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Antibacterial Activities (Bacitracin A and Polymyxin B) of Lyophilized Extracts from Indigenous *Bacillus subtilis* Against *Staphylococcus aureus*

Marwan Y. Hussain*, Adnan A. Ali-Nizam and Samir M. Abou-Isba

Department of plant Biology, Faculty of Science, Damascus University, Syria

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Abstract

The antibacterial compounds, which are produced from *Bacillus* species, have a strong inhibition ability, and the composition of these compounds have not been elucidated. The aim of the present study is to explore the antibacterial activity of crude lyophilized extracts, then isolate and characterize these antibacterial. *Bacillus subtilis* strains were isolated from Damascus zone and identified by two methods which are the API CHB 50 and 16S DNA. *Staphylococcus aureus* strains were isolated from clinical samples, and identified by API Staph 20 and coagulase test. The crude antibacterial was extracted by flasks content LB where incubated for 72 h. in shaker incubator at 37℃ at 150 rev/min, then centrifugated 12000 rev/minor 15 min. at 4 ℃ and filtrated the supernatants throw 0.45 µm, next frozen and lyophilized. The crude extracts were run on HPLC system to separate the antibiotics. Some strains of *B. subtilis* gave high activity against strains of *S. aureus* and others were not. Peptides families of antibiotics were separated from lyophilized extracts of *B. subtilis*, and then they were identified as bacitracin A and polymyxin B. This crude extract could function as antibacterial activity against *S. aureus* pathogens.

Keywords: Antibacterial, Staphylococci, Well diffusion, Crude, Lyophilized, Peptide.

1. Introduction

Staphylococcus aureus is responsible for a variety of infections, like carbuncles, impetigo, pneumonia, osteomyelitis, acute endocarditis, conjunctivitis, wounds, and urinary tract infections in man, women, and children (Brooks *et al.*, 2007; Wolff *et al.*, 2013).

S. aureus, especially methicillin resistance *S. aureus* (MRSA), is still one of the five most common causes of nosocomial infections; in addition, the infections are associated with surgical sites, wound infections, catheters and prosthetic implant. The increase of infections by these organisms has been correlated with the wide medical uses of prosthetic and indwelling devices and the growing number of immune compromised patients in hospitals (Cal *et al.*, 2005; Dockrell *et al.*, 2010; Owens and Fowler, 2012). More medical and pharmaceutical applications were benefited from the antibacterial which were produced from *Bacillus subtilis* (Pag and Sahl, 2002; Stein, 2005).

Many studies focused on the ability of Bacillus species to produce antibiotics and antifungals. In a study by Chen *et al.* (2008), the lipopeptide antibiotics were isolated and characterized from *Bacillus subtilis*, and, according to Chen *et al.*, these compounds could play as a biocontrol agent against a large spectrum of pathogens. There are many different support factors, like pH, time incubation, that have an effect on growth microbial cells, and production metabolites, like enzymes and antibiotics (Awais *et al.*, 2010). *Bacillus subtilis* was isolated from the soil, and showed activity against *S. aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* (Sethi *et al.*, 2013). The effects of the endophyte *B. subtilis* were investigated on the growth of cacao seedlings in which the root length and the leaf were increased up to 30% and 14%, respectively while in the stem height was increased up to 7.6% only. Besides, it also showed the strongest inhibition against the fungi *Colletotrichum* sp. and *C. gossypii* (Falcäo *et al.*, 2014). The antiviral activity of subtilosin from *Bacillus amyloliquefaciens* was studied and showed strong inhibition against the extracellular and total virus production of herpes simplex virus type 2 (Quintana *et al.*, 2014). Several antimicrobial substances and antibiotic macrolactin E were produced by *B. amyloliquefaciens* (Yuan *et al.*, 2014). Due to the prevalence of antimicrobial resistance among key microbial pathogens is increasing in worldwide. However, there are no specific reports available on the lyophilized extracts from *Bacillus* spp. against *S. aureus*. Therefore, the present study presents and characterizes some new strains of *B. subtilis* that can

* Corresponding author. e-mail: mrgoldeny@gmail.com.

produce antibiotics like bacitracin with their evaluation the antibacterial activity potential and characterizes the partially purified antibacterial compounds from these new strains.

