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A Broad-Spectrum Antibacterial Activity of Lyophilized Crude Extracts of *Bacillus Subtilis* against Clinical and Food-Borne Pathogens

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Abstract— *The aim of this research is to study the broad spectrum antibacterial activity of crude lyophilized extracts from Bacillus subtilis, then characterize these antibacterial activity. B. subtilis strains were isolated and identified by both API CHB 50 and molecular methods. Staphylococci strains, negative gram bacteria and Candida spp. were isolated from clinical and food samples and identified by biochemical and morphological tests. The crude antibacterial were extracted by flasks containing Lactose Broth LB, which incubated for 72 hours in shaker incubator at 37° ºC at 150 rev/min, then centrifuged 12000 rev/min or 15 min. at 4 ºC and filtrated the supernatants through 0.45 µm then frozen and lyophilized. The crude extracts were run on Diamonsil C18 column (5 µm, 250 × 4.6 mm) in HPLC system to separate the antibiotics and run on FTIR spectrum. Some strains of B. subtilis showed high activity against target strains, and others did not. The antibacterial activity of crude lyophilized extracts from B. subtilis (BS1A, BS2D, BS7SH, BS8SH) against S. aureus strains was between high (16-40 mm) and intermediate (8-14 mm). While the antibacterial activity was intermediate (8-14 mm) against Gram negative and Candida spp. strains. Two kinds of antibiotics were separated from lyophilized extracts of B. subtilis and identified to the peptides families, Bacitracin A and Polymyxin B.*

Keywords— *Antibacterial, Staphylococci, Listeria, Candida, crude, peptide.*

I. INTRODUCTION

Bacillus subtilis produces many antibacterial and antifungal peptides such as bacitracin and polymyxin and lipopeptide such as surfactin, iturin and fengycin with a great potential for biotechnological and biopharmaceutical applications (Schallmey *et al*., 2004; Stein, 2005; Sumi *et al*., 2015). *Staphylococcus aureus* especially methicillin resistance *S. aureus* (MRSA) and Coagulase Negative Staphylococci (CNS) are 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 implants (Chambers *et al*., 2012; Kainthola *et al*., 2015). Also Worldwide foodborne pathogens cause a lot of sufferings and deaths, mainly *S. aureus* and *Listeria monocytogenes* (Soni, 2015).

Candida spp. particularly, *Candida albicans* remains the most common cause of invasive fungal infections, such as superficial, subcutaneous and systemic infections. Candidiasis has become a major problem in hospitals where cause death in hospitalized adults and children in many countries worldwide (Guinea, 2014; Hirota, 2016).

Most research, are focused on the ability of Bacillus species to produce antibiotics and antifungals. Kuijk *et al*. (2011) studied and elucidated the molecular mechanism of action of the antimicrobial peptide subtilosin, which were extracted from *Bacillus amyloliquefaciens* against the foodborne pathogen *L. monocytogenes* Scott A, also Sethi *et al*. (2013) showed that *B. subtilis* which was isolated from the soil showed activity against *S. aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*, while Ndahi *et al.*(2013) studied the prevalence and antimicrobial sensitivity of *L. monocytogenes* and *S. aureus* which were isolated from raw meat and meat products, whereas in study of Ceresa *et al.*(2015) was appeared the ability of biosurfactants which were produced by *Lactobacillus brevis* was investigated to inhibit the biofilm formation of *C. albicans*, in addition in study of Haubert *et al.* (2015) about 50 isolates of *L. monocytogenes* were isolated from food and food environment and studied for antimicrobial resistance and genetic relationships, while in study of Das *et al.* (2016) it was evaluated the antimicrobial and antibiofilm of small molecules against *S. aureus* and *Pseudomonas aeruginosa*, also in study of Sharma and Sharma, (2017) *Bacillus licheniformis* which was isolated from indigenous traditional fermented beverage showed broad antagonism against most pathogens. However, the prevalence of antimicrobial resistance among key microbial pathogens is increasing at an alarming rate worldwide. This study presented the characterization of some new strains of *B. subtilis* which produce antibiotics and antifungal then were evaluated their activity against positive and negative bacteria and *C. albicans*.

