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EVALUATION OF ANTIFUNGAL ACTIVITY OF A NOVEL CHITINASE PROTEIN FROM *XENORHABDUS NEMATOPHILUS*

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Abstarct

Fusarium oxysporum f. sp. *Lycopersici and Alternaria alternata*, have been most common and destructive fungus of the tomato plants, they cause *Fusarium* wilt and Early blight disease in tomato respectively, which is the most economically important vegetable crop and used worldwide. Since, the chitin is the major component of the fungal cell wall and it is hydrolyzed by chitinases that results into inhibition of the fungal growth. A novel ~76 kDa protein from *Xenorhabdus nematophilu*s, was expressed in *E.coli* and purified. The recombinant purified protein shows both exo and endochitinase activity and strongly inhibit the hyphal growth of fungi *Fusarium oxysporum* f. sp. *Lycopersici and Alternaria alternate* in in-vitro. We have reported first time that a novel chitinase protein from *Xenorhabdus nematophilus* shows antifungal activity.

Key words: *Xenorhabdus nematophilus*, Chitinase, Chitin, *Fusarium oxysporum*, *Alternaria alternate*.

Introduction

Disease caused by fungi in crop plants is increasingly recognized as worldwide threat to food security as fungi is the most common cause of plant diseases. Fungi have long been known to comprise a wide spread threat to crop species. More than 10, 000 kinds of fungi can cause disease in plants among 100, 000 known species of fungi (Daniel *et al.*, 2013). Epidemic and persistent outbreaks of fungal infection in wheat (rust caused by *Puccinia graminis*), potatoes (late blight caused by *Phytophthora infestans*), rice (rice blast caused by *Magnaporthe oryzae*), maize (smut caused by *Ustilago maydis*) and soybean (rust caused by *Phakospora pachyrizi*) cause heavy loss of crop, although varying regionally but posing a growing threat to food security (Pennisi *et al.* 2010) (Table: List of fungal disease in major crop plants).

Diseases caused by fungal infection, presently destroy at least 125 million tones of the major crops, wheat, rice, potatoes and maize annually. The major crop losses and damage due to fungi cost $60 billion annually in global agriculture (Source: Agriculture Today: The National Agriculture Magazine, July 2012).

A soil born plant pathogen, *Fusarium oxysporum* f. sp. *lycopersici*, belongs to class Hyphomycetes, causes *Fusarium* wilt on tomato, which is the most economically important vegetable crop and used worldwide (Sudhamoy *et al.* 2009). More than 100 kinds of *Fusarium* Vascular wilt diseases are known worldwide affecting productivity of the tomato (Burgess *et al.* 2008). The *Fusarium* enters the plant through root tips (Sally *et al.* 2006) and can remain viable in the soil for very long period (Thangavelu *et al.* 2003). The mycelium starts growing in the xylem vessels very fast, where they cut off water supply that result into wilting (Stephen *et al*., 2003). The appearance of yellowed leaves and wilted is the characteristic symptoms of the disease. This disease may induce 30 to 40% yield loss and under favorable whether condition the yield loss may reach to 80% (Kiran *et al.* 2008; Kapoor 1988). 25–55% *Fusarium* wilt incidences have been recorded from various parts of India (Pandey and Gupta 2013; Asha *et al.* 2011)

Another fungus *Alternaria alternata*, which is the most common and destructive fungus of the tomato plants, it causes Early blight disease in tomato as well as in other crop plants. The fungus can cause disease on stem (collar rot), foliage (leaf blight) and fruit, and can result in severe damage during all stages of plant development (Sabriye 2011).

Fungicides are major component of the disease

management of agronomic crops, generally fungicides are applied to avert the epidemics disease caused by fungi in crops, it forms barrier to protect the crops from fungal diseases (Sallam *et al.* 2012). But, massive and repeated use of fungicides results into exertion of selection pressure on the fungal population and development of resistance strains of fungus, also have non target effects. Usage of fungicides selectively restrain the sensitive populating while allow the multiplication of resistance strain. Fungicides also get accommodated in crops and vegetable and result into public health issues and environmental risk (Richard, 2011).

Chitin is the main component of the cell wall of fungi and yeast and of the exoskeleton of crustaceans and insects, chitin is an abundant biopolymer composed of units of N-acetyl-D-glucosamine linked by b-1, 4 glycosidic bonds. The degradation of chitin is catalyzed by chitinases, among them, the chitinases from microorganisms are extremely important for the degradation of the chitin (Goodayg 1979).

