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ORIGINAL PAPER

Enhanced degradation of α-chitin materials prepared from shrimp processing byproduct and production of *N*-acetyl-D-glucosamine by thermoactive chitinases from soil mesophilic fungi

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Abstract Soil isolates of mesophilic *Penicillium monoverticillium* CFR 2, *Aspergillus flavus* CFR 10 and *Fusarium oxysporum* CFR 8 were cultivated in solid state fermentation (SSF) using wheat bran solid medium supplemented with α-chitin in order to produce chitinolytic enzyme. Under SSF cultivation, maximum enzymes (U/g IDS) production was 41.0 (endo-chitinase) and 195.4 (*β*-*N*-acetylhexosaminidase) by *P. monoverticillium*, 26.8 (endo-chitinase) and 222.1 (*β*-*N*-acetylhexosaminidase) by *A. flavus* and 13.3 (endo-chitinase) and 168.3 (*β*-*N*-acetylhexosaminidase) by *F. oxysporum* after 166 h of incubation. The crude endo-chitinase and *β*-*N*-acetylhexosaminidase derived from *A. flavus* and *F. oxysporum* revealed optimum temperature at 62 ± 1°C, but the enzymes from *P. monoverticillium* showed optimum temperature at 52 ± 1°C for maximum activity. Several fold increase in endo-chitinase and *β*-*N*-acetylhexosaminidase activities in the crude enzymes preparation was achieved after concentrating with polyethylene glycol. The concentrated crude chitinases from *P. monoverticillium*, *A. flavus* and *F. oxysporum,* respectively yielded 95.6, 96.6 and 96.1 mmol/l of *N*-acetyl-D-glucosamine (GlcNAc) in 48 h of reaction from colloidal chitin. While, the crude enzyme preparations of *P. monoverticillium*, *A. flavus* and *F. oxysporum* produced 10.11, 6.85 and 10.7 mmol/l of GlcNAc respectively, in 48 h of reaction from crystalline α-chitin. HPLC analysis of colloidal chitin hydrolysates prepared with crude chitinases derived from *P. monoverticillium*, *A. flavus* and *F. oxysporum* revealed that the major reaction product was monomeric GlcNAc (~80%) and a small amount of $(GlcNAc)_4$ (~20%), indicating the potential of these enzymes for efficient production of GlcNAc from α-chitin.

Keywords Soil fungi · Chitinases · Shrimp byproduct · Chitin degradation · *N*-acetyl-D-glucosamine

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Introduction

Chitin, is a non-toxic, biodegradable, biocompatible and high molecular weight linear homopolymer of *β*-1,4 linked *N*-acetyl-D-glucosamine (GlcNAc) and occurs widely in nature as a structural constituent in the exoskeleton of insects and crustaceans and in the cell wall of most fungi and some algae. Chitin exhibits structural similarity to cellulose and differs from it with the replacement of C-2 hydroxyl residue by acetamido group. Chitin is generally classified into three forms depending on the crystalline structure: α, *β*, and *γ*-chitins. The most abundant and easily

accessible form is α-chitin. However, α-chitin is chemically rigid as compared with other forms, due to the different antiparallel arrangement of chitin molecules in α-chitin that allows maximum intermolecular hydrogen bonding (Kurita 2006). It is believed that chitin is the second most abundant and renewable biopolymer on earth, next to cellulose, with an estimated annual potential of 100 billion tons. Chitin and its hydrolytic derivatives have high economic value owing to their versatile biofunctional properties and wide range of applications in biomedical, food and agro-chemical sectors (Jeon et al. 2000; Synowiecki and Al-Khateeb 2003; Suresh et al. 2011a). The number and variety of uses of chitin and its differnt derivatives are increasing rapidly. Although chitin is widely distributed in nature, the most practical source for isolating this versatile biopolymer commercially is seafood processing crustaceans byproducts (Synowiecki and Al-Khateeb 2003). It has been estimated that the annual global chitin production is at 150,000 tons (Subasinghe 1999). During the last decade, research on chitin has received great attention to upgrade and use of chitin in order to exploit the renewable resource and also to minimize seafood byproducts disposal problems (Jeon et al. 2000; Synowiecki and Al-Khateeb 2003; Kurita 2006; Suresh et al. 2011a). Shrimp processing is one of the important marine industry in India that generates considerable quantities (more than 100,000 tons) of solid byproducts in the form of head and body carapace (Mathew and Nair 2006). However, despite small quantity used for the extraction of chitin most of these valuable byproducts is discarded by ocean dumping (which causes severe environmental problems) or used as feed or manure. Therefore, attention must be paid to effective chitin utilization in order to manage shrimp processing byproducts issues.

