



BIOLOGICAL CONTROL OF SOIL -BORNE FUNGAL PATHOGENS

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ABSTRACT

Seventeen bacterial isolates have been successfully isolated and purified from soil rhizosphere samples collected from different agricultural areas Buhayra, Sharqia and Alqilyubia of Egypt, and screened for production of chitinase enzymes had the highest chitinolytic activities amongst those investigated. Isolate S3-C and S1-C were identified by biochemical, physiological tests and 16S rRNA gene primer as *Bacillus cereus* S3C, Gene Bank NCBI accession MK185696 and *Bacillus cereus* S1C accession MK185697. The production of chitinase by *B.cereus* S3C and *Bacillus cereus* S1C was optimized using colloidal chitin medium amended with 1% colloidal chitin at 30°C after five days of incubation. *B.cereus* S3C had potential for cell wall lysis of many phytopathogenic fungi tested such as *Fusarium oxysporum* and *Rhizoctonia solani* Potato phytopathogenic fungi by in vitro antagonistic test. The addition of *B. cereus* S3C chitinase was more effective than that of *B.cereus* S1C in increasing the resistance of Potato plants infected with various Soil- borne phytopathogenic fungi.

Keywords: Biological control, Soil borne, Fungal pathogens, *Bacillus cereus*, Chitinase, Characterization, Optimization, Antifungal activity.

INTRODUCTION

Chitin is an insoluble polysaccharide composed of linear chains of β -1, 4-N acetyl glucosamine (GlcNAc) residues that are highly cross-linked by hydrogen bonds. Chitin is one of the most plenty biopolymers on earth, next to cellulose. Chitin is

widely distributed in nature as it is found in the outer skeleton of insects, fungi, algae, crabs, shrimp, lobsters and in the internal structures of other invertebrates (Bhattachrya et al 2007).

We are facing in Egypt major issues when it comes to crop production due to the fungal diseases that infect the crops. Various kinds of fungal pathogens affect the soil in the crops yield. Major damage happens to the crops yield affect by the fungal species of *Alternaria*, *Aspergillus*, *Fusarium*, *Macrophomina* and *Sclerotium* in. For instance we find that severe damages occur to the rice crops where the soil infected by fungus as *Rhizoctonia Solani* and *Pyricularia Oryzae* which live endlessly in the soil as saprophytes. Some pathogens carried by the soil like *Fusarium* and *Vetiticillium* do exist merely in vascular tissue and shows higher impact of parasitic specialization. One destructive disease namely wilt disease caused by *Fusarium Oxysporum* that leads to severe losses and damages in crops such as tomato, potato, castor, chick pea and cotton.

Usually to overcome these fungal diseases, some precautions take place like using chemicals resorting to some cultural traditions in agriculture, breeding for resistance and genetic engineering of crops can be helpful. Yet, not all the previous measurements are sufficient to completely control fungal pathogens. Huge quantities of chemicals such as methyl bromide and methyl isothiocyanate (MIT) chloropicrin are being used to fight phytopathogenic fungi.

Subsequently, these chemicals had dramatic negative effects on health, soil salinity and affect passively the ecosystem. To avoid all the negative influences resulting by using chemicals, scientists thought of another method which is purely natural

and that is using antagonistic microorganisms. These antagonistic microorganisms do exist in a roots area of plants, and we can find them more in suppressive soils. In fact, these microorganisms limits the numbers of fungal pathogens by two ways: direct and indirect; one way is affecting fungi directly by producing secondary metabolites like antibiotics that are poisonous to fungal pathogens. On the other hand, some antagonistic microorganisms can produce hydrolytic enzymes like chitinases or glucanases that dissolve and destroy the cell walls of fungal pathogens. The second way is the indirect way; where microorganisms control pathogens by using some nutrients of the soil and confine the fungi growth. The process where we control the spreading of the fungal pathogen diseases using other microbes without causing issues to the environment is called biological control (Siddiqui et al 2001 and Altindag et al 2006).

Wide range of microorganisms like fungi, bacteria, vesicular, arbuscular mycorrhizal kinds have been tested and by evidence were proved to be biological control agents. Regarding the fungi proven to be agents of biological control, are *Trichoderma*, *Gliocladium*, *Ampelomyces*, *Talaromyces*, and *Rhizopus*. As for the bacterial types that show, greatly to be biological control agents are *Pseudomonas* and *Bacillus*. For example, *Serratia Marcescens* taken from soil were found to have great influence on fighting the diseases resulting by *Sclerotium Rolfsii*. Different species of *Bacillus* were involved in the biological control of fungal pathogen diseases. Several characteristics of these bacteria playing great role in identifying them to be naturally antagonistic including seeking nutrients in soil, releasing antibiotics and producing and discharging of hydrolytic enzymes.

It has been reported previously that those microorganisms, which are able to dissolve and destroy the cell wall of fungal pathogens, are the best to suppress their growth as they produce and release enzymes such as glucanases or chitinases.

