+	+	+	+	-
Endospore	+	+	+	+	-
Oxygen requirements	aerobic	aerobic	Aerobic	aerobic	aerobic
Benzidine reaction	+	+	+	+	-
Citrate Utilization	-	-	-	-	-
Voges-Proskauer Test	-	-	-	-	-
Growth at 50˚ C	+	+	-	+	-
Growth at 60˚ C	+	+	-	-	-

3.2. Antimicrobial Activity Test

The antimicrobial activity of the isolates was studied against different pathogens (Table 2). The isolates showed high to low antimicrobial activity. The results revealed that the ethyl acetate extract of the *B. stearothermophilus* isolate 1 has an inhibitory effect against *P. aeruginosa*, *P. mirabilis*, *S. aureus* and *S. pneumonia*, while the second isolate of *B. stearothermophilus* showed an inhibitory effect against *P. mirabilis*, *E. coli*, *S. aureus*, *S. pneumonia and K. oxytoca*. The third isolate, *B. firmus*, showed only an inhibitory effect against *P. mirabilis, whereas* the fourth isolate, *B. circulans* isolate, has an inhibitory effect *against P. mirabilis*, *E. coli*, *S. aureus*, *S. pneumonia* and *K. oxytoca*. On the other hand, the unknown isolate has an inhibitory effect against the gram-positive bacteria *S. aureus* and *S. pneumonia* only. The highest antimicrobial activity was achieved from the ethyl acetate extract of *B. circulans* isolate against *K. oxytoca* with an inhibition zone of 33.67± 0.88 mm, while the lowest antimicrobial activity was obtianed from the ethyl acetate extract of *B. stearothermophilus* isolate 2 against *S. aureus* with an inhibition zone of 17.67 ± 1.45 mm. *B. stearothermophilus* isolate 2 showed a broad antimicrobial activity against gram-positive and gram-negtive pathogens (*P. mirabilis*, *S. pneumonia* , *S. aureus*, *K. oxytoca*, *E. coli*). However, none of the five isolates showed any antimicrobial activity against *K. pneumonia*, *Enterobacter sp.*, *Salmonella* sp. and *P. vulgaris* or even against different yeasts employed.

Table 2. Antimicrobial activity of the five isolates against the tested pathogens

	Zone of inhibition (mm) *							
Pathogens/ Gram stain	*B. stearothermophilus* isolate 1	*B. stearothermophilus* isolate 2	*B. firmus*	*B. circulans*	Unknown isolate	Cl	C2	C3
S. pneumonia /Gram +ve	22 ± 2.08a	18 ± 1.73a	NI	24.33 ± 0.67a	20.67 ± 2.33a	19 ± 0.48a	20 ± 1.06a	23.77 ± 1.20a
Staph. aureus/Gram +ve	22 ± 2.08a	17.67 ± 1.45a	NI	25.76 ± 2.33a	24 ± 2.58a	18 ± 0.51a	20 ± 1.15a	23.66 ± 1.20a
P. mirabilis/Gram -ve	20.33 ± 1.76a	18.67 ± 1.86a	23.67 ± 0.88a	21.33 ± 0.88a	NI	18 ± 0.58a	20 ± 1.16a	22.76±1.20a
K. oxytoca/Gram -ve	NI	27.33 ± 3.18b	NI	33.67 ± 0.88b	NI	18 ± 0.58a	20 ± 1.11a	22.72 ± 1.20a
E. coli/Gram -ve	NI	32.33 ± 2.19b	NI	NI	NI	19 ± 0.57a	20 ± 1.16a	21.66 ± 1.20a
P. aeruginosa/Gram -ve	21.33 ± 1.03a	NI	NI	NI	NI	20 ± 0.68a	21± 1.13a	22.76±1.20a
K. pneumonia/Gram -ve	NI	NI	NI	NI	NI	19 ± 0.48a	20 ± 1.06a	23.77 ± 1.20a
Ent. aerogenes/Gram -ve	NI	NI	NI	NI	NI	18 ± 0.58a	20 ± 1.11a	22.72 ± 1.20a
S. typhi/Gram -ve	NI	NI	NI	NI	NI	19 ± 0.57a	20 ± 1.16a	21.66 ± 1.20a
P. vulgaris/Gram -ve	NI	NI	NI	NI	NI	20 ± 0.68a	21± 1.13a	22.76±1.20a