Based on chitinolytic activity chitinases have been divided into two main groups: Endochitinases (E.C 3.2.1.14) and exo-chitinases. The endochitinases randomly split chitin at internal sites, thereby forming the dimer dicetylchitobiose and soluble low molecular mass multimers of GlcNAc such as chitotriose, and chitotetraose. The exochitinases have been further divided into two subcategories: Chitobiosidases (E.C. 3.2.1.29) and 1-4-β-glucosaminidases (E.C. 3.2.1.30). Chitibiosidase involves in the progressive release of di-acetylchitobiose starting at the non-reducing end of the chitin microfibril (Gummadi, 2009).

Because of the characteristic of chitinases to hydrolyse the chitin, it could be effectively used in controlling the fungal growth and showed biocontrol potential. Several chitinases such as isolated from *Bacillus amyloliquefaciens* V656 shows inhibition against *Fusarium oxysporum* (Wang *et al.* 2010), another chitinase from Enterobacter sp. NRG4, showed an inhibitory potential against the growth of hyphal tips of *Fusarium moniliforme*, *Mucor rouxi*, *Aspergillus niger* and *Rhizopus nigricans* (Dahiya *et al.* 2005). Chitinase of *Monascus purpureus* CCRC31499 inhibited the growth of *Fusarium oxysporum* and *Fusarium solani* (Wang *et al.* 2002).

We have reported a novel ~ 76 kDa chitinase proteins from *Xenorhabdus nematophilus*, which shows fungal inhibition against *Fusarium oxysporum lycopersici and Alternaria alternate*. *X. nematophila* is non spore forming, Gram negative bacterium; dwell symbiotically in the gut of the soil nematode belongs to genus *Steinernema*. The association of bacteria nematode is highly toxic to several insect species and currently used as an agent of biological control of many insect species (Akhurst and Dunphy, 1993).

Materials and Methods

Source of *Xenorhabdus nematophilus* genome

The genomic DNA of *Xenorhabdus nematophila* 19061 was received from Prof. Nirupama Banerjee, International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi.

Chemicals

Most of the chemicals like Tris-HCl, potassium acetate, sodium acetate, EDTA, sodium dodecyl sulphate (SDS), sodium chloride, agarose, etc. were of molecular biology grade, antibiotics like ampicillin, kanamycin, dyes like Bromophenol blue, Ethidium Bromide and biochemicals like lysozyme, all the chemicals and column used in protein purification and estimation respectively were procured from Sigma Chemical Company, USA. Luria broth and agar powder were obtained from Hi-Media laboratories, Mumbai. Organic solvents like chloroform, isopropyl alcohol, acetone, etc. were procured from Fisher Scientific, Mumbai.

Plasmid spin miniprep kit, QIAquick PCR purification kit and QIAquick gel extraction kit were purchased from Qiagen Inc., Valencia, USA. pGEMT easy cloning kit was obtained from Promega Corporation, Madison, USA. All Restriction Endonucleases, T4 DNA ligase and Taq DNA Polymerase were purchased from MBI, Fermentas.

Development of bacterial expression vector

An amplicon of 1.9 kb of chitinase gene was amplified from genomic DNA isolated from *X. nematophila* with gene apecific primers having *Xho*I restriction site in forward primer and *BamH*I in reverse primer. The gene was first cloned in pGEMT easy vector and released by digestion with *Xho*I and *BamH*I to confirm the cloning. The chitinase gene was released from pGEMT-*chitinase* vector by digestion with *Xho*I and *Not*I. The pET28b was digested with *Sal*I and *Not*I to generate compatible ends and sub cloning. The restriction enzymes *Xho*I and *Sal*I generate compatible ends and after ligation the site *Xho*I/*Sal*I was lost. The subcloning was first confirmed by colony PCR with gene specific primers. The sub cloning was finally confirmed by *BamH*I, a fall out of 1.9 kb was observed. Restriction digestion with *Sal*I and *Not*I linearized the plasmid.

Expression and purification of recombinant protein

BL-21 harbouring the *pET28b-chitinase* construct were grown in LB medium grown till $O.D_{600}$ reached 0.5-0.6. 1 mM IPTG was used to induce the culture and was allowed to incubate at 28^0C for another 4-5 h. Cells were harvested after centrifugation at 8000 rpm for 15 min at 4^0C. The cells were washed, resuspended in lysis buffer (50 mM Tris, pH 80,200 mM Nacl) and was subjected for sonication for disruption and the lysate was allowed to centrifuge at 10,000 rpm for 20 min. The supernatant was collected as soluble fraction and the pellet was resuspended again in 10 ml lysis buffer. The resuspended pellet was subjected for sonication and centrifuged as before and finally washed with 1 M NaCl followed by centrifugation. The pellet was resuspended in lysis buffer and kept for 1 hour at 37^0C followed by centrifugation to collect the insoluble fraction. The Ni-NTA agarose (Qiagen, Germany) affinity column was equilibrated with 50 mM Tris, pH 8.0, 200 mM NaCl and the two fractions were loaded onto column. The column was first washed with 5 column volumes of the 50 mM Tris, pH 8.0, 200 mM NaCl buffer. The column was again washed with 5 column volumes of 50 mM Tris, pH 8.0, 200 mM NaCl with 25 mM Imidazole to remove non-specifically bound proteins. The recombinant protein was eluted with buffer conataining 50 mM Tris, pH 8.0, 300 mM NaCl and 200 mM Imidazole. The different fractions were resolved on a 12% SDS-PAGE. Fractions containing single band protein were pooled and dialyzed extensively against 10 mM Tris buffer, pH 7.5, at 4^0C. The protein was estimated by Bradford assay (Bradford, 1976). The purity of the final fractions was determined by SDS-PAGE (Sambrook *et al.*, (1989).

