DOI: 10.56511/JIPBS.2015.v2.i04.pp632-645

Journal of Innovations in Pharmaceuticals and Biological Sciences
www.jipbs.com

JIPBS

ISSN: 2349-2759

Research article

Potential antimicrobial activity of the antagonistic *Bacillus* strains to *Saccharomyces cerevisiae*

Salah H. Salem*[1], Hussein H. El-Sheikh[2], Mohamed M. Naguib[1], Yehia A. Heikal[3]

[1]Food Toxins and Contaminants Dept. National Research Center, Cairo- Egypt
[2]Botany and Microbiology Dept. Fac. of Science, Al-Azhar University, Cairo- Egypt
[3]Food Science Dept., Faculty of Agriculture, Ain Shams University, Cairo Egypt

Abstract

Members of the genus *Bacillus* are known to have several metabolites with biologically active property. Six *Bacillus* species; *B. Subtilis* NS4182-01**,** *B. subtilis* NS4182-06*, B. amyloliquefaciens* NS4182-03, *B. amyloliquefaciens* NS4182-07, *B. subtilis* NS4182-04and *G. stearothermophilus* NS4182-05**;** were tested for their activity against *Saccharomyces cerevisiae*, pathogenic bacteria and fungi. The cell free supernatant, its chloroform extract as well as methanol extracts of its HCl precipitates showed inhibition activity towards *S. cerevisiae*as well as antibacterial activity against the tested Gram positive and Gram negative bacteria with different inhibition zones diameters. The antagonism of whole *Bacillus* strains towards fungal species showed that the reduction percent of mycelial growth ranged from nil to as much as 51.4%, also chloroform and HCl extracts showed antifungal activity against tested fungi. The FT-IR analysis showed NH stretching vibration in protein, CH stretching vibration in aliphatic chain, C=O and NH bending vibration in amide I and amide II and the presence of lactone carbonyl group that typical for surfactin and fengycins families of lipopeptides. The fatty acids moiety of *Bacillus* extracts found to contain C14-C18 chain. It is to be concluded that finding lipopeptides producing *Bacillus* strains is of great importance from the industrial, pharmaceutical and biological control point of view.

Key words:*Bacillusstrains, Lipopeptides, S.cerevisiae*, Antibacterial, Antifungal and FT-IR.

***Corresponding Author: Salah H. Salem,**Food Toxins and Contaminants Dept. National Research Center**,** Cairo- Egypt.

1. Introduction

Members of the genus *Bacillus* are known to have several metabolites with biologically active molecules potentially inhibitory for phyto-pathogens growth. Their spore- forming ability also makes these bacteria some of the best candidates for developing efficient bio-pesticide products from a technological point of view [1] [2].

Bacillus strains produce gene- encoded antibiotics as well as a variety of small antibiotic peptides (<2000 Da) synthesized non-ribosomally [3]. These Peptide antibiotics also named

©JIPBS, All rights reserved

lipopeptides and are classified into three families depending on their amino acid sequence; iturins, fengycins or surfactins [2][4].

In recent years, there has been a considerable interest in using *Bacillus* strains for producing lipopeptides antibiotics like iturin A and surfactins as biocontrol agents due to their antagonistic and repressive activities against pathogens[5]. Iturin and fengycin display a strong antifungal activity. However surfactin are not fungitoxic by themselves but retain some synergistic effect on the antifungal activity of iturinA [6]. Ongena and Jacques [7] reported that the surfactins are powerful biosurfactants, which show antibacterial activity but no marked fungitoxicity (with some exceptions).Kim *et al.,* [8] stated that these amphiphilic cyclic bio-surfactants have many advantages: low toxicity, high biodegradability and environmentally friendly characteristics.

The antimicrobial peptides produced by *Bacillus subtilis* strains are of high potential for biological control applications[1]. Kumar *et al.,* [9] reported that the bacterium *Bacillus subtilis* produce a variety of antibacterial and antifungal antibiotics. Surfactins acyclic lipopeptides are one of the most effective biosurfactant known so far, which was first reported in *B. subtilis* ATCC- 21332 and it was named surfactins because of its exceptional surfactantactivity.

Many workers reported the antagonism of *Bacillus* strains against plant pathogens as antifungal agents; among them the antagonism of *B. Amyloliquefaciens* against *Rhizoctoniasolani* [10], *B. subtilis* YM 10-20 against *Penicilliumroqueforti*[11], *B. subtilis* GA1 against *Botrytis cinerea* [12], *B. subtilis* UMAF 6614 toward *Podosphaerafusca* [13] and *B. subtilis* EPCO16 against tomato wilt pathogen *Fusariumoxysporum*[14]. Salem *et al.,* [15] detected *Bacillus sp.* in baker's yeast which showed inhibitory effect against *S. cerevisiae.*

The aim of this work is to assess the antimicrobial activity of *Bacillus* strains towards different microorganisms and the attempt to partial characterization of the bioactive compound produced by these strains.

2. Materials and Methods

Microorganisms

Six *Bacillus* species were identified using 16SrRNA, in previous work by [15]; and were deposited in the Gene Bank sequence database, with accession numbers KP243195 for *B. Subtilis* NS4182-01, KP243197 for *B. amyloliquefaciens* NS4182-03, KP243198 for *B. subtilis* NS4182-04, KP243199 for *G. stearothermophilus* NS4182-05, KP273194 for *B. subtilis* NS4182-06 and KP273195 for *B. amyloliquefaciens* NS4182-07.

Cultivation of *Bacillus* strains

The strains were grown in Tryptic soya broth medium (TSB) at 37±2^{o}C in shaking incubator at 150 rpm for 24 hr., the broth was centrifuged at 8500Xg for 15 min to separate the bacterial cells and obtain the cell free supernatant (CFS). The cell free supernatant was assayed for its antimicrobial activity and also used for extraction of bioactive compounds using different solvents.

Extraction of bioactive compound from *Bacillus* strains

Based on the method of Kumar A. *et al.*,[9] the bioactive compounds were extracted by different solvents including n-Hexane, ethyl acetate, diethyl ether, chloroform, and butanol. The solvent (1:1 ratio) was

added to supernatant and the mixture was agitated for one hour. The extract was separated from the supernatant using separating funnel and centrifuged for 5 min at 3000 Xg then evaporated to dryness in rotary evaporator.