* Values represent the means ± standard error of the mean (SE) of triplicate measurements. P value < 0.0001. If the letters are similar (a with a or b with b) between two values this means there is no significant difference, while if the letters are different between two values this means there is significant difference. NI: No inhibition. C1, C2 and C3 are the control antibiotic disks (10 µg/disc; C1: Streptomycin, C2: Amoxicillin and

3.3. Effect of Temperature, pH, Nitrogen Sources and Carbon Sources on Isolates Growth and their Antimicrobial Activity

The effect of temperature on the isolates' growth showed that the isolates were able to grow at temperatures ranging between 28 ˚C and 40 ˚C. The optimum temperature for a higher growth was at 31 ˚C for the *B. circulans* isolate, *B. stearothermophilus* isolate 2 and the unknown isolate, while it was 37 ˚C for the *B. firmus* isolate and 28 ˚C for *B. stearothermophilus* isolate 1 (Table 3). The nitrogen source, pH value and temperature, which were needed for maximal yield of the antimicrobial activity, seem to differ among the bacterial isolates. The optimum temperature, pH, and nitrogen source for the five isolates are summarized in Table 3. After optimization of the isolates' growth, the maximum antibacterial activity was achieved from the cultures of *B. stearothermophilus* isolate 2 during growth at 31 ˚C temperature after seventy-two hours of incubation where the optical density of the culture and the zone of inhibition elevated significantly (Figure 1). Nevertheless, after ninety-six and 120 hours of culturing the isolates, the isolates started to cease as the optical density of the culture decreased noticeably. On the other hand, there was a high degree of variation in the level of antimicrobial activity when different carbon sources were tested in the medium. Glycerol was the most effective in increasing antimicrobial activity of *B. firmus* and *B. circulans* isolates followed by starch, sucrose, and glucose. Starch was the most effective in enhancing the antimicrobial activity of the two isolates of *B. stearothermophilus* and the unknown isolate followed by glycerol, sucrose, and glucose (Table 4).

Table 3. Optimum temperature, pH, and nitrogen sources for the growth of the five isolates.

Isolate	Temperature	Nitrogen source	pH
B. stearothermophilus isolate 1	28 ˚C	Peptone	7
B. stearothermophilus isolate 2	31˚C	Urea	8
B. firmus	37˚C	Peptone	7.5
B. circulans	31˚C	Peptone	8
Unknown isolate	31˚C	Yeast extract	7

Table 4. Effect of carbon sources on the isolate's antimicrobial activity.

* Values represent the means ± standard error of the mean (SE) of duplicate measurements. P value < 0.01. If the letters are similar (a with a or b with b) between two values this means there is no significant difference, while if the letters are different between two values this means there is a significant difference. NI: No inhibition.

	Diameter of inhibition zone (mm)* in pathogen cultures					
Isolate/carbon source	*P. mirabilis*	*S. pneumonia*	*Staph. aureus*	*K. oxytoca*	*E.coli*	*P. aeruginosa*
B. stearothermophilus isolate 1/ starch	23.23 ± 1.56b	23.23 ± 1.56b	23.23 ± 1.56b	23.23 ± 1.56b	23.23 ± 1.56b	23.23 ± 1.56b
B. stearothermophilus isolate 2/ starch	22.67 ± 1.76b	22.67 ± 1.76b	22.67 ± 1.76b	22.67 ± 1.76b	22.67 ± 1.76b	22.67 ± 1.76b
B. firmus/ glycerol	26.77 ± 0.30b	NI	NI	NI	NI	NI
B. circulans/ glycerol	25.33 ± 0.88b	26.67 ± 1.18b	25.76 ± 2.33b	35.67 ± 0.88d	NI	NI
Unknown isolate/ starch	NI	25.13 ± 1.88b	25.16 ± 1.13b	NI	NI	NI