2. Material and Methods

2.1. Collection the Samples of Soils and Water

Three different soil samples were taken from every site from the 15 -20 cm layer, then mixed to form a composite sample for each site. The samples were collected in the sterile polypropylene bags from cultivated and barren lands in, and around Damascus: Al-Tall, Doma, Khan-Alsheh, Meliha, Adawee, Jedaideh, and Yafour. Three different water samples were taken from three sites from Bab-Sharqi, the wastewater and one canal of Barada river in Damascus, had been collected in the sterile glass bottle. Finally, the samples were prepared for identification.

2.2. Isolation and Identification of B. subtilis

From soil, each 10 g of sample was suspended in 90 mL of sterile distilled water and shaken vigorously for 2 min, while from water; 10 mL of sample was suspended in 90 mL of sterile distilled water and shaken vigorously for seconds. Both of the samples were heated at 80 ℃ for 15 min in a water bath. Then the soil suspensions were diluted in sterile distilled water and 1 mL of dilutions from 10^{-1} to 10^{-6} were plated on Nutrient Agar (NA) (Abtec, England). The plates were incubated at 37 ℃ for 24 h. The identification of bacterial strains was performed both by microbiological, and biochemical methods, according to the Bergey's Manual 2009, by using API 50 CHB (BioMérieux, France) and results of tests were read by API 50 CHB web, in addition, the spore morphology, gram characteristics and motility tests were carried out (Benson, 2001; Logan and De vos, 2009).

2.3. Molecular Identification of B. subtilis

Bacteria was grown in 50 mL Lactose Broth (LB) (Merck, Germany) for 24 h then DNA extraction was done according to (Japoni *et al.*, 2004; Kalia *et al.*, 1999) with simple modifications. All *Bacillus* isolates were identified by 16SrDNA sequence and genomic DNA amplified using Gene Pro thermal cycler (Model TC-E-96G), with forward and reverse primers BacF (5`-GTGCCTAATACATGCAAGTC-3`) and BacR (5`-CTTTACGCCCAATAATTCC-3`) from Alpha DNA Company (Canada), were flanked a highly variable sequence region of 545 bp (Nair *et al.*, 2002). The reaction conditions consisted of one initial denaturation cycle at 94 ℃ for 3 min, 45 cycles of 94 ℃ for 1 min, 55 ℃ for 1 min and 72 ℃ for 2 min and a final extension step at 72 ℃ for 10 min then cold 4 ℃. The amplified products were run on a 1.25% agarose gel and visualized on a U.V. transilluminator. The nucleotide sequence analysis of the 16S rDNA of strains was identified at NCBI server using BLAST-n (www.ncbi.ncm.gov/blast), and the sequences compared. The sequences were submitted to Genbank and accession number obtained.

2.4. Isolation and Identification of S. aureus

Staphylococci strains were taken from patients hospitalized in three hospitals from different departments: Al-Mouwasat, University children hospital, and Dermal Venereal Diseases hospital (ear, eye, urine, cerebrospinal fluid (CSF), bronchial lavages, peritoneal fluid, pleural effusion, abscess, furuncle, bullous, pus, wound) and healthy people (human skin) in Damascus city and plated on NA and blood agar (BA) (Abtec, England) and mannitol salt agar (MSA) (Biolife, Italy). The plates were incubated at 37 ℃ for 24 h then morphology of cultures, Gram stain, production of catalase and coagulase slide test (Sigma, Germany) in addition, the API Staph was carried out for identification (Garcia and Isenberg, 2007; Colaninno, 2009).

2.5. Microbial Strains and Culture Conditions.

All strains were isolated between June 2013 to August 2014, seven strains of *B. subtilis* (six from soil, and one from wastewater), and 52 strains of *S. aureus* from different sources (patients and healthy skin) were used in the experiments. Bacterial strains were maintained on NA slants at 4℃ for a short time, and glycerin 20% in LB at −30℃ freezer for a long-time.