To obtain the acid precipitate extract; the cell free supernatant was acidified with 6N HCl to pH 2 and kept overnight at 4^{o}C to precipitate the antimicrobial compound. After centrifugation, the pellet was extracted with methanol and evaporated in rotary evaporator to dryness. The dry films dissolved in Dimethyl Sulfoxide (DMSO) and assayed for it activity by disk diffusion method [9] [16].

Antimicrobial assay

The CFS as well as the extracts obtained from different solvent were assayed for their antimicrobial activity, first against *S.cerevisiae* on YPD agar using disk diffusion method and those that showing inhibiting activity against *S. cerevisiae* were assayed against other microorganisms including pathogenic bacteria and toxigenic fungi. Antibacterial assay was conducted using disk diffusion method on nutrient agar according to Touré Y *et al.,* and Bauer AW *et al.,* [12], [17] [18]. Dimethyl Sulfoxide (DMSO) was used as negative control, while Doxycycline Hyclate(sigma- Aldrich) at concentration 0.1mg/ml was used as positive control. The antifungal activity of viable *Bacillus* strains was conducted on PDA plates using dual culture method as described by TouréY *et al.,*[12], while, the extracts were tested for antifungal activity using disk diffusion method. Dimethyl sulfoxide (DMSO) was used as negative control and Econazole (Sigma- Aldrich) at concentration 0.1 mg/ml was used as positive control.

Tested Microorganisms

The antibacterial assay was done against two Gram positive pathogenic bacteria; *Bacillus cereus* EMCC 1080 and *Staphylococcus aureus* ATCC 13565 and four Gram negative bacteria; *Escherichia coli* O157-H7 ATCC 51659, *Salmonella typhi* ATCC 15566 *Pseudomonas aeruginosa* NRRL B-272 *and Klebsiella pneumonia* LMD 7726.The strains were obtained from marine toxins Lab., Food toxins and contaminants dept., National Research Center and grown on nutrient agar slants at 37^{o}C for 24 hr. and kept in refrigerator at 4^{o}C until subsequent use.

The antifungal assay was done against six fungal species including *Aspergillusfalvus* NRRL 3357, *A. parasiticus* SSWT 2999*, A. carbonarius* ITAL 204*, A. ochraceus* ITAL 14*, Fusariumverticillioides* ITEM 10027 and *F. proliferatum* MPVP 328. The strains were obtained from applied mycology department, Cranfield University, UK.

Characterization of antimicrobial bioactive compounds

Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy (FTIR) was employed as reported by Kumar A *et al.,* [9] and Varadavenkates anand Murty [19]. The technique helps to explore the functional groups and the chemical bonds present in the crude extract (chloroform and HCl extracts). FTIR results were recorded on Jasco FTIR 6100. The spectra were scanned in the range of 400- 4000 cm^{-1}. The spectra were obtained using potassium bromide (KBr). Thus, (100 mg KBr) was mixed with sample (1 mg). The spectra were plotted as intensity versus wave number. The spectrum was studied to interpret the chemical nature of the bioactive fraction.

Gas Chromatography (GC)

To obtain the fatty acid methyl ester (FAME) a method based on [20] and [21] was used. Five mg of HCl and chloroform extracts were methylated with methanol acidified with 5% H_2SO_4 in at 90°C for 15 hr. in sealed tubes. The residue was extracted by n-Hexane and dried with anhydrous Na_2SO_4 and the hexane was concentrated under nitrogen to obtain extracts containing FAME.

Gas Chromatography (GC):Perkin Elmer Auto System XL equipped with flame ionization detector (FID) was used for analysis under the following conditions: Fused silica capillary column ZB-5 (60 m x 0.32 mm i.d), Oven temperature was maintained initially at 150°C and programmed from 150 to 240°C at rate 3°C/min. Injector temp. 230°C, Detector temp. 250°C, Carrier gas, Helium, flow rate 1 ml/min, Sample size 2µl, and split 1:10**.**

Statistical analysis:

Experiments was carried out in triplicate and the results was expressed as mean ±SE. Data were tested for statistical significance by analysis of variance (ANOVA) using SAS computer program by SAS Institute Inc., Cary, NC, USA).

3. Results and Discussion

Effect of bioactive compounds from *Bacillus* strains on *S. cerevisiae*

The cell free supernatant, chloroform and HCl extracts of *Bacillus* strains showed inhibition activity against *S. cerevisiae*, while other solvent extracts did not show any inhibition. Table (1) showed the effect of chloroform and HCl extracts of *Bacillus* strains on *S. cerevisiae*, also the CFS showed inhibition activity against *S. cerevisiae* (data published in previous work[15]). The chloroform extract of *B. subtilis* NS4182-06 showed the maximum inhibition against *S. cerevisiae* with inhibition zone of 17 mm which approximately equal the effect of 0.5 mg/ml of econazole solution with no significant difference between them while the chloroform extracts of the *B. amyloliquefaciens*NS4182-03 and *B. amyloliquefaciens*NS4182-07 showed the minimum inhibition zone with 12.3 mm each, which is higher than the effect of 0.1 mg/ml econazole. However; the HCl extract of *B. amyloliquefaciens*NS4182-07 showed the maximum activity with inhibition zone 14.3 mm. In general, the chloroform extracts of *Bacillus* strains showed higher activity over than that obtained by the HCl extracts.

Table1. Effect of *Bacillus* chloroform and HCl extracts on the growth of *S. cerevisiae*.

