

# Production of *N*-Acetylglucosamine Using Recombinant Chitinolytic Enzymes

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**Abstract** The pharmaceutically important compound *N*-acetylglucosamine (NAG), is used in various therapeutic formulations, skin care products and dietary supplements. Currently, NAG is being produced by an environment-unfriendly chemical process using chitin, a polysaccharide present in abundance in the exoskeleton of crustaceans, as a substrate. In the present study, we report the potential of an eco-friendly biological process for the production of NAG using recombinant bacterial enzymes, chitinase (CHI) and chitobiase (CHB). The treatment of chitin with recombinant CHI alone produced 8% NAG and 72% chitobiose, a homodimer of NAG. However, supplementation of the reaction mixture with another recombinant enzyme, CHB, resulted in approximately six fold increase in NAG production. The product, NAG, was confirmed by HPLC, TLC and ESI-MS studies. Conditions are being optimized for increased production of NAG from chitin.

**Keywords** *N*-acetylglucosamine · Chitin · Chitobiase · Chitinase · Gene cloning

## Introduction

Chitin is an insoluble linear polymer of  $\beta$ -1,4 linked *N*-acetylglucosamine (NAG) and is the second most

abundant biopolymers in nature after cellulose. It is a common constituent of shells of crustaceans, exoskeletons of insects, arthropods and fungal cell walls. It is abundant in the marine environment and the worldwide annual recovery of chitin from the processing of marine crustaceans is estimated to be 80,000 metric tons per annum [1].

The degradation of chitin produces various commercially important products including NAG [2–4]. The latter compound is used as an anti-inflammatory agent for the treatment of various diseases like osteoarthritis, inflammatory bowel disease and gastritis [5, 6]. It also possesses anti-tumor properties and has also been proposed for the treatment of autoimmune diseases. Glucosamine which is a derivative of NAG, has been shown to regenerate joint cartilage and is been used extensively in the treatment of osteoarthritis [7]. Presently, NAG is being derived using chemical processes by heating chitin in concentrated hydrochloric acid, which poses great technical and environmental concerns [8].

The enzymatic breakdown of chitin by chitinases has been reported from our laboratory [9, 10] as well as from others [11–15]. The chitinase gene (*chi*) of *Enterobacter* sp. NRG4 has already been cloned and characterized in our laboratory [10]. This recombinant CHI hydrolyzed swollen chitin into chitobiose (major fraction) and NAG (minor fraction). Chitobiose, which is a homodimer of NAG, can be hydrolyzed into NAG by the enzyme chitobiase, CHB. Many microbes including *E. coli* [16–18] are known to produce CHB. As the genome sequence of *E. coli* is known, its *chb* gene was cloned to produce large amount of recombinant CHB.

In the present study, we demonstrate the potential of NAG production from swollen chitin using a mixture of recombinant enzymes, chitinase (CHI; EC 3.2.1.14) and chitobiase (CHB; EC 3.2.1.52).

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## Materials and Methods

### Materials

All chemicals used in the study were purchased from Sigma-Aldrich (USA) and were of highest purity, IPTG was procured from USB Corporation (USA), Nickel–nitrilotriacetic acid (Ni–NTA) agarose from Qiagen (USA) and bacterial culture media from HiMedia Laboratories (India). All buffers were prepared in MilliQ™ water of 18.2  $\Omega$  resistivity and filtered with 0.22  $\mu$ m filters (Millipore).

### Bacterial Strains and Plasmids

The *E. coli* strains (K12, DH5 $\alpha$ , M15 and BL21(DE3)) with/without plasmids (pQE-30, pSTR1 and pMRT1) were routinely grown in Luria–Bertani (LB) medium supplemented with, if needed, appropriate concentrations of ampicillin (100  $\mu$ g/ml) and/or kanamycin (50  $\mu$ g/ml) (HiMedia Laboratories, India).

