KAVAKA 52: 52-61 (2019)

Characteristics and Applications of a Thermostable and Acidic Exochitinase of the Thermophilic Mould *Myceliophthora thermophila*

Bharti Rohatgi and T. Satyanarayana*
Department of Microbiology, University of Delhi South campus, Benito Juarez Road, New Delhi-110021
**Division of Biological Sciences & Engineering, Netaji Subhas University of Technology, Sector 3, Dwarka, New Delhi- 110021, India*
Corresponding author Email : tsnarayana@gmail.com
(Submitted on March 05, 2019; Accepted on June 10, 2019)

ABSTRACT

The thermophilic mould *Myceliophthora thermophila* (Apinis) van Oorschot produces an extracellular thermo-acid-stable chitinase, which has been purified to apparent homogeneity by affinity adsorption followed by hydrophobic interaction chromatography. The pure enzyme is a monomer with a molecular mass of 43.0 kD on SDS-PAGE and pI of 4.0. Based on MALDI-ToF-MS/MS and LC-MS analysis of peptides, it was identified as glycosyl hydrolase family 18 protein. The chitinase is optimally active at pH 4.0 and 55 °C with $T_{1/2}$ values of 9 and 3 h at 55 and at 70°C, respectively. The K_m and V_{max} values (for colloidal chitin hydrolysis) are 0.396 mg ml^{-1} and 25.25 nkats mg^{-1} S^{-1}. It is strongly inhibited by Hg^{2+}, Al^{3+} and Fe^{3+}, but stimulated by Mn^{2+}, Ca^{2+} and Ba^{2+}. The chitinase is tolerant to organic solvents and detergents. It liberates N-acetylglucosamine and chitobiose from colloidal chitin degradation and has a potential to degrade powdered chitin and chitosan. The high temperature optimum and thermostability makes it a suitable candidate for the production of pharmacologically important N-acetylglucosamine and chitobiose from chitin. The cell walls of spores and hyphae are disrupted in *Fusarium oxysporum*, *Curvularia* sp. and *Stachybotrys* sp. due to the degradation of chitin in the cell walls. The enzyme arrests hatching of eggs in the nematode, *Meloidogyne incognita* and induces mortality in the insect larvae of *Aedes aegypti* as well as mealy bug (*Maconellicoccus hirsutus*).

Keywords: Exochitinase, *Myceliophthora thermophila*, colloidal chitin, N-acetylglucosamine, chitobiose

INTRODUCTION

Chitin is the most underexploited biomass resource available on the Earth. Several million tonnes of chitin residues are produced annually from sea food processing industries worldwide. Recently soluble derivatives of chitin such as N-acetylglucosamine (NAG) and chitobiose have attracted attention particularly in the field of medicine. NAG has therapeutic potential in osteoarthritis, inflammatory bowel disease and skin hyperpigmentation (Chen *et al.*, 2010). Chitooligosaccharides have been proposed as anti-microbial agents, enhancers of the immune response and anticancer agents. The monomers and oligomers are currently produced by acid hydrolysis of chitin using concentrated HCl, which is an inefficient process and also has economical, environmental and technical concerns (Sashiwa *et al*., 2003).

Enzymatic chitin hydrolysis is not only mild and environment-friendly but is also more efficient as the extent of hydrolysis and the consistency of the products can be controlled (Waghmare and Ghosh, 2010). The enzymatic hydrolysis has also been shown to produce N-acetylglucosamine in relatively higher yields than the acid hydrolysis (Pichyankura *et al*., 2002). The process, however, is slow and requires long reaction times, during which hydrolysis reactors are susceptible to contamination (Kuk *et al*., 2005). The use of thermostable enzymes at higher reaction temperatures can minimize contamination risks (Berka *et al.*, 2011). Additional advantages of hydrolysis at elevated temperatures include enhanced mass transfer, reduced substrate viscosity and the potential for enzyme recycling (Margaritis and Merchant, 1986; Unsworth *et al*., 2007).

Interest in chitinolytic enzymes in the field of biological control has arisen due to their involvement in antagonistic activity against pathogenic chitin-containing pests. These pathogenic organisms can be controlled by degrading vital structures (peritrophic membrane and cuticle of insects, fungal cell walls, and eggshells of nematodes) where chitin plays a critical role and can be considered a target for biocides (Kramer and Muthukrishnan, 1997; Patil *et al*., 2000). The absence of chitin in plants and vertebrate animals allows the consideration of safe and selective 'target' molecules for control of chitin-containing pathogens. Several microbial chitinases have shown antagonistic activities against plant pathogenic fungi, nematodes and insect larvae (Siddiqui and Mahmood, 1996; Kramer and Muthukrishnan, 1997; Patil *et al*., 2000). Chitinases can be added as a supplement to the commonly used fungicides and insecticides to make them more potent and to minimize the application of harmful chemical components present in fungicides and insecticides (Bhushan and Hoondal, 1998). Unlike chemical fungicides and bacterial agents, the enzyme-based formulations need repeated application as they would not be viable and active for long time (Neeraja *et al*., 2010). Thus attention is needed on the stability of active enzyme for extended times during storage, transport and field application (Kim and Je, 2010). Stability is also beneficial for biocatalysis as prolonged shelf life of the catalyst reduces process costs. Attempts have, therefore, been made to increase the thermal tolerance of enzymes from fungi (Kim *et al*., 2008; Kim and Je, 2010).

Thermophilic fungi have high potential for industrial applications as a source of thermostable enzymes (Johri *et al*., 1999; Singh *et al*., 2016). Biomass-degrading enzymes from thermophilic fungi consistently demonstrate higher hydrolytic capacity (Wojtczak *et al*., 1987). Thermophilic fungal strains are also capable of rapid growth with minimized viscosity at relatively elevated growth temperatures, thereby enhancing productivity in fermentors (Jensen and Boominathan, 1997). Although chitinases have been reported from a few thermophilic fungi, the application potential of these thermostable chitinases in efficient chitin

degradation remains to be explored (McCormack *et al*., 1991; Guo *et al*., 2005; Li *et al*., 2010; Kopparapu *et al*., 2012).

Myceliophthora thermophilia (Apnis) van Oorschot is a thermophilic mould that grows optimally at 45°C and has attracted attention because of its potential to produce thermostable enzymes. It is capable of producing some industrially important hydrolytic enzymes such as phytase, lipase, cellulase, xylanase and others (Satyanarayana *et al*., 1985; Singh and Satyanarayana *et al*., 2006). The recently sequenced genome of *M. thermophila* isolate ATCC42464 harbours a large number (>210) of glycosyl hydrolases, and thus, the fungus is being considered as an all-purpose decomposer (Berka *et al.*, 2011). Although a 45 kD endochitinase and a 70 kD exochitinase have been reported from this fungus based on ESI-MS/MS analysis of extracellular proteins (Visser *et al.*, 2011), a detailed investigation on biochemical characteristics and application potential of the chitinases has not yet been reported. In this investigation, a chitinase from *Myceliophthora thermophila* was purified, characterized and its applicability in generating chitooligosaccharides has been shown. The biocontrol potential of this thermostable chitinase is also being reported for the first time.

MATERIALS AND METHODS

Source of the thermophilic mould

Myceliophthora thermophila was isolated from a soil sample collected from Gujarat state of India on Emerson's YpSs agar (Emerson, 1941) supplemented with 0.5 % chitin at 45 °C. The mould has been maintained at 4 °C and as glycerol stock at -20 °C.

