Isolation and Characterization of *Bacillus* spp. with Antagonistic Activities for Biological Control

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Received 08 / 10 / 2013
Accepted 08 / 12 / 2013

الخلاصة

تم عزل 47 عزلة بكتيرية مختلفة مأخوذة من 13 عينة تربة مختلفة وكانت 5 من هذه العزلات عائدة لجنس *Bacillus subtilis* والتي أظهرت فعالية تثبيطية تجاه أنواع مختلفة من الفطريات المرضية للنبات والإنسان وتم ترميز هذه العزلات البكتيرية بـ B1 و B2, B3, B4 وB5. تم انتخاب أفضل عزلة من خلال فعاليتها التثبيطية ضد أنواع مختلفة من الفطريات الممرضة للنبات والإنسان وكانت هذه العزلة هي B4 وكان التثبيط بسبب إنتاج هذه العزلة مواد أو مركبات مضادة للفطريات. حددت الظروف المثلى لإنتاج هذه المركبات حيث كانت تنمية البكتريا في درجة 28°م لمدة خمسة أيام و بتضبيط قيمة الأس الهيدروجيني عند 7. كذلك استخلصت المادة الخام من البكتريا وتم اختبار فعاليتها التثبيطية ضد أنواع مختلفة من الفطريات والتي أظهرت قيم مختلفة من التثبيط من خلال قياس قطر التثبيط للفطريات وكان الفطر *Alternaria alternata* الأكثر تحسسا تجاه البكتريا وكذلك تجاه مستخلص البكتريا. من خواص هذا المستخلص انه كان ذائبا في الماء والمذيبات العضوية وان فعاليته لم تتأثر عند معاملة هذا المستخلص بالحرارة. لذا من الممكن استخدام هذه العزلة البكتيرية وكذلك مستخلصها كعامل سيطرة حيوية فعال تجاه الفطريات المرضية ويعتبر كبديل للأدوية الكيميائية والتي لها آثار جانبية ضارة.

ABSTRACT

We isolated 47 different bacterial isolates from 13 different soil samples, five of them showed antifungal activities belonged to Gram positive bacteria *Bacillus subtilis* and were designated as B1,B2,B3,B4 and B5 and they were screened to choose the better one whose activity appear to be high against different kinds of plant and human pathogenic

fungi which it was the isolate B4. It's activity is due to the production of antifungal compound(s). Also the optimum conditions for the production of the antifungal compound were determined by growing the antifungal isolate at 28°C for 5 days at pH 7. The crude extract containing the active compound was extracted and tested against different types of pathogenic fungi and showed different values of activities by measuring the inhibition zone and it was found that the fungus *Alternaria alternata* was the most sensitive. The crude extract is soluble in water and organic solvents and the activity of the extract was not affected by heat treatment. Thus the isolate B4 and its extract could be used as a potent biological control agent against these pathogenic fungi rather than chemicals which have harmful side effects.

INTRODUCTION

The genus *Bacillus* is among the most widely distributed groups of microorganisms in nature. Though the main reservoir of *Bacillus* species is considered to be in soil. (1,2).

Many *Bacillus* spp. are capable of producing antibiotics of these bacitracin, polymyxin, tyrocidin, gramicidine (1,3). Most of the antibiotics produced are classified as peptide antibiotics and exhibit a range of spectra. They are active against Gram-positive bacteria whereas some inhibit Gram-negative bacteria and others exhibit antifungal properties (4). The potential application of bacilli is their capability of producing antifungal compounds which could be used as biological control (5).

Biological control based on microorganisms to suppress plant disease, offers a powerful alternative to synthetic chemicals. The abuse of chemical pesticides or fungicides to cure or prevent plant diseases has caused soil pollution and detrimental effects in humans. It is desirable to replace chemical pesticides with materials that possess the following three criteria: high specificity against the targeted plant pathogens; easy degradability after effective usage; and low mass production cost (6,7).

The genus *Bacillus* is comprised of Gram-positive, rod-shaped, spore-forming bacteria which are well known for their ability to produce a diverse array of antimicrobial compounds. Of particular interest is the ability of certain strains to produce antifungal compounds. Such organisms have the potential for application in agriculture where they can be used as biocontrol agents against selected plant pathogenic fungi. The use of a gram-positive *Bacillus* species as a biocontrol agent is relatively rare, and has received less intensive study than the use of gram-negative bacteria (5).