II. MATERIAL AND METHODS

2.1 Isolation and Identification of B. subtilis

During the period between 6/2014 - 6/2015, three different soil samples were taken from each site from cultivated and

barren lands in and around Damascus then collected in the sterile polypropylene bags: Al-Tall, Doma, Khan-Alsheh, Meliha and Adawee. The sample of soils were mixed to form composite sample, and all soils were used in all experiments were collected from the 15 -20 cm layer. Three different water samples were taken from three sites from Bab-Sharqi, the wastewater and one canal of Barada river in Damascus, and collected in the sterile glass bottles.

Soil and water suspensions were diluted in sterile distilled water after being heated at 80ºC for 15 min., then 1 ml of dilutions from 10^{-1} to 10^{-6} were plated on nutrient agar NA. The plates were incubated at 37ºC for 24 hour The identification of bacterial strains was performed both by microbiological, and biochemical methods also, spore morphology, gram characteristics and motility according to the Bergey's Manual 2009, by using API 50 CHB (BioMérieux, France) then results of tests were read by API web (Logan and De vos, 2009)

2.2. Molecular Identification of B. subtilis

DNA extraction was done according to Japoni *et al.* (2004) with simple modifications. All *Bacillus* isolates were identified by 16S rDNA 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 94ºC for 3 min., 45 cycles of 94ºC for 1 min., 55ºC for 1 min and 72ºC for 2 min., and a final extension step at 72ºC for 10 min. then cold 4ºC. 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 were 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.

2.3 Clinical and Food Bacterial Strains

A total of 23 staphylococcal strains were isolated from clinical samples (ear, urine, wound, pus, bronchial lavages, abscess, furuncle), which were collected from Al-Mouwasat hospital in Damascus city.

The isolates were biochemically identified upon API Staph. (BioMérieux, France) according to the Bergey's Manual (2009), also conventional methods that included Gram staining, tests for colonial morphology, catalase test, in addition coagulase slide test (Sigma, Germany) according to the manufacturer's instructions (Schleifer and Bell, 2009; Tille, 2014).

As for gram-negative strains, were identified by morphological tests on selective medium like EMB agar for Enterobacteriaceae and cetrimide agar for *Pseudomonas aeruginosa* and biochemical tests (sugar utilization, starch utilization, casein hydrolysis, indole production, citrate utilization, oxidase production, catalase production, nitrate reduction, gas production from glucose). The strains were stored on NA agar slants at 4ºC and in glycerol-containing nutrient broth NB 20% (v/v) at -30ºC freezer until further analysis and were subcultured on nutrient agar (Brenner and Farmer, 2005; Garrity *et al*., 2005) .

For isolates of food-borne pathogens, *S. aureus* was isolated from cheese by Baird Parker Agar incubated at 37ºC for 24-48 h. Staphylococci produce dark gray to black colonies due to tellurite reduction; staphylococci that produce lecithinase break down the egg yolk and cause clear zones around respective colonies. An opaque zone of precipitation is formed due to lipase activity (Rola *et al*., 2016). However, *L. monocytogenes* was isolated from meat where 25 g of meat samples were homogenized with 225 ml Listeria Enrichment Broth (Merck, Germany). For a homogenous distribution of the sample in the medium, was shaken for 2-3 min. then Inoculated media were incubated at 30ºC for 24-48 h. After 24 and 48 h. pre-enrichment process, the culture was made on Listeria identification Agar Base (PALCAM) and Listeria Oxford Medium Base. The planted plates were incubated for 48 h. at 37 ºC under aerobic conditions; and then examined by Henry method of oblique lighting. At the end of the incubation, they were evaluated as typical colonies with black haloes of 1-3 mm in diameter. The Listeria were identified by gram staining, catalase production, oxidase activity, H2S production using Triple Sugar Iron Agar (MERCK, Germany), and mobility test in SIM media at 25ºC and 35ºC. Isolates were biochemically confirmed as Listeria spp. The biochemical characteristic of examined samples were determined by using the API Listeria system (BioMérieux, Marcy l' Etoile France) to identify the Listeria' strains (Jamali *et al*., 2013; Wellinghausen, 2015).

Clinical yeast isolates were obtained from patients in Dermal and Venereal Diseases hospital. The various clinical specimens were collected and analyzed by standard microbiological procedures with Sabouraud's dextrose agar supplemented with chloramphenicol and gentamicin and incubated at 37ºC for 48 h. Yeast isolates were identified on ID Candida Chromogenic medium (ID Candida-Biolife Italy) based on the following criteria (*C. albicans* green colonies; *C. glabrata* light white colonies) (Jorgensen and Pfaller, 2015). All strains of Bacteria and fungi were isolated between 6/2014–6/2015.