Chitinase assay

Chitinase activity of the purified recombinant protein was determined by chitinase assay Kit procured from Sigma Aldrich, USA. 4-methylumbelliferone (4MU) was released by the hydrolysis of substrates. 4-methylumbelliferone is a fluorescent molecule and it is measured by fluorimetrically at an excitation wavelength of 360 nm and an emission wavelength of 450 nm in the basic pH environment.

For the detection of different chitinolytic activities, three types of substrates were used. 4-methylumbelliferyl N, N'-diacetyl-β-D-chitobioside, is the first one, which was used for the detection of chitobiosidase activity of the enzyme. Chitobiosidase activity is a type of exochitinase activity. 4-methylumbelliferyl N-acetyl-β-D-glucosaminide is the second one which was also used for the detection of exochitinase activity (β-N-acetylglucosaminidase activity). For the detection of endochitinase activity of the recombinant purified chitinase, the third substrate 4-Methylumbelliferyl β-D-N, N', N''-triacetylchitotriose was used. Manufacture's protocol was used for the chitinase assay.

A single standard concentration was used to calculate the chitinase activity. The fluorescence of 350 ng (6.65µmole/ml) standard was measured and then the following equation was used:

$$\text{Units/ml} = \frac{(FLU_{sample} - FLU_{blank}) \times 6.65 \times 0.45 \times DF}{FLUs_{tandard} \times \text{time} \times V_{enz}}$$

Evaluation of in vitro antifungal assays

Evaluation of antifungal activity of purified recombinant chitinase protein was carried out with *Fusarium oxysporum* f. Sp. *lycopersici* (Accession No. ITCC 1322) and *Alternaria alternate* (Accession No. ITCC 7360) on PDA (Potato dextrose agar) medium plate. The fungal cultures were procured from Division of plant pathology, IARI, New Delhi and it was maintained on PDA medium plate at 22 ± 3^0C. Different amount of purified recombinant chitinase protein were diluted in 10 mM Tris buffer, pH 7.5. Preparation of spore suspension of both the fungi and the experiment were carried out by using slightly modified protocol of Mondal *et al.*, (2003). Four Wells were made onto a single PDA plates for each fungus. 50 µl each of *F. oxysporum* f. Sp. *lycopersici* and *A. alternate* suspension (10^6 spores/ml) was used to fill the all the four wells of the two respective plates for each fungus and allowed to incubate at 28^0C overnight. After overnight incubation, two well on a single plate was treated with two different amount of chitinase protein (diluted in 50 µl of 10 mM Tris buffer, pH 7.5) one well was filled with heat deactivated chitinase protein and another one was filled with 10 mM tris buffer, pH 7.5 alone for each fungus. The wells filled with buffers were taken as control. The plates were further allowed to incubate, for both the fungi (*F. oxysporum* f. Sp. *lycopersici* and *A. alternate*) radial growth was recorded at every 24 hrs for 4 days.

Inhibition of hyphal growth % = [Diameter of the fungal colony around the well treated with the different concentrations of chitinase protein/diameter of the fungal colony around the well treated with the control (Buffer or heat deactivated protein)] × 100

Statistical analysis

The fungal inhibition experiments were carried out in triplicates. Results were analyzed by the Student's t test. Significance was defined as $P < 0.05$.

Results

Development of bacterial expression vector

The chitinase gene was released from pGEMT-*chitinase* by restriction digestion with *Xho*I and *Not*I. It was cloned in pET28b vector at *Not*I and *Sal*I restriction

Table: List of fungal diseases in major crops (Mohiddin *et al.* 2010).

