Global Journal of Molecular Sciences 2 (2): 57-66, 2007
ISSN 1990-9241


# Isolation and Identification of Rhizosphere Soil Chitinolytic Bacteria and their Potential in Antifungal Biocontrol

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**Abstract:** Four hundred bacterial isolates were isolated from rhizosphere of some plants collected from Egypt and screened for production of chitinase enzyme. Only four isolates designated MS1, MS2, MS3 and MS4 were the most potent chitinolytic bacterial species. SDS-PAGE analysis of vegetative and sporulated cells of the four isolates revealed that the protein profile of the four isolates were different from each other in their banding pattern and were identified as *Bacillus licheniformis, Stenotrophomonas maltophilia, Bacillus licheniformis* and *B. thuringiensis*. *In vitro* MS1 and MS3 were the most active species, so they suppressed the growth of all tested pathogenic fungi (*Rhizoctonia solani, Macrophomina phasiolina, Fusarium culmorum, Pythium sp, Alternaria alternata and Sclerotium rolfsii*). Also, MS3 produced the highest level of chitinase enzyme (1.27 µ/ml) after 4 days incubation as compared with the other isolates. In green-house experiment, *B. licheniformis* (MS3) significantly reduced the damping off disease caused by *Rhizoctonia solani*, in *Helianthus annus* using the seed coat or soil draing treatments.

**Key words:** Antagonistic activity . chitinase . *Bacillus licheniformis* . damping off . phytopathoginic fungi

## INTRODUCTION

Potential use of naturally occurring bacteria, actinomycetes and fungi replacement or supplements for chemical pesticides have been addressed in many studies [1-4]. Chitin, a homopolymer of ß-1,4-linked N-acetyl-D-glucosamine residues, is the most abundant renewable resource after cellulose. It is widely distributed in nature as a structural component of crustacea, fungi, protozoa and insects. The annual global yield of chitin is assumed to be 1 to 100 billion metric tons, making chitin the second most abundant polysaccharide on the earth. Chitinases (EC 3.2.1.14) are glycosyl hydrolases, which catalyze the degradation of chitin. These enzymes are present in a wide range of organisms such as bacteria, fungi, insects, plants and animals. Chitinases are divided to family 18 and family 19 of glycosyl hydrolases on the base of their amino acids sequences [5, 6]. Screening and isolation of organisms capable of producing chitinase is usually done on a medium containing chitin. The chitinases of the above-mentioned organisms play important physiological and ecological roles.

Invertebrates require chitinases for partial degradation of their old exoskeletons [7] and plants as a defense mechanism against fungal pathogens [8]. Bacteria produce chitinases mainly to degrade chitin and utilize it as an energy source. In addition, some chitinases of chitinolytic bacteria, such as the *chiA* gene produced from *Serratia marcescens* and *Enterobacter agglomerans* are potential agents for the biological control of plant diseases caused by various phytopathogenic fungi [9, 10]. The latter enzymes inhibit fungal growth by hydrolyzing the chitin present in the fungal cell wall. Antifungal proteins such as chitinases are of great biotechnological interest because of their potential use as food and seed preservative agents and for engineering plants for resistance to phytopathogenic fungi [11].

Biological control of damping-off in crops caused by *Rhizoctonia solani* has been reported all over the world using application of antagonistic fungi and bacteria isolated from coastal soils [12-14]. The aim of this study was to isolate chitinolytic bacteria from Egyptian rhizosphere soils and to screen their antagonistic activity against some fungal pathogens.

## MATERIALS AND METHODS

**Sample sites and microbial strains:** Rhizospheric soil samples of maize, wheat and rice were collected from Giza, Helwan and Mansoura, for isolation of

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chitinolytic bacteria. Bacterial reference strains used in this study, *Bacillus thuringiensis var. kurstaki* and *Serratia marcescens* were obtained from Microbial Molecular Biology Laboratory, Agricultural Genetic Engineering Research Institute, Giza. The phytopathogenic fungi *Rhizoctonia solani, Fusarium culmorum, Macrophomina phasiolina, Sclerotium rolfsii* and *Pythium* sp. were kindly provided by Plant Pathology Department, Faculty of Agriculture, Alexandria University and maintained on potato dextrose agar medium.