Examinations were done to these antagonistic microorganisms that releasing chitinases and were well used to fight and confine the fungal diseases. These microorganisms produce and discharge chitinase enzymes which dissolve the chitin polymer existing in the cell wall of fungal mycelia and leads to their collapse.

Genes encoding for these chitinases have been cloned from various bacterial and fungal species. In earlier times, different species of *Bacillus* were proved to release chitinase enzymes. The *Bacillus* species releasing chitinase are *B. licheniformis*, *B. stearothersophilous*, *B. circulans*, *B. coagulans*, *B. megaterium* and *Bacillus cereus*.

Moreover, *Bacillus thuringiensis* that forms and discharge chitinase could immensely reinforce the insecticidal activity against *Spodopetra littorals* by 2.4 fold (Mostafa et al 2003).

In the present study, chitin degrading bacteria were isolated from soil samples collected from agricultural fields in Egypt. Optimization and characterization of chitinases, which were produced by the most potent species, *Bacillus cereus* were studied. These studies also included an examination of the antagonistic activity of the produced chitinases against some fungal pathogens and their effectiveness in increasing resistant of potato cultivars infected with various phytopathogenic fungi.

We are still studying the most potent chitinolytic bacterial isolate *Bacillus cereus* S3c for detection of the chitinase gene using CHI specific PCR primers and Cloning of the chitinase gene from the most potent isolate. Comparing the nucleotide sequence of chitinase gene isolated from the selected isolate with those of some other chitinase. Then, studying the effect of the selected bacterial isolate in the suppression of common fungal diseases in potato cultivars under greenhouse conditions. Scaling up the production of chitinase using fermentation technology and optimizing of fermentation protocol for the commercial production of chitinase.

MATERIALS AND METHODS

1. SAMPLES COLLECTION

Soil samples from agricultural fields (Buhayra, Sharqia and Alqilyubia of Egypt) were collected with clean shovels and transferred in to clean plastic bags. Three rhizosphere soil samples were randomly chosen and removed into plastic bags, transported to the laboratory within 12 hours and analyzed the same day.

2. PREPARATION OF COLLOIDAL CHITIN

Colloidal chitin was prepared as described by (Hsu and Lockwood, 1975) from crab shell chitin (Sigma). Forty grams of bleached chitin were dissolved in 400 ml of concentrated HCl by stirring for 30-50 minutes. The chitin was precipitated as a colloidal suspension by mixing the solution with two liters of 4°C distilled water. After 48 hours, the supernatant was decanted and the precipitate was

filtered to remove large pieces of undissolved chitin. The precipitate was centrifuged and washed with 0.2% K_2HPO_4 , and the supernatant was removed in 5 to 6 repeated cycles. The resulting hydrolyzed chitin precipitate was used in the preparation of the chitin agar medium.

3. Isolation of bacteria from rhizosphere soil

From each soil samples one gram (dry weight) were suspended in 100 ml sterile water and were shaken on a rotatory shaker for 90 min at 30°C. 5 ml of each suspension was incubated at 80°C for 10 minute to isolate spore formers bacteria (Travers et al 1987).

The isolation was initiated by making ten-fold dilutions of these suspensions in sterile distilled water. A 0.1 ml aliquot of the serially diluted suspension was spread-plated on LB agar medium in triplicate. The spread-plate cultures were incubated for 24 h. at 30 °C. Ten to fifteen representative colonies, with different morphological appearances, were selected from the countable plates and re-streaked on a new plate but of the same media to obtain pure colonies. Dominant colonies were included in the isolation procedure. A total of 17 isolates originally obtained in this manner were maintained on LB agar slants.

3.1. Isolation of chitin degrading bacteria

For isolation of chitinase-producing microorganisms, a mineral medium containing colloidal chitin as sole carbon and energy source was used. Bacterial isolates were screened on agar plates containing colloidal chitin and incubated at 30°C for two days. Colonies surrounded by zones of clearance against the creamy background were regarded as chitinase-producing bacteria and were maintained at 4°C on agar plates.

4. Identification of selected chitin degrading bacterial isolates

4.1. Morphological tests

Morphological characteristics were analyzed according to the Bergey's Manual of Systematic Bacteriology (Sneath, 1986). The selected two different colonies, S1-C and S3-C were restreaked on LB agar plates for further purification. The colony morphological characters as color, form, configuration, margin, appearance, elevation and production of diffusible and non- diffusible pigments,

was examined on LB medium. The Gram's stain was used to differentiate between Gram positive and Gram negative bacteria. Motility was determined by dotting each isolate onto motility plate, after incubation the isolate was scored as motile if the colony was observed to spread out from the incubation site.