β-1,4 linked *N*-acetyl-D-glucosamine, the basic structural unit of chitin is known to have various biological functions, and has attracted special attention for the treatment of osteoarthritis (Tamai et al. 2003), inflammatory bowel diseases (Salvatore et al. 2000) and gastritis (Gindzienski et al. 1971) and in food as supplement (Aloise et al. 1996; Jeon et al. 2000; Sashiwa et al. 2001; 2003; Synowiecki and Al-Khateeb 2003; Kuk et al. 2005a; Kurita 2006). Generally, GlcNAc is produced by the hydrolysis of chitin with concentrated HCl and acid hydrolysis has several disadvantages, including low yield, formation of acid wastes, high production cost and deacetylation of products (Sashiwa et al. 2002; 2003; Kuk et al. 2005a; 2005b; Binod et al. 2007). Consequently, enzymatic hydrolysis of chitin for the production of GlcNAc has gained much attention, in order to apply mild reaction conditions and product consistency (Aloise et al. 1996; Sashiwa et al. 2003; Kuk et al. 2005a; 2005b; Binod et al. 2007; Larsen et al. 2011). The enzymatic hydrolysis of chitin to GlcNAc is carried out by chitinolytic enzyme system, which contains two fractions: 1) endo-chitinase (EC 3.2.1.14), and 2) *β-N*-acetylhexosaminidase (EC 3.2.1.52) and the action of which is known to be synergistic and consecutive. The combined action of these enzymes results in the hydrolysis of insoluble chitin polymer into its low molecular weight soluble derivatives (Suresh and Chandrasekaran 1999; Binod et al. 2007; Suresh et al. 2011a). Chitinases have been found widely in a variety of organisms, including various species of fungi (Suresh and Chandrasekaran 1998; Dahiya et al. 2005; Binod et al. 2007; Suresh et al. 2011a; 2011b). However, commercial exploitation of chitinases for the production of GlcNAc is currently limited owing to the high cost and also their activity and stability (Suresh et al. 2011a, Wang et al. 2011). Recently, production of GlcNAc from chitin materials using bacterial chitinases derived from *Bacillus* sp. (Thamthiankul et al. 2001), *Aeromonas hydrophila* H-2330 (Sashiwa et al. 2002), *Aeromonas* sp. (Kuk et al. 2005a; 2005b) and *Serratia marcescens* (Aloise et al. 1996) and fungal chitinases derived from *Penicillium aculeatum* and *Trichoderma harzianum* (Binod et al. 2007) have been reported. Besides, chitinases extracted from liver of squids (Matsumiya 2004) and non-chitinase commercial enzymes (Sukwattanasinitt et al. 2002) were also used for the production of GlcNAc from chitin materials. In order to obtain high yield of GlcNAc by one step enzymatic hydrolysis of chitin, the enzyme preparation must contain relatively low endo-chitinase and high *β-N*-acetylhexosaminidase activities (Aloise et al. 1996; Synowiecki and Al-Khateeb 2003; Binod et al. 2007). Since, the highly crystalline nature of chitin makes it less available for chitinolytic enzymes for its degradation, the thermoactive chitinases have an advantage and can play efficient chitin hydrolysis during the production of its lower derivatives (Haki and Rakshi 2003; Suresh et al. 2011a). Therefore, there is a need for research into enzymatic production of GlcNAc to find a more efficient enzyme preparation

and method. In the present study we report the enhanced production of GlcNAc by one step hydrolysis of α-chitin materials using thermoactive chitinases from soil isolates of mesophilic fungi.

Materials and methods

ρ-nitrophenyl-*N*-acetyl-β-D-glucosaminide (ρ-NPGlcNAc), ethylene glycol chitosan and GlcNAc from Sigma Chemical Co. (St. Louis, USA), high-performance liquid chromatography (HPLC) standard for *N*-acetyl chitooligosaccharides from Associates of Cape Cod, Inc. (East Falmouth, MA, USA), polyethylene glycol (PEG), potato dextrose agar (PDA) from Himedia (Mumbai, India) were used. All other chemicals and reagents were of the analytical grade.

Chitinolytic enzyme production by solid state fermentation (SSF)

Microorganisms used in the study were soil isolates of mesophilic *P. monoverticillium* CFR 2 (Suresh et al. 2011a), *A. flavus* CFR 10 (Maheswari 2010) and *F. oxysporum* CFR 8 (Elakkiaselvi 2010). These strains were maintained on PDA slants at 4°C and subcultured every 2 months. The inoculum was prepared by growing the fungi on PDA slants at 30 ± 2°C for 5 days and the spores were dispersed in distilled water containing 0·1% (w/v) Tween 80 by scraping with a sterile inoculation loop. The concentrations of spore suspension were adjusted to 9.4×10^8 colony forming unit/ml (cfu/ml) and used as inoculum.

