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Isolation, Specificity, and Application of β -N-Acetylhexosaminidases from *Penicillium crustosum*

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Abstract: β -N-acetylhexosaminidases have great potential in applied biocatalysis owing to their ability to act on a wide range of natural and modified substrates. In this work, β -N-acetylhexosaminidases from four *Penicillium crustosum* strains (PcHex) were studied. The production strains showed the highest enzymatic activity in the culture medium after 11–14 days of cultivation. The specific activity of the isolated and purified PcHex of hydrolysis of 4-nitrophenyl-N-acetyl- β -D-galactopyranoside was 15–20 U/mg protein. All isolated β -N-acetylhexosaminidases showed similar pH-activity profiles, with the optimum pH being 4.0–5.0 and the optimum temperature being 40 °C–50 °C. Apart from standard substrates, two synthetic substrates (5-bromo-4-chloro-3-indolyl-N-acetyl- β -D-galactoside and 2-chloro-4-nitrophenyl- β -D-galactopyranoside) were tested and successfully hydrolyzed using β -N-acetylhexosaminidase preparations. Maximum activity toward the fluorogenic substrate 4-methylumbelliferyl-N-acetyl- β -D-galactopyranoside (4MU- β -GalNAc) was observed for enzyme preparations from PcHex1 (15.38 U/mg protein) isolated from swab samples of books in the Slovak National Museum in Martin (Slovak Republic) and stored in our laboratory. The same enzyme preparation was used for the selective hydrolysis of β -anomer of 4MU-GalNAc from an anomeric mixture of 4MU- α / β -GalNAc. Thus, pure α -anomer (with the total yield being 90%) was separated from the mixture, which suggests the application potential of these enzymes.

Keywords: *Penicillium crustosum*; β -N-acetylhexosaminidase; biochemical characterization; synthetic glycosides; chemo-enzymatic synthesis



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1. Introduction

β -N-acetylhexosaminidases (Hex, EC 3.2.1.52) are a class of exo-glycosidases catalyzing the hydrolysis of the terminal β -linked N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) residues from glycoconjugates. They are ubiquitous in most living organisms (both prokaryotic and eukaryotic) and participate in several biological processes depending on the type of organism and their cellular localization. According to their origin, Hex can hydrolyze not only β -N-acetylhexosaminides but also β -glycosides [1]. These enzymes have gained attention owing to their structure, function, and specificity toward various substrates. They have great potential in applied biocatalysis based on transglycosylation or reverse hydrolysis reactions. In particular, fungal and recombinant Hex are capable to act over a broad range of natural and modified substrates and have been extensively used for synthetic purposes. Kinetically controlled transglycosylation reactions are predominantly used in synthetic applications. Different approaches and experimental conditions, such as high acceptor concentrations, the addition of co-solvents, choice of enzyme source, and removal of the transglycosylation product, have been chosen to promote the transglycosylation and prevent hydrolysis. Because of their synthetic potential, Hex have received great attention in the preparation of new bioactive glycosides (glycostructures, glycoconjugates) showing important effects on various biological processes such as immunoactivity, cancer metastasis, or hormone regulation [2,3].

To expand the current group of natural β -*N*-acetylhexosaminidases, we investigated the production and properties of fungal β -*N*-acetylhexosaminidases from *Penicillium crustosum* that have not been studied so far. The fungus *P. crustosum* was first isolated by Thom in 1930 [4]. Since then, it has been extensively used as an active producer of pectolytic and cellulolytic enzymes [5], a novel lipase [6], and schizophyllan degrading β -glucanase [7]. Moreover, *P. crustosum* has been used for the production of secondary metabolites such as roquefortine, penitrem, viridicatols, terrestric acid, and andrastin [8]. However, to the best of our knowledge, none of the previous studies have reported the production of β -*N*-acetylhexosaminidases by *P. crustosum*. Burtseva et al. [9] assessed active glucanases and glucosidases from 92 strains of fungi from various marine habitats, one of which was *P. crustosum*, which, however, showed no β -*N*-acetylhexosaminidase activity [9]. Thus far, the potential application of Hex in the production of α -anomers from synthetic substrates by enzymatic hydrolysis of the anomeric mixture has been described in the literature only twice, that is, once using bacterial Hex in the preparation of α -anomer from GlcNAc [10], and the other, using fungal Hex in the preparation of 4-nitrophenyl-*N*-acetyl- α -D-galactopyranoside (4-NP- α -GalNAc), which is our previous study [11].

