Purification and Characterization of a Thermophilic Fungal Recombinant Chitinase, Overexpressed in *Saccharomyces cerevisiae* –Suitable For Bioconversion of Chitin Wastes

Muthu Prasad	Peramachi Palanivelu
Department of Molecular Microbiology, School of Biotechnology, Madurai Kamaraj University	Professor, Medical Biotechnology CARE, Chettinad University, Kelambakkam, Chennai

ABSTRACT *A chitinase gene of the thermophilic fungus, T. lanuginosus, was cloned and overexpressed in Saccharomyces cerevisiae. The overexpressed recombinant chitinase was purified by ammonium sulphate precipitation, ion exchange and hydrophobic interaction column chromatographic techniques. The enzyme was purified 20-fold with a specific activity of 69817. The recombinant chitinase was homogeneous as judged by SDS-PAGE and the molecular mass of the protein was ~ 39 kDa. The purified chitinase was optimally active at pH 6.0 and at 60 ˚C. K_M and V_{max} of the purified recombinant were found to be 0.66 mM and 81.6 moles/min/mg protein, respectively. The enzyme was found to be highly thermostable and retained about 80% of the activity even after 3 h at 50 ˚C. The enzyme was found to be glycosylated. MALDI-MS analysis confirmed that the purified enzyme belongs to an extracellular, family 18 chitinase type. Atomic force microscopic data clearly indicate the degradation of the native chitin polymer by the recombinant purified chitinase to much smaller units. Efficient hydrolysis of native chitins by the recombinant chitinase suggests potential industrial applications for this enzyme requiring chitin processing at elevated temperatures. CD spectra of the native and denatured recombinant chitinase suggest that the beta structures play an important role on the stability of the enzyme at high temperatures.*

Introduction

Chitin is one of the most abundant renewable biopolymers in nature. About 10^{11} tons of chitin is produced annually from aquatic sources alone (Ramaiah et al. 2000). Chitin is a linear, homopolymer of N-acetylglucosamine (GlcNAc) residues linked by β- 1, 4 glycosidic bonds. Invertebrate exoskeletons and fungal cell walls and shells of crustaceans are the major sources of chitins in nature (Aranaz et al. 2009). Chitinases cleave the β-1, 4 glycosidic linkages of chitin and the carbon and nitrogen source thus contribute to the recycling of chitin in nature. The chitinases are classified into two distinct glycoside hydrolase families, viz., GH18 and GH19 based on their amino acid sequences, 3D structures, sensitivity to inhibitors and catalytic domains Henrissat and Bairoch, 1993). The retention types, family 18 chitinases, involve in the assimilation of chitin whereas the inverting types, family 19 chitinases, mainly involve in defensive mechanism in plants to ward off fungal infections (Tews et al. 1997; Kawase et al. 2006).

Thermostable enzymes are gaining wide industrial and biotechnological interest in various industrial applications (Demirjian et al. 2001). Biotechnological processes involving high temperatures have many advantages, namely higher yield due to increased activity of enzymes, minimum possibility for contamination and higher solubility of substrates and products at higher operational temperatures (Haki and Rakshit, 2003). Many thermostable enzymes, isolated from several thermophilic organisms, find applications for various industrial, medical and environmental applications (Dahiya et al. 2005).

Thermostable chitinases have been mainly isolated from large number of thermophilic microorganisms such as hyper thermophilic archae bacteria and thermophilic eubacteria. However, there are only a few reports on the chitinases from thermophilic fungi, e.g., *Talaromyces emersonii* (McCormack et al. 1991), *Thermomyces lanuginosus* (Gou et al. 2008; Prasad and Palanivelu 2012), *Thermoascus aurantiacus var. levisporus* and *Chaetomium thermophilum* (Li et al. 2010).

Molecular cloning and characterization of the chitinase gene from the thermophilic fungus, *Thermomyces lanuginosus* (ATCC 44008) have been reported by Palanivelu and Lakshmi (2013). The recombinant chitinase was further cloned into a yeast expression vector and overexpressed in *S. cerevisiae* and the properties of the recombinant chitinase were reported (Prasad and Palanivelu, 2012). In this communication, purification and characterization of the recombinant chitinase is described and also its suitability for bioconversion of chitin waste is established.

Materials and Methods
Chemicals

Chitin, ρ-nitrophenol, 4-Nitrophenyl- β-D-N, N'-diacetylchitobiose, dimethyl sulfoxide, bovine serum albumin, Trichoderma *viride* chitinase, DEAE-Sepharose, phenyl agarose, β-mercaptoethanol and CTAB were purchased from Sigma Aldrich Chemical Company, USA. All other chemicals used were of analytical grade purchased from Indian manufacturers.

Yeast and fungal strains and growth conditions

Thermomyces lanuginosus-RMB (ATCC 44008) was obtained from Dr. Ramesh Maheshwari, Indian Institute of Science, Bangalore. The chitinase gene sequence of *T. lanuginosus* has been deposited in GenBank (GenBank accession No. is JQ801444). *Saccharomyces cerevisiae* (SEY 2101) and the yeast vector, pLC9, were kindly provided by Prof. Del Castillo Agudo, University of Valencia, Spain. Growth and induction of the recombinant chitinase from the recombinant *S. cerevisiae* (YT_5), harboring the thermophilic fungal chitinase gene, is described already (Prasad and Palanivelu, 2012).

Assay of chitinase and protein estimation

The chitinase activity was assayed as described earlier

(Prasad and Palanivelu, 2012) using the chromogenic substrate, 4-Nitrophenyl-β-D-N, N'-diacetylchitobiose. Protein concentrations were estimated by Bradford's method using BSA as the standard (Bradford, 1976).

Purification of the overexpressed recombinant chitinase

The inoculum of recombinant yeast culture (YT_5) was prepared by growing the cells in 25 ml YEPD medium at 30 ˚C for ~ 15 h at 180 rpm in a gyratory shaker. The expression of the recombinant chitinase was achieved by sub culturing 2% of the inoculum into 1000 ml of induction medium containing colloidal chitin and incubated at 30 ˚C in a gyratory shaker at 180 rpm for 4 days. The cells were harvested by centrifugation at 6000 rpm for 20 min at 4 ˚C and the cell free culture filtrate (960 ml) was used as the enzyme source. The crude enzyme was concentrated by ammonium sulphate precipitation at 90% saturation. The pellet was collected by centrifugation at 12,000 rpm for 15 min at 4 ˚C, and resuspended in 25 ml of 50 mM phosphate buffer, pH 6.5 and dialyzed overnight at 4 ˚C against 1000 ml of 10 mM phosphate buffer, pH 6.5.