2.4 Antimicrobial Crude Lyophilized Extraction Production Conditions

Shake flask fermentation method was used for bacterial antibiotic production as Bhatta and Kapadnis (2010) with some modification: For all strains of *B. subtilis*, production cultures were prepared in lactose broth LB (Merck, Germany). the flasks were inoculated with a fresh culture of *B. subtilis* by using the sterilized loop and incubated again for 72 h. at 37ºC ± 1ºC in shaker incubator (JSR, Korea) at 150 rev/min, until 15×10^7 CFU ml^{-1} by optical density OD (OD_{600} 0.5 = 5×10^7 CFU ml^{-1}). After incubation, cultures were centrifuged (Hittch, Germany) at 12000 rev min^{-1} for 15 min. at 4ºC, to get cell free supernatant, then 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°C between 6 - 8 h. then put in lyophilizer (Lyotrap, England) at -40°C, and less than 1 millibar for 48-72 h. until complete lyophilization, finally the crude lyophilized extract were weighed and stored at 2-8°C. moreover, these crudes were used to evaluate antimicrobial activity.

2.5 Antimicrobial Activity

Agar well diffusion method was used to check the antimicrobial activity of the crude lyophilized extract of *B. subtilis*. Mueller-Hinton agar MHA surface (Merck, Germany) plates were swab-inoculated with *S. aureus* were grown in nutrient broth NB and adjusted by OD (OD_{600} 0.5 = 1 $\times 10^8$ CFU ml^{-1}). Wells were made in the inoculated plates using sterile stainless still borer (diameter 6 mm). 20 µl of soluble crude lyophilized extract were added into wells (0.5 mg / µl), and the plates were incubated at 37°C for 24 h. Then inhibition zones were observed. The diameter of the inhibition zone was measured in mm with well size of 4 mm (Bhatta and Kapadnis 2010; Sethi *et al*., 2013).

2.6 Antifungal Activity

Agar well diffusion method was used for antifungal activity of the crude lyophilized extract of *B. subtilis*. 1ml (1×10^6 spores/ml) of test fungus was spread over the agar surface of the plate.

Wells were made using sterile stainless still borer (diameter 6 mm). 80 µl of soluble crude lyophilized extract were added into wells (0.5 mg / µl), and the plates were incubated at 37°C for 24 h. Then inhibition zones were observed and measured (Kumar *et al*., 2009; Sethi *et al*., 2013).

2.7 Determination the Antimicrobial Substance by HPLC

The crude lyophilized extract (water solution, 0.5 mg / µl), were detected by reversed-phase HPLC as follows. A 50 µl 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^{-1} 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 (Yuan *et al*., 2014).

2.8 Determination the Functional Groups of Crude Antibacterial Extracts 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 sample (5 mg). The spectra were plotted as intensity versus wave number.

2.9 Statistical Analysis

One Way Anova were performed using SPSS program software (ver. 17) to validate the signification of the results. The data are presented as means (± SD) of three replicates.

III. RESULTS

3.1 Isolation and Identification

For Bacillus and according to API 50 CHB testing and microscopic study the tests indicated that strains belong to *B. subtilis*. Partial sequence of 16S rDNA of *B. subtilis* (*BS1A, BS2D, BS3K, BS4M*, *BS5W, BS7SH, BS8SH*) were submitted to the Genbank database under accession numbers: KM052377, KM192148, KF792060, KM251459, KF792061, KM213762, KM189125 respectively. 16S rDNA gene sequence confirmed the obtained results of biochemical (Table I). By strains of Staphylococci and after confirmation the characteristics upon biochemical tests and API Staph. the results were submitted to 18 *S. aureus*. in addition, 5 *S. epidermidis* (Table II).

TABLE I. Characteristics of soils and the strains of *B. subtilis* from soil and water.

Sample	Source's sample	*B. subtilis*	Accession No.
Al-Tall	soil	*BS1A*	KM052377
Doma	soil	*BS2D*	KM192148
Khan-Alsheh	soil	*BS3K*	KF792060
Meliha	soil	*BS4M*	KM251459
Adawee	soil	*BS5W*	KF792061
Bab-Sharqi (canal of Barada)	waste water	*BS7SH*	KM213762
Bab-Sharqi (canal of Barada)	waste water	*BS8SH*	KM189125

TABLE II. Strains number of target organisms and source's sample.

