Isolation and identification of antifungal substances producer Bacillus

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THE ABSTRACT

Fifteen *Bacillus* isolates originally isolated from soil surfaces were evaluated for possible antagonistic activity against some of molds included, *Aspergillus*, *Penicillium*, *Fusarium and Rhizopus* species. Five of the isolates (1, 3, 10, 13 and 15) possessed noticeable antagonistic properties, and were effective in suppressing the growth of previous mentioned pathogens. The maximum Inhibition (52.6%) against *A. niger* was noticed by isolate No: 15 at supernatant concentration of 35% v/v**.** The most active isolate named MHS15 identified by 16S rRNA gene sequencing as *Bacillus subtilis* (accuracy of 100%) which was matched with the sequence of *B. subtilis* OH2377A which recorded in Genebank under the Accession Number of gbIKF030229.1.

Analysis of methanolic extract of *B. subtilis* MHS15 by RP-HPLC revealed that there were three isomers of iturin A at retention times of 3.025, 5.160 and 11.576 min which were conformable with the isomers formed from standard iturin A at the same retention times which were; 3.057, 5.178 and 11.753 min. Results obtained using the Electrospray ionization - Mass Spectrometry (ESI-MS) clarified that the three isomers of iturin A produced by *B. subtilis* MHS15 which had molecular weights of 1043.46, 1057.37 and 1071.48 Da respectively, reveals that they belong to iturin A2, A3 or A4 or A5 (which have the same M.W.) and A6 or A7 (which have the same M.W.) respectively. All isomers produced by *B. subtilis* MHS15 were found to have an inhibitory action against to the growth of *A. niger.*

Introduction

Biological control of plants by microorganisms is a very hopeful alternative to the increased consumption of chemical pesticides, which cost high and accumulate in plants, having different deteriorative effects on humans. Such chemicals can also be lethal to the useful inhabitants of soil (Leroux, 2003). Moreover, the presence of undesirable chemical compounds in different food linked with the appearance of fungicide-resistant strains of pathogens leads for an alternative which be non-polluting strategy for controlling plant diseases (Balhara *et al*, 2011), suppression of plant diseases caused by microorganisms due to the production of a wide range of antimicrobial compounds (Ongena *et al*., 2005b), competition in colonization for the nutrients with species nonstimulating for plant growth or even pathogenic (Bais *et al*., 2004; Timmusk *et al*., 2005) and enhancing of the host defense system by induced systemic resistance (ISR) (Ongena *et al*., 2005a). Plant stimulatory effects may be also obtained by an increased availability of nutrients for the absorption from the soil, for instance, nitrogen, phosphorus, amino acids (Idriss *et al*., 2002). Recently the attention has focused on the Gram-positive members of the aerobic, spore-forming genus *Bacillus*. Among them, *Bacillus subtilis* was found to, increase plant growth, and colonizes the root surface and inhibits fungal growth, such fungi might cause a wide range of important plant diseases that caused by the mycelia growth of *Aspergillus niger, Fusarium oxysporum*, *F. solani*, *Sclerotinia sclerotiorum*.and *Rhizoctonia solani* (Souto *et al*., 2004). The genus *Bacillus* has wide suppressive properties for more than 20 types of plant pathogens as a result to its ability to produce different antibiotics with an amazing variety of structures and activities (Stein, 2005). Those compounds include mostly peptides (surfactin, iturin and fungycin) that are either of ribosomal origin or are generated nonribosomally. The characteristics which determine their effectiveness are the wide spectrum of action and resistance to hydrolysis by proteolytic enzymes. Their activity is also resistant to high temperatures and a wide range of pH (Souto *et al*., 2004).

The aim of this study is to isolate and identify a locally *Bacillus subtilis* and to evaluate its antifungal activity against some pathogenic fungi.

