ISSN: 0067-2904
GIF: 0.851

Extraction and Characterization of Antifungal Substances from *Brevundimubsonas diminuta* and Their Effect on *Fusarium oxysporum*

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Abstract

Eighty one bacterial isolates were obtained from 53 soil samples of different plants rhizosphere. All the isolated bacterial were screened for antifungal effect against *Fusarium oxysporum* . Three isolates gave antifungal activity with inhibition zone ranged between (0.5-2.5 cm). Two isolates (Bd1 and Bd2) were *Brevundimonas diminuta*, while the third (Pf1) was *Pseudomonas fluorescence* . *B. diminuta* (Bd1) which used in this study isolated from *Raphanus sativus* gave the highest inhibition zone against *F. oxysporum*. Cell free supernatant of *B.diminuta*(Bd1) was better in antifungal activity than bacterial cells against *F. oxysporum.* The highest antifungal substance production was obtained from mineral salt broth containing 1% peptone after incubation at 35^0C for 7 days at pH=7. It was observed that no one of the three carbon sources (D- glucose , lactose and starch) and lipid sources (tween 80 , sun flower and olive oil) had an effect on antifungal substances production .Other factors were studied , *B.diminuta* (Bd1) produced protease, while did not produce chitinase and cellulase enzymes, also was unable to produce emulsifier substance but produced volatile compounds with antifungal activity against *F. oxysporum*. The results showed that chloroform at dilution 1:3 (v:v) was more efficient than methanol for antifungal substance extraction. The antifungal substances reduced the spores number of *F. oxysporum* using Malt Extract Agar after 7 days of incubation, while the bacterial cells reduced the spores number *F. oxysporum* in until no growth was observed after 9days.

Keywords: *Brevundimonas diminuta* , PGPR , biocontrol , antifungals, volatile compounds.

استخلاص وتشخيص المادة المضادة للفطريات والمنتجة من بكتريا *Brevundimonas diminuta* وتأثيرها على الفطر *Fusarium oxysporum*

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الخلاصة

تم الحصول على واحد وثمانين عزلة بكتيرية عزلت من ثلاثة وخمسين عينة من ترب جذور نباتات مختلفة . تم التحري عن قابلية جميع العزلات على انتاج مواد مضادة للفطر *Fusarium oxysporum* . اظهرت ثلاثة عزلات فعالية مضادة للفطر بمناطق تثبيط تراوحت بين0, 5 – 2, 5 سم . أظهرت النتائج ان عزلتين (Bd1 و Bd2) تعود للنوع *Brevundimonas diminuta* اما العزلة الثالثة (Pf1) فتعود الى النوع *Pseudomonas fluorecensce* . تم استخدام عزلة (Bd1) *B. diminuta* في هذه الدراسة والمعزولة من نبات الفجل ، والتي تمتلك أعلى فعالية تثبيط ضد الفطر *F. Oxysporum*. اظهر الرائق البكتيري للعزلة (Bd1) *B. diminuta* فعالية مضادة للفطر أعلى من خلايا البكتريا نفسها . اظهرت النتائج بان أعلى

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انتاجية للمواد المضادة كانت باستعمال الوسط الملحي السائل الحاوي على نسبة 1% ببتون كمصدر نايتروجيني بعد حضانة سبعة ايام بدرجة حرارة 35 °م لمدة سبعة ايام وعند الرقم الهيدروجيني 7. ايضا تم دراسة تأثير بعض المصادر الكربونية والدهنية على إنتاج المواد المضادة ولم يكن لأي مصدر كربوني (كلوكوز–D واللاكتوز و النشاء) ولأي مصدر دهني (tween 80 وزيت عباد الشمس وزيت الزيتون) أي تأثير على انتاج المواد المضادة. تم التحري عن قابلية البكتريا على انتاج بعض الانزيمات المضادة للفطر ، اذ تبين إن العزلة (Bd1) *B. diminuta* منتجة لانزيم البروتييز، بينما لم تكن قادرة على انتاج كلا من الكايتينيز والسيليوليز ، وغير منتجة لمادة الأستحلاب، لكنها أظهرت قابليتها على انتاج مركبات طيارة مضادة للفطر *F.oxysporum* لقد بينت النتائج ان الاستخلاص للمواد المضادة للفطر بالكلوروفورم بنسبة تخفيف 1:3 (حجم : حجم) أكفأ من الاستخلاص بالايثانول. أظهرت المواد المضادة للفطر تأثيرا ملحوظاً على أعداد سبورات الفطر *F.oxysporum* باستعمال وسط Malt Extract Agar ، اذ انخفضت ألأعداد بعد سبعة ايام حضن بينما اظهرت نتائج تاثير خلايا البكتريا انخفاضاً في أعداد السبورات ولم يلاحظ اي نمو بعد تسعة ايام.