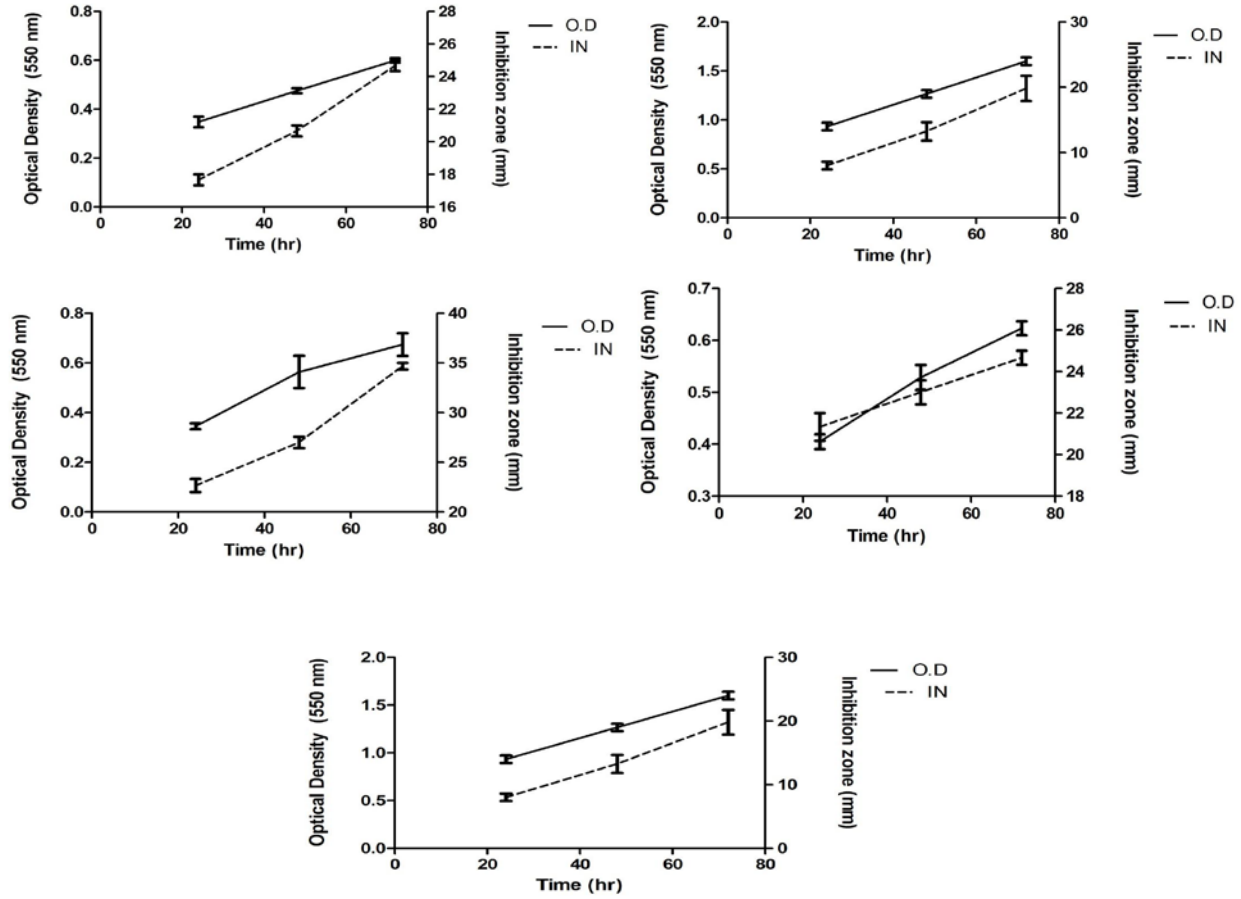

Figure 1. Time profile of the isolates' growth and their effect on the tested pathogens in agar- well diffusion method. A) *B. stearothermophilus* isolate 1 and its effect on *P. aeruginosa* B) Unknown isolate and its effect on *S. pneumonia*. C) *B. stearothermophilus* isolate 2 and its effect on *P. mirabilis*. D) *B. firmus* and its effect on *P. mirabilis*. E) *B. circulans* and its effect on *S. aureus*. Growth is represented by the absorbance value (OD_{550}) and the antimicrobial activity is represented by the zone of inhibition (mm). Values represent the means ± standard error of the mean (SE) of triplicate measurement. *P* value < 0.01.

3.4. Antimicrobial Activity vs. Extracts Concentration

To study the inhibitory effects of the five extracts, different concentrations of each extract were tested against the pathogens' growth. This was achieved after preparing a liquid culture of each pathogen containing different volumes of the ethyl acetate extracts. The antimicrobial activity was determined according to the optical density of the pathogen