



## Endochitinase 1 (Tv-ECH1) from *Trichoderma virens* has high subsite specificities for acetylated units when acting on chitosans

Franziska Bußwinkel<sup>a</sup>, Oscar Goñi<sup>b</sup>, Stefan Cord-Landwehr<sup>a</sup>, Shane O'Connell<sup>b</sup>, Bruno M. Moerschbacher<sup>a,\*</sup>

<sup>a</sup> Institute for Biology and Biotechnology of Plants, University of Münster, Schlossplatz 8, 48143 Münster, Germany

<sup>b</sup> Plant Biostimulant Group, Shannon Applied Biotechnology Centre, Institute of Technology Tralee, Clash, Tralee, County Kerry, Ireland

### ARTICLE INFO

#### Article history:

Received 16 March 2017

Received in revised form 14 December 2017

Accepted 14 March 2018

Available online 15 March 2018

#### Keywords:

GH18

Chitosans

Subsite specificities determination

### ABSTRACT

Chitosans with defined characteristics have been shown to possess reproducible bioactivities for numerous applications. A promising approach for producing chitosans with defined degrees of polymerization (DP), degrees of acetylation (DA), and patterns of acetylation (PA) involves using chitin-modifying enzymes. One such enzyme, the chitinase Tv-ECH1 belonging to the glycoside hydrolase (GH) family 18, seems to have an important role in the biocontrol properties of the fungus *Trichoderma virens*, suggesting its potential in generating novel chitosans for plant health applications. In this study, the Tv-ECH1 enzyme was overexpressed in the methylotrophic yeast *Pichia pastoris*, yielding large amounts (up to 2 mg mL<sup>-1</sup>) of purified recombinant enzyme of high activity, high purity, and high stability, making the system promising for industrial production of Tv-ECH1. The purified Tv-ECH1 chitinase displayed a wide optimal pH range from 4.5 to 6 and an optimal temperature of 37 °C. Detailed subsite specificity analyses revealed high preference for acetylated residues at all four subsites analyzed (−2, −1, +1, +2), making Tv-ECH1 a promising candidate for the biotechnological production of specific chitosan oligomers and for the characterization of chitosan polymers via enzymatic fingerprinting.

© 2018 Elsevier B.V. All rights reserved.

### 1. Introduction

Chitosan polymers, which are partially de-*N*-acetylated counterparts of chitin, have a wide range of bioactivities, including antimicrobial activity or the ability to make plants resistant to pathogens. Because their bioactivities may rely on their oligomeric breakdown products generated through the action of chitosanolytic enzymes that, for example, are present in the target tissues, a major focus of current chitosan research is on identifying chitinases (EC 3.2.1.14) and chitosanases (EC 3.2.1.132), characterizing their substrate specificities, and determining the structures of their products [1–3]. Depending on the hydrolytic enzyme, hydrolysis of chitosan polymers can result in oligomeric products exhibiting different degrees of polymerization (DP), degrees of *N*-acetylation (DA), and patterns of *N*-acetylation (PA), all of which may influence the molecules' physicochemical properties and their promising biological functionalities [4–7]. Thus, identifying chitinases and chitosanases that result in consistent oligomeric products is crucial for systematically producing oligomers with consistent bioactivities [8].

**Abbreviations:** DP, degree of polymerization; DA, degree of *N*-acetylation; D, Glucosamine; GH, glycoside hydrolase; PA, pattern of acetylation; A, *N*-Acetylglucosamine; Tv-ECH1, *Trichoderma virens* endochitinase 1; UHPLC, ultra high performance liquid chromatography; HILIC, hydrophilic interaction chromatography.

\* Corresponding author.

E-mail address: [moersch@uni-muenster.de](mailto:moersch@uni-muenster.de) (B.M. Moerschbacher).

One particularly promising source of chitinases are mycoparasitic fungi of the genus *Trichoderma*, which can contain up to 36 genes coding for chitinases of the glycoside hydrolase family GH18 [9]. Some of the most strongly conserved chitinases in the fungal kingdom are the abundantly expressed 42-kDa endochitinases from different *Trichoderma* spp., such as Tv-ECH1/CHT42 in *T. virens*, ECH42 in *T. atroviride*, and CHIT42 in *T. harzianum*. Although these chitinases are not exclusively specific for mycoparasites, previous work has proven that they are involved in the mycoparasitic lifestyle of *Trichoderma* [9–11]. Furthermore, mycoparasitic fungi are frequently used as biocontrol agents against various phytopathogens, and several studies have focused on examining if *T. virens* gets its biofungicide activity from the endochitinase Tv-ECH1. For example, one study proved that the expression of *Tv-ech1*, which shares 83% sequence identity with the well-characterized 42-kDa endochitinase gene *chit42* from *T. harzianum*, was induced when it contacted a cell wall preparation from the phytopathogen *Rhizoctonia*, indicating that the Tv-ECH1 endochitinase could be involved in the fungus's interaction with phytopathogens [12]. A different study examined how an extracellular 42-kDa chitinase was able to influence the biocontrol activity of *T. virens* using genetically manipulated strains of this fungus. In comparison to the wildtype, the two strains in which the chitinase gene was disrupted or constitutively over-expressed showed decreased and enhanced biocontrol activity against *R. solani*, respectively [13]. Furthermore, transgenic cotton lines expressing Tv-ECH1 showed enhanced resistance against *R. solani*

and *Alternaria alternata* [14]. However, Tv-ECH1 has not yet been thoroughly characterized: To date, only one study has performed purification and characterization on an endochitinase that was tentatively identified as Tv-ECH1 [15].

Although experiments on Tv-ECH1 are still lacking, different chitinolytic enzymes from *Trichoderma* have been previously cloned and expressed in *Escherichia coli* [16] or *Pichia pastoris* [17,18]. The methylotrophic yeast *P. pastoris* has emerged as a cost-effective expression system for the production of eukaryotic recombinant proteins, offering several advantages over prokaryotic and other eukaryotic expression systems, such as the ability to perform correct post-translational modifications, high productivity levels, and secretion into the culture media for easier recovery [19,20]. Therefore, in this study we aimed to heterologously express the Tv-ECH1 gene in *P. pastoris* to achieve efficient enzyme production. Then, we aimed to characterize the purified recombinant enzyme and its oligomeric chitosan hydrolysis products analyzing enzyme activity on biotechnologically produced oligomeric chitosan substrates with fully defined PA and the recently developed tool of mass spectrometric sequencing of the partially acetylated chitosan oligomers. These methods allowed us to determine the substrate specificities of the enzyme and the properties of the oligomeric products in unprecedented detail.

## 2. Material and methods

### 2.1. Substrates and strains

$\alpha$ - and  $\beta$ -chitin, chitosan 124 (DP ~2600, DA 1.51%) and 144 (DP ~650, DA 2.23%), were kindly provided by Dr. Dominique Gillet from Mahtani Chitosan (Veraval, India). Methods described by Schatz et al. [21] and Hirai et al. [22] were applied for determination of DP and DA. *N*-acetylglucosamine (A), was purchased from Sigma Aldrich (München, Germany), and A<sub>2-6</sub>, from Megazyme (Bray, Ireland). Partially acetylated chitosan tetramers with defined PA were generated by deacetylation of A<sub>4</sub> with the chitin deacetylases NodB, COD/VcCDA [23], PgtCDA [24] and PesCDA [25]. Deacetylation was monitored with MS<sup>1</sup> measurements. The preparation of colloidal chitin from  $\beta$ -chitin was performed following the protocols reported by Hsu and Lockwood [26]. To obtain chitosan series of increasing DA, chitosan batches 124 and 144 were chemically acetylated according to the protocol of Lamarque et al. [27].

*P. pastoris PichiaPink* Strain 1 from the *PichiaPink* Expression System (Invitrogen) was used for the expression of Tv-ECH1.

### 2.2. Construction of expression vector pPink $\alpha$ -HC::Tv-ech1 and *P. pastoris* transformation

For expression of Tv-ECH1 in *P. pastoris*, the *Tv-ech1* gene sequence (GenBank: AF397020.1) was slightly modified. The internal signal sequence (first 22 amino acids), predicted with SignalP [28] and TargetP [29], was excluded while 5' *Mly* I and 3' *Kpn* I restriction sites and a 3' His<sub>6</sub> tag (CACCATCATCACCACCAC) were included. GeneArt (Fisher Scientific, Regensburg, Germany) was provided with the modified sequence for codon optimization and gene synthesis. Transformation of *P. pastoris PichiaPink* Strain 1 with the expression vector pPink $\alpha$ -HC::Tv-ech1 was performed with the PEG 1000 transformation method for *Pichia* following the *Pichia* Expression Kit User Guide (Invitrogen). Along with pPink $\alpha$ -HC::Tv-ech1, the empty vector pPink $\alpha$ -HC was transformed as a negative control for further experiments.