***Bacillus* strains**	***S. cerevisiae* growth inhibition in mm (Mean± SE)**	
	Chloroform extract	HCl extract
B. subtilis NS4182-01	14± 0.91^{cd}	0.0± 0^{e}
B. subtilis NS4182-06	17.0± 1.78^{ab}	0.0± 0^{e}
*B. amyloliquefaciens*NS4182-03	12.3 ± 1.18^{cd}	10.3 ± 0.25^{d}
*B. amyloliquefaciens*NS4182-07	12.3 ±0.85^{cd}	14.3 ± 0.25^{b}
*B. subtilis*NS4182-04	13.8 ± 1.11^{cd}	11.7 ±0.63^{c}
G. stearothermophilus NS4182-05	14.5 ± 0.29^{bc}	10.0 ± 0.0^{d}
0.1 mg/ml Econazole	11.3 ± 0.5^{d}	11.3 ± 0.25^{c}
0.5 mg/ml Econazole	17.6 ± 0.75^{a}	17.6 ± 0.38^{a}

It is to be concluded that the HCl extracts didn't show more inhibition than that of chloroform extracts. In this respect, *B. subtilis* NS4182-06 is the most inhibitor strains for *S. cerevisiae* followed by *G. stearothermophilus* NS4182-05. Therefore, it is of importance to evaluate the antimicrobial inhibitory effect of these strains.

Antibacterial activity of *Bacillus* strains

Bacillus strains cell free supernatant, chloroform and HCl extracts that showed inhibition against *S. cerevisiae* were assayed for its antibacterial and antifungal activity. Figure1 showed the effect of *Bacillus* strains cell free supernatant, chloroform and HCl extract against Gram positive and Gram-negative bacteria. The HCl extract of *B. subtilis* NS4182-01 showed higher activity, over its corresponding cell free supernatant and chloroform extract, against all tested bacteria except *K. pneumonia* and *E. coli* which showed higher inhibition by CFS and chloroform extract respectively.

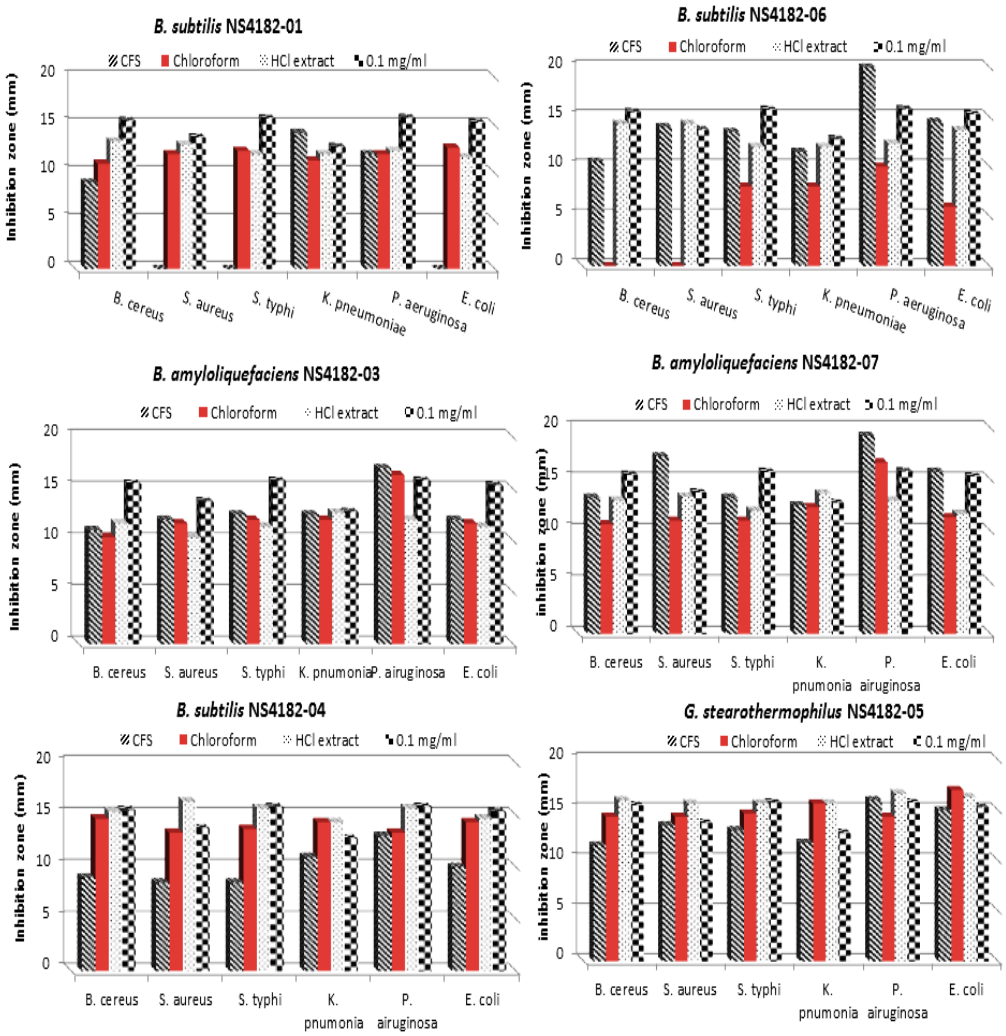


Figure 1. Antibacterial effect of *Bacillus* isolates cell free supernatant (CFS), chloroform and HCl extract compared with 0.1mg/ml doxycycline against Gram positive and Gram-negative bacteria.

The highest activity of *B. subtilis* NS4182-01 was recorded by its CFS against *K. pneumonia* with 14.3mm inhibition zone which is over the effect of 0.1mg/ml doxycycline (12.8 mm). The CFS of *B. subtilis* NS4182-06 showed higher activity against *S. typhi, P. aeruginosa* and *E. coli* with 13.5, 20, and 14.5 mm inhibition zones respectively. The higher activity against the other three tested bacteria was recorded by the corresponding HCl extract of the same isolate with 14.3, 14.3 and 12 mm for *B. cereus, S. aureus*and *K. pneumonia* respectively.

Also, CFS of *B.amyloliquefaciens*NS4182-03 showed activity higher than its corresponding HCl and chloroform extracts against *S. aureus, S. typhi, P. aeruginosa* and *E. coli*, while the higher activity against *B. cereus* and *K. pneumonia* was recorded by HCl extract of the same isolate. The highest inhibition zone was developed by CFS of *B. amyloliquefaciens* NS4182-03 against *P. aeruginosa* with 17mm inhibition zone, which is superior to the effect of 0.1mg/ml doxycycline. The same pattern of inhibition was obtained by *B. amyloliquefaciens* NS4182-07 as the CFS of this strain recorded inhibition activity higher than activity of its corresponding chloroform and HCl extract; against all tested bacteria except *K. pneumonia, which* showed the higher inhibition by HCl extract with 13.7 mm inhibition zone. The highest inhibition zone was developed by the CFS of *B. amyloliquefaciens* NS4182-07 against *P. aeruginosa* with 19.3mm inhibition zone which over the effect of 0.1mg/ml doxycycline.

