# Preparation of chitooligosaccharide from fungal waste mycelium by recombinant chitinase

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**Abstract**

This study aimed to develop an enzymatic method for conversion of chitin from fungal waste mycelia to chitooligosaccharides. The recombinant chitinase *Ll*Chi18A from *Lactococcus lactis* was over-expressed by *Escherichia coli* BL21 (DE3) and purified by affinity chromatography. The enzymatic properties of the purified enzyme were studied by chitin oligosaccharides. Waste mycelium was pre-treated by alkaline. The optimal conditions for hydrolysis of fungal chitin by recombinant chitinase were determined by Shales method. HPLC/ESI-MS was used to determine the content of N-acetylglucosamine and chitooligosaccharides after hydrolysis. The level of reducing sugar released from pretreated mycelium by chitinase increased with the reaction time during 6 days. The main product in the hydrolysates was chitinbiose. After hydrolysis by chitinase for 5 d, the yield of chitinbiose from waste mycelium was around 10% with purity around 70%. Combination of chitinase and snailase remarkably increased the yield to 24% with purity of 78%. Fungal mycelium which contains chitin is a new potential source for obtaining food grade chitooligosaccharides.

**Keywords:** Chitinase, Chitin, Waste mycelium, Hydrolysis, Chitobiose

## 1. Introduction

Chitin is the principal structural component of the cell wall of most fungi, insect exoskeletons, and the shells of crustaceans.[1] Its estimated annual production is $10^{10}$ to $10^{11}$ tons.[2] Chitin is a crystalline, insoluble and biocompatible polymer composed of β-1, 4-linked N-acetylglucosamine (GlcNAc) and glucosamine (GlcN). However, its oligomers are water-soluble and have numerous biological activities including lowering of blood cholesterol and blood pressure,[3] protective effects against infections,[4] controlling arthritis,[5] and enhancing antitumor properties[6] and anti-adhesion activity. They have many applications in food and biomedical industries.[5,7-10] They also have applications in agriculture to elicit protective responses in various plants and possess antimicrobial activities against a wide spectrum of phytopathogens.[11]

Chitin and its deacetylated derivative chitosan are the basic materials to produce chitooligomers. Besides the exoskeletons and shells of crustaceans,[12] the cell walls of most fungi are also good resources for chitin and chitosan. The cell walls of filamentous fungal contain up to 10-30% of chitin in the cell wall dry weight.[13] *Aspergillus* sp. and *Penicillium* sp. are extensively used for biotechnological production of antibiotics, enzymes and organic acids.[14] About 80,000 tons of *A. niger* waste mycelium results from annual world requirements of citric acid.[15] Fungal chitin can be extracted from fungal mycelial waste, which is almost costless and easy to obtain from fungal fermentation industries. Although chitin from *A. niger* is fully acetylated, it has a lower crystallinity (0.37) when compared to commercial chitin (1.25) from crustaceans,[15] thus it is a good renewable resource to produce chitooligomers.

Chitooligomers are converted enzymatically or chemically from chitin or chitosan. Compared with chemical hydrolysis, enzymatic conversion of chitosan/ chitin to oligomers is more environmentally friendly. Chitosan can be prepared from the waste *Aspergillus niger* mycelium by enzymatic conversion with lysozyme, snailase, neutral protease, and chitin

deacetylase to break down the cell wall, and to achieve deproteinization and deacetylation.[16] To date, however, the use of chitinase for specific preparation of chitooligomers from fungal waste mycelium has not been reported. Chitinases occur in actively growing chitinous fungi, where they serve to balance wall synthesis and wall lysis during growth. Chitinases are also produced by mycoparasites to inhibit mycelial growth, spore germination, and germ tube elongation,[17] degrading not only the tips of mature hyphae, but also the chitin-glucan complex of the cell wall and sclerotia.[18] Endochitinases (EC 3.2.1.14) cleave the linkage between GlcNAc-GlcNAc, GlcN-GlcNAc, or GlcNAc-GlcN in chitin chains to release smaller chitin oligomers of variable size.[19] Recombinant endochitinase 18A from *Lactococcus lactis*[20] is a food grade chitinase, which partially degraded α-chitin after 2 weeks reaction time. Compared with α-chitin, the degree of crystallinity of fungal chitin is lower because of presence of other polysaccharides and proteins.

This study therefore aimed to use the recombinant chitinase *Ll*Chi18A from *L. lactis* ssp. *lactis* IL1403 for conversion of fungal chitin into chitooligomers. To characterize the enzymatic properties of *Ll*Chi18A, the hydrolysates of oligosaccharides were analyzed. For conversion of fungal chitin, waste mycelia were pretreated by alkali and compared with acid or salt solution. The process of hydrolysis by chitinase was optimized and analysis of the hydrolysates from waste mycelia was determined by HPLC/ESI-MS. The yield of oligomers after hydrolysis by chitinase was compared with snailase and the combination of chitinase and snailase.