Enzyme production by SSF using commercial wheat bran (CWB) as solid substrate was carried out by the procedure reported earlier (Suresh et al. 2011a). Briefly, CWB procured from local market was washed twice with tap water (1:2 w/v) to remove the dirt and reducing sugars and dried at 55 ± 2°C for 12 h in a hot air drying oven (Kilburn, Mumbai, India). Ten gram of washed CWB in Erlenmeyer conical flask (250 ml) was moistened with 20 ml of tap water and autoclaved at 15 psi pressure for 30 min. Shrimp shell pure α-chitin powder was added at 1% (w/w) as an inducer. After cooling, the solid CWB medium was inoculated with 2 ml spore suspension and mixed with sterile glass rod to achieve a uniform distribution of inoculum throughout the medium. The inoculated flasks were then incubated at 30 ± 2°C for 166 h. The inoculated moist CWB medium had an initial moisture content of 75% (w/w) and pH 6.4 ± 0.2. After incubation, the moldy bran in each flask were mixed with 100 ml of chilled (4°C) acetate buffer (pH 5.6, 0.1 M) and agitated at 150 rpm on a temperature controlled orbital shaker (Technico, Chennai, India) for 20 min at 20 ± 2°C. The slurry was then filtered through dampened cheese cloth and the extracts were clarified by centrifugation at 10,000 rpm at 4°C for 20 min. The clear supernatant was used as crude enzyme source.

Assay of endo-chitinase

Endo-chitinase activity was assayed using ethylene glycol chitin as the substrate by the procedure described earlier (Suresh et al. 2011a). Briefly, the assay mixture consisting of 300 µl of 0.2% (w/v) ethylene glycol chitin, 150 µl of acetate buffer (pH 4.6, 0.1 M) and 50 µl of enzyme solution was incubated at 52 or 62 ± 1°C for 20 min. The reaction was terminated by heating the reaction mixture in boiling water bath for 10 min. Heat inactivated enzyme with substrate was used as blank. The amount of GlcNAc produced in the supernatant was estimated with ρ-dimethyl amino benzaldehyde reagent according to Ressing et al. (1955). One unit of endo-chitinase activity was defined as the amount of enzyme which released 1 µmol of GlcNAc under the reaction conditions and enzyme production in SSF was expressed in units/g of initial dry substrate (U/g IDS) (Suresh and Chandrasekaran 1999).

Assay of β-*N*-acetylhexosaminidase

β-*N*-acetylhexosaminidase activity was assayed using ρ-NPGlcNAc as the substrate by the procedure described earlier (Suresh et al. 2011a). Briefly, the reaction mixture contained 450 µl of 1 mM ρ-NPGlcNAc in acetate buffer (pH 4.6, 0.1 M) and 50 µl of enzyme solution. After incubation for 20 min at 52 or 62 ± 1°C, the reaction was stopped by adding 2 ml of 0.2 M Na_2CO_3 solution and the absorbance of ρ-nitrophenol released were measured at 420 nm. For blank Na_2CO_3 solution was added to the reaction mixture before adding substrate. One unit of enzyme activity was defined as the amount of enzyme which released 1 µmol of ρ-nitrophenol under the reaction conditions and enzyme production in SSF was expressed in U/g IDS (Suresh et al. 2011a).

Determination of optimum temperature for crude endo-chitinase and β-*N*-acetylhexosaminidase activity

The optimum temperature for endo-chitinase activity was determined using the standard assay described above by incubating the crude enzyme and assay substrate in acetate buffer (pH 4.6, 0.1 M) at different temperatures (32–72 ± 1°C). For β-*N*-acetylhexosaminidase, the assay substrate was prepared in buffers (acetate buffer, pH 4.6, 0.1 M) and the optimum temperature for enzyme activity was determined using the standard assay described above by incubating the crude enzyme at different temperatures (32–72 ± 1°C).

Enzymatic hydrolysis of chitin substrates

Concentration of enzymes

The crude enzyme extracts obtained from the SSF growth of *P. monoverticillium* CFR 2, *A. flavus* CFR 10 and *F. oxysporum* CFR 8 were filled into dialysis bag and sealed. The sealed dialysis bags were placed in a container containing PEG (mol. wt. 20,000) and stored at 4°C for 24 h. Later the concentrated enzymes were dialyzed with several batches of acetate buffer (pH 5.6, 0.1 M) at 4°C for 24 h in order remove the reducing sugars present. The concentrated and dialyzed enzymes were stored at –20°C until used for chitin hydrolysis experiments.

Preparation of α-chitin substrate

Pure α-chitin from fresh shrimp (*Penaeus* sp.) abdominal shells and colloidal α-chitin were prepared by the procedures reported (Suresh et al. 2011b). The purity of chitin prepared (Spinelli et al. 1974) and the chitin content (dry weight) in the colloidal chitin (Suresh et al. 2011b) was determined.

Chitin hydrolysis

The hydrolysis reactions were carried out in Erlenmeyer conical flask (25 ml) at 42 ± 2°C under stirring (~125 rpm) with a magnetic stirrer placed in a temperature controlled incubator (Technico, Chennai, India) by adding chitin substrates (10 mg/ml) viz. colloidal α-chitin (dry weight basis) and crystalline α-chitin powder (< 22 mesh size) in 3.5 ml acetate buffer (pH 4.6, 0.1 M) and 3.5 ml of concentrated enzyme preparation. Toluene (0.1 ml) was added to reaction mixture as antimicrobial agent. Aliquots were collected at different intervals and heated in boiling water bath for 10 min to terminate the enzyme activity. After removing the undigested material by centrifugation at 10,000 rpm for 20 min, the amount of GlcNAc in the supernatant was quantified spectrophotometrically using ρ-dimethyl amino benzaldehyde reagent according to Ressing et al. (1955).