Materials and Methods:

Isolation of *Bacillus* species

Methods used for screening various *Bacillus* strains were based mainly on the resistance of their endospores to elevated temperatures (Sadfi *et al*., 2001). Soil samples were placed in sterile plastic bags. Each soil sample

(20 g) was suspended with 20 ml of sterile distilled water in a sterile universal bottle. Soil suspensions were mixed and placed in a water bath with temperature adjusted to 100ºC for 5 min with gentle shaking. After heat treatment, heat-treated soil suspensions were incubated at room temperature for 2 h and serially diluted prior to plating on tryptone soya agar for isolation of single colonies. Plates were incubated at 37 ºC for 72 h. Different isolates were picked from the plates. (Kok-Gan *et al*., 2007). Different isolates were maintained on TSA slants at 4°C and subcultured each month (Rebib *et al*., 2012).

Extraction of bacterial supernatant

Each isolate was grown in TSB medium at 37˚C for 72 h. The supernatants of cultural media of different bacterial isolates were separated by centrifugation at 10 000 g for 20 min followed by filteration (sterilization) them through 0.21µm membrane filter to remove the bacterial cells. This supernatant considered as a crude antifungal agent extract and kept in sterilized plastic tube for determination of antifungal activity or further use **(**Tendulkar *et al*., 2007).

Antifungal activity of isolated bacteria

The antifungal activity against the tested pathogens was determined according to poisoned food technique (method of Grover and Moore 1962). The following tested fungi were used in this technique which was obtained from the Department of Plant Protection / College of Agriculture:

1. *Aspergillus flavus*
2. *Aspergillus niger*
3. *Fusarium solani*
4. *Fusarium spp*
5. *Penicillium gititatum*
6. *Penicillium spp*
7. *Rhizopus spp*

Poisoned food technique involves, preparing of 25 ml aliquots of sterilized potato dextrose agar media which supplemented separately with; 15 and 35%, v/v of sterilized bacterial supernatants as a crude antifungal agent extract. Media were poured in sterilized plates under aseptic conditions, allowed to cool and solidify. 6 mm discs (from the edge) of six days old culture from the tested fungi were inoculated at the centre of PDA Petri dishes. The plates were incubated at 26°C for 5-7 days. The Petri dishes containing media free of the supernatant served as control. After incubation, the colony diameters for the fungi at each plate were measured in millimeter (Satish, 2007**)**.

The percentage inhibition of mycelial growth was calculated using the formula: -

Percent inhibition = C – T / C * 100

Where C = Mycelial growth in control plate

T = Mycelial growth in treatment plate.

Identification of *Bacillus* isolates

The most active antifungal isolate was identified by Vitek 2 compact system which involved Forty six biochemical tests,in addition to 16S rRNA gene sequencing which was conducted by LGC Genomics Sequencing Service in Germany and the preparation of samples was done according to its instructions as follows:

Single colony from each bacterial sample were inoculated into 5 ml of nutrient broth and incubated at 37˚C on a rotary shaker at 200 rpm for 16-18 h, and bacterial suspensions were centrifuged. The precipitates were individually dissolved in 1 ml of 70% ethanol using eppendorf tubes. All steps were done under aseptic conditions. The total genomic DNA was extracted using the DNA Extraction Kit (Life Technologies Co., Germany).The forward primer F27 (5-AGAGTTTGATCATGGCTCAG-3) and reverse primer R1492 (5-TACGGTTACCTTGTTACGACTT-3) were used in PCR. The PCR was performed on an automated thermocycler device using the following parameters: denaturation at 94 ˚C for 4 min followed by 35 cycles of denaturation at 94 ˚C for 40 s, annealing at 50 ˚C for 50 s, elongating at 72 ˚C for 80 s and a final extension at 72 ˚C for 8 min. The 16S rRNA gene sequencing of isolate No. 15 were analyzed and aligned with the related sequences retrieved from GenBank database using The Basic Local Alignment Search Tool (BLAST) on The National Center for Biotechnology Information (NCBI).

Detection of antifungal active substances by RP- HPLC

The standard stock solution was prepared by dissolving 1mg/ml of iturin A obtained from *B. subtilis* MHS15. This solution was stored in dark glass bottle at 4 ºC and was stable for at least 1 month. Working standard solution was freshly prepared by dissolving suitable amount of the above solution in methanol 65% v/v for RP-HPLC and then directly injected into the HPLC (Yerra *et al*., 2008) Acid Precipitation and Methanol Extraction method (APME) was used as sample preparation for RP-HPLC (Phae and Shoda, 1991). In this method Forty milliliters of culture supernatant of *B. subtilis* MHS15 was acidified to a pH of 2.0 using HCl (0.5N) and then

incubated for 18 h at 4°C. Precipitates were collected by centrifugation at 8000 g for 10 min at 4°C and then extracted with 4 mL of methanol by shaking for 30 min. The methanol extract was collected by centrifugation at 8000 g for 10 min at 4°C.

