Biochemical and Biophysical Research Communications 459 (2015) 184–188

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Characterization and homologous overexpression of an N-acetylglucosaminidase Nag1 from *Trichoderma reesei*

Fei Chen [a, b], Xiu-Zhen Chen [a], Li-Na Qin [a], Yong Tao [a], Zhi-Yang Dong [a, *]

[a] State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, No. 1 West Beichen Road, Chaoyang District, Beijing 100101, China
[b] University of Chinese Academy of Sciences, Beijing 100049, China

ARTICLE INFO

Article history:
Received 4 December 2014
Available online 19 December 2014

Keywords:
Trichoderma reesei
NAGase
Nag1
cbh1 promoter
Homologous overexpression

ABSTRACT

Trichoderma reesei is thought to be a promising recombinant host for the production and secretion of complex proteins due to its ability to secrete large amounts of proteins. In this study we identified a functional N-acetyl-β-glucosaminidase (NAGase) gene Nag1 in *T. reesei*. Nag1, a putative gene encoding a GH 20 family NAGase in *T. reesei*, was cloned and homologous overexpressed in the *T. reesei* RutC30ΔU3 with a strong *cellobiohydrolase1* gene (*cbh1*) promoter. Nag1 was secreted in its active form and the highest expression level was around 499.85 IU/ml. Nag1 has a molecular mass of 80 kDa. The optimum pH and temperature were 4.0 and 60 °C, respectively.

1. Introduction

Chitin, an unbranched homopolymer of 1,4-β-linked N-acetyl-D-glucosamine (GlcNAc), is widely distributed and is believed to be the second most abundant and renewable source in nature, next to cellulose. Chitinases chitin-degrading enzymes and mainly composed of chitobiosidase, N-acetyl-β-glucosaminidase, β-D-glucosaminidase and chitosanase. They are widely distributed in a wide range of species, such as viruses, fungi, bacteria, plants and animals [1]. These enzymes play different roles in diverse organisms and have important physiological functions and potential applications in biological, environmental and agricultural fields [1,2].

N-acetyl-β-glucosaminidase (NAGase), is an indispensable member of the chitinolytic system and belongs to the glycoside hydrolase family 20 [3]; its substrates include chitobiose, higher analogs and glycoproteins [4]. It catalyzes the hydrolysis of β (1–4) linked N-acetylglucosaminyl residues from glyco-conjugates at the non-reducing end, producing different lengths of GlcNAc. With the cooperative action of NAGase, endochitinase, and exochitinase, chitin can be degraded into GlcNAc. In recent years, NAGase has been extensively studied due to its significance in many fields. For example, it has been reported that human NAGase in lysosomes can cleave the N-acetyl-β-D-galactosamine and its deficiency may cause neurodegenerative lysosomal storage diseases [5]. NAGase is proposed to be involved in fungal cell development, microorganism or parasite destruction, cell wall synthesis, modification and degradation during the autolysis [6]. There are many published studies on cloning, characterization and functions of NAGase in fungi; however, the production of fungal NAGase is low in the reported *Trichoderma harzianum* recombinant system [7]. Therefore, the construction of high-expression system is of great value and importance for higher NAGase productivity and industrial applications.

As one of the excellent cellulase producers, *Trichoderma reesei* is widely used in biomass-degradation for industrial applications; several mutant strains can produce up to 40 g/L of cellobiohydrolase I (CBH1) and accounts for approximately 50% of all secreted proteins from the strains [8]. To date, two chitinolytic enzymes in *T. reesei* with chitinase and β-D-glucosaminidase activities have been identified [2]. Purification, characterization, gene cloning and expression of the 46 kDa chitinase (Chi46) from the *T. reesei* PC-3-7 has been carried out in *Escherichia coli*. However, based on our analysis of the *T. reesei* genome-sequence, more than 20 putative chitinolytic enzymes has been identified and only 3 of them belong to the family 20 chitinases. Little is known about chitinolytic enzymes in *T. reesei* [1,6,9]. In the present study, we cloned a *nag1* gene encoding NAGase in *T. reesei* and homologous overexpressed this gene in the *T. reesei* strain RutC30ΔU3 with the strong *cellobiohydrolase 1* gene (*cbh1*) promoter. The characteristics of this NAGase are also described.