Molecular identification

The mycelial biomass was suspended in the extraction buffer (50 mM Tris-HCl, pH 8; 50 mM EDTA; 2% SDS; 1% Triton X 100) along with glass beads (diam. 0.5 mm) [1:1.6:0.6] and homogenized for 2 min. in a bead beater (20 sec pulses with intermittent cooling in ice). After centrifugation, genomic DNA extracted from the cell-free supernatant and purified according to Lee *et al*. (2000), has been used as a template for PCR amplification of ITS sequences.

For amplification of ITS sequence, universal primer pair ITS1 (TCCGTAGGTGAACCTGCGG) and 4 (TCCTCCG CTTATTGATATGC) was used. The resulting amplicon of 0.5 kbp was excised and purified with DNA extraction kit (Geneaid) and sequenced. The sequence was used for identifying the fungus with the help of the BLASTn program (www.ncbi.nlm.nih.gov/BLAST) and multiple-sequence alignments using Clustal W program.

Chitinase production

The mould was cultivated in 250 ml Erlenmeyer flasks containing Emerson's YpSs medium in which starch was substituted with colloidal chitin (0.5 %) and 0.1% lactose as inducers. The flasks were incubated at 45 °C and 250 rpm in an incubator shaker for 5 days, and the cultures were harvested by filtration and the cell- free filtrates were used in chitinase assays.

Chitinase assay and protein determination

The chitinase was assayed using colloidal chitin as the substrate prepared from commercial chitin according to Tanaka *et al*. (1999). The released reducing sugars were determined using dinitrosalicylic acid (DNSA) reagent (Miller, 1959) with N-acetylglucosamine (NAG) as the standard. One unit of chitinase is defined as the amount of enzyme that liberates 1 nmol of reducing sugar per second. The protein content in the enzyme samples was determined according to Lowry *et al.* (1951) using bovine serum albumin (sigma) as the standard.

Chitinase purification

The cell-free culture filtrate was concentrated by lyophilisation and re-suspended in 0.1 M acetate buffer (pH 4). The enzyme (114 nkats) was mixed with colloidal chitin (50 mg) and stirred on a magnetic stirrer at 4 °C for 4 hrs, and the colloidal chitin was then collected by centrifugation at 10,000 g for 10 min. and washed twice with ice-chilled deionised water. Desorption of chitinase was done with 0.1 M acetate buffer at 45 °C for 12 hours. Chitin was removed as pellet and the supernatant containing the enzyme was concentrated and further purified by hydrophobic interaction column (9x1.5 cm, bed volume 10 ml) chromatography using phenylsepharose matrix. The column was pre-equilibrated with 0.1 M acetate buffer (pH 4) followed by 1.0 M ammonium sulphate for binding the protein to a hydrophobic column. The proteins were eluted at 1 ml/min with a stepwise decreasing ammonium sulphate gradient from 1.0 to 0.0 M.

Determination of molecular weight and zymography

Sodium dodecylsulfatepolyacrylamide gel electrophoresis (SDSPAGE) was carried out according to Laemmli (1970) in 12 % polyacrylamide gel. The activity of the purified enzyme was demonstrated on native polyacrylamide gels. After electrophoresis, the separating gel was overlaid on the substrate gel containing 1 % colloidal chitin. Both gels were then incubated at 60 °C for 4 hours. Proteins were visualized after staining with 0.1% Coomassie brilliant blue R-250. Images of electrophoresis gels were recorded with GelDoc 2000 (Bio-Rad).

Determination of isoelectric point

Isoelectric focusing (IEF) was performed using BioRad Mini IEF Cell Model 111. The gel was cast with Bio- Lyte 3/10 ampholyte with pH operating range 3.7-9.3. The initial voltage was 100 V for 15 min followed by 200 V for 15 min. The voltage was finally increased to 450 V for 60 min. The focusing was carried out at 4 °C, and the gel was fixed in tricloroacetic acid and the bands were visualized after staining with Coomasie brilliant blue (0.04 %).

Peptide fingerprinting

The purified protein bands fractionated by SDS-PAGE were cleaved by trypsin, and the peptides were sent to The Centre for Genomic Application (TCGA), New Delhi for peptide mass spectrometric analysis by LC/MS (Agilent 1100 series 2D NanoLC MS). Mass spectrometry data were compared with data in the NCBI and Swiss Prot databases using the

Mascot search algorithm.

Characterization

Effect of pH and temperature for activity : The optimum temperature for the enzyme was determined by assaying the enzyme activity at different temperatures, while the heat stability was analyzed by measuring the residual activity after subjecting enzymes to 45 °C and 60 °C. The pH optimum for the enzyme activity was determined by conducting enzyme assay at different pH using different buffers [glycineHCl buffer (pH 3.05.0), NaH2PO4Na2HPO4 buffer (pH 6.08.0), borate buffer (pH 8.09.0), and glycine-NaOH buffer (pH 1012)] at 55 °C.

Kinetic studies : The effect of substrate concentration was studied by performing chitinase assay with different concentrations of colloidal chitin ranging between 0.1 % and 1.0 % and measuring the liberated reducing sugars by using DNSA. The kinetic constants K_m and V_{max} were determined graphically from the LineweaverBurk plot.

Effect of cations, inhibitors, solvents and detergents : The effect of various cations and modulators on the enzyme activity was assessed by incorporating them into the reaction mixtures at 1.0 and 5.0 mM. Similarly various solvents and detergents were incorporated into the reaction mixture at 10% concentration followed by chitinase activity.

Substrate specificity : The substrate specificity of the chitinase towards various natural substrates was measured by using crystalline powdery chitin, non-crystalline colloidal chitin and chitosan at 2 % concentration as substrates in the reaction mixtures. The activity against chromogenic derivative pNp-(Glc-NAc) was determined by incubating 50 µl of enzyme with 50 µl of 25 mM pNp-(Glc-NAc) in 100 mM acetate buffer (pH 4) at 55 °C for 30 min, and determining the released p-nitrophenol at 410 nm. One unit of enzyme activity is defined as the amount that liberates one µmole of p-nitrophenol per minute under the specified conditions.

Enzymatic hydrolysis of chitin : The effect of enzyme dosage on chitin hydrolysis was assessed by incorporating varied amounts of enzyme (20-140 U) in the reaction mixtures containing 100 mg swollen chitin. As the $T_{1/2}$ of chitinase at 55 °C was 9 hours, the initial enzyme concentration was replenished after 9 hours of incubation. The supernatants after centrifugation were analyzed using DNSA reagent. The yield of NAG was estimated from a calibration curve of NAG. The hydrolysis products in the supernatants were also analyzed by HPLC (Waters 600 E) using a carbohydrate column (carbohydrate column, 3.9 x 300 mm), with a mobile phase [acetonitrile : water (70:30)] at a flow rate of 1 ml/min using refractive index detector.

Determination of the biocontrol potential of the chitinase Activity against pathogenic fungi : Antifungal activity of the purified chitinase was estimated by a hyphal extension-inhibition assay as described by Kopparapu *et al*. (2012). The selected fungi were cultured in 100 x 15 mm Petri dishes containing 10 ml of potato dextrose agar. The plates were incubated at 30 °C until fungal growth was observed. Purified chitinase (35 U) was spotted on sterile paper discs (6 mm in diameter), at a distance of 0.5 cm away from the rim of the fungal colony. The plates were incubated at 30 °C for 72 h until mycelial growth envelopes disks and forms zones of inhibition around disks impregnated with chitinase.

Biocontrol against nematode eggs : Approximately equal sized egg masses of *Meloidogyne incognita* were taken in a microtitre plate (one egg mass per well) and 500 µl of purified enzyme (17.5 U) was dispensed into a well. Water and buffer (0.4 M acetate buffer, pH 4.0) were taken as control. The plate was incubated at 28 °C for 12 days and observed under microscope (16 X) after 3, 6, 9 and 12 days for hatching of eggs and appearance of juveniles. Juveniles in each well were counted.

