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Heterologous expression and characterization of purified partial endochitinase (*ech*-42) isolated from *Trichoderma harzianum*

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Chitinase gene from *Trichoderma harzianum* was cloned and hetrologously over expressed in M15 *Escherichia coli*. The recombinant protein of 42 kDa from *E. coli* was purified through Ni-NTA affinity column chromatography. The purified enzyme was active over broad range of pH (2.0 to 8.0) and temperature (10 to 60°C) with the peak activity at pH 5 (0.50 µg/ml) and 20°C with enzyme activity value (0.49 µg/ml). The purified protein fractions were tested for *in vitro* antifungal activity against different phytopathogens like *Fusarium oxysporum* f.sp. *lycopersici*, *Sclerotioum rolfsii*, *Alternaria brassicae* and *Alternaria brassicicola*. Purified endochitinase isolated from *T. harzianum* caused necrotic lesions, segmentation, branching and hyphal bursting at the concentration of 200 µg ml^{-1}.

Key words: Antifungal activity, *Trichoderma harzianum, Fusarium oxysporum* f.sp. *lycopersici, Sclerotioum rolfsii, Alternaria brassicae* and *Alternaria brassicicola.*

INTRODUCTION

Trichoderma spp. are among the most frequently isolated soil fungi, well known for their biocontrol ability against a wide range of plant pathogenic fungi (Howell, 2003; Sharma et al., 2011). There are various modes of action associated with the ability of *Trichoderma* spp. to control plant pathogens but the best characterized and studied mechanism is mycoparasitism which involves a group of genes that encode for lytic enzyme chitinases (EC 3.2.1.14), known to play important role in digesting chitin wall of the phytopathogenic fungi. Chitin, which is a significant component in the cell walls of large groups of fungi is made up of units of N-acetylglucosamine, linked together by 1,4-β-glycosidic bonds. Chitinases being chitin-degrading enzymes hydrolyze the β-1,4-glycosidic bonds between the N-acetyl glucosamine residues of chitin. There are many chitinase-producing organisms including bacteria (Ningthoujam et al., 2009), insects (Merzendorfer and Zimoch, 2003), plants (Salami et al., 2008), fungi (Rattanakit et al., 2007) and vertebrates (Tunc et al., 2008). Chitinases are classified into two families, family 18 and 19, based on the amino acid sequence of their catalytic domains. While family 18 includes

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Abbrevations: IPTG, Isopropyl β-D-1-thiogalactopyranoside; ***ech*-42,** endochitinase.

chitinases of viruses, fungi, bacteria, animals and some plants, family 19 includes all chitinases from plant origins and *Streptomyces griseus* (Patil et al., 2000; Dahiya et al., 2006). Chitinase secretion is one of the mechanisms used by *Trichoderma* to inhibit the growth of other fungi. *Trichoderma* chitinases belong to the glycosyl hydrolase family 18 and can be further grouped into class III and class V. Many chitinase genes from *Trichoderma* have been studied, including class III chitinases, such as *cht33* of *T. harzianum*, *cht33* of *T. atroviride* and *cht1* of *T. virens* and class V chitinase, such as *ech1*, *ech2* and *ech3* of *T. virens* (Kim et al., 2002; Markovich and Kononova, 2003; Duo-Chuan, 2006). Many biological control agents in the last few years were being tested and are commercially available in market. However, there is still considerable interest in finding more efficient strains, which differ considerably with respect to their biocontrol effectiveness. The main objective of the study was the heterologous expression and partially purified *ech*-42 endochitinase and *in vitro* antifungal property of purified protein against different phtyo-pathogenic fungi.

MATERIALS AND METHODS

Collection of *Trichoderma* spp. under test

Fungal cultures of *T. harzianum* were taken for isolation, cloning and characterization of gene coding endochitinase in Trichoderma. *T. harzianum* strain Th3 (ITCC: 5593), was specifically taken for this study from biocontrol laboratory, division of Plant pathology, IARI, New Delhi. The plasmid pQE-30 (Qiagen) was used as expression vector, and *E. coli* strain M15 was used as an expression host. The experimental *T. harzianum* was multiplied on potato dextrose agar media, with the combination of peeled potato: 250 g, dextrose: 20 g, agar: 15 g and distilled water: 1000 ml.