* Corresponding author. Fax: +86 1064807337.
E-mail address: dongzy@mail.im.ac.cn (Z.-Y. Dong).

http://dx.doi.org/10.1016/j.bbrc.2014.12.066
0006-291X/

2. Materials and methods

2.1. Strains and growth conditions

The *T. reesei* strain RutC30ΔU3 (uridine auxotrophic strain) was constructed and stored in our laboratory for the expression of Nag1. The *T. reesei* strain spores were maintained on potato dextrose agar (PDA) plates. Liquid minimal medium (MM) was prepared without peptone as described by Ilmen et al. [10]. When necessary, 10 mM uridine was supplemented with MM or PDA. The pH of MM was adjusted to 5.3 ± 0.2 with NH_4OH. *E. coli* DH5α were used as the host strain for plasmid propagation.

2.2. Gene cloning and sequence analysis

The fragments of Nag1 (without the signal peptide sequence) were amplified by PCR from the RutC30 genomic DNA by using primers jdz-F: 5′-GGAATTCATGTATCGGAAGTTGGCCGTCATCTC GGCCTTCTTGGCCACAGCTCGTGCTATGCTGCTGCCCAAGGCCGTCCT-3′ (contains *cbh1* gene signal peptide sequence) and jdz-R: 5′-GGACTAGTTCATGGGAAAAGGGCGCAGGCCGTC-3′ which contain *Eco* RI and *Spe* I sites, respectively. The resulting amplicons were digested with *Eco* RI and *Spe* I and cloned into the *Eco* RI and *Spe* I digested vector pSKcPT [11], a plasmid that contains the *T. reesei cbh1* gene promoter and terminator, to form the expression vector pSKtrNag1. Plasmid pSKpyr4 was constructed and stored in our laboratory for the transformation of the *T. reesei* strains [11].

2.3. Expression and purification

T. reesei protoplasting and transformation conditions were as described previously [12]. The *T. reesei* strain RutC30ΔU3 (an auxotrophic strain) was co-transformed with vector pSKtrNag1 and vector pSKpyr4, that bears the *pyr4* gene which complements uridine auxotrophic strains. The Nag1 expression transformants were screened on minimal medium.

Positive transformants were selected and further confirmed with PCR using primers Pcbh1F: 5′-ATGTTGTGAATCTGTGTCGGG-CAGG-3′ and PTrNag1R: 5′-GAGGGCGTGCAGCACGCCGGTCGAC-3′, that should generate a 1.6 kb fragment. About 10^7 spores of the *T. reesei* strain were added into 50 ml liquid minimal medium in a 250 ml flask and cultured for 48 h at 200 rpm at 28 °C, the mycelia were then filtered through 200 mesh sifter (30 μm pore diameter) and washed twice with carbon-free minimal medium. About 2 g of this culture was added to 50 ml of the cellulase-inducing minimal medium, in which 2% glucose (w/v) was substituted with 1% Avicel (Fluka), and grown for 6 days at 200 rpm at 28 °C to produce Nag1.

To obtain purified Nag1, the culture medium was centrifuged at 8000*g* at 25 °C for 10 min, ultrafiltration concentrators (Millipore) was used to concentrate the supernatant. Next a Hitrap Q column (GE) was used, followed by a superdex 200 column (GE), and then the samples were resuspended in 50 mM sodium acetate buffer, pH 5.0. The results were analyzed by 12.5% SDS–PAGE.

2.4. Enzyme activity assay

N-acetyl-glucosaminidase activity was assayed in triplicate as described previously by De Marco [13]. The absorbance was measured at 405 nm to determine the amount of p-nitrophenol produced using a standard curve. One unit of N-acetyl-glucosaminidase activity corresponded to the amount of enzyme required to produce 1 μmol of p-nitrophenol min^{-1}. To determine the substrate specificity of the chitinase, p-nitrophenol-N-acetyl-glucosaminide, p-nitrophenol-xylopyranoside, p-nitrophenol-mannopyranoside, p-nitrophenol-fucopyranoside, p-nitrophenol-galactopyranoside and p-nitrophenol-mannoside were used as substrates with the assay method described above.