4.2. Physiological and biochemical tests

Standard physiological and biochemical identification tests were carried out as described in Bergey's Manual of Systematic Bacteriology (Sneath, 1986). These include the growth in presence of different concentrations of NaCl (1-12%, w/v). The pH range of growth was determined on LB solid medium, initially adjusted to different pH 5-10 with 1N HCL or 1N KOH. Temperature range was determined as above by incubation at 25, 30, 37, 40, 50, 55 and 65°C. The Voges-Proskauer test was used (including demonstration of the production of acetylmethylcarbinol by the isolates and checking the final pH of the broth). The production of acid and gas from glucose, mannitol and lactose was a differentiating test between bacilli. Fermentative capacity was shown by the yellow color change of indicator in the whole medium, while oxidative formation of acid only occurred on the upper part. A blue color indicated alkalization. Gas production caused bubbles in the agar.

Hydrolysis of starch, casein, gelatin and urea, utilization of citrate, formation of indole, reduction of nitrate to nitrite, deamination of tryptophane and the catalase reaction were used to identify these isolated chitinolytic bacteria. Details of the media necessary for the performance of each of these testes for the differential and diagnostic characters are listed in the material section.

4.3. 16S rRNA sequencing and analysis

Amplification of 16S rRNA gene

Bacterial isolates were tested for species identity using the 16S rRNA sequencing method according to the methods of (Rochelle et al. 1995). 16SrRNA analysis was carried out using specific universal primers for 16SrRNA, forward primer 27 F 5'-AGAGTTTGATCMTGGCTCAG-3' and reverse primer 1492 R 5'-TACCTTGTTACGACTT-3'. Isolated DNA from pure bacterial culture was used as template. PCR was performed with a 50 µl reaction mixture containing 16S gene primer, DNA template buffer,

MgCl₂, dNTPs and Taq polymerase. PCR program was carried out in Bio-RAD i-cycler which comprises of three steps; 1) Denaturation at 94°C for 40 seconds; 2) Annealing at 51°C for 50 seconds; 3) Extension at 72°C for 1.5 minute all running for 35 cycle, with initial denaturation 94 °C for 4 minute and final extension 72 °C for 7 minute. PCR Products were analyzed by electrophoresis in 1.5% agarose gel then one of them is gel-purified using (Promega Wizard SV Gel and PCR Clean Up-system Kit cat. # A9282). 4 µL of the purified DNA PCR product was sequenced the gene; sequencing was done at **Macrogen (South Korea)**. Obtained sequence was edited to exclude the PCR primer binding sites and manually corrected with Chromas 2.3 (**Chromas version 2.3; www.technelysium.com.au.chromas.html**).

Analysis of 16SrRNA gene sequence, Phylogenetic tree

16SrRNA gene sequence was compared with already submitted in database using the standard nucleotide-nucleotide BLAST algorithm (**Altschul et al 1990**). Further, most similar sequences were aligned by MEGA7 software, using the same software phylogenetic tree was constructed to analyze. The evolutionary history was inferred using the Neighbor-Joining method (**Saitou N. and Nei M. et al 1987**). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (**Felsenstein 1985**). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (**Felsenstein 1985**). The evolutionary distances were computed using the Maximum Composite Likelihood method (**Tamura et al 2004**) and are in the units of the number of base substitutions per site. The analysis involved 19 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1545 positions in the final dataset. Evolutionary analyses [**Phylogenetic tree**] were conducted in MEGA6 (**Kumar et al 2016**).

5. Dual culture antifungal activity (assay of antifungal activity)

The antifungal activity was assayed *in vitro* by inhibiting the growth of phytopathogenic fungi on

PDA media (**Altindag et al 2006**). A mycelial plug (5 mm diameter) of *F.oxysporum* or *R.solani* Ware placed 2.5 cm away from the edging a petri dish and incubated at 30°C for one day. The bacterial inoculum of the two chitinolytic isolates S1-C, S3-C and ATCC 14580 was picked aseptically and streaked 4.5 cm away from the plug of *F.oxysporum* or *R.solani* on the same petri dish. The petri dish was incubated at 28 °C for five days. The antagonism was expressed by measuring the magnitude of interactions that could appear. The effect of the bacteria on the fungi was categorized in a graduated scale as follows: **N**=No antagonism (mycelium formation not different from control); **W**=Weak antagonism (partial inhibition of mycelium formation mostly appearing as a less dense mycelium than that in control); **M**=Moderate antagonism (almost complete inhibition of mycelium formation in the bacterial zone, but some hyphae escaped via growth through agar; **S** =Strong antagonism (complete inhibition and mycelium did not pass the bacterial zone) (**De Boer et al 1999**).

RESULTS AND DISCUSSION

1. Isolation, screening and identification of chitinolytic bacteria

1.1 Isolation of chitin degrading bacteria

Seventeen bacterial isolates have been successfully isolated and purified from a total of three rhizosphere soil samples, collected from rhizosphere of different commercial plants located in three different agriculture areas in Egypt; Buhayra, Sharqia and Alqilyubia of Egypt, during July 2018, the temperature of sampling day ranging from 35 - 36 °C. The bacteria were isolated using methods described by (**Travers et al 1987**) and others.

