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Process optimization, purification and characterization of a novel acidic, thermostable chitinase from *Humicola grisea*

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Abstract

An extracellular acidic and thermostable chitinase (HgChi) from thermophilic *Humicola grisea* was purified and characterized. Enhancement in chitinase production (Q_p = 2.9662 Ul^{-1} h^{-1}) was achieved through derivation of optimum fermentation conditions via central composite design. *H. grisea* observed to produce various isoforms of chitinase, among which the major expressed form has molecular mass of about 50 kDa. Purified chitinases exhibited optimal activity at pH 3.0 and 70 ^{0}C. Chitinase showed notable stability at increasing temperatures. Half-life of chitinase is 169.06 min at optimum temperature. Chitinase has effectively catalyzed *N*-acetyl chitobiose $(GlcNAc)_2$, and *N*-acetyl chitotriose $(GlcNAc)_3$ and colloidal chitin. Purified chitinase from *H. grisea* showed high affinity towards colloidal chitin as evident by its comparatively lower K_m value. Presence of metal ions viz. Mn^{2+}, Co^{2+}, NH_4^+ and Mg^{2+} significantly increased the chitinase activity. Thin layer chromatography (TLC) analysis revealed the significant hydrolyzing competence of HgChi for colloidal chitin, $(GlcNAc)_3$ and $(GlcNAc)_2$ into oligomers and *N*-acetyl–D-glucosamine (GlcNAc). Thermostable chitinase appeared as potential candidate for efficient conversion of chitin to bioactive oligosaccharides at industrial scale.

Keywords: Chitinase, *H. grisea*, optimization, purification, thermodynamics, kinetics

1. Introduction

Chitin, the second most ubiquitous natural biopolymer on earth after cellulose, is a co-polymer GlcNAc and D-glucosamine (GlcN) subunits linked by β-(1,4) glycosidic bonds [1]. It is predominantly present in the exoskeletons or cuticles of invertebrates and in the fungal cell wall. About 10^{12}-10^{14} tons of chitin is produced annually in the biosphere with the major contributor being marine ecosystem [2]. Sea food processing industries are harvesting the marine resources to fulfill the requirements of mankind chiefly as food, resulting in the generation of huge amounts of chitin-containing waste in the coastal areas. Despite of its gigantic abundance, utilization of chitinous waste is still in its primitive stage. The key barrier in the utilization of chitin is its crystallinity and insolubility [3]. The utilization of chitinous waste could be expanded by its inter-conversion into derivatives viz. chitosan (CHS), chitooligosaccharides (COS), GlcN etc., with novel and improved characteristics. The bioconversion of chitin for generation of high-value commercial products could also provide an economic, sustainable and environmentally solution to coastal pollution reduction [4].

COS, the major degradation products of chitin are linear co-oligomers of GlcNAc and GlcN units in varying proportions. A range of bioactivities *viz*. antimicrobial, antioxidant, immune cell proliferation effects, gene therapy and tumor growth inhibition possessed by COS makes their production and application is a foremost area of research [5, 6]. COS can be produced by physical, chemical and biological routes, however, enzyme technology involving chitinases is one of the most promising approach to produce COS with specific degree of polymerization, acetylation/deacetylation and bioactivities.

Chitinase (EC 3.2.1.14), member of glycosyl hydrolase family have widely been employed and explored in the hydrolytic reactions to produce COS. In nature, chitinase has a broad distribution including bacteria, fungi, nematodes, plants, insects, fish and human with a vast range of functions including nutrition, morphogenesis, pathogenesis, parasitism, growth regulation, defense and immunity [7, 8]. Production and application of microbial chitinase from *Aspergillus flavus* H5 [9], *Trichoderma harzianum* P1 [10] and *Penicillium janthinellum* [11] has been extensively researched and patented. Recent advancements in chitin research have paved the way to utilize microbial-derived chitinase to produce well-defined COS via overcoming recalcitrance of chitin. However, the development of efficient and effective bioprocess for COS production is in its infancy due to the scarcity of potent chitinase producing organisms, high production cost, low activity and stability of available chitinase and partial understanding of its mode of action to control degree of acetylation/deacetylation and polymerization of COS [12]. So, it would be pertinent to explore and characterized novel extracellular chitinase producers, which may produce remarkably higher levels of enzymes. Moreover, the stability of enzyme at high temperature and pH can amplify their commercial exploitation as compared to their mesophilic and neutrally stable counterparts. Additionally, optimization of fermentation conditions could be helpful to decrease the production cost of enzyme. The optimized media and external factors play a crucial role in the several folds enhancement of enzyme production [13, 14]. Thermodynamic and kinetic parameters would provide additional information about factors affecting thermal stability of enzyme along with the suitable substrate for enhanced COS production.

In this perspective, process optimization for enhanced chitinase production from

thermophilic *H. grisea* was carried out employing central composite design (CCD). Subsequently, the enzyme was purified to homogeneity and kinetics of thermal inactivation of enzyme was studied by evaluating thermodynamic parameters. Kinetic parameters were also determined to get an idea about most suitable substrates, concentration range and catalytic efficiency of enzyme. Further, hydrolyzing efficiency of chitinase was evaluated using colloidal chitin, $(GlcNAc)_3$ and $(GlcNAc)_2$. The study would be advantageous to decipher the characteristic features of the chitinase that materializes its compatibility for bioconversion reactions.

2. Materials and Methods

2.1 Polymers, oligomers and chemicals

Shrimp shell chitin was procured from HiMedia, India. CM-sepharose, DEAE-sepharose, Calcofluor white M2R and COS with degree of polymerization (2-3) were obtained from Sigma-Aldrich Co., USA. TLC silica gel sheets were purchased from Merck, Germany. All other chemicals used were of analytical grade.

2.2 Microorganism

H. grisea ITCC 10,360.16 isolated and characterized in biocatalysis and bioprocessing laboratory, Department of Microbiology, Central University of Rajasthan, India from soil sample of Jaisalmer (26° 54′ 41.9796″ N, 70° 55′ 22.5408″ E), Rajasthan, India was used in the study. [15]. The strain was maintained on potato dextrose agar (PDA) slants, stored at 4°C and subcultured periodically.

2.3 Chitinase production

Chitinase production by *H. grisea* was carried out under submerged fermentation (SF) employing media containing an appropriate ratio of chitin and colloidal chitin as

carbon source [15]. Briefly, the SF was performed in Erlenmeyer flasks (250 ml) using 50 ml of medium contained (gl^{-1}): chitin, 7.49; colloidal chitin, 4.91; K_2HPO_4, 0.87; KH_2PO_4, 0.68; KCl, 0.19; NH_4Cl, 1.0; $MgSO_4.7H_2O$, 0.2 and yeast extract, 5.50 and the pH was adjusted to 6.5. After doing heat sterilization (121 °C, 20 min), the medium was inoculated with 4 discs (8mm diameter) of freshly grown fungal culture and incubated (45 °C, 150 rpm, 72 hours). The fermentation broth was centrifuged (8000 g, 10 min) and the supernatant was used as the enzyme source for further studies.

