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Identification of a Broad-Spectrum Antifungal Chitinase from *Bacillus Subtilis* Strain BC121.

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Research Article

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ABSTRACT

A chitinase enzyme, Chi25, was purified from the supernatant of cultured *Bacillus subtilis* strain BC121 by ammonium sulfate precipitation and chitin affinity chromatography. The purity of the enzyme was confirmed by SDS-PAGE and RP-HPLC analysis and the molecular weight of the enzyme was estimated to be approx. 25 kDa. The enzyme was stable over a pH range of 6–7.5, while the optimal pH for enzyme activity was 6.5. Chi25 was stable over a temperature range of 25–40 °C with 35 °C as the optimal temperature for activity. Bioassay of Chi25 demonstrated the inhibition of spore germination and hyphal extension of important plant pathogenic fungi. Results of the glasshouse trials revealed that the wild-type strain (BC121) reduced fusarium wilt incidence by 80%. Comparative analysis of both the wild-type strain (BC121) and the mutant strain of this bacterium (BC121M) revealed that the antagonism of the BC121 strain against plant pathogenic fungi both in vitro and in vivo is due to the Chi25 enzyme

INTRODUCTION

Chitin, a linear homopolymer of N-acetyl-D-glucosamine, is a major constituent of the cell walls of fungi, insect exoskeletons, and crustaceans. Chitinases (E. C. 3.2.1.14) are hydrolytic enzymes that are capable of hydrolyzing the chitin polymer into its oligomers. Bacteria, fungi, insects, crustaceans and higher plants produce chitinases for nutritional or defensive purpose [1,2,3]. These chitinolytic microorganisms as a whole and the chitinolytic enzymes they produce have potential applications in the biocontrol of both plant pathogenic fungi and insects [4,5], as well as in several other biotechnological areas [2]. Fungi, like *Trichoderma* and *Gliocladium* were shown to control soil-borne fungal pathogens[6,7]. Similarly, the rhizosphere bacteria, including *Bacillus, Pseudomonas, Serratia, Arthrobacter*, and *Enterobacter*, have also been shown to control fungal diseases[8,9,10].

The non-pathogenic soil *Bacillus* species offers several advantages over other bacteria since they can form endospores that can tolerate extreme pH, temperature, and osmotic conditions. These organisms colonize plant root surfaces, increase plant growth, and cause fungal mycelia lysis[11,12]. Previously, several strains of *Bacillus* have been described as effective antifungal agents and have been implemented in the biological control of many plant fungal pathogens [11,13]. Indeed, most of these biological control agents are target-specific and, therefore, are only effective against specific pathogens. The chitinolytic bacteria present in the region of the rhizosphere inhibit various fungal pathogens by degrading the cell walls of fungi and, therefore, are more efficient in controlling fungal diseases [14].

Previously, we have reported that a chitinolytic *Bacillus* sp. BC121, a soil bacterium, showed antagonistic activity towards *Curvularia lunata*[15]. In the present work, we find that the broad-spectrum antifungal activity of the BC121 strain against various plant pathogenic fungi is due to an extracellular

chitinase enzyme. Furthermore, the purification and characterization of the chitinase enzyme was performed.

MATERIALS AND METHODS

Bacterial cultures and Growth media

Bacillus sp. strain BC121, isolated from the rhizosphere soil samples, and its mutant strain BC121M produced by treatment with nitrosoguanidine solution (1 mg/ml NTG) suspended in 10 mM Tris maleic acid (pH 6.0) and reported to lose in its antifungal activity were used in this study[15]. To identify the phylogenetic analysis, 16S rDNA was PCR amplified using universal primer sets and the sequence was aligned with the Ribosomal RNA Database Project and from GenBank (http://www.ncbi.nlm.nih.gov/)[16]. For chitinase induction, bacteria were grown on chitin media[17] supplemented with 0.5% (wt/vol) colloidal chitin, 0.3% (wt/vol) yeast extract, 0.2% (wt/vol) $Na_2HPO_4.2H_2O$, 0.03% $NaH_2PO_4.2H_2O$, 0.07% (wt/vol) $MgSO_4$, 0.05% (wt/vol) NaCl, 0.05% (wt/vol) KCl, and 0.13% (wt/vol) $CaCl_2$.