Introduction

Fungal phytopathogens pose serious problems worldwide in the cultivation of economically important plants [1].Different methods have been used to control plant pathogens, being the most used cultural practices, resistant cultivars, chemical and biological control. Biological control is a natural and specific way to control pathogens and enhance crop yield by growth promoting attributes of friendly environment microorganisms [2].To increase crop yields, it is necessary to apply agrochemicals, which have several negative side effects. Chemical fungicides are extensively used in current agriculture, however, excessive use of chemical fungicides in agriculture has led to deteriorating human health, environmental pollution, and development of pathogen resistance to fungicide [3]. Since pathogen damage potentially causes large yield losses, the use of plant growth-promoting rhizobacteria (PGPR) with antifungal properties is an attractive alternative to the use of such xenobiotic compounds [4].Bacteria of diverse genera have been identified as PGPR, of which *Bacillus* and *Pseudomonas* spp. are predominant [5].In modern agriculture PGPR is used as bio-fertilizer as well as biological control agent against certain seed and soil-borne plant pathogens[6].The bacteria *Brevundimonas diminuta* is considered as an effective PGPR as it possess a number of traits useful for their establishment and proliferation in soil [7].The antifungal compounds production is most important mechanism of action for the effective biocontrol of soil borne diseases under green house or field conditions [8].Microbial antagonist strains capable of producing both nonvolatile compounds and volatile compounds (VOCs), which exhibit strong inhibitory activity against plant pathogens, have received much attention [9,10].Bacterial volatiles represent a source for new natural compounds that are interesting for man, since they can be used, for example, to improve human health or to increase the productivity of agricultural products [11].

The aim was to identify alternative PGPR for plant protection against *F. oxysporum*, which are less dependent on chemicals and environmentally friendly.

Materials and Methods

Samples Collection

Fifty three samples of rhizospheres soils of different plants from Baghdad city were collected under aseptic condition then transported to the laboratory until use.

Isolation of bacteria

One gm of each soil sample was added to 9 ml of D.W and mixed to homogenize and serial dilutions were prepared for each sample. About 0.1 ml of each dilution was spreaded on MacConkey agar plates and incubated at 30°C for 2 days.The bacterial colonies were purified by sub culturing on nutrient agar plates until pure culture was obtained.

Screening for antifungal substance producing bacteria

All bacterial isolates were tested for antifungal substance production according to [12]. Nutrient broth tubes (5ml) were inoculated with activated bacterial suspensions and incubated at 30 °C for 24hrs.Discs of sterilized filter paper NO.1(5mm) were saturated with bacterial suspensions (O.D = 0.3 at 600nm).Plates of Malt Extract Agar (MEA) pH 6, were inoculated with 10^6 fungal spores/ml of *F.oxysporum* by swabbing, the plates were leaved for 10 min.The saturated filter paper discs were

placed on the surface of the MEA plates and incubated at 30 °C for 7 days.The diameters of inhibition zones around the discs were measured.

Identification of bacterial isolate:

Bacterial was identification by used VITEK 2 compact device (Biomerienx USA) in Ibn Balady hospital laboratories, Ministry of health. This device contains 47-biochemical tests.

Determination of antifungal activity for bacterial cells and cell free supernatant Growth of bacterial isolate

Nutrient broth (250ml) was inoculated with 1% ml of activated bacterial suspension (selected isolate) and incubated at 30 °C for 24hrs.The cells were precipitated by centrifugation at 10000 rpm for30 min. The precipitated cells and supernatant were assayed for antifungal activity.

Antifungal activity assay

Antifungal activity assay was done according to [13]. Plates of MEA was mixed with (10% v/v) of activated bacterial cells suspension or with cell free supernatant and poured in sterilized petri dishes and left to solidified at room temperature.Mycelial discs (8mm in diameters) of *F.oxysporum* grown on Potato Dextrose Agar, were transferred to MEA (which mixed with bacterial cells or supernatant previosly), and incubated at 30 °C for 7days. Control plates (MEA without bacterial cells or supernatant) were inoculated with *F.oxysporum*. The radial growth of fungus was measured and percent inhibition was calculated as (control − treatment)/control × 100.

Determination of the cultural conditions effect on antifungal substances production by the selected bacterial isolate

Nitrogen sources

Five ml of mineral salt broth containing 1% of two nitrogen sources (pepton and beef extract) separately were inoculated with 0.1ml of activated bacterial suspension (O.D = 0.3 at 600nm) and incubated at 30 °C for 24hrs. The cells were precipitated by centrifugation for 30min at 10000 r.p.m.The supernatant were assayed for antifungal activity. Control: five ml of mineral salt broth containing 1% of the two nitrogen sources (0.5% pepton+ 0.5% beef extract).

Carbon and Lipid sources

Five ml of mineral salt broth with 1% nitrogen source containing 1% of different sources of carbons (D-glucose , lactose and starch) or 1% of different kinds of lipids (Tween 80 , Sunflower and Olive oil) were inoculated separately with 0.1ml of activated bacterial suspension (O.D = 0.3 at 600nm) and incubated at 30 °C for 24hrs.The cells were precipitated by centrifugation for 30 min at 10000 r.p.m. The supernatant were assayed for antifungal activity. Control : five ml of mineral salt broth containing 1% pepton.