Fungal pathogens	Disease	Crops
Rhizoctania solani	Sheath blight	Rice
Fusarium oxysporum f. sp lycopersici	*Fusarium* wilt	Tomato
Alternaria triticana	Leaf blight	wheat
Sclerotium rolfsii	Wilt	Potato
Sclerotinia sclerotiorum	Head rot	sunflower
Pythium aphanidermatum	Damping off	tomato
Alternaria alternata f.sp lycopersisci	Early blight	tomato
Alternaria solani	Early blight	tomato, potato
Phytophthora infestans	Late blight	potato
Ceratocystis paradoxa	Pine apple disease	sugarcane

site. The restriction enzymes XhoI and *Sal*I generate compatible ends. After ligation the *Xho*I/*Sal*I site was lost. Cloning was confirmed by restriction digestion of vector at *BamH*I restriction site and SalI/NotI restriction site. Digestion with *BamH*I gave a fall out of ~ 1.9 kb while digestion with *Not*I and *Sal*I linearized the plasmid because of loss of *Sal*I site (fig: 1).

Expression of chitinase protein

Different concentrations of IPTG (0.2 mM, 0.5mM, 0.7 mM and 1 mM) was added to induce the expression of the recombinant chitinase protein in *E. Coli* (BL-21) at temperature 37^{0}C after O.D$_{600}$nm reached 0.6. Cells were harvested after centrifugation at 8000 rpm for 15 min at temp 4^{0}C. The expression of recombinant protein was observed after 5 hours of induction. Expression of protein was observed at all the concentrations of IPTG. The recombinant protein of approximately ~76 kDa was observed in supernantant as well as in cell pellet after the lysis of the *E. coli* clones (fig: 2).

Purification of recombinant protein

To study the function of putative chitinase gene, it was expressed as fusion protein with a His tag at the N-terminus as described in the section of material and methods. Purification of the recombinant protein was done using Ni-NTA agarose. The recombinant protein present in the supernatant fraction was bound to the Ni-NTA agarose resin which was further washed with buffer containing NaCl. The purified fusion protein was eluted with imidazole containing buffer. Protein fraction at different stages was analyzed on 12% SDS-PAGE. A partially purified band (~76 KDa) was observed on a Commassie stained gel (fig: 3) shows the profile of the purified recombinant chitnase protein

The pooled fractions containing the purified protein were dialyzed against 10 mM Tris buffer (pH 7.5).

Chitinase assay

Three types of substrates were used for the estimation of various kinds of chitinolytic activities. For the detection of chitobiosidase activity, 4-methylumbelliferyl N, N'-diacetyl-b-D-chitobioside was used, while 4-methylumbelliferyl N-acetyl-β-D-glucosaminide was used for the exochitinase activity (β-N-acetylglucosaminidase activity) and 4-methylumbelliferyl β-D-N, N', N''-

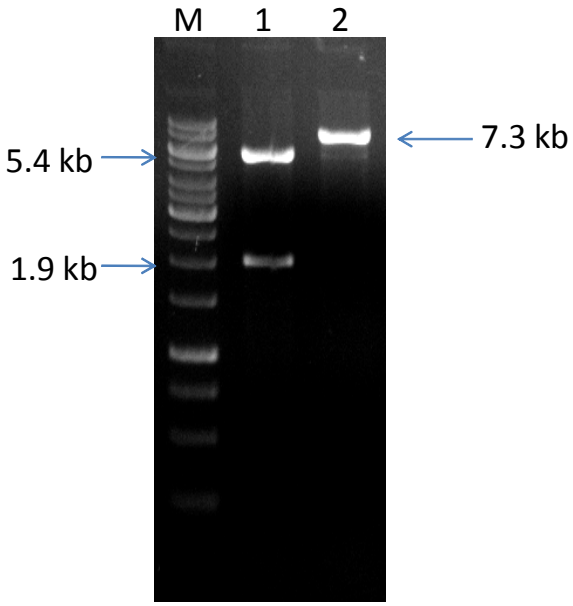

Fig 1: Restriction digestion of *pET28b-chitianse* construct. The *pET28b-chitinase* vector was digested with *BamH*I. A band of 1.9 kb was observed in the lane 1. Lane 2 shows only linearization of the vector when *pET28b-chitinase* construct was digested with sal1 and *Not*I

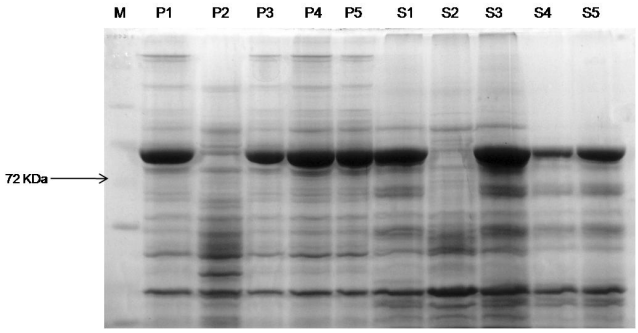

Fig 2: Induction of the recombinant protein in *E. coli* cells by IPTG. Both pellet and supernatant fractions were analyzed on a 12% SDS-PAGE. Lane M: protein marker, P1: 0.2 mM IPTG (pellet), P2: uninduced pellet fraction, P3: 0.5 mM IPTG (pellet), P4: 0.7 mM IPTG (pellet), P5: 1.0 mM IPTG (pellet), S1: 0.2 mM IPTG (supernatant), S2: uninduced supernatant fraction, S3: 0.5 mM IPTG (supernatant), S4: 0.7 mM IPTG (supernatant) and S5: 1.0 mM IPTG (supernatant).