2.4 Chitinase assay

Chitinase was assayed using colloidal chitin (1%) as a substrate by estimating the reducing sugar as described by Miller [16]. The assay was carried out by incubating enzyme and substrate in 1:1 ratio (v/v) at 70 °C for 60 min. Sodium phosphate buffer (100 mM, pH 6.0) was used for chitinase assay throughout the process optimization and citrate buffer (100 mM, pH 3.0) was employed for further activity calculations employing purified chitinase. One unit (U) of chitinase activity was defined as the amount of enzyme required to release 1µmol of GlcNAc per minute under the standard assay conditions.

2.5 Optimization of fermentation parameters by central composite design

The optimum fermentation conditions (pH, temperature and inoculum size) for enhanced chitinase production by *H. grisea* were derived by employing CCD under submerged conditions through the statistical software package "Design-Expert version 10", State-Ease Inc., Minneapolis, USA. The three variables were studied at five coded levels ($-\alpha$, -1, 0, +1, $+\alpha$, where $\alpha=2^{K/4}$, k is the number of variables) in a set of 20 different experiments performed in triplicates (Table 1). The response (Y) was taken

from the mean value of chitinase activity (Ul^{-1}). An experimental model was plotted through the analysis of variance (ANOVA) in order to establish a relation between independent variables and the observed responses. The relation revealed about the statistical significance of the model and its behaviour can be explained by the second-order polynomial equation for a three-factor system (Eq. 1):

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{ij=1}^{3} \beta_{ij} X_{ij} \quad (1)$$

Where Y is the predicted response, β_0 is offset term, β_i are coefficient for the linear terms, β_{ii} are the square terms, β_{ij} are the coefficient of the interactive terms. Most favourable levels of all the three independent variables were determined by Eq. 1 along with the analysis of the response surface contour plots. Conducting the chitinase production under the conditions predicted validated the model. Samples were drawn at specified intervals and the analysis for chitinase production was done as described earlier.

2.6 Purification of chitinase

The crude extracellular chitinase was subjected to three-step purification process comprised of concentration, cation and the anion exchange chromatography. The culture broth was centrifuged at 8000 x g for 10 min at 15 °C. The crude enzyme supernatant thus obtained was concentrated using activated dialysis membrane (10 kDa molecular weight cut-off, HiMedia, India) by applying sucrose bed. The concentrated chitinase obtained from the dialysis was assayed for chitinase activity and protein content. Further, the concentrated enzyme was loaded on to CM-sepharose (Sigma-Aldrich Co., USA) column equilibrated with citrate buffer (100 mM, pH 3.0). Sodium chloride

gradient (50-500 mM) was employed for the elution of protein (flow rate, 1.0 ml min^{-1}). Fractions were collected and analyzed for chitinase activity and protein content. Chitinase active fractions were pooled and further purified using DEAE-sepharose (Sigma-Aldrich Co., USA) column as mentioned above. The active fractions were pooled and used for the further studies. Bradford [17] method was used for protein estimation using bovine serum albumin as a standard and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli [18].

2.7 Zymogram analysis

For zymogram analysis, proteins were separated with 10% polyacrylamide native gel containing 0.1% colloidal chitin and after that the gel was immersed in citrate buffer (100 mM, pH 3.0) at 60 °C for 180 min followed by staining with 0.01% (w/v) Calcofluor white M2R [19] and washing with distilled water. Lytic zone was visualized by placing the gels on a UV-transilluminator.

2.8 Biochemical analysis of the purified chitinase

2.8.1 Determination of optimum pH and stability

The optimum pH for the purified chitinase was detected by analyzing its activity over a pH range of 2.0-11.0 using the following buffers: 100 mM hydrochloride acid/potassium chloride buffer (2.0), 100 mM citrate buffer (3.0-5.0), 100 mM phosphate buffer (6.0-8.0) and 100 mM carbonate-bicarbonate buffer (9.0-11.0). The stability of enzyme at varying pH was determined by pre-incubating the purified enzyme in the buffers mentioned above for 24 h at 4 °C and subsequently analyzed the

residual activity under standard assay conditions. The experiments were performed in triplicates and their mean value was utilized for activity calculations.

2.8.2 Effect of optimum temperature and stability of chitinase

The optimum temperature was determined by assaying the chitinase activity at various temperatures (30-90 °C) for 60 min in 100 mM citrate buffer (pH 3.0). The thermostability of the enzyme was determined by pre-incubating the enzyme at various temperatures ranging from 70 to 85 °C. Aliquots were withdrawn at various time intervals, allowed to cool and the enzyme assay procedure described above was followed.

2.8.3 Thermodynamics of chitinase inactivation

Activation energy ($E_{a,d}$) of the purified chitinase was assessed using the slope of Arrhenius plot. Half-life of the chitinase was estimated according to equation (2). Other thermodynamics parameters for chitinase activation of denaturation *viz*. free energy (ΔG_d), enthalpy (ΔH_d) and entropy (ΔS_d) were obtained by employing equations (3-5).

$$t_{1/2} = \ln (2)/ k_d \quad (2)$$

$$\Delta G_d = -RT \ln (k_d h / k_b T) \quad (3)$$

$$\Delta H_d = E_{a,d} - RT \quad (4)$$

$$\Delta S_d = (\Delta H_d - \Delta G_d) / T \quad (5)$$

here, k_d is the first-order rate constant (thermal deactivation constant) which was obtained from the slope of the relative activity (%) vs time at different temperatures, h is the Planck constant (6.626×10^{-34} Js), R is Gas constant (8.314 J mol^{-}

^{1} K^{-1}) , k_b is the Boltzmann constant (1.381×10^{-23} J K^{-1}) and T is thermodynamic temperature (K).

2.8.4 Determination of kinetic parameters

The kinetic parameters of the purified chitinase were determined by measuring the enzyme activity at different concentrations (0.5-2.5 mg ml^{-1}) of chitin oligomers (chitobiose, chitotriose) and colloidal chitin. The assay was conducted in triplicates and the various kinetic constants viz. V_{max}, K_m, k_{cat} were estimated through the linear regression plots of Lineweaver and Burk.

2.8.5 Effects of various metal ions and EDTA on chitinase activity

Effects of various metal ions (Na^+, K^+, NH_4^+, Ni^{2+}, Cu^{2+}, Ca^{2+}, Hg^{2+}, Cr^{2+}, Fe^{2+}, Co^{2+}, Ba^{2+}, Mn^{2+} , Zn^{2+}, Li^{2+}, Mg^{2+}) and EDTA on the chitinase activity was studied by pre-incubating the purified enzyme in the 100 mM citrate buffer (pH 3.0) with a metal ion (1 mM). Subsequent to incubation (1 h), residual enzyme activity was measured under the standard assay conditions. The activity assayed after dialyzed against citrate buffer (100 mM, pH 3.0) was taken as the control.