Confirmation of iturin fractions using Mass Spectrometry

The fractions detected as iturin A by RP-HPLC were selected to confirm the iturin A fractions at concentrations of (184.26, 19.61and 55.36 µg/ml) at retention times of 3.025, 5.160 and 11.576 minutes respectively.. Each sample has been dissolved in 1 ml methanol in eppendorf tubes. The major molecular ions [M+H] with M/Z was determined by Mass Spectrometry and the results were compared with the molecular weight of standard iturin.

Results and Discussion

Isolation on basis of spores forming

One of the main natural habitats for *Bacillus* species is the upper layers of the soil and plant rhizosphere (Earl *et al*., 2008). Therefore the selection of collected samples was conducted from a specific depth of Abu-Graib soil (5-7 cm). Fifteen distinct isolates have survived heat treatments at 100˚C for 5 min and that was due to their spore's resistance to high temperature (Sadfi *et al*., 2001). Heat survived isolates were subcultured on TSA medium.

Isolation according to antagonistic activity

Among the isolates tested for antagonism in vitro, only 5 bacterial isolates (1, 3, 10, 13, and 15) showed distinct antagonism compared with others against most of tested molds (Table 1) *Aspergillus* and *Penicillium* species were affected by bacterial supernatant more than *Fusarium* sp. *Rhizopus.* sp was not influenced by any of tested isolates which might be due to its rapid and wide spreading filamentous and branching hyphae, (Pusey 1989; Zheng *et al*., 2007) or this may be explained by the low ergosterol content of the *Rhizopus sp*. membrane (Schnurer 1993).The effective isolates were, Gram-positive, rod shaped and forming spores,. The maximum Inhibition (52.6%) against *A. niger* was noticed by the isolate No: 15 at supernatant concentration of 35% v/v (Fig 1**).** Thus *A. niger* was used for further antifungal activities tests. Inhibitory effect increased proportionally with supernatant concentrations; however an economic feasibility must be taken in account. The production of extracellular secondary metabolites screened by the active isolates might be the cause of inhibition action (Joseph, 2003).

Table 1: Isolation of *Bacillus* species according to their antifungal activity by supplementation of PDA medium with a supernatant (35% v/v) of each isolate.

		Inhibition (%)						
Isolate no.	Supernatant(%)v/v	*Aspergillus niger*	*Aspergillus flavus*	*Penicillium gititatum*	*Penicillium.spp*	*Fusarium solani*	*Fusarium.spp*	*Rhizopus.spp*
1	15	0	0	0	0	0	0	0
	35	28.4	31.6	32.8	30.7	24.2	19.6	0
2	15	0	0	0	0	0	0	0
	35	14.6	6.4	12.6	9.2	7.5	9.4	0
3	15	0	0	0	0	0	0	0
	35	40.5	38.8	30.0	34.6	30.4	27.6	0
4	15	0	0	0	0	0	0	0
	35	11.3	7.4	10.2	8.4	6.8	8.6	0
5	15	0	0	0	0	0	0	0
	35	14.4	12.6	8.4	10.5	7.5	6.5	0
6	15	0	0	0	0	0	0	0
	35	12.9	6.6	7.5	6.7	13.6	9.8	0
7	15	0	0	0	0	0	0	0
	35	7.6	9.6	4.9	6.4	12.8	10.0	0
8	15	0	0	0	0	0	0	0
	35	10.8	14.7	5.8	8.5	5.0	8.4	0
9	15	0	0	0	0	0	0	0
	35	10.4	7.6	16.4	13.6	8.5	12.4	0
10	15	0	0	0	0	0	0	0
	35	40.0	36.4	34.6	38.3	21.0	22.8	0
11	15	0	0	0	0	0	0	0
	35	9.3	12.6	6.8	7.4	10.5	8.0	0
12	15	0	0	0	0	0	0	0
	35	12.8	14.6	9.8	7.0	5.9	7.2	0
13	15	0	0	0	0	0	0	0
	35	41.4	35.4	38.5	39.5	32.4	32.1	0
14	15	0	0	0	0	0	0	0
	35	8.6	9.4	12.8	9.5	7.4	9.2	0
15	15	0	0	0	0	0	0	0
	35	52.60	44.8	42.8	44.0	36.2	39.3	0