## 2. Materials and Methods

### 2.1 Materials

Waste mycelium of *Aspergillus niger* was provided by Huangshi Xinghua Biochemical Limited Company (Huangshi, Hubei, China). 4-Methylumbelliferyll-D-N'N''N'''-triacetylchitotrioside [4-MU-$(GlcNAc)_3$], 4-methylumbelliferone (4-MU) and N-acetyl-D-

glucosamine were purchased from Sigma-Aldrich (St Louis, USA). The plasmid pETM11-*Ll*Chi18A was kindly provided by Gustav Vaaje-Kolstadt from the Norwegian University of Life Sciences. *Escherichia coli* BL21 (DE3) was purchased from Invitrogen Co (Carlsbad, US). Chitooligosaccharides with different degrees of polymerization (DPs) from 2 to 6 (chitobiose, chitotriose, chitotetraose, chitopentaose, and chitohexaose) were purchased from BZ Oligo (Qingdao**,** Shandong, China). Silica gel plates were purchased from Marine chemical (Qingdao, Shandong, China). All the reagents used were of analytical or chromatographic grade. Snailase was purchased from Huashun Biotechnology Co. Ltd (Wuhan, China). Colloidal chitin was prepared from chitin (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) as described[21]. Water-soluble chitosan was prepared by N-acetylation from chitosan to achieve a degree of acetylation of 40 – 60%[22].

**2.2 Enzyme expression and purification**

The plasmid pETM11-*Ll*Chi18A was cloned in *Escherichia coli* BL21 (DE3). *E.coli* BL21 (DE3) harboring pETM11-*Ll*Chi18*A* was incubated at 37 ℃ in Luria-Bertani (LB) medium containing 50 mg $L^{-1}$ kanamycin to $OD_{600nm}$ of 0.6. Isopropy-β-D-thiogalactoside (IPTG) was added to a concentration of 0.05 mM. Cultivation was continued for another 12 hours at 20 ℃ and 250 rpm. Cells were harvested by centrifugation, resuspended in PBS buffer (pH 7.4), and disrupted by ultrasonication. The supernatant obtained by centrifugation at 12,000 ×g for 10 min was loaded to a Ni-NTA column (1 ml, Sangon, Shanghai, China) and equilibrated with 100 mM Tris/HCl (pH 8.0). The recombinant protein was eluted with elution buffer containing 100 mM imidazole in 100 mM Tris/ HCl (pH 8.0). The peak containing chitinase was collected, concentrated by ultrafiltration with a molecular cutoff of 10 kDa at 4,000 ×g for 20 min, and stored at -20 ℃. The purity and the molecular weight of recombinant *Ll*Chi18A were determined by SDS-PAGE (10%). The protein content was measured by Bradford method using bovine serum albumin (BSA) as standard.[23]

**2.3 Enzymatic conversion of chitooligomers by recombinant chitinase 18A**

The specific enzyme activity of purified *Ll*Chi18A was measured by using 4-MU-$(GlcNAc)_3$ as a substrate at pH 3.8 and 37 °C[20]. Reactions contained 2.0 nM chitinase 18A in 50 mM HAc-NaAc buffer (pH 6.5) and were incubated at 40 °C for 5 min. Reactions were also performed with colloidal chitin and water-soluble chitosan at a concentration of 0.1% to determine whether the optimum pH and the optimum temperature of *Ll*Chi18A were dependent on the substrate.

Reactions were also performed with each chitin oligosaccharides with different DPs from 2 to 6 at a concentration of 10 g $L^{-1}$. Enzymatic reactions were stopped after 5 to 10 min by heating at 100 °C for 5 min. The carbohydrates in the reaction mixtures were identified by thin-layer chromatography (TLC) using silica gel plates. Chitin oligosaccharides with different DP from 2 to 6 served as standards. TLC plates were developed with a solvent system of n-propanol: methanol: ammonia: water =5: 4 : 2: 1 (v/v). Carbohydrates were visualized by spraying 0.5% diphenylamine and 0.5% aniline in 95% ethanol solution and by heating them in an oven at 80 °C for 30 min.

The quantification of substrates and products of enzymatic conversion were achieved with a Supelcosil LC-NH2 column (250mm×4.6mm, 5um, Sigma Aldrich, Oakville, Canada) coupled with a UV detector (195 nm). Separations were conducted on an Agilent 1200 series LC system (Agilent Techologies, Palo Alto, CA). Samples were eluted using isocratic mobile phase with acetonitrile/water at 70:30 or 75:25 (v/v) at a flow rate of 0.3 mL $min^{-1}$ at 30 °C. The reaction products were quantified by using GlcNAc and chitin oligosaccharides with different DPs from 2 to 6 as external standards.