2.9 Time-course hydrolytic properties of chitinase from *H. grisea*

Hydrolytic efficiency of HgChi was analyzed employing TLC using colloidal chitin, $(GlcNAc)_3$ and $(GlcNAc)_2$ as the substrates. The reaction mixture containing 0.1% (w/v) substrate prepared in 100 mM citrate buffer (pH 3.0) with 0.3 U ml^{-1} of the purified chitinase was incubated at 70 °C. Aliquots (100 µl) were withdrawn at various time intervals and the reaction was stopped by boiling for 10 min, followed by centrifugation (10000g, 5 min). The samples were concentrated, spotted on to a silica gel plate (TLC Silica gel 60, Merck, Germany) and developed in a solvent system [20],

followed by spraying with aniline-diphenylamine reagent [20]. The TLC sheets were analyzed after heating in an oven for 10 min at 150 °C.

3. **Results and Discussion**

3.1 Determination of optimum fermentation conditions

Development of an economic and efficient bioprocess for enzyme production relies on cost-effective production medium and optimum fermentation conditions. The optimum level of fermentation medium and conditions can be obtained through the statistical optimization methods viz. response surface methodology (RSM) that allows study of effects of various factors at a time along with determination of their interactive effect on production. Effect of three fermentation variables (pH, temperature and inoculum size) on chitinase production was evaluated by CCD with the help of five coded levels of three variables (k=3). The CCD design and the corresponding responses are shown in Table 2. By applying ANOVA on the experimental data, the results of CCD were put into the following second-order polynomial equation in order to explain chitinase production as a function of studied variables (Eq. 6).

$$Y = 374.41 - 40.08\,A + 17.71\,B - 299.29\,C + 1.20\,AB + 4.07\,AC + 5.88\,BC - 4.24A^2 - 0.48\,B^2 + 2.26\,C^2 \quad (6)$$

Where, Y represents chitinase production (Ul^{-1}) and A, B and C are coded values of pH, temperature and inoculum size, respectively.

The very low probability value [(Pmodel F) < 0.0001] of the Fisher's F test for the model proved the significance of the model. ANOVA for chitinase production indicated the '*F* value' to be 13.86, which illustrated the high significance of the model in approximating the response surface of the experimental design. Corresponding

probability of failure value (>*F*) showed that there were only 0.01 % chances for the occurrence of this value due to noise. The goodness of fit of the model was examined by coefficient of determination (R^2) and it was found to be 0.9258, which can be used to explain up to 92.58 % variability of the response. The 'adequate precision' value of 14.19 directed an adequate signal and suggested that the model can be used to navigate the design space. The low value of coefficient of variance (CV = 11.89) indicated that experiments were conducted with high precision. The ''lack of fit test'' compares the residual error to the pure error from replicated design points. The significance of each coefficient on the response was evaluated by Student's t-test and Probability of failure value. Model terms with 'Prob>[F]' values < 0.05 were considered significant while insignificant terms have values > 0.10. According to the model, linear terms of pH and temperature have noteworthy effect on chitinase production. Additionally, the squared term of temperature and the interaction of temperature with inoculum size also significantly affected chitinase production (*P*-value < 0.05). The uncoded optimum values calculated from the model equation were 6.49, 47 ^{0}C and 5 discs for pH, temperature and inoculum size, respectively.

Employment of RSM for optimization studies also provides an insight about the effect of interaction of independent variables on the response. Interaction between temperature and inoculum size observed to have notable effects on chitinase production by *H. grisea* (Fig. 1a, b). It is evident from the response surface plot that the effect of temperature is more pronounced on chitinase production as compared to inoculum size (Fig. 1b). Likewise, inoculum is considered as one of the most important factors affecting enzyme production during fermentation processes. Increase in both the inoculum size and temperature positively affected chitinase production but up to a

certain extent. Addition of inoculum beyond a definite range may led to early exhaustion of rapidly utilizable carbon source in the medium that further resulted into excessive biomass production and lower levels of enzymes. Also, extremely high temperature may be detrimental to cell growth and may led to formation of some other proteins required for survival of cell rather than production of desired molecules i.e. enzymes. The utilization of statistical means to optimize fermentation conditions had exploited by many researchers successfully to increase the enzyme titres over the one factor at a time optimization [21, 22].

Estimating chitinase production using derived conditions did verification of the model equation. The optimized conditions had resulted into actual maximal chitinase production of 213.57 ± 6.4 Ul^{-1} and was observed to be in good agreement with the theoretically predicted values by the quadratic model (202 Ul^{-1}) (Fig. 2). Optimization of process parameters had led to 1.24 folds enhancement in chitinase titres. *Lacanicillium muscarium* CCFEE-5003 showed similar chitinase production (243 ± 17.3 Ul^{-1}) following employment of optimized substrate concentration and pH conditions (through RSM) in a bioreactor [23]. Our results are in good agreement with the study. However, *Bacillus licheniformis* AT6 (505.26 ± 22.223 Ul^{-1}) [24] and *Pseudomonas fluorescens* HN1205 (1030 Ul^{-1}) [25] exhibited relatively enhanced chitinase production titres following optimization.

3.2 Chitinase purification and molecular mass determination

Extracellular HgChi (50 kDa) was purified from the crude culture supernatant by employing a three-step purification process consisting of concentration followed by cation and anion exchange chromatography (Table 3, Fig. 3). Patil et al. [26] also

reported production of extracellular chitinase (40 °C, 120 rpm and 96 h) from thermophilic *Penicillum ochrochloron* and purified through DEAE-cellulose anion exchange chromatography followed by molecular mass determination (64 kDa) through SDS-PAGE. The molecular mass of the purified fungal chitinase lies in the range of 30-150 kDa [27-30].

3.3 Effects of pH on chitinase activity and stability

Purified chitinase exhibited maximum activity at pH 3.0 (Fig. 4a). A sharp decline in chitinase activity was observed at all other pH ranges studied. Enzyme's pH stability analysis had shown that the enzyme retained about 90% of its activity at pH 3.0 following 24 h of incubation (Fig. 4b). Moreover, at higher pH 9.0-11.0 enzyme was able to retain more than 80% of activity. Mander et al. [31] had also reported high pH (5.0-13.0) stability of the purified chitinase obtained from *Streptomyces anulatus* after the incubation for 48 h at 4 °C. Chitinase exhibiting high acid or base tolerating capacity has also reported by several researchers [32, 33]. The higher stability of chitinase from *H. grisea* at low pH advocates its applicability as a suitable bio-catalytic solution for large scale applications for bioconversion of chitin waste to the value-added COS.

3.4 Thermal kinetics of chitinase

The purified chitinase showed maximum activity at 70 ^{0}C. Additional incubation at higher temperatures led to reduction in enzyme activity. Time-dependent thermal inactivation kinetics of enzyme was studied by incubating it at various temperatures (70-85 ^{0}C) to get an idea about thermal stability of enzyme at higher temperatures. It was observed that the enzyme retained notable activity at relatively higher temperatures.