1.2. Screening of chitin degrading bacteria using plate assay

The screening of chitin degrading bacteria was examined by spreading inoculum of each colony using puncturing on plates having minimal salt medium along with colloidal chitin as a sole source of carbon and energy. The microorganisms degrading chitin formed colonies of 1-2 mm. in diameter circled by clear zones due to chitinase activity.

Two isolates of a total number of 17 isolates exhibited different zones sizes of degradation of colloidal chitin on solid chitinase-inducing medium,

some of these isolates showed highly chitinolytic activity others exhibited limited activity evidencing differences in their chitinolytic enzymes. In this assay the authentic chitinase producer *Bacillus licheniformis* Chester 1901AL (ATCC 14580) was used as a positive control. Two isolates designated S3-C and S1-C were selected for identification and for further studies (Figure 1) from the seventeen bacterial isolates that exhibited chitinase activity.

2. Identification of selected chitin degrading bacterial isolates

2.1. Morphological and Biochemical Strains identification

Identification including morphological observation, microscopic examination and physiological characteristics was used to identify the two local isolates to the species level. The results summarized in tables (Table 1, 2) showed that the two isolates S3-C and S1-C had smooth colonies. The results of microscopic examination revealed that isolates S3-C and S1-C are gram-positive (figure 2), motile, rod shaped and spore forming bacteria. No differences between S3-C and S1-C cells have been observed; both form motile rods of equal sizes (1.2 by 5.0 µm) and contain only one terminal endospore (Table1, 2).

Table 1. Morphological characters of colonies

Bacterial isolates	Colony morphology				
	Shape	Elevation	Edge	Color	Surface
S1-C	Irregular	Flat	Branched	White	Smooth
S3-C	Irregular	Flat	Branched	White	Smooth

Isolate S3-C and S1-C have the same colony morphology.

Table 2. Microscopic examination of cells

Bacterial isolates	Cell morphology and character				
	Form	Gram reaction	Endospore	Parasporal bodies	Motility
S1-C	Rod	+	+	-	+
S3-C	Rod	+	+	-	+

Isolate S3-C and S1-C have the same cell morphology and character Form: Rod, Gram reaction: (+), Endospore :(+), and Motility: (+).

Table 3. A Physiological and biochemical characteristic of chitinolytic bacterial isolates S1-C and S3-C.

Characteristics	Bacterial isolates	
	S1-C	S3-C
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	-	+
Nitrate to nitrite	+	+
Gas from nitrate	-	-
Formation of indole	-	-
O/F Media		
• Glucose	+F	+F
• Sucrose	+F	+F
• Mannitol	+F	+F
• Lactose	+F	+F
Acid from		
• D-glucose	+	+
• D-mannitol	-	-
• Inositol	-	-
• D-sorbitol	-	-
• L-rhamnose	-	-
• D-sucrose	+	-
• D-melibiose	-	-
• L-arabinose	-	-
H₂S production	-	-
Blood hemolysis	+	+
Growth at		
• 5 °C	-	-
• 30 °C	+	+
• 40 °C	+	+
• 50 °C	+	+
• 55 °C	-	-
• 65 °C	-	-

Table 3.The tow new strains were positive in the following examinations: Catalase, anaerobic growth, hydrolysis of casein, lipase, growth at 30°C and at 40°C.All strains were negative in the following examinations : Formation of indole, Ornithine decarboxylase, Gas from nitrate, growth at 5°C, growth at 65°C and H₂S production. (ND= not determined).