2.6. Growth and Production of Antimicrobial by Lyophilization

Production of antimicrobial lyophilized crude carried out according to Haddad *et al.* (2009) and Bhatta and Kapadnis (2010) with some modifications. After preparing 1000 mL of LB (Merck, Germany), the media were allocated in separated Erlenmeyer flasks 500 mL, each one containing 100 mL of LB and autoclaved, the flasks were inoculated with a fresh culture of *B. subtilis* by using the sterilized loop and incubated for 72 h at 37 ℃ ± 1 ℃ in shaker incubator (JSR, Korea) at 150 rev/min until cell numbers reach 15 $\times 10^7$ CFU/mL by optical density OD (OD_{600} 0.5 = 5 $\times 10^7$ CFU/mL) (WPA CO8000, Biochrome England). After growth, culture media were centrifuged (Hittch, Germany) at 12000 rev/min for 15 min at 4 ℃ and the supernatants were filtered through 0.45 µm membranes (Sartorius, Germany). The resulting filtrates (pH 7.0–8.0) were collected in special vials and put in freezer −30 ℃ between 6 - 8 h then put in lyophilizer (Lyotrap, England) at −40 ℃ and less than 1 millibar for 48-72 h until complete lyophilization, finally the crude lyophilized extract was weighed and stored at 2 - 8 ℃ and these crudes were used to evaluate antimicrobial activity.

2.7. Antimicrobial Activity

The antimicrobial activity of the crude lyophilized extract of *B. subtilis* was checked by agar well diffusion method. Mueller-Hinton Agar MHA surface (Merck, Germany) plates were swab-inoculated with *S. aureus* were grown in Nutrient Broth (NB) (SRL, India) and the turbidity of the cultures of *S. aureus* were adjusted by OD (OD_{600} 0.5 = 1 $\times 10^8$ CFU/mL). Wells were made in the inoculated plates using sterile stainless still borer (diameter 6 mm), then 20 µL of the crude lyophilized extract was added into wells (0.5 mg/µL) and after the plates were incubated at 37 ℃ for 24 h, the diameter of the inhibition zone was measured in mm with well size of 4 mm, in addition, the standard antibiotic disc was used as positive control (Kumar *et al.*, 2009; Bhatta and Kapadnis, 2010).

2.8. Determination the Minimum Inhibition Concentration (MIC) of Lyophilized Extracts

MIC of the crude lyophilized extract against *S. aureus* were determined by broth dilution method, according to the Clinical and Laboratory Standards Institute (CLSI, 2007). The crude was dissolved in distilled water (0.5 mg/µL) and double dilution of the compound was made in NB. Then 1 mL inoculum (10^8 CFU/mL) of *S. aureus* was added separately to an equal volume of two-fold dilutions of respective antimicrobial solution. For positive control, 1 mL inoculum (10^8 CFU/mL) of target organisms was added to 1 mL growth medium without antimicrobial. All the tubes were incubated for 24 h at 37 °C.MICs were determined as the lowest concentration of a compound that showed no visible growth of the organism (Bhatta and Kapadnis, 2010).

2.9. Statistical Analysis

Statistical analysis (One Way ANOVA) was performed using SPSS program software, version 17 to validate the signification of the results. The data are presented as means (± SD) of three replicates.

2.10. Partial Purification of Extracted Antimicrobial Substance by HPLC

Antibiotics in the crude lyophilized extract (water solution, 0.5 mg/µL), were detected and quantified by reversed-phase HPLC as follows: A 50µL sample was injected into the HPLC column [PREP-ODS C18, 250×4.6 mm]. The temperature of the column was maintained at 25°C throughout the experiment. The mobile phase components were solution (A) 0.1% trifluoroacetic acid (TFA) in water and solution (B) 0.1% TFA in acetonitrile. The compounds were eluted at a flow rate of 1 mL/min with a linear gradient of solvent B increasing from 0 to 50%. The elution pattern was monitored at 268 nm, pooled fractions were collected and concentrated (Chen *et al.*, 2008; Snyder *et al.*, 2010; Yuan *et al.*, 2014).

2.11. Determination of Functional Groups of Crude Antibacterial Extracted by FTIR

The FTIR (Fourier transform infrared) spectrum was recorded using FTIR 4200 (Jascoo, Japan). The spectra were scanned in the range of 400 – 4000 cm^{-1}. The spectra were obtained using potassium bromide pellet technique. Potassium bromide (AR grade) was dried under vacuum at 100°C for 48 h. Thus, 100 mg KBr was mixed with 5 mg of sample. The spectra of intensity versus wave number were plotted.

3. Results

3.1. Isolation and Identification of B. subtilis

Table 1 shows the number of strains of *B. subtilis* and characteristics of soil: color, humidity, and sources, *B. subtilis* was able to isolated from the soil of Al-Tall, Doma, Khan-Alsheh, Meliha, Adawee, Jedaideh except from Yafour and be isolated from wastewater of Bab-Sharqi. The strains of *S. aureus*, which were isolated from pathogen samples and healthy human skin were showed in Table 2 and Table 3.