The dialyzed sample was applied onto a pre-equilibrated (10 mM phosphate buffer, pH 6.5) DEAE-Sepharose column (30 ml) at the rate of 30 ml/h. The flowthrough (6×5 ml) and washthrough (10×6 ml) were collected at the same flow rate. Chitinase activity was assayed in all these fractions. Flowthrough fractions (4-6) and washthrough fractions (1-4) showing good amount of activity were pooled and made up to 1 M ammonium sulphate by adding solid ammonium sulphate and loaded at a flow rate of 30 ml/hr onto a pre-equilibrated phenyl agarose column (10 ml) with 150 ml of 10 mM phosphate buffer, pH 6.5, containing 1 M ammonium sulphate The flowthrough (5x10 ml) and washthrough (10x5 ml) were collected. Chitinase activity was assayed both in the flowthrough and washthrough fractions. As no activity was detected, the enzyme was eluted by a negative gradient using 1 M ammonium sulphate and MilliQ water at the same flow rate as mentioned above. Fractions (50x2 ml) were collected. Chitinase activity and absorbency at 280 nm were determined in all the eluted fractions. The protein profile of all the active fractions was analyzed in 10% SDS-PAGE and the homogeneity was confirmed by both silver staining and Coomassie brilliant blue staining methods. The homogeneous fractions were pooled and the chitinase activity and protein concentration were determined in the pooled fraction. The homogeneous preparation was used for all further studies.

Analysis by SDS–PAGE and glycoprotein staining

The homogeneity of the recombinant chitinase was analyzed by SDS–PAGE (Laemmli, 1970). The eluted, active fractions were boiled for 5 min with sample loading buffer and electrophoresed in 10% polyacrylamide gel. The gel was stained by silver staining method. The molecular mass of the purified protein was determined using known standard protein molecular weight markers. To determine the presence of carbohydrate moiety in the purified chitinase, glycoprotein staining was done according to (Palanivelu, 2009).

TLC analysis of chitin hydrolysis

Chitin hydrolytic ability of the recombinant thermostable chitinase was analyzed by thin layer chromatography. The reaction was carried out by incubating 1 ml of 1% colloidal chitin with 100 µl of the chitinase enzyme (1500 U) at 50˚C for 6 h. Equal units of commercial *Tricho-*derma viride chitinase was used to compare with the recombinant chitinase. Proper controls were maintained for the experiments. Aliquots (100 µl) of the reaction mixtures were taken at different time intervals (1h; 2h; 3h and 6h) and analyzed by silica gel thin layer chromatography (TLC). The samples of 20 µl from each aliquot were spotted along with N-acetyl D-glucosamine (NAG) as standard. The solvent system contains n-butanol: ethanol: water at the ratio of 50:25:25 (v/v) for separation. The chitin hydrolytic products were detected by spraying with aniline-diphenylamine reagent (2 ml of aniline; 2 g of diphenylamine; 100 ml of acetone and 15 ml of 85% phosphoric acid). After spraying, the TLC sheets were baked at 120˚C for 10 min.

Effects of pH and temperature on the recombinant chitinase activity

Effect of pH of the recombinant chitinase was determined by measuring the chitinase activity between pH 4.0 and 8.0 using 0.2 M buffers under standard assay conditions (sodium acetate buffer pH 4.0 to 6.0 and Na-K phosphate pH 6.0 to 8.0).

The optimum temperature of the chitinase activity was determined by assaying the enzyme at various temperatures between 30 ℃ and 70 ℃ in 0.1 M of phosphate buffer, pH 6.0. Thermostability of the recombinant chitinase was evaluated by pre-incubation of the enzyme at 50 ℃ in same buffer for 6 h and the residual activity was determined at different time intervals under standard assay conditions.

Kinetic parameters of the enzyme

The kinetic parameters such as V_{max} and K_M of the purified recombinant chitinase were determined using the substrate, 4-Np-(GlcNAc)$_2$ at different concentrations between 0 and 125 µg/ml. The assay of chitinase was performed under standard assay conditions. The V_{max} and K_M values were obtained from Lineweaver–Bürk plot using EZ-Fit5 enzyme kinetics program (Perrella Scientific., Amherst, USA).

Effects of metal ions and other additives on the recombinant chitinase activity

Effect of metal ions on the purified recombinant chitinase activity was determined by assaying the enzyme in presence of following metal ions: Ag^+, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Na^+, Pb^{2+} and Zn^{2+} at the final concentration of 10 mM under standard assay conditions. Control was maintained without the metal ion.

Effect of the chelating agent, EDTA, on the recombinant chitinase activity was determined at 1 mM and 10 mM concentrations. Effect of the denaturing agent, urea (0.5 M and 3.0 M) and reducing agents such as dithiothreitol and β-mercaptoethanol (1 mM and 10 mM) on the recombinant chitinase activity were studied. Effects of various detergents on the purified chitinase activity were determined by addition of CTAB, Tween 80, Triton X-100 and SDS at the final concentrations of 0.1% and 1% in the reaction mixture. Proper controls were maintained for all these experiments.

Mass spectrometry and protein identification

The purified chitinase was excised from the SDS-polyacrylamide gel and analyzed by MALDI-MS. The trypsin digested peptide mass spectrum was analyzed by using MASCOT search engine. The NCBInr database was used with the peptide tolerance of ± 0.4 Da to identify homologous proteins from the database.

Atomic force microscopy (AFM) analysis

The colloidal chitin was incubated with the purified recombinant chitinase to investigate the surface changes on the chitin polymer during digestion. The reaction mixture contained 100 µl of the 1% colloidal chitin with 50 µl of the purified chitinase (87.27 Units). A control was maintained with assay buffer instead of the enzyme. The reaction was carried out at 50 °C for 30 min and the samples were coated on the UV treated cover slips under aseptic condition. The samples were dried and the surface topography of the chitin before and after digestion was monitored under AFM.

CD spectroscopic analysis

The secondary structure of the chitinase was determined by UV circular dichroism (CD) spectroscopy, using a Jasco J-810 spectropolarimeter at room temperature in 10 mM sodium phosphate buffer (pH 7.0) in the presence of chitin (0.5 µg) and the membrane mimicking solvent, trifluoroethanol (TFE, 50%). The protein was denatured at 100° C for 60 min and the secondary structure of the denatured protein was also determined. The protein (300 ng) was loaded into a 0.1 cm quartz cell and its ellipticity was scanned between 190 and 300 nm. Each spectrum obtained was the average of two scans. The secondary structure of the chitinase was estimated by K2D3 algorithm with the input wavelength range of 190-240 nm from the recorded spectra (Louis-Jeune, 2011).