Analysis of chitin hydrolysates by HPLC

HPLC analysis of hydrolysates was performed on a HPLC system containing a LC-10AT pump and RID-10A RI detector (Shimadzu, Japan) and UBondapak™ NH_2 3.9 × 300 mm column (Water, Ireland). After, the enzymatic hydrolysis of colloidal chitin, insoluble material was removed by centrifugation at 10,000 rpm. Ten milliliter of sample was injected into the HPLC system using a mixture of acetonitrile and water (65:35) as the mobile phase and a flow rate of 0.6 ml/min. The standard used was a mixture includes *N*-acetyl-D-glucosamine, di-*N*-acetyl-chitobiose, tri-*N*-acetyl-chitotriose, tetra-*N*-acetyl-chitotetrose, penta-*N*-acetyl-chitopentose and hexa-*N*-acetyl-chitohexaose.

Analytical methods

The moisture (AOAC 2000), the pH of CWB substrate (Suresh and Chandrasekaran 1999) and the GlcNAc (Ressing et al. 1955) were determined.

Statistical analysis

Statistical significance of experimental data was performed by the Analysis of variance (ANOVA) techniques using the statistical software STATISTICA (1999). All experiments were conducted in triplicate and the mean values are reported.

Results and discussion

SSF cultivation production of chitinases and concentration

SSF cultivation of *A. flavus* CFR 10 produced maximum activity of 26.8 U/g IDS of endo-chitinase and

222.1 U/g IDS of β-*N*-acetylhexosaminidase after 166 h of incubation. Similarly, *F. oxysporum* CFR 8 formed maximum endo-chitinase activity of 13.3 U/g IDS and β-*N*-acetylhexosaminidase activity of 168.3 U/g IDS after 166 h of incubation. At the same time, *P. monoverticillium* CFR 2 was recorded maximum activity of 41.0 U/g IDS of endo-chitinase and 195.4 U/g IDS of β-*N*-acetylhexosaminidase. Chitinases from three fungi not differed significantly ($P \leq 0.05$) in their endo-chitinase and β-*N*-acetylhexosaminidase activity levels. In this study we had used wheat bran as the solid substrate for the fungal chitinases production by SSF, because, wheat bran is the most preferred and widely exploited substrate in SSF production of fungal enzymes due to its favorable properties for mold growth (Suresh and Chandrasekaran 1999; Nampoothiri et al. 2004; Suresh et al. 2011a; 2011b). Naturally, chitinase enzyme plays various important biological and physiological roles in microorganisms. In fungi, chitinases play roles in autolysis, nutrition, morphogenesis, symbiosis and pathogenesis. Biosynthesis of chitinases by fungi is also involved in degradation and recycling of enormous quantities of chitinous materials deposited in the biosphere by dead fungi and invertebrates (Gooday 1990). Though, chitin is the major component in the cell of most fungal species, many fungi syntheses extracellular chitinolytic enzyme complex and these enzyme complex have shown potential applications in various fields including in the bioconversion of chitinous byproducts of shellfish processing industry to its monomer or defined *N*-acetyl chitooligosaccharides (Binod et al. 2007; Suresh et al. 2011a). SSF has been reported to be an economical alternative to conventional submerged fermentation for cost effective production of high titres of microbial chitinases (Suresh and Chandrasekaran 1998; 1999; Nampoothiri et al. 2004; Binod et al. 2007; Suresh et al. 2011a). SSF production of fungal endo-chitinase by *Beauveria bassiana* (Suresh and Chandrasekaran 1998; 1999), *P. aculeatum* and *T. harzianum* (Binod et al. 2007), *T. harzianum* (Nampoothiri et al. 2004) and β-*N*-acetylhexosaminidase by *F. oxysporum* (Konstantinos et al. 2004, Elakkiaselvi 2010) and *P. monoverticillium* CFR 2 (Suresh et al. 2011a) and *A. flavus* (Maheswari 2010) have been reported.