3.2. Identification of B. subtilis by API 50 CHB and Molecular Method

The microscopic study of bacterial cultures showed these strains to be Gram-positive rods, less than 1 µm in diameter. All strains sporulated aerobically without swelling of the cell and produced catalase. These data indicated that tested strains belong to *Bacillus* genus. Additional testing with API 50CHB kit identification resulted that all strains were *B. subtilis*. Partial sequence of 16S rDNA of *B. subtilis* (*BS1A, BS2D, BS3K, BS4M, BS5W, BS6J, BS7SH*) were submitted to the Genbank database under accession numbers KM052377, KM192148, KF792061, KM251459, KF792060, KM189125 and KF792061, respectively. In addition, the results of 16S rDNA gene sequence confirmed with the obtained results of biochemical tests.

Table 1. Characteristics of soils and the strains of *B. subtilis* from soil and water.

Sample	Source's sample	Color	Humidity	*B. subtilis*
Al-Tall	soil	black	little wet	*BS1A*
Doma	soil	black	wet	*BS2D*
Khan-Alsheh	soil	white	dry	*BS3K*
Meliha	soil	black	wet	*BS4M*
Adawee	soil	black	wet	*BS5W*
Jedaideh	soil	black	dry	*BS6J*
Yafour	soil	red	little wet	0
Bab-Sharqi (canal of Barada)	waste water	-	-	*BS7SH*

3.3. Antimicrobial activity

Table 2 shows that two crude extracts (*BS1A*, *BS2D*) from strains of *B. subtilis*, which had been isolated from the soil and *BS7SH*, which had been isolated from wastewater, have an intermediate to a high antibacterial activity in which they gave a bigger zone against most of *S. aureus* strains (8-40 mm), whereas another crude extracts from strains of *B. subtilis*, which were isolated from the soil (*BS3K*, *BS4M*, *BS5W*, *BS6J*), did not give antibacterial activity against most of these strains of *S. aureus*.

As shown in Table 3, most of *S. aureus* strains have between intermediate to good sensitivity with diameter inhibition zone (8-25 mm) facing the crude extracts from strains of *B. subtilis* (*BS1A*, *BS2D*) and (*BS7SH*), while the crude extracts from strains of *B. subtilis* (*BS3K*, *BS4M*, *BS5W*, *BS6J*) did not have antibacterial activity against *S. aureus* strains except *S31*, *S36*, *S43*, *S47*.

3.4. MIC of crude lyophilized extract

The minimum inhibitory concentration of the antimicrobial compound in the crude lyophilized extract was found to be 5-7.5 mg/mL for *S. aureus*.

3.5. Statistical Results

According to the statistical results, the differences between the antibacterial activity against *S. aureus* were subjected to One Way ANOVA statistical test and it

showed that there is a significant difference between some strains of *B. subtilis* towards *S. aureus* (*P<0.05*) and there is no significant difference between others (*P>0.05*) (Table 2 and Table 3).

Table 2. The mean, ±SD, and results of Anova$^{(a,b,c..)}$ of the diameters of inhibition zone (mm) of crude lyophilized extract against *S. aureus* strains which isolated from patients as test organisms.

