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Overexpression of a Chitinase Gene from the Thermophilic Fungus, *Thermomyces lanuginosus* in *Saccharomyces cerevisiae* and Characterization of the Recombinant Chitinase

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Abstract

A chitinase gene from the thermophilic fungus, *Thermomyces lanuginosus* ATCC 44008 has been cloned and overexpressed in *Saccharomyces cerevisiae* SEY 2101. The recombinant chitinase was produced as soluble secreted protein. The enzyme activity was found to be maximum on fourth day at 30°C in the induction medium. The overexpressed chitinase displayed optimum activity at pH 6.5 and at 60°C. The recombinant chitinase attributed remarkable thermostability by holding more than 60% of the enzyme activity after 6 h at 50°C. The molecular mass of the overexpressed chitinase was 42 kDa as measured by SDS-PAGE. The kinetic parameters such as K_M and V_{max} of the enzyme were found to be 0.147 mM and 814 mmoles/min/mg protein, respectively. Synthesis of the recombinant chitinase was strongly repressed by glucose. The eukaryotic translational inhibitor, cycloheximide in the induction medium showed 10% of higher activity whereas 30% of the activity was inhibited by transcriptional inhibitors, viz. 8-azaguanine and 8-hydroxyquinoline. The overexpressed recombinant chitinase may find potential application in pharmaceutical industry to prepare chitooligosaccharides.

Keywords: *Saccharomyces cerevisiae*; *Thermomyces lanuginosus*; Recombinant chitinase; Overexpression; Thermostability; Chitooligosaccharides

Introduction

Chitin is one of the most abundant renewable biopolymers on the biosphere. It is the primary structural component of fungal cell walls and arthropod exoskeletons. Chitin is the homopolymer of β-1, 4 linked N-acetyl glucosamine [1]. Chitinases are the enzymes which involve in the degradation and effective recycling of insoluble chitin in nature by hydrolyzing the β-1, 4 glycosidic linkages. Based on amino acid sequences of the catalytic domains, chitinases are classified into two phylogenetically distinct families (GH18 and GH19), which are evolutionarily unrelated and have variations in protein sequence and structure [2]. Chitinases are ubiquitous and found in a broad range of taxons with diversified functions [3]. There are many reports on the controlled expression of microbial chitinases with chitin or its oligomers as inducers and glucose as repressor [4].

In the recent years, thermostable enzymes isolated from thermophilic microorganisms have gained wide industrial, medical, environmental and biotechnological applications due to their inherent stability to high temperatures and wide range of pH optima [5]. They have many advantages including reducing the risk of contamination by mesophilic microorganisms, higher process yield due to increased solubility of substrates and products [6]. Thermostable chitinases are required to digest the chitin at higher temperatures due to the crystalline nature of the chitin [7]. Thermostable chitinases have been isolated from hyperthermophilic archaeon such as *Thermococcus chitonophagus* [8], *Thermococcus kodakaraensis* [9] and *Pyrococcus furiosus* [10] and thermophilic bacteria such as *Aeromonas* sp [11], *Bacillus* sp [12-15], *Clostridium thermocellum* [16], *Microbispora* sp [17], *Paenibacillus* sp [18], *Streptomyces thermoviolaceus* [19]. However, there are only limited reports on chitinases from the thermophilic fungi [20-22].

Molecular cloning and characterization of the chitinase gene from the thermophilic fungus, *Thermomyces lanuginosus* ATCC 44008 was established by Palanivelu and Lakshmi (in press). The thermophilic fungal chitinase gene was cloned into a yeast centromeric plasmid, pLC9 under the control of sucrose promoter and transformed into *Saccharomyces cerevisiae* SEY 2101 (YT_0). Among the 11 transformants obtained, two of them (YT & YT_8) were growing also on chitin containing media and zone of clearance was found on chitin agar plate. These two strains were further analyzed and found that YT_5 produced tremendous quantity of chitinase and 99.9% of the enzyme was secreted into the culture medium (unpublished data). This communication describes in details further characterization of the yeast transformant, YT_5 and properties of the recombinant chitinase.