Results

Purification of recombinant chitinase

The thermophilic fungal chitinase was found to be overexpressed in *S. cerevisiae*. Almost all the overexpressed recombinant chitinase (99.9%) was found in the culture medium. Therefore, the culture medium was used as the enzyme source for purification of the enzyme. The enzyme from the culture supernatant was concentrated by ammonium sulphate precipitation and dialyzed against phosphate buffer overnight and the dialyzed sample was passed through a DEAE-Sepharose column. The enzyme did not bind to the column and most of the enzyme was found in the flowthrough and washthrough fractions. When the pooled active fractions from the DEAE-Sepharose column was subjected to hydrophobic interaction chromatography on a phenyl agarose column, the enzyme bound to the matrix very strongly. The elution profile of the enzyme from the phenyl agarose column is shown in Fig 1. The summary of purification of the overexpressed recombinant chitinase is shown in Table 1.The enzyme was purified by 20 fold from the crude culture filtrate and the specific activity of the purified enzyme was 69817 Units/mg with the overall recovery of 11%. Active fractions (31- 36) were pooled and the homogeneity of the pooled fractions was ascertained by SDS–PAGE.
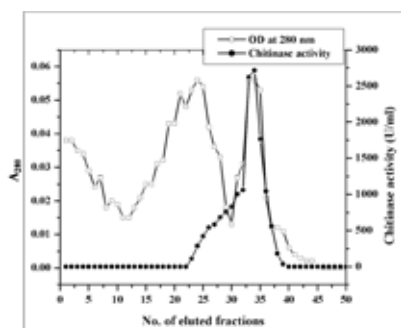

Fig.1 Elution profile of the recombinant chitinase from phenyl agarose column

Table 1 Summary of purification of the thermophilic fungal recombinant chitinase, overexpressed in S. cerevisiae

Purification step	Total volume (ml)	Total activity (Units)	Total protein (mg)	Specific activity	Fold purification	Recovery (%)
Crude extract	950.0	183,006	53.1	3446	1.0	100
$(NH_4)_2SO_4$ precipitation	27.3	56,644	15.6	3631	1.1	31
Dialysis	29.5	46,785	12.6	3713	1.1	26
DEAE -Sepharose	44.6	35,729	5.3	6741	2.0	20
Phenyl -agarose	12.0	20,945	0.3	69817	20.0	11

The molecular mass of the purified recombinant chitinase was found to be ~39 kDa, as determined by SDS-PAGE (Fig. 2). The progress of the purification process is shown in Fig. 2A. The recombinant chitinase expressed in *S. cerevisiae* was found to be glycosylated (Fig. 2B)
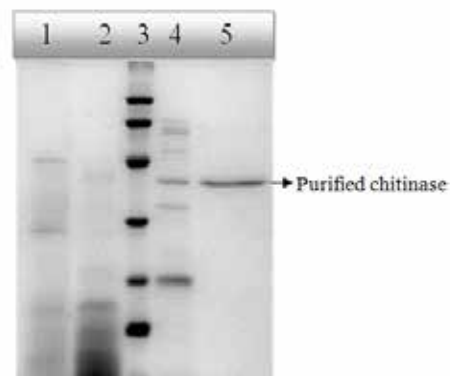

Fig.2A SDS-PAGE analysis of the purified of recombinant chitinase
Lane 1. Culture filtrate
Lane 2. Dialyzed sample after ammonium sulphate precipitation
Lane 3. Medium range protein molecular weight markers (97.4; 66; 43; 29; 20.1; 14.3 kDa)
Lane 4. Pooled active fraction from DEAE-Sepharose column
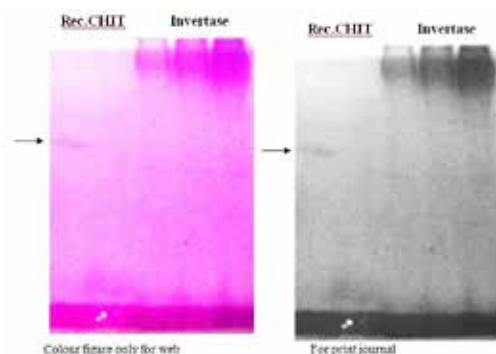Lane 5. Pooled active fraction from phenyl agarose column

Fig.2B SDS-PAGE and glycoprotein staining of the purified of recombinant chitinase

The arrow mark indicates the recombinant chitinase stained for glycoprotein

Optimum pH of the purified chitinase

The effect of pH on the enzyme activity was studied at different pH values from 4.0 to 8.0. The enzyme was maximally active between pH 6.0 and 7.0. The enzyme was found to be more stable at acidic pH values and exhibited more than 80% of activity at pH 4.0 and only about 60% activity was found at pH 8.0. The activity profile of the purified chitinase at different pH values is shown in Fig. 3.
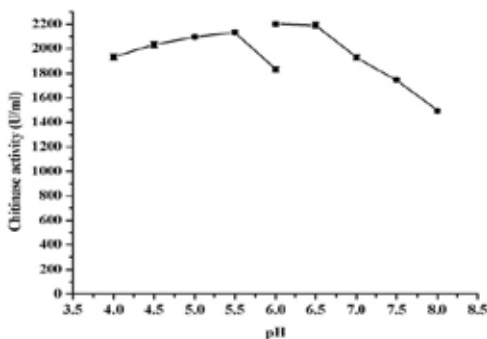

Fig. 3 Effect of different pHs of the purified recombinant chitinase

Chitinase activity was determined by assaying the enzyme in 0.2 M sodium acetate buffer (pH 4.0 to 6.0) and Na-K phosphate (pH 6.0 to 8.0).