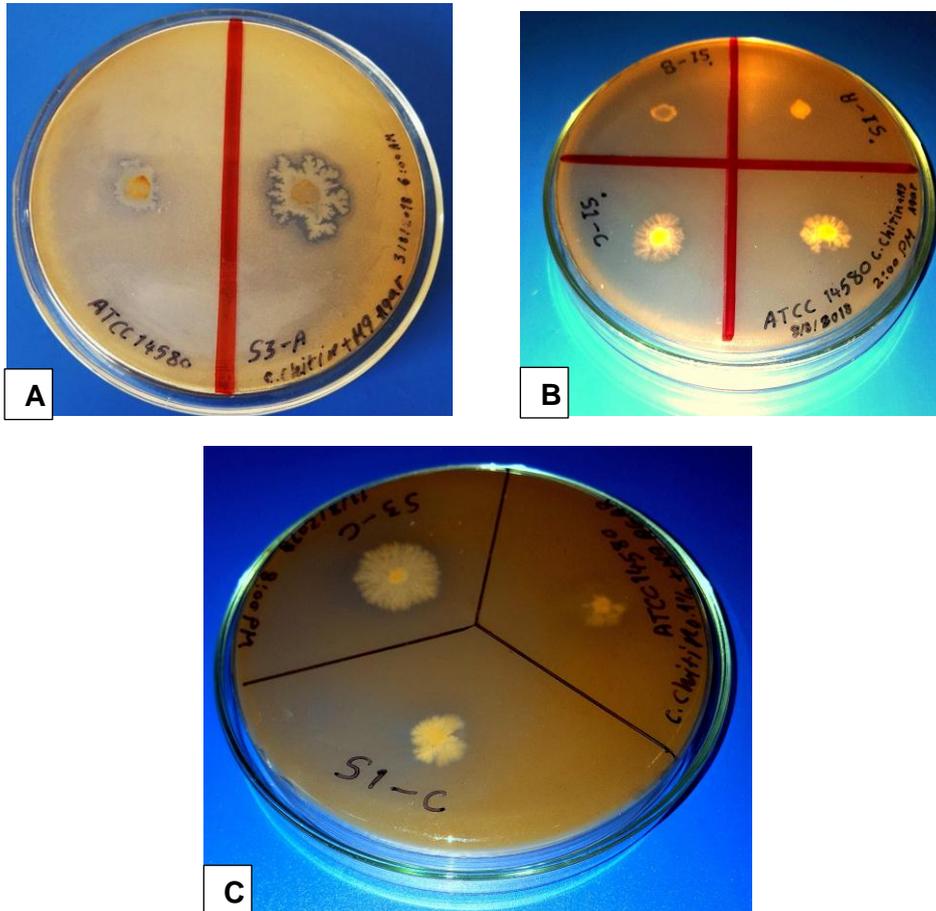


Fig. 1. Plate assay showing chitinase activities of local isolates on M9 media supplemented with 1 % (w/v) colloidal chitin. The formation of clear zones is apparent with isolates S3-C (image A,C) and S1-C (Image B, C) compared to other isolates showing limited clear zones or no clear zones with ATCC14580 as Control .

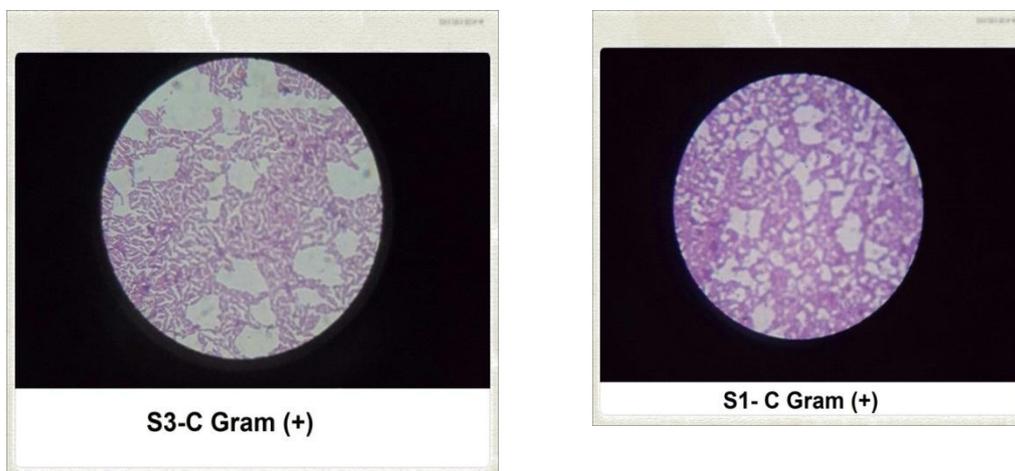


Fig. 2. Isolate S3-C and S1-C Bacteria and have the same morphology Rod shape and Gram (+).

Based on morphological, physiological and biochemical results summarized in tables 1, 2, 3, the isolates S1-C and S3-C were identified to the species level. The outcomes of these examinations were similar to those of Bergey's Manual of Systematic Bacteriology (Sneath, 1986).

Moreover, the isolates S1-C and S3-C proved to have the same outcomes of biochemical examinations and identified as *Bacillus Cereus*. However, definite evidence for the affiliation of the isolates with the species *B. cereus* was obtained from sequencing the 16S rRNA gene.

2.2. 16S rRNA sequencing and analysis

To assure the identification of isolates S3-C and S1-C; an analysis of gene sequence of 16S rRNA has been executed. The sequence alignment using BLAST (Basic Local Alignment Search Tool) software for the comparison of up to 980 bp (Figure 4) of S3-C and S1-C (Figure 5) gave high homology (99%) with *Bacillus cereus* strain AHSM124 16S rRNA gene partial sequence accession: MK074711.1, *Bacillus cereus* gene for 16S rRNA partial sequence, strain: DLTA4.32 accession: LC208134.1, *Bacillus cereus* gene for 16S rRNA partial sequence, strain: SBABrB5 accession: LC189361.1, *Bacillus cereus* gene for 16S rRNA partial sequence, strain: SBFWS5 accession: LC189359.1, *Bacillus cereus* strain BA8 16S rRNA gene, partial sequence accession: KU510055.1, *Bacillus sp.* GG3 (2015) gene for 16S rRNA partial sequence accession: LC055679.1, *Bacillus cereus* strain LP13_RH08 16S rRNA gene, partial sequence accession: KT427911.1, *Bacillus sp.* (in: Bacteria) strain CZW-12 16S rRNA gene, partial sequence accession: MG866081.1, *Bacillus cereus* strain CZW-5 16S rRNA gene, partial sequence accession: MG866079.1 and *Bacillus sp.* (in: Bacteria) strain 18JY8-13 16S rRNA gene partial sequence accession: MK101106.1.