Cloning and sequencing of endochitinase gene

DNA sequence of 1,476 base encoding endochitinase was successfully cloned with accession no JN798187. The deduced amino acid sequence of endochitinase of *T. harzianum* was seen homologous to other *Trichoderma* species (82-97%) identity (Sharma et al., 2012). The nucleotide sequence having 1 to 268 bp open reading frame that has high homology with other reported Chit42 belonging to the *Trichoderma* spp. The amino acid N-terminal sequence showed a putative signal peptide for the possible secretion of the protein. The amplicon was then digested with restriction enzymes and was ligated into pre-digested pQE-30 expression vector. The recombinant vector was transformed into chemical competent cells of *E. coli* M15 by heat shock method (Froger and Hall, 2007). The transformants were selected on Luria-Bertani (LB) agar supplemented with ampicillin (100 $\mu g.ml^{-1}$). The positive clones were screened by colony PCR. The recombinant plasmids were extracted by plasmid extraction kit (Qiagen) and selection was carried out by restriction analyses and sequencing.

Expression in *Escherichia coli* and purification

The *E. coli* strain M15 harboring the pQE-Chi42 vector was grown at 37°C. When the OD 600 increased from 0.8 to 1.0, isopropyl-β-D-thiogalactoside (IPTG) inducer was added to a final concentration of 0.1 mM. The culture was further incubated for 6 h. Cells were harvested by centrifugation and stored at -20°C. Previously stored cells at -20°C were transferred to ice and kept on ice for 30 min to thaw. The cells were resuspended in lysis buffer (50 mM Na-phosphate buffer pH 8.0, 300 mM NaCl, and 10 mM imidazole). Lysozyme was added at a final concentration of 1.0 mg ml^{-1} to facilitate the lysis. The cell lysis was performed mechanically using sonicator equipped with sonication microtip, using six 10 s bursts at 200 to 300 Watt, with a 10 s cooling period between each burst. After lysis, cell debris were removed by centrifugation at 20 000 *g*. Total soluble proteins were applied to Ni-NTA matrix column (Qiagen) and Chi42 was purified following the instruction of the manufacturer (Qiagen). Purity of the Chi42 was further confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 12.5%) for analysis of purified protein fractions.

Enzymatic assay for chitinase

Chitinase activity is determined colorimetrically by detecting the amount of N-acetylglucosamine (GlcNac) released from the colloidal chitin substrate. Colloidal chitin (Sigma Chemicals Co., USA) was used as a substrate (Wen et al., 2005). 0.3 ml of 1% colloidal chitin in acetate buffer (50 mM, pH 6.0) with 1 ml of enzyme were mixed and incubated at 30°C for 30 min. The hydrolysis reaction was terminated by adding 0.6 ml of dinitrosalicylic acid (DNS) reagent. The mixture was kept in a boiling water bath for 15 min, chilled and centrifuged to remove the insoluble chitin. The resulting adduct was measured in UV double beam spectrophotometer at 540 nm (Miller, 1959). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1 mol of N-acetyl D-glucosamine per ml in minutes.

Effect of temperature, pH on purified enzyme activity

Chitinase activity was assayed at different pH values (pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0) using buffers, such as citrate-phosphate buffer (100 mM, pH, 2.5 to 7.0), sodium phosphate buffer (100 mM, pH, 7.0-8.0), and glycine-NaOH buffer (100 mM, pH,8.5 to 10.0). The purified enzyme was incubated at a range of temperatures (10, 20, 30 40, 50 and 60°C) to check for the enzyme activity.

Antifungal activity assay

Cylinder plate method (Jhonson and Curl, 1972) was used to make wells in the medium. Plug colony of *F. oxysporum* f. sp. *lycopersici* , *S. rolfsii, A. brassicae* and *A. brassicicola* were grown on petri dishes containing 1.5% potato dextrose agar (PDA). After three days of growth when colony diameter was 4 to 5 cm, wells were filled with 50 µL of enzyme solution with different concentration of purified enzyme ranging from (50,100, 150, 200 µg ml^{-1}) in triplicates. Plates were then observed after 16 h. Abnormal growth of hyphae and morphological changes were noted through light microscopy.