S. aureus No.	Source's sample	BS1A	BS2D	BS3K	BS4M	BS5W	BS6J	BS7SH
S1	CSF	40.66±1.15^{a}*	23.33±1.15^{b}	8±0^{c}	10±0^{c}	8±0^{c}	12±2.0^{c}	12±0^{c}
S2	Ear	29.33±1.15^{a}	12.66±1.15^{b}	-	-	-	-	8.66±1.15^{b}
S3	pus	30.33±0.57^{a}	15.33±1.15^{b}	-	-	-	-	8.66±1.15^{b}
S4	Bronchial lavages	-	-	-	-	-	-	8±0
S5	Pus	28.66±1.15^{a}	14.33±0.57^{b}	-	-	-	-	8.66±1.15^{c}
S6	Ear	13.33±2.88^{a}	14±0^{a}	-	-	-	-	9.33±1.15^{a}
S7	bronchial lavages	-	16.66±1.15^{a}	-	-	-	-	10±0^{b}
S8	Abscess	-	-	-	-	-	-	-
S9	Ear	-	18.66±1.15^{a}	-	-	-	-	10.66±1.15^{b}
S10	Pus	-	18.66±1.15^{a}	-	-	-	-	12.66±1.15a
S11	Pus	14±0^{a}	10.66±1.15^{a}	-	-	-	-	10.66±1.15^{a}
S12	Pus	-	15.33±1.15^{a}	-	-	-	-	11.33±1.15^{a}
S13	Furuncle	11.33±1.15^{a}	12±2.0^{a}	-	-	-	-	8.66±1.15^{a}
S14	Pus	-	-	-	-	-	-	10±0
S15	Abscess	21.33±1.15^{a}	10.66±1.15^{b}	-	-	-	-	10.66±1.15^{b}
S16	Abscess	13.33±1.15^{a}	14.66±0.57^{a}	-	-	-	-	11.33±1.15^{a}
S17	Urine	15.33±1.15^{a}	`16±5.29^{a}	9.33±1.15^{b}	10.66±1.15^{b}	10.66±1.15^{b}	9.33±1.15	13.33±1.15^{a}
S18	Urine	11.66±2.88^{a}	12.66±1.15^{a}	8.66±1.15^{a}	8±0^{a}	-	-	8.66±1.15^{a}
S19	Abscess	11.33±1.15^{a}	14±0^{a}	-	-	-	-	8.66±1.15^{a}
S20	CSF	17±2.64^{a}	10±0^{b}	-	-	-	-	10.66±1.15^{b}
S21	Ear	-	10±0^{a}	-	-	-	-	10±0^{a}
S22	Eye	-	10±0^{a}	-	-	-	-	10.66±1.15^{a}
S23	pleural effusion	-	-	-	-	-	-	10±0
S24	Urine	-	15.33±1.15^{a}	-	-	-	-	10.66±1.15^{b}
S25	Urine	28±2^{a}	15.33±1.15^{b}	-	-	8±0^{c}	-	10±0^{d}
S26	Peritoneal fluid	12.66±1.15^{a}	-	-	-	-	-	8.66±1.15^{a}
S27	Pus	-	11.33±1.15^{a}	6±0^{b}	-	-	-	10±0^{a}
S28	Ear	12.66±1.15^{a}	10.66±1.15^{a}	-	-	-	-	10±0^{a}
S29	Wound	-	11.33±1.15^{a}	-	-	-	-	10±2.0^{a}
S30	CSF	-	14.66±1.15^{a}	-	-	-	-	11.33±1.15^{a}

Sensitive: ≥16 mm, intermediate sensitive: 10 – 15 mm, non-sensitive: 4 - 6 mm, (-) no inhibition zone.

**Different letters in the same line indicate significant differences among the samples*

Table 3. The mean and ±SD and results of Anova[(a,b,c..)] of the diameters of inhibition zone (mm) of crude lyophilized extract against *S. aureus* strains which isolated from (skin's infections, healthy skin) as test organisms.

S. aureus	Source's sample	BS1A	BS2D	BS3K	BS4M	BS5W	BS6J	BS7SH
S31	Eczema	12.66±1.15[a]*	8.66±1.15[a]	11.33±1.15[a]	9.33±1.15[a]	-	-	12.66±1.15[a]
S32	common Bullous	9.33±1.15[a]	-	-	-	-	-	-
S33	Bullous	11.33±1.15[a]	13.33±1.15[a]	-	-	-	-	-
S34	Furuncle	8.66±1.15[a]	11.33±1.15[a]	10±0[a]	9.33±1.15[a]	-	-	10.66±1.15[a]
S35	Bullous	18.66±1.15[a]	-	-	-	-	-	8.66±1.15[b]
S36	Ulcers	25±0[a]	22±2.0[a]	10.66±1.15b	10.66±1.15[b]	11.33±1.15[b]	10.66±1.15[b]	14±0[b]
S37	Bullous	-	12±0[a]	-	-	-	-	8.66±1.15[a]
S38	Bullous	10±0[a]	12±2.0[a]	-	-	-	-	14.66±1.15[a]
S39	Bullous	-	10.66±1.15	-	-	-	-	11.33±1.15[a]
S40	Furuncle	10.66±1.15[a]	14±0[b]	-	-	-	-	8.66±1.15[a]
S41	Face	12.66±1.15[a]	12±0[a]	-	-	-	-	10.66±1.15[a]
S42	Face	13.33±1.15[a]	12.66±1.15[a]	8±0[b]	8±0[b]	-	-	13.33±1.15[a]
S43	Hand	15.66±0.57[a]	10±0[b]	14±0[a]	10.66±1.15[b]	10.330.57[b]	8.66±1.15[b]	16.33±0.57[a]
S44	Face	-	-	-	-	-	-	-
S45	Face	16.66±1.15[a]	14.66±1.15[a]	-	-	-	-	12±0[a]
S46	Hand	-	-	-	-	-	-	10.66±1.15
S47	Face	17.33±2.30[a]	15.33±1.15[a]	8.66±1.15[b]	7.33±1.15[b]	8.66±1.15[b]	11.33±1.15[b]	13.33±1.15[c]
S48	Face	-	10.66±1.15[a]	12±0[a]	10.66±1.15[a]	-	-	10.66±1.15[a]
S49	Around mouth	16.66±1.15[a]	14.66±1.15[a]	-	-	-	-	10±0[b]
S50	Around mouth	12.66±1.15[a]	10±0[b]	9.33±1.15[b]	8±0[b]	-	-	12.66±1.15[a]
S51	Hand	10.66±1.15[a]	16.66±1.15[b]	8.66±1.15[a]	9.33±1.15[a]	-	-	10±0[a]
S52	Face	-	-	-	-	-	-	11.33±1.15

