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Research article

Role of chitinase produced from *Azospirillum brasiliense* in degradation free and snail chitin in soil

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ABSTRACT

Azospirillum brasiliense Aw-1 isolated from soil of plant roots was used for production of chitinase and degradation of soil and snail chitin. Soil contained chitin was treated with the bacterial cells and cell free extract (crude chitinase). The level of reducing sugars (as an indicator for chitin degradation) was measured. Significant increase in the reducing sugars was observed after treatment with chitinase (631.6 μg/ml) and bacterial cells (846.6 μg/ml) as compared with the control (305 μg/ml). Azospirillum brasiliense Aw-1 chitinase was used for treatment snail (chitin containing organism). This treatment caused many changes in snail shell and death the animal. These results showed that the possibility of using the chitinase to overcome the ability of chitin containing organisms to cause the infection.

Keywords: *Azospirillum brasiliense,* **chitinase, reducing sugars, snail.**

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INTRODUCTION

Chitin is a white, hard, inelastic polysaccharides, which consists of a linear poly-B-1,4-N-acetyl-Dglucos-amine. It is considered the second most abundant polysaccharide on the earth and can be found mainly in the cuticles of insects, shells of crustaceans and cell walls of most fungi [1]. Chitinase is one of the hydrolytic enzymes, which plays a major role in the biological processes in soil, such as degradation of organic compounds and liberating or recycling of nutrients such as nitrogen, phosphorus, sulphur and other essential metals [2].

Chitinase plays a vital role in the biological control of many plant diseases by degrading the chitin polymer in the cell walls of fungal [10]. Production of microbial chitinase has received worldwide attention in both industrial and scientific communities because its wide spectrum of applications [3].

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There are many application of chitinase including production of medical compounds, insect control, and control of plant pathogenic fungi [4].

Chitinase is one of the important mechanisms that are involved in the antagonistic activity of bio-control agent [5]. Chitinase is one of the hydrolytic enzymes, which are major components of biological soil processes, such as the degradation of organic compounds, their mineralization and the liberation or recycling of nutrients including nitrogen, phosphorus, sulphur and other essential metals [6].

The aim of this study is detecting the effect of chitinase produced from *A. brasiliense* that isolated from soil of plant roots on soil and snail chitin, which may apply in reducing the ability of snails to infect plant.

MATERIALS AND METHODS

Sample collection

Sixty-eight samples of soil surround plant roots (wheat, maize, barley and rice) were collected from Baghdad, Mousl and Babylon in sterile containers and transported to the laboratory and preserved at 4oC until using.

Isolation of *Azospirillum*

One gram of each sample was added to 9 ml of distilled water. Serial dilution with sterile distilled water up to 10-8dilutions was done. One ml of the diluted sample (10^{-6} to 10^{-8}) was inoculated in test tube contain 9 ml of selective nitrogen free bromothymol blue (5 %) semisolid media, consisted of 5.0 gm malic acid, 0.5 gm K_2HPO_4, 0.2 gm $MgSO_4$.7 H2O, 0.1 gm NaCl, 0.002 gm $CaCl_2$, 0.002 $Na_2MoO_4.2H_2O$, 0.01 Na_2MoO_4.2H2O, 0.015 $FeCl_3.6H_2O$, 0.0001 Biotin, 4.5 KOH, 1.75 agar and 2 ml bromothymol blue all these components was completed to 1 liter with sterile distilled water.

All tubes were incubated at 32 ^oC for 48 h. The change of color of nitrogen free bromothymol blue semisolid medium from green to blue with formation of white pellet after 24 h and raise to the top after 48 h confirmed to grow of *Azospirillum*. The bacteria was purified on Rogo Congo medium (5.0 gm malic acid, 0.5 gm K2HPO4, 0.2 gm MgSO4.7 H_2O, 0.5 gm NaCl, 0.5 gm yeast extract, 0.015 gm $FeCl_3.6H_2O$, 4.8 gm KOH, 20.0 gm agar dissolved in 1 litter distilled water) and incubated at 37 ^oC for 72 h and preserved on Nitrogen free bromothymol blue semisolid or agar medium

Identificationof *Azospirillum*

Azospirillum Isolates were identified by morphological features, microscopic examination and biochemical tests. The last included growth in 3% NaCl and at pH 6 and 7.5, oxidase and catalase test, requirement of biotin for bacterial growth and consuming of different carbon sources in bacterial growth.