Biocontrol against insects : The biocontrol efficacy of the enzyme samples against 3rd instar larvae of *Aedes aegypti* and grape mealy bugs was determined at National Chemical Laboratory, Pune by Dr. M.V. Deshpande and his coworkers. Five larvae of *Aedes aegypti* were kept in 1:5 dilution of enzyme in tap water and observed for 5 days. Corrected mortality was calculated by Abbott's formula (Abbott, 1925):

$$\% \text{ Mortality} = (\% \text{ Kill in treated} - \% \text{ kill in control}) / (100 - \% \text{ Kill in control}) \times 100$$

For bioassay with *Maconellicoccus hirsutus*, 20 mealy bugs after treatment with lyophilized chitinase, were transferred on sprouted potato and observed for 7 days. Mortality was calculated as above.

RESULTS

The amplification of genomic DNA using the primer pair ITS1 and ITS4 resulted in the fragments of approximately 500 kbp. This fragment consisting of the complete ITS region, including ITS1, 5.8S rRNA and ITS2, was sequenced (Genbank Accession no. KT 287076). Based on the phylogenetic analysis using BLAST, the thermophilic fungal strain was identified as *Myceliophthora thermophila*.

The chitinolytic enzyme was detected in the culture supernatant when *M. thermophila* was grown in the medium containing colloidal chitin as the substrate and N-acetylglucosamine as the inducer. More than 90 % of the activity was adsorbed on colloidal chitin. A summary of purification of chitinase is presented in **Table 1**. The purified protein was homogenous on SDS PAGE with molecular mass of 43.0 kD (**Fig. 1**). The comparison of results of native and SDS PAGE revealed that the chitinase is a monomer. Zymogram of chitinase showed a single zone of digested colloidal chitin (**Fig. 1**). The Iso-Electric-Focusing (IEF) gel revealed that it has isoelectric point of 4.0 (**Fig. 1**).

The purified protein was identified by MALDI-ToF (MS/MS) analysis by comparing the peptide fragments liberated by

Table 1. Purification of chitinase from *M. thermophila*

Purification steps	Total activity (nkats)	Total protein (mg)	Sp. activity (nkats/mg)	Yield (%)	Purification fold
Crude	113.89	62.9	1.81	100	1
Affinity adsorption	66.86	3.6	18.57	58.7	10.25
Hydrophobic interaction chromatography	16.5	0.21	78.57	14.54	43.4

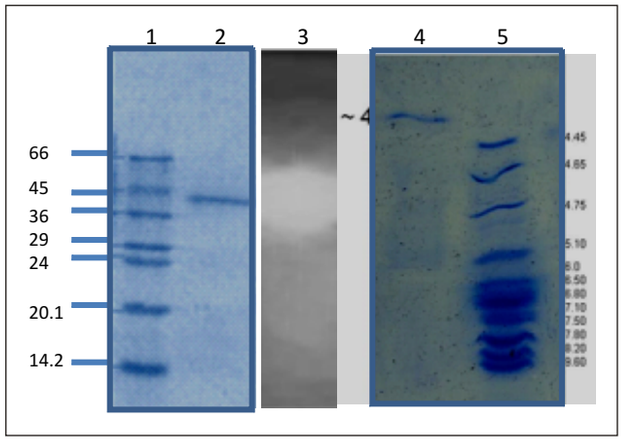

Fig. 1. Detection of molecular weight by SDS-PAGE and isoelectric focusing of the purified chitinase from *M. thermophila*. Lane 1 contains molecular mass markers, lane 2 contains the purified chitinase, lane 3 shows the zymogram, lane 4 shows the purified protein and lane 5 contains pI markers

trypsin digestion with the calculated masses of expected peptides. The spectra matched 11 tryptic peptides that could be correlated with a glycosyl hydrolase family 18 protein from *Myceliophthora thermophila* ATCC 42464 (GenBank Accession No. AE O58299) with 42 % sequence coverage and ion score of 78 in mascot search (**Table 2**). LC-MS analysis of the tryptic peptides also revealed identity of the purified chitinase to glycosyl hydrolase family 18 protein from *Myceliophthora thermophila* ATCC 42464 with 19 % sequence coverage and a score of 565.

Table 2. Peptides identified in the purified chitinase* of *M. Thermophila*

Peptide position	Mass of peptide (m/z)	Amino acid sequence of the matched peptides
38–56	2253.1027	R.QSSGYKNIVYFTNWGIYGR.N
44–56	1602.8098	K.NIVYFTNWGIYGR.N
57–79	2675.4187	R.NYQPDQLPASQLTHVLYSFANIR.S
128–151	2458.2830	K.TLLSIGGWTYSATFPAAASTAESR.A
275–298#	2476.3830	K.ALSDYVAAGVDPAKIVLGMPIYGR.S
289–298	1118.6019	K.IVLGMPIYGR.S
299–330	3470.8011	R.SFEATDGLGKPFTGVGQGSWESGVWDYKVLPR.A
331–352	2336.0971	R.AGATVQYDEEAGATYSYDPATR.E
331–364#	3713.8269	R.AGATVQYDEEAGATYSYDPATRE LISFDTVDMVK.K
374–387	1531.7051	K.GFAGSMFWEASADR.T

***** Calculated mass, 46.181 and calculated pI, 5.11

Peptides identified by LGMS/MS

LSIGGWT: Consensus sequence motif known to play a role in substrate binding

The temperature and pH optima for the activity of chitinase are 55 °C and 4.0, respectively (**Fig. 2**). The chitinase retained 100% activity when exposed to 45 °C for 12 hours. The $T_{1/2}$ values of the chitinase are 9 h at 60 °C and 3 h at 70 °C. The apparent K_m and V_{max} values of the pure chitinase (colloidal chitin) are 0.396 mg ml^{-1} and 25.25 nkats mg^{-1} s^{-1}, respectively.

The cations Mn^{2+}, Ca^{2+} and Ba^{2+} stimulated chitinase activity, while Hg^{2+}, Al^{3+} and Fe^{3+} strongly inhibited chitinase at 5mM concentration (**Table 3**). EDTA has no effect on chitinase, while β-mercaptoethanol, PMSF, Woodward's reagent K and N-bromosuccinamide inhibited chitinase activity to a varied extent (**Table 3**). In the presence of methanol, hexane, ethanol, butanol, acetone, toluene and isoamyl alcohol, the enzyme retained 63-85 % activity (**Table 3**). The chitinase is resistant to detergents and displayed 76 % residual activity in the presence of 10 % SDS (**Table 3**).

Table 3. Effect of various modulators on chitinase activity

Effect	Relative enzyme activity (%)	
Control	100	
Cations	**1mM**	**5mM**
Cu^{2+}	98.05	84.76
Fe^{2+}	106.20	90
Zn^{2+}	98.74	113.26
Al^{2+}	80.11	-
Hg^{2+}	63.45	-
Mg^{2+}	110.32	103.61
Mn^{2+}	119.59	**166.66**
Ba^{2+}	109.55	**138.37**
Co^{2+}	101.1	102.56
Ca^{2+}	113.24	**135.43**
Na^{+}	104.34	102.26
Fe^{3+}	87.97	-
Inhibitors		
Mercaptoethanol	31.28	9.83
Dithiothretol	63.91	41.64
Iodo acetic acid	96.38	54.88
N-Ethylmaleimide	86.59	65.86
N-Bromosuccinimide	84.91	27.43
Woodwards reagent K	86.32	52.11
EDTA	101.12	98.93
PMSF	59.15	50.15
Solvents	**(10%)**	
Methanol	76	
Hexane	85	
Ethanol	75	
Butanol	65	
Acetone	63	
Toulene	97	
Isoamyl alcohol	73.8	
Detergents	**(10%)**	**(20%)**
Tween 80	108.42	87.37
Tween 40	100.86	97.51
Tween 20	119.01	86.33
Triton-X-100	120.14	87.51
SDS	76.45	50.8

The purified *M. thermophila* chitinase displays maximum activity on colloidal chitin. The relative activity of the chitinase on colloidal chitin was 2.08-fold and 1.88-fold higher than on the powered (crystalline) chitin and deacetylated chitosan (**Table 4**). The activity towards synthetic substrate p-nitrophenyl N-acetyl-β-d-glucosaminide (PNP-GlcNAc) substrate was also detected inferring an associated chitobiase activity with the chitinase (**Table 4**).