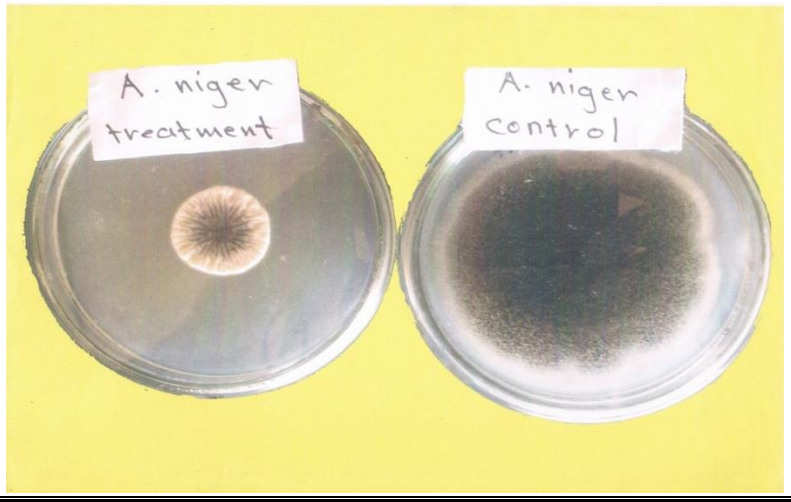


Fig 1: Inhibition of *Aspergillus niger* grown on potato dextrose agar plus 35% v/v of the cell free filtrate of the isolate No 15.

Identification tests of selected bacterial isolates using 16S rDNA

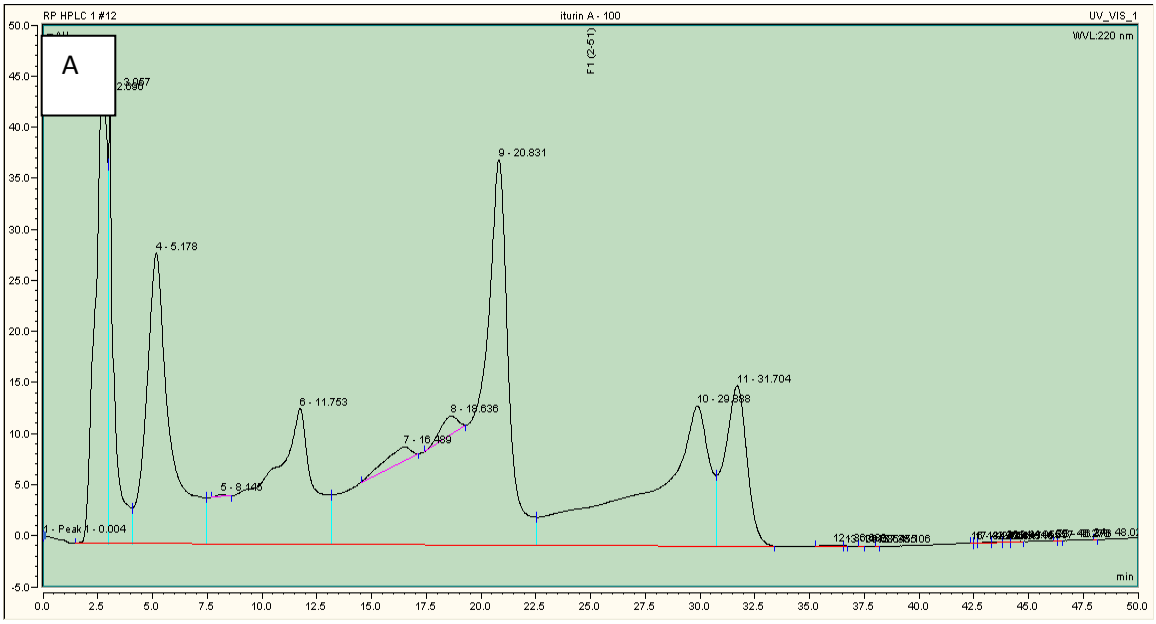
Among several strains of *Bacillus* which isolated from soil, one strain (No.15) out of others showed prominent antifungal activity and it was identified as *Bacillus pumilus* by vitek 2 system. Using 16S rRNA gene analysis, this strain was identified as belonging to the species *subtilis* and had 100% similarity with *B. subtilis* OH2377A on the basis of cataloging Genbank database under accession number of ID: gbIKF 030229.1. It has been given a code number of MHS15 to distinguish it from other isolates.