To determine the optimum pH for Nag1, the enzyme activity was measured from pH 3.0 to 8.0 with the assay method described above. The buffer solutions (50 mM) used was sodium acetate (pH 3.0–5.0) and sodium phosphate (pH 6.0–8.0). To determine the optimum temperature for Nag1, the enzyme activity was measured from 30 °C to 80 °C with the assay method described above. To determine the effect of temperature on the stability of the enzyme activity, the enzyme was incubated at temperatures ranging from 20 °C to 80 °C for 30 min, and then the residual activity was assayed by the method described above. To determine the enzyme activity to the chitin oligosaccharides, the optimum pH and optimum temperature was assayed by the method described above, and then TLC assays described previously by Powning [14] were used to investigate the effects of Nag1 on the degradation of chitin oligosaccharides.

3. Results

3.1. Sequence comparisons of a putative NAGase

Our search of the *T. reesei* genome database revealed that three encoding putative NAGases genes were proposed in the *T. reesei* genome. The deduced amino acid sequence of all three putative family 20 NAGases genes are Nag1 (protein ID: 21725), Nag2 (protein ID: 23346) and Nag3 (protein ID: 105931). Among them, Nag1 with different functionally-characterized GH20 enzymes from human, bacteria, and fungi were aligned and compared (Fig. 1). The results showed that the deduced amino acid sequence of Nag1 was very similar to the sequence of the *Trichoderma atroviride* Nag1 (Ta Nag1) with 87% identity. The key amino acids residues involved in catalytic function and substrate binding were also identical [15,16].

3.2. Cloning of the nag1 gene

In order to express the *nag1* gene in *T. reesei* under the control of the *cbh1* promoter, the targeting plasmid pSKtrNag1 was constructed by inserting the coding region of the *nag1* gene into pSKcPT vector, including the 1.9 kb *cbh1* promoter and signal peptide region and 2.2 kb *cbh1* terminator region. Briefly, *nag1* gene was amplified by PCR from genomic DNA of the *T. reesei* strain RutC30 using the primers described above, and then ligated with the vector pSKcPT fragment. The resulting vector, pSKtrNag1, was confirmed by sequencing.

3.3. Expression and purification of the recombinant Nag1

Targeting vector, pSKtrNag1, was then transformed into genomic DNA of the *T. reesei* strain RutC30ΔU3 using standard transformation methods mentioned above. The positive transformants were selected by assaying for mitotic stability of the inserted recombinant *nag1* expression cassette and uridine auxotrophic complement gene *pyr4* as mentioned above in the methods. They were further confirmed with a 1.6 kb fragment detected by PCR using primers Pcbh1F and PTrNag1R.

Proteins secreted from several positive transformants, namely U3-chi1 to U3-chi4, were analyzed by SDS–PAGE. The results show the molecular weight of Nag1 is approximately 80 kDa (Fig. 2A). MALDI-TOF mass spectrometry was performed to further confirm Nag1, and the amino acid sequence was as expected. The CBH I protein could be detected, except the secreted proteins of the U3-chi3 (Lane 4), and *cbh1* genes were not amplified from the DNA of U3-chi3, which demonstrated that *cbh1* was knocked out during

the homologous recombination by the plasmid pSKtrNag1. Additionally, U3-chi3 demonstrated higher target protein activities among these transformants. Also, with the convenience for protein purification, U3-chi3 was selected for further study.

The recombinant protein from clone U3-chi3 was purified with the methods mentioned above. The results of SDS–PAGE showed that the recombinant proteins were purified (Fig. 2B).

3.4. Biochemical and catalytic characterization of the recombinant Nag1

P-nitrophenyl-N-acetylglucosaminide was used as the substrate to determine the optimum pH of the recombinant Nag1. The highest activity was obtained at pH 4.0, and more than 60% of the enzymatic activity was retained between pH 3.5 and 6.0 (Fig. 3A). To further analyze the effects of pH on the stabilization of Nag1, the enzyme was incubated at different pH conditions, and the time-courses of inactivation were measured. The results indicated that the recombinant Nag1 activity was almost unchanged after incubation at pH 5.0 for 60 min, while retaining about 80% activity between pH 4.0 and 6.0. The activity decreased dramatically with a pH higher than 6.0 (Fig. 3B).