cultures measured at 550 nm and the inhibition zone (mm) using the agar-well diffusion method. It was noticed that when the concentration of the ethyl acetate extract increases in the pathogen's liquid culture, the optical density at 550 nm of all bacterial cultures decreased to more than 50 % (Figure 2). Noticeably, when a 1000 µl of the ethyl acetate extract was used, the growth of pathogens ceased as the reading of OD_{550} was close to zero. In addition, when the concentration of the ethyl acetate extract increases in the agar wells from 40 µl to 100 µl, the zone of inhibition (mm) increased and doubled against all pathogens throughout the experiment especially in the case of *K. oxytoca* and *S. pneumonia* (Figure 3).

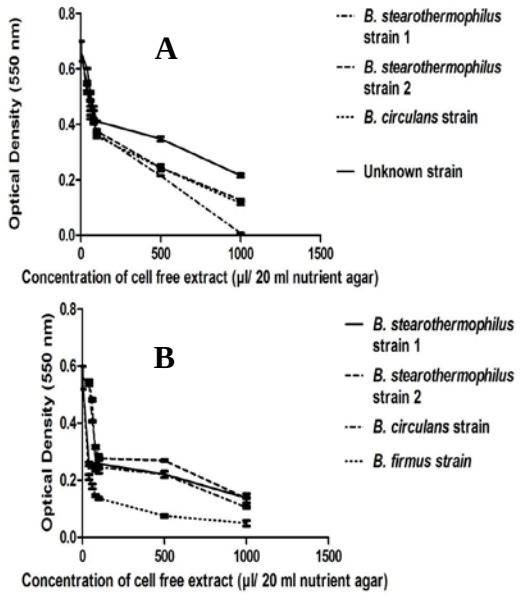

Figure 2. Effect of the ethyl acetate extract concentration of the isolates against tested pathogens A) *S. pneumonia* B) *P. mirabilis* in liquid cultures. The OD_{550} represents the pathogen growth after incubation for 48 hrs. Values represent the means ± standard error of the mean (SE) of duplicate measurement. *P* value < 0.01.