**2.4 Pretreatment and hydrolysis of fungal waste mycelia**

In order to compare the alkali pretreatment with acid and neutral solutions, the fungal waste mycelia was firstly sifted, washed to remove medium components and freeze-dried. Pretreatment was carried out by stirring with 0.5 M NaOH, 0.5 M HCl or 0.6 M NaCl at ambient temperature for 0, 4, 8, 12, and 24 h. The mycelia were filtered and washed with

distilled water to neutral pH. Reducing sugars released during pretreatment were monitored by Shales method[24] using N-acetylglucosamine (GlcNAc) as standard. The content of protein released from fungal waste mycelia was measured by Bradford method as indicated above. FTIR spectra were employed to analyze the chemical structure of waste mycelium after alkali pretreatment and recorded in powder in KBr discs in the range of 4000-400 $cm^{-1}$ on a Nicolet AVA2TAR360 FTIR spectrophotometer (Madison, USA).

**2.5 The effects of pH, temperature, and reaction time on hydrolysis by chitinase**

The effects of pH, temperature, and reaction time on chitinase activity were determined using fungal mycelia after 8 h alkali pretreatment as substrate. To determine optimum temperature, wet mycelium with a water content of 73.7% was mixed with 10 volumes of phosphate buffer. *Ll*Chi18A was added to a level of 0.6 mg of chitinase per g of dry mycelium. Mixtures were incubated for 6 h at temperatures ranging from 27 °C to 57 °C at the pH value of 7.0. To determine the optimum pH, pretreated mycelium and *Ll*Chi18A were incubated in 20 mM acetate buffer with a pH ranging from 3.0 to 6.0, or in 20 mM phosphate buffer with a pH ranging from pH 6.0 to 9.0 at 50 °C. The effect of the reaction time was assayed in 20 mM phosphate buffer (pH 7.0) at 37 °C. Chitinase activity was monitored by sampling every 24h over 6 days. The contents of reducing sugars in the hydrolysates were monitored by Shales method. Values given are the average of three replicates and data are expressed as mean ± standard deviation.

**2.6 Identification and quantification of the chitinase hydrolysis products from waste mycelia**

The pretreated waste mycelium was hydrolyzed by 3 mg of *Ll*Chi18A to 1 g of dry substrate in 20 mM acetate buffer (pH 6.5) at 50 °C for 5 d. The enzymes were inactivated by heating at 100°C for 5 min. The reducing sugars released in the hydrolysates were measured by Shales method as indicated above. The contents of reducing sugar containing GlcNAc were measured by Elson-Morgan method[25] using GlcNAc as standard.

174 **2.7 Hydrolysis by chitinase after 2 h hydrolysis by snailase**

175 The pretreated waste mycelia was hydrolyzed by 4% snailase (Enzyme/ dry Substrate, w/w)
176 in 20 mM acetate buffer (pH 5.4) at 50 ◦C for 2 h and then heated at 100 ◦C for 5 min to
177 inactivate the enzyme. The mycelium was harvested by centrifugation and *Ll*Chi18A was
178 added into the precipitate as indicated above. Incubation of mycelium without addition of
179 chitinase served as control. The reducing sugar and the content of reducing sugar containing
180 GlcNAc were measured as above.

181 **2.8 Identification and quantification of the hydrolysates from waste mycelia**

182 The quantification of the hydrolysates from waste mycelia was performed by HPLC as above.
183 The hydrolysates from waste mycelia were analyzed by a 1200 series HPLC (Agilent
184 Technologies, Santa Clara, US) linked to a 6420 Triple Quad MS system (Agilent
185 Technologies, Palo Alto, CA) with external standards. Electrospray ionization (ESI) was
186 performed to identify the main product in the hydrolysate in negative mode with the optimum
187 ion source parameters as follows: ion spray voltage, -6000 V; curtain gas, 25; declustering
188 potential, -45 V; focusing potential, -170 V, source temperature, 550 ◦C; scanning over a mass
189 range of *m/z* 100-1500. Glucose, GlcN, GlcNAc, and chitin oligosaccharides with DPs from 2
190 to 6 were used as external standards.

191 **2.8 Yield and estimated purity of chitobiose obtained from waste mycelium**

192 To determine the yield of chitobiose from waste mycelium by *Ll*Chi18A and combination of
193 *Ll*Chi18A and snailase, chitobiose contents in the hydrolysates were measured by HPLC. The
194 content of reducing sugar containing GlcNAc was measured by Elson-Morgan method as
195 indicated above. The purity of the hydrolysates was calculated as the percentage of the area
196 of chitobiose to the total peak area of carbohydrates in the chromatogram. The chitin content
197 of waste mycelia was measured by chemical alkali-acid extraction and was determined as
198 20% (w/w) in the dry waste mycelia. The yield of oligomers was calculated as follows.

$$Yield\ (\%) = \frac{Chitin\ oligosaccharides\ in\ the\ dry\ hydrolysate\ [g]}{dry\ mycelium\ [g]\frac{0.2\ g\ chitin}{g\ dry\ mycelium}}$$

## 3 Results

### 3.1 Enzyme purification

The chitinase *Ll*Chi18A was over-expressed by *E. coli* BL21 (DE3) which was induced by IPTG and purified by affinity chromatography. The purity of the recombinant enzyme was determined by SDS-PAGE. In agreement with prior observations, the purification protocol yielded a single protein with Mw 54 kDa.