Data presented in Table 1 confirmed that both endo-chitinase and β-*N*-acetylhexosaminidase activity of chitinase preparations from three fungi significantly ($P \leq 0.01$) increased after concentrating with PEG followed by dialysis. The enzyme concentrate of *P. monoverticillium* CFR 2 showed a 12.0 fold increase in endo-chitinase and 52.1 fold increase in β-*N*-acetylhexosaminidase activity as compared to the original enzyme activity found before concentration. Similarly, the enzyme concentrate of *A. flavus* CFR 10 exhibited a 14.8 fold increase in endo-chitinase and 51.1 fold increase in β-*N*-acetylhexosaminidase activity after concentration. At the same time, the enzyme concentrate of *F. oxysporum* CFR 8 showed an increase of 34.6 fold in endo-chitinase and 62.1 fold in β-*N*-acetylhexosaminidase activity as compared with original enzyme activity observed before concentration (Table 1). Enzymatic degradation of chitin is carried out by chitinolytic enzyme system, which contains two fractions (endo-chitinase and β-*N*-acetylhexosaminidase) and the action of which is known to be synergistic and consecutive. The endo-chitinase fraction is responsible for the hydrolysis of insoluble chitin polymer into *N*-acetyl chitooligosaccharides, which, in turn, further hydrolyzed by β-*N*-acetylhexosaminidase fraction to soluble GlcNAc (Suresh and Chandrasekaran 1999; Binod et al. 2007; Suresh et al. 2011a). It has been reported that, in order to produce high amount of GlcNAc by one step enzymatic hydrolysis of chitin, the enzyme preparation must contain both fractions (endo-chitinase and β-*N*-acetylhexosaminidase) of chitinolytic system and also the enzyme preparation must contain relatively low endo-chitinase and high β-*N*-acetylhexosaminidase activities (Aloise et al. 1996; Synowiecki and Al-Khateeb 2003; Binod et al. 2007). Kuk et al. (2005b) observed an increased yield of GlcNAc with an increase in enzyme amount during the hydrolysis of swollen chitin with chitinolytic enzyme of *Aeromonas* sp. GJ-18. Studies reported by various researchers confirmed that fungal chitinases having high ratio of endo-chitinase to β-*N*-acetylhexosaminidase activity release almost exclusively GlcNAc from chitin (Bruno et al. 2003; Suraini et al. 2008). As shown in Table 1, the enzyme preparations from three fungi contain low endo-chitinase and high β-*N*-acetylhexosaminidase activity and the ratios of endo-chitinase to β-*N*-acetylhexosaminidase activity is not varied significantly ($P \leq 0.01$). Thus, it is confirmed that the enzyme preparations from *P. monoverticillium* CFR 2, *A. flavus* CFR 10 and

Table 1 Endo-chitinase and *β-N*-acetylhexosaminidase activities of crude chitinolytic enzyme preparations of *P. monoverticillium* CFR 2, *A. flavus* CFR 10 and *F. oxysporum* CFR 8 before and after concentration

Organisms	Total volume (ml)		Enzyme activity (U/ml)				Ratio E/*β*-NAH[a]
	BC[b]	AC[c]	Endo-chitinase		*β-N*-acetylhexosaminade		
			BC	AC	BC	AC	
P. monoverticillium	845.0	53.5	4.09	48.98	19.54	1018.4	0.048
A. flavus	1000.0	49.0	2.64	39.09	22.20	1134.0	0.034
F. oxysporum	730.0	48.0	1.33	46.05	16.83	1045.0	0.044

a = Ratios of endo-chitinase to *β-N*-acetylhexosaminidase activity of concentrated enzyme preparation

b = Before concentration

c = After concentration

F. oxysporum CFR 8 will have advantages in one step enzymatic hydrolysis of α-chitin substrates for the production of GlcNAc.

Temperature optima for crude endo-chitinase and *β-N*-acetylhexosaminidase activity

As shown in Fig. 1, the crude enzyme preparations of *A. flavus* CFR 10 and *F. oxysporum* CFR 8 showed optimum temperature for maximum endo-chitinase and *β-N*-acetylhexosaminidase activity at 62 ± 1°C. The crude enzyme preparation of *P. monoverticillium* CFR 2, however, showed maximum endo-chitinase and *β-N*-acetylhexosaminidase activity at 52 ± 1°C. In general most of the fungal chitinases showed their optimum temperature below 50°C. Essentially chitin is a heterogeneous polysaccharide with different crystalline forms (α, *β* and *γ*) and characterized by variable levels and patterns of acetylation (Bruno et al. 2003). The high crystalline nature of chitin, especially α from prevent its hydrolysis and make the enzyme less active (Haki and Rakshi 2003). α-Chitin is the most abundant and easily available form of chitin found in nature. It has been reported that, thermoactive chitinases have advantages and can be effectively utilized for chitin degradation for GlcNAc and *N*-acetyl chitooligosaccharides production (Haki and Rakshi 2003; Suresh et al. 2011a). Therefore, we suggested that, the thermoactive chitinase preparations of *P. monoverticillium* CFR 2, *A. flavus* CFR 10 and *F. oxysporum* CFR 8 will have potential in α-chitin hydrolysis.

Chitin hydrolysis and production of GlcNAc

The α-chitin prepared from fresh shrimp abdominal shell had 97.5 ± 0.5% purity and contained 0.15 ± 0.05% (w/w) ash. The colloidal chitin prepared form pure α-chitin had 83.0 ± 0.5% (w/w) moisture with 17.0 ± 0.5% chitin content (w/w, dry weight basis) and a yield of 50 ± 1.0% (w/w).