Temperature

Five ml of mineral salt broth containing 1% pepton was inoculated with 0.1 ml of activated bacterial suspension (O.D = 0.3 at 600nm) and incubated at different temperatures (30,35,40 and 45°C) for 24hrs.The cultures were centrifuged for 30min at 10000 rpm.The supernatants were assayed for antifungal activity.

pH

Five ml of mineral salts broth containing 1% pepton was prepared at different pH value (3,5,6,7,8,9,11,13) adjusted by HCl (1N) and NaOH (1N) , and inoculated with 0.1 ml of activated bacterial suspension (O.D = 0.3 at 6000 rpm) and incubated at 35°C for 24hrs.The cultures were centrifuged for 30min at 10000 rpm).The supernatants were assayed for antifungal activity.

Detection of some antifungal enzymes produced by the selected isolate

Protease production

Plate of Skim Milk Agar was streaked with activated bacterial culture and incubated at 35°C for 24hrs. , clear zone around the colony (hydrolysis of milk) indicate positive result [14].

Chitinase production

Plate of Chitin Agar medium was streaked with activated bacterial culture and incubated at 35°C for 14 days, clear zone around the colony (hydrolysis of chitin) indicate positive result [15].

Cellulase production

Plates of cellulose Agar medium was streaked with activated bacterial culture and incubated at 30°C for 14 days, clear zone around the colony (hydrolysis of cellulose) indicate positive result [16].

Detection of emulsifier production by selected bacterial isolate

One ml of cell free supernatant was added to 1ml of olive oil (equal volumes v:v) , mixing with vortex for 2min. , the tube was left for 24 hrs. at room temperature. The formation of emulsifier layer indicate the positive result [17].

Detection of volatile compounds produced from selected bacterial isolate

Plate of MEA was divided into two parts by removing a part of the medium (5 mm width) from the middle of the plate longitudinally.One of the two parts was inoculated with activated bacterial isolate ,and the other part was inoculated with mycelial disc (8mm in diameter) of *F.oxysporum*.The control was MEA plate inoculated with the fungus (mycelial disc) only.The plates were sealed with parafilm and incubated at 30°C for 7days.The radial growth of fungus was measured and the percentage of the inhibition was calculated [18].

Extraction of crude antifungal substances from selscted isolate

Mineral salts broth (500 ml) containing 1% pepton , was inoculated with 1% ml of activated bacterial culture (O.D = 0.3 at 600 nm) and incubated at 35°C in the shaking incubator for 24hrs.The culture was centrifuged for 30 min at 10000 r.p.m.The cell free supernatant was concentrated by poly ethylene glycol (PEG).The concentrated supernatant was extracted with two solvents (chloroform and methanol) as following :

The concentrated supernatant was diluted by chloroform and methanol at ratio of (1:1 , 1:2 and 1:3 V/V) and left for 3 hrs.Two layers were appeared , the upper and the lower layers were evaporated at 25°C for 48 hrs. and assayed for antifungal activity.Control : plate of MEA was inoculated with mycelial disc (8mm in diameter) of *F.oxysporum*.

Effect of antifungal substance produced from selected bacteria on spore numbers of *F.oxysporum*

Mineral salt broth (250 ml) containing 1% pepton, pH 7 , was inoculated with 1% ml of activated bacterial culture (O.D= 0.3 at 600 nm) and incubated at 35°C in the shaking incubator for 24 hrs.The culture was centrifuged for 30 min at 10000 rpm.MEA was mixed with (10% v/v) of the supernatant and poured in sterilized petri dishes and left to solidified at room temperature. 4 - The control was MEA. Slants of *F.oxysporum* growth was suspended in 5 ml of sterile distilled water .Serial of dilutions were made and numbers of spores in each dilution was counted in a haemocytometer [19]. Both MEA plates (test and control) were inoculated with 0.1 ml of spore suspension from (10^{-4}) dilution and incubated at 30°C for 7 days. The CFU of *F.oxysporum* for both (test and control) was estimated.

Determination the effect of bacterial cells on growth of fungi in soil

Test (T): Three repeats of 50 gm of sterilized soil in sterilized containers were inoculated with 10^6 fungal spore/gm and 10^8 bacterial cells/gm.

Controls: Bacterial control (CB) was prepared by using 50gm of sterilized soil in sterilized containers inoculated with 10^8 bacterial cells/gm. **Fungal control** (CF) was prepared by using 50gm of sterilized soil in sterilized containers inoculated with 10^6 fungal spores/gm. All the treatments were incubated at 30°C for three weeks , the number of bacteria was estimated every three days by using Most Probable Number Method (1gm of soil diluted in set of MacConkey broth tubes), and the viable count of the fungus was estimated also every three days by inoculating PDA plates by spreading method and estimating the CFU.