Sensitive: ≥16 mm, intermediate sensitive: 8 – 15 mm, non-sensitive: 4 - 7 mm, (-) no inhibition zone.

**Different letters in the same line indicate significant differences among the sample*

3.6. Partial Purification by FTIR

The FTIR spectrum exhibited characteristic absorption valley at 3361 cm^{-1} (amide bonds) and valley at 1649 cm^{-1} (carbonyl bonds C=O) and valley at 3300 cm^{-1} (carboxyl group) and valley at 3500 cm^{-1} (hydrogen bonded OH groups) and valley at 1456 cm^{-1}(carbon-carbon bond C=C) and it perhaps indicates the existence of a cyclical peptides. The valley at 2965 cm^{-1} shows C-H stretching, and valley at 1338 cm^{-1} shows C-O bond and valley at 2360 cm^{-1} shows S-H hydrogen sulfide bond. All indicated that substance contains peptide bonds (Figure 1 and Figure 2). So the characterizations of crude lyophilized extracts were showed in Table 4.

3.7. Partial Purification of Extracted Antimicrobial Substance by HPLC

The standard of bacitracin A and polymyxin B was run on HPLC. Bacitracin A gave peak with retention time 7.88 min while polymyxin B gave peak with retention time 28.36 min, then crude lyophilized extracts were purified by HPLC and were attributed to the peptides, like Bacitracin A. About 4 to 6 peaks were appeared in each extract, then, eluted fractions were collected and concentrated. The peak of bacitracin A purified from the strains *BS1A*, *BS2D*, *BS7SH* with retention time 8.65 and 9.57 min. (Figure 3 and Figure 4) and concentration 50, 50.20, 50.25 µg, respectively, and correlates the antibacterial activity of its crudes extracts against *S. aureus* strains. However, there is no peak of bacitracin A appeared for the strains *BS3K*, *BS6J*, *BS5W*, and *BS4M* which correlates with antibacterial activity of its crudes extracts. The peaks of polymyxin B were appeared in these extracts *BS1A*, *BS2D*, *BS7SH* with retention time 28.36 and 28.72 min. (Figure 3 and Figure 4), then the fractions of peaks were collected and concentrated which were 114, 112 and 114.7 µg, respectively, but the other peaks were unknown because the standards were not available. Thus, these strains of *B. subtilis* which isolated from soil and wastewater can be added to the limited number of *B. subtilis* strains were reported to co-produce peptides previously.

Table 4. Physical and chemical properties of crude lyophilized extract from *B. subtilis*.

Properties	Results
Color	light yellow to brownish when lyophilized and brown after soluble in water.
Nature	light, smooth, brittle, special scent, porosity, morphous
Solubility	water soluble, methanol, and soluble in 50% methanol-ethanol, but insoluble in chloroform, and Absolute ethanol

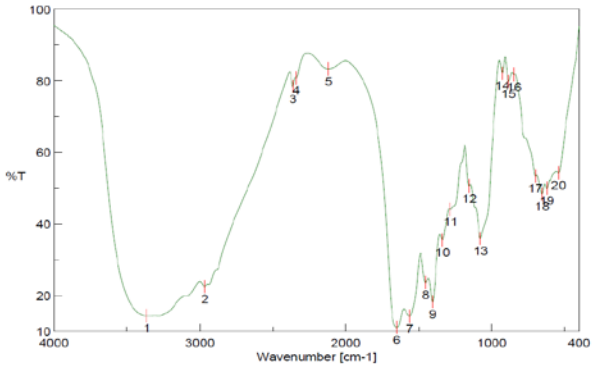

Figure 1. FTIR spectra of the Antibacterial compounds of crude lyophilized extract from *B. subtilis* was isolated from soil's Doma.