RESULTS

Purification of endochitinase

Chitinase cDNA was cloned in a prokaryotic expression vector named pQE-30. The cloning strategy was designed such that the protein containing an additional N-terminal methionine residue and C-terminal 6x-His Tag

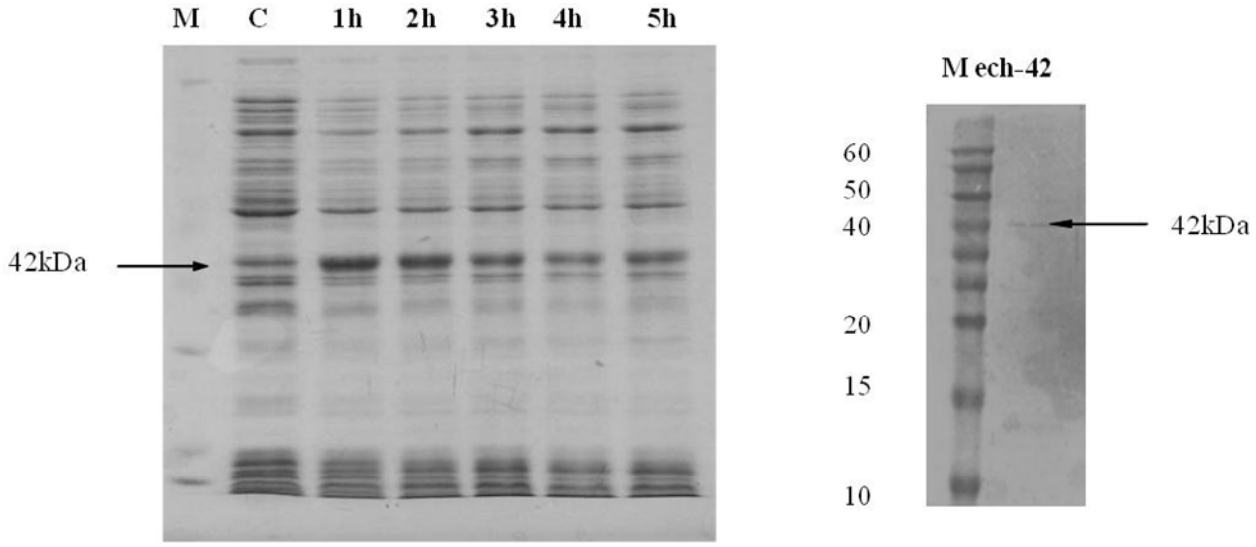

Figure 1. Hetrologous expression of endochitinase in *E. coli* after 5 h of induction with IPTG and enzyme purification through Ni-NTA column.

would be produced. Expression of the active chitinase was optimized by inducing it with IPTG inducer at different time of induction. After SDS-PAGE, the chitinase was regenerated by the removal of SDS with purified Triton X-100. After purification with Ni-NTA coloumn to bind to 6x His Tag the recombinant protein was found to be 42 kDa on SDS-PAGE as shown in Figure 1. The purified recombinant protein fractions extracted from pQE-30 with low amounts having protein (mg/mL) 1.01, 0.94, 1.20 respectively.

Effect of pH

The effect of pHs and stability for chitinase based on enzyme activity were examined at 28°C by varying pHs of the reaction mixture (pH 2 to 8) using different buffers. The enzyme was found most active between pH 3.0 to 6.0 with enzyme activity (0.30 µg/ml at pH 3.0, 0.37 µg/ml at p H 4.0, 0.57 µg/ml at p H 5.0 and 0.50 µg/ml at p H 6.0 respectively. Beyond this there is indeed loss of enzyme stability it found relatively stable at pH 7.0 and p H 8.0 with the enzyme activity (0.39 µg/ml and 0.30 µg/ml respectively). Chitinases, including the one under study showed a pH optimum in the acidic range (Figure 2).

Effect of temperature

When the enzyme was kept at various temperatures for 30 min in an acetate buffer (pH 5.0) the chitinase activity was most active at 20°C with the enzyme activity (0.49 µg/ml). The activity starts decreasing from 30°C (0.43 µg/ml) to 40°C (0.27 µg/ml). Above 40°C, the activity decreased further at 50°C (0.18 µg/ml) and was reduced to 0.06 µg/ml at 60°C (Figure 3).

Inhibition of fungal growth

Inhibition zone of 42 kDa endochitinase isolated from *T. harzianum* against different phytopathogens like *F. oxysporum f.sp. lycopersici*, *S. rolfsii*, *A. brassicae* and *A. brassicicola* was formed at the concentration of 200 µg ml^{-1}. No inhibition zone was found between 50 to 100 µg ml^{-1}. Microscopic examination revealed that enzyme caused necrotic lesions, branching, hyphal bursting at the concentration of 200 µg ml^{-1} (Figures 4 to 7).