### Cloning of Chitobiase (*chb*) Gene

The genomic DNA of *E. coli* K12 strain was extracted from harvested log phase culture by HiPurA™ Bacterial and Yeast Genomic DNA Purification Spin Kit (HiMedia laboratories, India). The *chb* gene was amplified from *E. coli* genomic DNA using *Taq* DNA polymerase (Fermentas, Germany) and a set of modified primers (Forward, 5'-ATGCGGGAGCTCGTGGGTCCAGTAATGTTGGATGTCG-3'; Reverse, 5'-ATGCGGCTGCAGTTAGTGACCTGCTTCTCTTCCTGC-3') containing *Sac*I and *Pst*I restriction sites (Bold letters). These primers were designed based on the sequence of *nagZ* gene of *E. coli* K12 strain accessed from NCBI's database. The thermal cycling was achieved in MyCycler™ (Biorad Laboratories, USA) according to the following program: 94°C/4 min, 35 cycles of 94°C/30 s, 60°C/40 s, and 72°C/3 min and a final extension at 72°C for 10 min. The amplified *chb* amplicons were ligated in pQE-30 with T4 DNA ligase (Fermentas, Germany) (16°C, overnight in a cooling water bath) after restriction digestion. The ligation product was transformed into competent *E. coli* DH5 $\alpha$  cells prepared by CaCl<sub>2</sub> method [19] and plated on LB agar plates supplemented with ampicillin (100  $\mu$ g/ml).

### Expression, Solubilization and Purification of Recombinant CHB

The *chb*<sup>+</sup> recombinant plasmid (pSTR1) was transformed into competent *E. coli* M15 cells followed by incubation at 37°C to monitor the overexpression and purification of the

recombinant protein. *E. coli* M15 carrying *lacI* plasmid pREP4 and pSTR1 was induced for overexpression with 0.5 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside (IPTG) at the mid-exponential growth phase and incubated at 20°C for 18 h. For purification of His-tagged *E. coli* recombinant CHB, IPTG-induced cells were harvested by centrifugation (8,000 rpm/20 min/4°C) and resuspended in 20% (w/v) lysis buffer (50 mM Tris–HCl, pH 7.0, 300 mM NaCl, 1 mM phenylmethylsulfonyl fluorid and 10 mM imidazole). Resuspended cells were lysed using a probe type ultrasonicator (Vibra-Cell, USA) with pulse-rest cycle (30 cycles; 10 s pulse with 10 s rest at 4°C). After centrifugation (13,000 rpm/30 min/4°C), the supernatant was allowed to bind to Ni–NTA agarose matrix (2 ml) equilibrated with lysis buffer followed by the washings with 5 column volumes of lysis buffer having imidazole (20 and 30 mM, respectively). Purified CHB was eluted with lysis buffer amended with 200 mM imidazole. 1 ml fractions were collected and checked for purified protein on 10% SDS-PAGE. The fractions showing purified protein were then pooled and dialyzed against 20 mM Tris–HCl, pH 7.0 and again checked by SDS-PAGE. Protein concentrations were determined by bicinchoninic acid (BCA) method using bovine serum albumin (BSA) as a standard. Recombinant CHI was induced, overexpressed and purified by using optimized protocol [10].

### Sequencing of Cloned DNA Fragments

The homogeneity of cloned *chb* gene was confirmed by DNA sequencing of pSTR1 using universal M13 primers and the sequence was submitted to GenBank.