In this research, we aimed to explore the cultivation conditions, biochemical properties, and substrate specificity of β -*N*-acetylhexosaminidases isolated from four *P. crustosum* (henceforth referred to as PcHex) strains. The ability of PcHex to hydrolyze modified glycosides used as synthetic substrates was also investigated, which has not yet been reported. Furthermore, the purified enzyme was used in the specific hydrolysis of β -anomer from chemically synthesized anomeric mixture of the fluorogenic substrate 4-methylumbelliferyl-*N*-acetyl- α / β -D-galactopyranoside (4MU- α / β -GalNAc).

2. Materials and Methods

2.1. Chemicals and Culture Media

4-Nitrophenyl-*N*-acetyl- β -D-glucopyranoside (4NP- β -Glc/GalNAc), 2-nitrophenyl-*N*-acetyl- β -D-glucopyranoside (2NP- β -Glc/GalNAc), 4-methylumbelliferyl-*N*-acetyl- β -D-galactopyranoside (4MU- β -GalNAc), 2-chloro-4-nitrophenyl- β -D-galactopyranoside (2Cl-4NP- β -Gal), and 5-bromo-4-chloro-3-indolyl-*N*-acetyl- β -D-galactoside (X- β -GalNAc) were purchased from Carbosynth, Ltd. (Compton, UK). The anomeric mixture of 4-methylumbelliferyl-*N*-acetyl- α / β -D-galactopyranoside (α : β anomer ratio = 4:3) was kindly provided by SynthCluster Co., Ltd. (Bratislava, Slovakia). All other chemicals were of analytical grade.

The cultivation medium contained the following constituents (g/L; pH 6.0 $\pm$ 0.2): KH₂PO₄, 3.0; NH₄H₂PO₄, 5.0; (NH₄)₂SO₄, 2.0; yeast extract, 0.5; NaCl, 15.0; MgSO₄, 0.5; *N*-acetylglucosamine, 5.0. MgSO₄ and *N*-acetylglucosamine were added after sterilization. Malt extract agar (MEA) contained the following constituents (g/L; pH 5.3 $\pm$ 0.2): malt extract, 20.0; peptone, 1.0; glucose, 20.0; agar, 20.0; stock solution of trace elements, 1 mL/L (0.5 g of CuSO₄·5H₂O, 0.1 g of ZnSO₄·7H₂O in 100 mL of dH₂O). Czapek yeast autolysate agar (CYA) contained the following constituents (g/L; pH 7.3 $\pm$ 0.2): sucrose, 30.0; NaNO₃, 3.0; KH₂PO₄, 1.0; KCl, 0.5; MgSO₄·7H₂O, 0.5; FeSO₄·7H₂O, 0.01; agar, 15.0. These media were sterilized in an autoclave at 121 °C for 20 min.

2.2. Microorganisms

The strain *P. crustosum* PcHex1 was isolated from samples taken by swabbing books in the Slovak National Museum in Martin (Slovak Republic) and stored in our laboratory. The strains *P. crustosum* CCF 3210 and CCF 3437 were obtained from the Culture Collection of Fungi (CCF), Department of Botany, Charles University in Prague (Czech Republic). The strain *P. crustosum* NRRL 62558 was obtained from the Agricultural Research Service Culture Collection (NRRL) in Peoria, Illinois. The strains were stored on MEA plates at 4 °C.

2.3. Cell Cultivation and Purification of β -N-Acetylhexosaminidase

The medium (100 mL) in 500-mL flasks was inoculated with a suspension of spores in 0.1% (*v/v*) Tween 80. Cultivations were carried out in an orbital shaker at 28 °C and 200 rpm. After cultivation, the mycelium was removed by filtration, and the clarified medium was used for enzyme purification with cation exchange chromatography (Fractogel® EMD SO_3^- ; Merck, Darmstadt, Germany) using the ÄKTA Purifier protein chromatography system (GE Healthcare, Uppsala, Sweden) according to the method described by Slámová et al. [12]. The purified enzyme was concentrated with an ultrafiltration membrane (Amicon® Ultra-4 10K, Merck, Kenilworth, NJ, USA) and stored in 1 M $(\text{NH}_4)_2\text{SO}_4$ at 4 °C. Protein quantification was performed using the Bradford assay calibrated for bovine serum albumin.

