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The efficacy of Chitinolytic bacteria as a bioinsecticide against *Tuta absoluta* (Lepidoptera: Gelechiidae)

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Abstract
Chitin is the main component of insect cuticle also, it presents in the gut membrane. Thus the use of chitinolytic bacteria or their metabolites to control insect pests is very interesting. In the present work, chitinolytic bacterial isolates were isolated and identified to use them as a bioinsecticide to control the tomato leaf miner insect *Tuta absoluta.* A total of 18 chitinolytic bacterial isolates were obtained from 6 soil samples. Two isolates were found to be more active in chitinase activity in both broth and solid colloidal chitin medium. These isolates were designated IS16 and IS17. Based on sequence of the 16S rRNA gene, IS16 isolate was similar to *Bacillus subtilis* and IS17 isolate was similar to *Lysobacter enzymogenes.* The efficacy of both bacterial isolates was tested against *Tuta absoluta* by leaf dipping bioassay method. The treatments were supernatant that contains chitinase enzyme and original bacterial culture that contain bacterial cells and chitinase enzyme and bacterial cells solution. The obtained data revealed that the culture which contains cells and chitinase had larvicidal activity more than other treatments. More deformation was observed in larvae when were treated by supernatant which contains chitinase enzyme. This study recommended that the chitinolytic bacteria could be used in IPM programs to control tomato leaf miners for decreasing chemical pesticide usage.

Keywords: bioinsecticides, chitinolytic bacteria, *Tuta absoluta,* 16S_ rRNA

Introduction
The use of biological agents to control crop pests holds great promise as an alternative to the use of chemical pesticides. Secondary metabolites and microorganism crude enzymes were used to control the population of crop pests (Kramer, *et al*. 1997) [13]. In nature, chitin is abundant as a structural compound in cuticles, especially in insects (Arakane and Muthukrishnan 2009) [2]. The growth and development of insects depend heavily on the construction and remodeling of chitin structures (Merzendorfer and Zimoch 2003) [15]. The chitin structure is a polysaccharide of an amino sugar (N-acetyl-glucosamine) linked together by β-1, 4-glycosidic bonds (Chuan, 2006) [8]. There are several bacteria and fungi able to produce chitinase enzymes that are able to degrade the chitin. (Kramer and Muthukrishnan 1997; Nurdebyandaru *et al.* 2010) [14, 18]. The damage caused by chitinase to the peritrophic membrane in the insect gut leads to a significant reduction in the use of nutrients and thus a decrease in insect growth (Terra and Ferreira 2005) [22]. As a result, chitinase in the insect diet will reduce the growth of insects (Otsu *et al*. 2003; Fitches *et al*. 2004) [19, 11]. Chitinolytic bacteria can be used as bio-control agents to control many crop pests. (Wang *et al*. 2006; Nurdebyandaru *et al*. 2010) [5, 18]. Fan *et al.* 2007 [10] mentioned that the over-expression of chitinase by an entomopathogenic microorganism may increase the mortality in treated insects (Fan *et al.* 2007) [10]. The aims of this work are isolation and identification of bacterial isolates that produce chitinase enzymes, determining the best isolates that secrete chitinase enzymes, evaluating the efficacy of the best isolates against the tomato leaf miner insect *Tuta absoluta* (Lepidoptera: Gelechiidae).

Materials and Methods
Bacterial isolation
Six soil samples were collected from different locations in the agriculture college's farm, Mansoura University, Mansoura, Egypt. These samples were put in sterile plastic bags and transported to the microbiology lab. to isolate the chitinolytic bacteria by using the selective medium, chitin colloidal agar (CCA medium) as described by Choi *et al.* 2004 [7]. Serial dilutions from soil samples were prepared in sterile water. CCA medium (0.5% colloidal chitin, 0.2% Na_2HPO_4, 0.1% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 0.05% $MgSO_4$ $7H_2O$, 0.05% $CaCl_2$ $2H_2O$, 0.05% yeast extract and 2% agar per liter) was autoclaved and poured in Petri plates then allow it to solidification. A 0.2 ml from each concentration were spread on the CCA plate and incubated at 28 ºC to 72 hrs. The isolates showing a clear zone around the colony have been selected and purified using fresh CCA medium (Choi *et al.* 2004) [7]. The degradation efficiency (DE) of the isolates was calculated to select the best isolates as described by Qureshi *et al.* (2012) [21] using the following formula:
DE % = ((Clear zone diameter – Growth diameter) ⁄Growth diameter) x100

Chitinase activity
The bacterial isolates that have a clear zone around their colonies on CCA medium were grown in colloidal chitin broth medium (CCB) at 28 ºC to 5 days under shaking. After incubation periods, cultures were centrifuged at 10000 rpm/20min. A 150µl from the supernatant was added to 300µl of 0.3% CCB and 150µl of 0.1M phosphate buffer (pH 7.0) and incubated at 37 ºC under shaking 120rpm/60min then centrifuged (10000rpm/5min.).