The effects of temperature on enzyme activity were also performed. The highest activity was obtained at 60 °C. Above this temperature the activity greatly decreased (Fig. 3C). The thermal stability of Nag1 was determined at different temperatures from 30 °C to 60 °C after incubation for 8 h. The results show that Nag1 retained most of its activity at temperatures below 50 °C (above 90% at 30 °C and 40 °C, and 85% at 50 °C). Even at 60 °C, Nag1 could conserve its activity up to 60% after incubation for 8 h (Fig. 3D).

With the optimized conditions, the kinetic parameters of the hydrolytic activity of Nag1 were assessed. The results show the specific activity of Nag1 was 319.89 IU/mg after purification. Km and kcat/Km values with p-nitrophenol-N-acetyl-glucosaminide were 69.41 ± 4 M and 1023 ± 23 M^{-1} s^{-1}, respectively.

To determine the substrate specificity of the Nag1, p-nitrophenol-N-acetyl-glucosaminide, p-nitrophenol-xylopyranoside, p-nitrophenol-mannopyranoside, p-nitrophenol-fucopyranoside, p-nitrophenol-galactopyranoside, and p-nitrophenol-mannoside were used as substrates. The recombinant Nag1 only used p-nitrophenol-N-acetyl-glucosaminide as its substrate.

The TLC assays results are shown in Fig. 3E. Lane 1 is GlcNAc, Lane 2 is chitin oligosaccharides without Nag1, and Lane 3 is chitin oligosaccharides that mixed with Nag1 and incubated in optimum reaction conditions. GlcNAc was increased and the other lengths of chitin oligosaccharides were obviously decreased. These results indicate that chitin oligosaccharides are a natural substrate that can be digested by Nag1.

The recombinant Nag1 activities were tested in the presence of different metal ions at a 1 mM concentration. The results indicate