### Enzyme Assays

**Chitobiase activity:** The CHB activity was determined by measuring the release of *p*-nitrophenol from *p*NP-NAG (*p*-nitrophenyl- $\beta$ -*N*-acetyl-D-glucosaminide) (Sigma-Aldrich, USA) as described by Cheng et al. [16] with slight modifications. A 400  $\mu$ l of 1 mM *p*NP-NAG in 50 mM Tris–HCl (pH 7.0) was added to 0.5  $\mu$ g of enzyme and total volume was made to 900  $\mu$ l with 50 mM Tris–HCl (pH 7.0). The reaction was stopped after 1 h incubation at 37°C by addition of 100  $\mu$ l of 1.25 M K<sub>2</sub>CO<sub>3</sub>. The amount of *p*-nitrophenol liberated was estimated by measuring the absorbance at 420 nm. One unit of CHB activity is defined as the amount of enzyme required to liberate 1  $\mu$ mol of *p*-nitrophenol per hour at 37°C.

**Chitinase activity:** The swollen chitin (HiMedia Laboratories, India) prepared by the method of Moneral and Reese [20] was used as substrate. To 1.0 ml of chitin suspension (5 mg/ml, pH 7.0), 500  $\mu$ l of recombinant CHI was added and incubated at 37°C/20 min followed by

centrifugation (8000 rpm/10 min). The supernatant so obtained was used to estimate the amount of reducing sugars liberated [21]. One unit of chitinase is defined as the amount of enzyme required to liberate 1  $\mu$ mol of reducing sugars in 1 h.

**Zymography:** The oligomeric state of the recombinant CHB was determined by zymographic analysis. The activity staining was performed by incubating the native gel on agarose having 200 mM *p*NP-NAG at 37°C.

#### Characterization of the Recombinant CHB

The optimum temperature and pH for CHB activity were determined by performing the enzyme assays at different incubation temperatures (30–80°C) and pH (4.0–10.0) using different buffers (Acetate, Tris–HCl, Carbonate bicarbonate, 50 mM). Thermostability profile was analyzed by incubating the enzyme at 37°C and 50°C for different duration and the residual activity was subsequently determined under standard assay conditions. The enzyme kinetics were studied by elucidation of the  $K_m$  and  $V_{max}$  of recombinant CHB by Lineweaver–Burk representation of the Michaelis–Menten model.  $k_{cat}$  and  $k_{cat}/K_m$  were also calculated.

#### Hydrolysis of Swollen Chitin and Identification of Endproducts

The recombinant CHB and CHI were added in different ratios (1:3, 1:6, 3:1, 6:1, 10:1) i.e. 50:150; 50:300; 150:50; 300:50 and 5000:50  $\mu$ g to 50 mg swollen chitin in 50 mM Tris–HCl (pH 7.0) and 5% glycerol in a volume of 10 ml and incubated at 37°C/24 h. After incubation the reaction was stopped by immersing the reaction tubes in boiling water for 5 min. Reaction mixtures were then centrifuged (8,000 rpm/10 min) and supernatant was used to check for NAG.

**HPLC:** The hydrolytic products of chitin, obtained after treatment with recombinant enzymes, were resolved by high performance liquid chromatography (Shimadzu, USA). The HPLC system was supported with LC10AT HPLC pumps, RID10A detector and Shimpack CLC-NH<sub>2</sub> column (4  $\times$  250 mm) from Shimadzu. 20  $\mu$ l of the sample volume was injected and eluted with 75% (v/v) acetonitrile in water with a flow rate of 1.0 ml/min.

**TLC:** Each reaction mixture was applied 10 times (1  $\mu$ l each) onto a Silicagel 60 F<sub>254</sub> aluminium sheet (Merck, Germany) and chromatographed in a mobile phase comprising isopropanol:ethanol:H<sub>2</sub>O::5:2:1 (v/v). The products were detected by spraying 10% sulphuric acid in ethanol on the TLC plate followed by baking at 180°C/3 min. The standard mixture of 10 nmol NAG (G1) and chitobiose (G2) was also run alongside.