### 3.2 Enzymatic properties of recombinant chitinase 18A

Enzymatic properties of purified recombinant *Ll*Chi18A were determined with colloidal chitin, water-soluble chitosan, fluorescent substrate, and chitin oligosaccharides. Using 4-MU-$(GlcNAc)_3$ as a substrate, *Ll*Chi18A exhibited a specific activity of 1060 $U{\cdot}mg^{-1}$ and an optimal activity at pH 3.8 and 37°C, in agreement with Vaaje-Kolstad *et al.* [20]. The metal ions $Zn^{2+}$, $Cu^{2+}$, $Co^{2+}$ and $Mn^{2+}$ strongly inhibited *Ll*Chi18A activity at a concentration of 10 mM. However, the hydrolysis rate of 4MU-$(GlcNAc)_3$ was increased by the addition of 1 mM EDTA, 1 mM $Na^{2+}$ or $Mg^{2+}$, or 5 mM $Ba^{2+}$ or $Li^{+}$ (data not shown). With water-soluble chitosan as substrate, the optimum temperature and pH shifted to 60°C and 7.0, respectively. With colloidal chitin as substrate, optimum conditions were 40°C and pH 5.0. The $V_{max}$ for colloidal chitin is much lower (78 times) than that of water-soluble chitosan, indicating that the degradation of insoluble chitin is much slower than that of soluble substrate.

To determine the release of oligosaccharides by chitinase, chitin oligosaccharides with DP from 2 to 6 were used as substrates and products obtained after 5 min of reaction were visualized by TLC (Fig. 1). Chitobiose was not hydrolyzed but chitin oligosaccharides with a DP more than 3 were hydrolyzed by *Ll*Chi18A. The main product was chitobiose and formation of GlcNAc was also observed. The yield of monosaccharide was higher when

oligosaccharides with uneven DP, chitotriose and chitopentaose, were used as substrate (Table 1). In agreement with results from Figure 1, *Ll*Chi18A did not hyrolyse chitobiose and the main product in all reactions was chitobiose. The percentage of monosaccharide as product decreased with oligosaccharides with longer DP, and was higher when substrates with an even-numbered DP. Taken together, the use of defined chitooligosaccharides suggests that chitobiose is the dominant product of chitin hydrolysis with *Ll*Chi18A.

### 3.3 Pretreatment of fungal waste mycelia for production of chitooligosaccharides

Pretreatment is necessary prior to enzymatic hydrolysis of chitin because the strong crystallinity and insolubility of chitin in an aqueous environment.[19] To achieve deproteinization of the mycelia, and to break down the rigid structure of the mycelia cell wall to increase the accessibility of the substrate to the enzyme, waste mycelia were pretreated with acid, alkaline, or NaCl solution. We compared alkaline extraction, acid extraction, and extraction with neutral NaCl solution to remove some water-soluble, acid-soluble, or alkali-soluble components. The hydrolysis of cell wall material was assayed by quantification of the contents of protein and reducing sugar in the supernatant (Figs. 2A and 2B, respectively). Compared with treatment with 0.5 M HCl or 0.6 M NaCl, the release of the reducing sugar and protein obviously increased during the treatment with 0.5 M NaOH until 8 and 12 h. Alkaline pretreatment for 12 h was used to prepare the alkali insoluble fraction.

The water content of waste mycelium was 74%, the chitin content in the dry matter was determined as 20%. The water content of mycelia after alkaline pretreatment was 69% and the chitin content in the dry matter increased to 28%. The recovery of chitin after alkaline pretreatment was 89%. The increase of the chitin content is attributable to the removal of alkali soluble components including proteins, lipids, pigments, nucleotides and other soluble intracellular metabolites after sequential washing and filtration steps.

### 3.4 Characterization of waste mycelia after alkali pretreatment

To provide a preliminary confirmation that alkali treated waste mycelia contain chitin, the

FTIR spectrum of commercial chitin (Fig. 3A) was compared to the spectra of waste mycelia after alkali pretreatment (Fig. 3B). The peaks at 1577 and 1569 $cm^{-1}$ were assigned to the N-H bending vibrations of the amide II band. The peaks at 1642 and 1643 $cm^{-1}$ were assigned to the carbonyl stretching of secondary amides (amide I band). Waste mycelium showed peaks at 928, 849 and 819 $cm^{-1}$ which were assigned to a (1, 3)-D-glucan.[26] The FTIR spectrum of waste mycelia indicated that the polymer skeleton of the waste mycelia after alkali pretreatment was composed mainly of (1, 3)-D-glucan which connected to branches contains chitin.