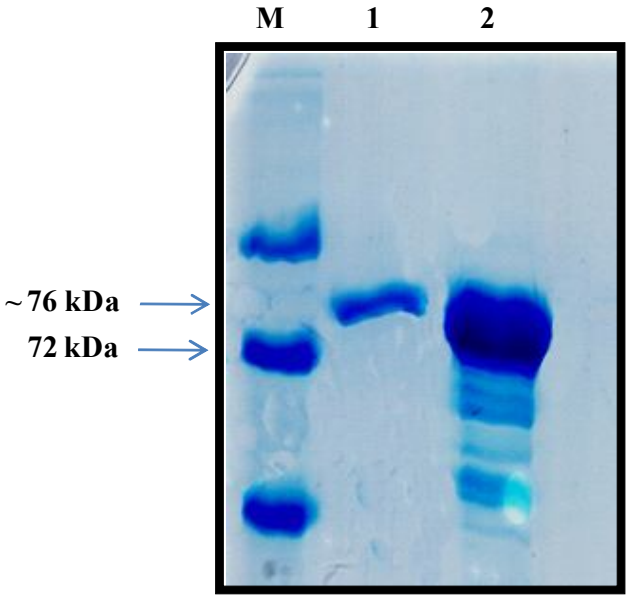

Fig 3: Fig 11: Ni-NTA purified recombinant chitinase protein from *E. coli* cells. The recombinant protein was purified on a Ni-NTA affinity column as described in materials and methods. The purified protein was checked on a 12% SDS-PAGE.

Lane M: Protein Marker profile.

Lane 1: Flow through of other proteins after recovery of chitinase.

Lane 2: Purified and dialyzed protein of ~76 kDa

triacetylchitotriose was used for the endochitinase activity of the enzyme. The chitobiosidase activity of the *X. nematophila* chitinase was very low (0.41 units/mg protein) while the β-N-acetylglucosaminidase activity and endochitinase activity were 22.67 units/mg and 15.03 units/mg protein, respectively (fig: 4).

Efficacy of chitinase protein on fungus *Fusarium oxysporum lycopersici*

Fungus *Fusarium oxysporum lycopersici* was grown in potato dextrose agar medium. The spores of fungal strain were collected and suspended in sterile potato dextrose broth. Four wells (a, b, c and d) were made in the potato dextrose agar plate. All the wells were filled with 50 µl aliquots of broth suspension containing 10^6 spores. The wells were left for overnight to grow the spores at 28^0C. To observe the inhibition of fungal growth by purified chitinase protein. Different amount of diluted protein, 10 µg and 5 µg were added to the wells a and b respectively, while 10 mM Tris buffer (pH 7.5) and heat deactivated chitinase protein (10 µg) were added to the well c and d respectively. Growth of fungal hypea was monitored every 24 hours for 4 days. 63 to 74 % hyphal growth inhibition was observed at 5 µg and 10 µg of chitinase protein respectively, 10 mM Tris buffer (pH 7.5) and heat deactivated chitinase protein were taken as control where growth inhibition was not observed (fig: 5).

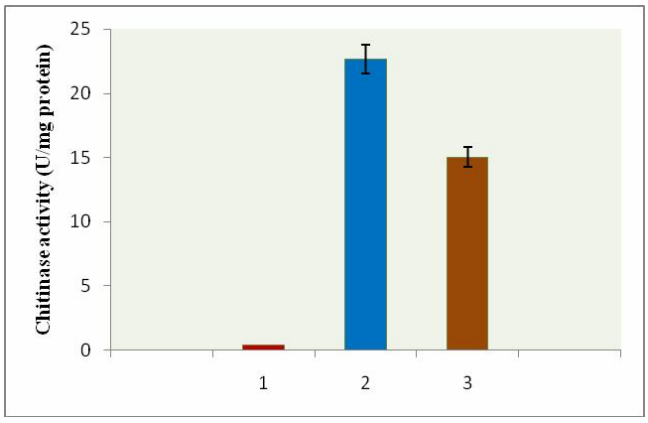

Fig 4: Different chitinolytic activities of *Xenorhabdus nematophila* chitinase.