Figure 2 shows the time scale formation of GlcNAc from colloidal α-chitin using crude chitinase preparations of three fungi. The chitinase preparations from *P. monoverticillium* CFR 2, *A. flavus* CFR 10 and *F. oxysporum* CFR 8 produced maximum GlcNAc yield of 95.6, 96.6 and 96.1 (mmol/l), respectively at 48 h of hydrolysis. The rate of GlcNAc release was very rapid at initial period of hydrolysis up to 24 h incubation and recorded about 95.4% (91.2 mmol/l), 94.1% (91.0 mmol/l) and 93.9% (90.2 mmol/l) of GlcNAc yield, respectively by the enzyme preparations of *P. monoverticillium* CFR 2, *A. flavus* CFR 10 and *F. oxysporum* CFR 8 (Fig. 2). Significant ($P \leq 0.05$) increase in GlcNAc was not observed in subsequent 48 h of incubation. The slow rate of GlcNAc production after 24 h of incubation by these enzyme preparations might be due to immobilization of enzymes by binding to chitin substrate. Similar observations have been reported earlier (Ilankovan et al. 2006; Suresh et al. 2011a). A maximum GlcNAc yield of 66–77% (mol/mol) from pulverized α-chitin after 10 days of incubation with crude enzyme from *A. hydrophila* H-2330 was reported (Sashiwa et al. 2002). A study by Kuk et al. (2005b) reported that when swollen chitin incubated with crude chitinase

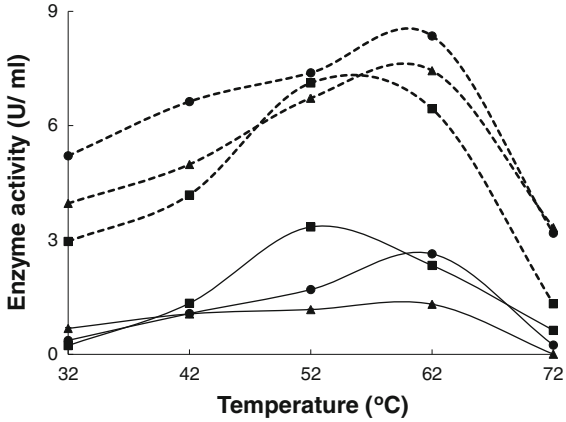

Fig. 1 Effect of temperature on the activity of crude endochitinase (—) and *β-N*-acetylhexosaminidase (···) of *P. monoverticillium* CFR 2 (*filled square*), *A. flavus* CFR 10 (*filled circle*) and *F. oxysporum* CFR 8 (*filled triangle*)

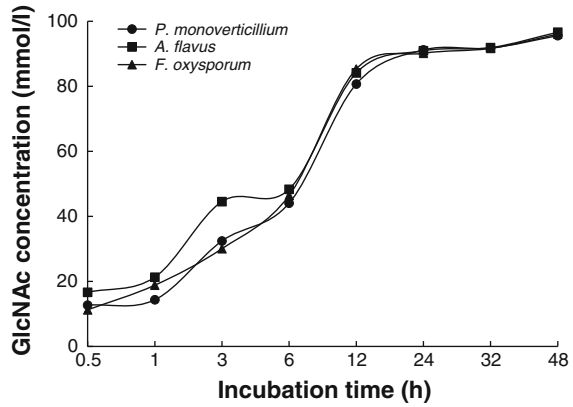

Fig. 2 Concentration of *N*-acetyl-D-glucosamine (mmol/l) produced from colloidal α-chitin using crude chitinolytic enzyme preparations of *P. monoverticillium* CFR 2, *A. flavus* CFR 10 and *F. oxysporum* CFR 8

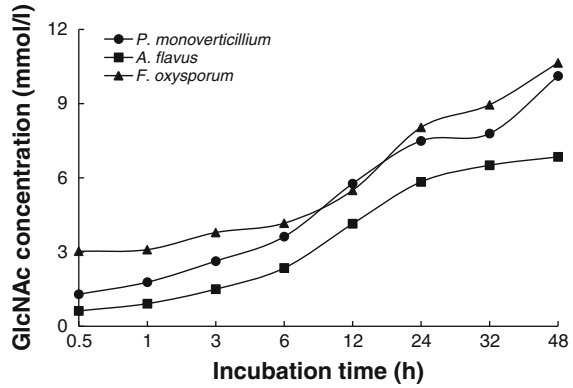

Fig. 3 Concentration of *N*-acetyl-D-glucosamine (mmol/l) produced from crystalline α-chitin using crude chitinolytic enzyme preparations of *P. monoverticillium* CFR 2, *A. flavus* CFR 10 and *F. oxysporum* CFR 8

preparation of *Aeromonas* sp. GJ-18 at 40°C, chitin was hydrolysed to 83.0 and 94.9% yield of GlcNAc at 5 and 9 days, respectively. In an another study by Kuk et al. (2005a) reported that the enzyme from *Aeromonas* sp. produced GlcNAc as a major product with 74% yield from swollen chitin at 45°C in 5 days of incubation. Thus, the GlcNAc yield reported in the present work is found to be highly significant as compared to GlcNAc yield of earlier reports (Sashiwa et al. 2002; Kuk et al. 2005a; 2005b) as they took higher incubation time (5–10 days) for obtain highest GlcNAc yield.