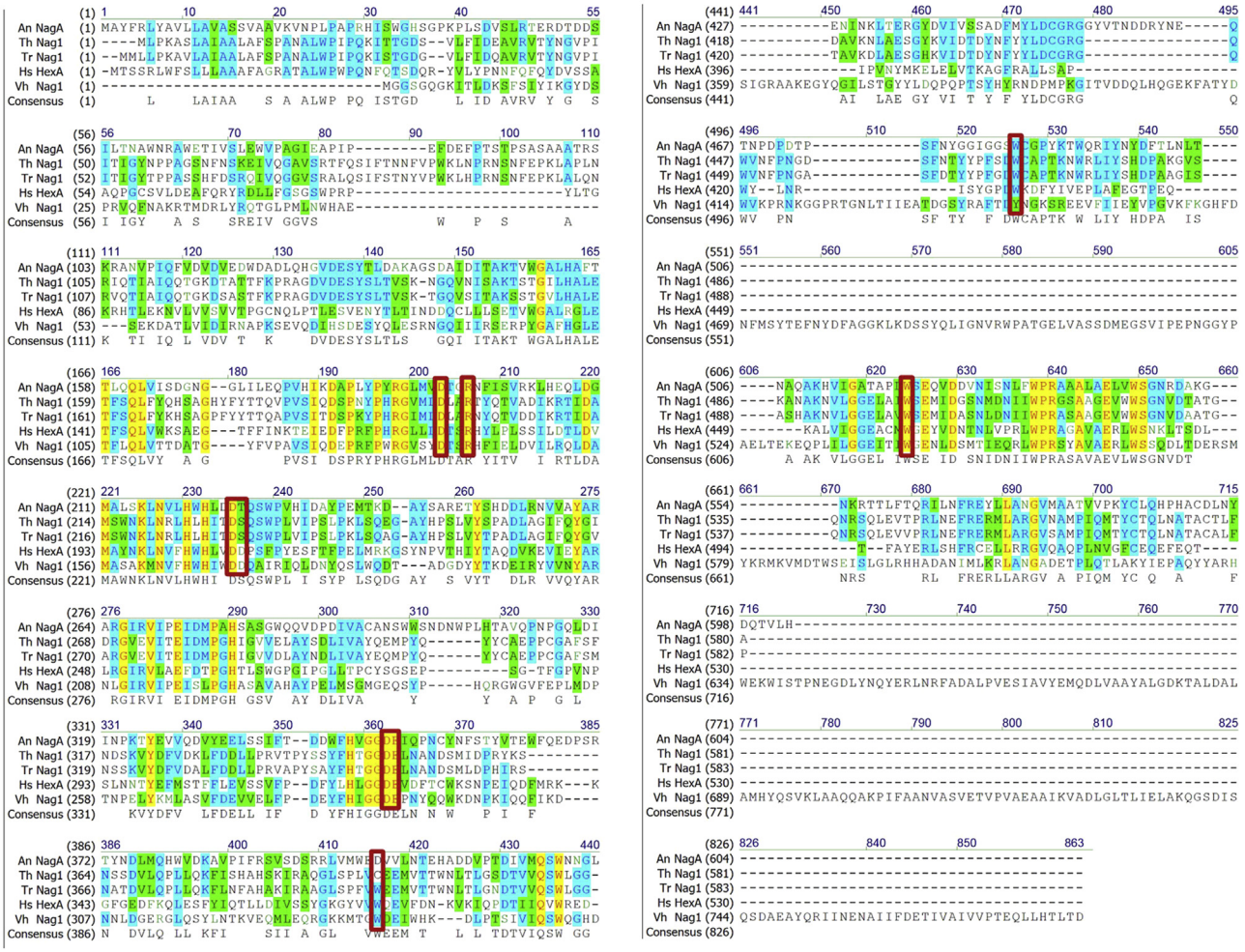

Fig. 1. Sequence alignment of Nag1.

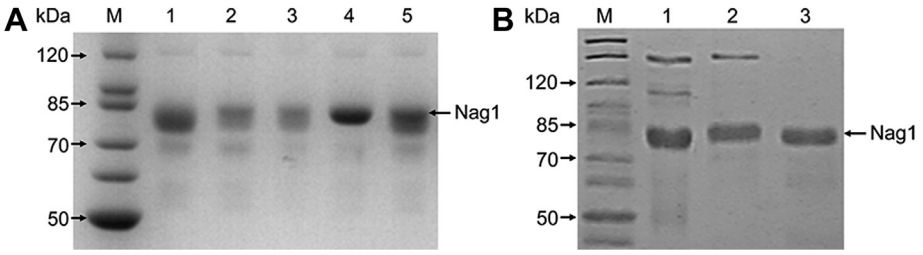

Fig. 2. Expression and purification of Nag1. (A) SDS–PAGE for different transformants: Lane 1, supernatant of RutC30ΔU3; Lane 2–5, supernatant of recombination strain U3-chi1 to U3-chi4. (B) SDS–PAGE analysis of NAGase: Lane 1, secreted proteins after concentration of reconstructed strains U3-chi3; Lane 2, soluble fraction after using of a Hitrap Q column; Lane 3, purified NAGase.

that Co^{2+}, Ca^{2+}, Cu^{2+}, Mg^{2+}, and Zn^{2+}could not affect its activities, while Fe^{3+} and Al^{3+} could inhibit its activities to 60% and 70%, respectively (Fig. 3F).

4. Discussion

Although in mycoparasitic fungi, the chitinolytic systems mainly focus on *T. harzianum*. To date, several chitinases of *T. harzianum* have been purified, including endochitinases, chitobiosidases and N-acetyl-glucosaminidases. The fungal chitinase genes are well expressed in *E. coli*, yeast, and plants. However, there is not much data on the expression of Nag1, or the production of the NAGase has been too low [1]. The key amino acids residues involved in catalytic function and substrate binding are much conserved in the species from bacteria to humans. The analysis of the Nag1 amino acid sequence showed that the key amino acid residues were identical [15,16]. With the application of the *cbh1* promoter, our results show that the U3-chi3 could not express CBH I and had a higher Nag1