Fungi are primary causes of grain loss, and some of them produce compounds that are toxic when consumed (8). Fungal diseases of plants are usually controlled by some combination of cultural practices, use of fungicides, and host plant resistance. Fungicides are the primary means of fungal disease control, but their use is currently controversial because investigation have indicated potentially undesirable environmental side effects (9).

According to these, this study was undertaken to isolate and characterize *Bacillus* spp. that exhibit broad spectrum of antifungal activity that could be used as potent biological control agent.

MATERIALS AND METHODS

Pathogenic fungal strains

Different pathogenic fungi were used in this study as test fungi, and as follows:

a- *Penicillium nalgiovense*, *Fusarium graminearum*, *Alternaria alternata*, *Rhizoctonia solani* were taken from Biology Department/College of Science/ Mosul University.

b- *Macrophomina phaseolina*, was taken from Plant Protection Department/College of Agriculture/ Mosul University.

c- Human pathogenic *Aspergillus niger*, *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Candida albicans* and toxin-producing *Aspergillus flavus* from Biology Department/College of Science/ Mosul University.

Collection of the samples

Different soil samples (about 13 soil samples) were collected for the isolation of the Gram positive antifungal bacteria and these samples were taken from the rhizosphere soils of different garden plants in Mosul city such as the rhizosphere of flowers and orange trees.

Isolation and identification of bacteria

Serial dilutions were done for each soil sample (in which 1 gram of the sample is diluted in 9 ml of sterile distilled water, then 1ml of this dilution is added to another 9 ml of distilled water and the same thing is repeated until we reach the dilution of 10^{-6}), then 0.1 ml of the dilutions 10^{-4} and 10^{-5} of each sample was cultured on 9cm Petri dish of nutrient agar and incubated at 30ºC for 24 hour. After that each different single colonies were tested against pathogenic fungi, and the antifungal bacterial isolates were taken and recultured for identification and further studies.

The suspected isolates showed preliminary antifungal activities were subjected to several microscopical tests (shape of cells, shape of spores, swelling of cells), morphological tests (color, size of colonies,

edge of colonies, consistency of colonies etc…), biochemical tests (citrate utilization, oxidaze, indol, catalase, gelatin hydrolysis) in addition to the Gram stain, pigment production, swarming on plate, motility test, blood hemolysis, and all of these tests were done according to (10,11,12,13).

Antifungal bioassay test

In order to examine the antagonistic properties of bacterial isolates against the pathogenic fungi, and according to (14), a dual culture technique was carried out which is the simplest method to detect antifungal activity. In this technique an agar block (5 mm diameter) of 5-day-old culture of fungal pathogen was placed in the centre of plate containing potato dextrose agar (PDA) or Sabouraud dextrose agar in the case of human pathogenic fungi (15). A loopful of 24-h-old culture of the tested bacteria was inoculated at 2 cm juxtaposed to the pathogen. A cork borer plug of fungal pathogen was inoculated centrally on PDA (or Sabouraud dextrose agar) plates, and some plates were left without inoculation of the bacteria juxtaposed to the fungal pathogen served as control. The plates were incubated at 28± 1°C for 5 days and inhibition of fungal growth was measured (16).

Determination of the optimum conditions for the production of antifungal compound(s)

Different conditions of incubation periods (4,5,6,7,8 days), temperatures (28,29-37°C), and pH values (6,7,8) were used to determine the optimum conditions for the production of the antifungal compounds from the bacterial isolates.

Extraction and partial characterization of the active antifungal compounds

Extraction of the active antifungal compounds and testing their activities against the used fungi were carried out according to (16) and also in order to characterize the nature of these compounds, the following method was described:

Nutrient broth (N.B.) medium was inoculated with a loopful of fresh (24-hour old) culture of the bacterial isolate and was incubated at 28±1°C for 5 days. It was then centrifuged at 7000 rpm. for 15 min, and the supernatant was finally passed through a millipore filter paper (0.2 µm porosity) to get cell-free culture filtrate that contain the antifungal compound (16).