The hydrolysis was maximum at enzyme concentration of 80 U /100 mg chitin. Increasing the enzyme dosage further had very little observable effect on the yield of reducing sugars. The hydrolysis products could be recovered by removing insoluble substrate by centrifugation, and the supernatant could be lyophilized for obtaining NAG and chitobiose in powder form.

The end products of colloidal chitin hydrolysis are N-acetylglucosamine and chitobiose, suggesting an exo-type

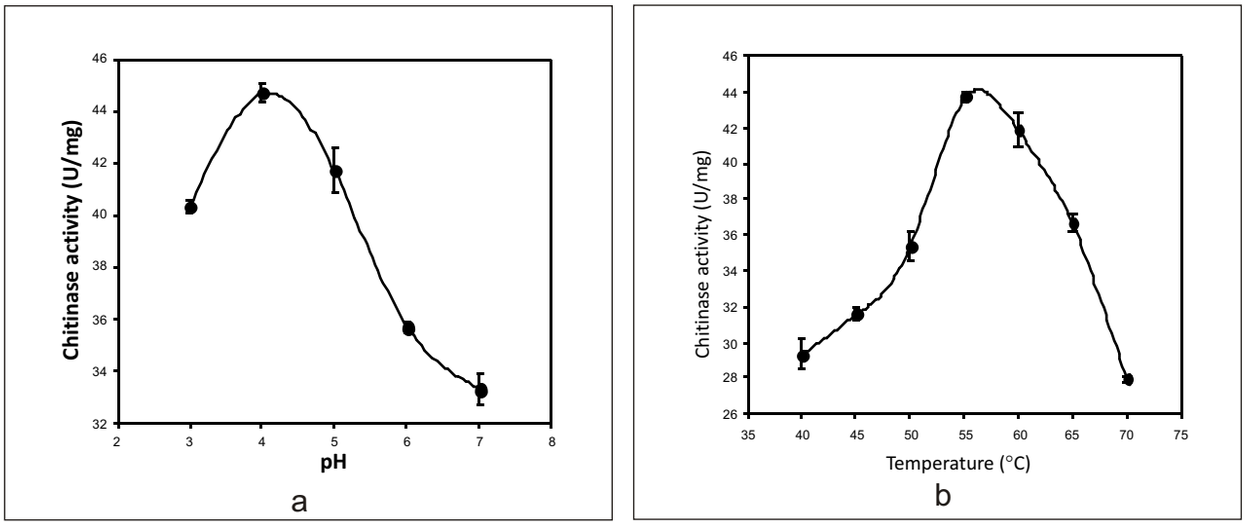

Fig. 2. Effect of (a) pH and (b) temperature on chitinase activity

Table 4. Substrate specificity of *M. thermophila* chitinase

Substrate	Specific activity (U/mg)
Colloidal chitin	9.6
Powdery chitin	4.6
Chitosan	5.1
pNp-GlcNAc[a]	7.2

Table 5. HPLC analysis of the end products liberated by the action of exochitinase of *M. thermophila* on colloidal chitin

Time (h)	NAG(%)	Chitobiose(%)
3	14	86
6	38	62
12	43	57

action of *M. thermophila* chitinase (**Table 5**). A high yield of chitobiose was attained after 3 h of the reaction and it declined with the concomitant increase in NAG. The hydrolysate consisted of 43 % NAG and 57 % chitobiose after 12 h of

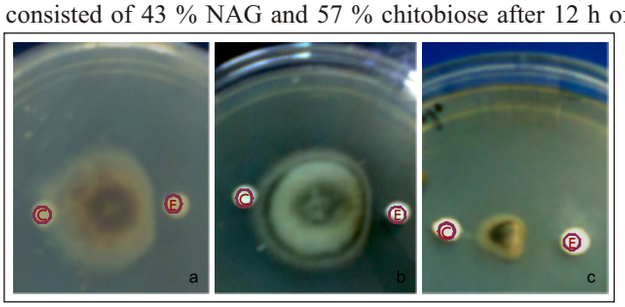

Fig. 3. Inhibition zones of chitinase against (a) *Fusarium oxysporum* (b) *Curvuleria* sp. and *Stachybotrys* sp. (c)

hydrolysis (**Table 5**).

The chitinase inhibited hyphal growth of *Fusarium oxysporum*, *Curvularia* sp. and *Stachybotrys* sp. as depicted by inhibition zones around disc impregnated with 35 U of pure chitinase (**Fig. 3**). The lysis of hyphal walls and resultant appearance of pores was evident after chitinase treatment of *Fusarium oxysporum* and *Stachybotrys* sp. mycelium. The spores of *Curvuleria* sp. appeared distorted under scanning electron microscopy (**Fig. 4**).

Both crude culture filtrate and purified chitinase effectively inhibited hatching of *Meloidogyne incognita* eggs. About 400 juveniles hatched from one egg mass in each well that served as control. However, when an egg mass was treated with chitinase, there was a complete inhibition of hatching of eggs

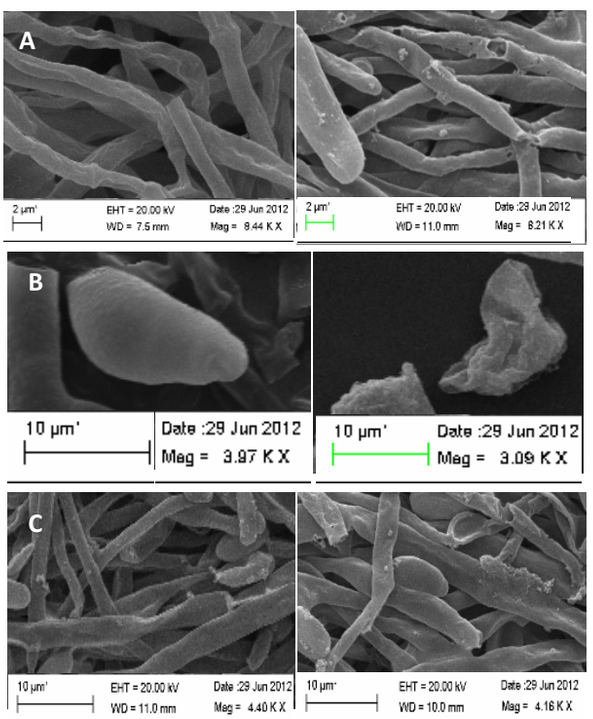

Fig. 4. Scanning electron micrographs showing control (left) and chitinase treated (right) mycelium of *Fusarium oxysporum* (A); spores of *Curvularia* sp. (B) and (C) mycelium of *Stachybotrys* sp.