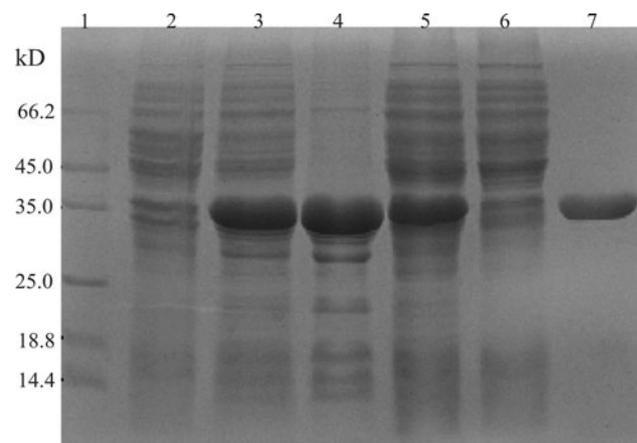
**Mass Spectroscopy:** The molecular mass of the hydrolyzed end-products were analyzed by ESI-MS (Electrospray Ionization Quadrupole Time-of-Flight Mass Spectrometry, Q-TOF, Waters-Co., USA). The ratio of mass to charge (*m/z*) in the range 100–500 units was scanned with exposure for 1 s/step and an interscan duration of 0.1 s/step. During ESI-MS analysis, the quadrupole scan mode was used under a capillary voltage of 2.8 kV, source block temperature at 80°C, pump flow of 10  $\mu$ l/min and desolvation (solvent removal) temperature of 200°C.

## Results and Discussion

#### Cloning, Expression and Purification of Recombinant CHB

The structural gene, *chb*, of chitobiase from *E. coli* K12 was PCR amplified and cloned in pQE-30. The cloning of *chb* gene in pQE-30 was confirmed by restriction enzyme digestion, colony PCR and sequencing. Forward and reverse DNA sequencing of the *chb* gene showed 100% identity with nucleotide sequence present in the genome of *E. coli* K-12 (NCBI database). The *chb* gene nucleotide sequence was submitted to GenBank and appears with accession number GQ906590.

The recombinant CHB was expressed using IPTG (1 mM) in *E. coli* M15. A thick band corresponding to the expected size of  $\sim$ 38.5 kD was observed in SDS-PAGE after coomassie-blue staining (Fig. 1). The densitometric analysis of the gel indicated the protein to be expressed to



**Fig. 1** Overexpression and purification of recombinant CHB. Lane 1 standard protein molecular weight markers; lane 2 whole cell extract from uninduced *E. coli* M15; lane 3 whole cell extract from *E. coli* M15 after induction; lane 4 total cellular soluble extract after induction; lane 5 IB after induction; lane 6 Ni-NTA affinity column flowthrough; lane 7: purified CHB, respectively