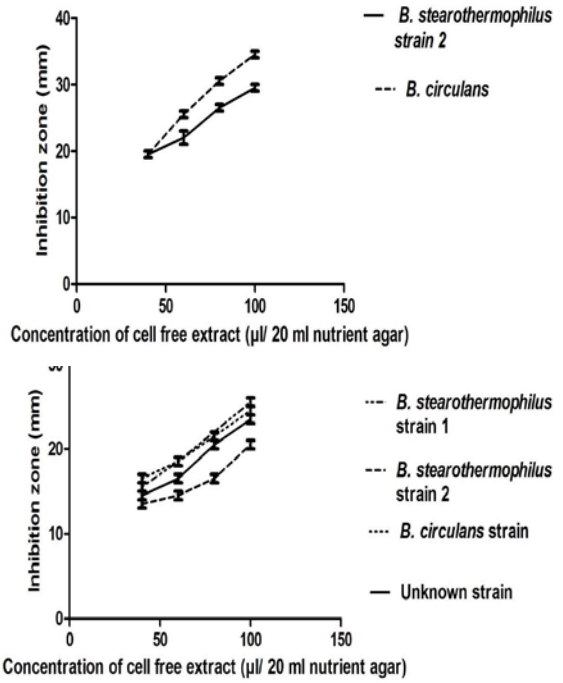

Figure 3. Effect of the ethyl acetate extract concentration of the isolates against tested pathogens A) K. oxytoca B) S. pneumonia using the agar-well diffusion method. Zone of inhibition (mm) represents the antimicrobial activity of the isolate. Values represent the means ± standard error of the mean (SE) of duplicate measurements. *P* value < 0.01.

4. Discussion

Bavishi *et al*. (2017) maintain that in searching for new antibiotics, relatively simple and rapid methods have been developed for screening microorganisms for antibiotic producing abilities. The emergence of antibiotic resistance and the need for better, broad-spectrum antibiotics is always in high demand. In the present study, antibiotic-producing bacteria were isolated from a local soil sample. As a result, there is an urgent need for developing new drugs which are effective against current antibiotic-resistant pathogens. Most antibiotics used today are isolated and extracted from microbial sources. The Hashemite University area was selected for its unique arid Badia climate which contains environmental pressures that might increase the availability of antimicrobial activity of microorganisms. These stress conditions may affect the metabolism of these microorganisms and enhance their antimicrobial activity.

The antimicrobial spectrum of the active substance produced from the five isolates, determined by the agar diffusion method, inhibited the growth of strong gram-positive and gram-negative bacteria. This could be due to the nature of the isolates' growth in their habitats and the stress conditions they live in; where most antibiotic-producing microorganisms are found, life is competitive. Leifert *et al*. (1995) stated that bacteria produce antimicrobial substances that function as self-defense against other organisms or as bio-control activity. As reported by Lancini and Prrenti (1982), the inhabitance must compete for carbon, nitrogen, and phosphate needed for their growth. A successful competition may be insured by the inhibition of the growth of other organisms through the production and secretion of substances interfering with their metabolism (antibiotics). In comparison to the three controls which were used, namely streptomycin, amoxicillin, and bacteriocin, there has been a significant difference in the activity of the ethyl acetate extract of *B. stearothermophilus* isolate 2 against *K. oxytoca* with an inhibition zone of 27.33 ± 3.18 mm and *E. coli* with an inhibition zone of 32.33 ± 2.19 mm. Also, there has been a significant difference in the activity of the ethyl acetate extract of *B. circulans* isolate against *K. oxytoca* with an inhibition zone of 33.67 ± 0.88 mm.

In this study, throughout the screening for antimicrobial-producing microorganisms, five bacterial species were isolated from soil samples collected from the Hashemite University and identified as *B. firmus*, *B. circulans* and two of the *B. stearothermophilus* isolates, while the fifth isolate is still unknown. Soil samples are commonly employed in discovering antibiotic- producing organisms. The production of antibiotic microorganisms from the soil is affected by many factors including nitrogen and carbon sources. It is well known that *Bacillus* sp. in general is able to produce several kinds of antibiotics such as gramicidin, tyrocidine, bacitracin, mycobacillin, surfactin, bacilysin, bacilysocin, and subtilin (Mannanov and Sattarova, 2001).