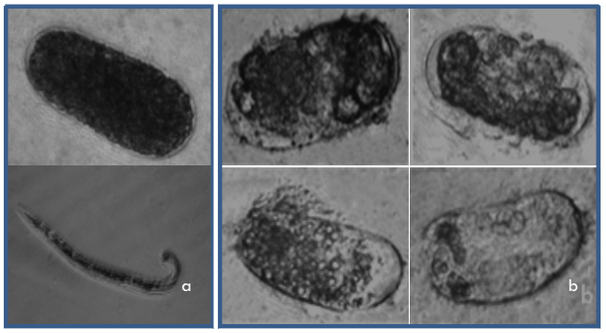

Fig. 5. Chitinase treatment of nematode eggs (a) control showing normal eggs and hatched juveniles and (b) Unhatched eggs showing clearance of egg cytoplasm after enzyme treatment.

(**Table 6**). The microscopic observation of eggs revealed appearance of vacuoles, partial degradation of egg shell and clearance of egg cytoplasm (**Fig. 5**).

It was observed that the chitinase from *M. thermophila* has larvicidal potential against the 3rd instar larvae of *Aedes aegypti*. The pure chitinase as well as crude culture filtrate of *M. thermophila* exerted 50 and 75% mortality of the larvae, respectively (**Table 7**). The crude enzyme extract of *M. thermophila* is also active against grape mealy bugs (*Maconellicoccus hirsutus*). The concentrated extract with 1 U chitinase caused 50% mortality in 7 days (**Table 8**).

DISCUSSION

The thermophilic fungus isolated in this investigation was identified as *Myceliophthora thermophila* based on morphology. The ITS sequence showed high similarity (99 %) to those of *Myceliophthora* species. It produces a chitinase extracellularly when grown in the presence of chitin as in *Thermomyces lanuginosus* (Guo *et al*., 2005) and *Talaromyces emersonii* (Hendy *et al*., 1990). The soluble sugars like glucosamine, N-acetylglucosamine and lactose induced chitinase in *M. thermophila* as reported by Tiunova *et al*. (1983) and Ulhoa and Peberdy (1991) in *Trichoderma viride* and *T. harzianum,* respectively.

Although *M. thermophila* has been reported to produce chitinase based on molecular data, this is the first report on purification and characterization of a chitinase from the fungus. The purified chitinase appeared as a single homogenous band on SDS PAGE with an apparent molecular mass of 43 kD. Usually, fungal endochitinases are monomeric proteins (Kopparapu *et al.*, 2012). The molecular mass for most of the fungal chitinases has been reported to vary from 27 to 190 kD. The reported molecular masses of thermophilic fungal chitinases, however, fall in the narrow range of 43-48 kD (Guo *et al*., 2005; Li *et al*., 2010; Kopparapu *et al*., 2012). The molecular mass of the chitinase is comparable to those reported for other thermophilic and mesophilic fungi: 43.7 kD from *Paecilomyces thermophila*, 43 kD from nematophagous fungi, *Verticillium chlamydosporium* and *V. suchlasporium* (Tikhonov *et al*. 2002), and 43-45 kD from *Metarrhizium anisopliae* (Leger *et al*., 1996). The acidic pI of the chitinase compares well with that of *Metarrhizium anisopliae* (pI 4.8) [Leger *et al*. 1996]. *Talaromyces emersonii* produced four chitinases with pI values in the range of 3.6 - 4.6 (Hendy *et al*., 1990).

Table 6. Hatching of *Meloidogyne incognita* eggs in the presence of chitinase

Days of Incubation	Number of juveniles		
	Control	Buffer	Enzyme
3	66±5	71±12	5±2
6	161±8	168±14	10±1
9	346±16	329±10	11±2
12	405±22	408±15	10±2

Table 7. Bioassay with 3rd instar larvae of *Aedes aegypti*

Treatment	Chitinase (U/ml)	Protein (mg ml^{-1})	Larval mortality (%)
Crude	0.87	1.11	75
Purified	0.7	0.393	50

Table 8. Bioassay with *Maconellicoccus hirsutus*

Treatment	Chitinase (U)	Lipase (U)	Protein (mg ml^{-1})	Mortality (%)
Concentrated enzyme extract	0.2	1	5	35
Concentrated enzyme extract	1.07	5	25	50

Generally fungal chitinases belong to the family 18 of glycosyl hydrolases (Seidl, 2005). The chitinase was identified by MALDI-ToF MS/MS and LC-MS as a glycosyl hydrolase family 18 protein from *M. thermophila* ATCC 42464. Furthermore, the calculated values for molecular mass and pI of *M. thermophila* ATCC 42464 protein are close to the experimental values being reported in this investigation. Chitinases from thermophilic fungi are composed of a single catalytic domain that is similar to other fungal chitinases (Li *et al*., 2010). Alignment of amino acid sequences of the chitinases from thermophilic fungi, *Thermoascus aurantiacus* var. *levisporus* and *Chaetomium thermophilum, Paecilomyces thermophila* and the most similar other fungal chitinases revealed that these chitinases share the LSIGGWT and DXXDXDXE motifs that are considered to be substrate-binding site and a chitin-catalyzing domain, respectively (Watanabe *et al*., 1993; Hollis *et al.*, 2000; Li *et al*., 2010). One of these two sequence motifs (LSIGGWT) is also present in the identified peptides from the chitinase of *M. thermophila*.

Fungal chitinases are active in the pH range of 4.0-7.0. The pH optimum for the activity of chitinase of *M. thermophila* is 4.0, which is very close to those of *Thermomyces lanuginosus* (4.5) [Guo *et al.*, 2005] and other thermophilic fungal chitinases (McCormack *et al.*, 1991; Li *et al*., 2010]. The acidic pH optimum will be advantageous for industrial application as chitin extraction from the shell waste involves demineralization with dilute acid. The optimum temperature for the activity of *M. thermophila* chitinase is 55 °C, which is similar to the chitinase of *Thermomyces lanuginosus* (Guo *et al*., 2005) and *Bacillus licheniformis* strain JS (Waghmare and Ghosh, 2010). The optimum temperatures for the chitinases of *Thermoascus aurantiacus* and *Chetomium thermophilum* are 50 and 60 °C, respectively (Li *et al*., 2010).

The K_m value of 0.39 mg ml^{-1} colloidal chitin is comparable with the earlier reports (Kudan and Pichyangkura, 2009; Watanabe *et al*., 2003). Low K_m values indicate high affinity of the enzyme to the substrate, which makes the enzyme significant for industrial use as the substrate to product conversion rate is high for enzymes with low K_m values (Ahmed *et al*., 2007).

High stability of chitinase is generally considered an economic advantage in industrial processes because of the reduced enzyme turnover. Most fungal chitinases have an optimum activity at 20-40 °C and are not stable at high temperatures (Li, 2006). Some mesophilic fungal chitinases are reported to have high temperature optimum but are not stable for long at their temperature optima (Binod *et al*., 2005). However, chitinases from thermophilic fungi have both high optimum temperature for activity and high thermostability (Guo *et al*., 2005; McCormack *et al*., 1991; Li *et al*., 2010). A chitinase of *Talaromyces emersonii* has maximum activity at 65 °C with a half-life of only 20 min at 70 °C (McCormack *et al*., 1991). In this investigation, the chitinase retained 100 % activity when exposed to 45 °C for 12 hours. The $T_{1/2}$ values of chitinase are 9.0 h at 60 °C and 3 h at 70 °C. This chitinase has higher thermostability than other chitinases reported from thermophilic fungi. The chitinase from *Thermomyces lanuginosus* SY-2 has a $T_{1/2}$ of only 25 min at 65 °C and less than 10 min at 70 °C (Run-fang *et al*., 2008). Chitinases from *Thermoascus aurantiacus* var. *levisporus* and *C. thermophilum* have half life values of 10 min and 50 min at 70 °C, respectively (Li *et al*., 2010). The higher thermostability of the chitinase from *M. thermophila* makes it a potent candidate for industrial chitin hydrolysis.