### 3.5 Optimization of recombinant chitinase 18A for conversion of chemical pretreated waste mycelia into chitooligosaccharides

Recombinant chitinase 18A showed different enzymatic properties between different substrates, especially between the fluorescence substrate and polymeric substrates. The effects of temperature, pH, and reaction time on the activity of *Ll*Chi18A using alkali pretreated fungal mycelium as substrate are shown in Fig. 4. The enzyme showed high activity at a broad range of temperatures ranging from 30 ℃ to 60 °C (Fig. 4A). When soluble fluorescent substrate was used, the enzyme exhibited an optimal activity at 37°C. This result indicates *Ll*Chi18A is less heat sensitive when associated with chitin, exhibiting activity in the range of 25 to 80°C when using colloidal chitin as substrate. Elevated temperatures destroys inter- and intramolecular hydrogen bonds of chitin, and thus alter substrate-enzyme interactions which may lead to improved enzyme stability. The enzyme was active in the range of pH value from 3.0 to 9.0. The maximum activity was observed at pH 7.0 (Fig. 4B). As shown in Fig. 4C, the enzyme remained active over 6 days, indicating that *Ll*Chi18A is stable at 37 °C. The optimal reaction time could thus be extended to more than 6 days.

### 3.6 Hydrolysis of waste mycelium by chitinase

Because the combination of purified chitinase and β-1,3-glucanase has strongly synergistic

effect on the degradation of fungal cell walls.[27] The snail gut enzyme may contain numerous enzyme activities including chitinase[27] and β-glucanase[28]. Chitinase, a commercially available snailase and their combination were used in the hydrolysis of waste mycelium, respectively. Waste mycelium was converted by 0.03% chitinase, 4% snailase, or 4% snailase for 2 h followed by inactivation of snailase and conversion with *Ll*Chi18A. Catalysis by chitinase requires only one GlcNAc residue at either side of linkage because of the substrate-assisted catalytic mechanism of GH18 enzymes. The specificity of the hydrolysis was initially estimated by quantification of reducing sugars (all reducing sugars), and by quantification of GlcNAc (reducing sugars contain GlcNAc) with the Elson-Morgan method. After 2 h of hydrolysis with chitinase, 0.4 g $L^{-1}$ reducing sugar and 0.1 g $L^{-1}$ GlcNAc were released. The ratio increased over hydrolysis time from 40% at 1 d to 67% at 5 d. Snailase released 1.8 g $L^{-1}$ reducing sugars and 0.4 g $L^{-1}$ GlcNAc after 2 h with ratio of 22% and it didn't change a lot (21%) after 1 d. The results indicated that the specificity of *Ll*Chi18A is higher than snailase and thus favors production of more pure chitin oligosaccharides.

To obtain a more accurate quantification of reaction products, chitin hydrolysates from waste mycelia were analyzed by HPLC coupled to ESI-MS detection and to a DAD detector. The HPLC chromatogram after 4 d of conversion is shown in Fig. 5. The first peak around 6.2 min was the solvent peak. The peaks of glucose and GlcNAc (7.6 and 9.6 min) were not obvious in the hydrolysate solution. The major peak at 11.4 min was identified as chitobiose with a molecular weight peak at m/z 423.1 and fraction peaks at m/z 322.1 and 219.0 on the ESI-MS spectrum. DAD and ESI-MS analyses thus both verified that chitobiose was the main product in the hydrolysates obtained with *Ll*Chi18A, indicating that the endochitinase from *L. lactis* releases predominantly chitobiose from fungal chitin in waste mycelia.

The yield and estimated purity of different hydrolysis methods by *Ll*Chi18A with or without combination of snailase were analyzed by HPLC and are shown in Table 1. The yield calculated by two methods (HPLC and Elson-Morgan method) did not show major

differences. The yield and purity of chitobiose after hydrolysis of 5 d by *Ll*Chi18A were about 10% and 70%, respectively. The yield of chitobiose by snailase after hydrolysis of 2 h was around 24%. However, the purity of chitobiose in the hydrolysate was only 40%. Hydrolysis by snailase for 2 h, followed by heat-inactivation and hydrolysis by *Ll*Chi18A provided a yield of about 23.5% and an estimated chitobiose purity of 78%.

## 4 Discussion

A chitin-glucan complex from waste mycelia is normally obtained by alkali hydrolysis, followed by washing, filtration and drying.[26] The alkali insoluble fraction from the cell wall of *A. niger* is mainly composed of chitin and β-(1,3)-glucan polysaccharides. After alkali pretreatment, the residue obtained mainly contains chitin-glucan complex, the ratio of chitin to glucan in the chitin-glucan complex ranges from 25: 65 to 60: 40.[29] This chitin-glucan complex has been determined to be Generally Recognized as Safe by the U.S. Food and Drug Administration (GRAS Notice No. GRN 000412). Fungal mycelial waste is an environmental friendly alternative source of chitin or its oligomers. Additional, β-glucan can also be isolated from the chitin-glucan complex which finds applications in biomedicine.