1 - chitobiosidase activity,

2 - β-N-acetylglucosaminidase activity,

3 – endochitinase activity

Efficacy of purified protein on fungus *Alternaria alternata*

For fungal inhibition assay, potato dextrose agar medium was used to grow fungus *Alternaria alternata*. The spores were collected from fungal strain grown on potato dextrose agar plate and were suspended in sterile potato dextrose broth. a, b, c and d wells were made in the potato dextrose agar plate. All the wells were filled with 50 µl aliquots of broth suspension containing 10^6 spores of the fungus *A. alternata*. After filling the wells with spores, the wells were left for overnight to allow the growth of the hyphae at 28^0C. Later different amount of diluted chitinase protein were used to estimate the fungal growth inhibition. 10 µg of chitinase protein were added to the well a and 5 µg of protein to the well b, respectively. Tris buffer 10 mM (pH 7.5) and heat deactivated chitinase protein (10 µg) was added to the well c and d respectively. After every 24 hours, the growth of the fungal hyphae was recorded for 4 days. 41 to 58 % hyphal growth inhibition was observed at 5 µg and 10 µg of chitinase protein respectively, where inhibition is less. Tris buffer (pH 7.5) and heat deactivated chitinase protein were taken as control where growth inhibition was not observed (fig: 6).

Discussion

Chitin is the major component of the fungal cell wall, which is the outermost part of the fungal cells; it provides plasticity and rigidity to the fungal cell wall which is required for the normal growth and survival of the fungus. With the tremendous increase in the knowledge of the architecture of cell wall of fungi led to the identification of various enzymes which remodel the components of cell wall (Latge 2007). Chitin is composed of *N*-acetylglucosamine (GlcNAc) residues linked by β-1-4

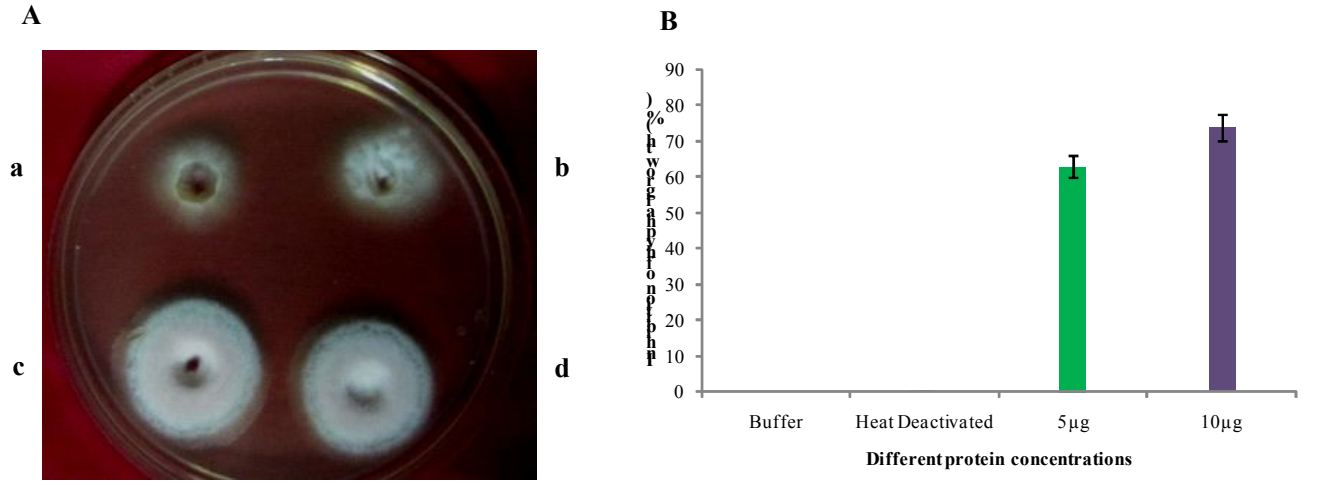

Fig 5: Purified chitinase protein inhibits the growth of *Fusarium oxysporum lycopersici*
(**A**) Treatment of wells with control and different concentration of chitinase protein
Well a: 10µg of chitinase,
Well b: 5µg of chitinase,
Well c: Buffer as control (10 mM Tris buffer pH 7.5)
Well d: Heat deactivated chitinase (10 µg)
(**B**) Histogram showing percentage (%) inhibition of hyphal growth at different concentration of chitinase protein and controls (Buffer and heat deactivated protein).