Antifungal activity

The antifungal activity of BC121 was tested by dual-plate assay and dual-liquid culture assay as described earlier[15]. Test fungal cultures and a bacterial cell suspension of BC121 were inoculated onto a nutrient agar plate at a distance of 4 cm. The plates were incubated at 37 °C and examined daily for the formation of an inhibition zone. In the dual-liquid culture assay, the mid-log phase bacterial culture (6 h) containing 10^7 CFU/ml was co-inoculated with the fungal culture (10^7 spores/ml) grown for 6, 12, and 24 h. Fungal cultures (10^7 spores/ml) grown without bacterial cultures served as controls. After 48 h of incubation at 30 °C, cultures were passed through pre-weighed Whatman No. 1 filter paper, left to dry for 24 h at 70 °C, and the differences in the dry weights between mycelia grown both with and without the bacterial strains were measured. Experiments were performed in triplicate and repeated twice. The growth of bacterial cells in the dual cultures was assayed by plating the serially diluted samples on LB-agar plates.

Extraction and purification of chitinase enzyme

Colloidal chitin was prepared by treating purified chitin powder (S.D. Fine chemicals, India) with cold HCl (10 N) and 50% aqueous ethanol as previously reported[18]. The bacterial culture was grown in chitin media containing 0.5% colloidal chitin for 72 h at 30 °C in an orbital shaker. The cell free supernatant was concentrated by ammonium sulphate precipitation (80%) and was collected into 10 mM sodium phosphate buffer (SP buffer, pH 6.0). The protein precipitate was passed through the chitin column (1.5 × 15 cm), which was pre-equilibrated with the same buffer. The column was washed with 10 mM SP buffer (pH 6.0) and 10 mM sodium acetate buffer (pH 5.5). Proteins bound to the chitin matrix were eluted with 10 mM acetic acid (pH 5.0) and the pH was adjusted to 7.0 with 1 M NaOH. The enzyme concentrate was applied to the Sephadex G-50 column (1.5 × 20 cm) and eluted with 10 mM SP buffer (pH 7.0) at a flow rate of 1.0 ml/min. Twenty micrograms of the purified proteins were analyzed using a hypo-reverse phase column (RP-318: 250 mm × 4.6 mm, Bio-Rad, Hercules, CA, USA) equilibrated with 0.1 trifluoroacetate in de-ionized water. The protein was eluted with a gradient of two degassed buffers for 70 min. at a flow rate of 1 ml/min and monitored at 280 nm and checked on 12% Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli[19].

Enzyme assay

The chitinase activity was evaluated using chromogenic oligomers of N-acetyl-β-D-glucosamine: *p*-NP-(GlcNAc), *p*-NP-$(GlcNAc)_2$, *p*-NP-$(GlcNAc)_3$, and *p*-NP-$(GlcNAc)_4$, according to the method of Roberts and Selitrennikoff (19). The reaction mixture of 10 µg of purified enzyme in 25 µl SP buffer (10mM, pH 7.0) and 25 µl stock solution (5 mM) of one of the three above-mentioned substrates was incubated at 37 ºC for 1 h. The amount of *p*-nitrophenol (*p*NP) released was determined from the absorbance at 410 nm (molar extinction coefficient, 17,700). Chitinase activity was also measured by a reduction in turbidity of the reaction mixture containing colloidal chitin and purified enzyme, according to Tronsmo and Harman[20].

Effect of culture conditions

The effect of substrate on chitinase production was studied using 0.5% purified chitin, 0.5% glycol chitin, 0.5% swollen chitin, or 0.5% colloidal chitin as substrate in the chitin synthetic media and chitinase activity was determined under standard assay conditions[20]. To determine temperature and pH tolerance,

the enzyme in 10 mM SP buffer (pH 7.0), was treated for 1 h at a temperature range of 20 to 65 °C and in pH from 3.0 to 12.0.