This study employed purified recombinant *Ll*Chi18A from *L. lactis* IL1403 for conversion of chitin from fungal mycelium to chitinbiose. Glycolyl hydrolase (GH) family 18 chitinases including *Ll*Chi18A employ a catalytic mechanism referred to as the substrate-assisted double displacement mechanism.[30,31] For the processive GH18 enzymes ChiA and ChiB from *Serratia marcescens*, products from chitosan degradation were predominantly even-numbered oligomers, which is indicative of progressive action.[32] *Ll*Chi18A was described as non-processive enzyme with endo-activity that releases predominantly disaccharides from chitosan.[19] The present study also suggests a predominant endo-activity by demonstrating that even-numbered and uneven-numbered oligosaccharides are produced from defined oligosaccharides. In contrast, chitobiose was the major product of chitin hydrolysis.

*Ll*Chi18A revealed maximum activity at pH 3.8 when using 4-MU-$(GlcNAc)_3$ as a substrate.

However, when waste mycelium was used as a substrate, the effects of pH and temperature on the chitinase activity showed broad range and *Ll*Chi18A particularly displayed a higher activity at elevated temperatures. The yield of chitobiose was low when residual mycelium was treated with *Ll*Chi18A only for 5 days. The yield was increased substantially by treatment of the mycelium with 4% snailase for 2 h, followed by hydrolysis with chitinase. Snailase contains several glycosyl hydrolases including cellulase, β-glucanase and chitinase activities, correspondingly, snailase treatment of pretreated mycelium resulted in chitin hydrolysis but the low content of GlcNAc in the hydrolysate indicated that other polysaccharides were hydrolyzed as well. Use of chitinase in combination with snailase was more effective than using chitinase alone as it increased both the yield and the purity of chitobiose. Degradation by active snailase first may have partially solubilized chitin in the fungal cell wall.[28] Bacterial chitinases are supported by chitin-binding proteins. For example, the chitin binding protein LlCBP33A from *L. lactis* ssp. *lactis* IL1403 increased the hydrolytic efficiency of LlChi18A to both α- and β-chitin.[20] The chitin-binding protein CBP21 from *Serratia marcescens* acts on the surface of crystalline chitin, where it introduces chain breaks and generates oxidized chain ends, thus promoting further degradation by chitinases.[33] Nevertheless, past studies aiming at enzymatic degradation of purified α-chitin achieved degradation of less than 20% after 2 weeks of reaction.[20] Chemical degradation achieved virtually quantitative conversion of chitin. However, reaction products contained a mixture of chitin monomers, dimers, trimers and tetramer.[34]

Past studies employed the fermentative conversion of crab shell waste for production of chitin**.** Fermentation of crab shells with *Lactobacillus paracasei* KCTC-3074 and *Serratia marcescens* FS-3 was reported as a feasible step for the production of chitin from crab shells.[35] The use of microbial chitinases for conversion of fungal waste mycelium, however, has not been reported. Previous methods for the enzymatic conversion of chitin from fungal waste mycelia produced chitosan by lysozyme, snailase, neutral protease, and chitin

deacetylase[16], or used chemical conversion of fungal mycelium to obtain (1, 3)-α-D-glucan[26]. This study achieved the production of chitobiose with considerable yield and high purity. First step, sift and washing were employed and then alkali treatment (0.5 M NaOH) for 8 h to remove the protein and other saccharides in the waste mycelium to obtain the chitin-glucan complex. This alkali treatment also aimed to open the molecular chain of chitin in the waste mycelium[26]. Subsequently, *Ll*Chi18A was employed to hydrolyse the chitin-glucan complex to chitobiose as main product of hydrolysis.

Short-chain chito-oligosaccharide and their derivatives have good solubility in water, moisture absorption, bacteriostatic action and promoting hair growth, and other functions. They can be used as edible sweetener in patients with diabetes or obesity, and also improve the food structure, the water retention of food and adjust the water activity of food.[36] Chitobiose has also been used for synthesis of galactosylated oligosaccharides with potential use to prevent pathogen adhesion[37]. The enzymatic conversion of fungal chitin thus may be a feasible approach to convert waste mycelia to a valuable product with potential food and biomedical applications.

## 5 Conclusion

Waste mycelium of *A. niger* obtained from industrial production of citric acid is an suitable feedstock to produce chitooligosaccharides. The highest yield of oligomers was 25 % of chitin in the dry mycelium and the main product was chitobiose. The process developed in this study provides a novel process to produce chitooligosaccharides from fungal waste mycelium. Moreover, it develops a new way for the comprehensive utilization of post-fermented biomass.