DISCUSSION

Fungi of genus *Trichoderma* has been long recognized for their ability to act as a biocontrol agents against plant pathogens (Harman, 2006). Most of these studies are based on characterization of genes and a few involved in purification of proteins. In the present study, an attempt was made to demonstrate *in vitro* over expression of the product of the gene coding endochitinase *ech*-42 in *E. coli* which is a part of the chitinolytic enzyme system of fungi and mechanism of biocontrol. The investigation leads us to separate a 42 kDa protein of *T. harzianum* on SDS-PAGE which was later purified to homogeneity as also been reported by Harighi et al. (2007). The activity of *ech*-42 was examined at different temperatures (20 to 60°C) with an optimum of 0.49 µg/ml at 20°C. The enzyme seems to retain activity at 60°C which supports the finding of Harighi et al. (2007) who reported the heat

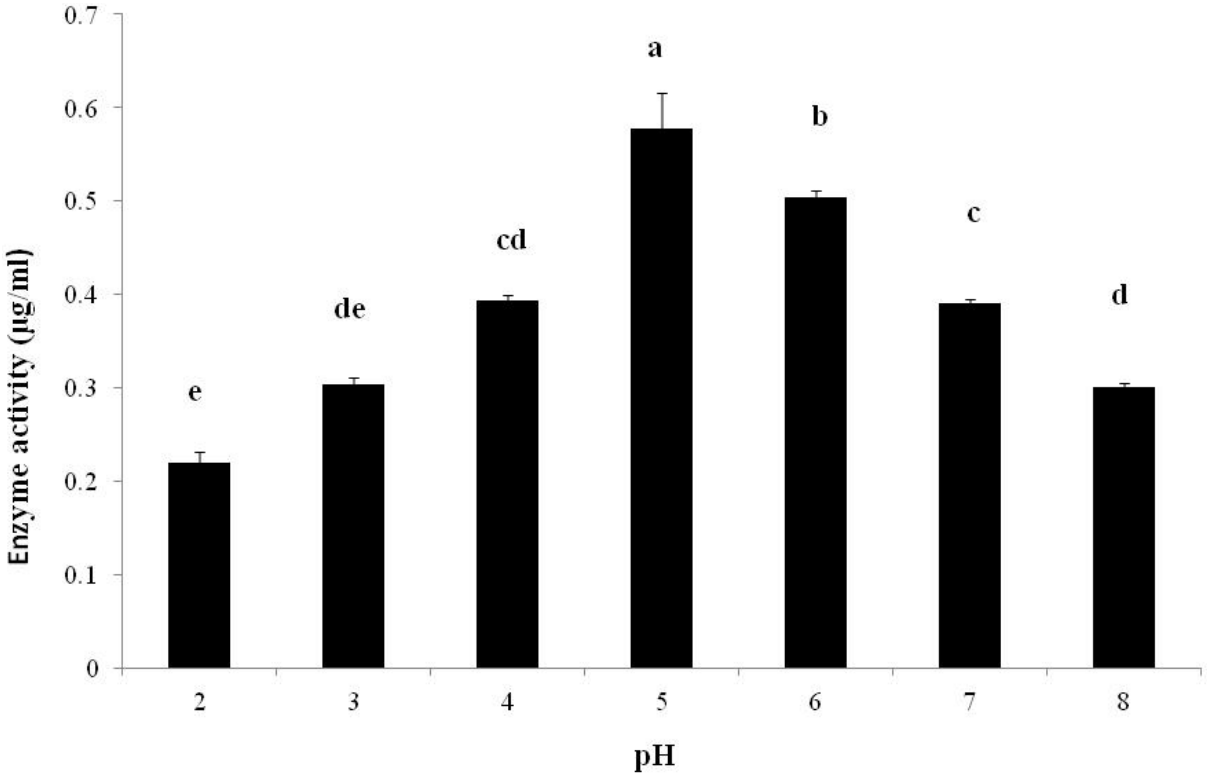

Figure 2. Influence of p H (2 to 8) on chitinase* activity. The values are the means ± S.E., for each p H , in each column followed by the same letter are significantly Different (P ≤ 0.05) from each other according to Duncan's Multiple Range (DMR) test.