Strains No.	target organism	Source's sample
S1	*S. aureus*	pus
S2	*S. aureus*	Ear
S3	*S. aureus*	pus
S4	*S. aureus*	bronchial lavages
S5	*S. aureus*	Pus
S6	*S. aureus*	Ear
S7	*S. aureus*	bronchial lavages
S8	*S. aureus*	Abscess
S9	*S. aureus*	Ear
S10	*S. aureus*	Pus
S11	*S. aureus*	Pus
S12	*S. aureus*	Pus
S13	*S. aureus*	Furuncle
S14	*S. aureus*	Pus
S15	*S. aureus*	Abscess
S16	*S. aureus*	Abscess
S17	*S. aureus*	Urine
S18	*S. aureus*	Urine
S19	*S. epidermodis*	wound
S20	*S. epidermodis*	pus
S21	*S. epidermodis*	Ear
S22	*S. epidermodis*	Urine
S23	*S. epidermodis*	Ear
S24	*S. aureus*	chees
S25	*L. monocytogenes*	meat (13 M/ ATCC 98 A6)
S26	*E.coli*	Urine
S27	*Klebsiella spp.*	Urine
S28	*Pseudomonas aeruginosa*	ear
S29	*C. albicans*	Vagina
S30	*C. albicans*	Vagina
S31	*C. glabrata*	Skin

While Gram negative bacteria were confirmed both morphological and biochemical tests then representative of

three strains including *E. coli*, *Klebsiella spp*. and *Pseudomonas aeruginosa* (Table II). Whereas Fungi three strains were identified as followed 2 *C. albicans* and 1 *C. glabrata* (Table II).

However, for strains were isolated from food and after biochemical and morphological tests of examined samples by using the API Listeria. and API Staph. in addition, molecular identification, then Listeria calcified in ATCC under number *Listeria monocytogenes* 13 M/ ATCC 98 A6, but one strain of *S. aureus* (Table II).

Yeast isolates were identified on ID Candida Chromogenic medium (ID Candida-Biolife Italy) based on the following criteria (*C. albicans* green colonies; *C. glabrata* light white colonies) (Table II).

3.2 Antimicrobial Activity

The characterizations of crude lyophilized extracts were showed in (Table III), while the table IV A, B shows the diameter of inhibition zone of crude lyophilized extract from *B. subtilis* against target strains, and shows that two crude extracts from strains of *B. subtilis* which isolated from the soil (*BS1A*, *BS2D*) have intermediate to high antibacterial activity, where gave bigger zone against most of strains (8-40 mm), while (*BS7SH, BS8SH*) which isolated from wastewater have intermediate antibacterial activity (8-14 mm), whereas another crude extracts from strains of *B. subtilis* which were isolated from the soil (*BS3K*, *BS4M*, *BS5W*) didn't give antibacterial activity against most of these strains (Table IV A, B). But the antibacterial activity of crude lyophilized extracts from *B. subtilis* (*BS1A*, *BS2D, BS7SH, BS8SH*) against Gram negative and Candida spp. strains was intermediate (8-14 mm) (Table IV).

TABLE III. 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
Yield	7 – 8 g. from each strain
Solubility	water soluble, methanol, and soluble in 50% methanol-ethanol, but insoluble in chloroform, and Absolute ethanol

3.3 Statistic Results

By the statistic results, the differences between the antibacterial activity against tested strains were subjected to One Way Anova statistical test, and it showed that there is a significant difference between some strains of *B. subtilis* toward test organisms ($P<0.05$), and there is no significant difference between others ($P>0.05$) (Table IV).

3.4 Partial Purification by FTIR

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 bond OH groups), and valley at 1456 cm^{-1}(carbon-carbon bond C=C), and it perhaps indicate to a cyclical peptides. The valley at 2965 cm^{-1} shows C-H stretching, and valley at 1338 cm^{-1} shows C-O bond. All indicated that substance contains peptide and lipopeptide bonds (Fig. 1).

TABLE IV (A). The mean, ±SD, and results of Anova[(a,b,c..)] of the diameters of inhibition zone (mm) of crude lyophilized extract against target organisms which isolated from patients.