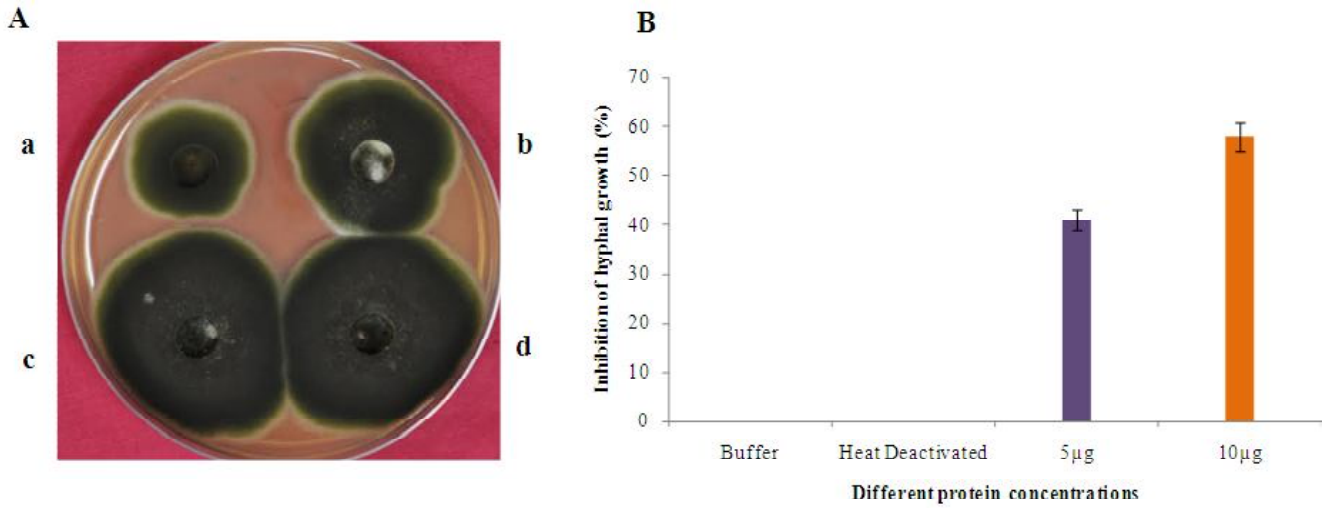

Fig 6: Purified chitinase protein inhibits the growth of *Alternaria alternata*
(**A**) Treatment of wells with control and different concentration of chitinase protein
Well a: 10µg of chitinase,
Well b: 5µg of chitinase,
Well c: Buffer as control (10 mM Tris buffer pH 7.5)
Well d: Heat deactivated chitinase (10 µg)
(**B**) Histogram showing percentage (%) inhibition of hyphal growth at different concentration of chitinase protein and controls (Buffer and heat deactivated protein)

glycosidic bonds forms a linear homopolymer. Chitinases extracted from several sources such as Bacteria (Kawase *et al.* 2006; Yasir *et al.* 2009), plants (Leah *et al.* 1991; Verburg and Huynh 1991; Ye and Ng 2005), fungi (Howell 2003) and Insect (Koga *et al.* 1997) have shown strong antifungal activity against different fungi.

In our study, we have demonstrated that a recombinant chitinase protein of size ~ 76 kDa, having both β-N-acetylglucosaminidase (Exochitinase activity) and endo chitinase activity, showed strong antifungal activity against fungi *Fusarium oxysporum f. sp lycopersici* and *Alternaria alternate*. Results obtained in our fungal

inhibition experiments corresponding to the chitinolytic activity we observed in purified recombinant chitinase protein. The strong antifungal activity possessed by the chitinase protein in our study could be because of the ability of showing both exo and endo chitinase activity, whereas, it was previously reported that the chitinases from bacterial origin, generally being a exochitinases have restricted accessibility to the chitin as a substrate, therefore not much effective against the hydrolyzing the fungal cell wall and inhibition (Roberts and Selitrennikoff 19987). Therefore, chitinases which has both endo and exochitinase activity of bacterial origin could be more effective against inhibiting fungal growth, like in our case, and could be a potent agent in complete hydrolyzing the chitinous structure, thus useful in controlling the fungal pathogen. Form our results, it could be concluded that the chitinas eprotein fromm X. *nematophilus* showed strong antifungal activity against *Fusarium oxysporum f.* sp *lycopersici* and *Alternaria alternate*. The chitinase protein could be expressed in transgenic plants as potent fungicidal candidates in near future for controlling fungal pathogens and can also be applied in integrated pest management practices.

References

Akhurst, R.J. and G.B. Dunphy (1993). Parasites and pathogens of insects (Beckage N, Thompson S, and Federici Beds) **2**: 1-23.

Asha, B.B., S. Chandra Nayaka, A.C. Udayashankar, C. Srinivas and S.R. Niranjana (2011). Biological control of *Fusarium* wilt of tomato. *Int. J. Microbiol Res.,* **3**:79–84

Burgess, L.W., T.E. Knight, L. Tesoriero and H.T. Phan (2008). Diagnostic manual for plant diseases in Vietnam, ACIAR, Canberra, 126-133,.