The cations Mn^{2+}, Ca^{2+} and Ba^{2+} stimulate chitinase activity, while Hg^{2+}, Al^{3+} and Fe^{3+} strongly inhibit chitinase. The chitinases from *Ralstonia* sp. A471 (Sutrisno *et al*., 2004) and *Bacillus* MH-1 (Sakai *et al*., 1998) are also activated by Mn^{2+} and Ca^{2+}, and the chitinase from bacterium C4 was activated by Mn^{2+} (Yong *et al*., 2005). EDTA did not inhibit the chitinase significantly, indicating that metal ions are not required for the activity. *M. thermophila* chitinase is inhibited by a carboxyl group modifier Woodward's reagent K as in *Streptomyces thermoviolaceus* OPC-520, suggesting that the carboxyl group of aspartate and/or glutamate probably plays a role in catalysis (Tsujibo *et al*., 2000). The chitinase is susceptible to N-bromosuccinamide as in the chitinases of *Streptomyces thermoviolaceus* OPC-520, *Pseudomonas* sp. YHS A-2 and *Enterobacter* sp. NRG4 indicating the importance of tryptophan residue(s) for substrate binding or catalysis (Lee *et al*., 2000). The chitinase displays tolerance to organic solvents such as toluene, hexane, methanol and others. High levels of thermal stability are correlated positively with the stability in the presence of organic solvents (Cowan, 1997).

Substrate specificity studies of different chitinases have shown that colloidal chitin is the best substrate among different forms of crystalline and amorphous chitin or chitosan (Waghmare and Ghosh, 2010; Dai *et al*., 2011). Two recombinant chitinases from *Thermoascus aurantiacus* var. *levisporus* and *Chaetomium thermophilum* showed higher activities on colloidal chitin than powered chitin and chitosan (Li *et al*., 2010) as in *M. thermophila*. Activity on synthetic substrate p-nitrophenyl N-acetyl-β-d-glucosaminide (PNP-GlcNAc) was detected inferring that a chitobiase activity is associated with the chitinase of *M. thermophila* as in *Metarhizium anisopliae* (Kang *et al*., 1999) and *Streptomyces* sp. NK1057 (Nawani and Kapadnis, 2004).

Hydrolysis of colloidal chitin with *M. thermophila* chitinase resulted in the liberation of NAG and chitobiose like that of *B. licheniformis* SK-1 (Pichyankura *et al*., 2002). *M. thermophila* chitinase produces chitobiose as the major hydrolysis product initially with small amounts of NAG. Chitobiose was the major end product of chitin hydrolysis by the chitinase of *Pyrococcus kodakarensis* KOD1 (Tanaka *et al*., 1999) and *B. licheniformis* (Waghmare and Ghosh, 2010). In the hydrosylate of *M. thermophila* chitinase, the proportion of NAG increased and that of chitobiose decreased with time. A similar observation was made with the exochitinase Chi71A from *Bacillus thuringiensis* subsp. *pakistani* (Thamthiankul *et al*., 2001). The chitin oligosaccharides are known to have biological functions such as antitumor activity and elicitor action (Kuk *et al*., 2005). Also chitobiose is important among chitooligosaccharides as it has been reported that chitobiose and chitotriose are appreciably absorbed from the gastrointestinal tract when given orally, while higher oligomers are not (Chen *et al*., 2005).

It is well established that chitinases are important components of plant pathogenesis related (PR) proteins and the disease resistance is related to their capacity to degrade cell wall of chitin containing pests such as fungi. The chitinase of *M. thermophila* inhibits the growth of *Fusarium oxysporum*, *Curvularia* sp. and *Stachybotrys* sp. Scanning electron microscopy revealed peeling off of the spore walls and pores in the mycelium. Chitinase was identified as the antifungal agent in the cell free culture filtrate of fluorescent pseudomonads that inhibited mycelial growth of *Fusarium oxysporum* f. sp. *dianthi*, the causative agent of vascular wilt of carnation (Ajit *et al*., 2006). Chitinases from *Trichoderma aureoviride* DY-59 and *Rhizopus microsporus* VS-9 inhibited microconidial germination in *Fusarium solani* (Nguyen *et al*., 2008).

Apart from fungi, nematode egg shell is composed of chitin. The egg is the most resistant stage in the nematode life-cycle and is a threat for agricultural crops as they remain in soil for long periods (Gortari and Hours, 2008). The chitinase of *M. thermophila* effectively inhibits hatching of *Meloidogyne incognita* eggs. The microscopic observation of eggs revealed clearance of egg cytoplasm. The developing eggs and juveniles of *Globodera pallida* (potato cyst nematode) become vacuolated and transparent suggesting hydrolysis of the egg and juvenile contents following treatment with chitinase and protease (Tikhonov, 2002). Incubation of *Meloidogyne incognita* eggs in the presence of chitinase from *Lecanicillium psalliotae* significantly inhibited egg hatching in *in vitro*. Approximately 38.2 % of eggs did not develop or hatch when treated with chitinase, and chitinase and protease, thus, hatching rate was reduced by 56.5 % (Gan *et al*., 2007).

The chitinase of *M. thermophila* has larvicidal potential against the 3rd instar larvae of *Aedes aegypti*. The purified chitinase and crude culture filtrate of *M. thermophila* caused 50 and 75 % mortality of the larvae, respectively. The higher mortality observed with crude preparation can be attributed to the contribution of other enzymes (protease and lipase) of the culture filtrate as reported by Mendonsa (1996).

The chitinase of *M. thermophila* is also active against grape mealy bugs (*Maconellicoccus hirsutus).* The chitinase induced mortality after 7 days. Katke and Balikai (2008) reported that *Verticillium lecanii* and *Metarhizium anisopliae* were effective in managing grape mealy bug. A chitinase from *Trichoderma harzianum* has been shown to negatively affect growth and morphogenesis of *Helicoverpa armigera*, when used in feed or topically applied (Binod *et al*., 2007).

The exochitinase of *M. thermophila* is a promising biocatalyst for the production of NAG and chitobiose from chitin residues, therefore, the gene that encodes this chitinase was cloned and heterologously expressed in *Pichia pastoris* (Dua *et al*., 2017). The high temperature optimum and the thermostability of chitinase are added advantages for its application in the recycling of chitinous residues from food industries at elevated temperatures, thereby increasing hydrolytic rates and reducing contamination risks. This chitinase has also a broad spectrum biocontrol potential against fungi, nematodes and insects.

ACKNOWLEDGEMENTS

One of us (BR) is grateful to Indian council of Medical Research, Govt. of India for the award of Junior and Senior Research Fellowship during the course of this investigation. Thanks are also due to Dr. M.V. Deshpande (National Chemical Laboratory, Pune) and Dr. Uma Rao (Indian Agriculture Research Institute, New Delhi) for their help in assessing biocontrol potential of chitinase against insect larvae and nematodes, respectively.

REFERENCES

Abbott, W.S. 1925. A method of computing the effectiveness of insecticide. *J. Econ. Ent.* **182**: 265-267.

Ahmed, S., Jabeen, A. and Jamil, A. 2007. Xylanase from *Trichoderma harzianum*: Enzyme characterization and gene isolation. *J. Chem. Soc. Pak.* **29**: 176-182.

Ajit, N.S., Verma, R. and Shanmugam, V. 2006. Extracellular chitinases of fluorescent pseudomonads antifungal to *Fusarium oxysporum* f. sp. *dianthi* causing carnation wilt. *Curr. Microbiol*. **52**:310-316.