Optimum temperature and thermostability of the purified chitinase

The enzyme showed a broad optimum temperature. The maximum chitinase activity was found to be at 60°C. A sharp decline in activity was observed after 60 °C (Fig. 4). Thermal stability of the purified chitinase was determined by incubating the enzyme at 50 °C for 6 h. The enzyme was found to be quite stable at 50 °C and retained more than 80% of the activity even after 3 h incubation. The activity started declining after 3 h and again got stabilized (Fig. 5).
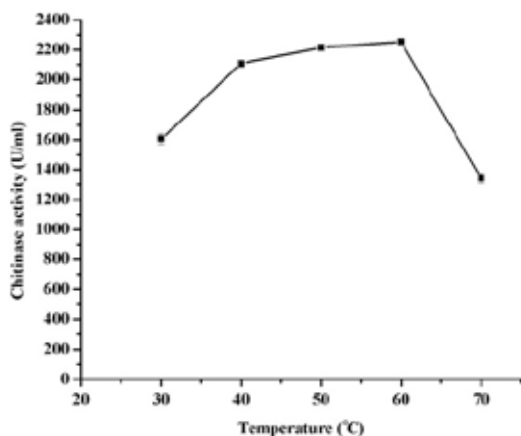

Fig. 4 Effect of different temperatures on the purified recombinant chitinase

Chitinase activity was determined by assaying the enzyme at various temperatures between 30 °C and 70 °C in 0.1 M of phosphate buffer, pH 6.0.
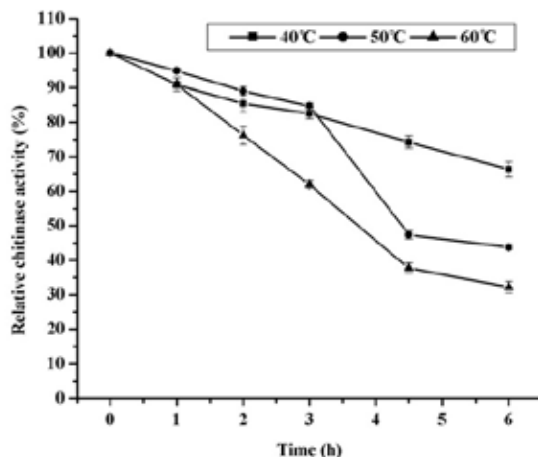

Fig. 5 Thermal stability of the purified recombinant chitinase

The purified enzyme in 0.1 M of phosphate buffer, pH 6.0, was incubated at 40, 50 and 60 °C. Residual activity was determined at various time intervals under standard assay conditions.

Kinetic parameters of the purified chitinase

The initial velocity of the enzyme was determined with increasing in concentrations of the substrate. The purified recombinant chitinase followed Michaelis-Menten kinetics. The K_M and V_{max} values were found to be 0.66 mM and 81.6 moles/min/mg protein, respectively as determined by Lineweaver – Bürk plot using Ez-Fit5 enzyme kinetics software (Fig. 6).
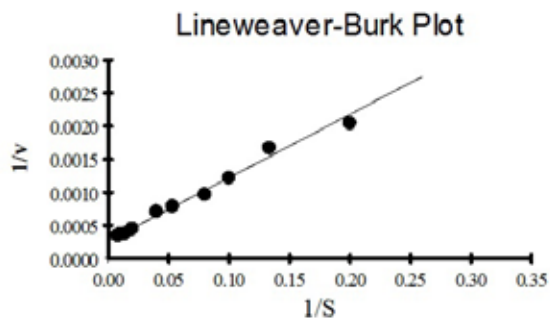

Fig. 6 Lineweaver- Bürk plot for the purified recombinant chitinase

The purified recombinant enzyme was assayed using the substrate, 4-Np-(GlcNAc)₂ at different concentrations between 0 and 125 µg/ml.

Effect of metal ions and other additives on the activity of the purified chitinase

The effect of metal ions, denaturants and detergents were studied on the purified chitinase. The catalytic activity of chitinase in the presence of different metal ions was examined at the final concentration of 10 mM. Most of the metal ions including the heavy metal ions such as Cu^{2+}, Pb^+ and Cd^+ showed enhanced enzyme activity whereas only silver ions showed a marked decrease in activity of the enzyme (Table 2). Out of the various metal ions and reagents analyzed on the activity of the purified recombinant chitinase indicates that some of the divalent metal ions sig-

nificantly increased the enzyme activity. Ag ions inhibited ~33% of the recombinant chitinase activity at 10 mM.

Table 2 Effect of metal ions on the purified thermophilic fungal recombinant chitinase from S. cerevisiae

Metal ion (10 mM)	Relative activity (%)
Control	100 ± 0.1
Ca^{2+}	135 ± 1.4
Zn^{2+}	135 ± 1.4
Cu^{2+}	133 ± 1.2
Fe^{2+}	119 ± 0.7
Co^{2+}	95 ± 0.9
Mg^{2+}	90 ± 2.3
Mn^{2+}	98 ± 1.4
Ag^+	67 ± 1.0
Na^+	95 ± 1.2
Pb^+	112 ± 1.1
Cd^+	129 ± 1.1

The effect of various detergents on the chitinase activity was studied at two different concentrations. The recombinant chitinase showed enhanced activity (up to 34%) with the detergents like Tween 80, Triton X-100 and CTAB (Table 3). The enzyme was found to be more stable towards 1% SDS, as compared to the native enzyme (Table 3). The reducing agents such as β-mercaptoethanol and dithiothreitol and EDTA at 1 mM and 10 mM did not show any significant effect on the activity of the recombinant enzyme (Table 4).

Table 3 Effect of detergents on the purified thermophilic fungal recombinant chitinase from S. cerevisiae

Detergent	Relative activity (%)
Control	100 ± 0.1
Tween 80 (0.1%)	130 ± 0.4
Tween 80 (1%)	133 ± 1.3
Triton X-100 (0.1%)	132 ± 1.0
Triton X-100 (1%)	134 ± 1.0
CTAB (0.1%)	126 ± 0.6
CTAB (1%)	128 ± 0.8
SDS (0.1%)	95 ± 1.0
SDS (1%)	89 ± 0.4

Table 4 Effect of additives on the purified thermophilic fungal recombinant chitinase from S. cerevisiae

Additives	Relative activity (%)
Control	100 ± 0.1
Urea (0.5 M)	95 ± 1.3
Urea (3 M)	60 ± 1.5
EDTA (1 mM)	92 ± 1.1
EDTA (10 mM)	90 ± 1.8
DTT (1 mM)	97 ± 0.9
DTT (10 mM)	90 ± 2.4
β-Mercaptoethanol (1 mM)	94 ± 0.8
β-Mercaptoethanol (10 mM)	80 ± 0.9

TLC analysis of chitin hydrolysis
On incubation with chitin, the recombinant chitinase released predominantly the dimer viz., the chitobiose, along with lesser amount of NAG, but on the other hand the *T. viride* chitinase released predominantly the monomer, viz., NAG as the major product of hydrolysis. Two different substrate sources viz. Himedia chitin (commercially available) and shrimp cell chitin (lab preparation from shrimp shell were used as the substrate for the mesophilic and thermophilic enzymes. Irrespective of the sources used, the results were same (Figs. 7.1 and 7.2).