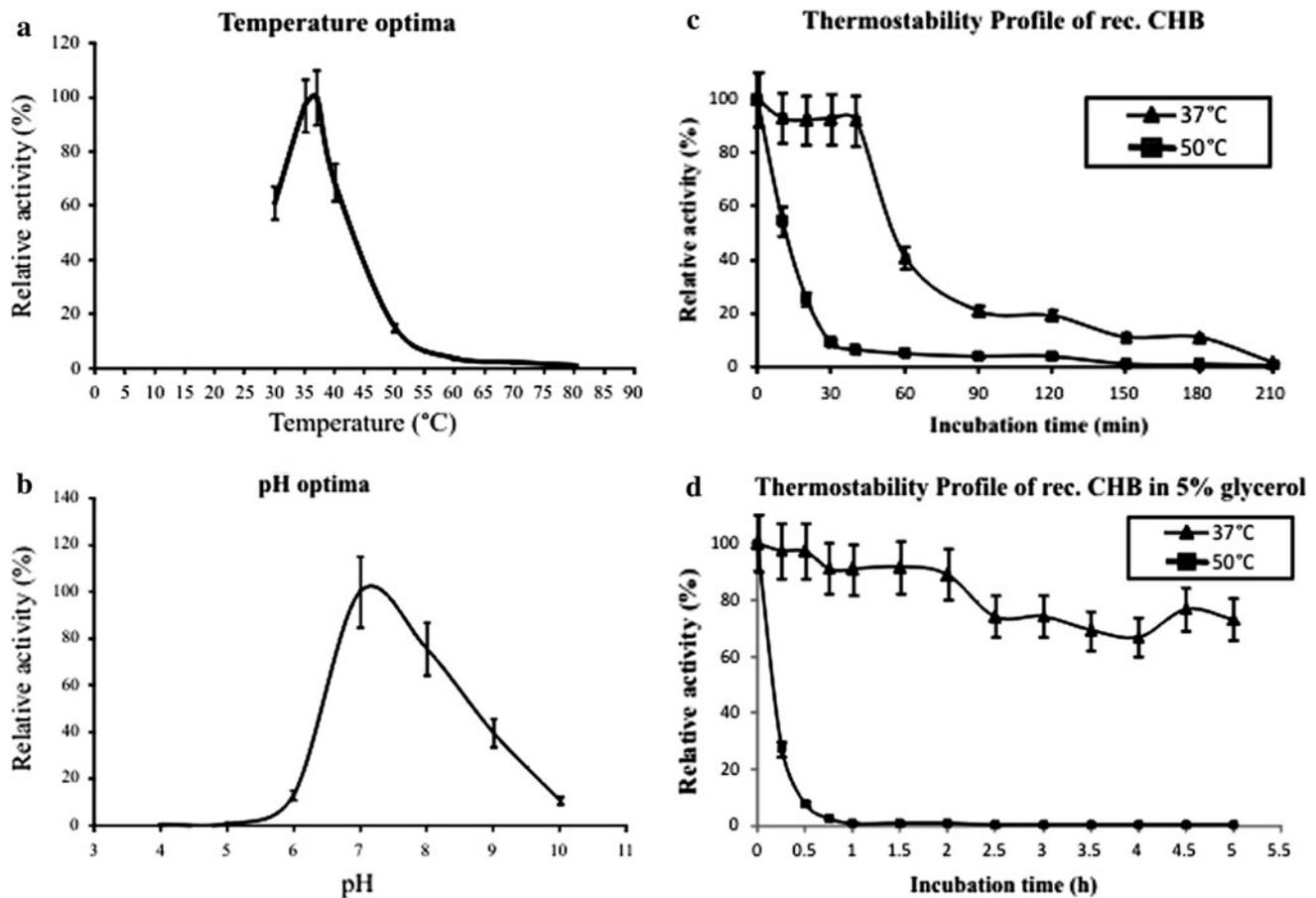
**Table 1** Purification profile of recombinant CHB

	Total volume (ml)	Total enzyme activity (U) <sup>a</sup>	Total protein content (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude CHB	11.5	977,500	97.75	10,000	100	1
Purified CHB	6	594,000	18	33,000	60.8	3.3

<sup>a</sup> 1 U was defined as the amount of enzyme that catalyzes the release of 1  $\mu$ mol of pNP in 1 h at 37°C

levels as high as 35% of the total protein. The induction studies carried out at an incubation temperature of 37°C revealed that over-expressed recombinant CHB was present in the cell pellet and very little amount was going to soluble fraction, thus, forming inclusion bodies (IB). Lowering the incubation temperature and IPTG concentrations in the nutrient medium have been shown to produce functionally active and soluble recombinant proteins in *E. coli* [22, 23]. The optimization of induction conditions [temperature (20°C)/time (18 h)/IPTG (0.5 mM)] resulted in production of soluble recombinant CHB in the

cell free supernatant. This recombinant CHB was purified using affinity chromatography column packed with Ni-NTA matrix. The purified protein was homogenous as indicated by a single band on SDS-PAGE (Fig. 1). The recombinant CHB was purified 3.3 folds with 60.8% recovery and a specific activity of 33000  $\mu$ mol/h/mg (Table 1). These values are higher than reported earlier (528  $\mu$ mol/h/mg) [18] using recombinant CHB from *E. coli* K-12 AB1157. The native PAGE and zymography data is in corroboration to earlier reports [17] that recombinant CHB exist as a monomer.