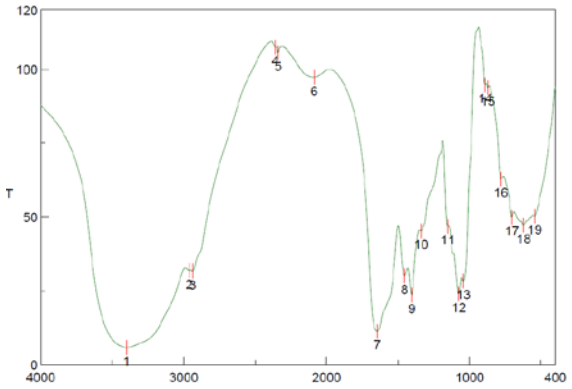

Figure 2. The FTIR spectrum of the Antibacterial compounds of crude lyophilized extract from *B. subtilis* was isolated from wastewater Bab-Sharqi

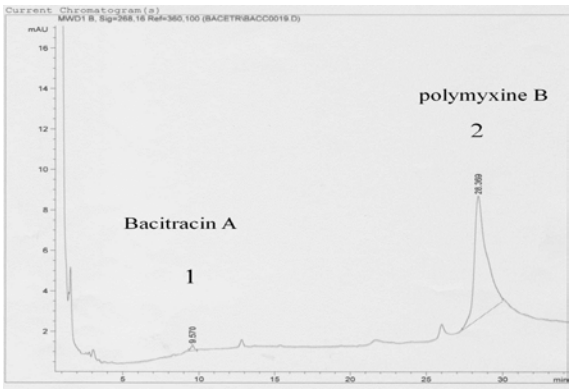

Figure 3. Reversed-phase HPLC chromatograms of antibiotics produced by *Bacillus subtilisBS1A, BS2D*. Peaks marked 1 is Bacitracin A, and 2 is Polymyxin B.

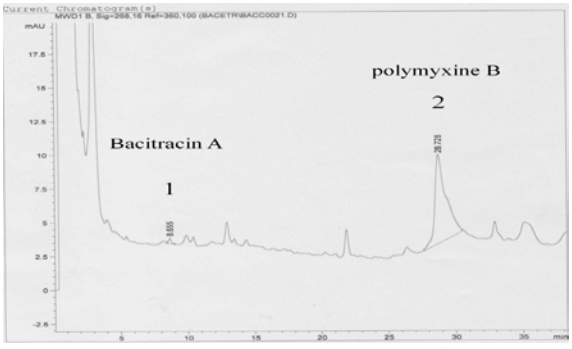

Figure 4. Reversed-phase HPLC chromatograms of antibiotics produced by *Bacillus subtilis BS7SH*. Peaks marked 1 is Bacitracin A, and 2 is Polymyxin B.

4. Discussion

To the best of our knowledge, the reports on antibacterial activity from *B. subtilis* against *S. aureus* pathogens especially are few. In our study, as shown in as Table 2 and Table 3, the crude lyophilized extract from *B. subtilis BS1A*, *BS2D* and *BS7SH* showed a high antibacterial activity against *S. aureus* and the mean of diameters of inhibition zone was 8 - 40 mm, with 62.5% percent for *BS1A*, and 76.78% for *BS2D*, while percentage for *BS7SH* was 89.28%. ,whereas the crude extracts from *BS1A* and *BS2D* showed good antibacterial activity against *S. aureus* which isolated from skin's infections, like bullous, ulcers, furuncles, and isolated from microflora of skin, like face and hand, with 74.07% percent for *BS1A* and 77.77% for *BS2D* (Table 2 and Table 3). On the other hand, the crude extracts from strains *BS3K*, *BS4M*, *BS5W* and*BS6J* did not give antibacterial activity against most of these strains of *S. aureus* except *S1*, *S17*, *S36*, *S43*, *S47*, in addition, the crude extracts from *BS3K* and *BS4M* were better than *BS5W*and *BS6J*, which gave intermediate activity against *S1*, *S17*, *S18*, *S31*, *S34*, *S36*, *S42*, *S43*, *S47*, *S48*, *S50*, *S51* (Table 2 and Table 3). At the same time, there were some strains of *S. aureus* resistant to the crude extracts, even the crudes extracts of species *BS1A*, *BS2D* and *BS7SH*, like *S8*, *S44*and resistant to the crudes extracts of *BS1A* and *BS2D)* but sensitive to the crude extracts of *BS7SH*, such as *S4*, *S14*, *S23*, *S46*, *S52*.