2.4. Enzyme Assay

β -N-acetylhexosaminidase activity was measured spectrophotometrically using 4NP- β -GalNAc as the substrate at a starting concentration of 4 mM. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of nitrophenol per minute in 50 mM citrate–phosphate buffer at pH 4.5, 35 °C, and under shaking (550 rpm) using a ThermoMixer® Comfort Shaker (Eppendorf, Cambridge, UK). Aliquots of 20 μL were taken at various time intervals and added to 2.0 mL of 0.1 M Na_2CO_3 , and the amount of nitrophenol released was determined spectrophotometrically at 420 nm. All experiments were performed in triplicate. The results of spectrophotometric measurements were confirmed with high-performance liquid chromatography (column—Poroshell 120 EC-C18, 3×100 mm, 37 °C, mobile phase—methanol–water (15:85, $0.5 \text{ mL} \cdot \text{min}^{-1}$), detector diode array detector VL+). The enzyme activity toward the substrates 4NP-GlcNAc and 2NP-Glc/GalNAc was determined in the same manner.

Alternatively, other chromogenic and fluorogenic substrates were used to investigate substrate specificity, such as 4NP- β Glc/GalNAc, 2NP- β -Glc/GalNAc (the same procedure as that for 4NP- β -GalNAc), 4MU- β -GalNAc, 2Cl-4NP- β -Gal, and X- β -GalNAc (experimental conditions described in Table 1).

Table 1. Experimental conditions for the tested substrates.

Substrate	Substrate Concentration	Reaction Volume	Enzyme Activity	Spectrophotometric Determination (Wavelength)	HPLC Determination		
					Column	Mobile Phase	Flow Rate
4MU- β -GalNAc	4 mM	2 mL	0.2–0.3 U	347 nm	Nucleodur C18ec, 250×4 mm, 30 °C	0.5% o-phosphoric acid (A), acetonitrile (B); gradient: 0–5 min, held at 21% B; 5–10 min, 21–40% B; 10–13 min, held at 40% B; 13–55 min, 40–21% B. Water (A), acetonitrile (B); gradient: 0–10 min, 40–80% B; 10–15 min, 80–40% B; 15–20 min, held at 40% B. Water (55%), acetonitrile (45%).	1 mL/min
X- β -GalNAc	0.5 mM	2 mL	0.1–0.7 U	-	Nucleodur C18ec, 250×4 mm, 30 °C		0.7 mL/min
2Cl-4NP- β -Gal	1 mM	2 mL	0.2–0.3 U	420 nm	Nucleodur C18ec, 250×4 mm, 30 °C		0.7 mL/min

The enzyme activity of β -N-acetylhexosaminidases was calculated from the linear part of a plot where the hydrolysis course is represented as a function of the concentration of aglycon released and time. One unit of enzyme activity was defined as the amount of enzyme that releases 1 μmol of aglycone per minute. The specific activity was calculated as the enzyme activity divided by the amount of protein used for hydrolysis. All experiments were performed in triplicate.

2.5. Resolution of the Anomeric Mixture of 4MU- β -GalNAc by Enzyme Hydrolysis

The reaction mixture contained the substrate 4MU- α/β -GalNAc (4.5 mM solution in citrate–phosphate buffer, 50 mM, pH 4.5, 10 mL) and the purified enzyme Pchex1 (0.22 U).

Hydrolysis was performed in 50 mL glass reactors at 35 °C for 4 h under stirring at 450 rpm. The reaction was stopped by heating the mixture solution to 100 °C for 5 min. The α -anomer of 4MU-GalNAc was isolated by extraction with organic solvents according to the protocol established by Hronská et al. [11].

3. Results and Discussion

3.1. Production, Purification, and Characterization of β -N-Acetylhexosaminidases from *P. crustosum* Strains

β -N-acetylhexosaminidases are an important member of the binary chitinolytic system of filamentous fungi, which is responsible for the formation of septa and hyphenation of fungal cells. The activity and excretion of β -N-acetylhexosaminidases into the extracellular space are constantly controlled by the cell and depend on the stage of the fungal growth [13]. To our knowledge, no studies have investigated the enzymatic activity of Hex in *P. crustosum* strains. In this work, Hex activity was tested in four strains of *P. crustosum*. The strain *P. crustosum* PcHex1 was obtained from a set of microscopic fungal isolates from damaged archived materials of the Slovak National Museum in Martin (Slovak Republic). Colonies of the strain grown on CYA medium appeared a grayish green color with white margins (Figure 1A). Additionally, they had asymmetrically branched conidiophores with a typical warty stem, and the conidia were smooth and oval, arranged in 3.0–4.0 μ m chains when observed under the microscope (Figure 1B). Based on the micromorphological and colony characteristics, the strain PcHex1 was identified as *P. crustosum* according to the Culture Collection of Fungi (CCF, Prague, Czech Republic). The other tested *P. crustosum* strains were obtained as isolates from international collections of microorganisms.