**Fig. 2** Effect of temperature and pH on recombinant CHB activity. **a** Temperature dependent activity profile. **b** pH dependent activity profile. The data has been represented as % relative activity with highest activity observed at 37°C and pH 7.0 taken as 100%.

**c** Thermostability profile of recombinant CHB at 37°C and 50°C. **d** Thermostability profile of recombinant CHB with 5% glycerol at 37°C and 50°C. Each point representing mean  $\pm$  SE of three independent measurements

## Biochemical Characterization

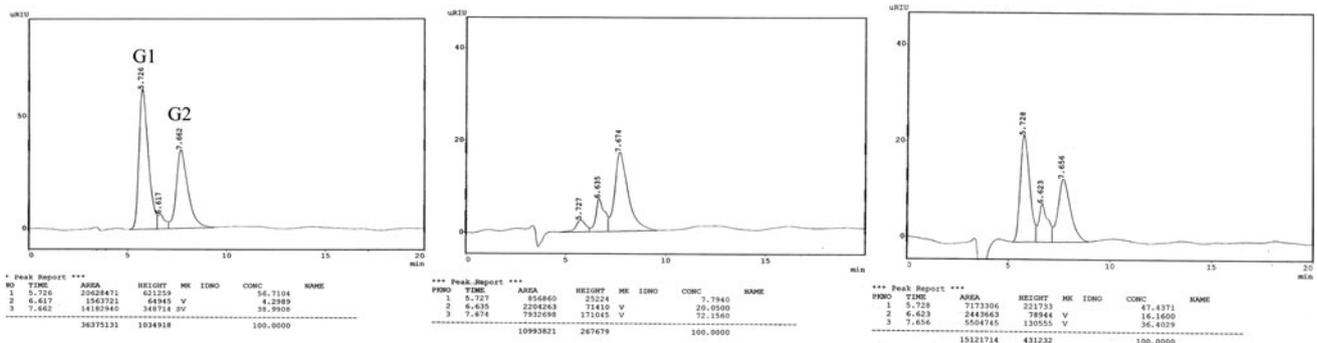
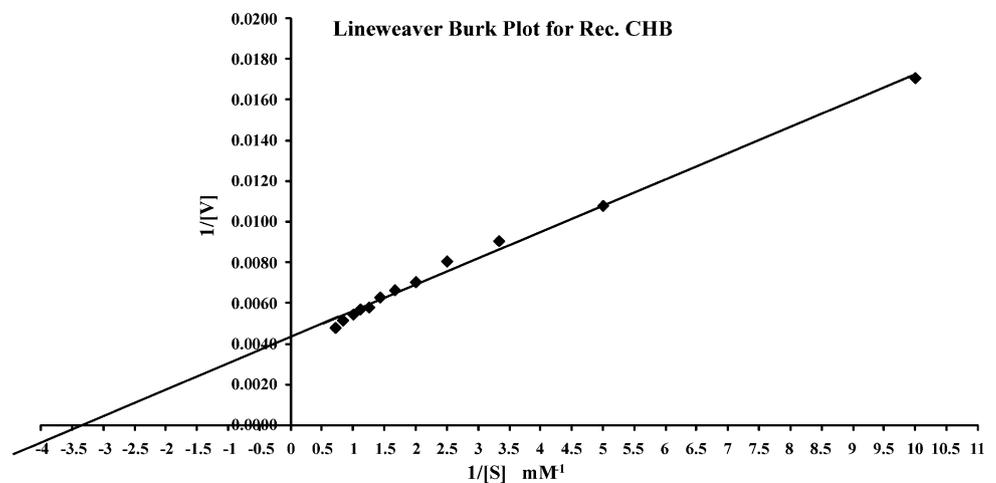
The observed optimum temperature (37°C, Fig. 2a) and pH (7.0, Fig. 2b) for recombinant CHB enzyme was consistent with earlier *E. coli* native enzyme reports (i.e. 37°C and pH range 6.8–7.7) [17, 18]. The recombinant CHB lost 60% of the activity at 37°C after 1 h incubation (Fig. 2c) which revealed that the enzyme might be thermo-labile which could be due to structural instability of the protein. This prediction was further supported by using the ProtParam server (<http://expasy.org/tools/protparam.html>) which is used for the computation of various physical and chemical parameters for a protein. However, the thermostability of recombinant CHB was enhanced (75% residual activity after 5 h at 37°C) by the addition of 5% glycerol (Fig. 2d) and the estimated half life was 24 h. The stability was enhanced only at 37°C in 5% glycerol while no effect of glycerol was observed at 50°C. The stability increased as the glycerol is known to shift the native protein assembly to more compact states and also inhibits protein aggregation during the refolding of many proteins along with acting as a cryoprotectant. The enzyme was reasonably stable at 4°C