Results and Discussion

Isolation of bacteria

Eighty one bacterial isolates were obtained from 53 soil samples, after culturing on MacConkey agar .This medium is selective for gram negative bacteria and differentiate between lactose fermenter and non lactose fermenter [20]. The result showed that 51 isolates were non lactose fermenter which appeared as pale colonies and 30 isolates were lactose fermenter which appeared as pink colonies.

Screening for antifungal substance producing bacteria

Eighty one bacterial isolates were screened for antifungal effect against *F.oxysporum*, the results showed that only three isolates gave antifungal activity with inhibition zone ranged between (0.5-2.5cm), and the isolate isolated from (*Raphanus sativus*) gave the highest inhibition zone (2.5cm) Table-1.

Table 1-The antifungal effect of bacterial isolates against *F.oxysporum*

Isolate number	Diameter of inhibition zone (cm)
1	2.5
2	1.8
3	2.0

Identification of bacterial isolates by VITEK

All the isolates which gave inhibition zones were identified by VITEK2 compact device. The results showed that two from the three isolates were *B.diminuta* (Bd1) and (Bd2) which isolated from (*Raphanus sativus*) and (*Triticuma estivum*) respectively , and one isolate was *Pseudomonas fluorescence* (Pf1) isolated from (*Anethum graveolens*).

Determination of antifungal activity for bacterial cells and cell free supernatant

To determine which was more effective, the cell or supernatant of *B.diminuta* (Bd1) for antifungal activity against fungi. The results showed that the cell free supernatant was more effective than bacterial cells on ***F.***oxysporum growth. The inhibition growth percentage of *F.oxysporum* when treated with bacterial cells after 7 days was (47%) while it was (52.9%) when treated with supernatant.

The activity of supernatant indicated that the antifungal substances were produced extracellularly and the inhibition by supernatant was higher than bacterial cells may be , the antifungal substance liberated previously from cells in supernatant ,while the bacterial cells need time to multiply and produce the antifungal substance.

Many studies mentioned about using cell free supernatant in the antifungal assay. Wang *et al*(2012) [13] revealed that *Lactobacillus plantarum* IMAU10014 had marked inhibitory capacity against *Phytophthora drechsleri* , the media incorporating cell-free culture at a concentration of 8%,10% (v/v), was used for the anifungal assay.

Effect of different cultural conditions on antifungal substances production from *B.diminuta* (Bd1)

Effect of nitrogen sources

As shown in Figure-1, *B.diminuta* (Bd1) can utilize two kinds of nitrogen source but the antifungal substance production from mineral salt broth containing pepton was slightly higher than beef extract and the inhibition of *F. oxysporum* growth was (58.8%) and (57.6%) respectively comparing with the control (52.9%) in which the bacteria was grown in mineral salt broth containing the two nitrogen sources.

Foster (1962) [21] mentioned that , one substrate, readily attacked by the microorganism, is utilized for rapid growth , while the second substrate , requiring enzymatic adaptation before it can be utilized , is not attacked until the first substrate has been depleted from the medium ; this explain our results in which the antifungal substance production was better when one kind of nitrogen source was added to the culture media comparing with control.

The results indicated that nitrogen source in the media plays a vital role in production of antifungal substance. Nitrogen is one of the important factors that effected bacterial growth , bacteria require nitrogen for metabolic pathways , also nitrogen enhance and increase bacterial growth , and required for biosynthesis of an important molecules such as (nucleic acid , protein and other important components), [22].

According to Teodoro and Martins (2000) [23] , results showed that addition of 0.5% yeast extract or 1% peptone to the liquid medium shortened the lag period and increased both the dry weight of the cell and the enzyme synthesis by thermophilic Bacilli sp. Isolated from soil. Peptone which is an enzymatic digest of animal protein is the principal source of organic nitrogen for the growing bacteria [24].

Leifson and Hugh (1954) mentioned that *B. diminuta* grows very readily in a simple pepton solution.

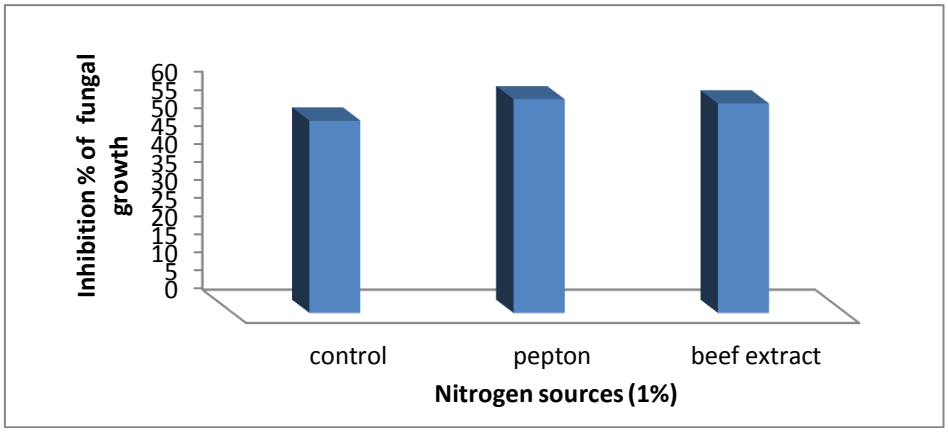

Figure 1- The effect of nitrogen sources on antifungal substance produced from *B.diminuta*(Bd1) against *F.oxysporm* after culturing on MEA and incubated at 30^0C for 7days.