Determination the ability of *Azospirillum* to produce chitinase (Qualitative assay)

Chitin agar medium consisted of 0.1 gm colloidal chitin, 0.5 gm yeast extract, 0.01 gm $MgSO_4$.7 H2O, 0.6 gm KH_2PO_4, 0.5 gm NaCl, 0.1 gm K_2HPO_4 and 2 gm agar dissolved in 100 ml distilled water [2]. Post sterilizing the medium by autoclave and poured in Petri dishes the medium was inoculated with bacterial culture and incubated at 30^oC for (3-7) days. A positive result was observed as a clear zone around the colonies. The diameter of colony and clear zone were measured. The ratio of clear zone diameter to colony diameter was measured.

Production of chitinase in liquid medium

Chitinase production medium consisted of 0.1 gm colloidal chitin, 0.01 gm $MgSO_4.7H_2O$, 0.6 gm KH_2PO_4, 0.5 gm NaCl and 0.1 gm K_2HPO_4, these materials was dissolved in 100 ml distilled water [2]. This medium post sterilizing by autoclave was inoculated with 1 ml of bacterial culture for 24 h and incubated at 30 ^oC for 5-7 days in a rotary shaker. The culture was centrifuged at 10,000 rpm for 30 min, the supernatant was collected (crude enzyme) and the chitinase activity was measured in the supernatant.

Estimation of chitinase activity (Whitaker and Bernhard, 1972)

Preparation of standard curve of glucose

A standard curve of glucose was established by preparing serial concentrations of glucose (100-800 μg/ml), one ml of Dinitrosalasilic acid (DNSA, 1%) was added to 1 ml of glucose solution and incubated in boiling water bath for 5 min and cooled by water. Five ml of distilled water was added to each tube and the optical density was measured at 540 nm [7].

Measurement of chitinase activity

0.1 ml of enzyme solution was added to 0.9 ml of colloidal chitin solution %1 (1 gm colloidal chitin in 100 ml of potassium phosphate buffer (0.2 M, pH 6) and incubated in water bath at 35 ^oC for 10 min. The reaction was stopped by adding DNSA solution and incubated in boiling water bath for 5 min. The mixture was cooled by water and then 5 ml of distilled water was added. The optical density was measured at 540 nm [7]. One unit of chitinase activity was defined as the amount of enzyme that liberated 1 μmol of the reducing sugar (glucose) per min at pH 6 and 30 ^oC. This experiment was done in laboratory conditions.

Determination of chitinase effect on soil chitin

One gram of chitin was added to 100 gm air-dried soil and sterilized by autoclave. To measure the concentration of reducing sugars in the soil (as a parameter for chitin hydrolysis), 5 gm of soil was taken and 10 ml of distilled water was added, The mixture was shacked well and left for 30 min, the supernatant was centrifuged at 5000 rpm for 15 min. Reducing sugars were estimated by using the DNSA method [7].

The soil was treated with the bacterial cells suspension (optical density 0.9 at 540 nm), and other samples of soil was treated with cells extract (crude chitinase), the concentration of reducing sugars was measured at different intervals of time (0, 2, 5 and 7 days).

Effect of chitinase on chitin containing organisms (snails)

Many snails were collected from soil and divided into 2 groups (5 snails in each) first group was treated with chitinase by spraying 0.1 ml of crude enzyme by dropping with syringe, and another group was left without treatment (control). Both groups were left at room temperature in sterile container and the changes in snail features were recorded.

RESULTS AND DISCUSSIONS

Isolation and identification of *Azospirillum*

Fifty two isolates of bacteria were obtained from sixty eight samples of soil, after culturing on *Azospirillum* selective medium. Seventeen isolates obtained from soil of wheat roots, fourteen isolates obtained from soil of barley roots, twelve and nine isolates were obtained from soil of rice roots and maize roots, while no isolate was obtained from soil of vegetables (spinach, onions and radish). The growth characteristics of these isolates on *Azospirillum* selective medium and RC medium indicated that the isolates were classified as a member of genus *Azospirillum* [9,10]. When these isolates were further identified by morphological and biochemical tests, the results showed that they were identified as strains of A. brasiliense.

Qualitative assay of chitinase in *A. brasiliense* growth

Most *A. brasiliense* isolates were able to produce chitinase enzyme. The hydrolysis zones of chitin ranged between 1.7- 5.2 mm, *A. brasiliense* (Aw 1, Aw 5, Aw 6, Aw 13, Ar 3 and Am 5) isolates isolated from wheat, rice and maize gave superior hydrolysis zones compared with others. This may be attributed to the adaptation of bacteria in the soil to chitin, which is generally found in soil as waste of insects and other organisms, these wastes induce the microorganisms to produce chitinase. While, the lowest hydrolysis ratio was found in isolate Am -7 (1.7 mm) and isolate Ar-6 that isolated from maize and rice toot, respectively (**table 1**).