In Vitro Antifungal activity of Chi 25

The antifungal activity for the purified enzymes was estimated using dual-plate assay and microtitre plate assay as described earlier[15]. Test fungal cultures and purified protein suspension taken on sterile Whatman filter paper discs were placed on nutrient agar plates, incubated at 35 ºC and observed for formation of an inhibition zone. For the microtitre plate assay, the fungal spore suspension (500 spores/80 µl of 10 mM SP buffer, 0.1% Triton X-100, pH 6.5) and the purified chitinase enzyme, Chi25 (10 µg/20 µl of 10 mM SP buffer pH 6.5) were incubated at 35 ºC and the fungal growth was recorded after 24 h at 405 nm.

Biocontrol activity of the BC121 strain

F. oxysporum f. sp. *ciceris* was chosen as a model pathogen that causes wilt disease to chick pea plants (*C. arietinum*). Seed surfaces of chickpea cultivar JG62 (a highly susceptible genotype) were disinfected with 0.5% $HgCl_2$ for 2 min and washed thoroughly with sterile distilled water. The bacterial cells (10^8 CFU/ml) were mixed with 0.5% carboxymethyl cellulose (CMC) in 1:3 ratios and used to mix with 10 g of seeds. Clay pots (15-cm diameter) with an autoclaved soil mixture [clay loam/peat, 2:1 (vol/vol)] were infested with fungal mycelia (1 g/kg of soil). In each pot ten seeds treated *B. subtilis* BC121 or *B. subtilis* BC121M were sown. The non-bacterized (coated with CMC alone) sown in separate pot was taken as control. After emergence, seedlings were thinned to five plantlets per pot and maintained in the greenhouse at 25–30 °C with regular watering. After 40 days, the healthy and wilted plants were counted and the pooled data was analyzed using the Fisher least significant difference test.

RESULTS

Antifungal activity of bacterial strains

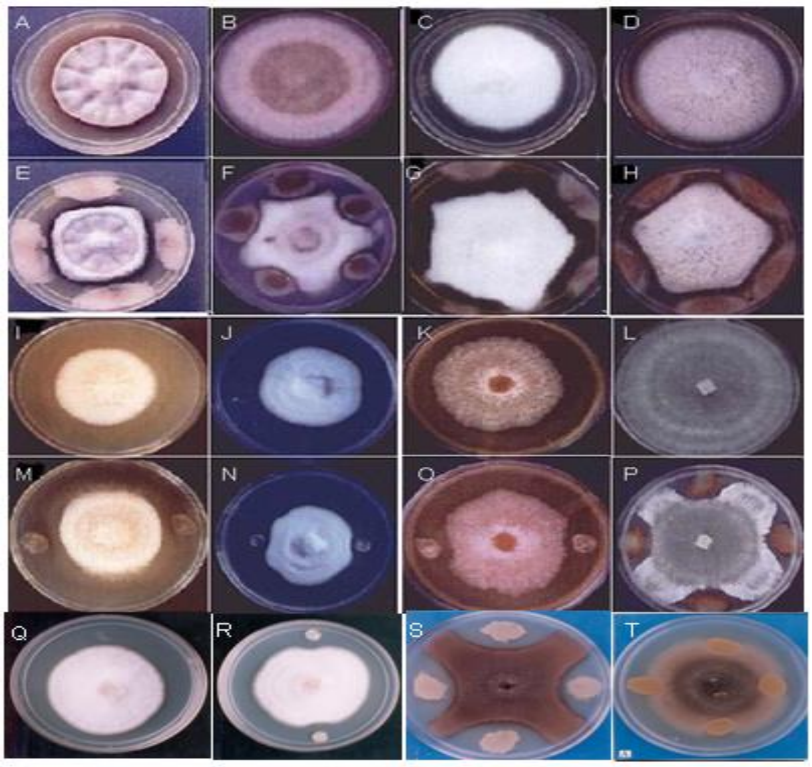

Figure 1: Antagonism of *B. subtilis* strain BC121 to plant fungal pathogens.