### 2.3. Chitinase expression

In order to identify the clone with the highest expression of Tv-ECH1, expression experiments were carried out with eight *P. pastoris PichiaPink* Strain 1 pPink $\alpha$ -HC::Tv-ech1 clones along with the negative control *P. pastoris PichiaPink* Strain 1 pPink $\alpha$ -HC. Experiments were

performed in 100 mL BMGY media using shake flasks. After 24 h of incubation at 30 °C and 250 rpm, chitinase expression was induced by methanol reaching a final concentration 0.5% (v/v) in yeast cultures. Methanol feeding was carried out every 24 h over a total of 144 h. Production of Tv-ECH1 was performed at lab-scale (200 mL) using shake flasks and scaled up in a 5 L bioreactor (Sartorius, Nümbrecht, Germany) according to the *Pichia* Fermentation Process Guidelines (Version B, 053002, Invitrogen). For the two fermentation batches produced, the pH used for the methanol-fed batch phase was either pH 5.0 or pH 6.0. Crude chitinase was harvested by centrifugation (14,000× g, 20 min) and stored at 4 °C until further analysis. To avoid microbial growth, sodium azide was added to a final concentration of 0.05% (w/v).

### 2.4. Purification of Tv-ECH1

Purification of Tv-ECH1 was achieved by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography using an ÄKTA pure liquid chromatography system (GE Healthcare, Freiburg, Germany) and Ni-NTA superflow cartridges (Qiagen, Hilden, Germany). Prior to purification, *Pichia* crude extract was concentrated and equilibrated in binding buffer (20 mM Na<sub>3</sub>PO<sub>4</sub>, 500 mM NaCl, 20 mM imidazole) by ultrafiltration. A linear gradient of imidazole (20–500 mM) was used for enzyme elution. Purified proteins were stored in 50 mM sodium acetate buffer (pH 5.5) and 50% (v/v) glycerol at –20 °C. Protein concentrations were determined by the Bradford [30] method using a protein-dye reagent (Bio-Rad, Hercules, California, USA). SDS-PAGE (12% (w/v) acrylamide gels) was carried out according to Laemmli [31]. For visualization, proteins were either stained with 0.1% (w/v) Coomassie Brilliant Blue G250 solved in 40% methanol, 5% acetic acid [32] or transferred onto a nitrocellulose membrane by the semi-dry transfer method [33], followed by immunodetection with His-Tag Monoclonal Antibody (mouse-IgG, Novagen, Merck, Darmstadt, Germany) and goat-anti-mouse-IgG-HRP (horseradish peroxidase)-conjugate (Sigma-Aldrich). Enhanced chemiluminescence was used for the visualization of immuno-complexes.

### 2.5. Chitinase activity assays

Chitinase activity in crude extract was detected with a fluorescent assay for endochitinase activity using the substrate 4-methylumbelliferyl-chitotriose (4MU-CHI3; Sigma Aldrich, St. Louis, Missouri, USA). Reaction mixtures (150  $\mu$ L) containing the enzyme extract in appropriate dilutions, 70 mM sodium acetate buffer (pH 5.0) and 48  $\mu$ M substrate were incubated in the dark at 37 °C and 180 rpm for 1 h. Enzymatic reactions were stopped by the addition of 100  $\mu$ L of 0.2 M sodium carbonate. Immediately, the release of free 4MU was measured by fluorescence spectrophotometry with excitation and emission wavelengths of  $\lambda = 360$  nm and  $\lambda = 460$  nm, respectively, using a Varioskan Flash instrument (Fisher Scientific, Waltham, Massachusetts, USA). Different concentrations (1.25–10 nM) of 4MU suspended in 0.2 M sodium carbonate were used as standards. Units of activity (U) were defined as nmol 4MU released per minute. Chitinase activity of purified Tv-ECH1 on various chitin/chitosan substrates was determined by measuring the increase of reducing sugar units using the protocol described by Horn and Eijsink [34].

### 2.6. Biochemical parameters and kinetic analyses

Reaction mixtures (200  $\mu$ L) containing 50 mM buffer, 0.62  $\mu$ g mL<sup>-1</sup> Tv-ECH1, and varying concentrations of colloidal chitin were incubated at 700 rpm and varying temperatures for 1 h (to determine the biochemical parameters) or 20 min (for the kinetic analysis). The pH optimum was analyzed at 37 °C, while the temperature optimum was determined in sodium acetate buffer (pH 4.5). For the kinetic analysis, 0.0625 to 5 mg mL<sup>-1</sup> substrate was incubated with Tv-ECH1 in 50 mM sodium acetate buffer (pH 4.5) for 20 min. Using a non-linear regression curve available in SigmaPlot (Systat Software, San Jose, CA), the

Michealis–Menten equation was fitted to the data. Enzyme stability was evaluated over 168 h by incubating  $12.5 \mu\text{g mL}^{-1}$  Tv-ECH1 at pH 4.5 (50 mM sodium acetate buffer) and  $37^\circ\text{C}$ ; chitinase activity was analyzed for aliquots at different time points.

### 2.7. Substrate preferences

The enzyme activity on insoluble substrates was analyzed with reaction mixtures (200  $\mu\text{L}$ ) made up of  $1 \text{ mg mL}^{-1}$  substrate (colloidal chitin,  $\alpha$ - and  $\beta$ -chitin),  $0.625 \mu\text{g mL}^{-1}$  enzyme, and 50 mM sodium acetate buffer (pH 4.5). Mixtures were incubated at  $37^\circ\text{C}$  and 700 rpm for 24 h. Two series of chitosans with increasing DA were used to assess the dependency of hydrolytic activity on the substrate's DA. Reaction mixtures (40  $\mu\text{L}$ ) containing  $1 \text{ mg mL}^{-1}$  of these soluble substrates,  $1.25 \mu\text{g mL}^{-1}$  enzyme, and 50 mM ammonium acetate buffer (pH 4.5) were incubated at  $37^\circ\text{C}$  for 10 min. The degree of scission was defined as relative fraction of glycosidic linkages cleaved by the chitinase. The availability of new reducing ends is used as a measurement for the amount of cleavages. For the determination of the smallest cleavable unit by Tv-ECH1, reaction mixtures (30  $\mu\text{L}$ ) containing  $1 \text{ mg mL}^{-1}$   $A_{2-6}$ ,  $1.25 \mu\text{g mL}^{-1}$  Tv-ECH1, and 50 mM ammonium acetate buffer (pH 4.5) were incubated at  $37^\circ\text{C}$  and 250 rpm for 24 h. Enzymatic reactions were stopped with one volume of 10% (v/v) ammoniac and an additional 10 min of incubation at  $95^\circ\text{C}$ . Products generated during the hydrolysis of  $A_{2-6}$  were evaluated with HP-TLC as described previously by Hamer et al. [35]. For this purpose samples were freeze-dried, resuspended in distilled water and 30  $\mu\text{g}$  of hydrolysis products applied for analysis.

### 2.8. Subsite specificity analyses of Tv-ECH1 using UHPLC-ELSD-ESI-MS<sup>n</sup>

Subsite specificities were determined by two approaches both analyzing chitin and chitosan oligomers by UHPLC-ESI-MS<sup>n</sup>. Analyses were performed with a Dionex Ultimate 3000RS UHPLC system (Thermo Scientific, Milford, USA) coupled to an ESI-MS detector (amaZon speed, Bruker, Bremen, Germany) following the protocol described previously [23]. For mass spectrometric analysis, products generated during the hydrolysis with Tv-ECH1 ( $1.25 \mu\text{g mL}^{-1}$ ) at  $37^\circ\text{C}$  and pH 4.5 (50 mM ammonium acetate buffer) were freeze-dried and resuspended in distilled water to obtain final concentrations of  $1 \text{ mg mL}^{-1}$  (hydrolysis of polymers) or  $0.5 \text{ mg mL}^{-1}$  (hydrolysis of chitin/chitosan tetramers).