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424

425 **FIGURE LEGENDS**

426 **Fig. 1** TLC spectra of (GlcNAc)n standards hydrolysed by chitinase 18A. GlcNAc,
427 $(GlcNAc)_2$ / DP2, $(GlcNAc)_3$ / DP3, $(GlcNAc)_4$ / DP4, $(GlcNAc)_5$/ DP5, or $(GlcNAc)_6$ / DP6
428 were used as substrates; the retention factor of the standards is indicated to the left of the TLC
429 plate.

430 **Fig. 2** Contents of protein (A) and reducing sugar (B) released by pretreatment with 0.5 M
431 NaOH (■), 0.5 M HCl (▲) and 0.6 M NaCl (▼).

432 **Fig. 3** FT-IR spectra of commercial chitin (A) and the products from alkali pretreatment (B).

433 **Fig. 4** The effects of temperature (A), pH (B), and reaction time (C) on the activity of
434 recombinant chitinase 18A on hydrolysis of pretreated waste mycelia (n=3).

435 **Fig. 5** HPLC analysis of hydrolysate from waste mycelia by chitinase. The first peak around
436 6.2 min was the solvent peak. Peaks were identified by LC/MS and by external standards;
437 chitobiose elutes at 11.4 min.

438

439 Table 1. Molar percentage in the hydrolysates from different oligosaccharides from HPLC
440 (n=2)

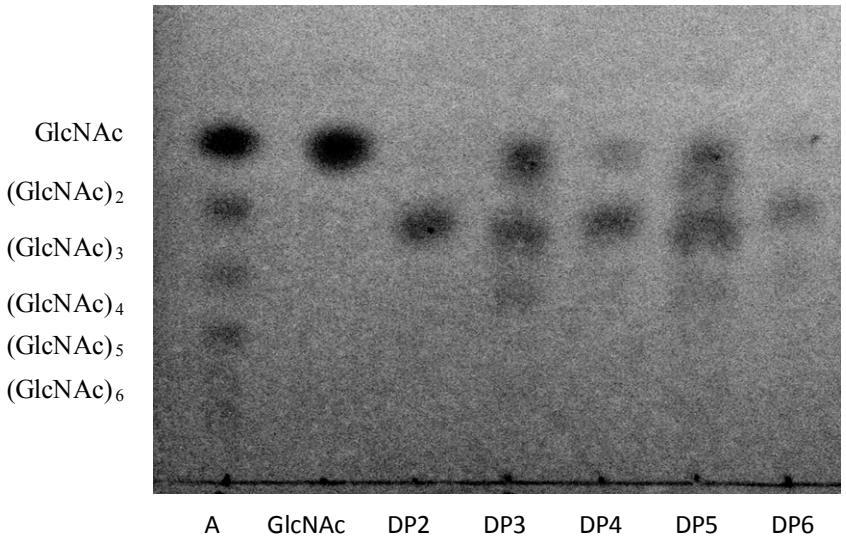
| % | Mono | Dimer | Trimer | Tetramer | Pentamer | Hexamer |
|---|---|---|---|---|---|---|
| $(GlcNAc)_2$ | | 100±0 | | | | |
| $(GlcNAc)_3$ | 38±5 | 44±9 | 18±5 | | | |
| $(GlcNAc)_4$ | 20±3 | 75±3 | 5±2 | | | |
| $(GlcNAc)_5$ | 32±5 | 60±10 | 8±2 | | | |
| $(GlcNAc)_6$ | 10±1 | 59±5 | 19±5 | 9±9 | | 3±1 |

441
442

443 Table 2. The yield and purity of chitobiose by chitinase and snailase by HPLC and Elson-
444 Morgan method (n=3)

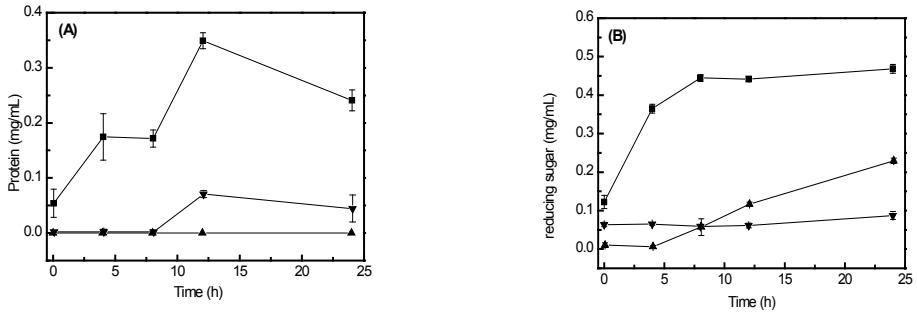
| method | Yield of chitobiose (%) | | |
|---|---|---|---|
| | 0.3% chitinase | 4% snailase | 0.3% chitinase after snailase |
| HPLC | 9.1 | 22.6 | 24.3 |
| Elson-Morgan | 11.3 | 24.9 | 23.2 |
| method | Purity of chitobiose (%) | | |
| | 0.3% chitinase | 4% snailase | 0.3% chitinase after snailase |
| HPLC | 69.2 | 39.5 | 78.1 |