Fungal culture grown on nutrient agar medium (control), *C. graminicola* (A), *A. alternata* (B), *F. oxysporum* f. sp. *ciceris* (C), *F. moniliforme* (D), *R. solani* (I), *F. oxysporum* f. sp. *ricini* (J), *S. rolfsii* (K), and *F. semitectum* (L), *B. subtilis* strain BC121 inhibiting the growth of fungi, *C. graminicola* (E), *A. alternata* (F), *F. oxysporum* f. sp. *ciceris* (G), *F. moniliforme* (H), *R. solani* (M), *F. oxysporum* f. sp. *ricini* (N), *S. rolfsii* (O), and *F. semitectum* (P).

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The BLAST search analysis of the 16S rDNA genes of the two bacterial strains showed maximal alignments to the *B. subtilis* subgroup and hence they were named as *B. subtilis* BC121 and *B. subtilis* BC121M. *B. subtilis* BC121 was found to inhibit the growth of all fungal pathogens tested in the dual-plate assay, including *A. alternata, C. graminicola, F. moniliforme, F. oxysporum* f. sp. *ciceris, F. oxysporum* f. sp. *ricini, F. semitectum, R. solani*, *and S. rolfsii.* A clear inhibition zone was observed between the mycelia and the bacterial culture (FIG. 1). After 10 days of incubation, the bacteria grew over the fungal mycelial mat and no hyphal growth was observed in the inhibition zone. Strain BC121M showed absolutely no inhibition of any of the fungi tested. The dual-liquid culture assay indicated that the *B. subtilis* strain BC121 retarded the fungal growth by 60% when compared with the controls (FIG. 2). Microscopic observations of the mycelia grown with *B. subtilis* BC121 revealed the presence of abnormal hyphae with swellings and distortions (FIG. 3), and the mycelial surface was colonized with bacterial cells. On the other hand, the mycelia grown with the BC121M culture showed normal mycelial features similar to control mycelia. The serially diluted samples of dual cultures showed similar number of bacterial cells, indicating the similar growth rate of the strain BC121 and BC121M in dual cultures (data not shown).

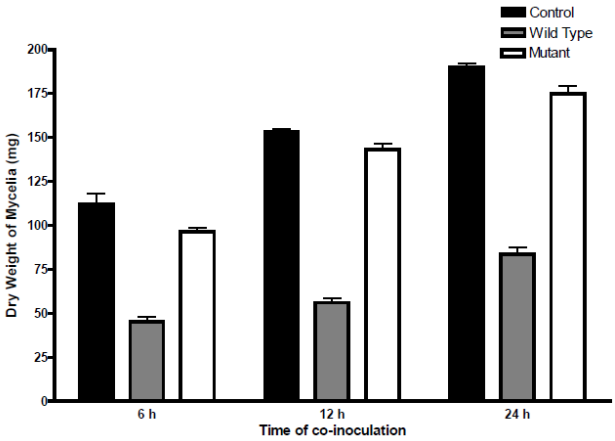

Figure 2: Effects of bacterial cells (BC121 and BC121M) on the growth of *F. oxysporum* f. sp. *ricini* in dual-liquid culture.

Assay was done by co-inoculating the log-phase growth bacterial culture into 6 h, 12 h and 24 h-grown fungal cultures as described in Materials and Methods. The fungal mycelium was grown in Czapeck-Dox medium for 48 h at 30 °C without bacterial cells (-■-), with *B. subtilis* BC121 (-■-), or with *B. subtilis* BC121M (-□-). The data represents mean values of three experiments run in replicates each time.