Materials and Methods

Chemicals, strains and plasmids

Chitin, ρ-nitrophenol, 4-Nitrophenyl- β-D-*N, N'*-diacetylchitobiose, dimethyl sulfoxide, bovine serum albumin, 8-azaguanine, cycloheximide and *Trichoderma viride* chitinase were purchased from Sigma Aldrich Chemical Company, USA. All other chemicals used were of analytical grade purchased from Indian manufacturers. The auxotrophic, sucrose mutant strain YT_0 (Genotype: MAT α, *Ura* 3-52, *leu* 2-3, *leu* 2-112, *Ade* 2, *Suc* 2-Δ9) and the shuttle vector, pLC9 that harbours the *SUC2* gene from *Saccharomyces cerevisiae* were kindly provided by Prof. Del Castillo Agudo, University of Valencia, Spain.

The chitinase gene sequence of *T. lanuginosus* ATCC 44008 has been deposited in GenBank and the GenBank accession number is JQ801444.

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Growth of *Saccharomyces cerevisiae* and induction of recombinant chitinase

The yeast strains, YT_0 and YT_5 were cultured in YEPD medium (1% yeast extract, 1.5% mycological peptone, 2% dextrose and agar 2% for plates) containing the supplements (Adenine, Leucine and Uracil at 40 µg/ml) at 30°C for about 12-16 h. Minimal medium containing different carbohydrates were used to study the expression of recombinant chitinase as reported by Delgado et al. [23] with minor modifications. The minimal medium contained 0.17% yeast nitrogen base, 0.5% $(NH_4)_2SO_4$ and the supplements, pH 6.5. For chitinase induction, 0.2% colloidal chitin or 2% sucrose was used as sole carbon source. Colloidal chitin was prepared according to Sandhya et al. [24].

Effect of carbon sources on the production of recombinant chitinase

Effect of various carbon sources on the growth and production of chitinase by the YT_5 and YT_0 were studied in 25 ml minimal medium containing the supplements as stated above and 2% glucose or 2% sucrose or 0.2% colloidal chitin for 7 days by continuous shaking of 180 rpm at 30°C. From each culture, the samples were collected at 24 h intervals and the growth was measured at 600 nm. Then the cultures were centrifuged at 6000 rpm for 10 min at 4°C and the clear supernatants were used as the enzyme source to quantify the chitinase activity.

Assay of chitinase activity and protein estimation

The chitinase activity was quantified using 4-Nitrophenyl-β-D-*N, N'*-diacetylchitobiose as substrate. The substrate (2.5 mg/ml) was prepared in dimethyl sulfoxide and in 100 mM phosphate buffer, pH 6.5 [25]. The assay was carried out according to instructor's manual (Sigma, CS0980) and Chernin et al. [26] with few modifications. The reaction mixture in a total volume of 100 µl contained 20 µl substrate (50 µg/ml) with 10 µl enzyme in 70 µl assay buffer. The tubes were incubated for 30 min at 37°C and the reaction was stopped by the addition of 900 µl of 4% Na_2CO_3. The absorbancy was measured at 405 nm and activity was calculated based on ρ-nitrophenol standard. One unit (U) of the enzyme activity is defined as one µmol of ρ-nitrophenol released per minute under standard assay conditions. Protein concentrations were estimated by Bradford's method with bovine serum albumin as standard [27].

Effects of pH and temperature on recombinant chitinase activity

The effect of pH on chitinase activity was studied in the pH ranges between 4.0 and 8.0 using 100 mM acetate buffer (pH 4.0 to 6.0) and 100 mM Na-K phosphate buffer (pH 6.0 to 8.0). The pH stability was studied by precipitating 1.0 ml of enzyme samples with $(NH_4)_2SO_4$ at 90% saturation on ice. After 30 min the pellets were collected by centrifugation at 12,000 rpm for 15 min at 4°C. The pellets were resuspended in equal volume of buffers of different pHs 4.0 – 8.0 then stored at 4°C. Chitinase activity was measured once in 7 days for three weeks and compared with the control for relative stability.

