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ORIGINAL ARTICLE

Purification and characterization of a novel antifungal endo-type chitosanase from *Anabaena fertilissima*

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Abstract A novel antifungal chitosanase from *Anabaena fertilissima*, strain RPAN1, was characterized as a prelude to its use in biocontrol. The culture grown at 8:16 h L:D photoperiod showed highest chitosanase/antifungal activity under environmental and nutritional conditions of 43 μM of P level, pH 9.0 and temperature of 27°C. The transcriptional level of chitosanase encoding gene (*cho*) measured using quantitative real-time PCR (qRT-PCR) also indicated increased expression levels under the same optimized conditions. Under these conditions, *cho* encoding chitosanase was purified which exhibited a specific activity of 822 U/mg. The chitosanase activity measured using different substrates showed the highest activity against colloidal chitosan. HPLC profile of the products of enzyme activity with different chitosan oligosaccharides revealed the production of dimer units $(GlcN)_2$ or more, confirming the endo-type nature of the purified chitosanase. The optimum pH and temperature of the purified enzyme was 7.5 and 27°C, respectively. Further, the enzyme was stable in the pH range of 5.5–9.0 up to 12 h and temperature between 27 and 50°C up to 3 h. The enzyme was strongly inhibited by Ag^{+}, Fe^{3+} and Hg^{2+} and stimulated by Cu^{+2} and Zn^{2+}. The investigation revealed significant features regarding the stability of the chitosanase enzyme from *A. fertilissima* under a broad range of pH and temperature which can help in its effective use in biocontrol.

Keywords Antifungal activity · Chitosanase · Cyanobacteria · HPLC · q-RT PCR

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Introduction

Cyanobacteria constitute an extremely diverse group of photosynthetic prokaryotes exhibiting variability in physiological, morphological and developmental characteristics. The chemicals excreted/secreted by these organisms are known to provide them with a competitive advantage and permit proliferation in the specific environments (Tonk et al. 2005). Various strains of cyanobacteria are known to produce intracellular and extracellular metabolites with diverse biological activities such as antibacterial, antifungal, cytotoxic, algicidal, immunosuppressive and antiviral (Ray and Bagchi 2001). Although the use of cyanobacteria as a biofertilizer has been demonstrated (Silva and Silva 2007), very few reports on its use as biocontrol agent are available (Manjunath et al. 2010).

Chitosan is a deacetylated derivative of chitin, a linear polymer of β-1,4-linked N-acetylglucosamine residues. In nature, chitosan is present only in the cell walls of a limited group of fungi such as the genera *Rhizopus*, *Absidia* and *Fusarium* (Alfonso et al. 1992). Chitosanases (EC 3.2.1.132) are the glycosyl hydrolases that catalyze the hydrolysis of the β-1, 4-glycosidic linkage of chitosan to

yield chitosan-oligosaccharides. The oligomers produced by the enzymatic hydrolysis of chitosan are very attractive for use in food in addition to agricultural and pharmaceutical applications, because of their various biological activities, including antitumor and antibacterial effects (Moon et al. 2007). Several chemical and physical techniques have been utilized to obtain chitosan chains with varying degree of polymerization (Popa-Nita et al. 2009). Chitosanases in either free or immobilized form have also been intensively studied using different biophysical techniques (Dennhart et al. 2008; Kuroiwa et al. 2009).

To date, several chitosanolytic enzymes have been purified and characterized from bacteria, fungi and plants. Chitosanases have been classified into seven glycoside hydrolase (GH) families mainly GH-3, GH-5, GH-7, GH-8, GH-46, GH-75 and GH-80, based on the amino acid sequence similarity of their catalytic domains (Ando et al. 2008; Bueno et al. 1990; Gupta et al. 2010; Ike et al. 2007; Johnsen et al. 2010; Park et al. 1999; Shimosaka et al. 1996; Tanabe et al. 2003). Most of them catalyze the endo-type cleavage of chitosan but their mechanism can be transformed into exo-type by protein engineering (Yao et al. 2008). Many glycoside hydrolases including chitosanases, cellulases, xylanases, and licheninases are classified as GH-3, GH-5, GH-7 and GH-8 enzymes. But the GH-46, GH-75, and GH-80 glycosyl enzymes are exclusively chitosanases (Lee et al. 2007).