In the first approach, two chitosan polymers with DA of 32% and 60% ( $1 \text{ mg mL}^{-1}$ ) were hydrolyzed for different periods of time. Reactions were stopped by the addition of sodium hydroxide solution. Since remaining polymers became insoluble at alkaline pH, it was possible to separate them by centrifugation. The supernatant was used for further analyses. LC-MS<sup>1</sup> (quantification) and LC-MS<sup>2</sup> (sequencing) measurements were performed with the oligomers generated to calculate the relative frequencies of de-*N*-acetylated (D) and acetylated (A) units at the first two positions at the reducing and non-reducing ends, corresponding to subsites  $-2$ ,  $-1$ ,  $+1$ ,  $+2$  during hydrolysis. Samples were re-*N*-acetylated with [<sup>2</sup>H<sub>6</sub>]acetic anhydride, labeled with <sup>18</sup>O at the reducing end, mixed with [<sup>13</sup>C<sub>4</sub> <sup>2</sup>H<sub>6</sub>]standards, and analyzed by UHPLC-HILIC-ESI-MS<sup>n</sup> [36] (Appendix, Table A1).

In the second approach chitosan tetramers which possessed D-units at specific subsites (DAAA, ADAA, AADA, AADD;  $0.5 \text{ mg mL}^{-1}$ ) were hydrolyzed for 0, 15, 60, and 1440 min. After a linear correlation between  $A_2$  concentration and corresponding peak area was confirmed by mass spectrometric analyses with defined amounts of  $A_2$  (0.0039  $\mu\text{g}$  to 0.25  $\mu\text{g}$ ), LC-MS<sup>1</sup> was used to calculate the amount of  $A_2$  generated by Tv-ECH1 for each tetramer and time point. Samples were separated via HILIC using a flow rate of  $0.4 \text{ mL min}^{-1}$  and a column oven temperature of  $35^\circ\text{C}$ . During the first 0.5 min, 100% (v/v) buffer A (80% (v/v) acetonitrile, 20% (v/v) H<sub>2</sub>O, 10 mM ammonium formate, 0.1% (v/v) formic acid) was used, followed by an increase of buffer B (20% (v/v) acetonitrile, 80% (v/v) H<sub>2</sub>O, 10 mM ammonium formate, 0.1% (v/v) formic acid) to a final

concentration of 75% (v/v) over 10 min and column re-equilibration over 2.5 min. Mass spectra were acquired for a target mass of  $m/z$  425.

## 3. Results

### 3.1. Heterologous expression of Tv-ECH1 in *P. pastoris*

Cloning of the expression vector pPink $\alpha$ -HC::Tv-*ech1* and its transformation into *P. pastoris* resulted in the successful expression of a functional chitinase enzyme. Shake flask expression experiments targeting the identification of the best-expressing clone were performed by analyzing the total soluble protein content, protein expression profiles, and total chitinase activity (data not shown). Once chosen, the best-expressing clone was used to produce Tv-ECH1 with *P. pastoris* PichiaPink Strain 1 pPink $\alpha$ -HC::Tv-*ech1* at the lab scale (200 mL) using shake flasks and at a small pilot scale using a 5 L-bioreactor. Shake flasks produced  $116 \text{ mg L}^{-1}$  of protein, while the bioreactor achieved a maximum of  $2 \text{ g L}^{-1}$  with high chitinase activity. Purity analysis of the crude Tv-ECH1 enzyme revealed a low amount of contaminant protein present in the crude mixture. Gel electrophoresis and immunoblotting confirmed that Ni-NTA purification successfully removed contaminant protein (Appendix, Fig. A1). Some differences were observed between the profile of proteins in SDS-PAGE when samples of crude Tv-ECH1 from the shake flask and bioreactor were compared. The shake flask expression sample had two protein bands of putative chitinase with slight size differences, whereas the bioreactor fermentation had one single protein product at the expected molecular mass of 45 kDa. Anti-His tag immunoblotting of the purified Tv-ECH1 clearly identified both protein products generated during shake flask expression as being derived from the Tv-ECH1 vector construct. Sequencing of both shake flask protein products by mass spectrometry did not show any differences in their amino acid sequence, and confirmed that the *S. cerevisiae*  $\alpha$ -mating factor secretion sequence was successfully cleaved from both mature proteins.

For a first estimation of optimal fermentation conditions, bioreactor fermentation was performed in two batches, maintaining the pH either at 6 or 5. Evaluation of the specific activity of Tv-ECH1 chitinase in harvested culture supernatants revealed two times higher values for pH 5 ( $9.6 \text{ U mg total protein}^{-1}$ ) than for pH 6 ( $4.9 \text{ U mg total protein}^{-1}$ ).

### 3.2. Characterization of Tv-ECH1

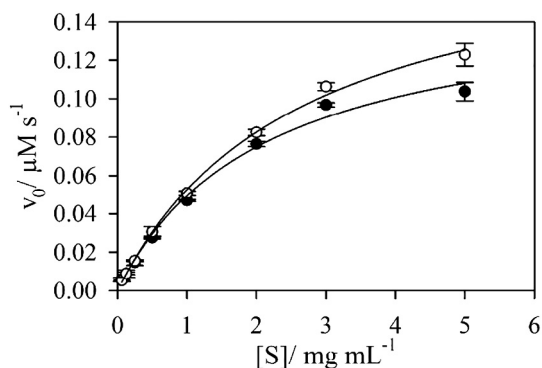
#### 3.2.1. Optimal reaction conditions and kinetic analysis

The determination of biochemical parameters for recombinant Tv-ECH1 revealed a broad pH optimum from 4.5 to 6 and an optimal reaction temperature around  $37^\circ\text{C}$  (Appendix, Fig. A2). The temperature profile showed a noticeable sharp decrease in hydrolytic activity at temperatures higher than  $45^\circ\text{C}$ . However, Tv-ECH1 remained stable for at least 50 h at pH 4.5 and  $37^\circ\text{C}$  (Appendix, Fig. A3), defined as optimal reaction conditions. When stored as purified protein in glycerol containing buffered solution at  $-20^\circ\text{C}$ , the enzyme was stable over a storage period of two years. Tv-ECH1 produced by bioreactor expression was found to have  $K_M$  and  $k_{cat}$  values of  $2.10 \pm 0.31 \text{ mg mL}^{-1}$  and  $8.08 \pm 0.53 \text{ s}^{-1}$  respectively, acting on colloidal chitin as a substrate (Fig. 1). Similar values ( $K_M = 2.62 \pm 0.22 \text{ mg mL}^{-1}$ ;  $k_{cat} = 10.03 \pm 0.42 \text{ s}^{-1}$ ) were determined for enzyme produced by shake flask.

#### 3.2.2. Substrate preferences

To assess the enzyme's substrate preferences, the hydrolytic activity of Tv-ECH1 was analyzed on the crystalline substrates  $\alpha$ - and  $\beta$ -chitin, the more accessible colloidal chitin, and soluble chitosans which possessed various DPs and DAs. To determine the smallest unit cleavable by Tv-ECH1, its activity was evaluated on  $A_{2-6}$  oligomers.

With final degrees of scission below 0.02, Tv-ECH1 showed almost no activity on the crystalline  $\alpha$ - and  $\beta$ -chitin. However, this value might underestimate activity on chitin as larger, insoluble chitin fragments might be missed by the reducing end assay used. Colloidal chitin



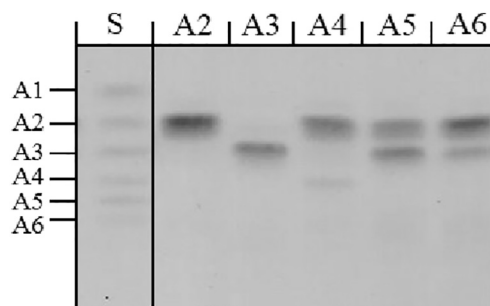
**Fig. 1.** Kinetic analysis of Tv-ECH1 produced by the bioreactor (filled symbols) or the shake flasks (open symbols). Hydrolysis reactions were carried out with colloidal chitin as a substrate under optimal reaction conditions for 10 min. Error bars represent standard deviations of triplicate determinations.

was hydrolyzed by the chitinase to a higher extent (Fig. 2), confirming the substrate's better accessibility. Based on their solubility, chitosans are expected to be highly accessible substrates. The DA-dependency of chitosan hydrolysis by Tv-ECH1 was analyzed with two chitosan batches which were re-*N*-acetylated to increasing DA values (Fig. 2). Results confirmed that the hydrolytic activity of Tv-ECH1 depended on the substrate's DA. In contrast and as expected, the enzyme's hydrolytic activity was not found to be influenced by the substrate's DP.