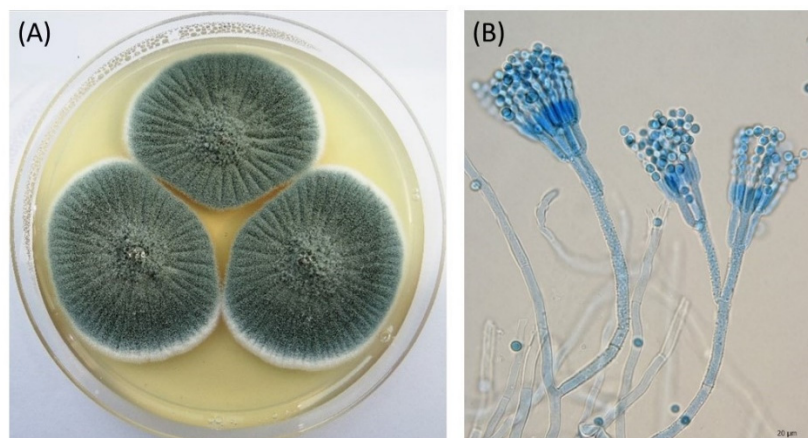


Figure 1. Colony morphology (A) and microscopic characteristics (B) of *Penicillium crustosum* PcHex1. The scale bar represents 20 μ m.

The strains of *P. crustosum* were grown in a culture medium containing *N*-acetylglucosamine as the β -N-acetylhexosaminidase inducer for 15 days. The highest level of enzyme activity in the medium was achieved after 11–12 days of cultivation for the *P. crustosum* strains CCF 3437 and PcHex1 and after 13–14 days for the strains CCF 3210 and NRRL 62558 (typically 0.15–0.25 U/mL and 9–11 U/mg protein, respectively; Figure 2). These results are comparable with those of previous studies wherein the specific activity of fungal Hex from *P. oxalicum* CCF 1959 and *Aspergillus versicolor* CCF 2491 was 10.8 U/mg protein [14] and 11 U/mg protein (0.4 U/mL), respectively [15]. The enzyme was purified using cation exchange chromatography and stored in 1 M ammonium sulfate at 4 °C (SDS-PAGE electrophoresis is shown in Figure S1). The specific activity of the stock enzyme was typically 15–20 U/mg protein, and after 6 months, the decrease was not more than 5% of its original activity.

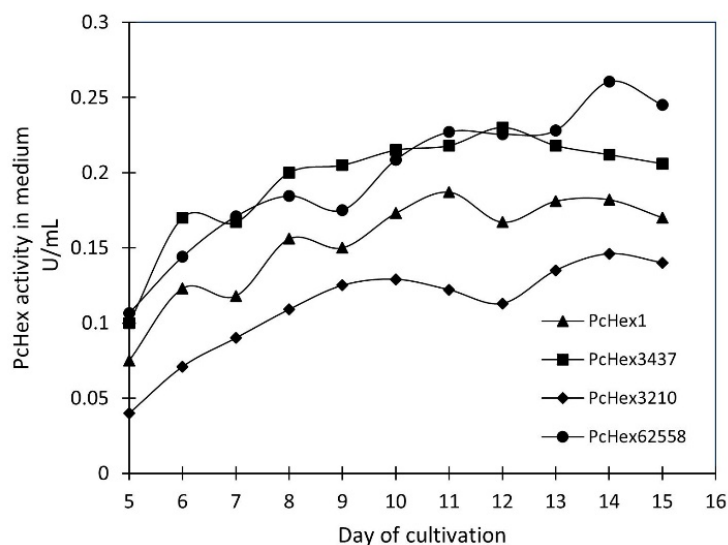


Figure 2. Time course of the extracellular Hex activity of PcHex3437, PcHex3210, PcHex1, and PcHex62558 in the culture medium containing 0.5% *N*-acetylglucosamine as the inducer.