The HgChi temperature optima (70 °C) was comparable to the patents related to the thermostable chitinase from recombinant *Vibrio parahemolyticus* strain (45 °C) [34] and *Brevibacillus later osporus* LAK 1210 (70 °C) [35].

Thermodynamic parameters viz. half-life, free energy, enthalpy and entropy predicts about the stability of enzyme against thermal denaturation. Half-life of the purified chitinase (i.e. enzyme thermostability indicator) at its optimum temperature was 169.060 min which was observed to reduce following increment in incubation temperature (Table 4). First-order rate constants (k_d) for thermal denaturation of HgChi at various temperatures were determined from the slopes of the first order plot (% relative activity *vs* time). In order to achieve complete denaturation of enzyme a least amount of energy is compulsory which is known as activation energy of denaturation ($E_{a,d}$). The activation energy of denaturation for HgChi was estimated to be 108.91 kJ mol^{-1} (Fig. 5). At optimum temperature for HgChi, the values of ΔG_d, ΔH_d, and ΔS_d were 100.079 kJ mol^{-1}, 111.765 kJ mol^{-1} and 34.069 J mol^{-1} K^{-1}, respectively (Table 4). The positive values of ΔS_d suggested the deactivation of chitinase following disruption of non-covalent bonds at high temperatures. There is no significant correlation was observed between ΔG_d and temperature as there is no considerable decrease in ΔG_d value with increasing temperature (Table 4). Similar relation was also observed for the enthalpy of activation for the thermal inactivation of HgChi with increasing temperature. The results are in agreement with the studies performed to investigate the thermal denaturation of chitinase [36], chitin deacetylase [37] and serine protease [38].

3.5 Kinetic parameters of chitinase

Kinetic parameters viz. V_{max}, K_m, k_{cat} and k_{cat} / K_m of purified chitinase were determined through Lineweaver-Burk plot using various substrates i.e. colloidal chitin,

$(GlcNAc)_2$ and $(GlcNAc)_3$. The purified chitinase showed significant affinity towards the substrates studied. $(GlcNAc)_2$ and $(GlcNAc)_3$ appeared to be preferable substrates by enzyme for catalysis. The affinity of purified chitinase towards $(GlcNAc)_2$ and $(GlcNAc)_3$ was almost similar, as evident from their K_m values (0.30 mg ml^{-1} for $(GlcNAc)_2$ and 0.33 mg ml^{-1} for $(GlcNAc)_3$). The enzyme also exhibited similar V_{max} values for the substrates (1.29 µmol min^{-1} for $(GlcNAc)_2$ and 1.25 µmol min^{-1} for $(GlcNAc)_3$) (Table 5). HgChi exhibited highest affinity towards colloidal chitin as suggested by its lower K_m value (0.11 mg ml^{-1}) (Table 5). However, the relatively less V_{max} value (0.84 µmol min^{-1}) for colloidal chitin could be attributed to its more crystalline nature as compared to the oligomers that restricts the substrate accessibility. The values of Michaelis-Menten constant and specificity constant signifies substantial catalytic activity of the purified chitinase towards $(GlcNAc)_2$ and a decrease in the enzyme affinity was observed with increase in the degree of polymerization. Prasad and Palanivelu [39] studied kinetics of a 42 kDa recombinant chitinase from thermophilic fungus *Thermomyces lanuginosus* and reported K_m and V_{max} values of 0.147 mM and 814 mM min^{-1} mg^{-1} employing colloidal chitin as the substrate. HgChi appeared to be posses better affinity towards colloidal chitin with lower K_m (0.11 mg ml^{-1}) and higher k_{cat} (14 s^{-1}) compared to the chitinase from *Penicillium ochrochloron* (K_m, 1.3 mg ml^{-1}; k_{cat}, 2.37 s^{-1}) [40].

3.6 Effects of metal ions on enzyme activity

Metals can play role as co-factors during enzyme catalysis and may enhance activity of certain enzymes. Effect of various metal ions and EDTA on chitinase activity was assayed to detect the metal ion requirement for catalysis. Among the different metal ions studied Mn^{2+}, Co^{2+}, NH_4^+ and Mg^{2+} appeared as activators of chitinase activity,

however, the chitinase activity was inhibited by Hg^{2+}, Ca^{2+}, Cu^{2+}, K^{+} and EDTA at 1 mM concentrations (Fig. 6). Among the activators, Mn^{2+} and Co^{2+} were significantly enhancing the chitinase activity up to 173% and 144% respectively. Ni^{2+}, Na^{+} and Li^{2+} had no considerable effect on the chitinase activity. In case of inhibitory metal ions, Ca^{2+} and Hg^{2+} were appeared to be the significant inhibitors of chitinase activity, whereas, Fe^{2+} and Cr^{2+} were reported to moderately inhibit chitinase activity. Chitinase activity inhibition by EDTA suggested that the enzyme is a metallo-protein. Chitinase activity was observed to be highly affected by Hg^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+} and Co^{2+}. Yang et al. [40] had reported enhancement in chitinase activity by Ca^{2+} and Ni^{2+} ions, however in the present study Ca^{2+} has appeared as inhibitor of enzyme activity and Ni^{2+} had no noteworthy effect on the chitinase activity. Fe^{2+}, K^{+} and Hg^{2+} were reported to be inhibitors of chitinase activity whereas; Mg^{2+} was reported to be activator of chitinase [26, 41]. The results from the present study showed good agreement with the previous research findings.

3.7 Hydrolytic properties of *H. grisea* chitinase

The hydrolytic efficiency of HgChi was investigated on colloidal chitin, $(GlcNAc)_3$ and $(GlcNAc)_2$. The hydrolyzing efficiency of HgChi to produce COS was evaluated by analyzing the hydrolytic products by TLC using $(GlcNAc)_3$, $(GlcNAc)_2$ and GlcNAc (Sigma-Aldrich, USA) as standards. The HgChi was able to efficiently hydrolyzed colloidal chitin to generate $(GlcNAc)_3$, $(GlcNAc)_2$ and GlcNAc following incubation at 70 ^{0}C (30-60 min) (Fig. 7a). Further increase in incubation period (i.e. 60-300 min) had resulted into augmentation of GlcNAc concentration due to the hydrolysis of $(GlcNAc)_3$ and $(GlcNAc)_2$. The HgChi was also capable of hydrolyzing $(GlcNAc)_3$ into $(GlcNAc)_2$ and GlcNAc following incubation for 5- 30 min (Fig. 7b). Additional elongation in