Endoglucanases/chitinases/chitosanases are identified as key enzymes which can be employed as biocontrol agents to restrict the growth of phytopathogens (Adams 2004). The successful use of *Pseudomonas* sp., *Serratia marcescens*, *Trichoderma* sp. *Bacillus* sp. and *Streptomyces* sp. producing chitinases/endoglucanases has already been demonstrated and hence can be employed as biocontrol agents against phytopathogenic fungi (El-Mougy et al. 2011; Ganiger et al. 2009; Gupta et al. 2011; Huang et al. 2005; Quecine et al. 2008; Someya et al. 2005; Weller 2007). The chitosanase-producing microbes such as *Bacillus pumilus*, *Bacillus cereus* D-11, *Amycolatopsis* sp. CsO-2, *Sphingobacterium multivorum*, and *Penicillium chrysogenum* have shown excellent potential in the biocontrol of *Rhizopus oryzae*, *Fusarium oxysporum*, *Fusarium solani*, and toxic molds (Fukamizo et al. 1996; Gao et al. 2008; Martin et al. 2010; Matsuda et al. 2001; Saito et al. 2009). Some bacteria such as *Burkholderia gladioli* and *Streptomyces coelicolor* produce both chitinases and chitosanases that can act synergistically against cell walls of fungi (Bentley et al. 2002; Shimosaka et al. 2000, 2001). Previously, we have demonstrated the potential role of hydrolytic enzymes (chitosanase, cellobiase, CMCase, etc.) and its correlation with fungicidal activity in several *Anabaena* strains (Prasanna et al. 2008). Also, chitosanase homologues and microcystin-like compounds have been identified in two promising strains, *Anabaena laxa* and *Anabaena iyengarii*, which exhibit fungicidal activity (Prasanna et al. 2010). In our recent investigation, we have also characterized a putative antifungal chitosanase gene (*cho*) belonging to the GH-3-like family in *A. fertilissima* and have shown that the transcriptional level of *cho* increased under high dark period (8:16 h L:D photoperiod) (Gupta et al. 2010). In the present study, the other environmental and nutritional conditions were optimized for obtaining effective activity of the enzyme which was then characterized for its specific properties.

Materials and methods

Organism and growth conditions

The axenized culture of *Anabaena fertilissima* strain RPAN1 was selected from the previous investigation (Gupta et al. 2010) for the current study. It was grown and maintained in BG11 medium (Supplementary Table 1) (Stanier et al. 1971) at 27±1°C under 8:16 h light:dark cycle. The intensity of white light used was 50–55 μmol photons m^{-2} s^{-2}.

The phytopathogenic fungus (*Fusarium oxysporum*) used for evaluating antifungal activity was obtained from the Indian Type Culture Collection, Division of Plant Pathology, IARI, New Delhi. The fungal strain was grown on Potato Dextrose Agar (PDA) Medium and maintained at 28±2°C for 3–4 days in a temperature controlled incubator.

Chitosanase and antifungal activities

Both chitosanase and antifungal activities were measured in the culture filtrates of *A. fertilissima* under each treatment condition (pH, temperature and P levels) independently as per the method described previously (Gupta et al. 2010). The protein content was calculated according to (Lowry et al. 1951), using bovine serum albumin as the standard.

Effect of environmental/nutritional factors

An experiment was set up to study the effect of different environmental/nutritional factors such as temperature, pH and phosphorus (P) on the chitosanase/antifungal activity. 10% inoculum of the 20- 25-day-old culture was used uniformly for evaluating the effect of temperature (15–40°C), pH (4.0–12.0) and P levels (43–344 μM). The K levels were maintained by addition of KCl to provide equivalent balancing counter ions in the BG-11 medium (Ray and Bagchi 2001). The cultures grown under normal P concentration (172 μM), pH (7.5) and temperature (27±1°C) were treated as control for both molecular and biochemical analyses.

Expression profiling of chitosanase gene (*cho*)

DNA-free intact RNA (10 μg) from 28-day-old cultures was isolated from each sample separately (Tri-reagent; Sigma) and then subjected to cDNA synthesis using Stratagene High fidelity 1st strand cDNA synthesis kit, as per the manufacturer's instruction. The expression level of *cho* was evaluated by quantitative real-time RT-PCR (qRT-PCR) according to the method described previously (Gupta et al. 2010). The bacterial 16S rRNA gene was used as a reference gene for qRT-PCR.

Purification and characterization of chitosanase

Genomic DNA was isolated using an Ultraclean plant DNA isolation kit (MoBio, Carlsbad, CA, USA). from *A. fertilissima* culture grown under the optimized conditions. PCR amplification of the genomic DNA was performed using *cho* specific primers ChoF1 5'-ATGC CAGCATTGCAGAGAC-3' and ChoR1 5'-TTAAAAA CAACAAGCGATCGCC-3' (Gupta et al. 2010) for isolating the full-length chitosanase-encoding gene. After sequence validation, the *cho* was cloned into the pIVEX glutathione *S*-transferase (GST) fusion vector (Roche) and the specific recombinant *cho* encoding chitosanase was purified as per the methodology described in our earlier investigation (Gupta et al. 2010). The purified chitosanase was used for the detailed characterization. Chitosanase activity of the purified chitosanase was measured as per the method described previously (Gupta et al. 2010). For the determination of K_m and k_{cat}, the kinetic assays with 0.5-ml reaction mixtures were set up containing eight different concentrations (0.02–0.8 mg ml^{-1}) of chitosan in eight replicates using the microtiter plate. The protein concentration and reaction time was adjusted to obtain the similar hydrolysis for all the samples. The liberation of reducing sugars was measured and K_m and k_{cat} were calculated using the non-linear least-square fitting procedure for the Michaelis–Menten equation in Prism software (version 5.0 for Windows; San Diego, CA, USA).