**Isolation and identification of bacteria:** Suspensions were made by adding 5g of soil to 50ml sterile basic salt solution. Ten fold dilutions of these suspensions were plated on Luria-Bertani (LB) agar. Only colonies from the highest dilution of the soil suspensions were selected for isolation of bacteria. For enrichment of chitinolytic bacteria, a minimal salt medium containing a colloidal chitin as sole carbon and energy source was used. The medium consisted of $Na_2HPO_4$, 6g; $KH_2PO_4$, 3.0g; $NH_4Cl$, 1g; NaCl, 0.5g; yeast extract, 0.05g, colloidal chitin 1.0% (w/v) agar, 15g and distilled water, 1000 ml and incubated at 30ºC. Colloidal chitin was prepared by the method of [15] from crab shell chitin (Sigma). Isolations were performed under aerobic conditions. The isolation was initiated by adding 0.5 g (wet weight) soil to LB medium (25 ml). In order to obtain pure cultures, samples of chitin degrading enrichment cultures were streaked on LB agar. Colonies showing zones of clearance against the creamy background were regarded as chitinase-producing and restreaked until pure cultures were obtained. The selected chitinolytic bacteria chosen for further studies were identified.

**One-dimension protein electrophoresis analysis:** Protein analysis was done by sodium dodecyl sulfate (SDS)-polyacrylamid gel electrophoresis (PAGE) with 10% gels [16]. 100 µl of either bacterial suspenations of vegetative and sporulated cells or crude supernatants proteins were mixed with 100 µl of 2X sample buffer, boiled for 2 min, then load. The gel were stained with 0.25% coomassie brilliant blue R-250 (Bio-Rad), destained with 10% acetic acid and 7% methanol.

***In vitro* antifungal activity:** The antifungal activity was assayed *in vitro* by inhibiting the growth of phytopathogenic fungus on PDA media [17]. The bacterial inoculum of the four chitinolytic isolates MS1, MS2, MS3 and MS4 was picked aseptically and streaked in the center of petridish. Fungal inocula consisted of agar disc (1cm diameter) punched out with sterilized corkborer from the growing margin of colonies was placed on either site of bacteria inoculated plates. The Petri plates were incubated at 28 ºC for five days. The diameter of hyaline inhibition zones were measured and experiment was repeated three times

**Chitinase assay:** Chitinase activity was determined colorimetrically by detecting the amount of *N*-acetylglucosamine (GlcNAc) released from a colloidal chitin substrate [18]. Four 500 ml flasks containing 250 ml chitin medium were inoculated by the four selected isolates and incubated at 30ºC on a rotary shaker 180 rpm for 5 days; flasks were removed every 24 hours and the chitinase was measured colorimetrically The reaction mixture consisted of equal volumes (0.2 ml) of culture supernatant and 0.5% colloidal chitin in 50 mM acetate buffer, pH 6.0. The reaction was performed at 37°C for 30 min and was measured at 585 nm. N-acetyl glucosamine (GlcNAc) (Sigma, USA) was used as the standard, with a molar adsorption coefficient of $2.4\times10^3$ l per mole-centimeter. One unit of chitinase activity was defined as the amount of enzyme that released 1 µmol GlcNAc or its equivalent from colloidal chitin in 1 min.

**Fungal inoculum preparation:** Fungal inoculum was prepared by growing the fungus on PDA agar for 7 days at 25°C. A 500 ml flasks was filled upto 1/3 of its volume with barley grains and was completed with distilled water then allowed to stand for 2 hr., after which time the water was decanted and the grains were autoclaved for 15 min. Four disks of agar from the margin of actively-growing colony of the fungus was introduced into the flasks and stored for 2 weeks at 25°C.

**Bacterial inoculum preparation:** Isolate MS3 was grown in 2 litre of nutrient broth on a rotary shaker at 30°C and 180 rpm for 24 h. The culture broth was centrifuged in 50 ml capacity sterile plastic tubes at 5000 rpm for 10 min. The pellets were re-suspended in sterile water to give a final concentration of $10^7$ and $10^{10}$ cfu/ml using the viable plate count method and optical density measurement at 600. Each concentration was divided into two equal portions. One portion was used in seeds treated (dipped for 10 min in bacterial suspensions containing $10^7$-$10^{10}$ cells $ml^{-1}$); and the other portion was used to drainage the soil of plants with 50 ml of each bacterial suspension concentration ($10^7$-$10^{10}$ cells $ml^{-1}$).