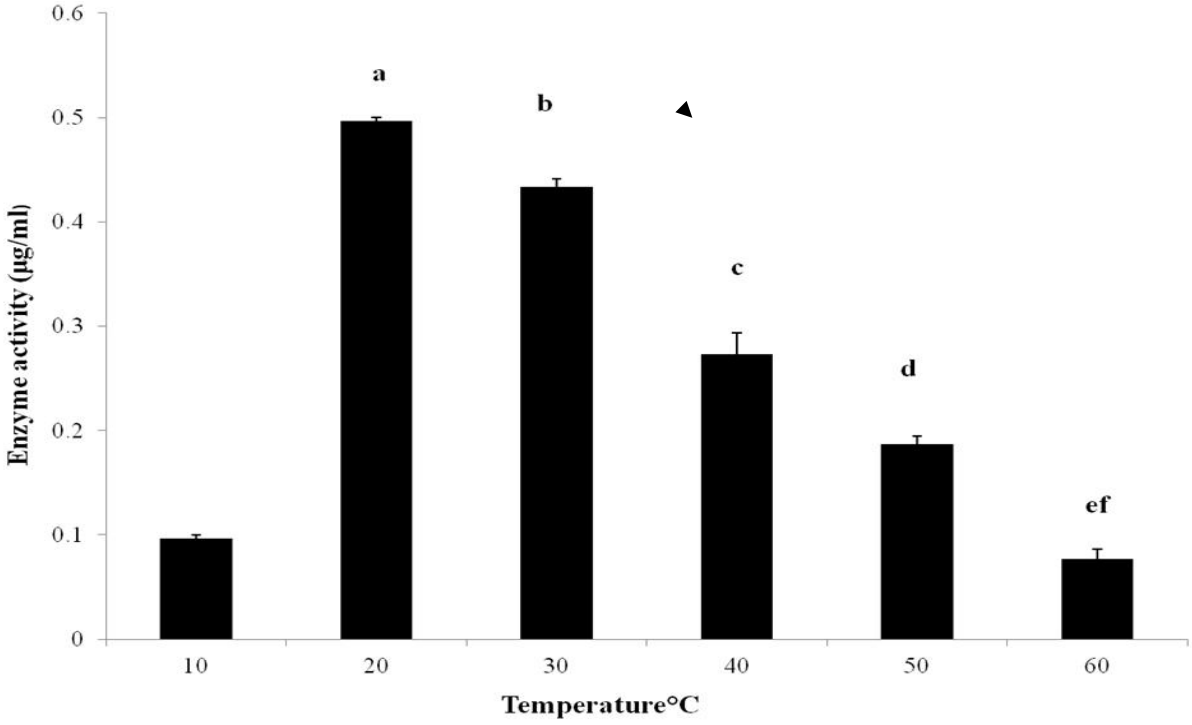

Figure 3. Influence of temperature 10 to 60°C on chitinase activity. The values are the means ± S.E. for each value in each column followed by the same letter are significantly different (P ≤ 0.05) from each other according to Duncan's multiple range (DMR) test.