Substrate specificity and nature of the purified chitosanase

The purified chitosanase was incubated independently with different derivatives of chitin (colloidal chitin and peptidoglycan) and chitosan (glycol and colloidal chitosan) and the chitosanase activity measured (Gupta et al. 2010). The exo/endo nature of the purified chitosanase was evaluated by measuring the chitosanase activity using different chitosan oligosaccharides $(GlcN)_2$–$(GlcN)_6$ (Sigma Aldrich). The relative activity of glycol chitosan was considered as a reference.

HPLC profiling of the reaction product from the chitosan oligosaccharide

The nature of the purified chitosanase was further confirmed by analyzing the degradation products of glycol chitosan and several oligosaccharides $(GlcN)_2$–$(GlcN)_6$, independently using high-performance liquid chromatography (HPLC) as per the method described previously (Gupta et al. 2010).

Effect of pH and temperature on the purified chitosanase

The effect of different pH on the activity and stability of enzyme was evaluated by pre-incubating the enzyme in buffers at different pH (4.0, 5.5, 6.5, 7.5, 9, 10, 12) and various incubation times (0, 1, 5, 12 and 24 h) at room temperature (25°C). McIlvaine buffer (pH 4.0–-8.0), Tris-HCl buffer (pH 9.0–10.0), and 0.05 M Na_2HPO_4 plus 0.1M NaOH buffer (pH 10.0–12.0) were used to create a range of pH values in the reaction mixtures (Park et al. 1999). After the incubation time, the chitosanase activity was measured independently from each sample. The data is represented in the terms of percentage of residual activity as a function of pH values and application times, considering a 100% activity for chitosanase at initial time (t=0).

The effect of temperature on the activity and stability was measured by pre-incubating the enzyme in the McIlvaine buffer (pH 7.5) at different temperatures (15–50°C) and various incubation times (1–12 h). After the incubation time, the chitosanase activity was measured. The data is represented in the terms of percentage of residual activity as a function of temperature values and application times, considering a 100% activity for chitosanase at initial time (t=0).

Effect of metal ions and compounds

The different metal ions (Ag^+, Hg^{2+}, Cu^{2+}, Mn^{2+}, Fe^{3+}, K^+, Na^+, Pb^{2+}, Mg^{2+}, Ba^{2+}, Zn^{2+}), compounds (β-mercaptoethanol, dithiothreitol and glutathione) and solvents (ethanol and acetone) were chosen to evaluate their effect on the chitosanase activity. The 1-mM final concentration of the respective metal ion and compound was added to the reaction mixture and then the activity was measured. The relative activity was expressed as percentage ratio of the specific activity (U ml^{-1}) of the purified chitosanase with metals and compounds vis-a-vis without metals and compounds.

Statistical analyses

All the data of chitosanase and antifungal activities was recorded in triplicate. The ANOVA (analysis of variance)

was performed using the MSTAT-C statistical package to evaluate the significant difference among the means. Ratios of relative *cho* expression were calculated using the method of Pfaffl (2001). Statistical analyses to identify significant differences from the control were performed using REST software (Pfaffl et al. 2002).

Results

Measurement of chitosanase and antifungal activities under environmental/nutritional factors

The measurement of chitosanase/antifungal activity in the extracellular filtrates of *A. fertilissima* under different conditions of pH, temperature and phosphorus (P) levels was undertaken to optimize the condition leading to highest activities. At 86 μM of P, enhancement of 10 and 5% was recorded; while at 43 μM of P, an increase of 21 and 22 increase was observed in chitosanase and antifungal activities, respectively, as compared to that of control. In contrast, no significant change was observed under the highest P levels (Supplementary Table 2).

The measurement of chitosanase and antifungal activities at different pH values (4.0–12.0) under optimized P concentration (43 μM) showed a significant increase of 24 and 10%, respectively at high pH (9.0) as compared to that of control. Both the activities were drastically reduced at pH values below control and above 9.0 (Supplementary Table 2). The data recorded at different temperature regimes under optimized P concentration (43 μM) and pH (9.0) showed a decrease in chitosanase/antifungal activity at sub/supra optimal temperatures. For instance, at 15°C, decreases of 54 and 65 % in chitosanase/antifungal activity, respectively, were observed as compared to control; while, at 50°C, decreases in both activities of 76 and 82% were recorded (Supplementary Table 2).