Effect of carbon sources

Effect of different carbon sources on antifungal substance produced by *B.diminuta* (Bd1) was studied . It was observed that no one of the three sources of carbon were utilized by *B.diminuta* (Bd1) , because the inhibition percentages of the fungal growth in the presence of D-glucose , lactose and starch were approximately the same (57.6% , 58.8% and 58.2%) respectively as in the presence of pepton (58.8%) which used as control, Figure-2. These results agree with Gilardi (1978) [25] who referred to the inability of *B.diminuta* to utilize these carbon sources and most carbohydrates.

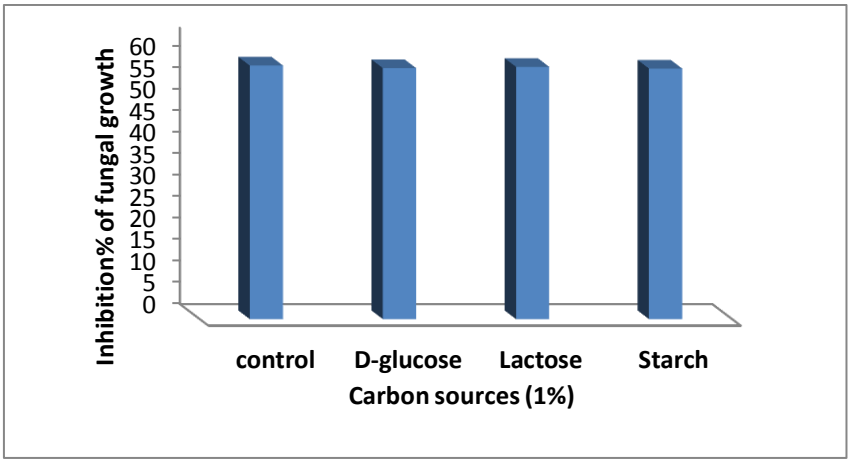

Figure 2- The effect of carbon sources on antifungal substance produced from *B.diminuta*(Bd1) against *F.oxysporm* after culturing on MEA and incubated at 30^0C for 7days.

Effect of lipid sources

The results illustrated that no effect of lipid sources on antifungal production from *B.diminuta* (Bd1) comparing with control which contain pepton only, the inhibition percentages of *F.oxysporum* growth were (58.6% , 58.5% and 58.2%) in the presence of (tween 80 , sunflower and olive oil) respectively, Figure-3.

That means *B.diminuta* (Bd1) did not utilize the lipid sources and this agree with Segers *et al.*(1994)[26] which refer to the inability of this bacteria to utilize lipids and a negative reaction obtained for lipase when used Tween 80 as substrate.

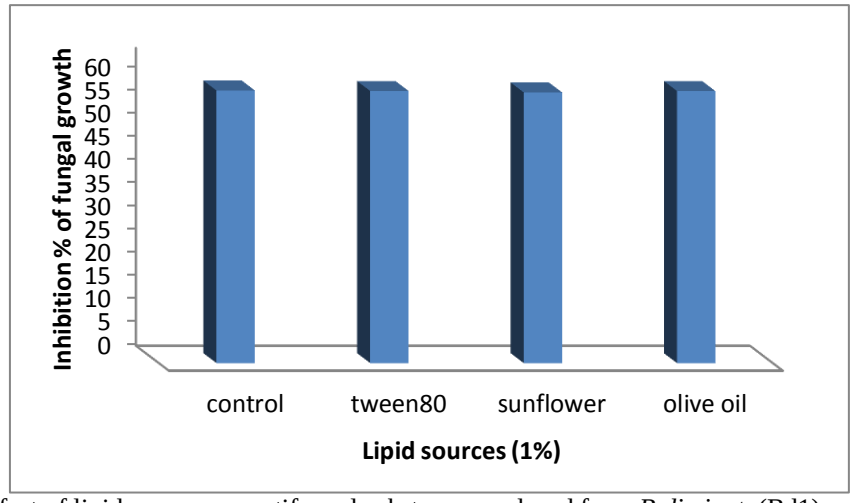

Figure 3-The effect of lipid sources on antifungal substance produced from *B.diminuta*(Bd1) against *F.oxysporm* after culturing on MEA and incubated at 30^0C for 7days.

Effect of temperature

B.diminuta (Bd1) revealed higher antifungal production at 35^0C , resulted in the highest inhibition percentage of *F.oxysporum* growth, (58.2%) , while the other temperatures (25^0C , 30^0C , 40^0C and 45^0C) decreased the antifungal production, the inhibition percentages were (48.2% , 49.4% , 52.9% and 51.7%) respectively, Figure-4.