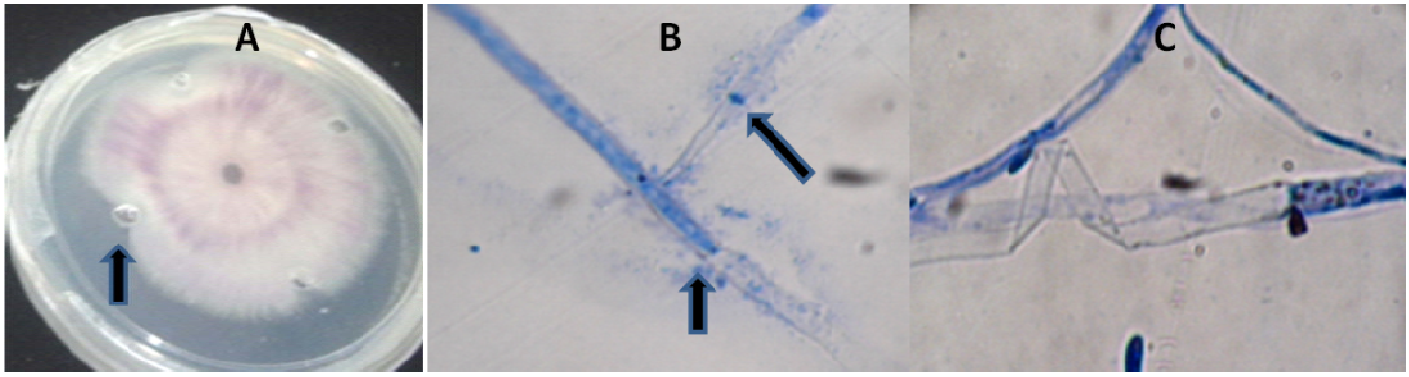

Figure 4. (A) Effect on growth of *Fusarium oxysporum f.sp. lycopersici* in response to different concentrations of endochitinase. Wells contain 50 µl of enzyme solution in µgml^{-1} from top in clockwise direction (50, 100, 150 and 200). **(B)** Cellular bursting was observed at 200 µgml^{-1} concentration. **(C)** Lysis of the hypha and loss of cellular components at 200 µgml^{-1}.

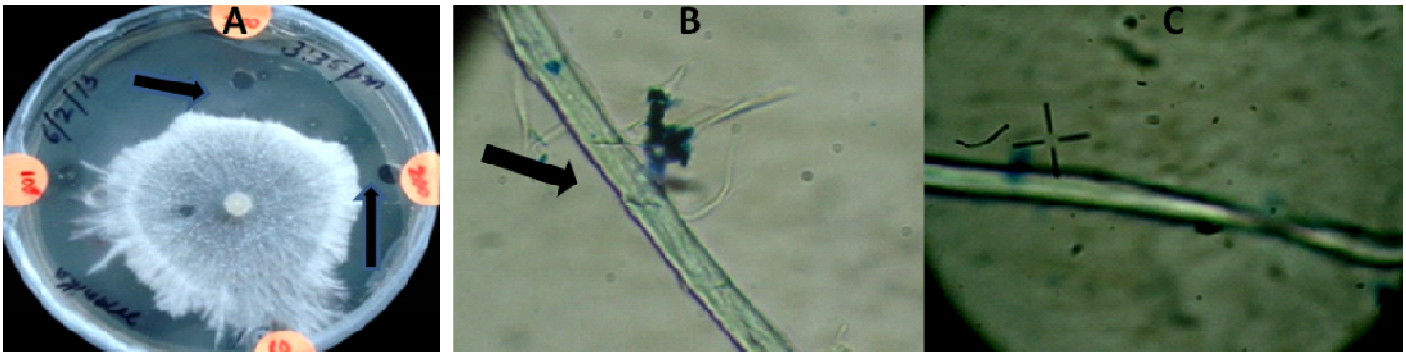

Figure 5. (A) Effect on growth of *Sclerotium rolfsii* in response to different concentrations of endochitinase. Wells contain 50 µl of enzyme solution in µgml^{-1} from top in clockwise direction (200, 100, 150 and 50). (B) Enzyme disturbance seen in the form of hyphal swelling at 200 µgml^{-1}. (C) Enzyme disturbance in the form of necrotic lesion and hyphal burst is visible at enzyme concentration of 200 µgml^{-1}.

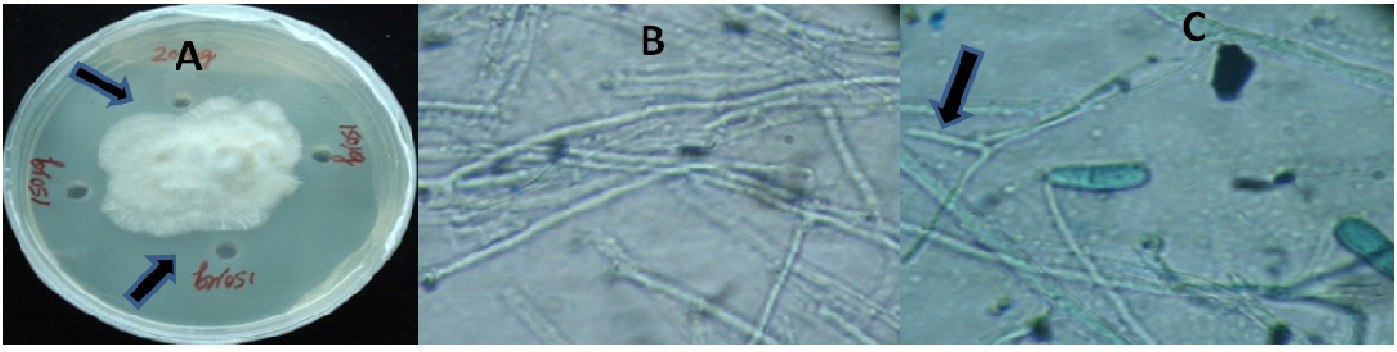

Figure 6. (A) Effect on growth of *Alternaria brassicicola* in response to different concentrations of endochitinase.Wells contain 50µl of enzyme solution in µgml^{-1} from top in clockwise direction (200,150,10050) (B+C) Enzyme disturbance seen in the form of branching but it is stable to recover as we did not find any sign of cellular bursting at 150- 200 µgml^{-1}.