Analysis of standard iturin A and extracted antifungal agents of *B. subtilis* MHS15.

The most commonly employed technique for detection of antifungal agent produced by *B. subtilis* MHS15 is Reversed Phase Chromatography, which results in separation of each lipopeptide structure based on polarity (Yerra *et al*., 2008). Separation of standard iturin and correspond isomers of the extracted materials of *B. subtillus* MHS15 have been achieved with methanol - water (65% v/v) as isocratic elution. As shown in Fig (2) standard iturin A contain 7 peaks No. as; 3, 4, 6, 7, 9, 10 and 11 suggest to be; A2, A3, A4, A5, A6, A7 and A8 at retention times; 3.057, 5.178, 11.753, 16.489, 20.83, 29.88, 31.70 respectively. The purified crudes of *B. subtillus* MHS15 had three main peaks (4, 5 and 7) RP-HPLC had also the same number of main peaks (6, 8 and 12) which were observed at elution times comparable with those observed for standard iturin A and they might be the isomers of iturin A at retention times; 3.025, 5.16 and 11.57 for *B. subtillus* MHS15 (Table 2 and Fig 2, A and B). Different parameters for RP-HPLC analysis were varied, such as temperature of analytical column (in the range of 20-30 ºC). Flow-rate and the type of column affect on quantity of separated compounds, and sample solvent (Yerra *et al*., 2008). RP-HPLC is an excellent method for the separation of lipopeptides (Aguilar, 2004). Although methods like thin layer chromatography (Desai & Banat, 1997), ion exchange chromatography (Mukherjee *et al*., 2006), gel permeation chromatography (Mukherjee *et al*., 2009) and ultrafiltration (Sen & Swaminathan, 2005) have been used for the separation and identification of lipopeptide biosurfactants. These techniques have a serious limitation as they do not separate individual analogue present in the crude lipopeptide mixture.

Table 2: RP-HPLC analysis for standard iturin, *B. subtilis* MHS15

Standard iturin	A2	A3	A4	A5	A6	A7	A8
Relative time (min)	3.057	5.178	11.753	16.489	20.83	29.88	31.70
***B.subtilis* MHS15**	A2	A3	A4				
Relative time (min)	3.025	5.160	11.57				