Nucleotide sequence accession numbers

Sequences obtained for *Bacillus cereus* S3-C and *Bacillus cereus* S1-C in this study were deposited in GenBank under the following accession numbers: 16S rRNA gene partial sequence; **MK185696** and **MK185697**.

Phylogenetic analysis

Phylogenetic tree (Figure 6) was drawn with both new isolate and its closest relative which terminate on the basis of highest score obtained in the MEGA6 result, tree constructed for the determination of evolutionary relatedness. New isolates designated as S3-C and S1-C were found to be closely related to accessions MK074711.1 and accessions LC208134.1 respectively.

Definite evidence for the affiliation of the isolates S3-C and S1-C with the species *Cereus* was obtained from PCR (figure 3) and sequencing the 16S rRNA gene:

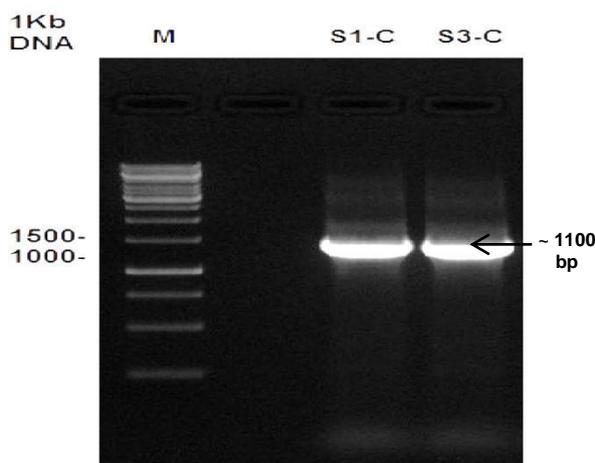


Fig. 3: Amplification of 16SrRNA gene from of isolates S3-C and S1-C. M: 1kb DNA ladder GeneRuler™ 1 kb Fermentas, catg. # SM0311, lane (S1-C): amplification of 16SrRNA gene from **Isolate S1-C** and lane (S3-C): 16SrRNA gene amplification form **Most Potent Isolate S3-C**.

3. Dual culture antifungal activity of the two selected chitinolytic isolates

Studies have been made via hyphal extension inhibition examinations to prove the effectiveness of chitin degrading bacteria isolates against fungal pathogens such as *Rhizoctonia solani* and *Fusarium oxysporum*. that infecting plants and cause damping off disease. The results demonstrated that isolate S3-C was more effective than isolate S1-C in inhibition of fungal mycelium comparing with control, therefore it is considered as the most potent effective isolates against all the examined phytopathogens.

>New Isolate S3-C 16s rRNA 27F 980bp

TTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCATAAGAGTGGG
ATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTGAACCGCATGGTTCGAAATTGAAAGG
CGGCTTCGGCTGTCACTTATGGATGGACCCGCTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACC
AAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAG
ACTCTACGGGAGGCAGCAGTAGGGAATCTTCGCAATGGACGAAAGTCTGACGGAGCAACGCCG
CGTGAGTGATGAAGGCTTTCGGGTCGTAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAAT
AAGCTGGCACCTTGACGGTACCTAACAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAA
TACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTC
TGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAG
AGGAAAGTGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGA
AGGCGACTTCTGGTCTGTAACCTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTAGAGGGTTTCGCCCTTATAGTGCTGA
AGTTAACGCATTAAGCACTCCGCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGAC
GGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTAATTGAAAGCAACGCGAAGAACCTTACCAGGT

Fig. 4. New Isolate *Bacillus cereus* S3-C 16 rRNA Partial Sequence.

>New Isolate S1-C 16s rRNA 27F 1075bp

CCAGGGCGGCTGCCTATACATGCAGTTCGAGCGAATGGATTAAGAGCTTGCTTCTTATGAAGTTAG
CGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCATAAGACTGGGATAACTCCGGGAAACCGG
GGCTAATACCGGATAACATTTGAACCGCATGGTTGAAATTGAAAGGCGGCTTCGGCTGTCACTT
ATGGATGGACCCGCTCGCATTAGCTAGTTGGTGAGGTAACGGCTACCAAGGCAACGAGCGTAG
CCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAG
CAGTAGGGAATCTTCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCTGAGTGATGAAGGCT
TTCGGGTCGTAAACTCTGTTGTTAGGGAAGAACAAGTCTAGTTGAATAAGCTGGCACCTTGACG
GTACCTAACAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGC
GTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCAC
GGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGAATTTC
CATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTCTGGTC
TGTAACCTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACG
CCGTAAACGATGAGTGCTAAGTGTAGAGGGTTTCGCCCTTATAGTGCTGAAGTTAACGCATTAAG
CACTCCGCCGGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAA
GCGGTGGAGCATGTGGTTAATTGAAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGA
CAACCTAGAGATAGGGCTTCTCCTTCGGGAGCAAATGACAGGTGGTGCAGGGTTGCTCACCT
CCTGCTGAAAATTTGGGTTAATCCC

Fig. 5. New Isolate *Bacillus cereus* S1-C 16 rRNA Partial Sequence.