To determine the minimal DP required for Tv-ECH1 to be able to hydrolyze the substrate's glycosidic linkages, A<sub>2-6</sub> oligomers were hydrolyzed for 24 h. Results presented in Fig. 3 show that incubating A<sub>2</sub> and A<sub>3</sub> with Tv-ECH1 did not result in any hydrolysis products. Thus, at least four A units need to be available for hydrolysis by Tv-ECH1. Hydrolysis of A<sub>4</sub> resulted in dimers, whereas A<sub>6</sub> was cleaved into dimers and trimers.

### 3.2.3. Subsite specificities

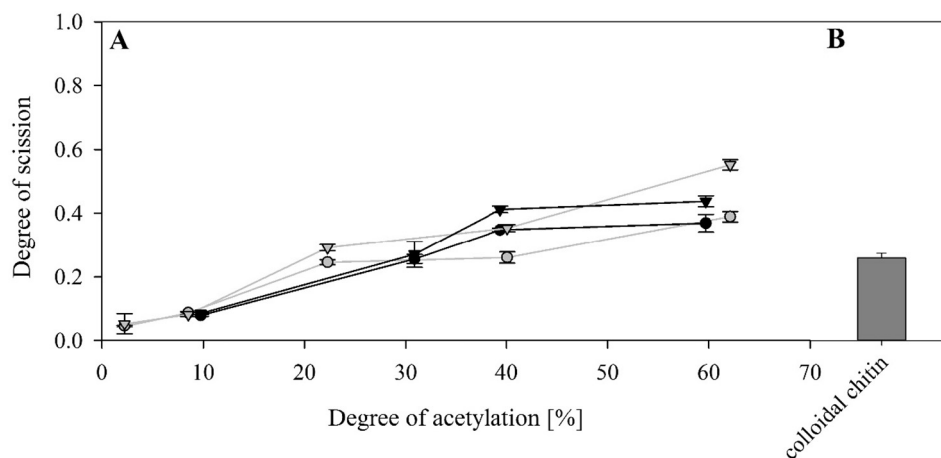
The mode of action of Tv-ECH1 was analyzed in detail by determining the subsite specificities of the enzyme's active site. In a first approach, two chitosan polymers with DAs of 32% and 60% were hydrolyzed with Tv-ECH1 over 24 h. Hydrolysates from different time points (5, 20, 60, 120, 1440 min) were analyzed using LC-MS<sup>1</sup> for quantifying and LC-MS<sup>2</sup> for sequencing the oligomers obtained. Sequencing of the oligomers reveals whether an acetylated (A) or a de-*N*-acetylated (D) unit had been present at the enzyme's subsites -2, -1, +1, and +2 during hydrolysis. Sugars which had been bound to subsites -1 and -2 will be found at the first and second position from the reducing end of the oligomer produced, whereas sugars which had been bound at



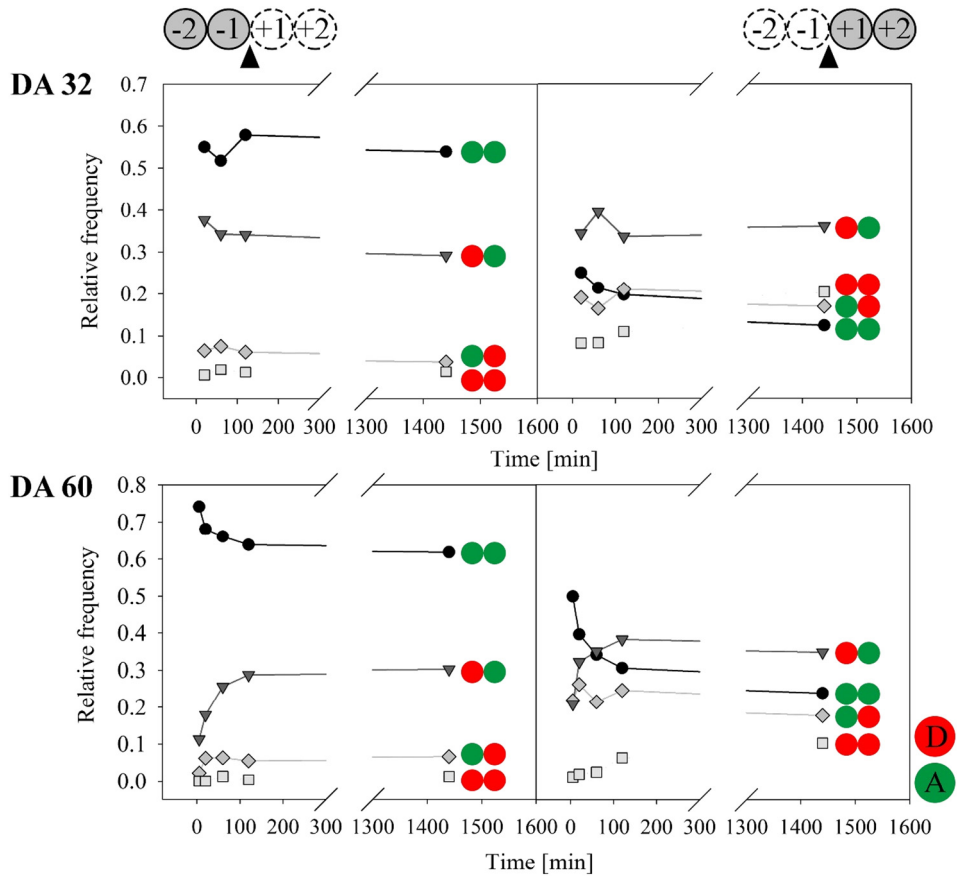
**Fig. 3.** Hydrolytic activity of Tv-ECH1 on A<sub>2-6</sub>. Different chitin oligomers were incubated with Tv-ECH1 (produced by bioreactor expression) at optimal conditions for 24 h. A<sub>1-6</sub> were applied as a standard (S).

subsites +1 and +2 will be located at the first and second position from its non-reducing end. Combining MS<sup>1</sup> and MS<sup>2</sup> results allows calculation of the frequencies of A- and D-units at and next to the reducing and non-reducing end of the oligomers produced (Fig. 4). The frequencies of the sequences DD and AD at the reducing ends of the products and, thus, at subsites -2 and -1 during enzymatic cleavage were found to be near zero during the whole period of hydrolysis, confirming that to hydrolyze the substrate, Tv-ECH1 needs A at subsite -1, as expected for a GH18 chitinase. At low DA, additional strong preferences for A-units at subsites -2 and +2, and initially also at subsite +1, were observed. Since the availability of A is generally high at high DA, this preference for A-units was then only visible at the early time points.

This first approach confirmed the necessity of A-units at subsite -1, but also indicated preferences for A-units at subsites -2 and +2, and less marked, +1. To confirm these results, a second approach was performed by hydrolyzing partially acetylated chitosan tetramers, which possessed D-units at defined positions (AAAA, DAAA, ADAA, AADA, and AADD), for 24 h. Enzyme activities on each tetramer were monitored via LC-MS<sup>1</sup> analyses after 15 min, 60 min and 24 h (Fig. 5). After 60 min, complete hydrolysis of A<sub>4</sub> by Tv-ECH1 was observed. Tetramers with D at the position next to the reducing end (ADAA) were not cleaved at all, again confirming the necessity of an A-unit at subsite -1. The activity of Tv-ECH1 on the other partially de-*N*-acetylated substrates was observed to be lower than on the completely acetylated tetramer. Hydrolysis products increased with increasing time of incubation, in the order of AADA > DAAA > AADD. After 24 h the fully acetylated as well as these two mono-deacetylated tetramers were completely degraded into dimers. Apart from ADAA, lowest relative activity of Tv-ECH1 was observed



**Fig. 2.** Hydrolytic activity of Tv-ECH1 depending on the substrates' DA. A: Two chitosan series (grey: DP 650, black: DP 2600) were hydrolyzed with Tv-ECH1, originating from bioreactor (circle) and shake flask (triangle). B: Activity of Tv-ECH1 on colloidal chitin. Soluble substrates were incubated with Tv-ECH1 (produced by bioreactor expression) at optimal reaction conditions (37 °C, pH 4.5, 750 rpm) for 10 min (A), insoluble ones for 24 h (B). Error bar represents standard deviation of triplicate determinations.