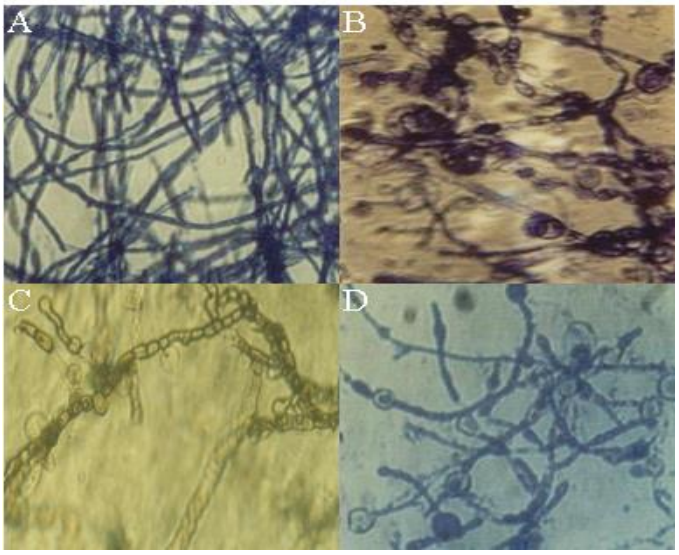

Figure 3: Light microscopic observations

(A) control mycelia of *F. oxysporum* f. sp. *ciceris* (B) mycelia of *F. oxysporum* f. sp. *ciceris* grown along with *B. subtilis* BC121, (C) Mycelia present in the inhibition zone showing lysis and fragmentation after interaction with Chi25, (D) spores grown with 10 µg of Chi25 showing poor germination and abnormal swelling.

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Purification of the chitinase enzyme

The total extracellular protein extract of *B. subtilis* BC121 was purified using chitin column chromatography and the fractions showing chitinase activity were further purified by gel filtration column using a Sephadex G-50 column. The eluted fractions were collected and stored at 4 °C. The reverse-phase HPLC analysis of the purified protein revealed a single peak at 280 nm at 15 min of elution time. The specific activity, purification and recovery of the enzyme at each purification step are summarized in Table 1. There was a 6.86-fold increase in specific activity of the purified chitinase compared with that of the crude extract. The purified protein fraction separated on 12% SDS-PAGE showed a single band that corresponded to approx. 25 kDa and it was named as Chi25 (FIG. 4).

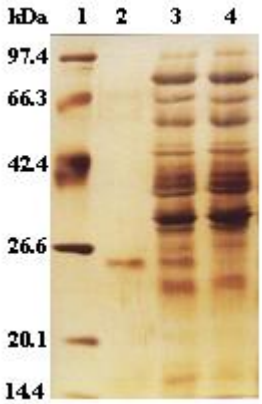

Figure 4: SDS-PAGE of purified chitinase.

Lane 1, molecular weight standard; Lane 2, purified chitinase; Lane 3, extracellular proteins of *B. subtilis* BC121 and Lane 4, extracellular proteins of *B. subtilis* BC121M.

Table 1: Purification of chitinase from the *B. subtilis* BC121 strain.

Step	Total protein (mg)	Total chitinase activity* (U)	Specific activity (U/mg)	Purification fold
Culture supernatant	2536	380	0.15	1.0
$(NH_4)_2SO_4$ ppt[a] (80%)	645	154	0.24	1.6
Chitin column eluate	490	142.1	0.29	1.93
Sephadex column eluate	31.9	32.8	1.03	6.86

[a]ppt, precipitate.
*Chitinase activity was determined by a reduction in the turbidity of the suspension of colloidal chitin as the substrate in conditioned media as mentioned in Materials and Methods.