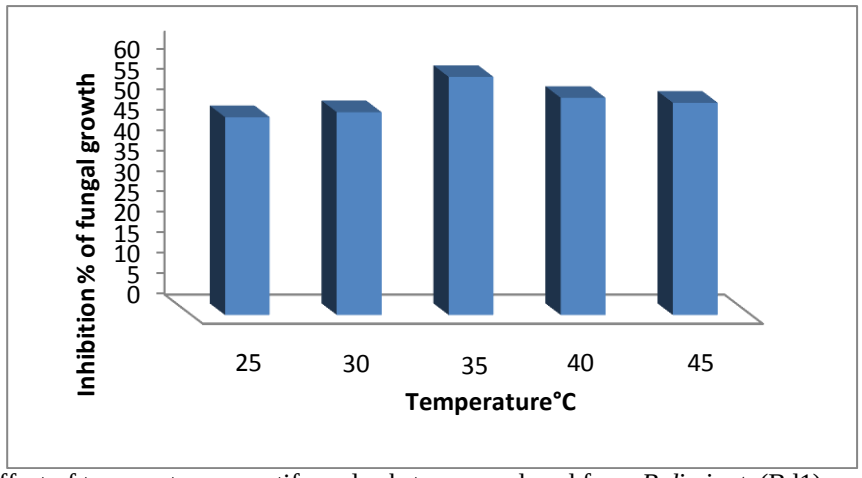

Figure 4- The effect of temperature on antifungal substance produced from *B.diminuta*(Bd1) against *F.oxysporm* after culturing on MEA and incubated at 30^0C for 7days**.**

Gebreel *et al.*(2008)[27] studied the effect of different temperatures on antifungal production by the three selected bacteria *T.inchonensis, C. nitrilophilus* and *C. cellulans* , it was found that the optimum temperature for antifungal production was 37°C. These results are approximately similar to above results, may be due to that moderate temperatures are suitable for growth and consequently for the activity of enzymes responsible for secondary metabolite biosynthesis.

Ray *et al.* (1992)[28] reported that the temperature regulate the synthesis and secretion of extracellular enzymes that responsible for biosynthesis.

Effect of pH

The results showed that *B.diminuta* (Bd1) produced antifungal substance at different pH value, the maximum production were observed at pH (6 and 7), the inhibition of *F.oxysporum* growth were (57.7% and 58 %) respectively, while the production was reduced at the other pH values (3, 5 , 8 , 9 , 11) , with inhibition (35.2% , 47% , 49.4% , 31.7% and 29.4%) as shown in Figure -5, and no effect on fungal growth at (pH 13) was observed, because *B.diminuta* (Bd1) could not grow at this pH. This indicates that, *B.diminuta* (Bd1) has the ablility to produce the antifungal substance within wide range of pH regardless the amount of the antifungal substance produced from the bacteria.

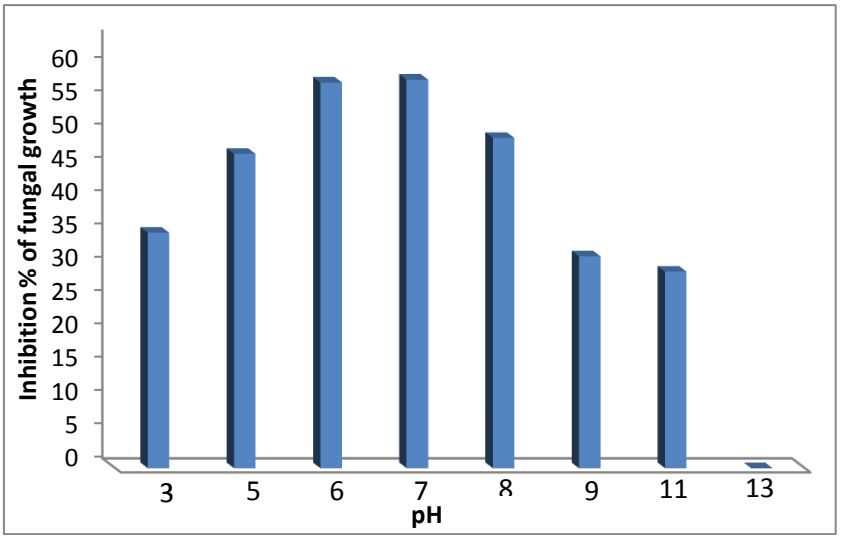

Figure 5-The effect of pH on antifungal substance produced from *B.diminuta*(Bd1) against *F.oxysporm* after culturing on MEA and incubated at 30^0C for 7days.

Changes in external pH affect many cellular processes such as the regulation of the biosynthesis of secondary metabolites [29]. The change in the production of antifungal compounds resulted from the effect of pH on an interfacial tension of lipid membrane [30].

Obviously, from our results, the antifungal substance production varies according to the cultural parameters and this agree with Laref *et. al.*,(2013)[31] who revealed that , temperature, pH and nutrients of the culture medium could influence the production of antifungal compounds by lactic acid bacteria(LAB). These factors change the metabolism of LAB; therefore change the effect of antifungal compounds on germination of conidia and growth of mycelia.