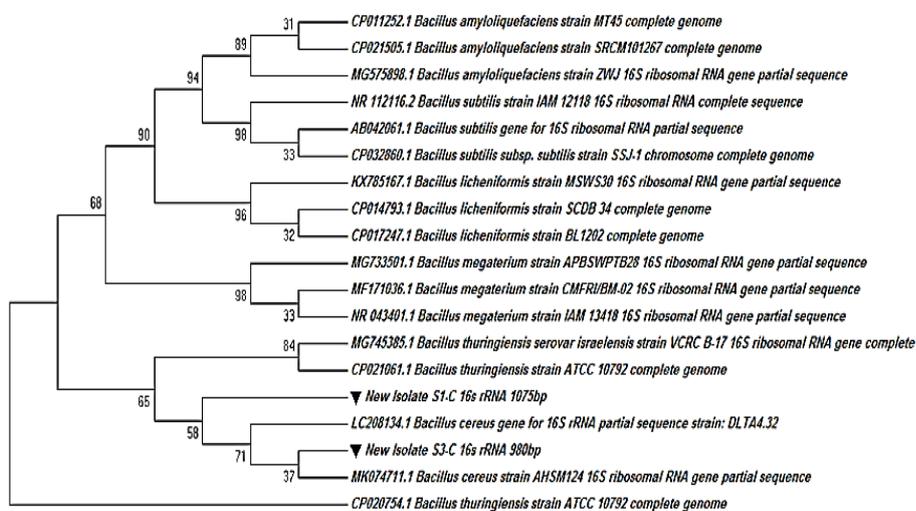


Fig. 6. Evolutionary relationships of taxa (phylogenetic Tree) of Two New Isolates *Bacillus cereus* S3-C and S1-C.

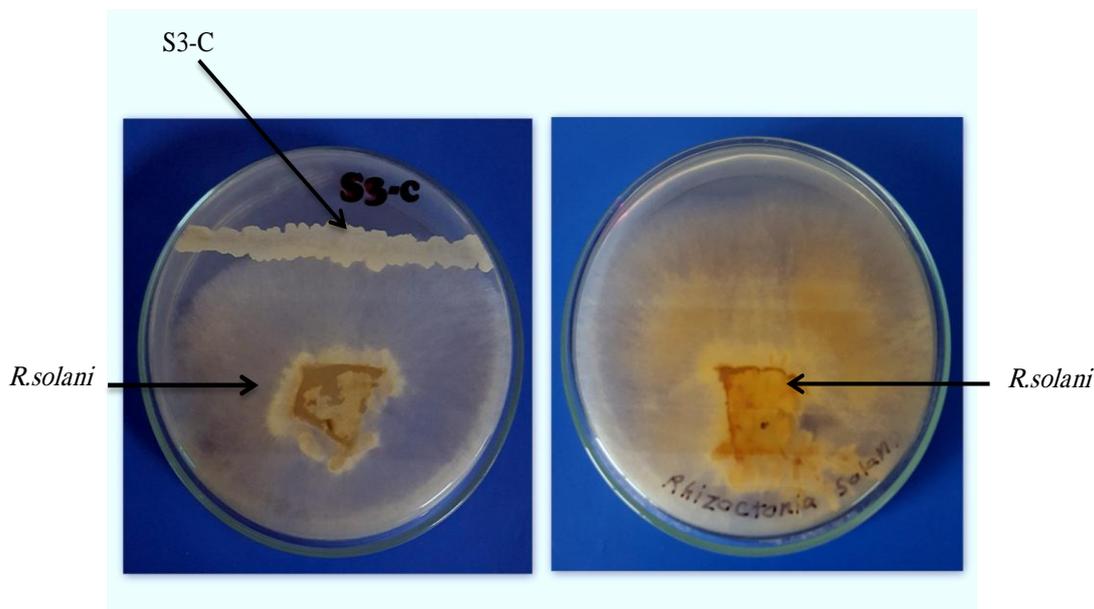


Fig. 7. In vitro antifungal activity of *Bacillus Cereus* S3-C against fungal pathogen *R.solani* compared with Control without bacteria on PDA medium at 35°C.



Fig. 8. In vitro antifungal activity of *Bacillus Cereus S3-C* against fungal pathogen *R. solani* compared with Control without Bacteria on PDA medium at 35°C.