Expression profiling of *cho* under different environmental/nutritional conditions

The results of biochemical characterization of the enzyme were validated by measuring expression levels of *cho* under similar environmental/nutritional conditions. The expression level was increased by 48 and 65% under low P concentrations (i.e. 86 and 43 μM, respectively), while under high P level (344 μM), a 5% down-regulation was observed as compared to that of control (Fig. 1a). Further, the expression profile measured at different pH values under optimum P concentration (43 μM) showed a 25% increase in the expression of *cho* at pH 9.0 as compared to that of control. Down-regulation of *cho* was observed at pH below control and above 9.0 (Fig. 1b). Additionally, the

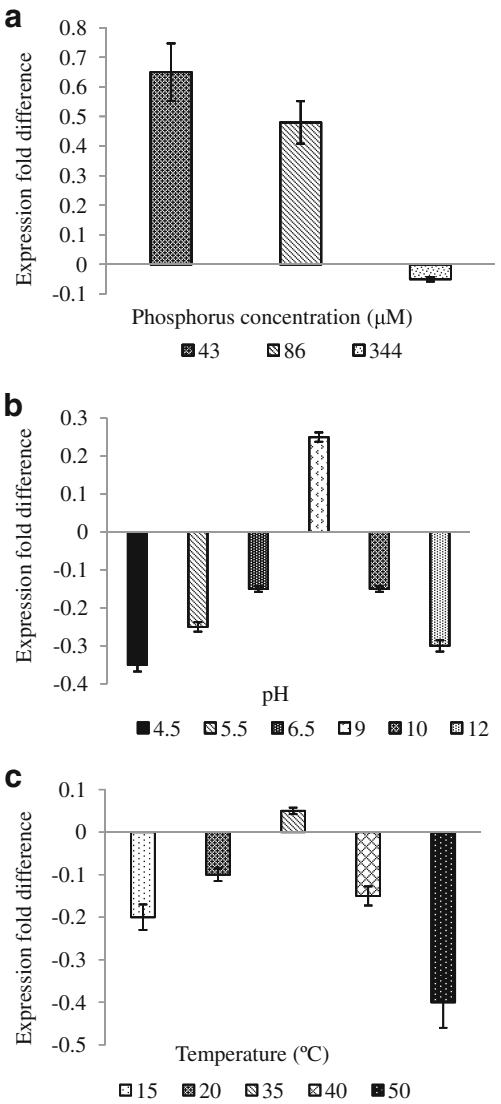

Fig. 1 Real-time expression profiles for *cho* from *A. fertilissima* under different environmental/nutritional conditions. **a** Phosphorus concentrations, **b**, **c** pH and temperature. Expression was compared to that of the control (0 on the *y* axis) for each treatment condition (see "Materials and methods"). *Bars* indicate the means of three technical and three biological replicates. The differences in the means were found to be statistically significant at a *P* value of <0.01 by using a one-way ANOVA test

data obtained at different temperature regimes under optimum P concentration and pH revealed 20, 10, 15 and 40% down-regulation at temperatures 15, 20, 40 and 50°C, respectively, as compared to control; while at 35°C, the level was at par with control (Fig. 1c).

Kinetic characterization, substrate specificity and reaction pattern of purified chitosanase

The recombinant chitosanase purified from chitosanase encoding gene (*cho*) showed specific activity of 822 U mg^{-1}. The

k_{cat} and K_m values of the purified chitosanase were 965 s^{-1} and 0.89 mg ml^{-1} of chitosan, respectively. In order to understand the substrate specificity, the chitosanase activity was measured against different derivatives of chitin and chitosan. Colloidal chitosan, CM-chitosan and colloidal chitin showed 13, 70 and 95% decreases in chitosanase activities, respectively, as compared to that of glycol chitosan (Table 1). The relative activity measured with different glucosamine oligomers (chitosan) showed no activity when chitobiose and chitotriose was used as a substrate; however, with chitotetrose and longer chitosan oligosaccharides, significant chitosanase activity was detected (Table 1). The reaction products of different glucosamine derivatives were also independently analyzed through HPLC (Fig. 2). The HPLC profile of the product obtained from chitotetrose and chitohexose revealed the formation of two and three oligomers of $GlcN_2$ (as indicated by the single peak of $GlcN_2$), respectively. The chitobiose and triose showed a single peak of $GlcN_2$ and $GlcN_3$ (Fig. 2). The reaction pattern of chitopentose was similar with earlier investigation (data not shown) (Gupta et al. 2010). The degradation product of chitosan was also analyzed which indicated the formation of chitosan oligosaccharides which were mainly longer than $(GlcN)_2$ (data not shown).