Detection of some antifungal enzymes produced by *B.diminuta*

Protease production

Proteolytic activity on skim agar medium was determined by observing the clear zone of hydrolysis around the colonies after incubation at 30^0C for 24 hrs. Chaia *et al.* (2000) [32] , reported that two proteases were detected from *B. diminuta*, 67 kDa and 50 kDa: both of them hydrolysed preferentially gelatin, but casein was also degraded and a slight hydrolysis was observed with hemoglobin. Yen *et al.* (2006)[33], reported that *P.aeruginosa* M-1001 produced a protease when it was grown in a medium containing shrimp and crab shell powder (SCSP) of marine wastes. An antifungal protease was purified from the culture supernatant to homology. The protease had a molecular weight of 38,000. Antifungal activity of this protease was found when using assay based upon inhibition of spores germination and hyphal extension of the fungal *F. solani*.

Chitinase and cellulase production

Plates of chitin agar medium and cellulose agar medium were streaked with *B.diminuta* (Bd1) and incubated at 35^0C for 14 days, the results showed that there was no clear zones around the colonies in the two media, indicating the inability of *B.diminuta* (Bd1) to produce chitinase and cellulase enzymes. Sindhu and Dadarwal (2001) [34], were reported that hydrolytic enzymes chitinases and cellulases produced by *Pseudomonas* sp. contribute to suppression of plant diseases by inhibiting growth of phytopathogenic fungi and also promote nodulation of legumes by rhizobia.

Ruchi *et al.*(2012) [35] mentioned that, the biocontrol activity of fluorescent *Pseudomonas* in addition to the wide range of metabolite production , may also be due to the production of different types of cell wall degrading enzymes like chitinase, protease / elastase and β-1,3 glucanase. These enzymes are supposed to degrade the cell wall of various bacterial and fungal plant pathogens.

Detection of emulsifier production by *Bdiminuta* (Bd1)

The results showed that *B.diminuta* (Bd1) was unable to produce emulsifier, no emulsification layer was formed.

Detection of volatile compounds (VOCs) produced from *B.diminuta* (Bd1)

The results showed that *B.diminuta* (Bd1) produced volatile compounds with antifungal activity against *F. oxysporum* growth, the inhibition was (50.5%) comparing with control. The VOCs decreased the length of fungal mycelia, and colonies seemed to be significantly reduced. The

inhibition of *F. oxysporum* by VOCs was about 40% compared with the control after 3 days, suggesting that the bacterial VOCs had a significantly inhibitory effect on fungal mycelia.

Raza *et al.* (2013)[18] mentioned that, the volatile compounds produced by strain *Trichoderma harzianum* SQR-T037 were highly effective to suppress the growth of *F. oxysporum* up to 9 days. The continuous growth inhibition of *F. oxysporum* was reached from 18 to 40 % from first to nine days

Extraction of the crude antifungal substance produced from *B. diminuta* (Bd1)

Two solvents were used for extraction of the antifungal substance, chloroform and methanol. The results showed that chloroform at dilution 1**:**3 (v:v) was more efficient in antifungal substance extraction than methanol , Table-2, all the antifungal substance was extracted at this dilution , two layers formed , the results showed that antifungal activity of lower layer (chloroform layer) effected the growth of *F.oxysporum* , inhibition percentage was (57.6%) , while a little effect appeared (12.9%) when treated with upper layer comparing with control.

Table 2- Extraction of antifungal substances produced by *B.diminuta* (Bd1)

Extraction by Chloroform			Extraction by Methanol		
Layer / Dilution (v:v)	Upper (inhibition % of ***F.oxysporm***)	Lower chlorofom layer (inhibition% of ***F.oxysporm***)	Layer / Dilution (v:v)	Upper methanol layer (inhibition% of ***F.oxysporm***)	Lower (inhibition % of ***F.oxysporm***)
1:1	29.4	35.2	1:1	29.4	11.7
1:2	25.8	44.7	1:2	29.4	17.6
1:3	12.9	57.6	1:3	31.7	15.2

Chloroform is an organic compound with formula $CHCl_3$. chloroform is also used in pesticide formulations, as a solvent for fats, oils, rubber, alkaloids, waxes, gutta-percha, and resins, as a cleansing agent, grain fumigant, in fire extinguishers, and in the rubber industry [36]. Methanol is the simplest alcohol, with the formula CH_3OH (often abbreviated MeOH) , and is a light, volatile, colorless, flammable liquid with a distinctive odor very similar to that of ethanol (drinking alcohol) , methanol is primarily used as an industrial solvent for inks, resins, adhesives, and dyes, it is also used as a solvent in the manufacture of cholesterol, streptomycin, vitamins, hormones, and other pharmaceuticals[37].

Effect of antifungal substances on spore number

The results showed that the spores of *F. oxysporum* was reduced from 10^4 to 3×10 spores comparing with control after 7 days of incubation at 30^0C , this means, the antifungal substance produced by *B.diminuta* (Bd1) affected the spores of this fungus.