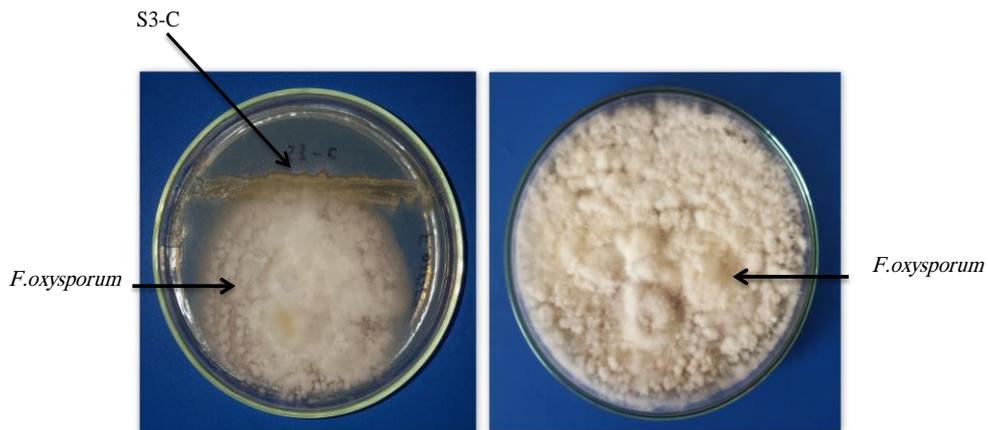


Fig. 9. In vitro antifungal activity of *Bacillus Cereus S3-C* agents fungal pathogen *F. oxysporum* compared with Control without bacteria on PDA medium at 35°C.



Fig. 10. In vitro antifungal activity of *Bacillus Cereus S1-C* agenst fungal pathogen *F.oxysporum* compared with Control without Bacteria on PDA medium at 35°C.

In contrast, fungal growth of *Rhizoctonia solani* was inhibited by isolates S3-C and S1-C (**Table 4**). The effective isolates taken into consideration are merely those capable of producing clear zones of inhibition or able to lyse the cell walls of fungal mycelium. Plates not treated with the bacterial isolates were used as a negative control. These plates were covered completely by the phytopathogens and showing no inhibition. Antifungal activity of *Bacillus cereus* S3-C and S1-C against *Rhizoctonia solani* and *Fusarium oxysporum* have been compared (figure 7, 8, 9 and 10). The former showed wide clear inhibition zone while the latter resulted in no inhibition zones.

Table 4. Symbols for reaction types: **M** stands for Moderate antagonism, **W** stands for weak antagonism and **S** stands for strong antagonism.

Bacterial isolates	The following fungal pathogens are fought by the inhibition activities of the isolates:	
	<i>R. solani</i>	<i>F. oxysporum</i>
S3-C	S	S
S1-C	M	M

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المقاومة الحيوية للفطريات الممرضة بالتربة

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الموجز

كما تم إختبار نشاط الكيتينيز للعزلتين *B.cereus S1C* و *B.cereus S3C* باستخدام بيئة أجار الكيتين بتركيز 1% كيتين والتحصين على 30 درجة مئوية لمدة خمسة أيام. كانت العزلة *B.cereus S3C* أعلى نشاطاً لتحليل جدار الخلية للعديد من الفطريات الممرضة بالتربة كفطر *Fusarium oxysporum* و *Rhizoctonia solani* وذلك على بيئة PDA ودرجة حرارة تحضين 30 درجة مئوية. فكانت إضافة *B.cereus S3C* أكثر فعالية من *B.cereus S1C* في زيادة مقاومة نباتات البطاطس المصابة بالفطرين المختبرين المحمولين بالتربة.

الكلمات الدالة: المقاومة الحيوية، أمراض التربة، الفطريات الممرضة، *Bacillus cereus*، إنزيم الكيتينيز، تقدير نشاط الميكروب لإفراز الإنزيم، تقدير نشاط الإنزيم في المكافحة الحيوية.

تم عزل سبعة عشر عزلة بكتيرية بنجاح من عينات تربة (منطقة الريزوسفير) لنباتات البطاطس من ثلاث محافظات مختلفة من مصر (الشرقية والبحيرة والقليوبية) وتم إختبارهم لنشاط إنتاج إنزيم الكيتينيز لتحديد أعلى العزلات نشاطاً لإنتاج الإنزيم بين تلك العزلات التي تم بحثها. وكانت العزلتين S3-C و S1C أعلى العزلات نشاطاً في إنتاج إنزيم الكيتينيز. وقد تم تعريف العزلتين S3-C و S1-C بواسطة الاختبارات البيوكيميائية والفسيلوجية والـ 16s rRNA Gene وكانت نسبة التشابه مع *Bacillus cereus* حوالي 99% وقد تم تعريفهم وتسجيلهم بالـ GeneBank International NCBI *Bacillus cereus S3-C* accession MK185696، *Bacillus cereus S1-C* accession MK185697.