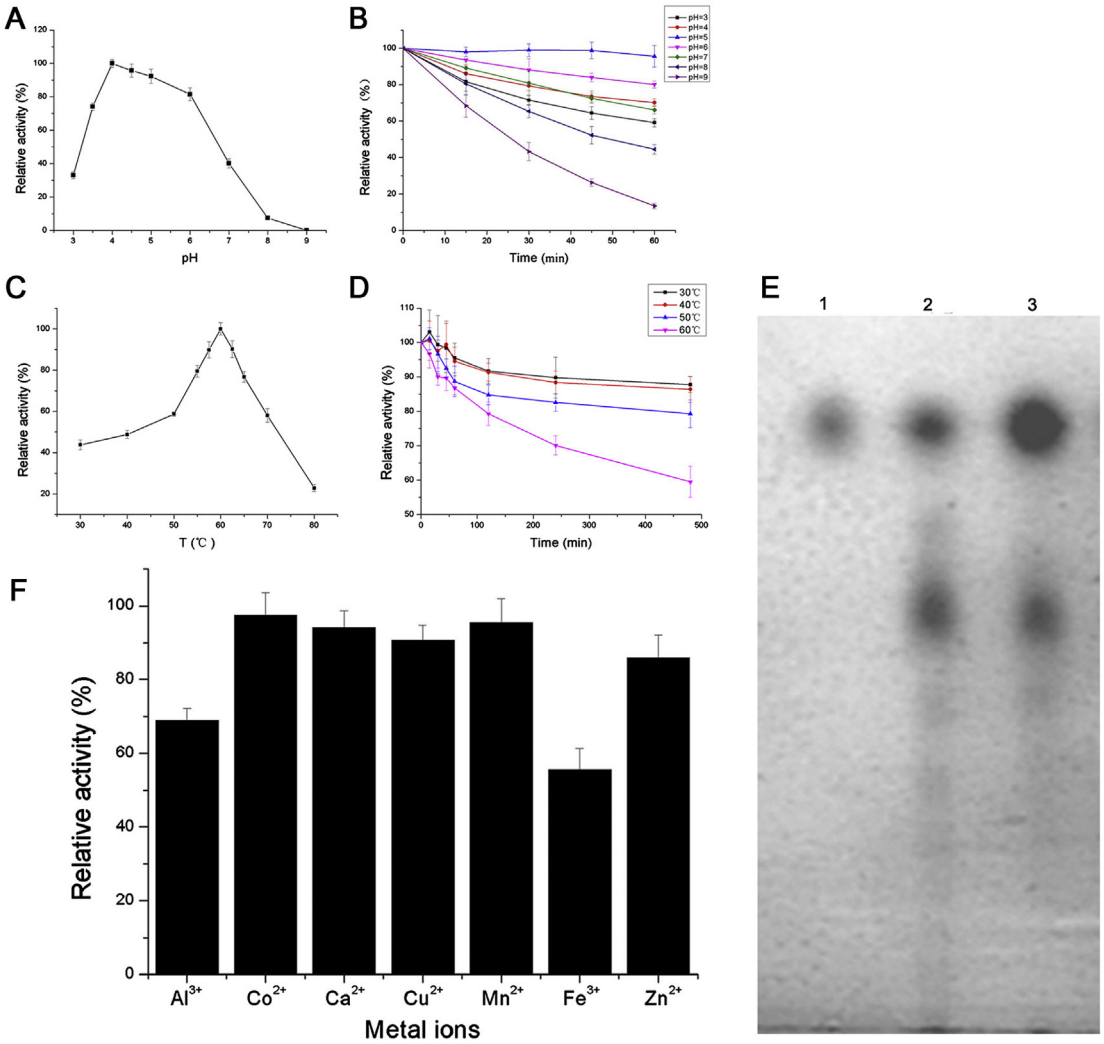

Fig. 3. The characteristics of Nag1. (A) Nag1 activities at different pH. (B) Time courses of residual Nag1 activities at different pH. (C) Nag1 activities at different temperatures. (D) Time courses of residual Nag1 activities at different temperatures. (E) The effects of Nag1 on the degradation of chitin oligosaccharides by TLC assay: Lane 1, GlcNAc; Lane 2, chitin oligosaccharides; Lane 3, chitin oligosaccharides with Nag1. (F) The effects of metal ions on Nag1 activity.

expression level around 499.85 IU/ml among the transformants. A recent study has shown that the lack of CBH I could improve the expression levels of the target protein [11].