showing 100% activity even after 2 months of storage. The apparent  $K_m$  was found to be 0.294 mM as calculated from Lineweaver–Burk plot (Fig. 3). The  $K_m$  value for *p*NP-NAG is in close proximity to the previous reports (0.310 mM) [18] while slightly higher  $K_m$  (0.43 mM) was reported by others [17]. The  $V_{max}$  for *p*NP-NAG was observed to be 227.27  $\mu\text{M}/\text{h}/\mu\text{g}$  of protein at 37°C. The value is higher than previous reports on *E. coli* [18]. This can be due to different enzyme properties such as enhanced activity because of different induction and reaction conditions and also different expression host. The  $k_{cat}$  for the recombinant CHB is 0.407  $\text{s}^{-1}$  and catalytic efficiency constant ( $k_{cat}/K_m$ ) is observed to be 1.384  $\text{s}^{-1} \text{mM}^{-1}$ .

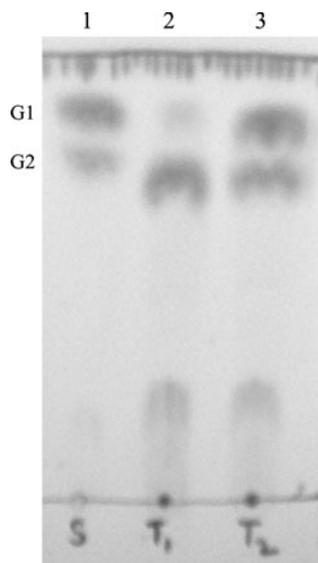
## Identification of Hydrolysis Products of Chitin

The analysis of hydrolytic products of swollen chitin produced by the action of recombinant CHI (300  $\mu\text{g}$ ) showed production of chitobiose (72%) and NAG (8%) as observed by HPLC. However, the experiment performed by using a mixture of recombinant CHI (300  $\mu\text{g}$ ) and recombinant CHB (50  $\mu\text{g}$ ) resulted in enhanced production of NAG

**Fig. 3** Lineweaver–Burk plot for recombinant CHB. Each data point represents the mean value of three different experiments



**Fig. 4** HPLC profiles. **a** Standard NAG (G1) and chitobiose (G2). **b** CHI treated substrate. **c** CHI and CHB treated substrate



**Fig. 5** TLC analysis of hydrolytic products. *Lane 1* Standard mixture of G1–G2; *lane 2*: CHI treated hydrolysis products; *lane 3*: CHI and CHB treated hydrolysis products