Several tests were done for partially characterizing the active crude extract such as heat treatment (100°C for 3 hours), nature of the compound(s) (protein or not),and solubility of the compound (s) in water and organic solvents (17,18).

RESULTS AND DISCUSSION

Isolation of antifungal bacteria

From the thirteen soil samples, 47 different bacterial isolates of suspected Gram positive bacteria were obtained. Each one of these isolates was preliminary tested against the fungus *Alternaria alternata* and *Macrophomina phaseolina* (later tested against the other fungi). Six different bacterial isolates showed an antifungal activities against the tested fungi.

Identification of bacterial isolates

The six bacterial isolates that showed antifungal activities were subjected to several morphological, physiological, and biochemical tests to identify these bacteria. The results revealed that five of the antifungal isolates belonged to the Gram positive bacteria and one belonged to the Gram negative bacteria. The Gram negative isolates were neglected, while the other five antifungal isolates appeared to belong to the genus *Bacillus* spp.

There are many researches concerning the isolation of different antifungal *Bacillus* isolates such as *B.subtilis* and *B.cereus* etc. (19, 20), the results showed that all antifungal isolates were able to grow in medium containing NaCl at concentration up to 5 and 7 %, also the Gram stain and other morphological, physiological, and biochemical tests indicated that all the five antifungal isolates were belonged to the genus *Bacillus subtilis* and they were designated as B1, B2, B3, B4, and B5, and these results were exactly in agreement with (10,11,12,13).

Antifungal Bioassay Test

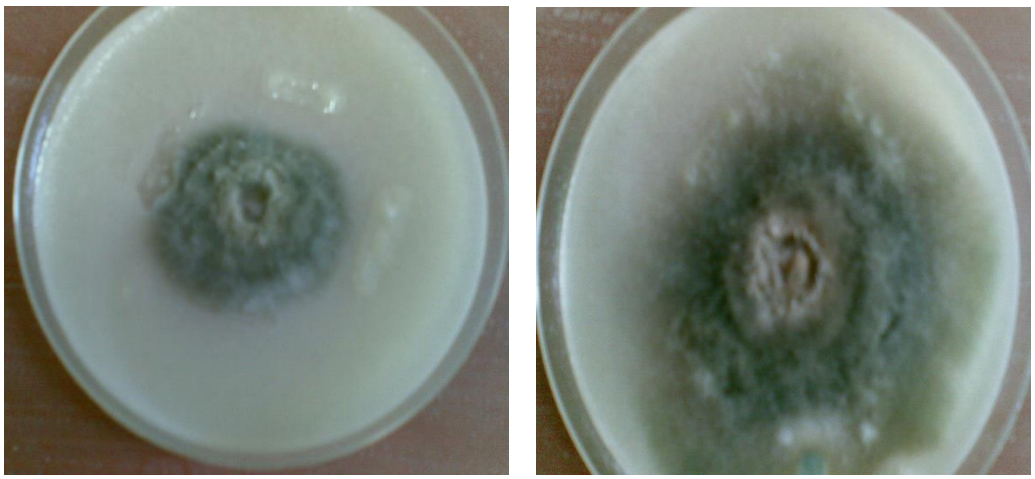
The *Bacillus* isolates showed preliminary antifungal activity against the fungus *Alternaria alternata* (figure 1) were screened to choose the best antifungal bacterial isolate and to complete the other further tests on it. It was found that the isolate *B. subtilis* (B4) is the most efficient because it showed the highest inhibition zone against the tested fungus and was 8 mm. while the bacterial isolates B1, B2, B3, and B5 showed inhibition zones of 5, 5, 4,and 3 mm respectively when tested against the same fungus. So the isolate B4 was selected and taken for further studies because of its higher efficiency and the other isolates were ignored.

The isolate B4 showed different inhibition activities when tested against different kinds of human pathogenic and phytopathogenic fungi (table 1).

Table 1: The antagonistic effect of *Bacillus subtilis* B4 on different pathogenic fungi.