Under the conditions of the present investigation, the active substances of the five isolates have possibly accumulated late in the growth cycle (stationary phase) in the culture reaching their maximum after seventy-two hours of incubation. The processing time needed for the maximal yield of the antimicrobial-substance production seems to be different among the bacterial isolates, 36-40, 72 and 120 hours which is in agreement with the results of Zheng and Slavik, 1999; Janisiewicz and Roitman, 1988; El-Banna and Winkelmann, 1998. Abdulkadir and Waliyu (2011) isolated *Bacillus lentus*, *Micrococcus roseus*, *Bacillus alvei*, *Enterobacter aerogens*, *and Bacillus pumillus* from soils and the inhibitory activities of the isolated microorganisms were checked against some of the important opportunistic microflora such as the *Staphylococcus aureus and Pseudomonas* species. Demain (1986) points out that in a batch culture, processes leading to the production of antibiotics are sequential; the cultures exhibit a distinct growth phase followed by a production phase, and timing depends on the nutritional environment presented to the culture. In this study, it was necessary to improve the process of antimicrobial production by studying the effect of temperature, pH, nitrogen source and carbon source. It was found that nitrogen and carbon supplementation was required for improving the antimicrobial activity and their sources seemed to differ among the bacterial isolates. Gutiérrez-Rojas *et al*. (2011) reported that the yeast extract was the best nitrogen sources for the *Azotobacter chroococcum* growth in comparison with meat extracts, NH_4Cl and $(NH_4)_2SO_4$. On the other hand, Dikin *et al*. (2007) reported that peptones had the most significant effect on the production of antimicrobial substances from bacteria against *Schizophyllum commune* FR. These findings are in agreement with the results of the current investigation where most of the *Bacillus* isolates preferred to utilize peptone as a nitrogen source (Table 3). Moreover, when the same experiment was repeated to study the effect of

different carbon sources on isolates growth and antimicrobial activity, it was found that glycerol and starch were the most preferred carbon sources to enhance the isolates' growth, and improve the antimicrobial activity. Bhattacharyya *et al*. (1998) maintain that glycerol supported the antibiotic production by *Streptomyces hygroscopicus* D1.5 against the tested pathogens better, while glucose was the most suitable carbon source for the maximum phenazine production by *Pseudomonas fluorescens* 2-97 as reported by Slininger and Shea-wilbur (1995). Moreover, Gutiérrez-Rojas *et al*. (2011) mentioned that sucrose was the best carbon source to support the *Azotobacter chroococcum* growth and metabolism. This all can be explained by the fact that rapidly metabolizable carbon sources are preferred for microbial growths, but may interfere with the biosynthesis of secondary metabolites. Demain (1986) studied the use of different carbon sources and found out that simple sources are depleted simply while complex ones are utilized during the antibiotic synthesis phase.

Furthermore, the effect of the ethyl acetate extract concentrations of the *Bacillus* isolates on the tested pathogens was investigated. It was found that when the concentration of the extract increases in the agar-well diffusion method or in the liquid-culture experiments, the growth of pathogens reduces to more than a half and causes more inhibitory effects. These results are in agreement with Sharma *et al*. (2015) and Cao *et al*. (2009) who studied the production and antimicrobial activity of a biosurfactant produced by *Bacillus* sp. They found that the antimicrobial activity increased with increasing the concentration of biosurfactant using the disk diffusion method. In previous studies, it was reported that the antibiotics produced by *Bacillus* species are more effective against gram-positive bacteria (Morikawa *et al*., 1992; Perez *et al*., 1993; Eltem and Ucar, 1998). However, in this study most of the isolates showed a remarkable antibacterial activity against gram-negative pathogens.

5. Conclusion

Five antibacterial-producing *Bacillus* sp. were isolated from the soil of the Hashemite University area and were measured for their antimicrobial activity against different bacterial pathogens. Four of these isolates were identified as *Bacillus* species and the last one is still unknown. The highest antimicrobial activity was achieved from the ethyl acetate extract of *B. circulans* isolate against *K. oxytoca* with an inhibition zone of 33.67 ± 0.88 mm, while the lowest antimicrobial activity was obtained from the ethyl acetate extraction of *B. stearothermophilus* isolate 2 against *Staphylococcus aureus* with an inhibition zone of 17.67 ± 1.45 mm. The highest antimicrobial activity was recorded from the extract of *B. stearothermophilus* isolate 2 against five bacterial pathogens including gram-positive and gram-negative strains. Further work is required to identify the antimicrobial metabolites of the isolates and access their efficacy after purification compared with currently available antibiotics.