The results presented in Fig. 3 shows the production of GlcNAc from crystalline α-chitin powder using the crude enzyme preparations of *P. monoverticillium* CFR 2, *A. flavus* CFR 10 and *F. oxysporum* CFR 8. A maximum amount (mmol/l) of GlcNAc of 10.11 (*P. monoverticillium* CFR 2), 6.85 (*A. flavus* CFR 10) and 10.7 (*F. oxysporum* CFR 8) was produced after 48 h of incubation. This result indicates the potential chitin degrading activity of these chitinase preparations towards crystalline α-chitin. Naturally microorganism is biosynthesis different chitinases for various purposes. Fungus, favors easily degradable carbon source such as colloidal chitin as compared to the raw shell and other complex media due to complex chemical composition (Suraini et al. 2008). Though, the GlcNAc yield from crystalline α-chitin (Fig. 3) is comparatively lower as compared to the yield of GlcNAc from colloidal α-chitin (Fig. 2), the use of crystalline α-chitin powder as substrate will have important economic advantages and offers great significance in environmental issues due to high acidic effluent from colloidal chitin preparation. Bruno et al. (2003) reported a maximum yield of 78% GlcNAc with 2% glucosamine and 10% $(GlcNAc)_2$ from langostino shells chitin using mixture of chitinases from fungi (*Trichoderma atroviride*) and bacteria (*Serratia marcescens)* at 32°C in 12 days of incubation.

Table 2 shows the relative production rate of GlcNAc from colloidal and crystalline α-chitin by the chitinase preparations of *P. monoverticillium* CFR 2, *A. flavus* CFR 10 and *F. oxysporum* CFR 8. The

Table 2 Relative production rate of *N*-acetyl-D-glucosamine (GlcNAc) from colloidal α-chitin (Co.ch) and crystalline α-chitin (Cr.ch) using crude chitinolytic enzyme preparations of *P. monoverticillium* CFR 2, *A. flavus* CFR 10 and *F. oxysporum* CFR 8

Reaction time (h)	Relative production rate[a] of GlcNAc					
	P. monoverticillium		*A. flavus*		*F. oxysporum*	
	Co. ch[b]	Cr. Ch[c]	Co. ch	Cr. ch	Co. ch	Cr. ch
0.5	100.00	100.00	100.00	100.00	100.00	100.00
1	44.30	36.30	39.09	34.00	29.86	48.98
3	6.52	8.18	6.22	6.91	6.23	13.34
6	2.40	2.97	2.87	2.20	2.02	6.07
12	0.65	0.93	0.82	0.63	0.55	2.30
24	0.29	0.36	0.38	0.22	0.26	0.79
32	0.22	0.26	0.28	0.15	0.19	0.53
48	0.14	0.13	0.18	0.09	0.12	0.30

a = The relative production rate on the n h was calculated from $Yield_n/(Yield_1 \times time) \times 100$ (Jung et al. 2007)

b = Colloidal α-chitin

c = Crystalline α-chitin powder

Table 3 Composition of colloidal α-chitin hydrolyzates produced using crude chitinolytic enzyme preparations of *P. monoverticillium* CFR 2, *A. flavus* CFR 10 and *F. oxysporum* CFR 8

Reaction time (h)	Product of colloidal chitin hydrolyzate (%)[a]					
	P. monoverticillium		*A. flavus*		*F. oxysporum*	
	GlcNAc[b]	$(GlcNAc)_4^c$	GlcNAc	$(GlcNAc)_4$	GlcNAc	$(GlcNAc)_4$
1	32.20	64.22	41.23	55.0	42.59	49.30
6	60.16	39.84	75.88	24.12	78.21	21.79
48	79.16	20.84	78.03	21.97	79.77	20.23

a = The concentrate was analysed by HPLC as described in the text

b = *N*-acetyl-D-glucosamine

c = Tetra-*N*-acetyl-chitotetrose

relative production rate of GlcNAc from colloidal α-chitin was significantly ($P \leq 0.01$) decreased to about 93% within 3 h of reaction by the enzyme preparations of all three fungi. Similarly, the relative production rate of GlcNAc from crystalline α-chitin powder was also decreased, respectively 92, 93 and 86% with in 3 h of reaction by enzyme preparations of *P. monoverticillium* CFR 2, *A. flavus* CFR 10 and *F. oxysporum* CFR 8. Subsequently, the relative production rate of GlcNAc from both colloidal and crystalline α-chitin was decreased by more than 99% during the 48 h of reaction by the enzyme preparations of all three fungi (Table 2). Similar observation was reported in the enzymatic production *N*-acetyl chitooligosaccharides from swollen chitin using the chitinases from *Paenibacillus illinosensis* KJA-424 (Jung et al. 2007). They reported that more than 44 and 87% decreases in relative production rate, respectively after 2 and 24 h of incubation.