445


GlcNAc
(GlcNAc)2
(GlcNAc)3
(GlcNAc)4
(GlcNAc)5
(GlcNAc)6
A
GlcNAc
DP2
DP3
DP4
DP5
DP6

446 **Fig.1**

447




448 **Fig. 2**

449

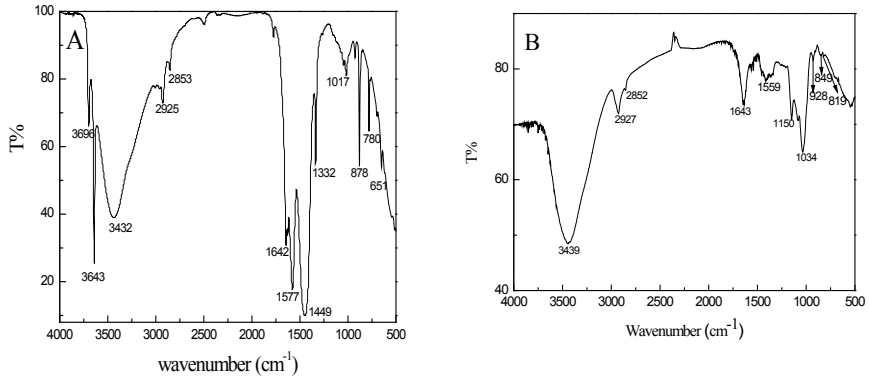
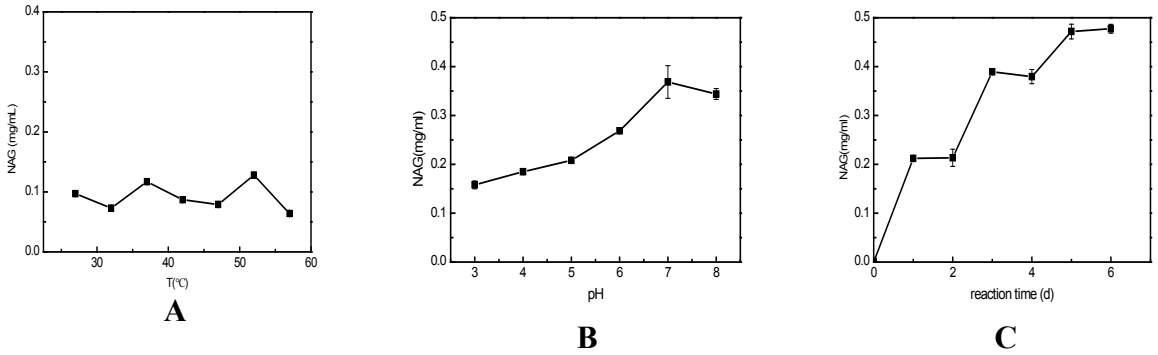

A
T%
wavenumber (cm-1)
3696
3643
3432
2925
2853
1642
1577
1449
1332
1017
878
780
651
B
T%
Wavenumber (cm-1)
3439
2927
2852
1643
1559
1150
1034
928
849
819

**Fig. 3**

452




453 **Fig. 4**

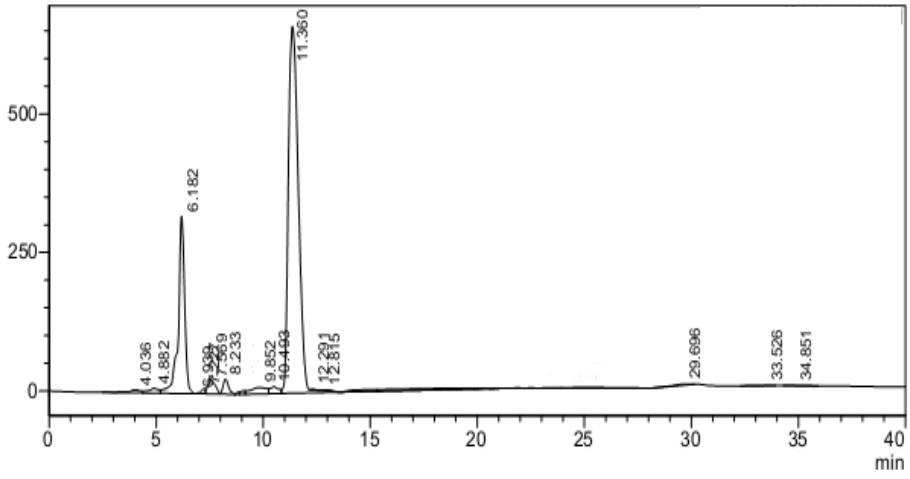

11.360
6.182
4.036
4.882
8.233
9.852
29.696
33.526
34.851
500
250
0
0
5
10
15
20
25
30
35
40
min

**Fig. 5**