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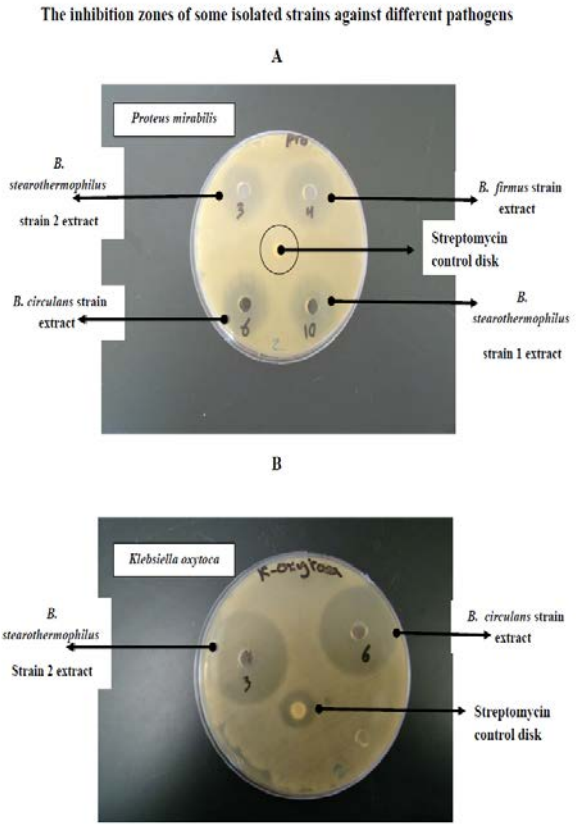
6. Supplementary Data



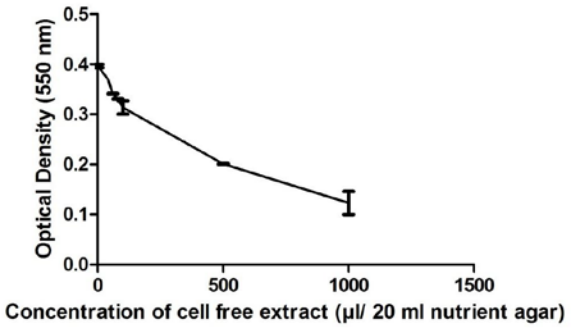


Figure. 1 Effect of ethyl acetate extract concentration on the antimicrobial activity of *B. stearothermophilus* isolate 1 against *Pseudomonas auroginosa* according to the optical density at 550 nm. Values represents the means ± standard error of the mean (SE) of duplicate measurement. *P* value < 0.01.

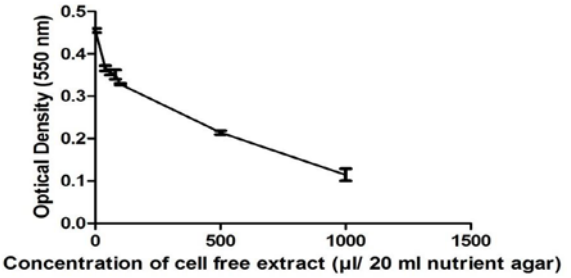


Figure. 2 Effect of ethyl acetate extract concentration on the antimicrobial activity of *B. stearothermophilus* isolate 2 against *E. coli*.