Strains No.	*BS1A*	*BS2D*	*BS3K*
S1	40.66±1.15[a]*	23.33±1.15[b]	8±0[c]
S2	29.33±1.15[a]	12.66±1.15[b]	-
S3	30.33±0.57[a]	15.33±1.15[b]	-
S4	-	-	-
S5	28.66±1.15[a]	14.33±0.57[b]	-
S6	13.33±2.88[a]	14±0[a]	-
S7	-	16.66±1.15[a]	-
S8	-	-	-
S9	-	18.66±1.15[a]	-
S10	-	18.66±1.15[a]	-
S11	14±0[a]	10.66±1.15[a]	-
S12	-	15.33±1.15[a]	-
S13	11.33±1.15[a]	12±2.0[a]	-
S14	-	-	-
S15	21.33±1.15[a]	10.66±1.15[b]	-
S16	13.33±1.15[a]	14.66±0.57[a]	-
S17	15.33±1.15[a]	`16±5.29[a]	9.33±1.15[b]
S18	11.66±2.88[a]	12.66±1.15[a]	8.66±1.15[a]
S19	21.33±1.15[a]	20.33±0.57[a]	-
S20	21±1[a]	17.66±0.57[b]	-
S21	27.33±1.15[a]	13.66±0.57[b]	-
S22	15±0[a]	10.66±1.15[b]	-
S23	16±0[a]	15.33±1.15[a]	10.66±1.15[b]
S24	10.66±1.15[a]	12.66±1.15[a]	10±0[a]
S25	9.33±1.15[a]	11.33±1.15[a]	-
S26	9.33±1.15[a]	11.33±1.15[a]	-
S27	-	-	-
S28	12±0[a]	10.66±0.57[a]	8±0[a]
S29	10.66±0.57[a]	10.66±0.57[a]	-
S30	-	-	-
S31	10.66±1.15[a]	10.66±1.15[a]	-

High sensitivity: ≥15 mm, intermediate sensitivity: 8 – 14 mm, resistant: 4 - 6 mm, (-) no inhibition zone.

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

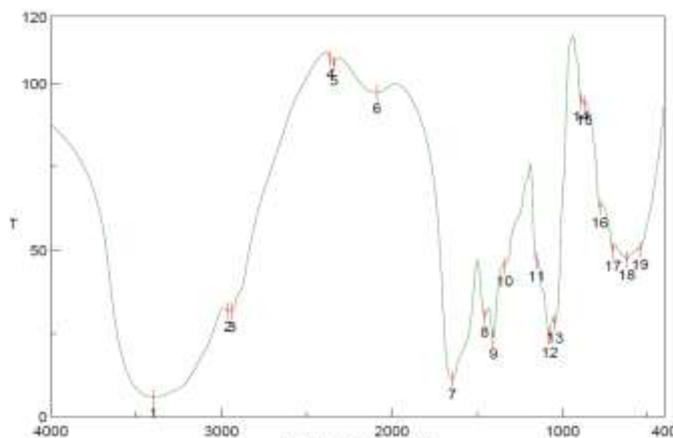

Fig. 1. The FTIR spectrum of the Antibacterial compounds of crude lyophilized extract from *B. subtilis* was isolated from wastewater Bab-Sharqi.

3.5 Purification by HPLC

Comparing with standard of Bacitracin A and Polymyxin B, the crude lyophilized extracts of the strains *BS1A*, *BS2D*, *BS7SH*, *BS8SH* purified and gave the peak of bacitracin A with retention time 8.65 min. (Fig. 2) and concentration 50.20,

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50.25 µg, and this agree with the antibacterial activity of its cruds extracts against *S. aureus* strains, but no peak of Bacitracin A was appeared from the strains *BS3K*, *BS6J*, *BS5W*, and *BS4M* which agreement with antibacterial activity of its crude extracts. While the peaks of polymyxin B were appeared in these extracts *BS1A*, *BS2D*, *BS7SH, BS8SH* with retention time 28.72 min. (Fig. 2), then collected and determined the concentration where 114, 7 µg but the other peaks were unknown because the standards were not available.