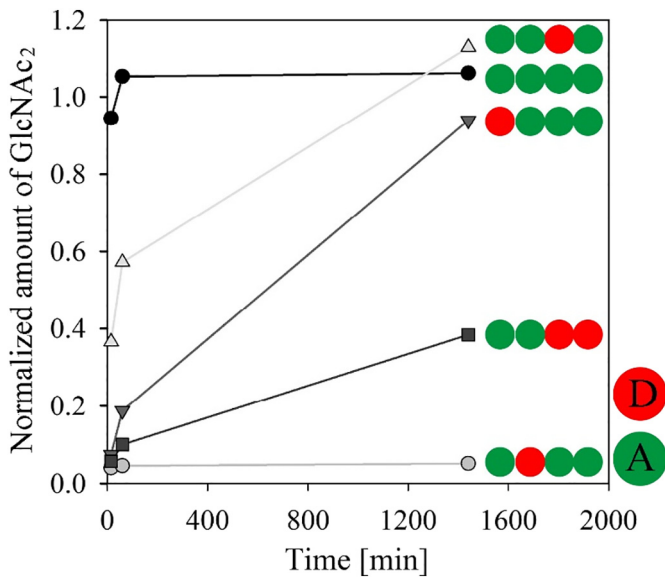
**Fig. 4.** Subsite specificities of Tv-ECH1 analyzed by quantification (MS<sup>1</sup>) and sequencing (MS<sup>2</sup>) of oligomeric hydrolysis products. Chitosan substrates (DA32% and 60%) were hydrolyzed for 5, 20, 60, 120, 1440 min at optimal reaction conditions. Relative frequencies of the sequences AA, AD, DA, and DD at the enzyme subsites –2 –1 and +2 +1 were determined by mass spectrometric analysis for each point in time of hydrolysis.

for the double de-N-acetylated substrate. The same results were achieved with the enzyme batch from bioreactor expression (data not shown).

#### 4. Discussion

##### 4.1. Heterologous expression of Tv-ECH1 using the *P. pastoris* expression system

Heterologous expression of Tv-ECH1 in *P. pastoris* resulted in secretion of a functional chitinase into the culture medium. Two expression scales, a lab scale (200 mL) system, using shake flasks, and small pilot scale, using a 5-L bioreactor, were studied to evaluate scalability of recombinant enzyme production. The induction of protein expression by methanol and the secretion of the enzyme into the culture medium were successful at both scales, resulting in 116 mg L<sup>-1</sup> protein for flask fermentation and 1–2 g L<sup>-1</sup> protein for bioreactor expression. Previous studies report similar protein amounts for the heterologous expression of chitinases in *P. pastoris* using flask or bioreactor expression [17,18,37]. The main difference observed between our two expression scales was the presence of two protein bands on SDS-PAGE gels when we analyzed samples from the shake flask expression system; a single protein band was observed for the bioreactor-generated enzyme. However, no differences were found in the amino acid sequence or kinetic properties of the two enzymes from the different expression scales. It was excluded that the size difference is based on a signal peptide which is not cleaved appropriately. Another possible explanation for the size difference is a variation in glycosylation due to fermentation conditions. However, in our study, we did not analyze protein glycosylation. The amount of protein produced by bioreactor fermentation was more than ten times higher than the amount achieved by flask expression, which is in-keeping with that observed for other proteins using the *Pichia* system [38]. A comparison of two conditions for fermentation (pH 5 or pH 6) revealed that pH 5 was better suited for the production of Tv-ECH1 as the lower amount of contaminating proteins leads to a



**Fig. 5.** Activity of Tv-ECH1 on specific chitin/chitosan tetramers. Tetramers were hydrolyzed with Tv-ECH1 (produced by shake flask expression), at optimal reaction conditions for 15, 60 and 1440 min. The amount of GlcNAc<sub>2</sub> generated was calculated via MS<sup>1</sup> and normalized against the maximal possible amount. It was taken into account that for the tetramer AAAA, the maximal possible amount of GlcNAc<sub>2</sub> is two times higher than for the other tetramers. Results shown are from one experiment and representative of two independent experiments.

higher purity in the crude supernatant. Clearly, this is a scalable expression system that allows for the generation of sufficient quantities of highly active enzyme for large scale chitosan oligomer production.

#### 4.2. Characterization of Tv-ECH1

Characterization of Tv-ECH1 revealed biochemical properties that are consistent with previous studies for related GH18 chitinases [16,17,39]. A variety of mostly bacterial GH18 chitinases have previously been analyzed concerning subsite preferences on partially acetylated chitosans using different approaches [40–42], but the mass spectrometric techniques we used give even deeper insight into subsite specificities [51]. Also, some GH19 chitinases, which are particularly abundant in plants but also occur in some bacteria, have been characterized in detail, including concerning their subsite specificities [49]. Tv-ECH1 is a fungal GH18 chitinase with a broad acidic pH optimum from 4.5–6 and a temperature optimum around 37 °C. The observed sharp decrease in hydrolytic activity at temperatures higher than 45 °C could be associated with low thermostability, which has already been reported for related chitinases. A similar decrease in activity at temperatures higher than 45 °C was shown by Di Pietro et al. [15] for an endochitinase which was purified from *T. virens* and later expected to be identical to Tv-ECH1 [13], and by Pérez-Martínez et al. [17] for the related chitinase ECH42 from *T. atroviride*. Moreover, CHIT42 from *T. harzianum* was shown to be inactivated at 50 °C [39]. Nevertheless, Tv-ECH1 shows stability for at least 50 h at optimal working conditions (pH 4.5, 37 °C). Stability at optimal working conditions is absolutely crucial for an application of the chitinase in the large scale biotechnological production of chitosan oligomers. This also offers the possibility of enzyme immobilization in production processes.

The kinetic analysis revealed  $K_M$  values of  $2.10 \pm 0.31 \text{ mg mL}^{-1}$  and  $k_{cat}$  values of  $8.08 \pm 0.53 \text{ s}^{-1}$  for Tv-ECH1 (produced by bioreactor expression) acting on colloidal chitin as a substrate. For the related chitinases ECH42 from *T. atroviride* and CHIT42 from *T. harzianum*,  $K_M$  values in a similar range ( $1.9 \text{ mg mL}^{-1}$  and  $1 \text{ mg mL}^{-1}$ ) were reported [17,39]. Bacterial chitinases, such as a chitinase from *Streptomyces* sp. DA11 [43] or Chi72 from *Bacillus licheniformis* SK-1 [44], display much lower  $K_M$  values of  $0.019$  and  $0.23 \text{ mg mL}^{-1}$ , respectively. Overall, these results show that 42-kDa endochitinases have low efficiency on the insoluble substrate colloidal chitin. On the crystalline substrates  $\alpha$ - and  $\beta$ -chitin, Tv-ECH1 showed even less activity than on colloidal chitin. The low activity of Tv-ECH1 on insoluble substrates is in-keeping with its classification in the subgroup A chitinases, which lack a chitin-binding domain; such domains are known to increase a chitinase's affinity for insoluble substrates [11,45]. However, it needs to be considered that only low-DP hydrolysis products of crystalline chitins would be soluble. Consequently, the reducing end assay might underestimate the extent of hydrolysis. As a soluble substrate, chitosan is more accessible for chitinases, allowing them to hydrolyze the substrate more efficiently. During analyses with a variety of chitosans with differing DPs and DAs, we showed that the hydrolytic activity of Tv-ECH1 on chitosan increased with increasing DA in a linear manner. In contrast, the DP did

not seem to have a significant impact on the hydrolytic activity, provided that at least four sugar units are available. Phylogenetic analyses, which associate Tv-ECH1 with the group of 42-kDa endochitinases, suggest an endo-type mechanism of hydrolysis [12,46]. However, to confirm the endo-type mechanism and to determine possible processivity of the enzyme, further experimentation would be required [47].