hydrolysis time (60-120 min) led to complete conversion of $(GlcNAc)_3$ into GlcNAc. HgChi also showed to notably hydrolyze $(GlcNAc)_2$ (Fig. 7c). Chitinase illustrated to completely hydrolyze $(GlcNAc)_2$ into $(GlcNAc)_2$ and GlcNAc within 3 min of incubation and complete conversion into GlcNAc within 10 min of incubation time. The results from TLC analysis could be used to deduce preliminary conclusions about mode of action of HgChi. TLC analysis suggested that HgChi is an endo-acting chitinase as it showed randon cleavage pattern and produce a mixture of $(GlcNAc)_3$, $(GlcNAc)_2$ and GlcNAc following hydrolysis. HgChi showed better hydrolyzing capacity towards colloidal chitin as compared to the extracellular chitinase from *Lecanicillium lecanii,* that reported to convert colloidal chitin into GlcNAc following 96 h of incubation at 40 °C [29]. The remarkable hydrolytic potential of HgChi could be attributed to its thermophilic nature that contributed in enhanced reaction rates. The hydrolyzing ability of HgChi towards colloidal chitin is comparable to the hydrolyzing efficiency of the recombinant chitinase developed from thermophilic *Themoascus aurantiacus* and *Chaetomium thermophilum* [42]. Chitinase from *T. aurantiacus* and *C. thermophilum* reported to hydrolyze colloidal chitin to produce $(GlcNAc)_2$ and GlcNAc following incubation for 30 min at 50 °C [42]. Yang et al. [30] has shown that acidic exochitinase from *Rhizomucor miehei* can effectively hydrolyze colloidal chitin, $(GlcNAc)_3$ and $(GlcNAc)_2$ into various chito-oligomers and monomers.

4 Conclusions

The present study concluded that the optimization of process parameters through RSM can enhance chitinase production. The chitinase produced by *H. grisea* can effectively hydrolyze chitin to produce oligosaccharides. Optimum activity of enzyme at relatively lower pH (3.0) and higher temperature (70 °C) along with its stability over a broad

range of temperature advocates its wide applicability from industrial point of view. High catalytic efficiency and specificity of HgChi supported its high industrial effectiveness and competence. Further enzyme structural studies would help to reveal its conformational stability and complexity of denaturation. Additionally, site-directed mutagenesis could be employed to enhance the activity and stability of chitinase at extreme operating conditions and also to deduce the structure-function relationships.

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Conflict of interest

The authors declare no conflicts of interest.

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Table 1. Concentration ranges of independent variables used in central composite design

Variable	Component	Range studied	Levels				
			-α	-1	0	+1	+α
A	pH	5.00-7.00	4.31	5.00	6.00	7.00	7.68
B	Temperature (^{0}C)	40.00-50.00	36.59	40.00	45.00	50.00	53.41
C	Inoculum size (No. of discs)	3.00-5.00	2.32	3.00	4.00	5.00	5.68

Table 2. Experimental design matrix for optimization of chitinase production using central composite design

Run	Independent variables (actual values)			Chitinase (U l^{-1})	
	pH	Temperature (^{0}C)	Inoculum size (No. of discs)	Observed	Predicted
1	7	50	5	165.23	173.22
2	6	45	4	133.63	132.62
3	6	45	2.32	122.19	125.72
4	5	40	3	149.46	143.21
5	6	45	4	128.01	132.62
6	5	40	5	68.74	92.057
7	6	45	4	135.23	132.62
8	6	53.40	4	141.78	145.37
9	6	45	5.68	158.30	152.30
10	6	36.59	4	58.47	52.41
11	5	50	5	200.43	194.12
12	4.32	45	4	169.80	155.17
13	5	50	3	112.39	127.64
14	6	45	4	136.78	132.62
15	7.68	45	4	73.93	86.10
16	7	50	3	112.03	90.45
17	7	40	3	73.93	81.97
18	6	45	4	131.21	132.62
19	6	45	4	130.45	132.62
20	7	40	5	60.62	47.10

Table 3. Purification profile of chitinase from *H. grisea*

Purification step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Yield (%)	Purification fold
Culture supernatant	50	11.72	4.86	2.41	100	1
Dialysis	10	5.26	1.91	2.75	44.88	1.14
CM-sepharose	14	3.12	17.31	0.18	26.62	0.07
DEAE-sepharose	20	2	0.22	9.09	17.06	3.77

Table 4. Thermodynamic parameters of chitinase from *H. grisea*

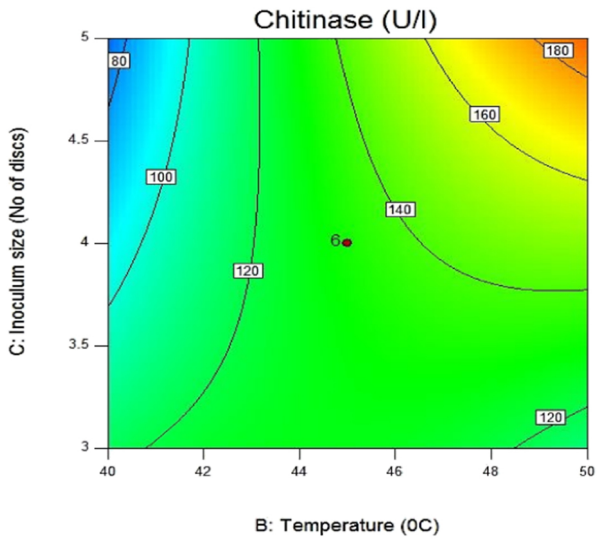
T (K)	$t_{1/2}$ (min)	ΔG_d (kJ mol^{-1})	ΔH_d (kJ mol^{-1})	ΔS_d (J mol^{-1} K^{-1})
343	169.060	100.079	111.765	34.069
348	93.668	99.871	111.806	34.296
353	60.802	100.080	111.848	33.337
358	32.850	99.709	111.889	34.030

Table 5. Substrate specificity and kinetic parameters of chitinase from *H. grisea*

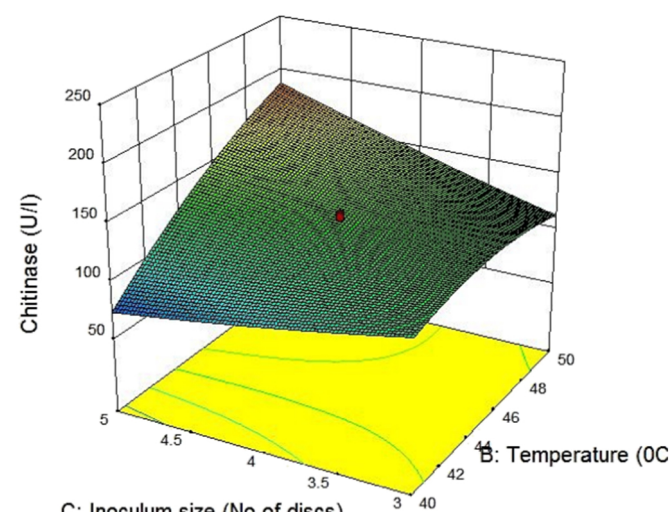
Substrate	Relative activity	Kinetic parameters			
		K_m (mg ml^{-1})	V_{max} (µmol min^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (mg ml^{-1} s^{-1})
$(GlcNAc)_2$	151	0.30	1.29	21.5	71.66
$(GlcNAc)_3$	145	0.33	1.25	20.83	63.13
Colloidal chitin	100	0.11	0.84	14	127.27

Highlights

- Optimization of fermentation conditions through RSM led to enhanced chitinase production (upto1.24 folds)
- 50 kDa highly acidic and thermostable chitinase was purified and characterized
- Chitinase showed high affinity and turnover number towards $(GlcNAc)_2$ and $(GlcNAc)_3$
- TLC analysis exhibited high hydrolytic affinity of endo-acting HgChi against colloidal chitin, $(GlcNAc)_2$ and $(GlcNAc)_3$ to produce chitooligosaccharides and *N*-acetylglucosamine.