The HCl extract of *B. subtilis* NS4182-04 recorded higher activity against all tested Gram positive and negative bacteria when compared with the corresponding CFS and chloroform extract of the same isolate. The highest activity was recorded with HCl extract against *S. aureus* with inhibition zone 16.3 mm, which is over than that developed by 0.1 mg/ml doxycycline (13.8 mm). Also, the HCl extract of *G. stearothermophilus* NS4182-05 showed the higher activity, over the corresponding CFS and chloroform extract, towards all tested bacteria except *E. coli* which showed the highest inhibition by chloroform extract of the same isolate with 17 mm inhibition zone which is superior to the effect of 0.1 mg/ml doxycycline (15.3 mm inhibition zone).

Considering that each mm of inhibition zone could be evaluated as one inhibition unit, the obtained results indicated that HCl extracts of *G. stearothermophilus* NS4182-05 and *B. subtilis* NS4182-04 was the most inhibitive strains to the tested bacteria followed by the CFS of *B. amyloliquefaciens* NS4182-07 and Chloroform extract of *G. stearothermophilus* NS4182-05.

Das *et al.,* [22] tested the antimicrobial activity of solvent extracted bio-surfactant derived from *Bacillus circulans*against Gram positive and Gram-negative bacteria. The inhibition zone was 17 mm for *Micrococcus flavus* and 15.33 mm for *B. pumilus* while the inhibition zone for *E. coli* NCIM 5138 was 14.6 mm and 12mm for *Klebsiellaaerogenes* NCIM 2098.Sihem*et al.,*[23]reported that the two *Bacillus* isolates exhibited antibiotic activity towards Gram negative bacteria *E. coli* and *P. aerugenosa* with 20.5 mm inhibition zone and against the Gram positive bacteria *M. luteus* with 14.25 mm, while the activity against *S. aureus* and *M. phlei* was not recorded. Baindara*et al.,* [24]isolated a bacterial strain producing two antimicrobial peptides, which identified as *B. subtilis* based on both

phenotypic and 16SrRNA gene sequence phylogenetic analysis. The antimicrobial peptide display a narrow spectrum activity against Gram-positive bacteria such as *L. monocytogenes*(MTCC839), *S. aureus* (MTCC1430), *S. mutans* (MTCC497) and *M. lutus* (MTCC106). *L. monocytogenes*(MTCC839), *S. aureus* (MTCC1430) are more sensitive strains, while no activity could be detected towards the tested Gram-negative bacteria.

Antifungal activity of *Bacillus* strains

Antagonism of whole bacterial culture against tested fungi

The antifungal activity of whole culture of *Bacillus* strain were determined using dual culture method with tested fungi on PDA plates figure (2). The reduction percent of mycelial growth was illustrated in table (2). The isolate *B. amyloliquefaciens* NS4182-07 showed the maximum reduction against *A. flavus*, *A. carbonarus* and *F. verticelloides* with percent 47.4%, 51.4% and 46.6% respectively, while the isolate *B. subtilis* NS4182-04 showed the maximum reduction for *A. parasiticus and A. ochraceus* with 49.4% and 39.1% respectively. The isolate *B. subtilis* NS4182-06 recorded a minimum reduction activity for all tested fungi. There are some significant differences between the effects of *Bacillus* isolates.

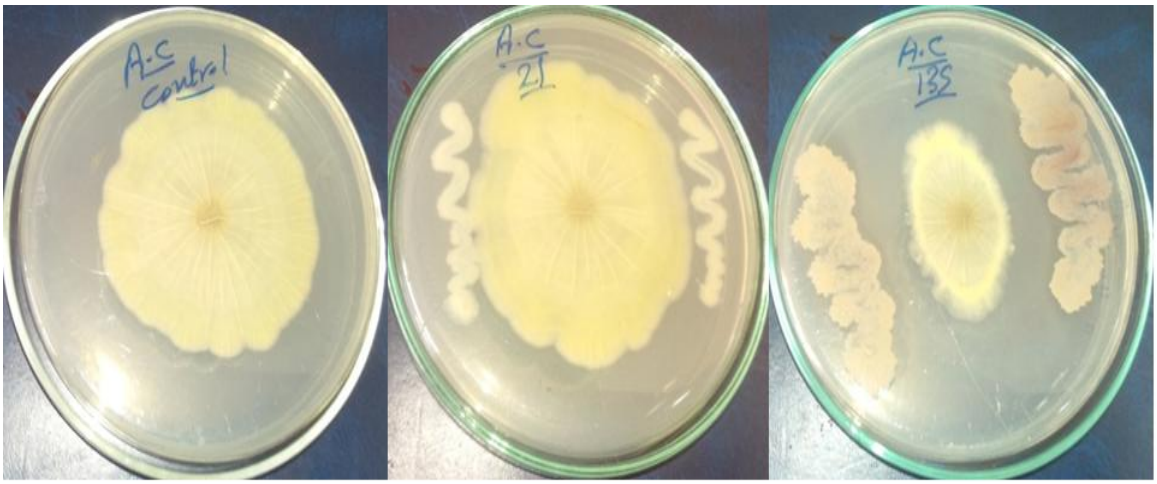


Figure 2. Antagonism between bacterial culture and tested fungi using dual culture method on PDA plates.