Berka, R.M., Grigoriev, I.V., Otillar, R. *et al.,* 2011 Comparative genomic analysis of the thermophilic biomass-degrading fungi *Myceliophthora thermophila* and *Thielavia terrestris*. *Nat. Biotechnol*. **29**:922-929.

Bhushan, B. and Hoondal, G.S. 1998. Isolation, purification and properties of a thermostable chitinase from an alkalophilic *Bacillus* sp. BG-11. *Biotechnol. Lett.* **20**:157-159.

Binod, P., Pusztahelyi, T., Nagy, V., Sandhya, C., Szakacs, G., Istvan, P. and Pandey, A. 2005. Production and purification of extracellular chitinases from *Penicillium aculeatum* NRRL 2129 under solid-state fermentation. *Enz. Microb. Technol*. **36**: 880-887.

Binod, P., Sukumaran, R.K., Shirke, S.V., Rajput, J.C. and Pandey, A. 2007. Evaluation of fungal culture filtrate containing chitinase as a biocontrol agent against *Helicoverpa armigera. J. Appl. Microbiol.* **103**:1845-1852.

Chen, A., Taguch, T., Okamoto, H., Danjo, K., Sakai K., Matahira, Y., Wang, M.W. and Miwa, I. 2005. Pharmacokinetics of chitobiose and chitotriose administered intravenously or orally to rats. *Biol. Pharm. Bull*. **28**:545-548.

Chen, J.K., Shen, C.R. and Liu, C.L. 2010. *N*-Acetylglucosamine: Production and Applications. *Mar. Drugs* **8**:2493-2516.

Cowan, D.A. 1997. Thermophilic proteins: stability and function in aqueous and organic solvents. *Comp. Biochem. Physiol*. **118**: 429-438.

Dai, D., Hu, W., Huang G. and Li, W. 2011. Purification and characterization of a novel extracellular chitinase from thermophilic *Bacillus* sp. Hu1. *Afr. J. Biotechnol*. **1013**: 2476-2485.

Dua, A., Joshi, S. and Satyanarayana, T. 2017. Recombinant exochitinase of the thermophilic mould *Myceliopthora thermophila* BJA: Characteristics and utility in generating N-acetyl glucosamine and in biocontrol of phytopathogenic fungi. *Biotechnol. Progr.* **33:** 70-80.

Emerson, R. 1941. An experimental study of life cycle and taxonomy of *Allomyces*. *Lloydia* **4**: 77-144.

Gan, Z., Yang, J., Tao, N., Liang, L., Mi, Q., Li, J. and Zhang, K.Q. 2007. Cloning of the gene *Lecanicillium psalliotae* chitinase Lpchi1 and identification of its potential role in the biocontrol of root-knot nematode *Meloidogyne incognita. Appl. Microbiol. Biotechnol.* **76**:1309-1317.

Gortari, M.C. and Hours, R.A. 2008. Fungal chitinases and their biological role in the antagonism onto nematode eggs: A review. *Mycol. Prog*. **7**:221-238.

Guo, R.F., Li, D.C. and Wang, R. 2005. Purification and properties of a thermostable chitinase from thermophilic fungus *Thermomyces lanuginosus*. *Acta Microbiologica Sinica* **45**: 270-274 in Chinese.

Hendy, L., Gallagher, J., Winters, A., Hackett, T.J., McHale, L. and McHale, A.P. 1990. Production of an extracellular chitinolytic system by *Talaromyces emersonii* CBS 814.70. *Biotechnol Lett*. **12**: 673-678.

Hollis, T., Monzingo, A.F., Bortone, K., Ernst, S., Cox, R. and Robertus, J.D. 2000. The X-ray structure of a

chitinase from the pathogenic fungus *Coccidioides immitis*. *Prot. Sci.* **9**:544-551.

Jensen, E.B. and Boominathan, K.C. 1997. Thermophilic fungal expression system. US Patent 5695985.

Johri, B.N., Satyanarayana, T. and Olsen, J. 1999. *Thermophilic Moulds in Biotechnology.* Kluwer Acad. Publ. Netherlands, pp. 354.

Kang, S.C., Park, S. and Lee, D.G. 1999. Purification and characterization of a novel chitinase from the entomopathogenic fungus, *Metarhizium anisopliae*. *J. Invertebr. Pathol*. **73**: 276-281.

Katke, M. and Balikai, R.A. 2008. Management of grape mealy bug, *Maconellicoccus hirsutus* green. *Ind. J. Entomol*. **70**: 232-236.

Kim, J.S. and Je, Y.H. 2010. A novel biopesticide production: attagel-mediated precipitation of chitinase from *Beauveria bassiana* SFB-205 supernatant for thermotolerance. *Appl. Microbiol. Biotechnol.* **87**:1639-1648.

Kim, J.S., Lee, H.Y., Chung, B.J. and Je, Y.H. 2008. Method for enhancing the thermal tolerance of entomopathogenic fungal spores, blastospores and enzymes. WIPO Patent WO/2008/063011.

Kopparapu, N.K. Zhou, P. Zhang, S., Yan, Q., Liu, Z. and Jiang, Z. 2012. Purification and characterization of a novel chitinase gene from *Paecilomyces thermophila* expressed in *Escherichia coli*. *Carbohydr. Res*. **347**: 155-160.

Kramer, K.J. and Muthukrishnan, M. 1997. Insect Chitinases: Molecular Biology and Potential Use as Biopesticides. *Insect Biochem. Mol. Biol*. **27**: 887-900.

Kudan, S. and Pichyangkura, R. 2009. Purification and characterization of thermostable chitinase from *Bacillus licheniformis* SK-1. *Appl. Biochem. Biotechnol.* **157**: 23-35.

Kuk, J.H., Jung, W.J., Jo, G.H., Kim, Y.C., Kim, K.Y. and Park, R.D. 2005. Production of N-acetyl-β-D-glucosamine from chitin by *Aeromonas* sp. GJ-18 crude enzyme. *Appl. Microbiol. Biotechnol.* **68**: 384-389.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* **227**: 680-685.

Lee, Y.M., Choi, Y.K., Min, B.R. 2000. PCR-RFLP and sequence analysis of the rDNA ITS region in the *Fusarium* spp. *J. Microbiol*. **38**: 66-73.

Leger, R.J., Joshi, L., Bidochka, M.J., Rizzo, N.W. and Roberts, D.W. 1996. Characterization and ultrastructural localization of chitinases from *Metarhizium anisopliae*, *M. flavoviride*, and *Beauveria bassiana* during fungal invasion of host *Manduca sexta*. cuticle. *Appl. Environ. Microbiol.* **62**: 907-912.

Li, A.N., Yu, K., Liu, H.Q., Zhang, J., Li, H. and Li, D.C. 2010. Two novel thermostable chitinase genes from thermophilic fungi: cloning, expression and characterization. *Biores. Technol.* **101**: 5546-5551.

Li, D.C. 2006. Review of fungal chitinases. *Mycopathologia* **161**: 345-360.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**: 265-275.

Margaritis, A. and Merchant R.F.J. 1986. Thermostable cellulases from thermophilic microorganisms. *Crit. Rev. Biotechnol.* **4**: 327-367.

McCormack, J., Hackett T.J., Tuohy, M.G. and Coughlan, M.P. 1991. Chitinase production by *Talaromyces emersonii*. *Biotechnol. Lett*. **13**: 677-682.