Chitinase (U/l)
C: Inoculum size (No of discs)
B: Temperature (0C)
80
100
120
140
160
180
120
6
5
4.5
4
3.5
3
40
42
44
46
48
50

(a)


Chitinase (U/l)
250
200
150
100
50
C: Inoculum size (No of discs)
5
4.5
4
3.5
3
B: Temperature (0C
40
42
44
46
48
50

(b)

Figure 1

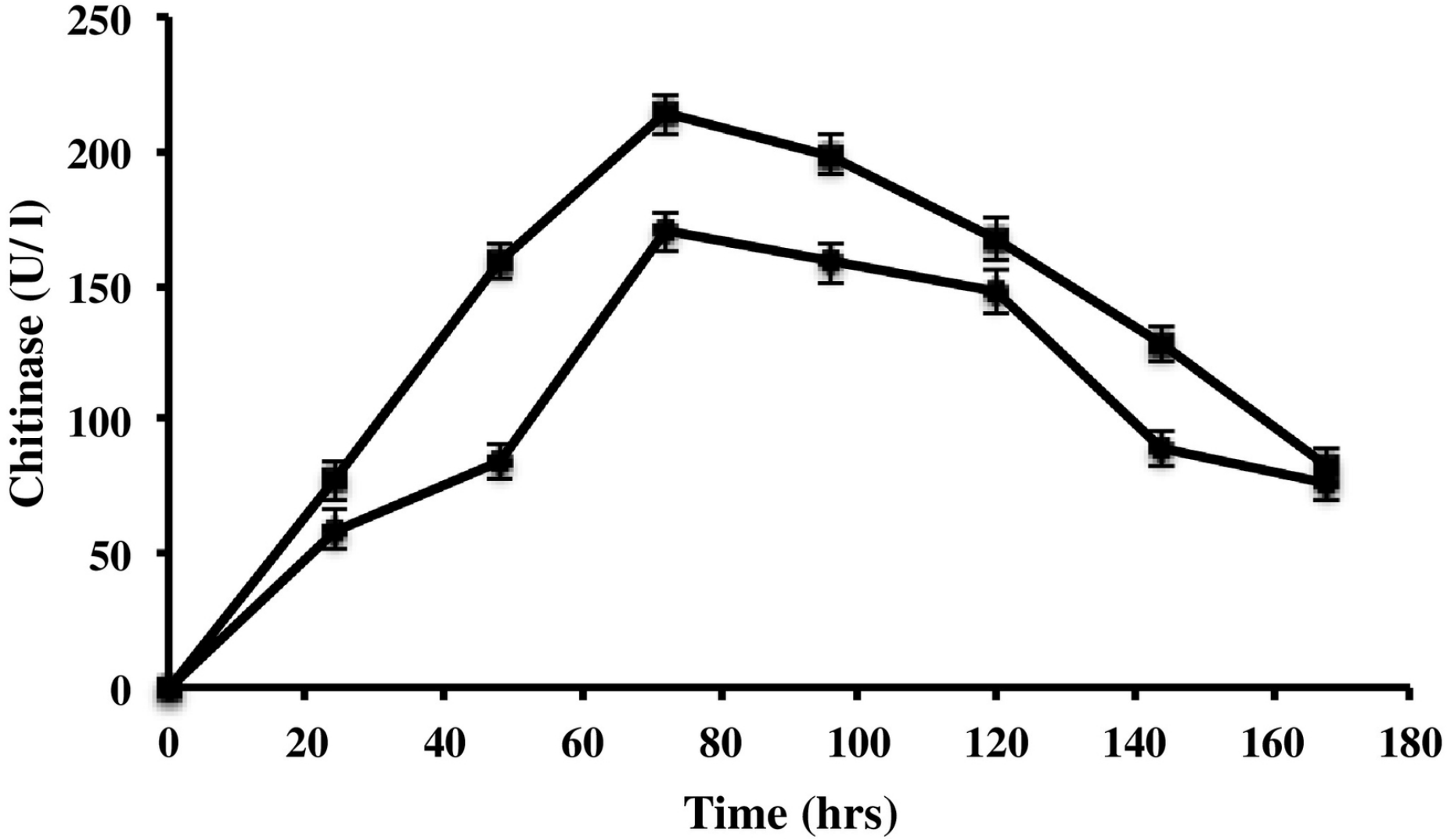

250
200
150
100
50
0
Chitinase (U/ l)
0
20
40
60
80
100
120
140
160
180
Time (hrs)

Figure 2

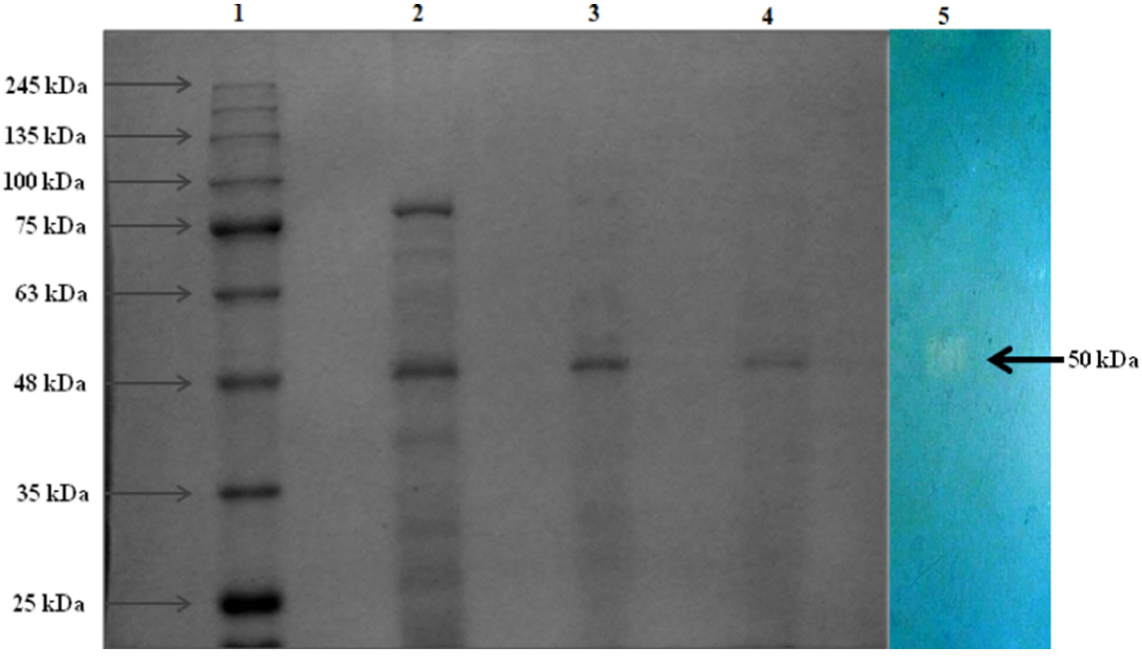
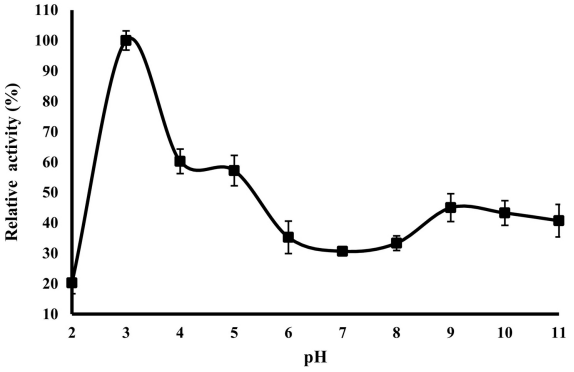
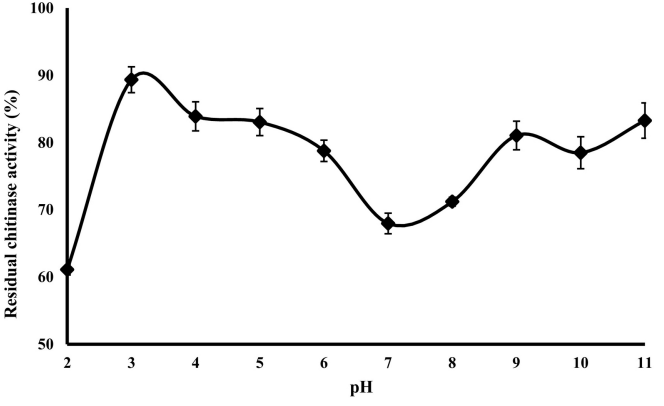