3.2. Effect of pH and Temperature on the Activity of β -*N*-Acetylhexosaminidases

In general, the pH optimum of fungal hexosaminidases lies in the acidic region [16], and it usually has quite a broad range. The maximum hydrolytic activity of all four PcHex strains tested was in the same pH range of 3.5–5.0 (Figure 3), which is similar to that of hexosaminidases isolated from *P. oxalicum* (pH optimum 2–4; [14]), *Talaromyces flavus* (pH 2.5–5.0; [12]), and *Aspergillus versicolor* (pH 3.0–6.0; [15]). PcHex3210 and PcHex62558 were more sensitive to minor pH changes, whereas PcHex3437 and PcHex1 showed good stability over the entire tested pH range.

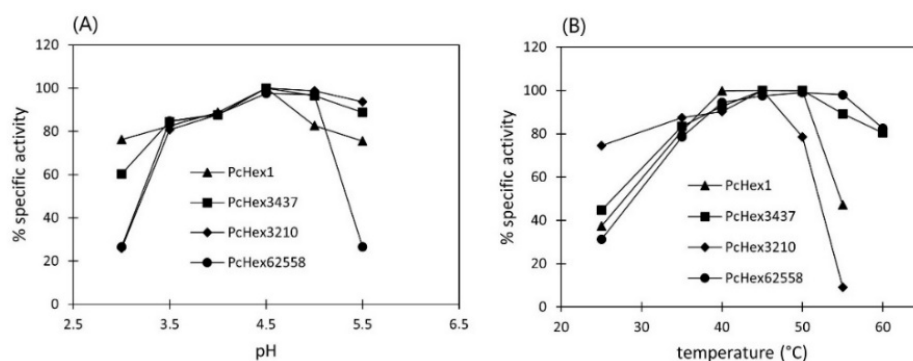


Figure 3. Effect of pH (A) and temperature (B) on the specific activity of Hex. Reaction conditions: reaction volume 2 mL, 4-NP- β -GalNAc concentration 4 mM, enzyme activity 0.2–0.3 U. The pH profile was investigated at 37 °C, and the temperature profile was investigated at pH 4.5.

The optimal temperature of fungal hexosaminidases also varies in a wider range, usually 35–60 °C [14,16]. Hex isolated from *P. crustosum* showed temperature optimum at around 40–50 °C. Both PcHex1 and PcHex3210 were sensitive to a temperature increase beyond the optimum, and they lost up to 90% of their activity at 55 °C. The temperature profile of PcHex3437 and PcHex62558 showed a broader temperature optimum; however, after 15 min of incubation at 55 °C, both enzymes started to lose their activity, and after 60 min of incubation, they became completely inactive.

3.3. Substrate Specificity toward 4NP- β -Glc/GalNAc and 2NP- β -Glc/GalNAc

β -*N*-acetylhexosaminidases are characterized by their inherent dual catalytic activity, which allows them to act over two substrates differing in the configuration of the

hydroxyl group on C-4 of the glycon, which may be *N*-acetylglucosamine (GlcNAc) or *N*-acetylgalactosamine (GalNAc). This specificity characteristic is commonly tested on substrates such as 4NP- β -GalNAc and 4NP- β -GlcNAc, which are largely accepted by a wide range of β -*N*-acetylhexosaminidases. Moreover, in this study, we used nitrophenyl glycosides with a nitro group binding to the phenyl group in position 2, namely, 2NP- β -GalNAc and 2NP- β -GlcNAc, because the GlcNAcase/GalNAcase ratio may also be affected by the aglycon structure [17]. In general, β -*N*-acetylhexosaminidases prefer D-glucose structures, with the GlcNAcase-to-GalNAcase ratio ranging from 1.5 to 4.0 [3].

As shown in Table 2, Hex produced by *P. crustosum* strains showed higher specific activity toward 2-nitrophenyl substrates, which indicated their preference for 2-nitrophenyl aglycon over 4-nitrophenyl, apart from the strain PcHex3437, for which the difference was not very significant. Concerning the preferences for glucose or galactose, Hex isolated from all four strains of *P. crustosum* exhibited a GalNAcase/GlcNAcase ratio similar to that of various species of *Penicillium*, namely, 0.9 for *Penicillium chrysogenum* [18], 1.2 for *Penicillium funiculosum*, or 1.15 for *Penicillium purpurogenum* [19], but still lower than that of *P. oxalicum* strains, for which the GalNAcase/GlcNAcase ratio usually ranged from 1.4 to 2.3 [19,20]. The determination of the preference for GalNAcase/GlcNAcase is a delicate matter, as the preference not only depends on the producing strain but also varies during microorganism cultivation and can be influenced by the presence of some inorganic salts or organic solvents [19].