Antifungal effect of chloroform and HCl extracts of bacterial strains

Figure (3) showed the antifungal activity of HCl and chloroform extracts of the *Bacillus* strains compared with the effect of 0.1mg/ml econazole solution. The HCl and chloroform extracts of *B. subtilis* NS4182-01 showed the same effect against *A. flavus, A. parasiticus* and *F. prolrferatum* with 8.7, 9.3 and 8.7 mm inhibition zone respectively. While, the chloroform extract showed the higher activity against *A. ochraceus* and *F. Verticelloides* with 8.7 and 10.7 mm inhibition zone, respectively. The chloroform extract of *B. subtilis* NS4182-06 showed higher activity against *A. ochraceus* and two tested *Fusarium* species, while the corresponding HCl extract of the same isolate showed higher activity against the other tested *Aspergillus* species. The HCl extracts of the isolates *B. amyloliquefaciens* NS4182-03 and *B. amyloliquefaciens*

NS4182-07 showed higher activity than its corresponding chloroform extracts against all tested fungi. The highest inhibition zone was recorded by HCl extract of *B. amyloliquefaciens* NS4182-07 against *A. ochraceus* with 16.7 mm, which was over than that developed by 0.1mg/ml econazole solution. Also; the HCl extracts of the isolates *B. subtilis* NS4182-04 and *G. stearothermophilus*NS4182-05 showed higher activity against all tested *Aspergillus* species; while the higher activity against tested *Fusarium* species was developed by the corresponding chloroform extracts of the same isolates. The highest inhibition zone was developed by HCl extract of *G. stearothermophilus* NS4182-05 against *A. ochraceus* with 18 mm inhibition zone, which was over than that obtained by 0.1 mg/ml econazole (11.3 mm). Considering that each mm of inhibition zone could be evaluated as one inhibition unit, the obtained results indicated that HCl extracts of *B. amyloliquefaciens* NS4182-07*and B. amyloliquefaciens* NS4182-03 is the most inhibitive strains to the tested fungi followed by the chloroform extract of the strain *B. subtilis* NS4182-06 and HCl extracts of *G. stearothermophilus* NS4182-05 and *B. subtilis* NS4182-04. From the antibacterial and antifungal assays, it is to be concluded that the HCl extracts of *G. stearothermophilus* NS4182-05, *B. subtilis* NS4182-04 followed by *B. amyloliquefaciens* NS4182-07, *B. amyloliquefaciens* NS4182-03 and chloroform extract of *B. subtilis* NS4182-06 was the most inhibitive strains to tested microorganisms.

The present results are in agreement with those reported by several authors. Ramayabharathi and Ragachander [14] assayed the antibiotic activity of crude extract from *B. subtilis* EPCO16 against *Fusariumoxysporum* and recorded an inhibition of mycelial growth to an extent of 46.04%. Romero *et al.,* [13] reported that the antifungal effects of cell free supernatant as well as the presence of antifungal compounds bacillomycin, fengycins, iturin A and surfactin are the key factors in antagonism of *B. subtilis* toward *Podosphaerafusca*.

Table 2. Effect of whole bacterial isolates on tested fungi by dual culture method

Isolates	Reduction of mycelial growth (%)					
	A. flavus	***A. carbonarus***	***A. parasiticus***	***A. ochraceus***	***F. verticelloides***	***F. prolrferatum***
B. subtilis NS4182-01	34.4±2.9^{b}	47.2±2.8^{ab}	28.9±4.4^{b}	26.8±3.2^{b}	40.7±4.7^{bc}	35.1±1.7^{ab}
B. subtilis NS4182-06	0.0±0.0^{c}	1.9±1.8^{d}	4.2±4.1^{c}	0.0±0^{c}	4.6±4.5^{d}	4.0±3.9^{c}
B. amyloliquefaciens NS4182-03	44.7±1.8^{a}	27.8±5.5^{c}	43.3±1.7^{a}	44.9±3.1^{a}	35.2±1.2^{c}	37.7±4.4^{a}
B. amyloliquefaciens NS4182-07	47.4±4.5^{a}	51.4±6.9^{a}	45.8±0.8^{a}	26.8±3.2^{b}	46.6±2.5^{ab}	32.8±4.0^{ab}
B. subtilis NS4182-04	46.1±3.2^{a}	39.3±4.0^{abc}	49.4±6.1^{a}	39.1±0.9^{a}	36.2±0.2^{c}	33.8±0.4^{ab}
G. stearothermophilus NS4182-05	44.7±1.8^{a}	35.0±1.6^{bc}	39.4±2.8^{ab}	28.7±1.3^{b}	51.0±1.0^{a}	26.7±2.3^{b}

Means with the same letter in the same column are not significantly different (Means ±SE).