A genome-wide analysis of *T. reesei* indicated the presence of numerous glycoside hydrolases-like coding cassettes, most of which the functions still remain to be elucidated [17]. Furthermore, the lack of understanding of the complex regulation networks for polysaccharide-carbon-source utilization remarkably limits the metabolic study and industrial applications of *T. reesei* [18]. Many factors, such as nutrient uptake, regulation of promoters, and growth or fermentation cycle, play an important role in the secretion and production of the enzyme. Moreover, some of metabolic intermediates, like oligosaccharides, might play critical roles as inducers or regulators of glycoside hydrolases secretion. Chitinase expression in fungi is thought to respond to degradation products that serve as inducers and to easily metabolize carbon sources that serve as repressors. While the chitinase from fungi has many advantages, such as inducible and exocytosis, and therefore it may play an important role in industrial production [19].

In this study we identified a functional gene Nag1. And by homo-overexpression of Nag1 in *T. reesei*, the production and specific activity is higher than that of native overproducing strain *T. harzianum* [20]. It is universally accepted that NAGase has broad substrate specificity and that NAGase from different fungus species may have different substrate specificities, which is of great value for industrial applications. It cleaves diacetylchitobiose and higher chitin polymers into GlcNAc monomers; it also splits some chromogenic substrates, such as p-nitrophenol-N-acetyl-glucosaminide and other p-nitrophenol-hexosaminides, to release p-nitrophenol [1]. In this study, we found that Nag1 used the p-nitrophenol-N-acetyl-glucosaminide as a substrate as expected, indicating that it does act as a NAGase. However, Nag1 could only utilize p-nitrophenol-N-acetyl-glucosaminide as its substrates, which suggested that Nag1 was only specific to a certain substrate and has very strict substrate specificity.

Most fungal NAGases, such as, *Aphanocladium album*, *Gliocladium virens*, and *Trichothecium roseum*, have been thought to be active between pH 4.0 and 7.0 [1]. The purified *T. reesei* Nag1 has the optimum enzymatic pH as low as 4.0, which was tested using p-nitrophenol-N-acetyl-glucosaminide as the standard substrate. This value was similar to that of the NAGases reported in *T. harzianum* and lower than other reported fungal NAGases [21]. As for optimum temperature, Nag1 in this study had a rather high optimum temperature of 60 °C, which was superior to other fungal NAGases that have the optimum activity at moderate temperatures ranging from 20 °C to 40 °C [1,4]. Furthermore, the fairly heat-stable property of Nag1 is comparable to the NAGases from thermophilic fungi such as *Thermomyces lanuginosus* and *Talaromyces emersonii* [2,8,13].

In conclusion, we identified a functional NAGase gene, *nag1*, encoding a GH 20 family NAGase in *T. reesei*. In addition the Nag1 homologous overexpression in *T. reesei* RutC30ΔU3 with the strong *cbh1* Nag1 promoter was successful with the highest expression level around 499.85 IU/ml. The thermo and pH stability, strict substrate specificity, high specific activity of Nag1 from *T. reesei*, indicate this enzyme has potential in a variety of industrial applications.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

This work was financially supported by the National Basic Research Program of China (No. 2011CB707402), the National Natural Science Foundation of China (30970073), and the Knowledge Innovation Program (No. KSCX1-YW-11B3) from the Chinese Academy of Sciences.