Table 2. Substrate specificity of the isolated β -*N*-acetylhexosaminidases.

	Specific Activity (U/mg Protein)					
	4NPGalNAcase	4NPGlcNAcase	Ratio 4NPGalNAcase/ GlcNAcase	2NPGalNAcase	2NPGlcNAcase	Ratio 2NPGalNAcase/ GlcNAcase
PcHex1	17.40 $\pm$ 0.52	16.61 $\pm$ 0.33	1.0	23.94 $\pm$ 1.13	20.05 $\pm$ 0.89	1.2
PcHex3437	15.31 $\pm$ 0.44	11.89 $\pm$ 0.23	1.3	16.40 $\pm$ 0.61	12.54 $\pm$ 0.43	1.3
PcHex3210	14.46 $\pm$ 0.24	18.76 $\pm$ 0.52	0.8	26.76 $\pm$ 0.83	21.52 $\pm$ 0.64	1.3
PcHex62558	15.81 $\pm$ 0.49	15.96 $\pm$ 0.51	1.0	20.32 $\pm$ 0.79	24.48 $\pm$ 1.09	0.8

Kinetic constants describing the hydrolysis of the substrates 4NP- β -GalNAc, 4NP- β -GlcNAc, 2NP- β -GalNAc, and 2NP- β -GlcNAc were determined for all four PcHex. The kinetics of the hydrolysis of 4NP- β -GalNAc, 4NP- β -GlcNAc, 2NP- β -GalNAc, and 2NP- β -GlcNAc was described using the Michaelis–Menten equation. The kinetic parameters k_{cat} (catalytic constant), and K_m (Michaelis constant) were determined using nonlinear regression from the initial reaction rate versus substrate concentration data using MATLAB R2022a (lsqnonlin procedure). When comparing the k_{cat}/K_m values shown in Figure 4, the most efficient catalyst overall was PcHex1, with the highest k_{cat}/K_m values for almost all tested substrates, except for 2NP- β -GlcNAc. The catalytic efficiency of PcHex1 was more than threefold higher than that of PcHex3437. Three of the four PcHex catalyzed the hydrolysis of 4NPGlcNAc the most efficiently. Only the strain PcHex3210 operated most efficiently with the substrate 2NP- β -GlcNAc.

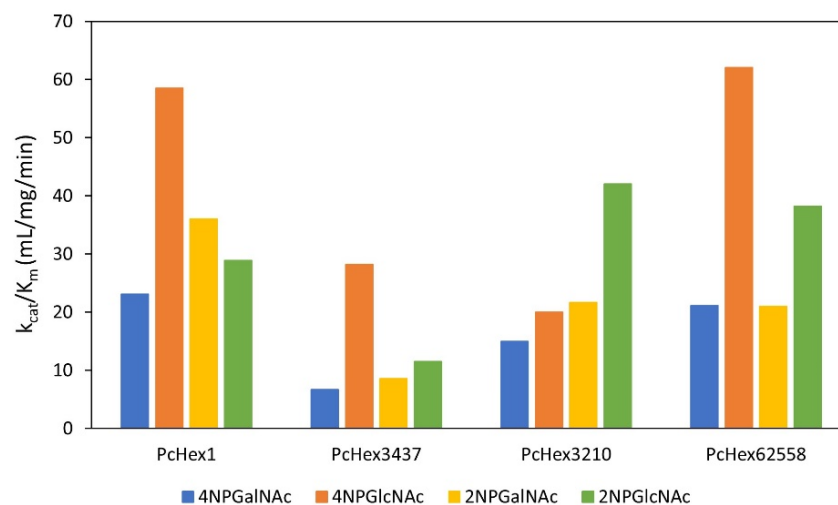


Figure 4. Kinetic parameter analysis describing hydrolysis of 4NP- β -Gal/GlcNAc and 2NP- β -Gal/GlcNAc by PcHex.