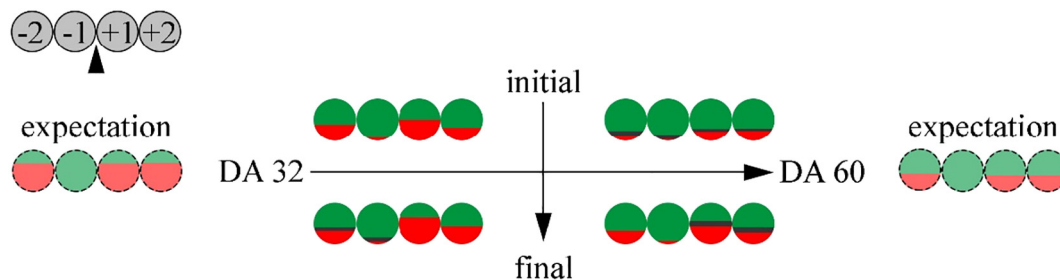
For hydrolysis of glycosidic linkages by GH18 chitinases with their substrate-assisted cleavage mechanism, the availability of an A-unit at subsite  $-1$  is an obligatory requirement. Consequently, an increase of hydrolytic activity with increasing DAs was expected, and this was confirmed by hydrolysis of the two series of re-*N*-acetylated chitosans. The mode of action was investigated in detail by analyzing the subsite specificities of Tv-ECH1 by mass spectrometric analyses with two different approaches. Cord-Landwehr et al. [36] has established the method of quantitative mass-spectrometric sequencing of chitosan oligomers and presented the cleavage specificity for ChiB on chitosans with DA 50 and 60%. The cleavage specificity of ChiB on chitosan DA 60% seems to be comparable to Tv-ECH1. However, to analyze the preference for A-units, chitosans with low DAs are the more appropriate substrate. Using chitosans with a DA of 32 and 60% for Tv-ECH1 we observed the necessity for an A-unit at subsite  $-1$  and further revealed preferences for an A-unit at subsites  $-2$  and  $+2$ , and to a lesser extent, at subsite  $+1$  (Fig. 6). As all GH18 chitinases require an A-unit at subsite  $-1$ , our results further confirm Tv-ECH1's assignment to this family. Even more importantly though, the finding of high subsite specificities for Tv-ECH1 makes the chitinase a promising candidate for different biotechnological applications. High cleavage specificity of the enzyme, especially during early time points of hydrolysis, suggests that it may be ideally suited for enzymatic fingerprinting analyses of chitosan polymers and oligomers [48]. It needs to be mentioned that a variety of glycosyl hydrolases with different cleavage specificities, amongst others plant GH19 chitinases [49], exist as candidates for fingerprinting analyses and especially the combination of enzymes with different specificities is important for this method [50].

#### 5. Conclusions

In this study, the successful heterologous expression of the chitinase Tv-ECH1 in *P. pastoris* was reported for the first time. The *P. pastoris* clone secreted 1–2 g L<sup>-1</sup> of Tv-ECH1 with high activity, high purity, and high stability into the fermentation media, making the system promising for industrial production of Tv-ECH1. Characterization of Tv-ECH1 revealed a high biotechnological potential of the chitinase. Specifically, the enzymatic cleavage of chitosan by Tv-ECH1 was found to be more specific than expected for a GH18 chitinase. This makes Tv-ECH1 a promising candidate for the production of specific chitosan oligomers. Also, chitinases with high subsite specificities are promising candidates for the characterization of chitosan polymers via enzymatic fingerprinting.

#### Acknowledgements

We would like to thank Celeste Brennecka for editorial support.



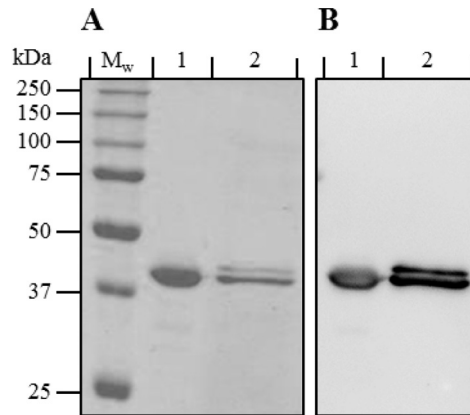
**Fig. 6.** Schematic representation of subsite specificities of Tv-ECH1. Chitosans of two DAs were hydrolyzed and products sequenced at initial (DA 32%, 20 min; DA 60%, 5 min) and final (1440 min) time points. Frequencies of A (green) and D (red) at subsites  $-2$  to  $+2$  are indicated as appropriately colored area in the circle. Grey areas represent the presence of unknown oligomers which could not be sequenced during MS analysis. The expectation shows the expected pattern of cleavage for a chitinase with cleavage specificity for A at subsite  $-1$  only, as dependent on the substrate's DA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

## Appendix A

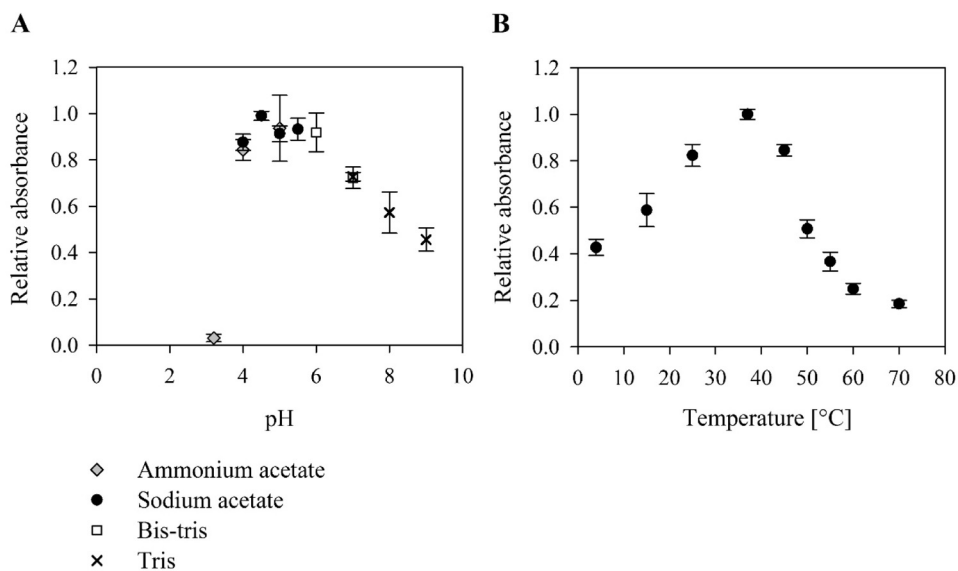
Table A1

Multiple reaction monitoring of UHPLC-ELSD-ESI-MS<sup>2</sup> with re-N-acetylated (R) and <sup>18</sup>O labeled chitosan oligomers.

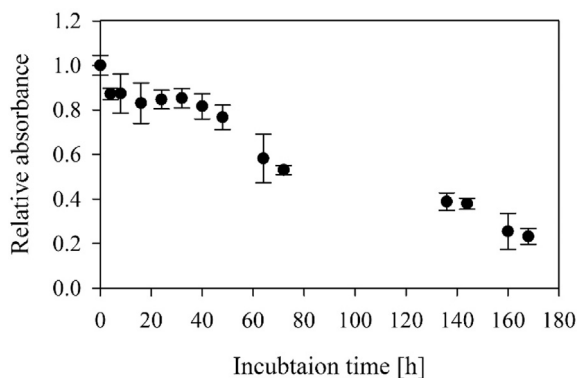
Elution time [min]	Target mass [m/z]	Precursor		Isolation Width	Reaction	
		Mass [m/z]	Sequence		Cut Off	Amplitude [%]
0–4.7	200	–	–	–	–	–
4.7–8	420	430.2	A1R1	2.0	116	85
8–11.4	630	633.2	A2R1	2.0	171	90
		636.2	A1R2	2.0	172	90
		836.3	A3R1	1.5	226	100
11.4–14	840	839.3	A2R2	1.5	227	100
		842.3	A1R3	1.5	227	100
		1039.4	A4R1	1.5	281	100
		1042.4	A3R2	1.5	281	100
14–16.6	1040	1045.4	A2R3	1.5	282	100
		148.4	A1R4	1.5	283	100
		1242.5	A5R1	1.5	335	110
		1245.5	A4R2	1.5	336	110
		1248.5	A3R3	1.5	337	110
16.6–18.5	1240	1251.5	A2R4	1.5	338	110
		1254.5	A1R5	1.5	339	110



**Fig. A1.** SDS-PAGE (12%) analysis of purified Tv-ECH1 via Coomassie staining (A) or immunoblotting (B). Three µg purified protein, originated from bioreactor (1) or flask (2) fermentation, was applied. Precision Plus Protein All Blue Standards (Bio-Rad) was used as protein standard. Expected Tv-ECH1 M<sub>w</sub> is 45.3 kDa.