References

[1] L. Duo-Chuan, Review of fungal chitinases, Mycopathologia 161 (2006) 345–360.
[2] M. Ike, K. Nagamatsu, A. Shioya, M. Nogawa, W. Ogasawara, H. Okada, Y. Morikawa, Purification, characterization, and gene cloning of 46 kDa chitinase (Chi46) from *Trichoderma reesei* PC-3–7 and its expression in *Escherichia coli*, Appl. Microbiol. Biotechnol. 71 (2006) 294–303.
[3] G.D. Tzelepis, P. Melin, D.F. Jensen, J. Stenlid, M. Karlsson, Functional analysis of glycoside hydrolase family 18 and 20 genes in *Neurospora crassa*, Fungal Genet. Biol. 49 (2012) 717–730.
[4] B. Amutha, J.M. Khire, M.I. Khan, Characterization of a novel exo-N-acetyl-β-D-glucosaminidase from the thermotolerant *Bacillus* sp. NCIM 5120, Biochim. Biophys. Acta – Gen. Subj. 1425 (1998) 300–310.
[5] D.J. Mahuran, Biochemical consequences of mutations causing the GM2 gangliosidoses, Biochim. Biophys. Acta – Mol. Basis Dis. 1455 (1999) 105–138.
[6] K. Slámová, P. Bojarová, D. Gerstorferová, B. Fliedrová, J. Hofmeisterová, M. Fiala, P. Pompach, V. Křen, Sequencing, cloning and high-yield expression of a fungal β-N-acetylhexosaminidase in *Pichia pastoris*, Protein Expr. Purif. 82 (2012) 212–217.
[7] C. Peterbauer, K. Brunner, R. Mach, C. Kubicek, Identification of the N-acetyl-D-glucosamine-inducible element in the promoter of the *Trichoderma atroviride* nag1 gene encoding N-acetyl-glucosaminidase, Mol. Genet. Genomics 267 (2002) 162–170.
[8] H. Durand, M. Clanet, G. Tiraby, Genetic improvement of *Trichoderma reesei* for large scale cellulase production, Enzyme Microb. Technol. 10 (1988) 341–346.
[9] T. Liu, T. Wang, X. Li, X. Liu, Improved heterologous gene expression in *Trichoderma reesei* by cellobiohydrolase I gene (*cbh1*) promoter optimization, Acta Biochim. Biophys. Sin. 40 (2008) 158–165.
[10] M. Ilmen, A. Saloheimo, M.-L. Onnela, M.E. Penttilä, Regulation of cellulase gene expression in the filamentous fungus *Trichoderma reesei*, Appl. Environ. Microbiol. 63 (1997) 1298–1306.
[11] L.-N. Qin, F.-R. Cai, X.-R. Dong, Z.-B. Huang, Y. Tao, J.-Z. Huang, Z.-Y. Dong, Improved production of heterologous lipase in *Trichoderma reesei* by RNAi mediated gene silencing of an endogenic highly expressed gene, Bioresour. Technol. 109 (2012) 116–122.
[12] M. Penttilä, H. Nevalainen, M. Rättö, E. Salminen, J. Knowles, A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*, Gene 61 (1987) 155–164.
[13] J.L. De Marco, M. Valadares-Inglis, C. Felix, Purification and characterization of an N-acetylglucosaminidase produced by a *Trichoderma harzianum* strain which controls Crinipellis perniciosa, Appl. Microbiol. Biotechnol. 64 (2004) 70–75.
[14] R. Powning, H. Irzykiewicz, Separation of chitin oligosaccharides by thin-layer chromatography, J. Chromatogr. A 29 (1967) 115–119.
[15] B.L. Mark, D.J. Mahuran, M.M. Cherney, D. Zhao, S. Knapp, M.N. James, Crystal structure of human β-hexosaminidase B: understanding the molecular basis of Sandhoff and Tay–Sachs disease, J. Mol. Biol. 327 (2003) 1093–1109.
[16] B.L. Mark, D.J. Vocadlo, S. Knapp, B.L. Triggs-Raine, S.G. Withers, M.N. James, Crystallographic evidence for substrate-assisted catalysis in a bacterial β-hexosaminidase, J. Biol. Chem. 276 (2001) 10330–10337.
[17] D. Martinez, R.M. Berka, B. Henrissat, M. Saloheimo, M. Arvas, S.E. Baker, J. Chapman, O. Chertkov, P.M. Coutinho, D. Cullen, Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. Hypocrea jecorina), Nat. Biotechnol. 26 (2008) 553–560.
[18] H. Jun, T. Kieselbach, L.J. Jönsson, Enzyme production by filamentous fungi: analysis of the secretome of *Trichoderma reesei* grown on unconventional carbon source, Microb. Cell Fact. 10 (2011) 68.
[19] L. Hartl, S. Zach, V. Seidl-Seiboth, Fungal chitinases: diversity, mechanistic properties and biotechnological potential, Appl. Microbiol. Biotechnol. 93 (2012) 533–543.
[20] R.N. Silva, S.P. da Silva, R.L. Brandão, C.J. Ulhoa, Regulation of N-acetyl-β-D-glucosaminidase produced by *Trichoderma harzianum*: evidence that cAMP controls its expression, Res. Microbiol. 155 (2004) 667–671.
[21] J. Li, J. Wang, S. Wang, M. Xing, S. Yu, G. Liu, Achieving efficient protein expression in *Trichoderma reesei* by using strong constitutive promoters, Microb. Cell Fact. 11 (2012) 84.