HPLC analysis of the hydrolytic product of colloidal α-chitin revealed that the crude chitinase preparations from three fungi produced mainly monomeric GlcNAc (78–80%) as the major end product and a small amount of $(GlcNAc)_4$ (~20%) in 48 h of incubation (Table 3). No other *N*-acetyl chitooligosaccharide was observed in 48 h incubation. Figure 4 shows the HPLC chromatogram of colloidal α-chitin hydrolysates of the enzyme preparations of *P.*

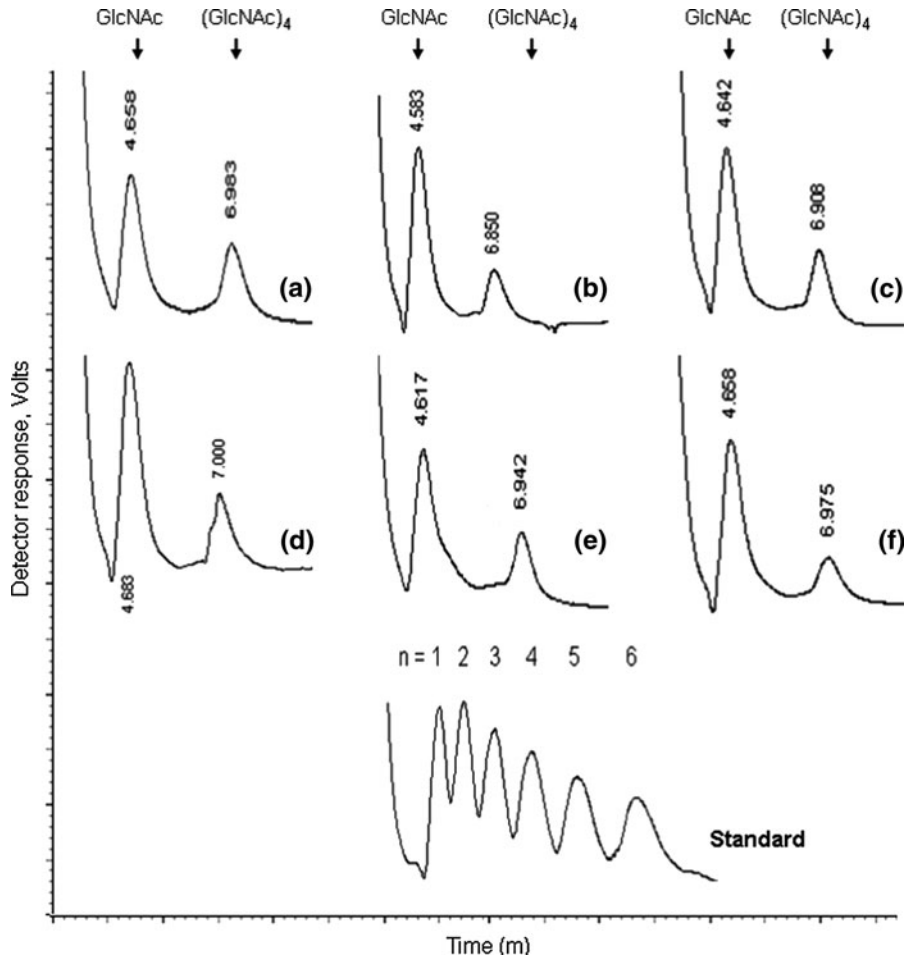

Fig. 4 HPLC chromatogram of colloidal α-chitin hydrolysates produced using chitinolytic enzyme preparations of different fungi, (**a**) and (**b**) represent respectively the 6 and 48 h of reaction product by enzyme preparation of *P. monoverticillium* CFR 2; (**c**) and (**d**) represent respectively the 6 and 48 h of reaction product by enzyme preparation of *A. flavus* CFR 10, (**e**) and (**f**) represent respectively the 6 and 48 h reaction product by enzyme preparation of *F. oxysporum* CFR 8. GlcNAc: *N*-acetyl-D-glucosamine and $(GlcNAc)_4$: Tetra-*N*-acetyl-chitotetrose. Standard: GlcNAc and $(GlcNAc)_{2-6}$ mixutre

monoverticillium CFR 2, *A. flavus* CFR 10 and *F. oxysporum* CFR 8. Kuk et al. (2005b) reported a yield of 62.8% GlcNAc and 4.1% $(GlcNAc)_2$ from swollen chitin hydrolyzed with crude chitinase preparation of *Aeromonas* sp. GJ-18 at 45°C within 5 days. Thus, the results of the present work shows the potential of thermoactive chitinase preparations of *P. monoverticillium* CFR 2, *A. flavus* CFR 10 and *F. oxysporum* CFR 8 for the application in seafood processing chitinous byproducts reclamation and preparation of high yield of bioactive GlcNAc.

Conclusion

Penicillium monoverticillium CFR 2, *A. flavus* CFR 10 and *F. oxysporum* CFR 8 produced high titres of thermoactive endo-chitinase and *β-N*-acetylhexosaminidase, indicating that these fungi are potential commercial source for thermoactive chitinases. Efficient production of GlcNAc was achieved by crude chitinolytic enzyme preparations of these three fungi within 48 h of hydrolysis from colloidal α-chitin. The HPLC analysis of colloidal α-chitin hydrolysates revealed that the chitinolytic enzyme preparations from three fungal cultures produced monomeric GlcNAc as the major end product suggesting their potential commercial application. However, further research is needed to establish the commercial production of chitinases by these fungi and their application for GlcNAc production from seafood processing chitinous byproduct.

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