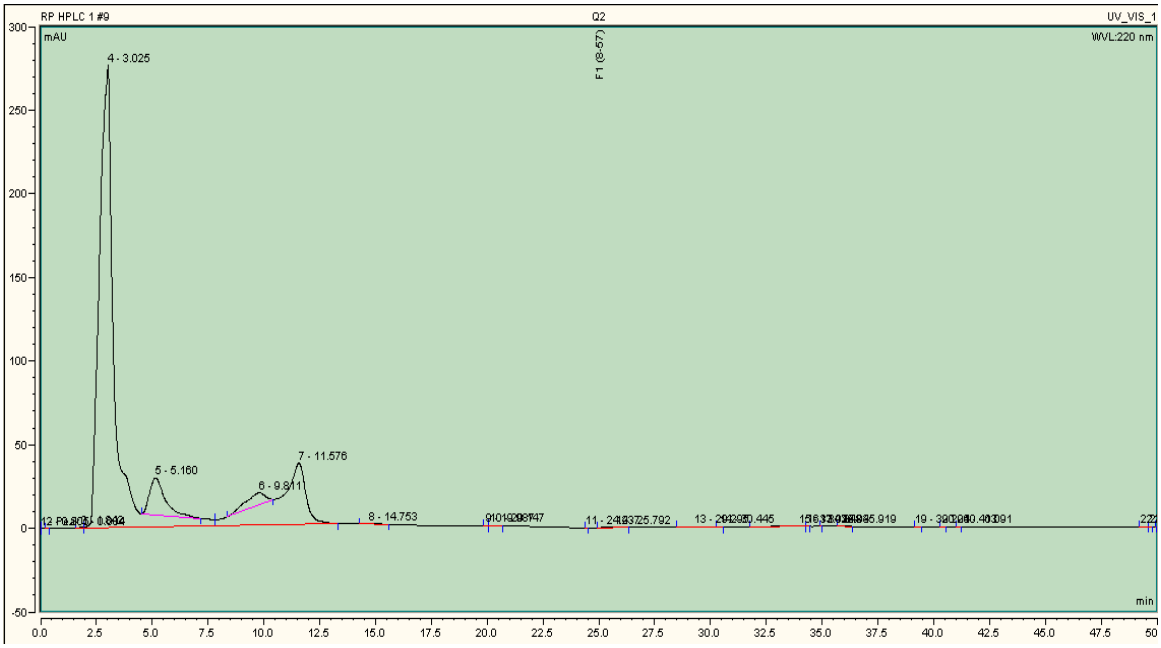


Fig 2: RP-HPLC chromatograms of standard iturin (A) and (B) the extract materials of *B. subtilis* Column: RP-C18. Eluent: methanol-water (65:35, v/v). Flow- rate: 1.0mL/min. Temperature: ambient tem. .Detection: 220nm.

Confirmation of RP- HPLC results:

The mass of molecules from crude extract of *B. subtilis* MHS15 eluted as HPLC
peak No. 3, 5 and 6 were found to be at *m/z* 1043.46, 1057.37, and 1071.48 Da which might belong to iturin A2, A3 or A4 or A5, A6 or A7 respectively (Yerra *et al.*,2008) as shown in Figs (3, 4 and 5) when analyzed by Electrospray Ionization Mass Spectrometry (ESI–MS).

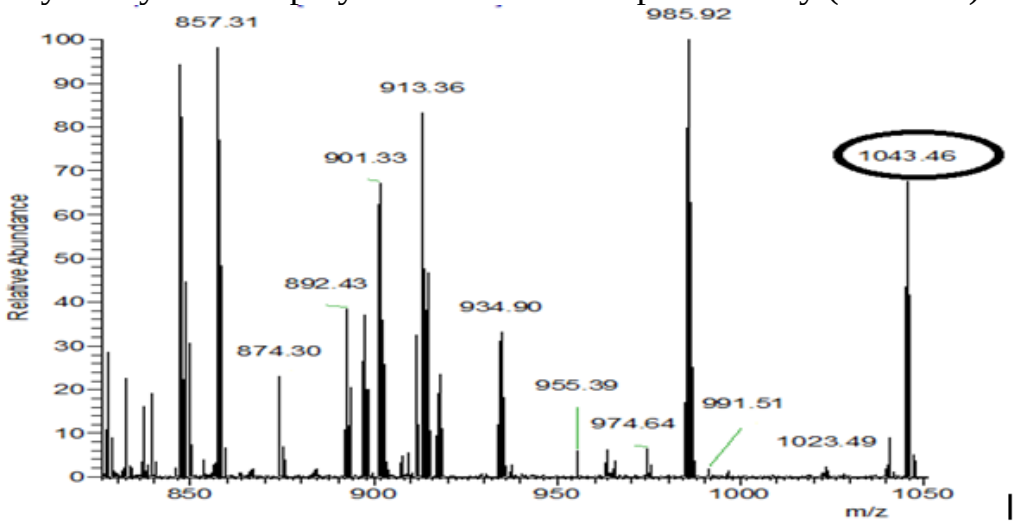


Fig 3: ESI-MS spectra of the methanolic fraction obtained by RP-HPLC (peak No.3)