Fig. 7 TLC analysis of the reaction products
The reaction products of the mesophilic and thermophilic enzymes were separated on silica gel plates and the plates were developed as described in the text
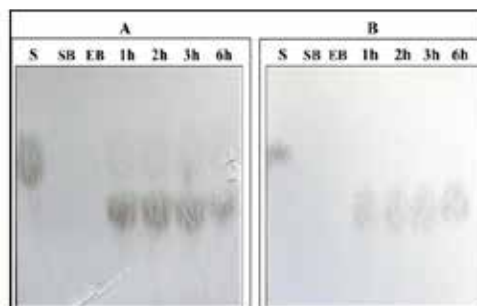

Fig. 7.1 TLC of chitin hydrolysis by the recombinant chitinase of *T. lanuginous*
A – Himedia Chitin B – Shrimp shell Chitin
Lane S: Standard [N-Acetyl glucosamine (NAG)]
Lane SB: Substrate blank
Lane EB: Enzyme blank
Lanes 1h-6h Tests -1h, 2h, 3h and 6h, respectively.

Fig. 7.2 TLC of chitin hydrolysis by chitinase of *T. viride*

A – Himedia Chitin B – Shrimp shell Chitin
Lane S: Standard [N-Acetyl glucosamine (NAG)]
Lane SB: Substrate blank
Lane EB: Enzyme blank
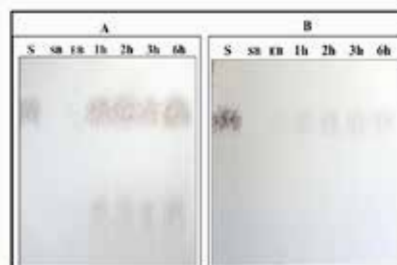Lanes 1h-6h Tests -1h, 2h, 3h and 6h, respectively.

Mass spectrometry and protein identification

The homogeneous chitinase band obtained from the SDS polyacrylamide gel was excised and analyzed by mass spectrometry. Determination of molecular mass and identification of the chitinase was performed using MALDI-MS analysis. Peptide mass fingerprinting (PMF) spectrum was searched against NCBI non-redundant protein sequence database (NCBI-nr) using the MASCOT search engine. Five internal peptides were found to be identical in both the chitinases which are shown in bold. The mass spectroscopy analysis of the purified recombinant chitinase revealed that the protein is similar to an extracellular chitinase family 18 type of *Bacillus* sp. The matched peptides between the recombinant chitinase and an extracellular chitinase from Bacillus sp. is shown in Fig. 8.

1 MLNKFKFICC TLVIFLLLPL APFQAQAANN LGSK **LLVGYW HNFDNGTGII**

51 KLREVSPK**WD VINVSFGETG GDRSTVVFSP VYGTDAEFKS DISYLK**SKGK

101 K**VVLSIGGQN GVVLLPDNAA K**QR**FINSIQS LIDKYGFDGI DIDLESGIYL**

151 **NGNDTNFKNP TTPQIVNLIS AIR**TISDHYG PDFLLSMAPE TAYVQGGYSA

201 YGSIWGAYLP IIYGVKDKLT YIHVQHYNAG SGVGMDGNNY NQGTADYEVA

251 MADMLLHGFP VGGNANNMFP ALRSDQVMIG LPAAPEAAPS GGYISPTEMK

301 KALDYIIKGI PFGGKYK**LSN ESGYPAFRGL MSWSINWDAK** NNFEFSNNYR

351 TYFDAIPLQK

Fig. 8 Peptide mass fingerprint data of the recombinant chitinase compared to that of matched peptides of **Bacillus cereus** chitinase

(Protein sequence coverage is 41% and the matched peptides are shown in bold).

Atomic force microscopy and surface topography of colloidal chitin with the purified chitinase

Atomic force microscopy (AFM) was utilized to detect the changes on the surface structure of the colloidal chitin after incubation with the enzyme for 60 min at 50 °C. The AFM image of colloidal chitin before and after digestion with the enzyme is shown in Fig. 9. The average roughness and the root mean square roughness of the chitin before and after digestion were 0.34 and 0.44 and 0.89 and 1.13, respectively. About 3 fold increase in the roughness was observed after the digestion process. Much smaller particles indicate the degradation of the chitin polymer.
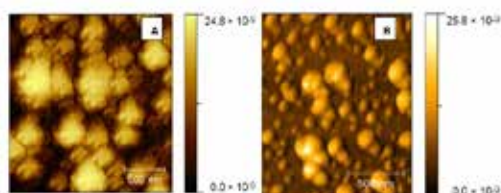

Fig. 9 Atomic force microscopic analysis of chitin hydrolysis by the purified recombinant chitinase

A. 100 µl of 1% colloidal chitin
B. 100 µl of 1% colloidal chitin and 50 µl of purified chitinase (87.27 U) after 60 min at 50° C

CD spectroscopic analysis of the purified recombinant chitinase

In order to find out the main contributory secondary structures for the thermostability of the thermophilic fungal recombinant chitinase, three different strategies were used. They were sequence based, structure based and CD spectroscopy based analyses. The UV-CD spectra of the native and denatured recombinant chitinases are illustrated in Fig. 10. UV-CD spectroscopic analysis of the purified chitinase was calculated using K2D3 algorithm (Louis-Jeune, 2011). The native enzyme showed a distinct pattern of α-helix by two negative bands of similar magnitude at 208 and 222 nm as compared to the standard spectrum of protein secondary structure (Greenfield, 2006). Interestingly, the denatured chitinase showed stabilized structure by exhibiting typical spectrum of β-strands with a negative band between 210 and 220 nm. The native enzyme contains 28% α-helix and 21.5% β-strands. On denaturation the chitinase have lost most of the α-helical structure (contains only 9.0%) but an increased level of the β-pleated structure (31.2%). Thus, this thermal unfolding found to have a major effect on the α-helical structure of the protein but not on the β-strands, which possibly plays an important role on the temperature stability of the thermostable chitinase at high temperatures. Secondary structure of the chitinase was also predicted based on sequence and 3D structure, using the bioinformatics tools such as SOPMA, DSSP and PDBsum. The in silico predictions of the secondary structure (α-helices and β-strands) of the chitinase were found to be closely similar to the experimental data (Table 5).
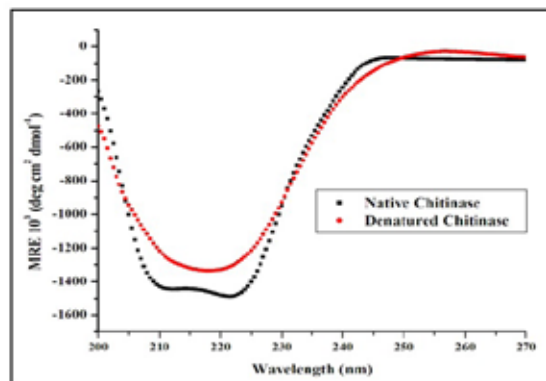