(47%) and reduction in chitobiose (36%) thereby suggesting that chitobiose was being hydrolyzed into NAG (Fig. 4). The recombinant CHB appeared to be stable during the course of the study. TLC analysis (Fig. 5) also revealed the conversion of chitobiose to NAG when both enzymes were used simultaneously. The formation of NAG and chitobiose was confirmed by ESI-MS analysis (Fig. 6) which revealed the production of NAG with mol wt.  $221.5 + 1.0$  and chitobiose with mol wt  $424.3 + 1.0$  (1.0 added for  $H^+$ ), along with certain other nonspecific

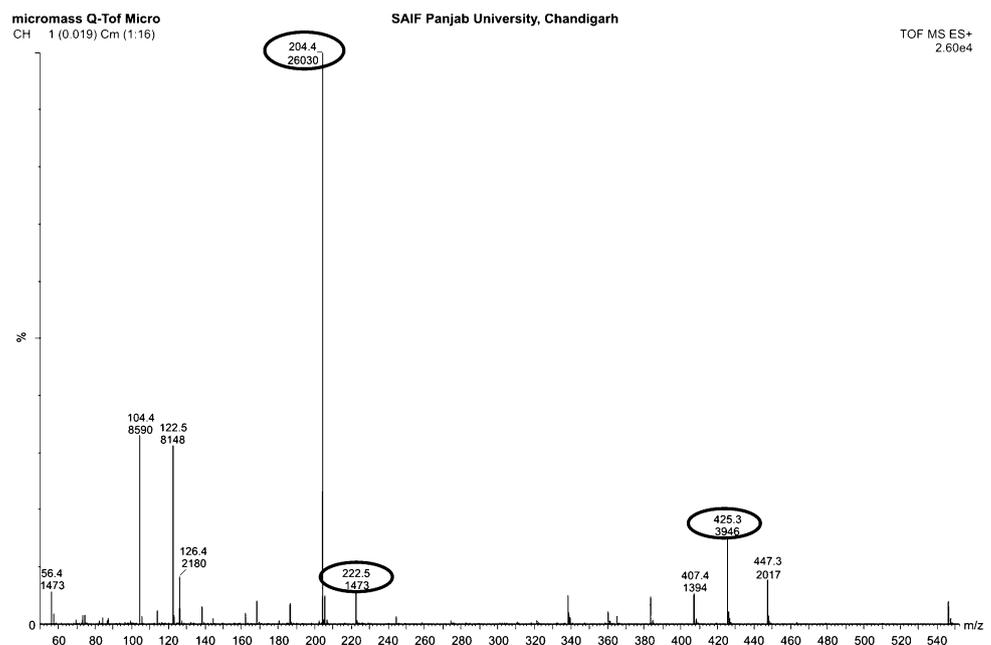
moieties. However, a hydroxal group from most of the NAG was removed during ionization process, yielding a product with molecular mass of 204.4.

To the best of our knowledge, this is the first report on the production of NAG from chitin using cocktail of recombinant enzymes from bacterial sources. There are a few reports [11, 14, 24] on the enzymatic conversion of chitin to NAG by the use of crude native enzymes. However, their protocols for the production and purification of chitin hydrolyzing enzymes are laborious, time-consuming and costly as compared to the process described here. The yield of NAG obtained by us is difficult to compare with others because of many variables, e.g. substrate used, source of enzymes, level of purification, incubation time/temperature and pH of the reaction buffer. The recombinant enzymes, used in our case, contain 6 $\times$ -His for one step purification. Attempts are going on in our laboratory for optimization of hydrolytic conditions for increased production of NAG from chitin.

## Conclusions

The structural gene for chitobiase, *chb*, of *E. coli* K-12 was cloned, over-expressed and the recombinant enzyme purified by affinity chromatography in a single step. The recombinant purified protein showed similar biochemical properties compared to native CHB. In this study we optimized the ratio (1:6) of recombinant CHB and CHI to enhance the hydrolysis of chitin and improve the NAG yield by six fold (approx. 47% from 8%).

**Fig. 6** ESI-MS graph of enzymatically hydrolyzed products of chitin



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