**Green-house experiment:** This was conducted to study the suppression of *Helianthus annus* damping off disease by the isolate MS3. Pots, 20 cm in diameter, filled with autoclaved sandy clay soil (1:1 v/v), were

aerated for one week. The soil was infested with the fungal inocula (10g $kg^{-1}$ soil) and left for one week. Ten sunflower seeds were sown in each pot and maintained in the greenhouse at 30±1°C. Two successive bacterial applications were made a week apart. The treatments were: plants inoculated with *Rhizoctonia solani* and MS3 isolate, plants inoculated with *Rhizoctonia solani* alone and control without fungal and bacterial inoculum. Pots were left for 4 days, after which time the percentages of pre-and post-emergence ssedlings, for each treatment were recorded for 20 days (3 replicates).

## RESULTS

**Isolation and screening of chitinolytic bacteria:** Screening of chitinolytic bacteria isolates was carried out by spread inocula of each colony on plates containing a minimal salt medium with colloidal chitin as a sole carbon and energy source. The chitin-degrading organism formed colonies of 1-2 mm in diameter, surrounded by clear zones indicating chitinase activity. Only 5% of 400 isolates exhibited different clear zones sizes. Out of these, four isolates designated MS1, MS2, MS3 and MS4 gave the widest clear zones (Plate 1).

**Identification of the selected chitinolytic bacterial isolates:** The results presented in Table 1-3 and the API Kit profiling, were matched with those presented in Bergey's Manual of Systematic Bacteriology [19]**.** These characteristics suggest that isolates MS1, MS3 and MS4 belong to the genus *Bacillus*, while the Gram negative isolate MS2 was identified to be *Stenotrophomonas maltophilia*. Furthermore the organism MS1 and MS3, although they are different in colonies morphology, they have the same results of biochemical test and identified as *Bacillus licheniformis*.

All strains were positive in the following tests: Catalase, anaerobic growth, hydrolysis of casein, lipase, growth at 30°C and 40°C. The isolates were negative in the following tests: Formation of indole, Ornithine decarboxylase, Gas from nitrate, $H_2S$ production, growth at 5°C, 10°C, 65°C and at NaCl, 12%.

**SDS-PAGE analysis of vegetative and sporulated cells:** The results of SDS-PAGE analysis of vegetative and sporulated cells of the four isolates and reference strains revealed that the four isolates were different from each other in their banding pattern as shown in Plate 2 (a). MS1 was slightly different when compared to the MS3 isolate; Furthermore, a common protein band (˜130 KDa) was observed for local isolate MS4 in the sporulated pattern. This band migrated in a similar position to the toxin bands produced by *Bacillus thuringiensis kurustaki HD1* as shown in Plate 2 (b) lane 3 and 4 which is active against *lepidopteran* insects.

**SDS-PAGE analysis of crude supernatants proteins:** Crude supernatant proteins secreted by the four bacterial isolates MS1, MS2, MS3 and MS4 in media supplemented by colloidal chitin as a chitinase inducer were fractionated on denaturing gels by electrophoresis (SDS-PAGE) and protein bands were clearly detectable

Table 1: Morphological characters of colonies

| Bacterial isolates | Colony morphology (from agar plates) | | | | |
|---|---|---|---|---|---|
| | Shape | Elevation | Edge | Color | Surface |
| MS1 | Irregular | Flat | Lobate | Brown | Wrinkled |
| MS2 | Round | Flat | Entire | Yellow | Smooth |
| MS3 | Irregular | Flat | Lobate | white | Wrinkled, slimy |
| MS4 | Irregular | Flat | undulate | white | Smooth |

Table 2: Microscopic examination of cells

| Bacterial isolates | Cell morphology and character | | | | |
|---|---|---|---|---|---|
| | Form | Gram reaction | Endospore | Parasporal bodies | Motility |
| MS1 | Rod | + | + | - | + |
| MS2 | Rod | - | - | - | + |
| MS3 | Rod | + | + | - | + |
| MS4 | Rod | + | + | + | + |