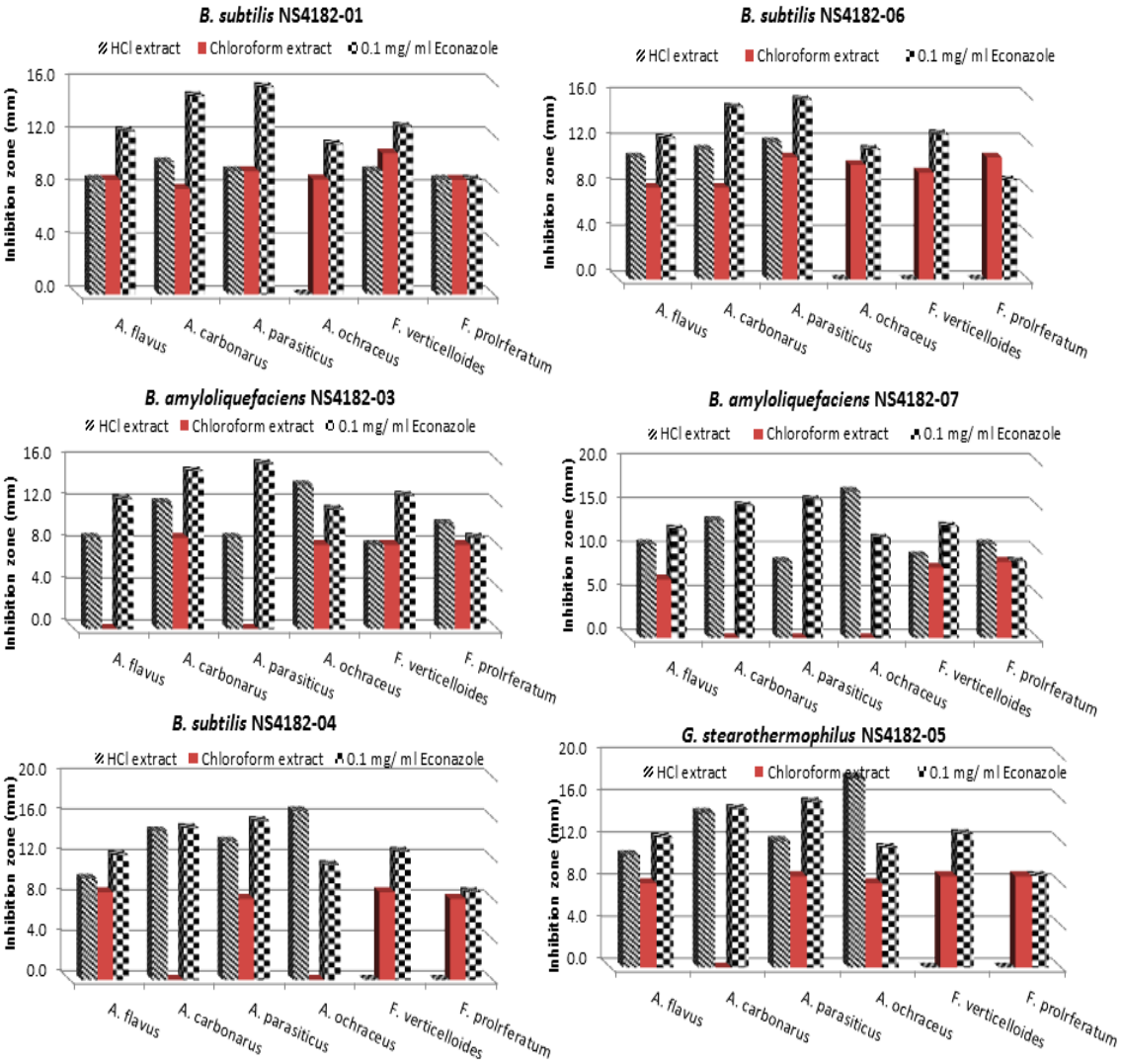


Figure 3. Antifungal activity of *Bacillus* strains chloroform and HCl extracts compared with 0.1 mg/ml Econazole solution against tested fungi.

Tourée*t al.,* [12]reported the antagonism developed by *B. subtilis* on PDA plates against fungal pathogens and recorded the percentage of reduction of mycelium expansion compared with control plates.The inhibition percent for *F. graminearum, F. oxysporum, A. flavus* and *A. niger* was 63%, 58%, 55% and 51%, respectively. Also, the inhibition of *Fusarium spp.* by *B. subtilis, B. circulans* and *B. amyloliquefaciens* with inhibition zone more than 5mm was reported by Hsieh F. C *et al.,*[25].

Chitarra*et al.,* [11]reported that the supernatant fluid of *B. subtilis* strain YM 10-20 inhibited the growth of *P. roqueforti, A. niger, A. parasiticus, A. flavus, Mucor sp.* and *F. clumorum,* while the growth of *Rhizopus sp.* was not affected and *F. cluorum* was the most sensitive fungus. Also, one fraction of HCl precipitate (separated by HPLC) showed inhibition zone against growth of *P. roqueforti* which revealed the same retention time and peak height when compared with HPLC profile of purified iturin A. Grover *et al.,* [16] assessed the in

vitro antagonism of phyto-pathogenic fungi by *B. subtilis* RP24 among these phyto-pathogenic fungi were *F. solani*, *F. oxysporum* and *A. niger* with inhibition zone 7.85, 8.25 and 8.50mm respectively.

Characterization of the antimicrobial bioactive compounds produced by *Bacillus* strains

Data obtained for the antibacterial and antifungal assays showed that the six *Bacillus* strains produced bioactive compounds with antibacterial and antifungal activity. This coincide with previous literature reporting that *Bacillus* species produce lipopeptides with antimicrobial activity, the main compounds of which is belonging to iturin, surfactins and fengycins families. Elucidation of the compound nature is achieved through chemical analysis. The FT-IR analysis gives an overall idea about the chemical groups and bonds present in the compound.

Fourier Transform Infra-Red (FT-IR)

Figure (4) shows the identical results obtained for the FT-IR analysis of the tested chloroform and HCl extracts of six inhibitory *Bacillus* strains. The absorbance at 3403 cm^{-1} indicates NH stretching in amide A of protein. The peaks around 2928 and 2864cm^{1} indicates Asymmetric and symmetric stretching of CH_2 in lipid. The absorbance at 1723cm^{-1} indicates the C=O stretching of carbonyl group. The peaks at 1653 and 1527 cm^{-1} indicates C=O stretching vibration in amide I and NH bending vibration in amide II respectively. The absorbance at 1455 cm^{-1} is indicates the presence of CH_2 in aliphatic chain. The absorbance at 1031cm^{-1} is due to C-O stretch. The recorded chemical groups and bonds demonstrate the presence of lipopeptides compound in all *Bacillus* chloroform and HCl extracts.

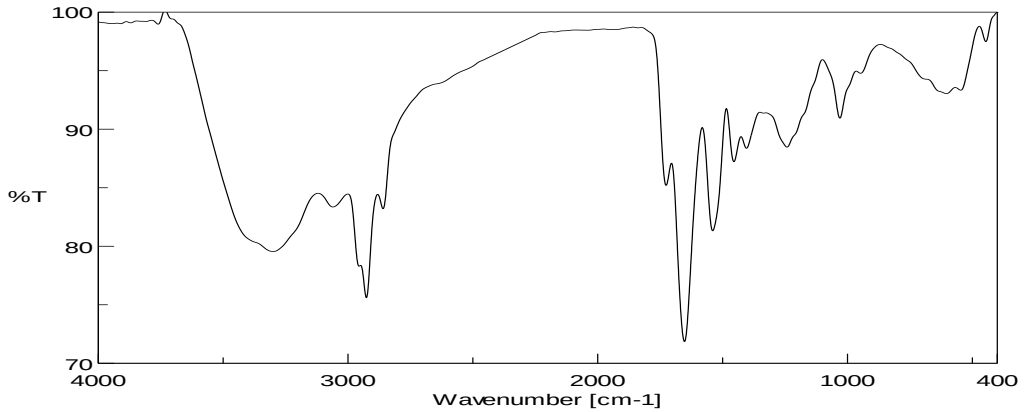


Figure 4.Fourier Transform Infra-Red(FT-IR) spectrum of antimicrobial compound from *Bacillus* strains.