The mixture of the supernatant, 500µl distilled water, and 1000µl Schales' reagent was boiled ten minutes. Schales' reagent (a mixture of 0.5 M sodium carbonate and 0.5 g/L potassium ferricyanide in water). Measuring absorbance at 420 nm was done to determine the enzyme activity using a spectrophotometer. One unit of enzyme activity was detected as the amount of enzyme which releases 1µmol of reduced sugar (N-acetyl glucosamine) per minute. (Toharisman *et al.* 2005).

Bacterial identification by 16S_rRNA

The specific primer P1F (5'-AGA GTT TGA TCC TGG CTC AG-3') and P2R (5'-TGA CTG ACT GAG GCT ACC TG-3') was used to identify both bacterial isolates according to Wahyudi *et al.* (2010) [24]. The setup of PCR condition was 35 cycles with a pre-denaturing step at 94°C for 2min, denaturing at 92°C for 30 sec, annealing at 55°C for 30sec, polymerization at 72°C for 1 min and post PCR at72°C for 5 min. (Baharuddin *et al.* 2010) [2]. Approximately 1300bp of the PCR product were purified and sequenced in Macrogen Inc., South Korea (www.macrogen.com/eng/). The Alignment of the 16S_rRNA sequences was done using the BLASTN program from the NCBI web site (http://www.ncbi.nlm.nih.gov).

The insect bioassay

Both bacterial isolates that have high production of chitinase enzyme were grown in 300 ml colloidal chitin broth medium (CCB) in 1 L conical flask for 8 days and 28 ºC under shaking incubation system. After the incubation period, half volume of the bacterial culture was centrifuged at 10,000 rpm for 10 minutes but the other bacterial culture still as it's. After centrifugation, the pellet of bacterial cells was mixed with 150 ml sterilized saline solution. The insect bioassay treatments were 150 ml of supernatant that contains chitinase enzyme (Sup) and 150 ml original bacterial culture that contain bacterial cells and chitinase enzyme (OBC) and 150 ml bacterial cells solution (BCS).

Laboratory bioassay was conducted by leaf dipping method as described by Miller *et al.* 2010, un-sprayed tomato leaves were taken and washed in freshwater. These leaves were dipped in the test treatment for 1 min. with gentle agitation, and then dry them in the air on tissue papers. After drying, the leaves were placed in Petri dishes (15 cm). Twenty individuals of 2nd instar larvae of *T. absoluta* were placed in each Petri dish. Four replicates were used for each treatment. Deformities in the larvae and mortality were recorded after 3, 5 and 7 days. The mortality percentage was corrected by Abbott's formula (Abbott 1925) [1].

Results

Isolation and purification of chitinolytic bacteria

Eighteen bacterial isolates were found in the collected soil samples able to degrade the colloidal chitin. The isolates were coded (From IS 01 to IS 18) and listed in Table 1 with their chitinase activities. Most of the isolates had a high activity of chitinase production specially isolates no IS16 and IS17 were more active as shown in Fig. 1.The degradation efficiency % (DE) of both isolates was 100% and 91%. Respectively also, chitinase activity (U/ml) reached 22 and 20 U/ml, respectively.

Table 1: Chitinase activities of bacterial isolates that had clear zone around their colonies on CCA medium.

Bacterial Isolates (Codes)	Diameter of growth (G) (cm)	Diameter of Clear zone (Cz) (cm)	Degradation efficiency (DE) %	Chitinase activity (U/ml)
IS 01	1.5	2.2	46.7	10
IS 02	1.1	1.7	54.5	12
IS 03	1.5	2.1	40.0	9
IS 04	1.0	1.6	60.0	13
IS 05	0.9	1.4	55.5	12
IS 06	0.8	1.2	50.0	10
IS 07	1.5	2.7	80.0	17
IS 08	1.5	2.4	60.0	13
IS 09	1.3	2.2	69.2	15
IS 10	1.3	2.0	53.8	12
IS 11	1.7	2.3	35.3	8
IS 12	1.5	2.1	40.0	9
IS 13	1.0	1.8	80.0	17
IS 14	1.2	1.9	58.3	12
IS 15	1.0	1.7	70.0	15
IS 16	1.3	2.6	100.0	22
IS 17	1.1	2.1	91.0	20
IS 18	0.9	1.6	77.7	17

DE % = ((Cz–G) ⁄G) x 100

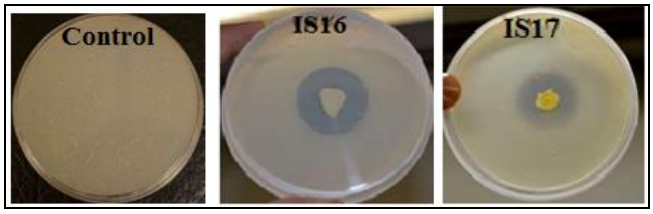

Fig 1: Colloidal chitin degradation by isolates IS 16 and IS 17 on CCA medium.