The chitinase activity was assayed at different temperatures between 30°C and 70°C with the interval of 10°C to investigate the optimum temperature for activity of the enzyme. Thermostability of the enzyme was determined at 50°C at different time points for up to 6 h and compared with the control and commercial *Trichoderma viride* chitinase under standard assay conditions.

Effect of substrate concentration and kinetic parameters

The initial velocity of the enzyme reaction was determined using different substrate concentrations between 2.5 µg/ml and 125 µg/ml under standard assay conditions. The chitinase activity versus substrate concentration was plotted and V_{max}, K_M values were calculated from Lineweaver–Burk plot using Sigma Plot (systat software Inc. USA).

SDS – PAGE analysis

The expression of the induced recombinant chitinase was analyzed by SDS–PAGE [28]. The culture filtrates were precipitated with 2 volumes of cold acetone on ice for 15 min and centrifuged at 12,000 rpm for 15 min at 4°C. The pellet was air-dried and resuspended in Milli Q water. The protein samples were boiled for 5 min with sample loading buffer. The proteins were distinguished in 10% polyacrylamide gel and stained overnight with Coomassie brilliant blue R-250. After destaining, the gel was documented.

Effect of eukaryotic inhibitors on chitinase expression

The regulation of the recombinant chitinase expression from YT_5 culture was studied with transcriptional inhibitors, 8-hydroxyquinoline (10 and 100 µg/ml) and 8- azaguanine (250 µg/ml) and a translational inhibitor, cycloheximide (CHI) at 50 and 100 µg/ml. The inhibitors were added to the induction medium and the culture was grown at 30°C in rotary shaker at 180 rpm for four days. Control was maintained without inhibitor. The growth of the culture at 600 nm, protein concentration and chitinase activity were assessed.

Results

Overexpression and repression of recombinant chitinase in *S. cerevisiae*

The transformed YT_5 and nontransformed YT_0 yeast strains were characterized for its growth on chitin as sole carbon source and production of chitinase and protein yeilds in the culture supernatant fluids. The YT_5 and YT_0 strains were grown in the induction medium and found that the YT_0 unable to grow significantly due to its inability to utilize colloidal chitin as sole carbon source (Figure 1). The recombinant chitinase was overexpressed by YT_5 in the minimal medium containing 0.2% colloidal chitin and maximum activity was found on fourth day. No significant chitinase activity was observed in the YT_0 (Figure 2) also shows that the protein profiles in the culture supernatant fluids of both the YT_5 and YT_0 yeast strains. Maximum protein yield was detected on the third day of the YT_5 culture and no significant amount of protein was found in the YT_0. The maximum chitinase activity of 191.12 U/ml and 528.52 U/ml was detected in 2% sucrose and 0.2% colloidal chitin containing medium, respectively on fourth day (Figure 3). The overexpressed recombinant chitinase was found to be strongly repressed by glucose. The minimal medium with 2% glucose produced only 1.8% of the chitinase activity. In the presence of glucose, YT_5 growth was found to be more than threefold higher than chitin induced culture. However, very little protein was found in the culture supernatant as compared to the chitin induced culture (Table 1).

Effect of pH and pH stability of chitinase activity

The effect of pH on chitinase activity from the YT_5 culture was studied. The chitinase activity exhibited optimum at pH 6.5 under standard assay conditions (Figure 4). Effect of pH on the enzyme stability was studied between pH 4.0 and 8.0. The enzyme was found to be stable in a wide range of pHs from 4.0 to 8.0 for 21 days (data

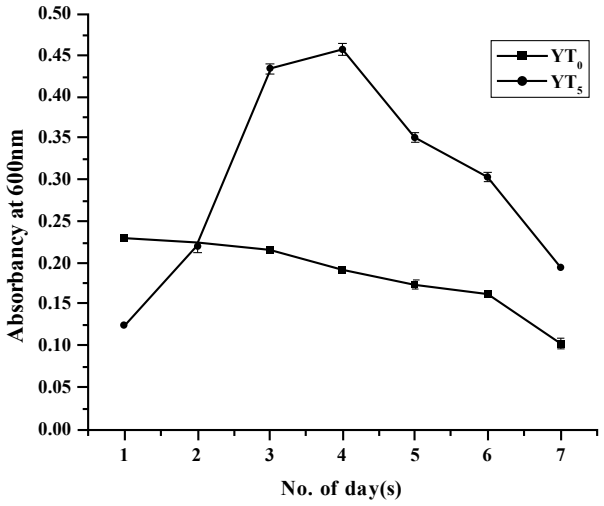

Figure 1: Growth of YT_0 and YT_5 in minimal medium containing 0.2% colloidal chitin.