Table 3. Chitinase levels produced by different isolates of *A.brasiliense*.

Bacterial isolates	Hydrolysis ratio (diameter of clear zone /colony diameter)	Bacterial isolates	Hydrolysis ratio (diameter of clear zone /colony diameter)
Aw-1	5.2	Aw-16	4.2
Aw-2	3.9	Aw-17	3.8
Aw-3	4.8	Ab-1	_
Aw-4	4.2	Ab-2	4.8
Aw-5	5	Ab-3	4.1
Aw-6	5	Ab4	3.8
Aw-7	4.1	Ab-5	_
Aw-8	4.5	Ab-6	_
Aw-9	4.8	Ab-7	4.1
Aw-10	4.3	Ab-8	3.7
Aw-11	3.3	Ab-9	3.9
Aw-12	3.7	Ab-10	3.8
Aw-13	5	Ab-11	4.2
Aw-14	4	Ab-12	4
Aw-15	3.9	Ab-13	3

Quantitative assay of chitinase produced from different isolates of *A. brasiliense*

Fifteen *A. brasiliense* isolates were selected according to their ability to produce chitinase (large hydrolysis zone) ranged between 3.8-5.2 mm. These 15 isolates were further examined to estimate their chitinase activities. The maximum activity (610 u/ml) was recorded in case of Aw-1 isolate, while the minimum activity (240 u/ml) was recorded in case of Am-3 isolate.

Effect of *A. brasiliense* (Aw-1) chitinase on soil chitin

A. brasiliense Aw-1 isolate was selected as this isolate produce the highest chitinase activity. The effect of chitinase and bacterial cells (Aw-1) on soil containing chitin was determined by measuring the reducing sugars in the soil before and after treating soil (contain chitin). It was observed that the *A. brasiliense* (Aw-1) cells was more effective than the chitinase enzyme in chitin degradation (**table 2**), since the reducing sugars liberated in soil treated with the bacterial cells was 890 μg/ml, while the reducing sugars liberated in soil treated with enzyme was 650 μg/ml. However the reducing sugars liberated in case of control was 305

μg/ml. This happened because the enzyme lost its activation due to the environmental conditions. The enzyme is protein sensitive to many environmental factors such as temperature, pH and metal ions [11]. While, the bacterial cells less sensitive to these factors because the bacterial cells protected by cell wall and that reflect on the producing of chitinolytic enzyme more effective than the free enzyme. Chitin is one of the most abundant biopolymers widely distribute in the marine and terrestrial environments. Chitinase enzyme has received increased attention due to its wide range of biotechnological applications, especially in agriculture for bio-control of phytopathogenic fungi and harmful insects [4,3].

Table 2. Effect of *A. brasiliense* Aw-1 cells and chitinase (crude enzyme) on soil chitin

	Concentration of reducing sugars in the soil (μg/ml)			
Treatment (triplicate)	Control conc.	After 2 days of treatment	After 5 days of treatment	After 7 days of treatment
Bacterial cells	305	625	710	840
	309	540	735	890
	301	560	710	810
Means ±S.d.				
	Control	2 days	5 days	7 days
	305± 4	575± 44.44	718.3 ±14.43	846.66 ±40.4
Concentration of reducing sugars in the soil (μg/ml)				
Chitinase (Crude extract)	305	525	545	620
	309	510	600	650
	301	490	585	625
Mean ± SD				
	Control	2 days	5days	7 days
	305	508.33±17.55	576.66±28.43	631.66±16.07

Chitinase is capable to hydrolyze insoluble chitin to its oligo and monomeric component found in a variety of organisms including fungi, insects and animals [12]. There was a significant difference ($P< 0.05$) between the control and the test (soil treated with chitinase or bacterial cells). The application of chitinolytic Azospirilla in soil not only has a benefit in fighting the fungal and insectal pests but also as a nitrogen fixer and plant growth promotor, it can cause more responses to *Azospirillum* inoculation in cereals and non cereal species, increase in total plant dry weight, in total amount of nitrogen in shoots and grains, in the total number of tillers, fertile tillers, and ears; earlier heading and flowering lime; increased number of spikes and grains per spike; increased grain weight; plant height; leaf size and higher germination rates [13].