3.4. Substrate Specificity toward Nonconventional Substrates

The specific activities of the purified PcHex were measured with three different nonconventional substrates: 4-methylumbelliferyl-2-acetamido-2-deoxy- β -D-galactopyranoside (4MU- β -GalNAc), 5-bromo-4-chloro-3-indolyl- N -acetyl- β -D-galactopyranoside (X- β -GalNAc), and 2-chloro-4-nitrophenyl- β -D-galactopyranoside (2Cl-4NP- β -Gal; Figure 5). They belong to the group of synthetic fluorogenic/chromogenic enzyme substrates, and the properties of Hex acting on these substrates have not yet been studied.

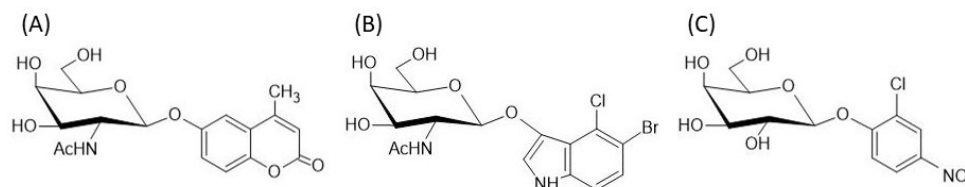


Figure 5. Structures of the synthetic substrates (A) 4MU- β -GalNAc, (B) X- β -GalNAc, and (C) 2Cl-4NP- β -Gal.

Most of the fluorogenic substrates contain a fluorescent coumarin heterocycle, such as 4-methylumbelliferone (4-MU), which is often used, for example, for the detection of glycosidases and phosphatases [21]. The fluorescence of 4-MU is strongly dependent on pH and reaches the maximum at pH 9–10 [22]. Derivatives of 4-methylumbelliferyl provide a more sensitive detection method than nitrophenyl glycosides, which are frequently used for carbohydrate-active enzymes [23]. 4-Methylumbelliferyl- N -acetyl- β -D-glucosamide has been successfully used for the early detection of fungal growth on various cultural heritage materials before it was visible to the human eye [24]. PcHex1 (15.38 U/mg protein) showed the highest specific activity against 4-MUGalNAc with 98% hydrolysis after 120 min. The other three enzymes showed threefold lower activity, approximately 5 U/mg protein (Table 3).

Table 3. Enzyme hydrolysis of 4MU- β -GalNAc, X- β -GalNAc, and 2Cl-4NP- β -Gal.

	Specific Activity (U/mg Protein)		
	4MU- β -GalNAc	X- β -GalNAc	2Cl-4NP- β -Gal
PcHex1	15.4 $\pm$ 1.2	0.181 $\pm$ 0.004	3.32 $\pm$ 0.29
PcHex3437	5.67 $\pm$ 0.51	0.171 $\pm$ 0.025	3.97 $\pm$ 0.30
PcHex3210	5.05 $\pm$ 0.47	0.221 $\pm$ 0.019	2.082 $\pm$ 0.009
PcHex62558	5.89 $\pm$ 0.42	0.287 $\pm$ 0.025	2.50 $\pm$ 0.21

X- β -GalNAc is a synthetic substrate consisting of *N*-acetylgalactopyranoside and aglycone 5-bromo-4-chloroindoxyl, which is commonly referred to as X. Indoxyl substrates are very popular among chromogenic substrates because of their intensive color, which appears when two indoxyl molecules dimerize under oxidative conditions. This compound is insoluble, which provides highly localized labeling of the target. Probably the best-known indoxyl substrate is 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-Gal), which is used in molecular biology applications to distinguish nonrecombinant transformant colonies (blue) from recombinant colonies carrying the Lac Z gene (white) [25]. The hydrolysis of X- β -GalNAc was catalyzed by all four purified PcHex, although their specific activity toward this substrate was significantly lower than that toward the other two substrates. The highest specific activity was observed for the enzyme PcHex 62558 (0.28 U/mg protein), with an almost 94% degree of hydrolysis after 22 h.