Some previous research studies used the liquid extracts, and *S. aureus* targets were isolated from clinical and food, while in the present study lyophilized extracts were used against clinical strains of *S. aureus*. It was found in a study by Aslim *et al.* (2002) and a study by Kim *et al.* (2003) that no effect of *B. subtilis* on *S. aureus*; in another study by Ouoba *et al.* (2007), it is showed that the *S. aureus* seemed resistance toward the antibiotic compounds which produced by *B. subtilis*. However, the studies of Motta *et al.* (2004), Fernandes *et al.* (2007), Sethi *et al.* (2013) and Ramachandran *et al.* (2014) reported that *B. subtilis* gave moderate activity against *S. aureus* and the diameters of inhibition zone varied from 8 to 16 mm. But Faheem *et al.* (2007) showed that Brevicin, which is isolated from *B. brevis*, gave a low activity against *S. aureus*. Whereas, Devi *et al.* (2008) showed that the antimicrobial activity from *B. clausii* was moderate against *S. aureus*. Also, the studies of Kuta *et al.* (2009), Mohammad *et al.* (2009), Bhatta and Kapadnis (2010), Moshafi *et al.* (2011) and Moore *et al.* (2013) showed that the antimicrobial compounds were produced from *B.subtilis* showed higher activity against *S. aureus* and the diameters of inhibition zone varied from 19 to 28 mm. On the other hand, Tabbene *et al.* (2010) characterized and identified a new antibacterial and antioxidant produced from *B. subtilis* and it has an activity against *S. aureus*, so Fuchs *et al.* (2011) isolated Entianin, which is a novel Subtilin from *B. subtilis* and gave a strong activity against *S. aureus* (MRSA).

These differences between the antibacterial activities of Bacillus species may be due to the production of antibiotics of each strain, or due to the environment in which they live, or may be due to the genetic differences which are important factors for the production of the antibiotics, and the biophysical and chemical factors in each soil.

By the results of FTIR, presence the amide bonds, carbonyl bonds (C=O), carboxyl group, carbon-carbon bond C=C and hydrogen bonded OH groups indicate to a polypeptide, where these results were similar to the results of Kumar *et al*. (2009) and Bhatta and Kapadnis (2010).

Few peptides antibiotics, produced by *B. subtilis*, are active against Gram-positive bacteria, as staphylococci; however, using HPLC, the antibiotics were isolated and purified from the crude lyophilized extracts of strains of *B. subtilis*. As mentioned above, these strains *BS1A*, *BS2D*, and *BS7SH* showed strong antibacterial activity against *S. aureus* strains. About 4-6 peaks appeared from every extract, two important peaks were showed, the first peak is for bacitracin A with retention time 8.65, 9.57 (Figure 3 and Figure 4) and the latter is for polymyxin B with retention time 28.36, 28.72 (Figure 3 and Figure 4) and the activity of second peak against *S. aureus* was lower when compared to peak of bacitracin, that is because polymyxin B is used for Gram-negative Bacteria while bacitracin is used for Gram-positive Bacteria. So, related to these results are some of studies that purified and identified the antibiotics, like subtilin, and antifungal, like iturin from *B. subtilis*, and other species (Smitha and Bhat, 2012; Ouoba *et al*., 2007).

The strains, which showed a high antibacterial activity, were the *BSIA*, *BS2D*, and *BS7SH*. These strains will be suitable to undertake further studies on the antimicrobial activity of the *B. subtilis* isolates in order to isolate, characterize and identify the antimicrobial compounds produced against the pathogens.

5. Conclusions

This crude extract could function as an antibacterial activity against *S. aureus* pathogens. A new approach for structural characterization of antibiotics from *B. subtilis* were provided in the present study which uses the crude lyophilized extract against *S. aureus*.

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