IV. Discussion

The species of genus *Bacillus* produce antibacterial, which can be used against animal and human pathogens. That, Tabbene *et al.* (2015) characterized and identified a new antibacterial and antioxidant produced from *B. subtilis* and it has an active against *S. aureus*, so Fuchs *et al.* (2011) isolated Entianin, which is a novel Subtilin from *B. subtilis* and gave a very active against *S. aureus* (MRSA). However, reports on antibacterial activity of crude lyophilized extracts from *B. subtilis* against bacteria and Candida pathogen strains are little. Current study, as Table IV A, B the crude lyophilized extracts from *B. subtilis* (*BS1A*, *BS2D*) and (*BS7SH, BS8SH*) showed between intermediate to high antibacterial activity against most of *S. aureus* and high activity against *S. epidermidis*, whereas the crude extracts from *B. subtilis* (*BS3K*, *BS4M*, *BS5W*) didn't give any activity against all target strains.

At the same time there are some strains of *S. aureus* are resistant to the crude extracts, even the crudes extracts of species (*BS1A*, *BS2D*) and (*BS7SH, BS8SH*) like *S. aureus* which isolated from bronchial lavages and abscess, also *Klebsiella spp*. which isolated from urine and *C. albicans* which isolated from vagina (Table IV A, B).

These differences between the antibacterial activities of *B. subtilis* strains may be upon to the production of antibiotics of each strain, or due to the environment, which live in it, or may be upon to the genetic differences which important factor for production the antibiotics and the biophysical and chemical factors in each soil.

The results in this research are agree in some results with previous and recent studies and disagree with other results, hence, Kim *et al.* (2003) appeared that no effect of *B. subtilis* on *S. aureus,* while Ouoba *et al.* (2007) showed that the diameters of inhibition zone against *S. aureus* was 3-5 mm and against E. coli was 0-3 mm and against *L. monocytogenes* was 1-3 mm, so Fernandes *et al.* (2007) showed the diameters of inhibition zone against *S. aureus* (MDR) was between 10.7 - 28.1 mm and against *P. aeruginosa* was between 10.3 - 12.1 mm, and against *E. coli* was 13.2-14.4 mm, also, as for Sharma & Gautam (2008) showed that the diameters of inhibition zone of *Bacillus mycoides* against *L. monocytogenes* was between 10 - 30 mm. while in study of Kuta *et al*. (2009) the diameters of inhibition zone of *B. subtilis* against *S. aureus, E. coli, Klebsiella spp.* was 19, 0, 17 mm respectively.

In study of Bhatta and Kapadnis (2010) was 25 mm against *S. aureus*, while as was reported from Sethi *et al.* (2013) the inhibition zone against *S. aureus, P. aeruginosa, E. coli, K. pneumonia* was 13.4, 13.8, 0, 0 mm respectively, but in study of Ramachandran *et al.* (2014) the diameters of inhibition zone of *B. subtilis* against *S. aureus* was between 0-14,5 mm, and against *S. epidermidis*, *P. aeruginosa, E. coli, K. pneumonia, C. albicans, C. glabrata* was 15.7, 17.33, 21.33, 15, 16. 83 and 20.5 mm respectively, whereas in study of Zhao *et al*. (2016) the diameters of inhibition zone of *B. subtilis* against *C. albicans* was between 24 - 29 mm.

TABLE IV (B). The mean, ±SD, and results of Anova$^{(a,b,c..)}$ of the diameters of inhibition zone (mm) of crude lyophilized extract against target organisms which isolated from patients.