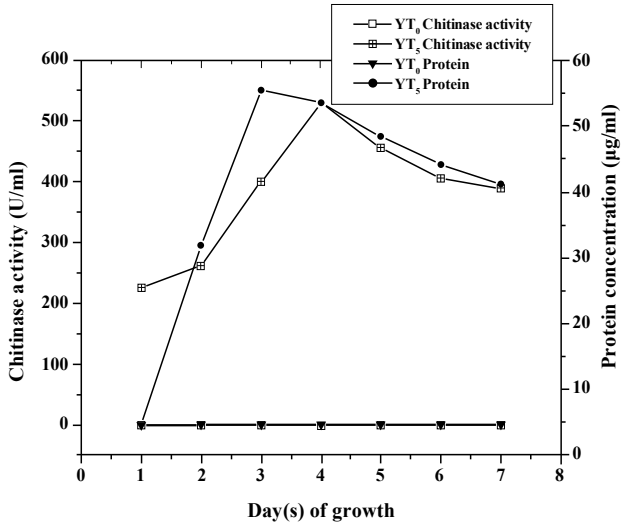

Figure 2: Recombinant chitinase activity and protein concentrations in YT_0 and YT_5.

not shown) and therefore, the enzyme can be stored at 4°C without significant loss of its activity.

Effect of temperature and thermostability of chitinase activity

The optimum temperature of the recombinant chitinase activity was examined between 30°C and 70°C and maximum activity was found at 60°C. About 50% of the activity was detected at 30°C whereas more than 65% of the activity was observed at 70°C (Figure 5). The thermostability of the enzyme was analyzed at 50°C for 6 h. As a result, 90.1% of the activity was found after 60 min and about 70% of the activity persisted after 3 h and after 6 h, more than 60% of the activity was detected. When compared to commercially available *T. viride* chitinase, recombinant chitinase from YT_5 held more than 20% of its activity at 50°C after 6 h (Figure 6).

Determination of kinetic parameters

Effect of substrate concentration on the enzyme catalyzed reaction was studied with increasing concentrations of the substrate. The enzyme reaction followed Michaelis-Menten kinetics and from the initial velocity, the kinetic constants were calculated. The K_M and V_{max} were 0.147 mM and 814 mmoles/min/mg of protein, respectively (Figure 7).

SDS – PAGE analysis has revealed a distinct band with a molecular mass of 42 kDa (Figure 8) which was not visualized in the YT_0. This molecular mass found to be nearer to the predicted molecular mass of 42, 668 Da based on the amino acid sequence.

Regulation of recombinant chitinase production in YT_5

The effect of eukaryotic inhibitors was evaluated on growth and chitinase production of the recombinant yeast. The transcriptional inhibitors (8-hydroxyquinoline and 8-azaguanine) inhibited the growth of YT_5 significantly (< 20%) but in the presence of CHI (100 µg/ml), more than 75% was observed. The protein profile was comparatively reduced to 6% and 20% at 100 µg/ml of 8-hydroxyquinoline and 250 µg/ml of 8-azaguanine, respectively when supplemented in the culture medium. Even though relative growth and protein concentrations were reduced in the presence of transcriptional inhibitors, chitinase activity was higher by 70%. In the presence of CHI (50 µg/ml) the chitinase established 10 ± 1.06% of higher activity than the control (Figure 9) but the growth and total protein had not been altered much by CHI (data not shown).