Fig. 10 CD spectra of the purified recombinant chitinase

Table 5 Summary of secondary structure analysis of the thermophilic fungal recombinant chitinase, overexpressed in S. cerevisiae

Secondary structure	Method of Analysis			
	CD Spectroscopy	SOPMA	DSSP	PDBsum
α-helix (%)	28.04	33.07	33.5	32.7
β-strand (%)	21.48	21.71	17.4	17.4
Others (%)	50.48	45.22	49.1	49.9

Discussion

As *Saccharomyces cerevisiae* comes under GRAS category of organisms, it is an ideal cloning host for expression of recombinant proteins especially for food and pharmaceutical industries. Chitinases play an important role in production of transgenic plants against fungal pathogens *and* in plant genetic engineering research. The chitinase gene of the thermophilic fungus, *T. lanuginosus*-RMB has been cloned and overexpressed in *S. cerevisiae* (Unpublished data from our lab). Very few fungal chitinases have been cloned and expressed in heterologous systems. Some of them are expressed in *E. coli* Kopparapu et al. 2012; Alias et al. 2009); Matroudi et al. 2008; Yu and Li, 2008; Ike et al. 2006; Hoell et al. 2005); some are in *P. pastoris* (Li et al. 2010; Wang and Yang, 2009) and only a few are expressed in *S. cerevisiae* (Jinzhu et al. 2005). The overexpression of the thermophilic fungal chitinase gene in *S. cerevisiae* and the properties of the recombinant chitinase have been already reported (Prasad and Palanivelu, 2012). For purification of the recombinant chitinase, the yeast transformant YT$_5$, harboring thermophilic fungal chitinase, was grown in one liter culture medium containing colloidal chitin as sole carbon source for 3 days. The enzyme was concentrated by ammonium sulphate precipitation and further purified to homogeneity by DEAE-Sepharose and phenyl agarose column chromatographic techniques.

The ammonium sulphate precipitation (at 90% saturation) resulted in the loss of 69% of the enzyme activity. Similar loss (77%) was reported by Guo et al., (2008) when the native chitinase was subjected to ammonium sulphate precipitation (at 80% saturation). It is interesting to note that similar problem was encountered with a polygalacturonase (pectinase) from the same organism. In this case, the ammonium sulphate (at 80% saturation) inactivated 80-90% of the enzyme activity (unpublished data from our lab). Both the enzymes act on similar polysaccharides and exhibit similar mode of action, (i.e.), releasing predominantly the dimer. Therefore, it is possible that higher concentrations of ammonium sulphate disrupt the 'highly hydrophobic substrate binding pocket' in both the enzymes and hence decrease the activity markedly. Since acetone precipitation also drastically inactivated the enzyme (~73%), ammonium sulphate step was used as a cost effective step for concentration and subsequent purification of the enzyme to homogeneity.

The enzyme was purified 20 fold from the crude culture filtrate and the specific activity of the purified enzyme was 69817 Units/mg with the overall recovery of 11%. The native chitinase from the same fungus was purified to 10.6 fold with an overall recovery of only 1.4% and with the specific activity of 35.51 Units/mg (Guo et al. 2008). As the recombinant chitinase was overexpressed, the specific activity and the yield were very high in the present study. Hydrophobic interaction chromatography yielded homogenous enzymes in both the cases suggesting a strong hydrophobic interaction in both the native (Guo et al. 2008) and recombinant chitinases (present study). Two thermophilic fungal chitinases expressed in *P. pastoris* were also purified using the same strategy (Li et al. 2010).

Molecular mass of the purified N-Terminal truncated recombinant chitinase in SDS-PAGE was found to be ~39 kDa as compared to the native chitinase of *T. lanuginosus* SY2 which was reported to be a 48 kDa protein (Guo et al. 2008). However, the molecular mass from amino acid sequence of the putative protein was ~42 kDa. The discrepancy observed in SDS-PAGE may be due to the compact nature of the highly hydrophobic, thermostable, thermophilic fungal protein after

purification. Most of the fungal chitinases had been reported in the molecular range of 44 – 48 kDa (Li et al. 2010). The mass spectroscopy analysis of the purified recombinant chitinase revealed that the protein is similar to an extracellular chitinase family 18 type of *Bacillus* sp. The MASCOT search did not pick up any fungal chitinases probably due to non availability of such data in the database. The catalytic region peptides that are matched in MASCOT, slightly differs in both the fungal and bacterial chitinases, e.g., DGI *DID* LES GIYL (in *B. cereus*) and DGL *DID* WEY PQDD (in T. lanuginosus). Interestingly both the chitinases use an E as the proton donor in catalysis (marked in italics). The substrate binding region is also highly conserved in both the chitinases, e.g., KVVLSIG-GQ (in B. cereus) and KVLLSIGGW in *T. lanuginosus*. However, the MASCOT search did not pick up any fungal chitinases probably due to non availability of such data in the database.

To observe the topological changes on the surface of chitin during digestion, the colloidal chitin was incubated with the purified recombinant chitinase and the chitin surface was analyzed using AFM. Marked changes in the topology were observed on the chitin and the chitin was degraded to much smaller chain lengths. Similar experiments with Streptomyces chitinase, after incubation for 7 days with shrimp shell chitin, showed significant changes on surface structure of the chitin (Hoang et al. 2011).

The optimum pH for the chitinase activity was found to be 6.0 and the enzyme displayed more stable activity in acidic conditions rather than in the alkaline conditions. The chitinase activity was found to be active in the range of pH 4.5 to 6.5. However, the optimum pH of the native chitinase from *T. lanuginosus* SY2 was found to be 4.5 (Guo et al. 2008). These findings were in close agreement to other fungal chitinases which are optimally active in the acidic ranges (Hoell et al. 2005; Li Duo- Chuan, 2006). The two thermophilic fungal chitinases viz., from *Thermoascus* auranticus and *Chaetomium thermophilum*, expressed in *P. pastoris,* were optimally active at pH values 8.0 and 5.0, respectively (Li et al. 2010). A recombinant enzyme from Trichoderma sp. expressed in E. coli was optimally active at pH 7.0 (Yu and Li, 2008). A B*acillus licheniformis* chitinase expressed in *E. coli,* was optimally active at pH 6.0 (Songsiriritthigul et al. 2010).