Strains No.	*BS4M*	*BS5W*	*BS7SH*	*BS8SH*
S1	10 ± 0^{c}	8 ± 0^{c}	12 ± 0^{c}	18.33 ± 1.15^{d}
S2	-	-	8.66 ± 1.15^{b}	16.66 ± 1.15^{c}
S3	-	-	8.66 ± 1.15^{c}	11.33 ± 1.15^{d}
S4	-	-	-	-
S5	-	-	8.66 ± 1.15^{c}	16.33 ± 1.15^{b}
S6	-	-	9.33 ± 1.15^{b}	9.33 ± 1.15^{b}
S7	-	-	10 ± 0^{b}	13 ± 1.73^{b}
S8	-	-	-	14 ± 0^{a}
S9	-	-	10.66 ± 1.15^{b}	10.66 ± 1.15^{b}
S10	-	-	12.66 ± 1.15^{b}	14.66 ± 1.15^{b}
S11	-	-	10.66 ± 1.15^{a}	10.66 ± 1.15^{a}
S12	-	-	11.33 ± 1.15^{a}	12.66 ± 1.15^{a}
S13	-	-	8.66 ± 1.15^{a}	8 ± 0^{a}
S14	-	-	10 ± 0^{a}	12.66 ± 1.15^{a}
S15	-	-	10.66 ± 1.15^{b}	12.66 ± 1.15^{b}
S16	-	-	11.33 ± 1.15^{a}	16.66 ± 0.57^{a}
S17	10.66 ± 1.15^{b}	10.66 ± 1.15^{b}	14.33 ± 1.15^{a}	14.66 ± 0.57^{a}
S18	8 ± 0^{a}	-	8.66 ± 1.15^{a}	10.66 ± 0.57^{a}
S19	10.0 ± 1^{b}	-	9.33 ± 1.15^{b}	12.66 ± 1.15^{b}
S20	-	-	11.66 ± 0.57^{c}	14.66 ± 0.57^{c}
S21	-	-	10 ± 0^{b}	12.66 ± 1.15^{b}
S22	-	-	10.66 ± 0.57^{b}	13.33 ± 1.15^{a}
S23	-	-	9.33 ± 1.15^{b}	12.66 ± 1.15^{b}
S24	5.33 ± 1.15^{b}	9.33 ± 1.15^{a}	10 ± 0^{a}	-
S25	-	-	8 ± 0^{a}	9.33 ± 1.15^{a}
S26	-	-	10.66 ± 0.57^{a}	10 ± 0^{a}
S27	-	-	-	-
S28	11.33 ± 1.15^{a}	-	11.33 ± 1.15^{a}	12 ± 0^{a}
S29	-	-	8 ± 0^{a}	10 ± 0^{a}
S30	-	-	12.33 ± 1.15^{a}	14.33 ± 1.15^{a}
S31	-	-	12 ± 0^{a}	14.66 ± 0.57^{a}

High sensitivity: ≥15 mm, intermediate sensitivity: 8 – 14 mm, resistant: 4 - 6 mm, (-) no inhibition zone.

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

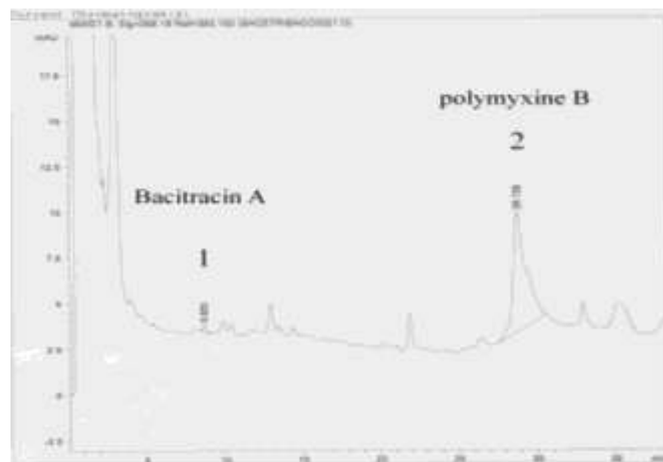

Fig. 2. Reversed-phase HPLC chromatograms of antibiotics produced by *Bacillus subtilis*. Peaks marked 1 is Bacitracin A, and 2 is Polymyxin B.

Few peptides antibiotics were produced by *B. subtilis* are active against Gram-positive bacteria as Staphylococci, however, by HPLC the antibiotics were isolated and purified from the crude lyophilized extracts of strains of *B. subtilis*. About 4-6 peaks appeared from every extract, two important peaks were showed, first one for bacitracin A with retention time 8.65 (Fig. 2) and second for polymyxin B with retention time 28.72 (Fig. 2), so related to these results some of researches purified and identified the antibiotics like subtilin and antifungal like iturin from *B. subtilis* and other species (Ouoba *et al.,* (2007; Smitha and Bhat, 2012).

V. Conclusion

Current research presents the antibacterial and antifungal activity of crude lyophilized extracts of *B. subtilis,* it is one of the few studies, which evaluate the antibacterial activity against bacteria and fungi were isolated from variable sources. The strains, which showed high antibacterial activity, will be suitable to undertake further studies on the antimicrobial activity of the *B. subtilis* isolates, so our knowledge, these strains of *B. subtilis,* which isolated from soil and wastewater, could be added to the limited number of *B. subtilis* strains reported to co-produce peptides.

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