Effect of pH and temperature on purified chitosanase activity

The residual activity of chitosanase measured at different pH values (4.0–12.0) and time points (1–24 h) showed highest increase of 19% at pH 7.5 during 1 h and it was stable in the pH range from 5.5 to 9.0 during 12 h (Fig 3a). However, the drastic drops in enzyme stability was observed at high acidic pH (<5.5) and basic pH (>9.0). At a pH value more than 9.0, chitosanase activity was decreased by 39–53 % after 1 h.

The studies on the role of temperature on the activity of purified chitosanase at different temperatures (15–50°C) revealed highest residual chitosanase activity of 96% at 27°C. The enzyme retained its activity at pre-incubation temperature of 30–40°C up to 12 h and at 50°C for 3 h. After 3 h, at 50°C, loss of activity up to 81 and 86% was observed in 6 h and 9 h, respectively (Fig. 3b).

Table 1 Substrate specificity of the purified chitosanase from *A. fertilissima*

Substrate	Relative activity (%)[a]
Glycol chitosan	100±1.3
Colloidal chitosan	87±1.4
Carboxymethyl chitosan	30±1.7
Colloidal chitin	5±0.5
Peptidoglycan	ND[b]
Chitosan oligosaccharides	
Chitobiose	ND
Chitotriose	ND
Chitotetrose	21±0.5
Chitopentose	24±1.5
Chitohexose	27±0.7
Chitoheptose	29±1.1

Values presented are means (±SEM) of three independent replicates

[a] Relative activity was expressed as rates relative to the activity of enzyme on glycol chitosan

[b] *ND* not detectable

Effect of various compounds and metal ions on chitosanase activity

Chitosanase activity measured in response to different metal ions showed that preincubation of the enzyme with Ag^{+}, Fe^{3+} and Hg^{2+} resulted in strongly inhibition by 81, 86 and 88%, respectively (Table 2). However, 25 and 20% higher chitosanase activity was observed on addition of Cu^{2+} and Zn^{2+}, respectively. However, chitosanase activity was completely lost on addition of β-mercaptoethanol, dithiothreitol and glutathione.

Discussion

Biological control of fungal plant pathogens represents an attractive and effective measure in the integrated disease management of crops. The role of enzymes such as chitinases, chitosanases, and endoglucanases has been widely explored for their potential as suitable options. In this context, the biocontrol potential of cyanobacteria, which are an important component of integrated nutrient management practices in the rice-wheat cropping system, can provide a useful approach. In our previous investigation, we identified a novel *cho* encoding antifungal chitosanase from *A. fertilissima* and showed that the transcriptional level of *cho* increased under the high dark period (8:16 h L:D). The specific chitosanase encoding protein was isolated and its functionality was confirmed by HPLC using chitopentamer as a substrate (Gupta et al. 2010). In the current study, besides 8:16 h L:D, the effect of other environmental/nutritional conditions (temperature, pH and phosphorous concentration) on antifungal chitosanase was evaluated in the same *A. fertilissima* strain. Further, the enzyme was purified under the optimized conditions and its specific properties such as kinetic parameters, substrate specificity, endo/exo nature using different chitosan oligosaccharides other than chitopentamer, effect of temperature, pH, metal ions and compounds were investigated. Chitosanase/antifungal activity from the extracellular filtrates of

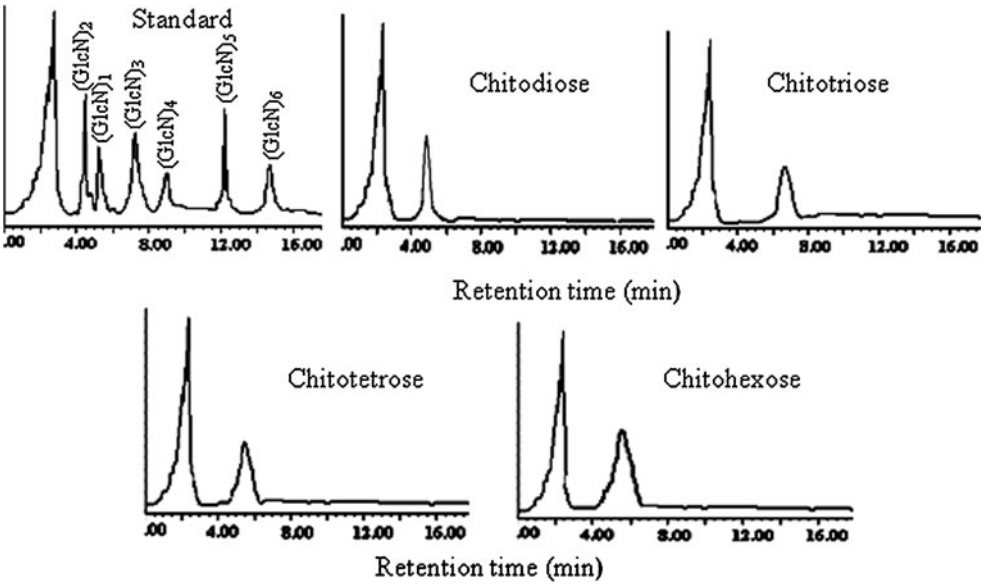

Fig. 2 HPLC-based profiles of purified chitosanase using different chitosan oligosaccharides