The present results coincide with that reported by Romero D *et al.,* [13]. The FT-IR analysis for active extracts from *Bacillus subtilis* strains showed bands in the range of 1630 to 1680 cm^{-1}, resulting from the stretching mode of the CO-N bond (amide I band), and at 1570 to 1515 cm^{-1}, resulting from the deformation mode of the N-H bond combined with C-N stretching mode (amide II band),both indicating the presence of a peptide component. The bands at 2855 to 2960

cm^{-1} were resulted from typical CH stretching vibration in the alkyl chain. They also observed band at 1730 cm^{-1} due to the lactone carbonyl absorption, which is typical for surfactins and fengycins families of lipopeptides.

Likewise, Yakimov*et al.,*[20]reported that the IR spectrum of lichenysin A from *B. licheniformis* BAS50 showed strong bands, indicating the presence of a peptide component at 3300 cm^{-1} resulting from the N-H stretching mode; at 1655 cm^{-1} resulting from the stretching mode of the CO-N bond, and at 1535 cm^{-1} resulting from the deformation mode of the N-H bond combined with the C-N stretching mode. The bands at 2960 to 2860 and 1470 to 1370 cm^{-1} resulting from the C-H stretching mode suggest the presence of an aliphatic chain. These results were considered as strong evidence that lichenysin A contains aliphatic and peptide-like moieties. The band at 1735 cm^{-1} was due to lactone carbonyl absorption. These patterns were similar to those of surfactins and lichenysin B.

The fatty acid composition of *Bacillus* strains extracts

The fatty acids percent of *Bacillus* chloroform extracts was illustrated in table (3). *Bacillus* strains chloroform extracts showed fatty acids with C14-C18 with percent differs from one strain to another and it is also different from that obtained by *Bacillus* HCl extracts. The fatty acid C18 was the abundant fatty acid in all strains with percent ranged from 31.98 to 100% for *B. amyloliquefaciens* NS4182-03 and *B. subtilis* NS4182-06 respectively. *G. stearothermophilus* NS4182-05 recorded the highest percent of fatty acid with C17 followed by *B. amyloliquefaciens* NS4182-03 with 47.43 and 42.25% respectively, while the highest percent of C14 was showed by *B. amyloliquefaciens* NS4182-07 with 35.26%.The fatty acid with C16 recorded the minimum percent of all *Bacillus* strains chloroform extracts.

Also, table (3) illustrates the fatty acids percent of HCl extracts of *Bacillus* strains. The strains *B. subtilis* NS4182-06, *B. amyloliquefaciens* NS4182-07 and *G. stearothermophilus* NS4182-05 showed the higher percent of fatty acids with carbon atom C16-C18. *G. stearothermophilus* NS4182-05 showed the highest percent of C17 and C18 with 14.28 and 66.68%, respectively, while the highest percent of C16 was obtained by *B. subtilis* NS4182-01 followed by *B. subtilis* NS4182-06 with 64.12 and 44.14% respectively. The strain *B. amyloliquefaciens* NS4182-03 was the only strain showed fatty acid of C22 with 6.1%.Also, *B. amyloliquefaciens* NS4182-07 was the only strain showed fatty acid of C20 with 6.56%. The higher percent of C14 was obtained by *B. amyloliquefaciens* NS4182-03 followed by *B. subtilis* NS4182-04 with 93.89 and 72.54%, respectively.

The obtained results confirmed that the lipid moiety is unique for each strain. Romero *et al.,* [13] reported that surfactin has a number of carbon atoms in fatty acid moiety of C13-C15, and fengycins C14-C18, together with iturin A C14-C15 (strain UMAF6639), bacillomycin D C14-C16 and bacillomycin L C17 (strain UMAF8561 and UMAF 6614), or bacillomycin L C14-C17 (strain UMAF6619) as reported in the present results. This leads to the fact that several lipopeptide compounds are metabolized by *Bacillus spp.*, which is capable to inhibit the growth of *S. cerevisiae*,and had a remarkable antimicrobial activity.

Table 3.Fatty acid composition of *Bacillus* strains Chloroform and HCl precipitate methanol extracts

Bacillus strains	Fatty acid percent of Chloroform extracts				Fatty acid percent of HCl extracts					
	C14	**C16**	**C17**	**C18**	**C14**	**C16**	**C17**	**C18**	**C20**	**C22**
B. subtilis NS4182-01	2.85	ND	6.26	90.9	ND	64.12	ND	35.88	ND	ND
B. subtilis NS4182-06	ND	ND	ND	100	ND	44.14	6.17	49.68	ND	ND
B. amyloliquefaciens NS4182-03	22.8	2.97	42.25	31.98	93.89	ND	ND	ND	ND	6.1
B. amyloliquefaciens NS4182-07	35.26	8.1	ND	56.65	ND	28.94	5.39	59.1	6.56	ND
B. subtilis NS4182-04	3.37	5.67	15.4	75.56	72.54	9.94	ND	17.51	ND	ND
G. stearothermophilus NS4182-05	ND	5.55	47.43	47.02	0.95	18.18	14.28	66.58	ND	ND

ND= Not Detected

Conclusion

Bacillus species are known to have several metabolites with biological active property. In a study for identification of inhibitory microorganisms associated with the production of baker's yeast, six *Bacillus* species were identified to be contaminants that inhibit the growth of *S. cerevisiae*. The antimicrobial activity of these strains was tested. The cell free supernatants of the tested strains along with the several solvent extracts of the supernatant were examined. Only chloroform and HCl precipitate methanol extracts have the antimicrobial activity. In general, the antibacterial effect was close to that of 0.1 mg/ml doxycycline and antifungal effect was close to the effect of 0.1 mg/ml econazole. Bioactive compounds produced by *Bacillus* species include iturin and fengycins families. FT-IR analysis of chloroform and HCl precipitate methanol extracts confirmed that it contains the main functional groups of lipopeptides.