Enzyme activity and stability

Chitinase activity was measured by a reduction in turbidity of the reaction mixture, colloidal chitin and the purified enzyme as previously described [21]. Chi25 had exhibited the specific activity of 1.03 U/mg. The chitinase activity was also confirmed using chromogenic oligomers of *p*-NP-(GlcNAc). The Chi25 produced *p*-nitrophenol from both the trimeric and tetrameric forms but not from the dimeric chito-oligosaccharides (FIG. 5). The release of pNP was measured and the specific activity of Chi25 was found to be similar with the values obtained in the above method. The effect of pH and temperature on chitinase activity of the Chi25 was conducted as described in materials and methods, wherein a continuous increase in chitinase activity was observed from pH 4.5 to 6.5, with a decrease in activity with a further increase in pH. The optimum pH for maximum chitinase activity was observed at 6.5 and stable over a pH range of 6–7.5 (FIG. 6A). Similarly, the effect of temperature on enzyme activity showed a continuous increase in chitinase activity from 25 to 35 °C with a decrease in activity after 40 °C with a total loss of activity at 55 °C. The maximum activity was observed at 35 °C and its stability was maintained between 25–40 °C as shown in FIG. 6B.

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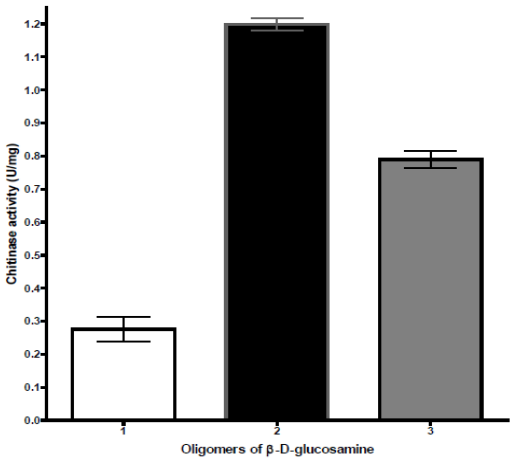

Figure 5: Chitinolytic activity of Chi25 from *B. subtilis* BC121.

Assay was done with (1) *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminidase (2) *p*-nitrophenyl-β-D-*N*-*N'*-diacetylchitobiose and (3) *p*-nitrophenyl-β-D- *N*-*N'N''*-triacetylchitotriose

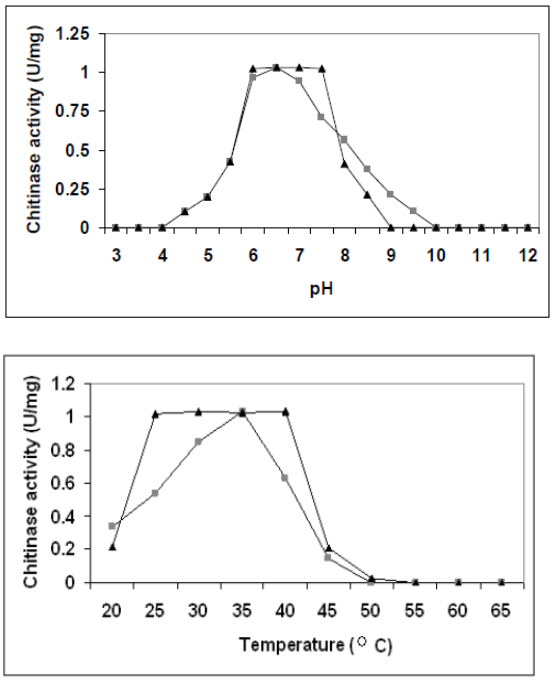

Figure 6: A. Effects of pH on the activity and stability of Chi25 (Top).

(-■-) Chitinase activity was measured at various pHs at 35 °C. (-▲-) Enzyme solution was incubated at various pHs at 37 °C for 1 h and residual activity was measured at pH 6.5 at 35 °C. (B). Effects of temperature on the activity and stability of Chi25 (Down). (-■-) Chitinase activity was measured at various temperatures at pH 6.5. (-▲-) Enzyme solution was incubated at various temperatures at pH 6.5 for 1 h and residual activity was measured at pH 6.5 at 35 °C.