Bacterial identification by 16S_rRNA

The sequence of 16S_rRNA gene was a continuous stretch of 1300 and 1500 pb to both bacteria isolates IS16 and IS17, respectively. The *16S_rRNA* gene sequences of related taxa were obtained from GenBank of the EMBL Nucleotide Sequence Database. The IS16 isolate had the highest degree of sequence similarity to *Bacillus subtilis* (97%) but the IS 17 isolate had the highest degree of sequence similarity to *Lysobacter enzymogenes* (99%).

Bioassay test on *Tuta absoluta*

The presented data in the bioassay test revealed that the mortality percentage of *T. absoluta* increased with increasing the days after treatment (Table 2). Also, the original bacterial culture (OBC) caused the highest mortality % followed by supernatant (Sup) and Bacterial cell solution (BCS) in both bacterial isolates. As well as, the obtained results from microscopic observation illustrate that the deformations of larvae exoskeleton increased with the treated larvae by supernatant (Sup) more than other treatments; however, the larvae still survive after 3 days (Fig. 1). In general, mortality percentage was increased by *B. subtilis* more than *L. enzymogenes* Table 2).

Table 2: Mortality % of treated 2nd instar larvae of *T. absoluta* by chitinolytic bacteria

Treatments		Mortality % after days		
		3 days	5 days	7 days
B. subtilis	OBC	20.6	35.7	71.4
	Sup	3.2	16.2	35.7
	BCS	13.4	25`.5	53.8
L. enzymogenes	OBC	16.7	26.4	58.7
	Sup	0.0	10.3	21.6
	BCS	10.2	17.8	43.4

OBC: Original Bacterial Culture, Sup: Supernatant, BCS: Bacterial Cells Solution

Fig 1: Symptoms of infection and exoskeleton deformation of *T. absoluta* larvae

Discussion

The chitinase enzyme able to degrade the cuticle layer and gut membrane of insects that led to weaker insects and increase insect mortality. The damage caused by chitinase to the membrane in the gut causes a decrease in nutrient consumption and consequently in the growth of insects (Terra and Ferreira 2005) [22]. In the present work, chitinolytic bacteria were isolated and identified to use them as a bioinsecticide to control the tomato leaf miner insect *T. absoluta.* A total of 18 chitinolytic bacterial isolates were obtained from 6 soil samples. Two isolates were found to be more active in chitinase activity in both broth and solid colloidal chitin medium. These isolates were designated IS16 and IS17. Based on sequence of the 16S rRNA gene, IS16 isolate was similar to *B. subtilis* and IS17 isolate was similar to *L. enzymogenes.* The previous studies revealed that these bacterial isolates could be produced chitinase enzymes and they could be used as biological control agents.

Production of chitinase has also been recorded in numerous Bacillus species, such as *B. Subtle* (Wang *et al*. 2006) [12], *B. cereus* (Huang *et al*. 2005) [12], *B. circulans* (Chen *et al*., 2004) [5], *B. licheniformis* (Waldeck *et al*. 2006) [25] and *B. megaterium* (Donderski and Brzezinska 2005) [9]. Also, *Lysobacter spp*. could produce protease, chitinase, and β-1, 3-glucanase where it showed strong chitinase activity *in vitro* as described by Qian *et al*. 2009 [20]. *Lysobacter spp* especially *L. enzymogenes* produce a wide range of compounds that have biological activity as lytic enzymes and antibiotics and thus have a high promise to plant protection as mention by Choi *et al*. 2012 [6].

Previous reports have shown that *Bacillus* spp. demonstrated their ability to degrade exoskeleton chitin of whitefly *Bemisia tabaci* and aphids *Myzus persicae* and *Aphis gossypii* (Mubarik *et al.* 2010; Nurdebyandaru *et al.* 2010) [17, 18]. Moreover, the results of Chandrasekaran *et al.* (2012) [4] reported that *B. subtilis* strain had a significant reduction against the tobacco cutworm *Spodoptera litura* by increasing the mortality of larvae and reducing their weight and growth. In conclusion, the results in this work recommended that using isolates *B. subtilis* and *L. enzymogenes* could be served as bioinsecticides for control tomato leaf miner *T. absoluta*.

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