References

1. Gordillo, M. A. and Maldonado, M. C: Purification of Peptides from *Bacillus* Strains with Biological Activity, Chromatography and Its Applications, Dr. SasikumarDhanarasu (Ed.); 2012; pp 202-224.
2. Youcef-Ali M., Chaouche N. K., Dehimat L., Bataiche I., Kara Ali M., Cawoy H. and Thonar P:Antifungal activity and bioactive compounds produced by Bacillus mojavensis and *Bacillus subtilis*. African Journal of Microbiology Research 2014; 8(6): 476-484,
3. Moyne AL., Cleveland TE., and Tuzun S:Molecular characterization and analysis of the operon encoding the antifungal lipopeptides bacillomycin D. FEMS. Microbiol.Lett.2004;234:43-9.
4. Perez-Garcia A., Romero D., and de Vicente A:Plant protection and growth stimulation by microorganisms: biotechnological applications of Bacilli in agriculture. Curr. Opinion Biotech.2011; 22: 187–193.
5. Bais H.P., Fall R., and Vivanco JM:Biocontrol of *Bacillus subtilis* against infection of Arabidopsis roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin

production. Plant Physiol. 2004; 134:307-319.

6. Maget-Dana R., Thimon L., Peypoux F., and Ptack M:Surfactin/IturinA interactions may explain the synergistic effect of surfactin on the biological properties of iturin A.Biochimie1992;74:1047-1051.
7. Ongena, M. and Jacques P:*Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. Trends Microbiol. 2007;16: 3115-125.
8. Kim PI., Bai H., Bai D., Chae H., Chung S., Kim Y., Park R., and Chi YT: Purification and characterization of a lipopeptide produced by *Bacillus thuringiensis* CMB26. J. Appl. Microbiol.2004; 97:942-949.
9. Kumar A., Saini P., and Shrivastava, J: Production of peptide antifungal antibiotic andbiocontrol activity of *Bacillus subtilis*. Indian J. Experimental Biol.2009; 47: 57-62.
10. Yu G.Y., Scnclair J.B., Hartman G.L., and Bertagnolli B.L: Production of Iturin A by *Bacillus amyloliquefaciens* suppressing *Rhizoctoniasolani*. Soil biology and biotechnology 2002; 34:955-963.
11. Chitarra GS., Breeuwer P., Nout MJ., van Aelst AC., Rombouts FM., and Abee T:An antifungal compound produced by *Bacillus subtilis* YM 10-20 inhibits germination of *Penicilliumroqueforti* conidiospores. J Appl Microbiol.2003;94(2):159-66.
12. Touré Y., Ongena M., Jacques P., Guiro A., and Thonart P: Role of lipopeptides produced by *Bacillus subtilis* GA1 in the reduction of grey moulddisease caused by *Botrytis cinereaon* apple. J. Appl. Microbiol.2004; 96:1151-1160.
13. Romero D., De Vicente A., Rakotoaly RH., Dufour SE., Veening JW., Arrebola E., Cazorla FM., KuipersOP., Paquot M. and Perez-Garcia A:The iturin and fengycin families of lipopeptides are key factors in antagonism of *Bacillus subtilis* toward *Podosphaerafusca* Mol Plant-Microbe Interact. 2007; 20: 430-440.
14. RamyabharathiSA., and Raguchander T: Efficacy of Secondary Metabolites Produced by *Bacillus subtilis* EPCO16 against Tomato Wilt Pathogen *Fusariumoxysporumf. sp. lycopersici*. Journal of mycol. and plant pathol.2014; 44: No.2.
15. Salem S. H., Naguib M. M., El-Sheikh H. H. and Heikal Y. A: Determination of the microbial profile of compressed baker's yeast and studying its symbiotic interactions. International Journal of Advanced Research 2015; 3(2): 303-312.
16. Grover M., Nain L., Singh S. B. and SaxenaA.K.: Molecular and Biochemical Approaches for Characterization of Antifungal Trait of a Potent Biocontrol Agent *Bacillus subtilis* RP24. CurrMicrobiol2010; 60:99–106.
17. Bauer AW., Kirby WM., Sherris JC., and Turck M: Antibiotic susceptibility testing by standardized single method. Am. J. Clin. Pathol., 1996; 45: 493-496.
18. EUCAST (2015). Disk Diffusion Method for Antimicrobial Susceptibility Testing. European Committee on Antimicrobial Susceptibility Testing.2015 (Version 5.0). www.eucast.org.
19. Varadavenkatesan H. and Murty V. R:Production of a lipopeptide biosurfactant by a novel *Bacillus sp.* and it's applicability to enhanced oil recovery. ISRN Microbiol.2013;3: 1-8.
20. Yakimov M., Timmis K., Wray V.and Fredrickson H:Characterization of a new lipopeptide surfactant produced by thermo tolerant and halo tolerant subsurface *Bacillus licheniformis* BAS50. Appl. Environ. Microbiol.1995;61: 1706–1713.
21. Smyth T. J. P., Perfumo A., McClean S.,Marchant R., and Banat I. M:Isolation and Analysis of Lipopeptides and High Molecular Weight Biosurfactants. K. N. Timmis (ed.), Handbook of Hydrocarbon and Lipid Microbiology, 2010. Springer-Verlag Berlin Heidelberg.
22. Das P., Mukherjee S. and SenR: Antimicrobial potential of a lipopeptides biosurfactant derived from a marine *Bacillus circulans*. Journal of Applied Microbiology 2008;104: 1675–1684.

23. Sihem, B. M., Rafik, E., Mathieu, F., Mohamed, C., and Lebrihi, A: Identification and partial characterization of antifungal and antibacterial activities of two *Bacillussp*. strains isolated from salt soil in Tunisia. African Journal of Microbiology Research, 2011; 5: 1599-1608.
24. Baindara P., Mandal S. M., Chawla N., Singh P. K., Pinnaka A. K. and Korpole S: Characterization of two antimicrobial peptides produced by a halotolerant *Bacillus subtilis* strain SK.DU.4 isolated from a rhizosphere soil sample. AMB Express 2013, 3:2. http://www.amb-express.com/content/3/1/2.
25. Hsieh F. C., Lin T. C., Meng M., and Kao S. S:Comparing Methods for Identifying *Bacillus* Strains Capable of Producing the Antifungal Lipopeptide Iturin A CurrMicrobiol, 2008; 56:1–5.