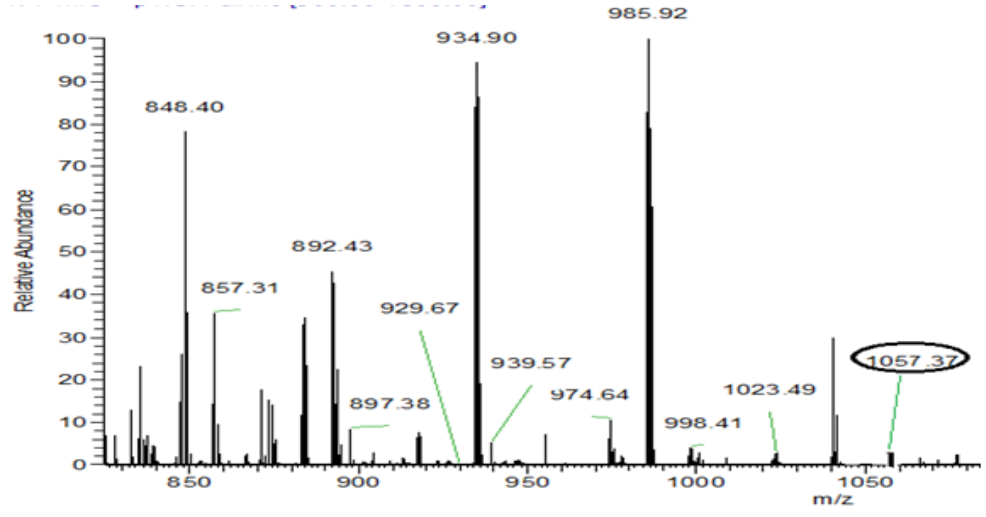


Fig 4: ESI-MS spectra of the methanolic fraction obtained by RP- HPLC (peak No.5)

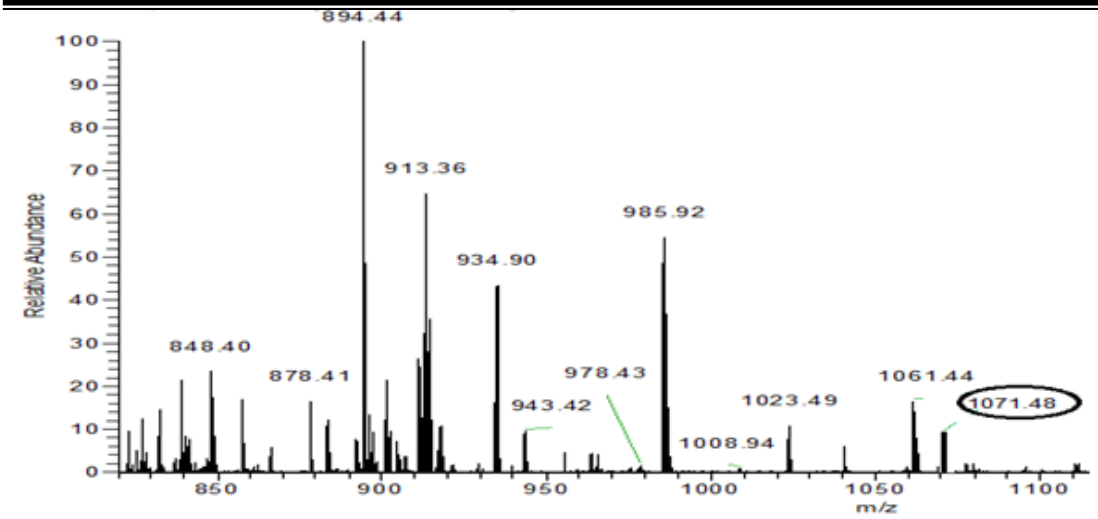


Fig 5: ESI-MS spectra of the methanolic fraction obtained by RP-HPLC (peak No.6)