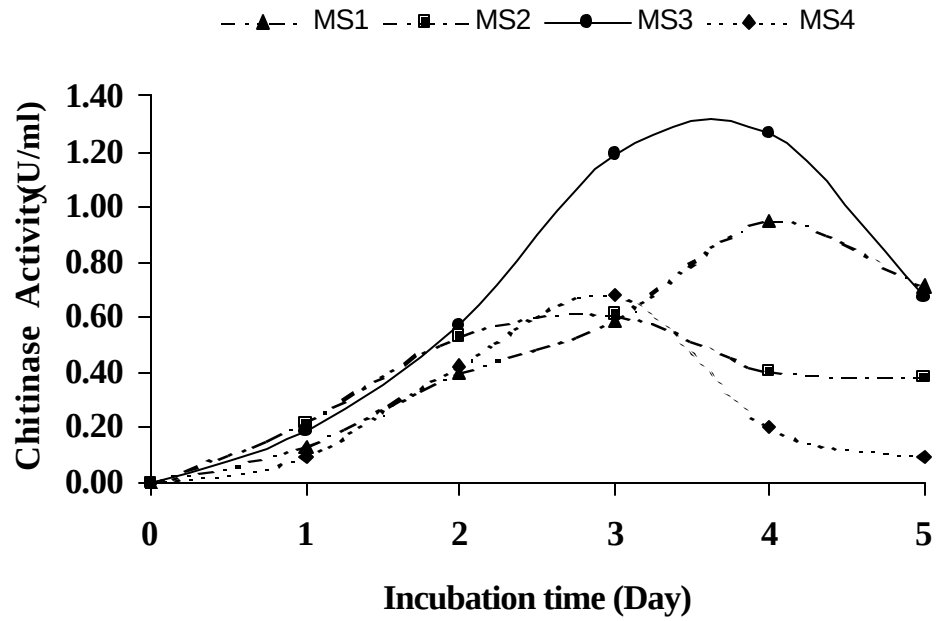


Fig. 1: Time courses of chitinase activity in culture supernatant of MS1, MS2, MS3 and MS4 grown in a medium supplemented with colloidal chitin as a sole carbon and energy source

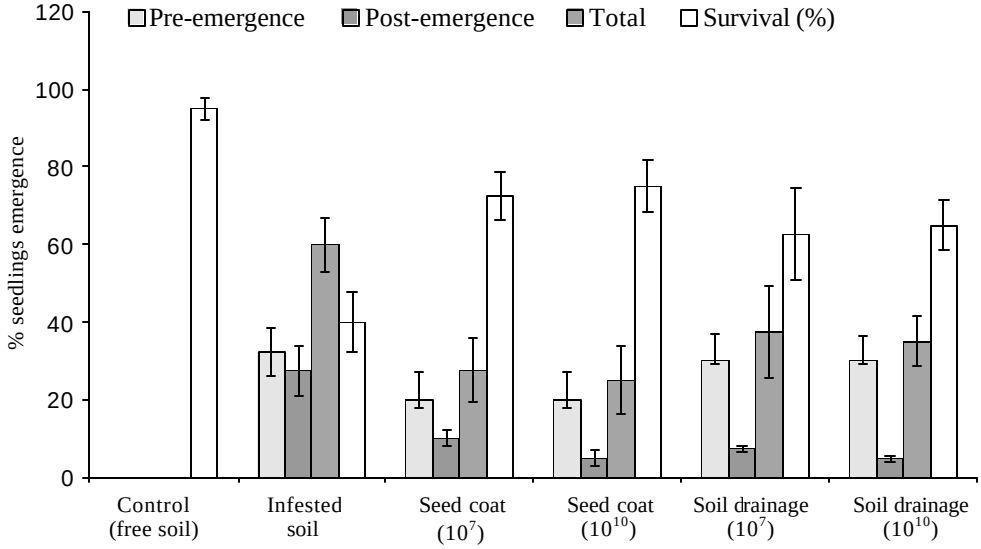


Fig. 2: Effect of different treatments of the chitinolytic bacteria *(B. licheniformis* MS3*)*, on the seed emergence and suppression of damping off diseases by *R. solani* in three weeks old *Helianthus annus* L. seedlings under greenhouse conditions

as shown in Plate 2 (c). The results revealed that both isolate MS1 and MS3 were almost identical. However, isolates MS2 and MS4 were different from each other and from the rest of isolates.

***In vitro* antifungal activity:** The *in vitro* antifungal activity of chitinolytic bacterial isolates against phytopathogenic fungi including *Rhizoctonia solani, Sclerotium rolfsii, Fusarium culmorum, Pythium sp*, (which cause plant damping off disease), *Alternaria alternata* (which cause leaf spot) and *Macrophomina phasiolina* (which cause root rot) have been studied using hyphal extention-inhibition assays. The results demonstrated that MS1 and MS3 isolates were the most effective isolates against all tested phytopathogens, where the largest inhibition zones were recorded (Table 4 and Plate 3).