Discussion

Fungal chitinases, especially from *Trichoderma* sp. had been cloned and expressed in *Escherichia coli.* However, the enzyme was produced as inclusion bodies [29,30]. In the present experiment, the recombinant chitinase was produced as soluble protein and interestingly almost all the enzyme (99.9%) was secreted into the culture medium. Recently, a thermophilic fungal chitinase from *Paecilomyces thermophila* was cloned and expressed *in E. coli* but the enzyme was reported as soluble protein [31]. Similar to present study, Jinzhu et al. [32] had shown that the *T. aureoviridae* chitinase was expressed in yeast by pYES2

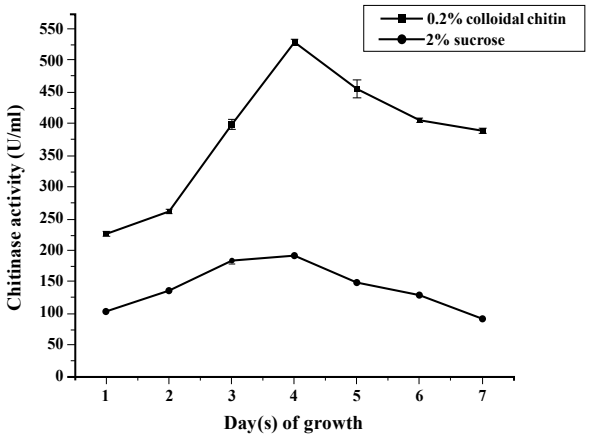

Figure 3: Chitinase activity in YT_5 with colloidal chitin and sucrose as inducers.

Carbon source	Growth (A_{600})	Protein (µg/ml)	Chitinase activity (U/ml)
0.2% Colloidal chitin	0.457	56.62	456.44
2% Glucose + 0.2% Colloidal chitin	1.327	6.97	7.97
2 % Glucose	0.699	0.87	8.60

Table 1: Effect of glucose on the production of recombinant chitinase in YT_5.

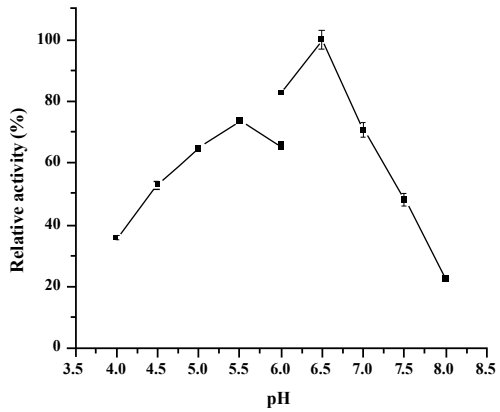

Figure 4: Effect of pH on the recombinant chitinase activity from YT_5.
Acetate buffer (0.1M), pH 4.0 - 6.0
Na-K Phosphate buffer (0.1M), H 6.0-8.0

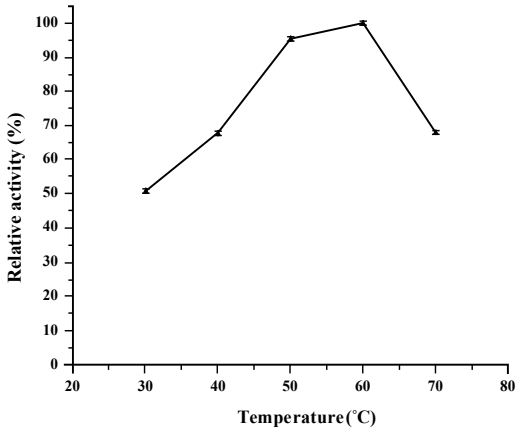

Figure 5: Effect of temperature on the recombinant chitinase activity from YT_5.

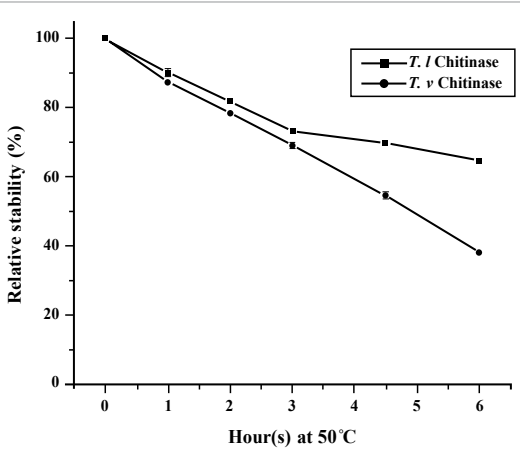

Figure 6: Temperature stability of recombinant chitinase and *T. viride* chitinase.