It is apparent that these isomers differed in their masses by multiples of 14 Da, which corresponds to the molecular weight of one CH2 group suggesting them to be members of a same family of iturins (Ana *et al*., 2011). On the basis of literature, these closely related isomers exist for each lipopeptide with different of fatty acid chain residues in their peptide ring which were identified as iturin A homologues (Vater *et al*., 2002; Yu *et al*., 2002; Cho *et al*., 2003; Gong *et al*., 2006). The observations of this study were in agreement with the Leenders *et al*. ,(1999) using Matrix-assisted lase desorption/ionization time-of-flight MS (MALDI-TOF MS) analysis, refer the secondary metabolites produced by some strains of *B. subtilis* with molecular masses between 1045 and 1123 Da corresponding to iturin and surfactin. The results obtained by Yu *et al*., (2002) were similar to the results of this study with that the major compounds produced by *B. liquefaciens* strain B94 with a molecular weight of 1042.5533 was identified as iturin A2. Hiradate *et al*.,(2002) using the strain *B. amiloliquefaciens* RC-2 attributed the compounds bioactivity to the production of iturin A2–A8 (m/z values 1043- iturin A2; 1057- Iturin A3–A5;1071- irurin A6 and A7; 1085- iturin A8).

Conclusion:

This study showed that antagonistic bacteria, which isolated from soil and identified as *B. subtilis* MHS15 according to 16S rRNA gene sequencing, exhibited strong antifungal properties against some of plant pathogenic fungi (*Aspergillus, Penicillium and Fusarium* genera) in vitro. Among the tested molds, *A. niger* was the most sensitive to the antifungal agents (iturin A) produced by *B. subtilis* MHS15, while no inhibition was noticed against

*Rhizopus spp.*2. The highest levels of antifungal agents produced by *B. subtilis* MHS15 were detected during bacterial stationary growth phase.

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عزل وتشخيص بكتريا *Bacillus* المنتجة للمواد المضادة للفطريات

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المستخلص:

تم الحصول على ١٥ عزلة من بكتريا *Bacillus* من التربة في كلية الزراعة واختبرت قدرتها التضادية تجاه بعض الفطريات التي اشتملت على *Aspergillus* ، *Penicillium* ، *Fusarium* و *Rhizopus*. وجد ان خمسة عزلات منها وهي (١، ٣، ١٠، ١٣، و ١٥) تمتلك فعالية مضادة تجاه تلك الفطريات وعملت على تثبيط نموها بشكل ملحوظ . وكان اكثر تلك العزلات فعالية هي العزلة التي رمز لها MHS15 والتي شخصت بتقنية تتابعات القواعد النيتروجينية لجين 16S rRNA بانها تعود الى *Bacillus subtilis* وبنسبة تشابه كاملة (١٠٠%) مع تتابعات القواعد النيتروجينية للجين نفسه لبكتريا *Bacillus subtilis* MHS15 المسجلة في بنك الجينات برقم التسجيل gbIKF030229.1 .

اقصى نسبة تثبيط (٥٢,٦%) تمت ملاحظته ضد *A.niger* كان باستخدام عزلة رقم ١٥ بتركيز راشح وقدره ٣٥% حجم/حجم. شخص العامل المضاد للفطريات المنتجة من قبل بكتريا قيد الدراسة باستخدام جهاز RP-HPLC فوجد انها . اوضح تحليل المستخلص المثيلي بطريقة RP-HPLC لراشح مزرعة البكتريا قيد الدراسة ان ثمت ثلاث صور ل iturin A تظهر في اوقات الاحتجاز ٣,٠٢٥ , ٥,١٦٠ و ١١,٥٧٦ دقيقة والتي كانت مطابقة لصور iturin A القياسي والتي ظهرت في وقت الاحتجاز ٣,٠٥٧ , ٥,١٧٨ و ١١,٧٥٣ دقيقة.

بينت نتائج التحليل بجهاز ESI-MS والتي تم الحصول عليها من تحلل iturin A المنتجة من قبل العزلة قيد الدراسة ان اوزانها الجزيئية تبلغ ١٠٤٣,٤٦، ١٠٥٧,٣٧ و١٠٧١,٤٨على التوالي. مما يشيرالى انها تعود الى iturin A2 و A3 او A4 او A5 (والتي تمتلك الوزن الجزيئي نفسه) و A6 او A7 (والتي تمتلك الوزن الجزيئي نفسه) على التوالي. ووجد ان جميع الصور التي تم تشخيصها من العزلة المحلية تمتلك فعالية مضاده تجاه *Aspergillus niger* .