stability of purified endochitinase. The enzyme activity was also determined at different pH (2 to 8). The highest activity was observed at pH 5 and enzyme was found stable from pH 5 to 7. These results are similar to the findings of Rashed et al. (2010) wherein the optimum temperature for *ech-42* at 35°C with maximum stability upto 55°C and highest activity at pH 6. Similarly there are other fungal chitinases (Chi 1,Chit 37,Chit 46) reported by

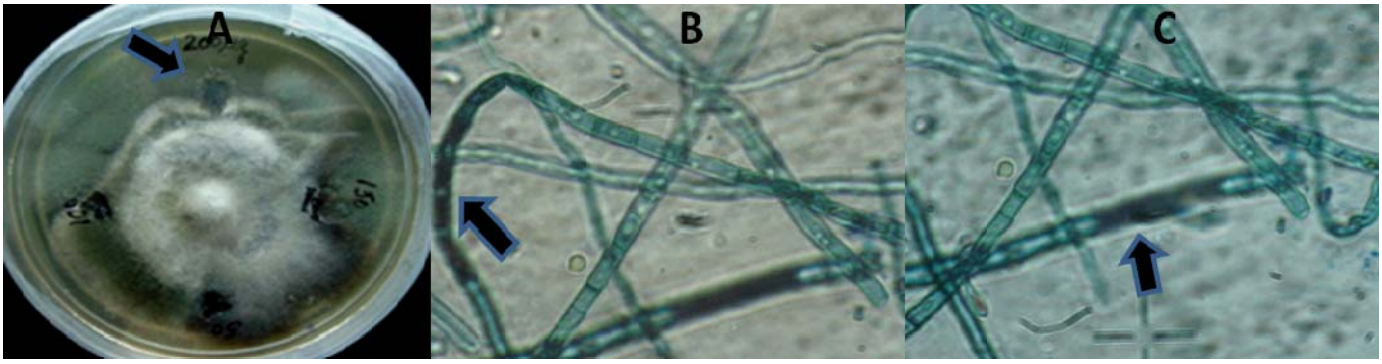

Figure 7. (A) Effect on growth of *Alternaria brassicae* in response to different concentrations of endochitinase. Wells contain 50 µl of enzyme solution in µgml^{-1} from top in clockwise direction (200, 150, 100 and 50). **(B)** Hyphal necrosis and swelling observed at concentration 200 µgml^{-1}. **(C)** Segmentation and enzyme disturbance observed.

several workers who found variable temperature and pH optimum (Gan et al., 2007; Caihong et al., 2007; Ike et al., 2006).

Investigations into the biological activity of *T. harzianum* clearly indicate strong reducing effect towards different phytopathogens *F. oxysporum* f.sp. *lycopersici*, *S. rolfsii*, *A. brassicae* and *A. brassicicola.* Microscopic examination revealed that the enzyme caused necrotic lesions, branching, hyphal bursting at the concentration of 200 µg ml^{-1} which is similar to the findings of Harjono and Widyastuti (2001), where pathogenic response of purified endochitinase of *T. reesei* on *Ganoderma philippii* was investigated. There are many workers who reported that the chitinases are substantially more active and effective against a wide range of fungi like Kaomek et al. (2003) who tested the antifungal activity of *L. leucocephala* chitinase against *Collectotrichum* sp., *Pestalestiopsis* sp. and *Fusarium* sp. and found it as good candidate for fungal inhibition.

Conclusion

It is concluded that 42-kDa endochitinase produced by *T. harzianum* has antifungal activity *in vitro*. The genes of *T. harzianum* coding for chitinolytic enzymes are attractive sources of these enzymes as their products can be used for combating with many phytopathogenic fungi. The enzymes like endochitinases can be used to add more biocontrol capabilities in wide series of microorganisms after further characterization. This could be an added advantage in bioprospecting novel antifungal or other compounds.

Conflict of Interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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