1
2
3
4
5
245 kDa
135 kDa
100 kDa
75 kDa
63 kDa
48 kDa
35 kDa
25 kDa
50 kDa

Figure 3


Relative activity (%)
110
100
90
80
70
60
50
40
30
20
10
2
3
4
5
6
7
8
9
10
11
pH

(a)


Residual chitinase activity (%)
100
90
80
70
60
50
2
3
4
5
6
7
8
9
10
11
pH

(b)

Figure 4

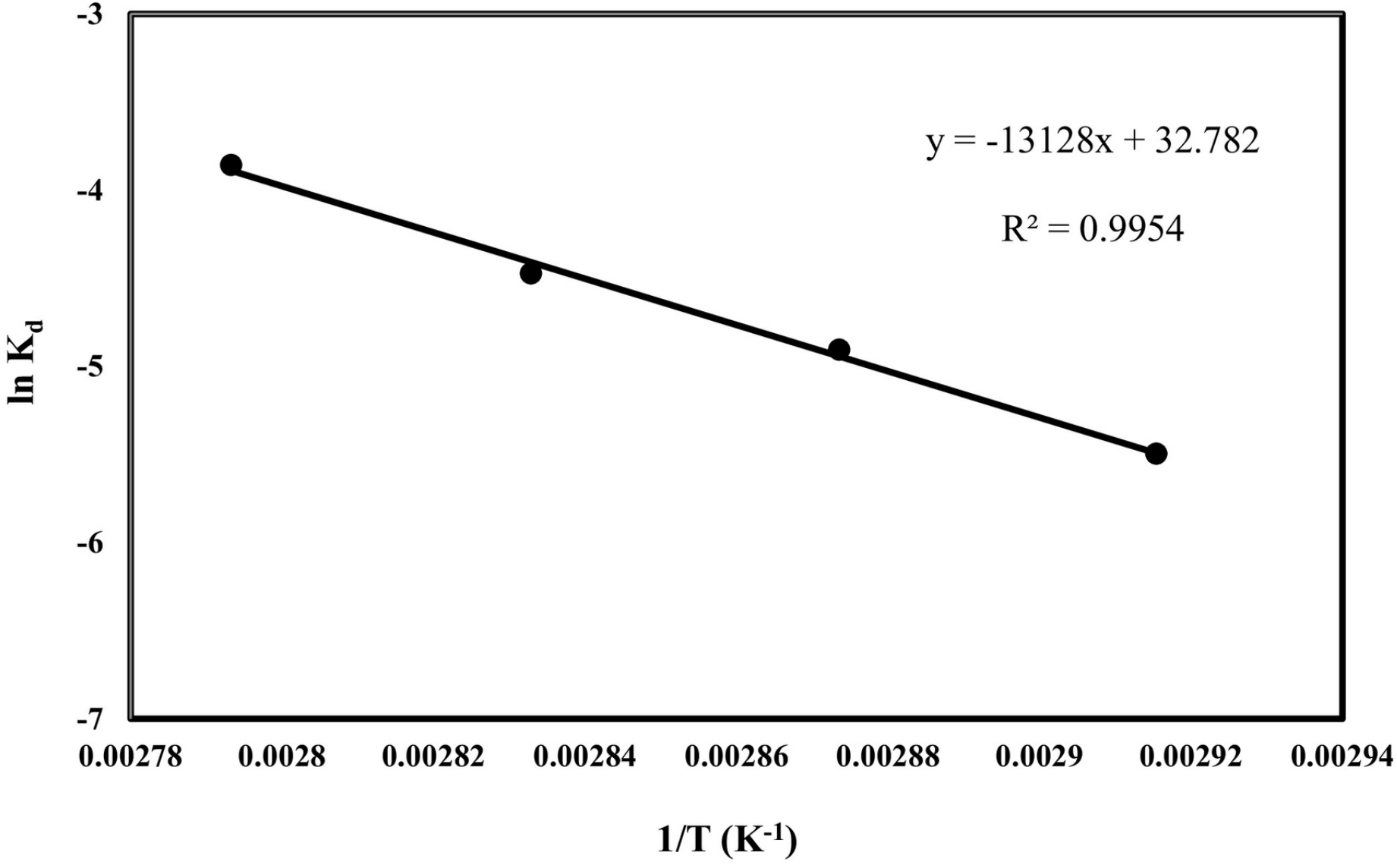

y = -13128x + 32.782
R² = 0.9954
ln K_d
-3
-4
-5
-6
-7
0.00278
0.0028
0.00282
0.00284
0.00286
0.00288
0.0029
0.00292
0.00294
1/T (K^-1)