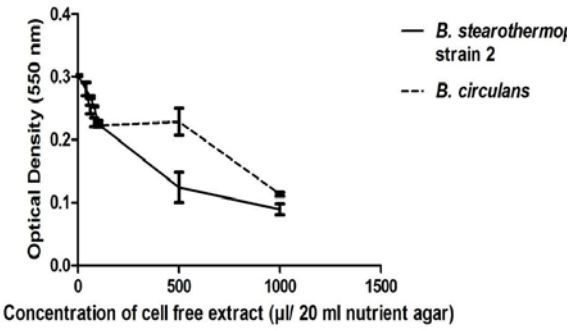


Figure. 3 Effect of ethyl acetate extract concentration on the antimicrobial activity of *B. stearothermophilus* isolate 2 and *B. circulans* against *K. oxytoca*.

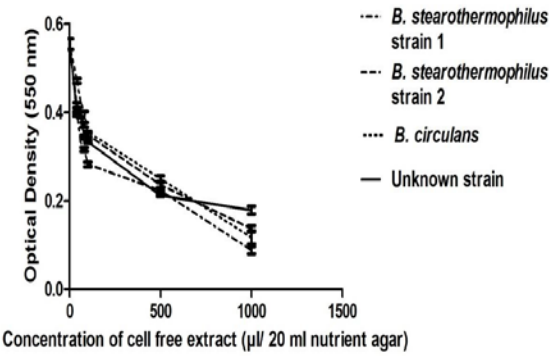


Figure. 4 Effect of ethyl acetate extract concentration on the antimicrobial activity of four isolates against *Staphylococcus aureus.*

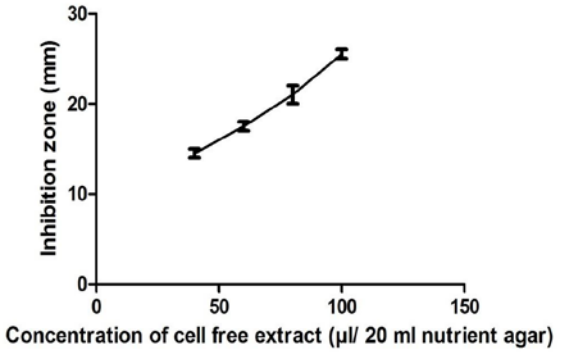


Figure. 5 Effect of ethyl acetate extract concentration on the antimicrobial activity of *B. stearothermophilus* isolate 1 against *Pseudomonas aeruginosa* according to the inhibition zone in mm. Values represents the means ± standard error of the mean (SE) of duplicate measurement. *P* value < 0.01.

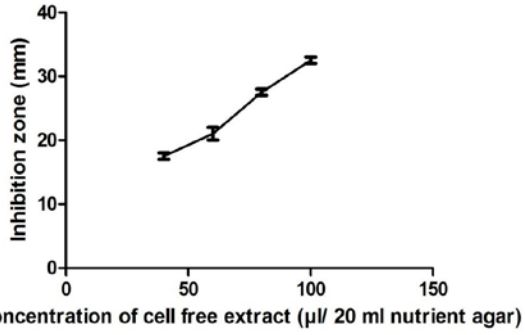


Figure. 6 Effect of ethyl acetate extract concentration on the antimicrobial activity of *B. stearothermophilus* isolate 2 against *E. coli*.

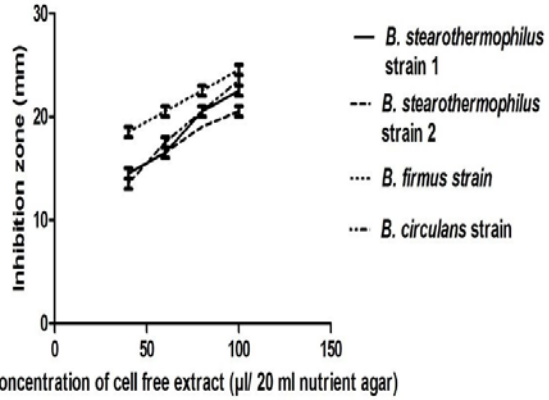


Figure. 7 Effect of ethyl acetate extract concentration on the antimicrobial activity of four isolates strains against *P. mirabilis*.