**Fig. A2.** Biochemical parameters of Tv-ECH1. Hydrolytic activity was analyzed on the substrate colloidal chitin (0.1%) using the reducing end assay. A: pH profile. Tv-ECH1 was incubated with the substrate at 37 °C for one hour using different 50 mM buffers. Three independent triplicate experiments were carried out. Error bars represent standard deviations. B: Temperature profile. 50 mM sodium acetate buffer (pH 4.5) was used and incubation was performed at different temperatures. Results are from one triplicate experiment. Error bars represent standard deviations.



**Fig. A3.** Stability of Tv-ECH1 at optimal reaction conditions (pH 4.5, 37 °C). During ~6 days of incubation, activity of Tv-ECH1 (produced by bioreactor fermentation) was assessed at different time points via reducing end assay using 0.05% chitosan (DP 700, DA 20%) as a substrate. All values are means of one triplicate experiment. Error bars represent standard deviations.

## References

- [1] B.B. Aam, E.B. Heggset, A.L. Norberg, M. Sørli, K.M. Vårum, V.G.H. Eijsink, Production of chito-oligosaccharides and their potential applications in medicine, *Mar. Drugs* 8 (2010) 1482–1517, <https://doi.org/10.3390/md8051482>.
- [2] W.-J. Jung, R.-D. Park, Bioproduction of Chito-oligosaccharides: present and perspectives, *Mar. Drugs* 12 (2014) 5328–5356, <https://doi.org/10.3390/md12115328>.
- [3] S.N. Das, J. Madhuprakash, P.V.S.R.N. Sarma, P. Purushotham, K. Suma, K. Manjeet, S. Rambabu, N.E. El Gueddari, B.M. Moerschbacher, A.R. Podile, Biotechnological approaches for field applications of chito-oligosaccharides (COS) to induce innate immunity in plants, *Crit. Rev. Biotechnol.* 35 (2015) 29–43, <https://doi.org/10.3109/07388551.2013.798255>.
- [4] A. Domard, A perspective on 30 years research on chitin and chitosan, *Carbohydr. Polym.* 84 (2011) 696–703, <https://doi.org/10.1016/j.carbpol.2010.04.083>.
- [5] E.N.J. Oliveira, N.E. El Gueddari, B.M. Moerschbacher, T.T. Franco, Growth rate inhibition of hytopathogenic fungi by characterized chitosans, *Braz. Dent. J.* (2012) 800–809.
- [6] M.H. Rahman, L.G. Hjeljord, B.B. Aam, M. Sørli, A. Tronsmo, Antifungal effect of chito-oligosaccharides with different degrees of polymerization, *Eur. J. Plant Pathol.* 141 (2015) 147–158, <https://doi.org/10.1007/s10658-014-0533-3>.
- [7] P. Vander, K.M. Vårum, A. Domard, N.E. El Gueddari, B.M. Moerschbacher, Comparison of the ability of partially N-acetylated chitosans and chito-oligosaccharides to elicit resistance reactions in wheat leaves, *Plant Physiol.* 118 (1998) 1353–1359, <https://doi.org/10.1104/pp.118.4.1353>.
- [8] N.E. El Gueddari, S. Kolkenbrock, A. Schaaf, N. Chilukoti, F. Brunel, C. Gorzelanny, S. Fehser, S. Chachra, F. Bernard, M. Nampally, T. Kalagara, P. Ihmor, B.M. Moerschbacher, Chitin and chitosan modifying enzymes: versatile novel tools for the analysis of structure-function relationships of partially acetylated chitosans, in: S.P.C. Filho, M.M. Beppu, A. Fiamingo (Eds.), *Adv. Chitin Sci* 2012, pp. 40–47.
- [9] S. Gruber, V. Seidl-Seiboth, Self versus non-self: fungal cell wall degradation in *Trichoderma*, *Microbiology* 158 (2012) 26–34, <https://doi.org/10.1099/mic.0.052613-0>.
- [10] S. Zeilinger, C. Galhaup, K. Payer, S.L. Woo, R.L. Mach, C. Fekete, M. Lorito, C.P. Kubicek, Chitinase gene expression during mycoparasitic interaction of *Trichoderma harzianum* with its host, *Fungal Genet. Biol.* 26 (1999) 131–140, <https://doi.org/10.1006/fgbi.1998.1111>.
- [11] V. Seidl, Chitinases of filamentous fungi: a large group of diverse proteins with multiple physiological functions, *Fungal Biol. Rev.* 22 (2008) 36–42, <https://doi.org/10.1016/j.fbr.2008.03.002>.
- [12] D.-J. Kim, J.-M. Baek, P. Uribe, C. Kenerley, D. Cook, Cloning and characterization of multiple glycosyl hydrolase genes from *Trichoderma virens*, *Curr. Genet.* 40 (2002) 374–384, <https://doi.org/10.1007/s00294-001-0267-6>.
- [13] J.M. Baek, C.R. Howell, C.M. Kenerley, The role of an extracellular chitinase from *Trichoderma virens* Gv29-8 in the biocontrol of *Rhizoctonia solani*, *Curr. Genet.* 35 (1999) 41–50, <https://doi.org/10.1007/s002940050431>.
- [14] C. Emani, J.M. Garcia, E. Lopata-Finch, M.J. Pozo, P. Uribe, D.-J. Kim, G. Sunilkumar, D. R. Cook, C.M. Kenerley, K.S. Rathore, Enhanced fungal resistance in transgenic cotton expressing an endochitinase gene from *Trichoderma virens*, *Plant Biotechnol. J.* 1 (2003) 321–336, <https://doi.org/10.1046/j.1467-7652.2003.00029.x>.
- [15] A. Di Pietro, M. Lorito, C.K. Hayes, R.M. Broadway, G.E. Harman, Endochitinase from *Gliocladium virens*: isolation characterization, and synergistic antifungal activity in combination with gliotoxin, *Phytopathology* 83 (1992) 308–313.
- [16] I.A. Hoell, S.L. Klemmsdal, G. Vaaje-Kolstad, S.J. Horn, V.G.H. Eijsink, Overexpression and characterization of a novel chitinase from *Trichoderma atroviride* strain P1, *Biochim. Biophys. Acta Protein Proteomics* 1748 (2005) 180–190, <https://doi.org/10.1016/j.bbapap.2005.01.002>.
- [17] A.S. Pérez-Martínez, A. De León-Rodríguez, L.J. Harris, A. Herrera-Estrella, A.P. Barba de la Rosa, Overexpression, purification and characterization of the *Trichoderma atroviride* endochitinase, Ech42, in *Pichia pastoris*, *Protein Expr. Purif.* 55 (2007) 183–188, <https://doi.org/10.1016/j.pep.2007.05.009>.
- [18] P. Yu, Y. Yan, Q. Gu, X. Wang, Codon optimisation improves the expression of *Trichoderma viride* sp. endochitinase in *Pichia pastoris*, *Sci. Rep.* 3 (2013) 1–6, <https://doi.org/10.1038/srep03043>.
- [19] R. Daly, M.T.W. Hearn, Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production, *J. Mol. Recognit.* 18 (2005) 119–138, <https://doi.org/10.1002/jmr.687>.
- [20] C. Rabert, D. Weinacker, A. Pessoa, J.G. Farias, Recombinant proteins for industrial uses: utilization of *Pichia pastoris* expression system, *Braz. J. Microbiol.* 44 (2013) 351–356, <https://doi.org/10.1590/S1517-83822013005000041>.
- [21] C. Schatz, C. Viton, T. Delair, C. Pichot, A. Domard, Typical Physicochemical Behaviors of Chitosan in Aqueous Solution, 2003 641–648.
- [22] A. Hirai, H. Odani, A. Nakajima, Determination of degree of deacetylation of chitosan by <sup>1</sup>H NMR spectroscopy, *Polym. Bull.* 94 (1991) 87–94.
- [23] S.N. Hamer, S. Cord-Landwehr, X. Biarnés, A. Planas, H. Waegeman, B.M. Moerschbacher, S. Kolkenbrock, Enzymatic production of defined chitosan oligomers with a specific pattern of acetylation using a combination of chitin oligosaccharide deacetylases, *Sci. Rep.* 5 (2015), 8716. <https://doi.org/10.1038/srep08716>.
- [24] S. Naqvi, S. Cord-Landwehr, R. Singh, F. Bernard, S. Kolkenbrock, N.E. El Gueddari, B. M. Moerschbacher, A recombinant fungal chitin deacetylase produces fully defined chitosan oligomers with novel patterns of acetylation, *Appl. Environ. Microbiol.* 82 (2016) 6645–6655, <https://doi.org/10.1128/AEM.01961-16>.
- [25] S. Cord-Landwehr, R.L.J. Melcher, S. Kolkenbrock, B.M. Moerschbacher, A chitin deacetylase from the endophytic fungus *Pestalotiopsis* sp. efficiently inactivates the elicitor activity of chitin oligomers in rice cells, *Sci. Rep.* 6 (2016) 38018, <https://doi.org/10.1038/srep38018>.
- [26] S.C. Hsu, J.L. Lockwood, Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil, *Appl. Microbiol.* 29 (1975) 422–426.
- [27] G. Lamarque, J.-M. Lucas, C. Viton, A. Domard, Physicochemical behavior of homogeneous series of acetylated chitosans in aqueous solution: role of various structural parameters, *Biomacromolecules* 6 (2005) 131–142, <https://doi.org/10.1021/bm0496357>.
- [28] T.N. Petersen, S. Brunak, G. von Heijne, H. Nielsen, SignalP 4.0: discriminating signal peptides from transmembrane regions, *Nat. Methods* 8 (2011) 785–786, <https://doi.org/10.1038/nmeth.1701>.
- [29] O. Emanuelsson, H. Nielsen, S. Brunak, G. von Heijne, Predicting subcellular localization of proteins based on their N-terminal amino acid sequence, *J. Mol. Biol.* 300 (2000) 1005–1016, <https://doi.org/10.1006/jmbi.2000.3903>.
- [30] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.* 72 (1976) 248–254.
- [31] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* 227 (1970) 680–685.
- [32] S. Fazekas de St Groth, R.G. Webster, A. Datyner, Two new staining procedures for quantitative estimation of proteins on electrophoretic strips, *Biochim. Biophys. Acta* 71 (1963) 377–391.
- [33] H. Towbin, T. Staehelin, J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, *Proc. Natl. Acad. Sci. U. S. A.* 76 (1979) 4350–4354.
- [34] S. Jarle Horn, V.G.H. Eijsink, A reliable reducing end assay for chito-oligosaccharides, *Carbohydr. Polym.* 56 (2004) 35–39, <https://doi.org/10.1016/j.carbpol.2003.11.011>.
- [35] S.N. Hamer, B.M. Moerschbacher, S. Kolkenbrock, Enzymatic sequencing of partially acetylated chitosan oligomers, *Carbohydr. Res.* 392 (2014) 16–20, <https://doi.org/10.1016/j.carres.2014.04.006>.
- [36] S. Cord-Landwehr, P. Ihmor, A. Niehues, H. Luftmann, B.M. Moerschbacher, M. Mormann, Quantitative mass-spectrometric sequencing of chitosan oligomers reveals cleavage sites of chitosan hydrolases, *Anal. Chem.* 89 (2017) 2893–2900, <https://doi.org/10.1021/acs.analchem.6b04183>.