culture grown under different environmental/nutritional conditions showed an increase in both activities under P limitation. Some researchers have reported that biocidal activity is enhanced with a rise in P levels in the growth media (Radhakrishnan et al. 2009; Ray and Bagchi 2001); however, the production of toxins and allelochemicals in cyanobacteria is known to be favored by a low level of P in the medium, which also regulates the quality/type of toxin produced (Oh et al. 2000). P being an essential element for growth, its low level may lead to low carbon fixation rates. It

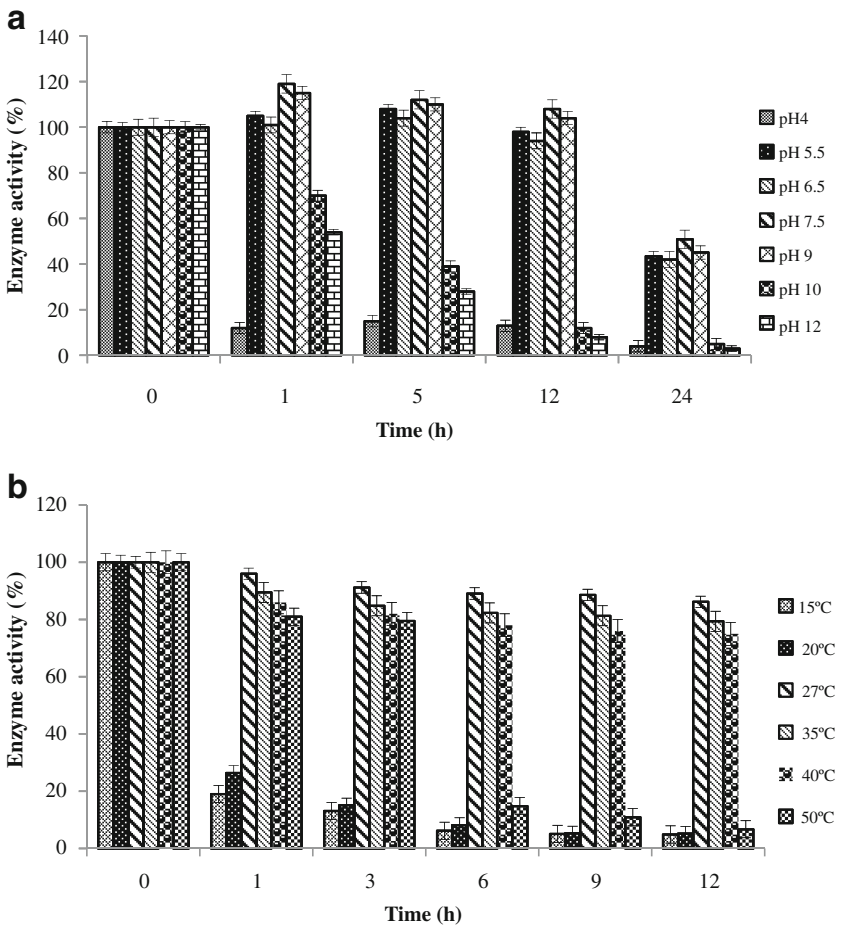

Fig. 3 Residual chitosanase activities at various pH and temperatures under different time points. For pH, activity was measured at 37°C for 15 min (**a**) and for temperature, at pH (7.5) for 15 min (**b**). Results for chitosanase stability were expressed in percentage of residual activity as a function of pH and temperature values and time points, considering a 100% activity for chitosanase at initial time (t=0)

Table 2 Effect of different metal ions and compounds on chitosanase activity

Metal ions and compounds	Relative activity (%)[a]
None	100±1.4
Mn^{2+}	84±2.3
Cu^{2+}	125±1.8
Zn^{2+}	120±2.4
Fe^{2+}	83±1.1
Fe^{3+}	14±1.9
K^{+}	79±2.0
Na^{+}	87±3.1
Ag^{+}	19±1.7
Ca^{2+}	90±2.5
Hg^{2+}	12±0.6
Mg^{2+}	92±1.8
Co^{+2}	82±2.4
β-mercaptoethanol	ND[b]
Dithiothreitol	ND
Glutathione	ND
Acetone	88±2.9
Ethanol	84±3.1

Values presented are means (±SEM) of three replicates

[a] Relative activity was expressed as rates relative to the activity of enzyme in the absence of any metal ion/ compound

[b] *ND* not detectable

can be surmised that the increased production of such allelochemicals/toxins/fungicidal enzymes may be providing a protective role against predators under such conditions.