Yuan *et al*. (2012)[38] *Bacillus amyloliquefaciens* NJN-6 produces volatile compounds (VOCs) that inhibit the growth and spore germination of *F. oxysporum***.**

Adebayo and Aderiye (2010)[39] revealed that germination of fungal spores and fungal growth were significantly reduced by the *Lactobacillus* cells and/or their cell-free filtrates, thus indicating the propensity of the use of these antifungal substances in bio-control.

The experimental results demonstrated the fungicidal effect of bacterial species and revealed the possibility of these bacterial species to be used as biocontrol agents against these fungal species.

Determination the effect of bacterial cell on growth of fungi in soil

Three treatments were used (CF, CB, T) to determine the effect of *B.diminuta* (Bd1) on growth of *F.oxysporum*, Figure-6A. Results noted that the effect of *B.diminuta* (Bd1) was clear on the growth of *F.oxysporum* in soil, it was reduced until there was no growth after 9 days comparing with fungal growth control(CF) which remain stable along the period of experiment.This means that the bacteria *B.diminuta* (Bd1) has antifungal activity which inhibited the spores of *F.oxysporum.*

The number of bacteria (test and control) elevated due to their multiplication, but the number reduced for both, maybe due to nutrition reduction and waste accumulation but the bacterial control survive for more days than the bacterial test can be explained to the competition between bacterial cells and fungus on nutrient, Figure-6B.

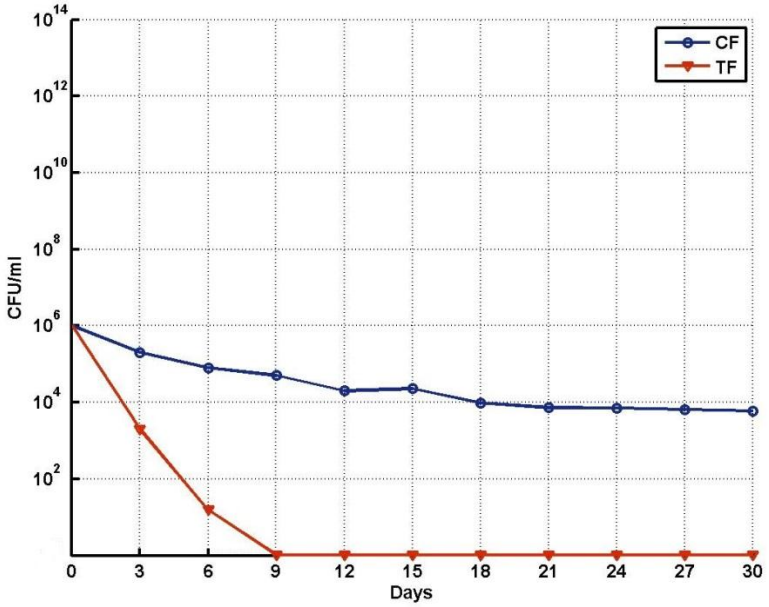

Figure 6A - The effect of *B.diminuta* (Bd1) on *F.oxysporum* growth. This figure clarify the inhibition of fungal spores by the bacteria , no growth appeared after 9 days. CF=Fungal Control, TF=Fungal Test.

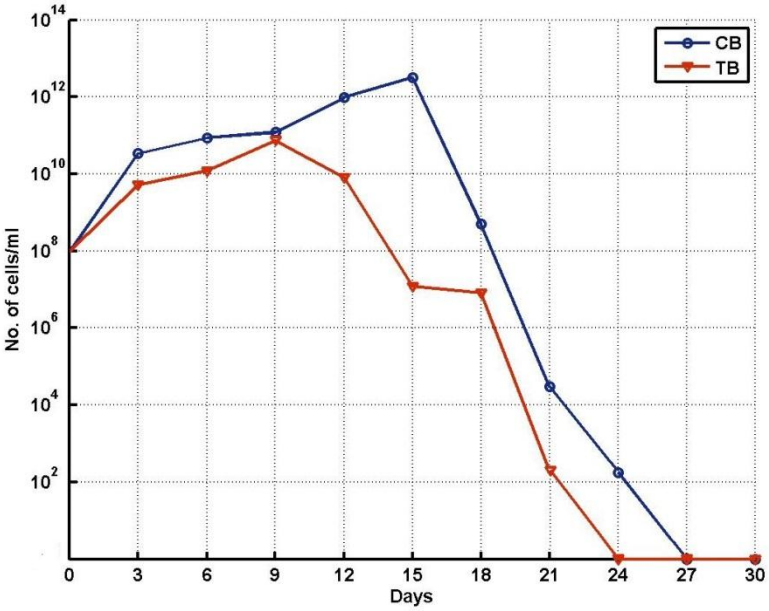

Figure 6B- Elevation and reduction of *B.diminuta* (Bd1) cells for test and control in soil. TB=Bacterial Test, CB=Bacterial Control.

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