vector under the galactose promoter. The chitinase production from *T. harzianum* was studied in respect to different substrates in various combinations and the results revealed that maximum production on chitin supplemented medium was found on the seventh day [33]. As reported in *Microbispora* sp. V2 [17], the YT_5 expressed recombinant chitinase maximally on fourth day of the culture. No significant chitinase activity was observed in the YT_0 (Figure 2). The chitinase expression was induced with 0.2% colloidal chitin and similar result was reported by Watanabe et al. [34] on the chitinase expression from *Bacillus circulans* WL-12 with 0.2% colloidal chitin and 0.5% yeast extract.

The recombinant chitinase production was strongly repressed by glucose. Similar results were reported for a chitinase production from *T. emersonii* [20] and other microbial chitinases [4]. Both sucrose and chitin induced the recombinant chitinase and maximum activity was observed on the fourth day. However, among them, chitin was found to be the better inducer of the chitinase from YT_5. This could also be due to stabilization of the recombinant chitinase by the unutilized chitin or its derivatives in the medium.

Many fungal chitinases have an optimum pH between pH 4 and 8 [35]. Hoell et al. [30] reported that *T. atroviride* strain P1 chitinase was active from pH 3 to 6 with an optimum of pH 5.0. *Thermococcus chitonophagus* chitinase, Chi70 was found to be active in a narrow range of pH 6.5 to 7.5 [8] but the recombinant chitinase from YT_5 holds activity in a broad range of pHs between 4.0 and 8.0 with an optimum activity at pH 6.5 and relatively 50% of the activity was observed at pH 4.5 and at pH 7.5 which corresponds to the *Chaetomium thermophilum* thermostable chitinase, CtCHIT1 [22]. The chitinase of *T. chitonophagus,* Chi70 showed only 50% activity after 24 h in the pH range 3–9 [8], whereas the recombinant chitinase was stable in the pH ranges from 4.0 to 8.0 for at least 3 weeks at 4°C.

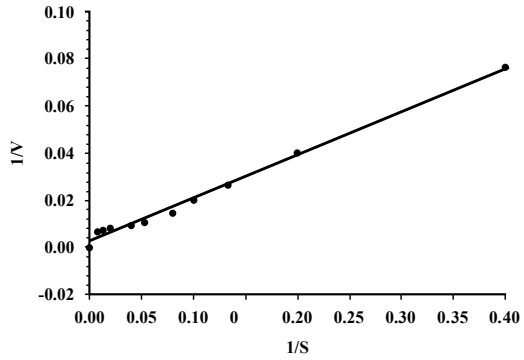

Figure 7: Lineweaver-Bürk plot of the recombinant chitinase from YT_5.

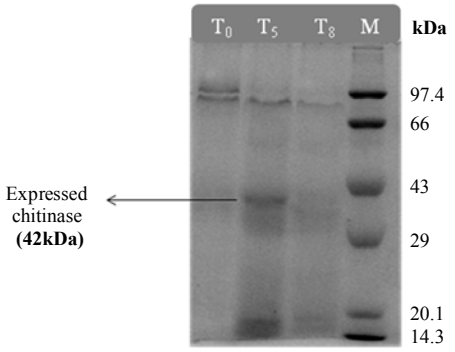

Figure 8: SDS-PAGE analysis of expression of recombinant chitinase from YT_5.
Lane 1- Culture filtrate of control (YT_0), anes 2 & 3- recombinant yeasts (YT_5 & YT_8)
Lane M: Protein molecular weight markers.