Dahiya, N., R. Tewari, R.P. Tiwari and G.S. Hoondal (2005). Production of an antifungal chitinase from Enterobactersp. NRG4 and its application in protoplast production. *World J. Microbiol Biotechnol,* **21**:1611-1616.

Goodyg, W. (1979). Biosynthesis of the fungal wall, mechanisms and implications. *In Fungal Wall and Hyphal Growth British Mycological Society Symposia,* **2**:203-223. Edited by J Burnett & A Trinci Cambridge : Cambridge University Press

Gummadi, S.N., D.S. Kumar, S.S. Dash and S.K. Sahu (2009). Industrially important carbohydrate degrading enzymes from yeasts: Pectinases, Chitinases and β-1,4-glucanases. In: Yeast Biotechnology: Diversity and Applications (eds. T. Satyanarayana and G. Kunze), Springer 673-691

Kapoor, I.J. (1988). Fungi involved in tomato wilt syndrome in Delhi, Maharashtra and Tamilnadu. *Indian Phytopathol.* **41**: 208–213.

Kawase, T., S. Yokokawa, A. Saito, T. Fujii, N. Nikaidou, K. Miyashita and T. Watanabe (2006). Comparison of enzymatic and antifungal properties between family 18 and 19 chitinase from S. coelicolor A3(2). *Biosci Biotechnol Biochem,* **70**: 988-998.

Kirankumar, R., K.S Jagadeesh, P.U. Krishnaraj and M.S. Patil (2008). Enhanced growth promotion of Tomato and nutrient uptake by plant growth promoting rhizobacterial isolates in presence of Tobacco Mosaic Virus pathogen. *J. Agric. Sci.,* **21**:309–311.

Latgé, J.P. (2007). The cell wall: a carbohydratearmour for the fungal cell. *Mol. Microbiol,* **66**:279–290

Leah, R., H. Tommerup, I. Svendsen and J. Mundy (1991). Biochemical and molecular characterization of three barley seed proteins with antifungal activity. *J. Biol. Chem.,* **66**: 1464-1573.

Pandey, K.K. and R.C. Gupta (2013). Virulence analysis of *Fusarium oxysporum* f. sp. lycopersicicausing tomato wilt in India. *J. Mycol. Plant Pathol,* **43**:409–413.

Roberts, W.K. and C.P. Selitrennikoff (1988). Plant and bacterial chitinases differ in antifungal activity. *Journal of General Microbiology,* **134**:169- 176.

Sabriye, Y., Y. Yusuf and K. Isa (2011). Evaluation of bacteria for biological control of early blight disease of tomato. *African Journal of Biotechnology,* **10(9)**: 1573-1577.

Sallam, M.A., Nashwa and A.M. Kamal (2012). Evaluation of various plants extracts against the early blight disease of tomato plants under greenhouse and field. *Plant Protection Science,* **48**:74-79.

Sally, A.M., C.R. Randal and M.R. Richard (2006). *Fusarium Verticillium* wilts of Tomato, Potato, Pepper and Egg plant. The Ohio State University Extension.

Stephen, A.F. and K.G. Andre (2003). *Fusarium* oxysporum. Department of Plant Pathology, CTAHR University of Hawaii at Manoa, 2A-FUOXY

Sudhamoy, M., M. Nitupama and M. Adinpunya (2009). Salicylic acid induced resistance to *Fusarium oxysporum* f. sp. lycopersici in tomato. *Plant Physiol Biochem,* **47**: 642–649

Thangavelu, R., A. Palaniswani and R. Velazhahan (2003). Mass production of Trichodermaharzianum for managing *Fusarium* wilt of banana. *Agricultural Ecosystem and Environment,* **103**: 259-263.

Verburg, J.G. and Q.K. Huynh (1991). Purification and characterisation of an antifungal chitinase from Arabidopsis thaliana. *Plant physio.,* **l95**:450-616.

Wang, S.L., W.J. Hsiao and W.T. Chang (2002). Purification and Characterization of an antimicrobial chitinas extracellularly produced by Monascuspurpureus CCRC31499 in a shrimp and crab shell powder medium. *J. Agric. Food Chem.,* **50**: 2249-2255.

Yasir, M., Z. Aslam, S.W. Kim, S.W. Lee, C.O. Jeon and Y.R. Chung (2009). Bacterial community composition and chitinase gene diversity of vermicompost with antifungal activity. *Bioresource Technology,* **100**:4396-4403.

Ye, X. and T.B. Ng (2005). A chitinase with antifungal activity from the Mung bean. *Protein Expr. Purif.,* **40**:230-236.