The optimum temperature for the recombinant chitinase was found to be 60 °C, whereas the native chitinase was optimally active at 55 °C (Guo et al. 2008). The *P. thermophila* enzyme expressed in E. coli was optimally active at 50 °C (Kopparapu et al. 2010). The recombinant chitinase from *T. virens* UKM-1 expressed in *E. coli* showed an optimum temperature of 50 °C at a pH of 6.0 (Alias et al. 2009). An endochitinase from a *Trichoderma* sp. expressed in *E. coli* BL 21 showed an optimum temperature of 35 °C and at pH (Yu and Li 2008). The *C. cuprum* chitinase, expressed in *P. pastoris* was optimally active an optimum temperature of 45 °C and at a pH of 5.8. The two novel thermophilic fungal chitinases expressed again in *P. pastoris* exhibited optimum temperatures of 50 °C and 60 °C.

The native and recombinant chitinases of *T. lanuginosus* exhibited high thermal stability which is important for industrial processing of chitins at high temperatures (Guo et al. 2008). The recombinant chitinase was found to be markedly stable and retained more than 80% of the activity at 50 ˚C even after 3 h. The recombinant chitinases from the thermophilic fungi viz., *T. aurantiacus* and C. thermophilum, which were expressed in *P. pastoris* were also found to be quite stable as expected, for example ~ 95% of the activity was retained after 60 min at 50 °C

and ~97% of the activity was retained at 60 ℃ after 60 min, respectively (Li et el. 2010). McCormack et al. (1991) reported that a chitinase from Taleromyces emersonii showed optimum activity at 65 ℃ and the half-life of the enzyme was 20 min at 70 ℃. The K_M and V_{max} values for the recombinant chitinase were 0.66 mM and 81.6 moles/min/mg protein, respectively. A lower K_M and higher V_{max} of the recombinant chitinase suggested that the purified enzyme has higher affinity for its substrate and a faster conversion rate. A B. licheniformis chitinase expressed in E. coli showed a K_M value of 0.03 mM with the V_{max} of 0.28 mM/min (Songsiriritthigul et al. 2010).

Out of the various metal ions and reagents analyzed on the activity of the purified recombinant chitinase indicates that some of the divalent metal ions significantly increased the enzyme activity. Similar to present study, Ca^{2+} enhanced the native chitinase activity of T. lanuginosus SY2 (Guo et al. 2008), Trichoderma virens chitinase expressed in E. coli, (Alias et al. 2009) and a bacterial chitinase, from Streptomyces roseolus (Jiang et al. 2012). Cu^{2+} and Zn^{2+} decreased the native chitinase activity to 4.97% and 39.5% at 50 mM, respectively and also the recombinant chitinase activities expressed in P. pastoris (Wang and Yang, 2009), whereas in contrast these metal ions enhanced the recombinant chitinase activity (present study). The inhibition of chitinase activity by Cu^{2+} was also reported for S. roseolus chitinase (Jiang et al. 2012). Trichoderma sp. chitinase showed enhanced activity with Cu^{2+} and decreased activity with Ca^{2+} and Zn^{2+} (Yu and Li, 2008). The native chitinase from T. lanuginosus SY2, was completely inhibited by Ag^+ (50 mM) (Guo et al. 2008), but in the present investigation, 33% of the recombinant chitinase activity was inhibited at 10 mM.

The recombinant chitinase showed enhanced activity (20%-30%) with the detergents like Tween 80, Triton X-100 and CTAB. The enhanced activity of the enzyme with the detergents could be due to greater accessibility and solubility of the substrate. The chitinase of T. virens UKM-1, Ech1 showed only 60% of activity with 1% Triton X-100 (Alias et al.2009). The recombinant enzyme was strongly inhibited by SDS (1%) like Paecilomyces thermophila chitinase, (Kopparapu et al. 2012) but the T. lanuginosus SY2 native chitinase, showed 73% of the activity (Guo et al. 2008). In contrast to the property of the native chitinase of T. lanuginosus SY2, the recombinant chitinase retained about 90% of the activity in the presence of 10 mM of EDTA. Urea at 3.0 M strongly inhibited (40%) the recombinant enzyme whereas the native chitinase was also inhibited by about 50% (Guo et al. 2008). Similar to the thermostable chitinase of Thermococcus chitonophagus, Chi70 (Andronopoulou and Vorgias, 2003), β-mercaptoethanol and dithiothreitol did not show any significant effect on the recombinant chitinase. Overproduction of the enzyme in S. cerevisiae, relative thermal and pH stability, stability of the enzyme activity in the presence of reducing agents, EDTA and detergents make this enzyme an ideal choice for industrial processing of chitin.

Efficient hydrolysis of native chitins from two different sources by the recombinant chitinase and releasing the chitobiose as the major product of hydrolysis suggests that this enzyme could be used for bioconversion of chitin wastes at elevated temperatures. Other thermostable chitinases from the thermophilic fungi viz. Thermoascus aurantiacus, Chaetomium thermophilum and Paecilomyces thermophila were also predominantly produced chitobiose from chitin (Li et al. 2010; Kopparapu et al. 2012).

It is clear from the CD spectra of the native and denatured chitinase that the B strands are the stabilizing factors of the chitinase, as they are seen even after denaturation at 100° C whereas, the α- helices collapsed at that tempera-

ture. It is interesting to note that the conserved catalytic residues, typical of the GH18 family chitinases were found in the β-strand regions of the recombinant chitinase which explains their activity at high temperatures.

In recent years fungal chitinases have attracted worldwide interests due to their great potential for multiple industrial applications, including bioconversion of chitin waste, an abundant biopolymer from crustacean food industry. However, the yield of this enzyme from naturally occurring microorganisms is often too low and does not satisfy the requirements for large scale industrial production. In the present study it is clear that the overexpressed thermophilic fungal recombinant chitinase can be easily purified with a single column chromatography and could be used for bioconversion of chitin waste and industrial production of chito-oligosaccharides.