Table 3: Physiological and biochemical characteristic of chitinolytic bacterial isolates

| | Bacterial isolates | | | |
|---|---|---|---|---|
| Characteristics | MS1 | MS2 | MS3 | MS4 |
| Catalase test | + | + | + | + |
| Anaerobic growth | + | + | + | + |
| Voges-Proskauer test | + | + | + | + |
| Methyl red test | - | - | - | + |
| PH in V-P Broth | | + | | |
| < 6 | + | - | + | + |
| > 7 | - | | - | - |
| Hydrolysis of | | | | |
| Casein | + | + | + | + |
| Gelatin | - | + | - | + |
| Starch | + | - | + | + |
| Chitin | + | + | + | + |
| Urea | - | - | - | - |
| Utilization of citrate | - | + | - | + |
| Lipase | + | + | + | + |
| B-galactosidase | + | + | + | - |
| Arginine dihydrolase | + | + | + | - |
| Lysine decarboxylase | - | + | - | - |
| Ornithine decarboxylase | - | - | - | - |
| Tryptophane deaminase | - | + | + | ND |
| Nitrate to nitrite | + | - | + | + |
| Gas from nitrate | - | - | - | - |
| Formation of indole | - | ND | - | ND |
| O/F Media | | | | |
| Glucose | +F | - | +F | - |
| Sucrose | +F | ND | +F | ND |
| Mannitol | +F | ND | +F | ND |
| Lactose | +F | ND | +F | ND |
| Acid from | | | | |
| D-glucose | + | - | + | + |
| D-mannitol | + | - | + | - |
| Inositol | + | - | + | + |
| D-sorbitol | - | - | + | + |
| L-rhamnose | - | - | - | + |
| D-sucrose | + | - | + | ND |
| D-melibiose | - | - | - | + |
| Amygdalin | + | - | + | ND |
| L-arabinose | + | - | + | - |
| $H_2S$ production | - | - | - | - |

MS1 = *Bacillus licheniformis*, MS2 = *Stenotropho-monas maltophilia*, MS3 = *B. licheniformis*, MS4 = *B. thuringiensis*, **ND**= not determined

**Evaluation of chitinase activity:** The chitinase enzymes secreted by the bacterial isolates MS1, MS2, MS3 and MS4 were evaluated using colorimetrical assay. The results indicated that the enzyme production showed growth relatedness, as the incubation period progressed then it slowed down gradually as shown in Fig. 1. The amount of enzyme increased up to day four of the cultivation. Strain MS3, gave the highest level of chitinase activity, with maximum enzyme production (1.27 U/ml) after 96 h.

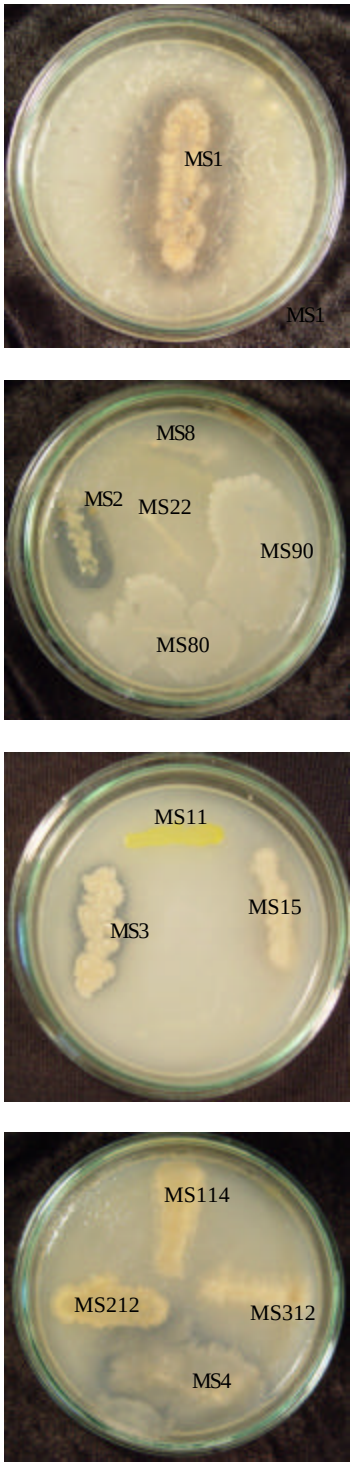


Plate 1: Plate assay showing the chitinase activities of local isolates on chitin medium supplemented with 1% (w/v) colloidal chitin. Note the formation of clearly visible zones on the plate with isolates MS1, MS2, MS3 and MS4 compared to other isolates which showed a little or no clear zones.