Bacterial isolate	Pathogenic fungi	Inhibition zone (mm)
Bacillus subtilis B4	*Alternaria alternata*	8
	Rhizoctonia solani	6
	Fusarium graminearum	5
	Penicillium nalgiovense	3
	Macrophomina phaseolina	6
	Aspergillus niger	3
	Aspergillus flavus	2
	Trichophyton mentagrophytes	3
	Trichophyton rubrum	2
	Candida albicans	0

From table 1, it was noticed that isolate B4 had a wide range of antagonism against different types of phyto and human pathogenic fungi, and the fungus *Alternaria alternata* was the most sensitive fungus among the tested fungi.



a) b)

Figure (1): Preliminary antifungal bioassay test of bacterial isolates against *Alternaria alternata* in (a) compared with the control of the fungus in (b).

Determination of the optimum conditions for the production of antifungal compounds

As mentioned previously different incubation periods (4,5,6,7,8 days), incubation temperatures (28,29-37°C), and pH values (6,7,8) were used to determine the optimum conditions for the production of the antifungal compounds from the selected bacterial isolate B4. These tests were done on the most sensitive fungus (*Alternaria alternata*) and the

results showed that the best conditions for the production was growing the antifungal isolate at 28℃ for 5 days at pH value of 7 because it showed the highest inhibition zone against the test fungus and as indicated in tables 2,3, and 4 and these results were in agreement with (14 and 16) which used different conditions to determine the optimum ones for the production of the antifungal compounds.

Table (2): Incubation time effect on the production of antifungal compound from the isolate B4.

Incubation Period (days)	Inhibition zone on *Alternaria alternata* (mm)
4	6
5	8
6	7
7	7
8	6

Table (3): Incubation temperature effect on the production of antifungal compound from the isolate B4.

Incubation temperature (°C)	Inhibition zone on *Alternaria alternata* (mm)
28	8
29	8
30	7
31	6.5
32	6
33	6
34	6
35	6
36	5.5
37	5

Table (4): pH value effect on the production of antifungal compound from the isolate B4.

pH value	Inhibition zone on *Alternaria alternata* (mm)
6	4.5
7	8
8	6

Extraction and partial characterization of the active antifungal compounds

As mentioned before and depending on (14) and (16), the crude antifungal compound(s) was extracted and tested against the different pathogenic fungi (table 5) that showed different inhibition values toward the tested fungi.

Table (5): the culture filtrate effect of the isolate B4 on different pathogenic fungi.

	Test fungi	Inhibition Zone (mm)
Culture filtrate effect of *Bacillus subltilis* B4	*Alternaria alternata*	6
	Penicillium nalgiovense	2
	Fusarium graminearum	3
	Rhizoctonia solani	4
	Macrophomina phaseolina	4
	Aspergillus niger	1.5
	Aspergillus flavus	1
	Trichophyton mentagrophytes	2
	Trichophyton rubrum	1
	Candida albicans	0

It was noticed from table 5 that the effect of the crude extract was lower when compared with the effect of the antifungal bacteria that are in direct contact with the fungi and this may be due to the presence of the fungi that induce and enhance the production of the antifungal compounds from the antifungal bacteria better than when the antifungal compounds were extracted, or the crude extract need further purification to get the active highly effective antifungal compound(s) (14,15,16).

On the other hand and according to (17 and18), the active crude extract that showed the antifungal activity was subjected to several tests to partially characterize it such as its nature, solubility in water and organic solvents etc…, and it was found that the compound was soluble in water and organic solvents such as acetone, butanol, ethanol, and others, and there was approximately no loss of antifungal activity when the active extract was subjected to heat treatment (100°C for 3 hours).

Also it was concluded that the optimum conditions for the production and extraction of the active compound was by growing the antifungal isolate at 28°C for 5 days at pH value of 7, and the compound was soluble in water and organic solvents and not affected by heat treatment that needs further several tests to complete identification and characterization of the compound such as HPLC, FTIR analysis, mass spectrophotometry, NMR, etc….(14) in order to know the formula of the compound that could be prospectively synthesized for traditional use. From all above results it was concluded that the isolate *Bacillus subtilis* B4 showed a wide range of antifungal activities against different types of phytopathogenic and even human pathogenic fungi that could be used as a natural potent biocontrol agent as an alternative to the chemical fungicides and we recommend to test its activity against the other different types of phytopathogenic and humanpathogenic fungi (and even pathogenic bacteria).

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