- [37] P. Yu, Y. Tang, Construction of the highly secreted endochitinase *Pichia pastoris* strain and the optimization of chitin-degrading conditions, *Carbohydr. Polym.* 89 (2012) 41–45, <https://doi.org/10.1016/j.carbpol.2012.02.034>.
- [38] S. Singh, A. Gras, C. Fiez-Vandal, J. Ruprecht, R. Rana, M. Martinez, P.G. Strange, R. Wagner, B. Byrne, Large-scale functional expression of WT and truncated human adenosine A2A receptor in *Pichia pastoris* bioreactor cultures, *Microb. Cell Factories* 7 (2008) 28, <https://doi.org/10.1186/1475-2859-7-28>.
- [39] J. De La Cruz, A. Hidalgo-Gallego, J.M. Lora, T. Benitez, J.A. Pintor-Toro, A. Llobell, Isolation and characterization of three chitinases from *Trichoderma harzianum*, *Eur. J. Biochem.* 206 (1992) 859–867, <https://doi.org/10.1111/j.1432-1033.1992.tb16994.x>.
- [40] S.J. Horn, A. Sorbotten, B. Synstad, P. Sikorski, M. Sorlie, K.M. Varum, V.G.H. Eijsink, Endo/exo mechanism and processivity of family 18 chitinases produced by *Serratia marcescens*, *FEBS J.* 273 (2006) 491–503, <https://doi.org/10.1111/j.1742-4658.2005.05079.x>.
- [41] A. Sorbotten, S.J. Horn, V.G.H. Eijsink, K.M. Vårum, Degradation of chitosans with chitinase B from *Serratia marcescens*, *FEBS J.* 272 (2005) 538–549, <https://doi.org/10.1111/j.1742-4658.2004.04495.x>.
- [42] C. Gorzelanny, B. Pöppelmann, K. Pappelbaum, B.M. Moerschbacher, S.W. Schneider, Human macrophage activation triggered by chitotriosidase-mediated chitin and chitosan degradation, *Biomaterials* 31 (2010) 8556–8563, <https://doi.org/10.1016/j.biomaterials.2010.07.100>.
- [43] Y. Han, B. Yang, F. Zhang, X. Miao, Z. Li, Characterization of antifungal chitinase from marine *Streptomyces* sp. DA11 associated with South China Sea Sponge *Craniella australiensis*, *Mar. Biotechnol.* 11 (2009) 132–140, <https://doi.org/10.1007/s10126-008-9126-5>.
- [44] S. Kudan, R. Pichyangkura, Purification and characterization of thermostable chitinase from *Bacillus licheniformis* SK-1, *Appl. Biochem. Biotechnol.* 157 (2009) 23–35, <https://doi.org/10.1007/s12010-008-8328-7>.
- [45] V.G.H. Eijsink, G. Vaaje-Kolstad, K.M. Vårum, S.J. Horn, Towards new enzymes for biofuels: lessons from chitinase research, *Trends Biotechnol.* 26 (2008) 228–235, <https://doi.org/10.1016/j.tibtech.2008.02.004>.
- [46] V. Seidl, B. Huemer, B. Seiboth, C.P. Kubicek, A complete survey of *Trichoderma chitinases* reveals three distinct subgroups of family 18 chitinases, *FEBS J.* 272 (2005) 5923–5939, <https://doi.org/10.1111/j.1742-4658.2005.04994.x>.
- [47] M. Kurasin, S. Kuusk, P. Kuusk, P. Va, Slow off-rates and strong product binding are required for processivity and efficient degradation of recalcitrant chitin by family 18 chitinases, 290 (2015) 29074–29085, <https://doi.org/10.1074/jbc.M115.684977>.
- [48] B.M. Moerschbacher, F. Bernard, N.E. El Gueddari, Enzymatic/mass spectrometric fingerprinting of partially acetylated chitosans, in: F. Rustichelli, C. Caramella, S. Senel, K.M. Varum (Eds.), *Adv. Chitin Sci*, vol. 11, 2011, pp. 185–191 (Venice).
- [49] C. Sasaki, K.M. Vårum, Y. Itoh, M. Tamoi, T. Fukamizo, Rice chitinases: sugar recognition specificities of the individual subsites, 16 (2017) 1242–1250, <https://doi.org/10.1093/glycob/cw043>.
- [50] M. Kohlhoff, A. Niehues, J. Wattjes, J. Bénéteau, S. Cord-Landwehr, N.E. El Gueddari, F. Bernard, G.R. Rivera-Rodriguez, B.M. Moerschbacher, Chitinase: a fungal chitosan hydrolyzing enzyme with a new and unusually specific cleavage pattern, *Carbohydr. Polym.* 174 (2017) 1121–1128, <https://doi.org/10.1016/j.carbpol.2017.07.001>.
- [51] T. Weikert, A. Niehues, S. Cord-Landwehr, M. Hellmann, B.M. Moerschbacher, Reassessment of chitinase substrate specificities and classification, *Nat. Commun.* 8 (2017) 1698, <https://doi.org/10.1038/s41467-017-01667-1>.