Table 4: Inhibition of phytopathogenic fungi mycelial growth on potato dextrose agar by chitinolytic bacterial isolates MS1, MS2, MS3 and MS4. Type of reaction; **N**=No antagonism; **W**=Weak antagonism; **M**=Moderate antagonism and **S**=Strong antagonism

| | Antifungal activity of the isolates against the following fungi | | | | | |
|---|---|---|---|---|---|---|
| Bacterial isolates | *R.solani* | *M.phasiolina* | *F.culmorum* | *Pythium sp* | *A.alternata* | *S.rolfsii* |
| MS1 | S | S | S | S | S | S |
| MS2 | N | W | M | S | M | S |
| MS3 | S | S | S | S | S | S |
| MS4 | N | W | N | M | M | S |

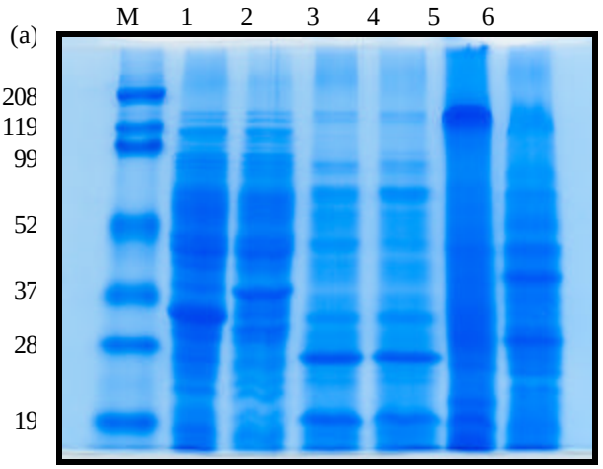


Plate 2a: SDS-PAGE analysis of total cellular proteins of vegetative cells of local isolates and authentic strain, stained with comassie brilliant blue. Lane M, represent high molecular weight protein standard ;Lane 1, MS1; Lane 2, MS3 ; Lane 3, MS4 ;Lane 4, *B.t kurustaki HD1;* Lane 5, MS2 ; Lane 6, *Serratia marcescens*

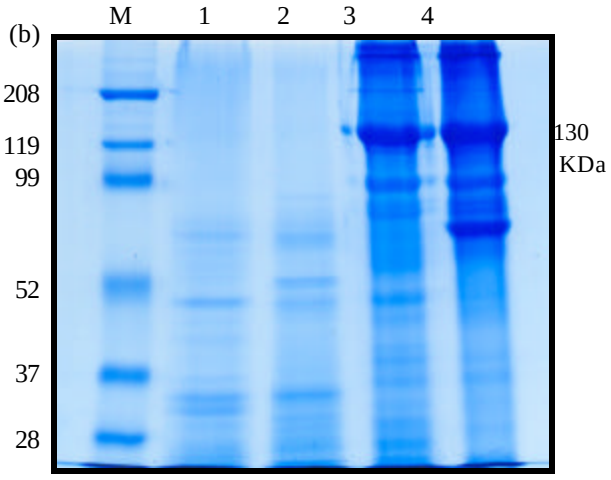


Plate 2b: SDS-PAGE analysis of sporulated cells proteins. Lane M, high molecular weight proteins standard ; Lane 1, MS1; Lane 2, MS3; Lane 3, MS4 ;Lane 4, *B.t kurustaki HD1*

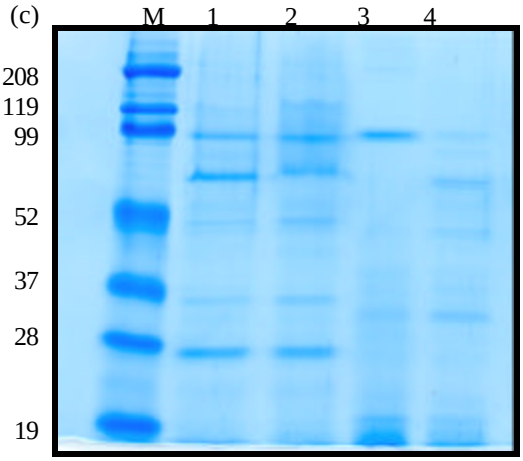


Plate 2c: SDS-PAGE analysis of the crude supernatant proteins secreted by the four bacterial isolates in media supplemented by colloidal chitin as a sole carbon source. Lane M, represent high molecular weight protein standard; Lane 1, MS1; Lane 2, MS3; Lane 3, MS2 and Lane 4, MS4