Mendonsa, E.S., Vartak, P.H., Rao, J.U. and Deshpande, M.V. 1996. An enzyme from *Myrothecium verrucaria* that degrades insect cuticle for biocontrol of *Aedes aegypti* mosquito. *Biotechnol. Lett.* **18**:373-376.

Miller, G.L. 1959. Use of dinitrosalicylic reagent for determination of reducing sugars. *Anal. Chem*. **31**: 426-428.

Nawani, N.N. and Kapadnis, B.P. 2004. Production dynamics and characterization of chitinolytic system of *Streptomyces* sp. NK1057, a well-equipped chitin degrader. *World J. Microbiol. & Biotechnol.* **20**: 487-494.

Neeraja, C., Anil, K., Purushotham, P., Suma, K., Sarma, P., Moerschbacher, B.M. and Podile, A.R. 2010. Biotechnological approaches to develop bacterial chitinases as a bioshield against fungal diseases of plants. *Critical Rev. Biotechnol*. **30**:231-241.

Nguyen, N.V., Kim, Y.J., Oh, K.T., Jung, W.J. and Park, R.D. 2008. Antifungal activity of chitinases from *Trichoderma aureoviride* DY-59 and *Rhizopus microsporus* VS-9. *Curr. Microbiol.* **56**:28-32.

Patil, R.S., Ghormade, V. and Deshpande, M.V. 2000. Chitinolytic enzymes: an exploration. *Enz. Microb. Technol*. **26**:473-483.

Pichyankura, R., Kudan, S., Kuttiyawong, K., Sukwattanasinitt, M. and Aiba, S. 2002. Quantitative production of 2-acetamido-2-deoxy-D-glucose from crystalline chitin by bacterial chitinase. *Carb. Res*. **337**:557-559.

Run-fang, G., Bao-sheng, S., Duo-chuan, L., Wen, M. and Qing, W. 2008. Purification and characterization of a novel thermostable chitinase from *Thermomyces lanuginosus* SY2 and cloning of its encoding gene. *Agri. Sci. China* **7**: 1458-1465.

Sakai, K., Yokota, A., Kurokawa, H., Wakayama, M., and Moriguchi, M. 1998. Purification and

characterization of three thermostable endochitinases of a noble *Bacillus* strain, MH-1, isolated from chitin-containing compost. *Appl. and Env. Microbiol*. **64**: 3397-3402.

Sashiwa, H., Fujishima, S., Yamano, N., Kawasaki, N., Nakayama, A., Muraki, E., Sukwattanasinitt, M., Pichyangkura, R. and Aiba, S. 2003. Enzymatic production of N-acetyl-D-glucosamine from chitin. Degradation study of N-acetylchitooligosaccharide and the effect of mixing of crude enzymes. *Carb. Polym*. **51**: 391-395.

Satyanarayana, T., Chavant, L. and Montant, C.H. 1985. Applicability of APIZYM for screening enzyme activity of thermophilic moulds. *Trans. Br. Mycol. Soc*. **85**: 727-730.

Seidl, V., Huemer, B., Seiboth, B. and Kubicek, C.P. 2005. A complete survey of *Trichoderma* chitinases reveals three distinct subgroups of family 18 chitinases. *FEBS J*. **272**: 5923-5939.

Siddiqui, Z.A. and Mahmood, I. 1996. Biological control of plant parasitic nematodes by fungi: a review. *Biores. Technol.* **58**:229-239.

Singh, B. and Satyanarayana, T. 2006. Phytase production by thermophilic mould *Sporotrichum thermophile* in solid state fermentation and its application in dephytinization of sesame oil cake. *Appl. Biochem. Biotechnol.* **133**: 239-250.

Singh, B., Focas-Fonseca, M.J., Johri, B.N. and Satyanarayana, T. 2016. Thermophilic moulds: Biology and applications. *Crit. Rev. Microbiol*. **42**: 985-1006.

Sutrisno, M., Ueda, Y., Abe, M., Nakazawa, K. and Miyatake 2004. A chitinase with high activity toward partially N-acetylated chitosan from a new, moderately thermophilic, chitin-degrading bacterium, *Ralstonia* sp. A-471. *Appl. Microbiol. Biotechnol*. **63**:398-406.

Tanaka, T., Fujiwara, S., Nishikori, S., Fukui, T., Takagi, M. and Imanaka, T. 1999. A unique chitinase with dual active sites and triple substrate binding sites from the hyperthermophilic archaeon *Pyrococcus kodakaraensis* KOD1. *Appl. Environ. Microbiol.* 65: 5338-5344.

Thamthiankul, S., Suan-Ngay, S., Tantimavanich, S. and Panbangred, W. 2001. Chitinase from *Bacillus thuringiensis* subsp. *Pakistani*. *Appl. Microbiol. Biotechnol*. **56**:395-401.

Tikhonov, V.E., López-Llorca, L.V., Salinas, J. and Jansson, H.B. 2002. Purification and characterization of chitinases from the nematophagous fungi *Verticillium chlamydosporium* and *V. suchlasporium*. *Fungal Genet. Biol*. **35**:67-78.

Tiunova, N.A., Zhloba, N.M. and Sidorova, I.I. 1983. Biosynthesis of β-glucanases and chitinases in a culture of mycophilic strain of *T. viride*. *Mikrobiologiya* **25**: 723-728.

Tsujibo, H., Hatano, N., Endo, H., Miyamoto, K., and Inamori, Y. 2000. Purification and characterization of a thermostable chitinase from *Streptomyces thermoviolaceus* OPC-520 and cloning of the encoding gene. *Biosci. Biotechnol. Biochem*. **64**: 96-102.

Ulhoa, C.J. and Peberdy J.F. 1991. Regulation of chitinase synthesis in *Trichoderma harzianum*. *J. Gen. Microbiol*. **137**: 2163-2169.

Unsworth, L.D., Oost, J., Koutsopoulos, S. 2007. Hyperthermophilic enzymes stability, activity and implementation strategies for high temperature applications. *FEBS J*. **274**: 4044-4056.

Visser, H., Vivi, J., Peter, P.J., *et al*. 2011. Development of a mature fungal technology and production platform for industrial enzymes based on a *Myceliophthora thermophila* isolate, previously known as *Chrysosporium lucknowense* C1. *Ind. Biotechnol*. **7**: 214-223.

Waghmare, S.R. and Ghosh, J.S. 2010. Chitobiose production by using a novel thermostable chitinase from *Bacillus licheniformis* strain JS isolated from a mushroom bed. *Carb. Res*. **345**: 2630-2635.

Watanabe, T., Ariga, Y., Sato, U., Toratani, T., Hashimoto, M., Nikaidou, N., Kezuka, Y., Nonaka, T. and Sugiyama, J. 2003. Aromatic residues within the substrate-binding cleft of *Bacillus circulans* chitinase A1 are essential for hydrolysis of crystalline chitin. *Biochem. J.* **376**:237-244.

Watanabe, T., Kobori, K., Miyashita, K., Fujii, T., Sakai, H., Uchida, M. and Tanaka, H. 1993. Identification of glutamic acid 204 and aspartic acid 200 in chitinase A1 of *Bacillus circulans* WL-12 as essential residues for chitinase activity. *J. Biol. Chem.* **268**:18567-18572.

Wojtczak, G., Breuil, C., Yamada, J. and Saddler, J.N. 1987. A comparison of the thermostability of cellulases from various thermophilic fungi. *Appl. Microbiol. Biotechnol.* **17**:82-87.

Yong, T., Hong, J., Zhangfu, L., Li, Z., Xiuqiong, D., Ke, T., Shaorong, G., Shigui, L. 2005. Purification and characterization of an extracellular chitinase produced by bacterium C4. *Annals Microbiol*. **55**:213-218.