Effect of substrate on Chi25 production

The effect of chitinase production by *B. subtilis* BC121 differed with the type of substrate used in the chitin media. Chitinase production was highest (0.29 U/mg) in media supplemented with 0.5% colloidal chitin, while chitinase activity was 0.16 U/mg, 0.12 U/mg, and 0.06 U/mg with swollen chitin, glycol chitin, and purified chitin as substrates, respectively.

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Antifungal activity of Chi25

The purified enzyme, Chi25 showed antifungal assay activity against fungal pathogens, *F. moniliforme*, *F. oxysporum* f. sp. *ciceris, F. oxysporum* f. sp. *ricini*, and *R. solani*. In the disc-plate diffusion assay, a clear inhibition zone was observed between the fungal mycelia and the paper discs loaded with 10 µg of Chi25 (FIG. 7). Microscopic study of the inhibition zone revealed the presence of mycelia with abnormal features such as swelling and damaged cell surfaces (FIG. 3B). The effect of Chi25 on fungal spore germination and hyphal growth was also studied in microtitre plates. After 24 h of incubation, the spores of *F. oxysporum* f. sp. *ciceris* treated with 10 µg of Chi25 showed abnormal swelling with poor growth of germ tubes. While the spores treated with SP buffer had normal growth (FIG. 3C). This study reveals the strong antifungal activity of Chi25.

Biocontrol activity of the BC121 strain

In order to investigate the biocontrol activity of *B. subtilis* BC121 to suppress *F. oxysporum* f. sp. *ciceris* in soil, a pot assay experiment was performed in glass house. After 40 days, the control plantlets of chickpea plant showed wilt symptoms, such as foliage droop caused by *F. oxysporum* f. sp. *ciceris*. The application of bacterial suspension of *B. subtilis* BC121 mixed with carboxymethylcellulose as a seed cover layer, suppressed the wilt symptoms by 80% compared to the control planlets. The disease level was significantly lower than that of the untreated plants ($p < 0.05$). The *B. subtilis* BC121M strain did not protect chickpea plants against the fungal pathogen and had similar wilt symptoms like control plantlets (FIG. 8).

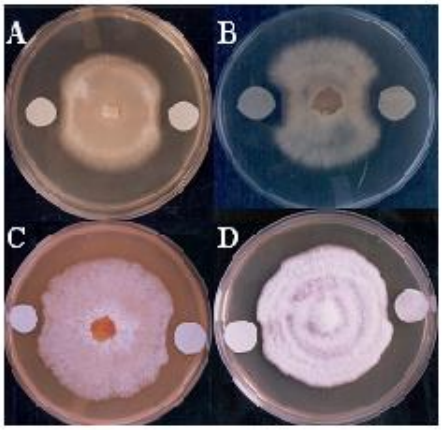

Figure 7: Antifungal activity of Chi25 of *B. subtilis* strain BC121.

Ten micrograms of Chi25 (in 100 µL of 10 mM SP buffer, pH 7.0) was placed on sterile filter paper discs and incubated against (A) *F. moniliforme*, (B) *F. oxysporum* f. sp. *ciceris,* (C) *R. solani,* and (D) *F. oxysporum* f. sp. *ricini.*

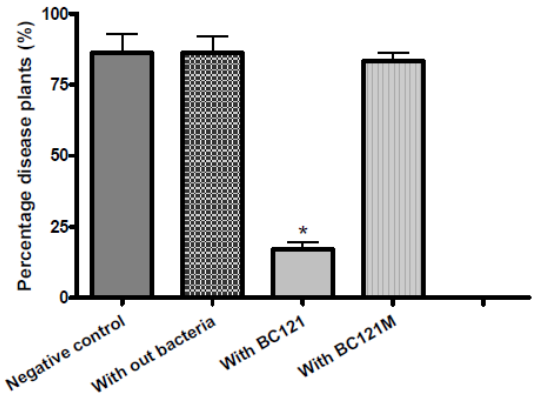

Figure 8: Biocontrol of wilt disease caused by *Fusarium oxysporum* f. sp. *ciceris*.