**Suppression of sunflower damping off disease by isolate MS3 under greenhouse condition:** The isolate MS3 was selected based on the previous results of chitinase activity and antifungal properties. Control plants not treated with bacteria but inoculated with *R. solani* alone rendered up to 60% damping-off incidence and the majority of plants completely dead. Plants inoculated with neither the pathogen nor the bacterial isolate also survived without any infection (Fig. 2). Plants treated by seed coat with bacterial concentration $10^7$ and $10^{10}$ cfu/cm improved the percentage of survival plants and caused an increase from 40% (control) to 72.5 and 75% (treatments), while plants treated by soil drainage with bacterial concentration $10^7$ and $10^{10}$ cfu/cm improved the percentage survival plants and caused an increase from 40% (control) to 62.5 and 65% (treatments). Results demonstrated the soil infected with *Rhizoctonia solani* and treated with *B. licheniformis* MS3 either by seed coat or soil drainage

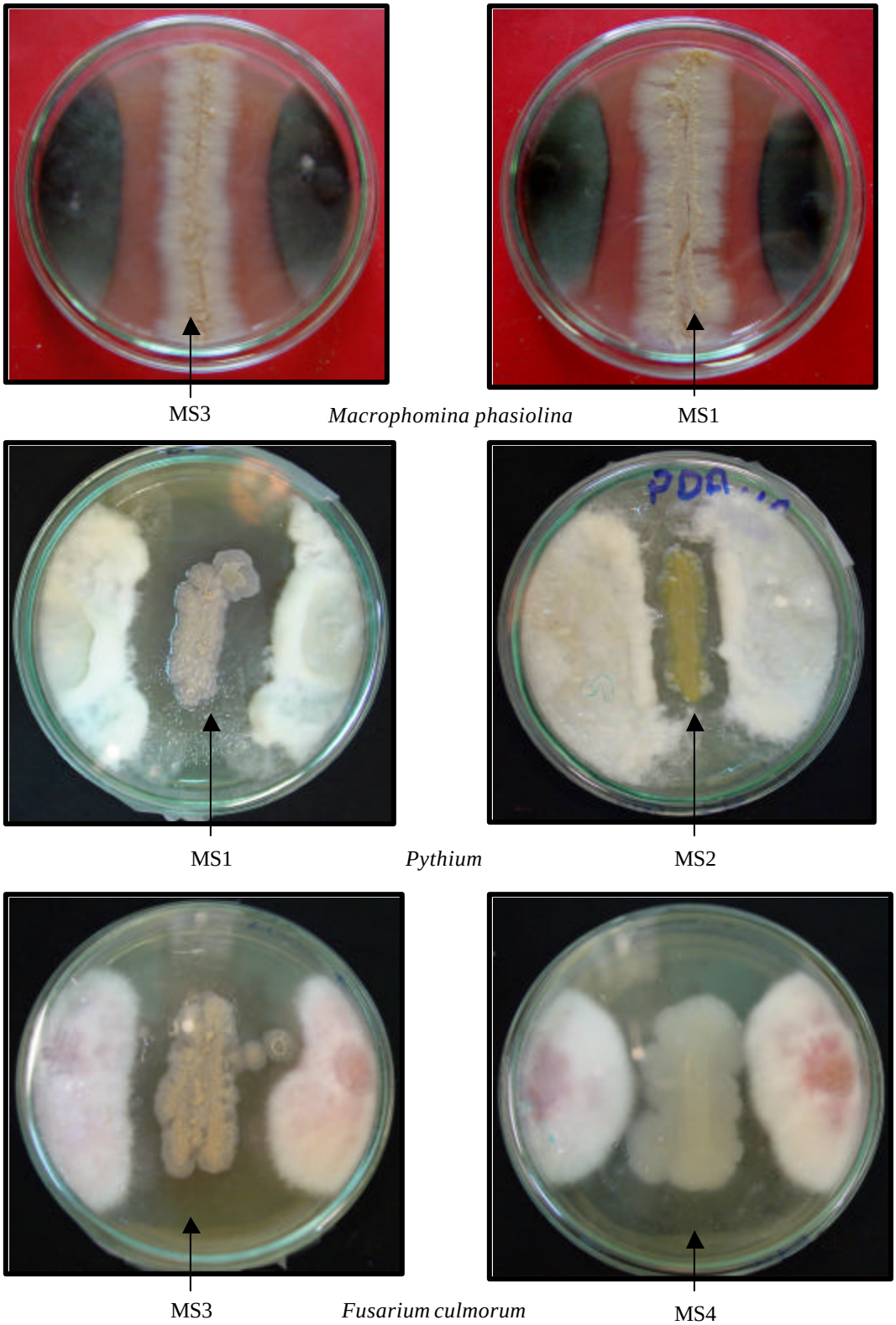


Plate 3: Inhibition of mycelial growth of *Macrophomina phasiolina*, *Pythium* and *Fusarium culmorum*, by chitinolytic bacterial isolates MS1, MS2, MS3 and MS4 *in vitro*. Note the formation of clearly visible inhibition zones on plates with isolates MS1, MS2 and MS3. Growth of phytopathogenic fungi was in PDA medium for 72 hours at 28ºC

significantly reduced damping off caused by *R. solani*. The seed coat treatment was more efficient than soil drainage treatment, where it induced high reduction in percentage of infection (from 60% to 25 and 27%) as compared with the pathogen alone.

## DISCUSSION

Detection of chitin-degrading bacteria from natural sources such as rhizosphere soil is useful in the isolation of bacteria that produce antifungal or other novel compounds. A high correlation between chitinolysis and production of bioactive compounds has been reported [20-22]. Microorganisms, which secret a complex of mycolytic enzymes, are considered to be possible biological control agents of plant diseases [23, 24].

Four hundred bacterial isolates have been isolated from rhizosphere of deferent plants collected from three different localities in Egypt. These isolates were subjected to study their chitinase activity using the plate assay. Of the 400 isolates tested, twenty exhibited chitinase activity. Furthermore, isolates MS1, MS2, MS3 and MS4 showed the highest chitinase activity compared to the other selected isolates and were identified as *Bacillus licheniformis*, *Stenotrophomonas maltophilia*, *Bacillus licheniformis* and *Bacillus thuringiensis* respectively according to the classical methods [25, 26]. *Bacillus thuringiensis* proved to be active against *Lepidopteran* insects [27].