Figure 5

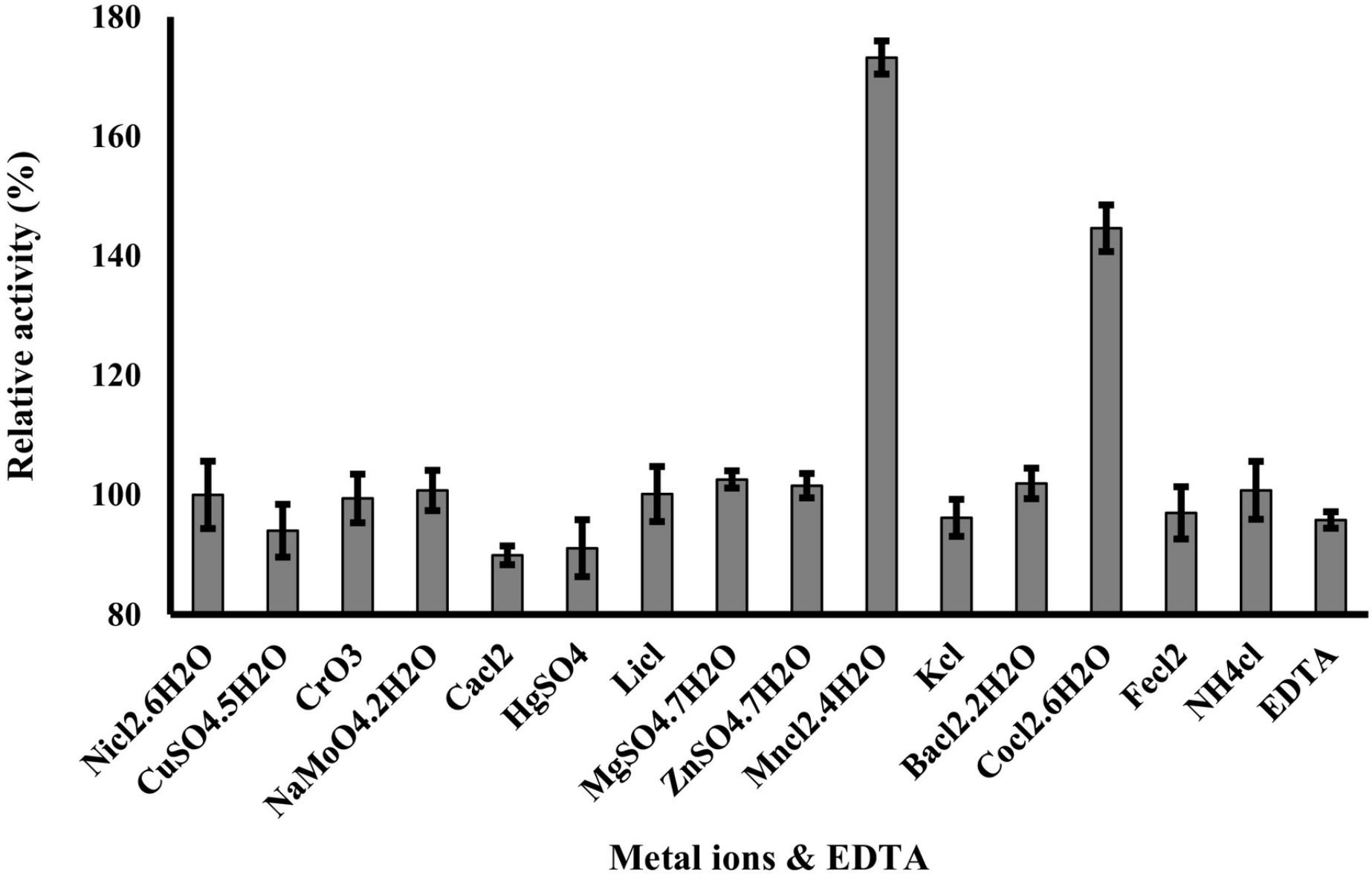
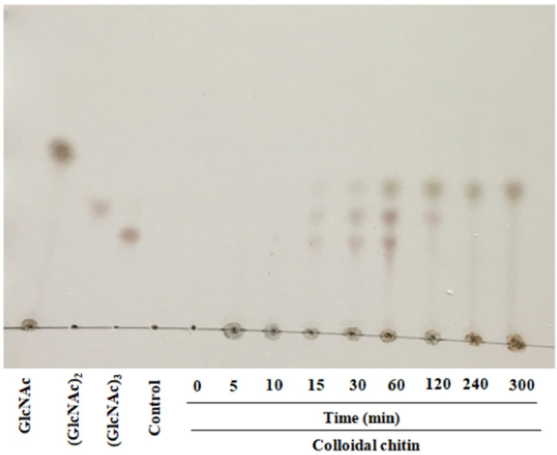
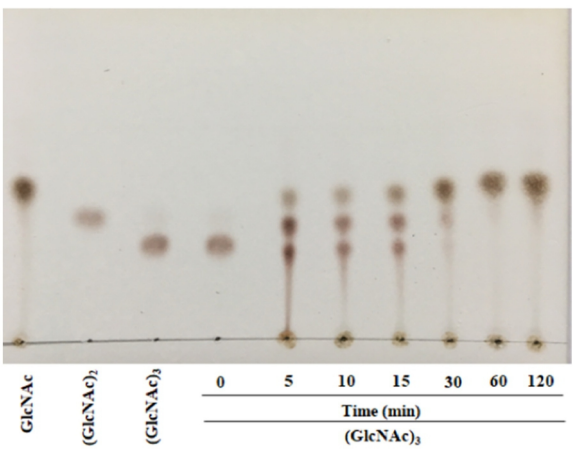

180
160
140
120
100
80
Relative activity (%)
Nicl2.6H2O
CuSO4.5H2O
CrO3
NaMoO4.2H2O
Cacl2
HgSO4
Licl
MgSO4.7H2O
ZnSO4.7H2O
Mncl2.4H2O
Kcl
Bacl2.2H2O
Cocl2.6H2O
Fecl2
NH4cl
EDTA
Metal ions & EDTA

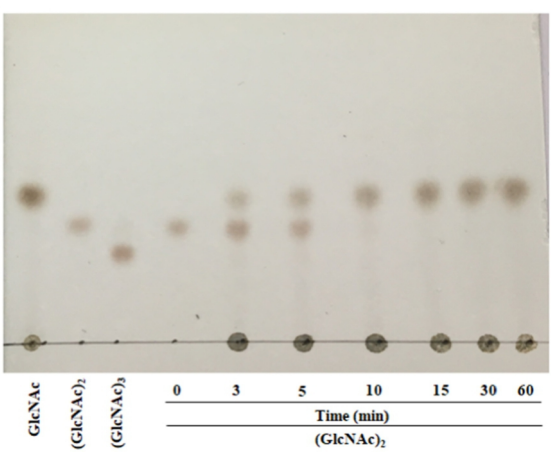
Figure 6


GlcNAc
(GlcNAc)2
(GlcNAc)3
Control
0 5 10 15 30 60 120 240 300
Time (min)
Colloidal chitin

(a)


GlcNAc
(GlcNAc)2
(GlcNAc)3
0 5 10 15 30 60 120
Time (min)
(GlcNAc)3

(b)


GlcNAc
(GlcNAc)2
(GlcNAc)3
0 3 5 10 15 30 60
Time (min)
(GlcNAc)2

(c)

Figure 7