REFERENCE Alias N, Mahadi M.N, Murad AMA, Bakar FDA, Mahmood NAN, and Illias RM (2009) Expression and characterization of Trichoderma virens UKM-1 endochitinase in *Escherichia coli*. World J. Microbiol. Biotechnol. 25: 561 – 572. | Andronopoulou E, Vorgias CE (2003) Purification and characterization of a new hyperthermostable, allosamidin-insensitive and denaturation-resistant chitinase from the hyperthermophilic archaeon, *Thermococcus chitonophagus*. Extremophiles 7, 43 – 53. | Aranaz I, Mengíbar M., Harris R, Panos I, Miralles B, Acosta N, Galed G, Heras A (2009) Functional characterization of chitin and chitosan. Curr. Chem. Bio. 3, 203-230. | Bradford, MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248 – 254. | Dahiya N, Tewari R, Tiwari RP (2005) Chitinase from Enterobacter sp. NRG4: Its purification, characterization and reaction pattern. Elec. J. Biotechnol. 8, 134 – 145. | Demirjian DC, Moris-varas F, Cassidy CS (2001) Enzymes from extremophiles. Curr. Opin. Chem. Biol. 5, 144 – 151. | Greenfield NJ (2006) Using circular dichroism spectra to estimate protein secondary structure, Nature Protocol, 1(6), 2876-2890. | | Guo RF, Shi, BS, Li, DC, Ma W, Wei Q (2008) Purification and characterization of a novel thermostable chitinase from *Thermomyces lanuginosus* SY2 and cloning of its encoding gene. Agric. Sci. China. 7, 1458 – 1465. | Haki GD, Rakshit SK (2003) Developments in industrially important thermostable enzymes: a review. Bioresource Technol. 89, 17 – 34. | Henrissat B, Bairoch, A (1993) New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem. J. 293, 781 – 788. | Hoang K, Lai, T, Lin, C, Chen, Y, Liau, C (2011) The chitinolytic activities of *Streptomyces* sp. TH11. Int. J. Mol. Sci. 12, 56 – 65. | Hoell I.A, Klemsdalb, SS, Vaaje-Kolstada G, Horn S.J Eijsink, VGH (2005) Overexpression and characterization of a novel chitinase from *Trichoderma atroviride* strain P1. Biochim. Biophys. Acta. 1748, 180 – 190 | Ike M, Nagamatsu K, Shioya, A, Nogawa, M., Ogasawara W, Okada H., Morikawa Y (2006) Purification, characterization and gene cloning of 46 kDa chitinase (Chi46) from *Trichoderma reesei* PC-3-7 and its expression in *Escherichia coli*. Appl. Microbiol. Biotechnol. 71, 294 – 303. | Jiang X, Chena D, Honga S, Wanga W, Chena S Zoub S (2012) Identification, characterization and functional analysis of a GH-18 chitinase from *Streptomyces roseolus*. Carbohydr. Polym. 87, 2409 – 2415. | Jinzhu S, Qian Y, Beidong L, Dianfu C (2005) Expression of the chitinase gene from *Trichoderma aureoviride in Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 69, 39 – 43. | Kawase T, Yokokawa S, Saito, A, Fujii, T, Nikaidou, N, Miyashita, K, Watanabe T (2006) Comparison of enzymatic and antifungal properties between family 18 and 19 chitinases from *S. coelicolor* A3(2). Biosci. Biotechnol. Biochem. 70, 988 – 998. | Kopparapu NK, Zhou P, Zhang S, Yan Q, Liu Z, Jiang Z (2012) Purification and characterization of a novel chitinase gene from *Paecilomyces thermophila* expressed in *Escherichia coli*. Carbohydr. Res. 347, 155 – 160. | Laemmli, UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227, 680 – 685. | Li Duo-Chuan (2006) Review of fungal chitinases. Mycopathologia. 161, 345 – 360. | Li A, Yu K, Liu H, Zhang J, Li H, Li D (2010) Two novel thermostable chitinase genes from thermophilic fungi: cloning, expression and characterization. Bioresource Technol. 101, 5546 – 5551. | Louis-Jeune C, Andrade-Navarro MA, Perez-Iratxeta C (2011) Prediction of protein secondary structure from circular dichroism using theoretically derived spectra. Proteins, 80, 374–381. | Matroudi S, Zamani MR, Motallebi M (2008) Molecular cloning of chitinase 33 (chit33) gene from *Trichoderma atroviride*. Braz. J. Microbiol. 39, 433 – 437. | McCormack J, Thomas HJ, Maria TG, Michael CP (1991) Chitinase production by *Talaromyces emersonii*. Biotechnol. Lett. 13, 677 – 682. | Palanivelu P, Lakshmi V (2013) Molecular cloning and characterization of a chitinase gene from the thermophilic fungus, *Thermomyces lanuginosus* – RMB. IOSR Int. J. Pharm. Biol. Sci. 5, 99-107 | Palanivelu P (2009) Analytical Biochemistry and Separation techniques, 4th ed., Twentyfirst century publications, Madurai, India. | Prasad M, Palanivelu P (2012) Overexpression of a chitinase gene from the thermophilic fungus, *Thermomyces lanuginosus* in Saccharomyces cerevisiae and characterization of the recombinant chitinase. J. Microbial. Biochem. Technol. 4, 86 – 91. | Ramaiah N, Hill RT, Chun J, Ravel J, Matte MH, Straube WL, Colwell RR (2000) Use of a chiA probe for detection of chitinase genes in bacteria from the Chesapeake Bay. FEMS Microbiol. Ecol. 34, 63 – 71. | Songsiriritthigul C, Lapboonrueng S, Pechsrichuang P, Pesatcha P, Yamabhai M (2010) Expression and characterization of *Bacillus licheniformis* chitinase (ChiA), suitable for bioconversion of chitin waste. Bioresource Technol. 101, 4096 – 4103. | Tewas I, Terwissscha van Scheltinga AC, Perrakis A, Wilson KS, Dijkstra BW (1997) Substrate-assisted catalysis unifies two families of chitinolytic enzymes. J. Am. Chem. Soc. 119, 7954 – 7959. | Wang Y, Yang Q (2009) Cloning and expression of a novel chitinase chi58 from *Chaetomium cupreum* in Pichia pastoris. Biochem. Genet. 47, 547 – 558 | Yu P, Li J (2008) Molecular cloning, sequence analysis, expression and characterization of the endochitinase gene from *Trichoderma* sp. in *Escherichia coli* BL21. World J. Microbiol. Biotechnol. 24, 2509 – 2516. | ***** |