Seeds of chickpea cultivar JG62 were coated with bacterial cells of BC121 strain or with BC121M strain were grown in a mixture of clay loam/peat, 2:1 (vol/vol) amended with *F. oxysporum* f. sp. *ciceris*. The negative control consisted of seeds coated without bacteria and grown in non infested soil. After 40 days of growth, the plants were scored for disease symptoms. Data were analyzed by a Fischer's least significant difference test ($p = 0.05$, $n = 12$). The star symbol denotes that it is statistically different from others.

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DISCUSSION

Several species of *Bacillus* have been reported as antifungal agents. *B. subtilis* strain GBO3 was shown to possess strong antifungal activity by producing a special class of pore-forming lipopeptides[22], *B. brevis* decreased the fusarial wilt of pigeon peas by producing several antibiotics[23]. *B. subtilis* AF1 has been shown to produce N-acetyl glucosaminidase enzymes that suppress the growth of *Aspergillus niger*[13]. However, the chitinolytic mechanism proved to be a major mechanism in the control of fungal pathogens[14]. Herein, we report a chitinolytic soil bacterium *B. subtilis* BC121 that has broad spectrum of antifungal activity against various fungal pathogens tested. Further, the purification and characterization of the chitinase enzyme, Chi25 from *B. subtilis* BC121 was carried out. The purified enzyme, Chi25 was shown to inhibit the growth of fungi tested. This may be due to the hydrolytic action of Chi25 on fungal cell wall component, chitin that is made up of with the units of N-acetyl-D-glucosamine along with sugars, proteins, lipids and polysaccharides[24]. The amount of chitin and the ratio of the other components vary with the culture of fungi[25]. Earlier reports showed that the chitinase activity differs with the chitin substrates[26]. The variations in the chitinase activity against different fungal cell walls can be explained by its inability to hydrolyze the modified Glc-N-GlcN linkages in the cell walls.

Two types of substrates, i.e., colloidal chitin and a set of chromogenic oligomers of N-acetyl-β-D-glucosamine were used to identify the chitinolytic activity of Chi25. It has hydrolyzed the colloidal chitin and formed clear halo regions on chitin-agar plates. According to current nomenclature practices[1], the chitinolytic enzymes are divided into three principal types: endochitinases are defined as enzymes that catalyze the random hydrolysis of 1,4-β linkages of GlcNAc at internal sites of the chitin polymer, exochitinases catalyze the release of diacetylchitobiose units from the chitin chain, and *N*-Ac-β-D-glucoaminidases act in an exo-splitting mode on diacetylchitobiose. In the present study, we observed that Chi25 forms *p*-nitrophenol from both the trimeric and tetrameric forms of N-acetyl-β-D-glucosamine and hence we concluded its activity as an endochitinase. According to Roberts and Selitrennikoff [3], plant and bacterial chitinases function as endochitinases and inhibit fungal hyphal elongation. In contrast, exochitinases from *Serratia marcescens, S. griseus*, and *P. stutzeri* have no effect on hyphal extension. However, the endochitinases from *S. marcescens* cause lysis of the hyphal tips of *Sclerotium rolfsii* and decrease disease incidence[9]. In this study we observed that the fungal spores incubated along with the Chi25 showed poor germination, swellings and distortions.

Further, antagonistic activity of *B. subtilis* BC121 was also confirmed in greenhouse trials. The wide range of chitinolytic activity between pH 6.0–7.5 and temperature 25–40 °C can be advantageous for the strain to be used as an antifungal agent. The mutant *B. subtilis* BC121M strain, which failed to inhibit fungal growth in vitro, neither produced Chi25 nor showed biocontrol activity in green house trial. Overall this study confirmed the antagonism of *B. subtilis* BC121 against fungal pathogens at the whole bacterium, crude extract, and purified enzyme levels. This antagonism has been conclusively shown to be due to the production of a chitinase enzyme of 25 kDa. Further, the isolation and characterization of their structural and regulatory gene of Chi25, will contribute to a better understanding and eventual improvement of biological control by *B. subtilis* BC121.

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