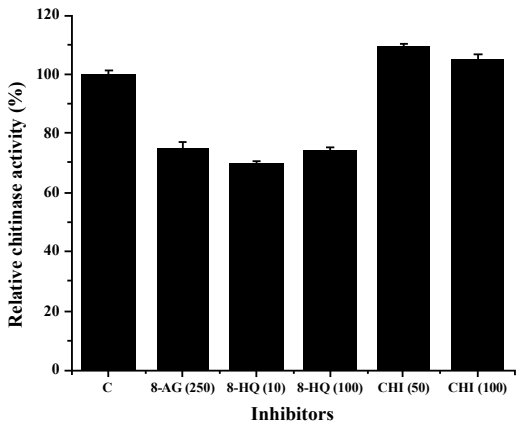

Figure 9: Effect of transcriptional and translational inhibitors on recombinant chitinase production.
C: Control, 8-AG: 8-Azaguanine (250 µg/ml), 8-HQ: 8-Hydroxyquinoline (10 and 100 µg/ml)
CHI: 50 and 100 µg/ml

The optimum temperature of the most fungal chitinases represented between 40 and 60°C. The recombinant chitinase of YT_5 was optimally active at 60°C which is close to 55°C of native chitinase from *T. lanuginosus* SY2 [21]. Two chitinases from *Talaromyces flavus* reported with an optimum temperature of 40°C showed decreased activity at temperature above 50°C [3]. The thermostable fungal chitinase from *T. emersonii* had maximum activity at 65°C [20]. The optimum temperature of 50°C and 60°C were reported of *T. aurantiacus* and *C. thermophilum* chitinases, respectively [22]. Bacterial thermostable chitinases had optimum temperature between 50°C and 60°C [11,17,18].

The recombinant chitinase was found to be remarkably stable at 50°C. The native chitinase from *T. lanuginosus* SY2 was also found to be stable at 50°C [21]. Other thermophilic fungal chitinases viz. TaCHIT1 and CtCHIT1 from the thermophilic fungus, *T. aurantiacus* and *C. thermophilum*, respectively were found to be more stable at higher temperatures [22].

The recombinant chitinase displayed higher substrate affinity with lower K_M value as compared to bacterial chitinases. To exemplify, the chitinase from *Pseudomonas aeruginosa* strain 385 showed higher K_M value (4.28 mM) with the same substrate [25]. These data showed that the recombinant chitinase is catalytically more efficient for degradation of chitin wastes and chito-oligosaccharides.

According to the amino acid sequence analysis with the chitinase of *T. lanuginosus* SY2, the recombinant chitinase was found to be truncated protein (data not shown). The molecular weight of the N-terminal truncated recombinant chitinase was found to be 42 kDa. The molecular mass of the native chitinase was reported as 48 kDa [21]. However, the truncated protein contained the substrate binding and catalytic domains intact and the N-terminal deletion did not affect the property of the enzyme. Molecular weight of other thermophilic fungal chitinases was also reported in the range of 44-48 kDa [22,31].

The transcriptional inhibitors inhibited the enzyme synthesis with 30% whereas CHI had not inhibited the enzyme synthesis significantly. In fact, 10% higher activity was found than the control, without addition of any inhibitors. But, Ulhoa and Peberdy [36] reported that *T. harzianum* chitinase synthesis was reduced 3 fold by the transcriptional inhibitor, 8-hydroxyquinoline (100 µg/ml) and 20 fold by CHI (20 µg/ml). However, superinduction phenomenon by CHI on translation process was reported of CYP1A1 and IL-6 on medium supplemented with 10 µg/ml of CHI from MCF10A cultures and in intestinal epithelial cells, respectively [37,38]. In this study, the ineffectiveness of CHI could be due to binding of CHI to the colloidal chitin in the medium because even 10 µg/ml of CHI inhibits almost completely the growth of the yeast in YEPD medium.

In conclusion, the overexpression of the thermophilic fungal chitinase gene in *S. cerevisiae* has been established. The colloidal chitin was found to be a better inducer for chitinase production. The overexpressed chitinase presents remarkable pH and thermal stability. Further experiments are in progress for complete analysis of this thermostable chitinase. The recombinant chitinase was very efficient in digesting chitin. These properties of the recombinant chitinase are valuable for the industrial processing of chitin, bioconversion of natural chitin wastes, production of single cell proteins and chitooligosaccharides, enzymatic conversion of chitin to ethanol and other biotechnological applications.

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