Effect of *A.* brasiliense (Aw-1) on chitin containing organisms (snails)

The result in **fig 1** describes the effect of *A. brasiliense* chitinase on the snails after an hour of treatment. It was observed full death of the snails, removal of the chitin layer and change in color from brown or black to white was observed. These features indicate the role of chitinase in degradation of chitin layers of snails and hence the harmful effect, which leaded to death of this organism.

The effectiveness of chitinase has important environmental and application benefits, it was used as a biomaterials (biocides) to eliminate pests and insects that cause large economic losses beside the toxicity of chemical pesticides that pose arisk on other organisms such as human and animal as well as the accumulation and the difficulty of decomposition in the soil leading to prolong survival in the environment and the continuation of its toxicity [4].

The importance of chitinolytic enzymes in insect, nematode and fungal growth and development received attention in regard to their development as biopesticides or chemical defense proteins in plants and microbial bio-control agents [1]. Biological control of some soil born fungal diseases has been correlated with chitinase production. Chitinase produces from Serratia marcescens had similar antagonistic effect to larvae of *Tribolium confusum* insect was reported in previous study [14]. Chitinase produced from *Bacillus* sp. and *Streptomyces* was used as insecticide and work to prevent the process of alienation [15]. The present study provides a good report about the possibility of using the chitinase in controlling the severity of plant pathogens.

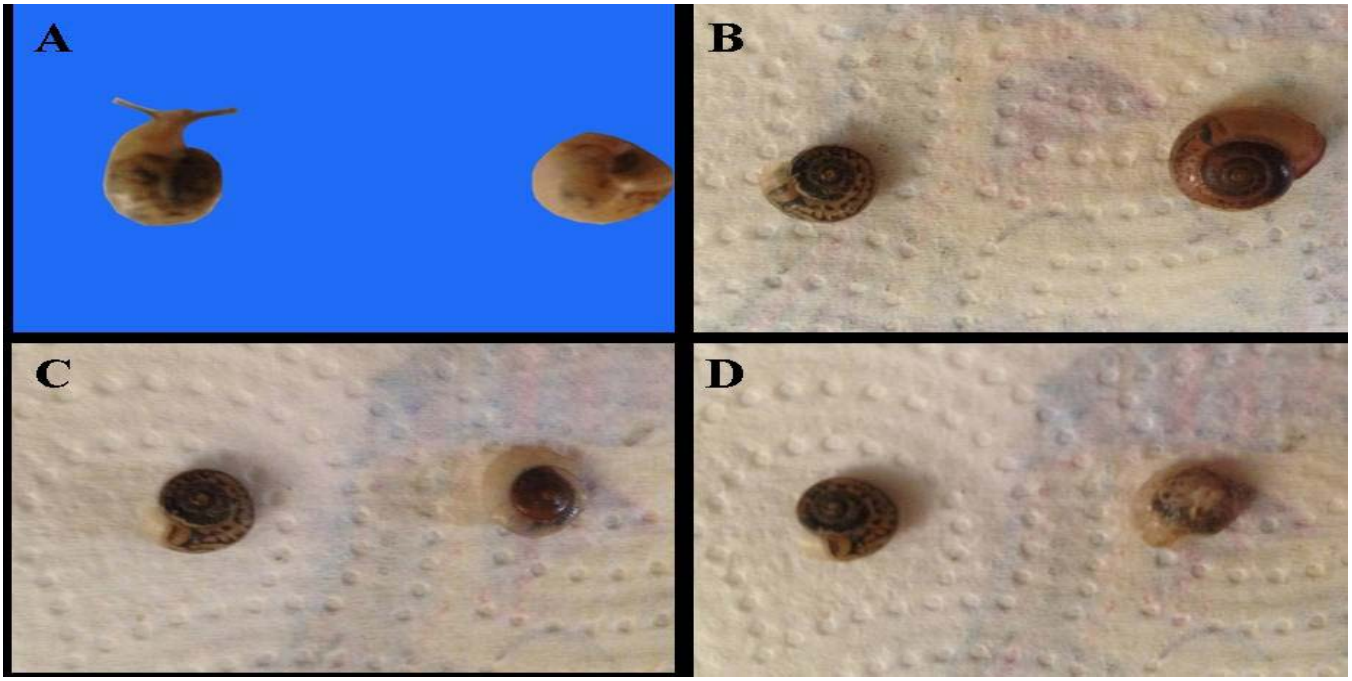

Fig 2. Effect of *A. brasiliense* (Aw-1) on chitin containing organism (snails). A, full death of the snail; B, degradation of the chitin layer; C, change in color; D, occurrence of alienation.

Conflict of interest:

The authors declare that they have no conflict of interests.

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