The measurement of chitosanase/antifungal activity at different pH values under optimized P concentration (43 μM) showed a significant increase at high pH values (9.0). High pH may affect cellular enzyme function and change the speciation of metals (McKnight et al. 2001). Since enzyme activities are pH-dependent, changes in extracellular pH are likely to affect intracellular pH and hence enzyme structure in algae (Taraldsvik and Myklestad 2000). The enhanced chitosanase/antifungal activity observed in *A. fertilissima* under high pH (9.0) may possibly be attributable to an increase in the activities of other enzymes regulating or linked to chitosanase production. The possibility also exists that elevated pH may affect metal speciation and reduce the bioavailability of iron (Lundholm et al. 2004; McKnight et al. 2001), which may in turn enhance chitosanase production.

Temperature exerts its effect in the natural environment both directly by its stimulatory or inhibitory effects on toxin production by individual cyanobacteria and indirectly by creating an ecological advantage for the cyanobacteria. Regarding the effects of most environmental variables, temperature influences the cyanobacteria in different ways. The optimal temperature for highest chitosanase/antifungal activity was recorded at 27°C. Several studies have revealed the highest toxin production in the cyanobacterial cultures grown at 15–30°C. For instance, Rapala and Sivonen (1998) found the highest anatoxin-a levels in the cultures of *Anabaena* at temperatures within the range of 15–30°C. Castro et al. (2004) also showed the highest toxin production in the cyanobacterium *Cylindrospermopsis raciborskii* cultures maintained at the temperatures of 19 and 25°C. However, Radhakrishnan et al. (2009) recorded the highest biocidal activity at temperature of 40°C. In our study, the optimal temperature for chitosanase production in *A. fertilissima* is 27°C. Low temperature generally related with lower metabolic rates may restrict the release of exogenous enzymes while the high temperatures may lead to loss in cell viability or denaturation of proteins (Castro et al. 2004). However, further investigation in this area is required to understand the exact mechanism of regulation of chitosanase activity by temperature. This finding was validated by measuring the expression profile of *cho* under the same environmental/nutritional conditions by quantitative real-time RT-PCR (qRT-PCR). The transcription level of *cho* was significantly increased under the same conditions as evident from the biochemical analyses. Based on biochemical and expression analyses, we can conclude that chitosanase/antifungal activity increases under low P (43 μM), pH (9.0) and temperature of 27°C.

The genomic DNA was isolated from the culture grown under the optimized environmental/nutritional conditions and chitosanase encoding gene (*cho*) amplified and sequenced for its validation. The recombinant *cho* specific chitosanase was isolated and purified, and used for further characterization. The specific activity (822 U mg^{-1}) of the purified chitosanase was comparatively higher than that of previously reported other chitosanases (Ando et al. 2008; Wang et al. 2008; Gao et al. 2008). The k_{cat} and K_m values of the purified chitosanase were comparatively lower than that of an antifungal chitosanase from *Bacillus cereus* (Gao et al. 2008), but at par with that of chitosanase isolated from *Bacillus* sp. strain KCTC 0377BP (Choi et al. 2004).

The purified chitosanase was incubated with different substrates in order to understand the substrate specificity of this enzyme. Among all the substrates used, the highest activity of chitosanase was obtained against colloidal chitosan, followed by glycol chitosan, which may be the specific substrates for this purified chitosanase. The chitosanase activity was also evaluated against different glucosamine residues. But significant activity was only obtained with chitotetrose and longer chitosan oligosaccharides. This indicates the endo-type nature of the purified chitosanase. The endo-type nature of the chitosanase was also validated by HPLC (Eom and Lee 2003; Gao et al. 2009; Jung et al. 2006; Yoon et al. 2002), which is the well-known method for

revealing the exo/endo nature of the chitosanase, through analyses of the glucosamine units produced during hydrolysis. Earlier, it has been suggested that the production of monomer unit (GlcN) reflects the exo-type of enzyme and dimer $(GlcN)_2$ or more units producer is indicative of the endo-type nature of the chitosanase (Choi et al. 2004; Eom and Lee 2003; Gao et al. 2009; Jung et al. 2006; Li et al. 2008; Park et al. 1999; Yoon et al. 2002). Marcotte et al. (1996) undertook X-ray analysis to reveal the endohydrolase activity of an antifungal chitosanase from *Streptomyces* N174, and postulated that Glu 22 acts as an acid and Asp 40 serves as a general base to activate a water molecule for an S_N2 attack on the glycosidic bond. Although our previous investigation revealed no monomer unit when chitopentamer was used as a substrate in the HPLC profile (Gupta et al. 2010), in the current study, the reaction products of other chitosan oligosaccharides such as chitobiose, chitotiose, chitotetrose and chitohexose along with chitosan were also evaluated. The results showed that the reaction mechanism of the purified chitosanase does not lead to the production of monomer units with any of the substrates tried. This confirms the endo-type nature of the enzyme, which is similar to that of the antifungal chitosanase isolated from *Bacillus cereus* D-11 (Gao et al. 2008) and other bacterial and fungal chitosanases (Choi et al. 2004; Li et al. 2008; Park et al. 1999). To the best of our knowledge, this is the first endo-type chitosanase reported from cyanobacteria.