Similarly, SDS-PAGE analysis was helpful in the identification of lactic acid bacteria from the genera *Lactococcus*, *Lactobacillus* and *Leuconostoc* isolated from Tenerife cheese [28].

Interestingly, we found that the four selected bacterial isolates were related to bacterial species that are known for their chitinolytic activity [29-32]. Members of the genus *Bacillus* are well known for their potential to secrete a number of degradative enzymes such as chitinase [33]. In addition, *Stenotrophomonas maltophilia*, produced chitinases in broth media containing chitin [34].

To test the ability of the selected chitinolytic isolates in suppression of mycelial growth of phytopathogenic fungi including *Rhizoctonia solani*, *Macrophomina phasiolina*, *Fusarium culmorum*, *Pythium sp*, *Alternaria alternata* and *Sclerotium rolfsii*, *in vitro* antifungal assay was conducted. It was found that the four chitinolytic isolates antagonized all the tested phytopathogens. MS1 and MS3 were the most effective isolates, where the largest inhibition zones were recorded. Our results are in agreement with those obtained by [35-39] who reported that chitinolytic bacteria were active against the fungal pathogens of pepper, sorghum and mango. *B. licheniformis* (MS3), gave the highest level of chitinase activity, with maximum enzyme production (1.27 U/ml) after 96 hours. These results are in accordance with [40, 41] who reported that *Bacillus licheniformis*, *B. chitinolyticus*, *B. cereus*, *B. ehimensis and Streptomyces griseus* gave the maximum amount of chitinase enzyme after 72-96 hours of incubation.

The results of green-house experiment indicated that the application of seed coat and soil drainage treatments significantly suppressed damping-off incidence by *Rhizoctonia solani* in sunflower seedlings. Seed coat treatment induced high reduction in percentage of infection and improved the percentage of survival plants significantly. These results are in acordance with [36, 42] who reported that *Bacillus licheniformis* may be a potential biocontrol agent against some plant diseases.

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