The data of chitosanase activity at different pH values (4.0–12.0) and time points (1–24 h) indicated that the optimum pH for isolated chitosanase was 7.5 and stable in the pH range from 5.5 to 9.0 during 12 h. Previous investigations revealed pH stabilities between 4.5 and 8 for chitosanase from *Aspergillus* sp. (Chen et al. 2005), 3 and 10 from *Bacillus* sp. (Sakihama et al. 2004), 5 and 8 from *Pseudomonas* sp. A-0 (Ando et al. 2008) and between 3 and 8 from *Streptomyces* sp. N17 (Shee et al. 2008). The differences in pH range for chitosanase stability might be explained on the basis of different composition in amino acid sequence of chitosanase, besides the nature of source. The optimal range of pH for the growth of most cyanobacteria is in the neutral to alkaline range, which validates the optimum pH for isolated chitosanase. The drastic drop in enzyme stability observed at high acidic and basic pH might be due to the pH denaturing effect on the three-dimensional structure of the chitosanase protein. More intramolecular repulsion at high acidic and basic pH may also lead to the unfolding of the protein, resulting in the loss of activity (Shee et al. 2008).

In order to evaluate the effect of temperature, the chitosanase activity measured at different temperatures (15–50°C) and time points (1–12 h) revealed optimum temperature for the purified chitosanase was 27°C and the activity was found to be stable up to 50°C and for 3 h. Thermostability of this purified chitosanase was closer to the other characterized chitosanases (Ando et al. 2008; Xu-fen et al. 2007). The decrease in enzyme activity on prolonged exposure to high temperatures in *A. fertilissima* chitosanase may be due to denaturation of chitosanase protein (Rivas et al. 2000).

In the current study, an interesting feature recorded was that, under in vivo conditions, the maximum activity of enzyme was observed at pH 7.5–9.0 and temperature 27°C; however, under in vitro conditions, the enzyme was found stable even at acidic pH (5.5) and temperature up to 50°C. The presence of cofactors/molecular chaperones under in vitro conditions may be responsible for the enhanced stability of chitosanase at supra optimal conditions.

The chitosanase activity measured in response to different metal ions revealed that it was strongly inhibited by Ag^{+}, Fe^{3+} and Hg^{2+} but stimulated by Cu^{+2} and Zn^{2+}. The dependence of the chitosanase activity on such metals ions has been recorded in several bacteria such as *Matsuebacter chitosanotabidus*, *Bacillus* sp. strain KCTC 0377BP, and *Pseudomonas* sp. A-01 etc (Ando et al. 2008; Choi et al. 2004; Park et al. 1999). Moreover, inhibition of chitosanase by Ag^{+}, Fe^{3+} and Hg^{2+} also indicated that thiol groups may be involved in the active catalytic site, which is essential for maintaining the three-dimensional structure of the active protein. This was further validated by evaluating the effect of thiol group inhibitors (β-mercaptoethanol, dithiothreitol and glutathione), and the results suggested that chitosanase activity was completely lost, suggesting again that sulfhydryl groups may be involved in the catalytic center of the enzyme (Oh et al. 1999). However, the chitosanase activity was significantly increased on addition of Cu^{2+} and Zn^{2+}, respectively. The exact mechanism behind this is not known. but one of the possible ways might be the interaction of both metal ions across the two imidazole nitrogen atoms on a common histidine residue of chitosanase. Copper might sit on the floor of a deep cleft and the zinc be completely buried in the protein. During catalysis, the copper is reduced with the substrate O_2^{-} to yield first O_2 then (by reoxidation) H_2O_2. Zn^{2+} in the protein is believed to increase the redox potential, implying it may also have a role in catalysis. Thus, in this way, both metal ions can increase the catalysis rate of the enzyme.

In the present scenario, most of the available information on chitosanases is related to their industrial applications. The data of chitosanase/antifungal activity under different environmental and nutritional factors suggested that *A. fertilissima* can perform as a biocontrol agent in tropical soils characterized by low P content (Oberson and Joner 2005). The maintenance of chitosanase activity over a wide range of pH and temperature emphasizes the promise of this organism under different soil ecologies. However, field

level evaluation is required to confirm its potential as a biocontrol agent.

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