Testing hydrolysis reactions using hexosaminidase with 2Cl-4NP- β -Gal as the substrate has not yet been described in the literature, although this substrate was used to measure the level of salivary α -amylase in athletes [26] and to analyze the correlation between pain and salivary α -amylase levels during dental treatment in children with Down syndrome [27]. During the hydrolysis of 2Cl-4NP- β -Gal, 2-chloro-4-nitrophenol is released, which causes a more intense yellow color than that generated by 4-nitrophenol. Hydrolysis of this substrate was surprising for us because, unlike other substrates assessed in this study, 2Cl-4NP- β -Gal lacks the *N*-acetyl group on galactopyranoside. This group is usually crucial for β -*N*-acetylhexosaminidases, as they are sensitive to its modification. However, fungal β -*N*-acetylhexosaminidases tolerate certain *N*-acyl-modified substrates that have sterical changes at C-2 (shorter or longer acyls, a hydroxyl group instead of a hydrogen atom) [17]. The hexosaminidase PcHex3437 could hydrolyze this substrate easily (enzyme activity: 3.97 U/mg), with the degree of hydrolysis reaching 96% in 30 min of reaction, similar to PcHex1.

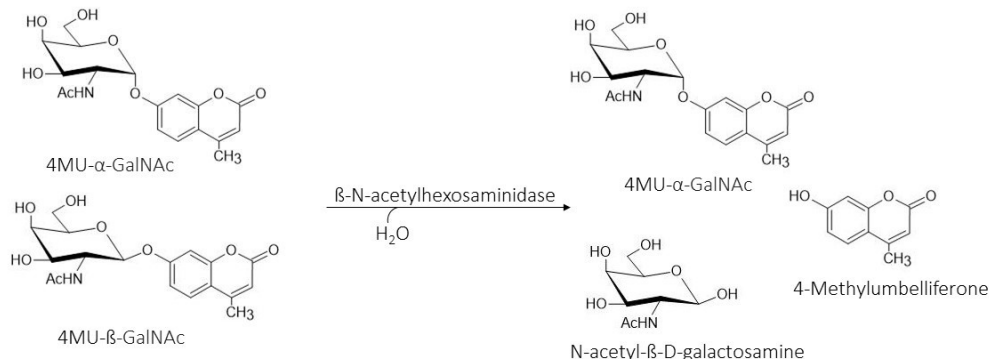
The observation of the ability to catalyze the hydrolysis of chlorinated derivatives of other than *N*-acetylated saccharides is so interesting that it would be useful to focus future research on verifying this ability using β -*N*-acetylhexosaminidases in a highly purified form and, this way, exclude the presence of contaminating activities of other enzymes whose presence in pre-purified preparations cannot be excluded with certainty.

3.5. Separation of an Anomeric Mixture of 4MU- α/β -GalNAc by Selective Hydrolysis

Fungal β -*N*-acetylhexosaminidases can selectively hydrolyze β -anomer in a mixture of α - and β -substituted GalNAc derivatives. It is a convenient method for preparing α -anomers of synthetic substrates suitable for the rapid identification and quantification of specific enzymes. This method is favorable because the enzymes are inexpensive and well-characterized, and the reaction is carried out under mild reaction conditions. After the hydrolysis reaction, α -anomer can be easily separated from the reaction mixture by extraction [11].

This is the first study to report the ability of hexosaminidases to efficiently hydrolyze β -anomer of 4MU-GalNAc in a chemically prepared anomeric mixture of 4MU- α/β -GalNAc (Scheme 1). The main product of this hydrolysis reaction, namely, 4MU- α -GalNAc, is a fluorogenic synthetic substrate, which allows the sensitive determination of α -*N*-acetylgalactosaminidase (α -NAGA), an important marker monitored in clinical practice. It

is naturally produced by the human body and is required by cells to degrade glycolipids and glycoproteins. Genetic mutations in the NAGA gene can cause a loss of enzyme activity, leading to a serious disease called Schindler's disease [28].



Scheme 1. Preparation of anomerically pure 4MU-α-GalNAc from a chemically prepared anomeric mixture of 4MU-α/β-GalNAc.

For the separation of the anomeric mixture, we chose the hexosaminidase from the *P. crustosum* strain PcHex1, which showed the highest specific activity toward the substrate 4MU-β-GalNAc (Table 2). The hydrolysis reaction was carried out under standard conditions in citrate–phosphate buffer (pH 4.5). After 4 h, 99.1% of the β-anomer was hydrolyzed (Figure 6). The reaction mixture consisted of α-anomer of 4-MU-GalNAc, 4-MU aglycone, and *N*-acetylgalactosamine. After double-solvent extraction with ethyl acetate, 98% of 4-MU was removed from the reaction mixture, leaving 91% of the α-anomer in the aqueous phase along with *N*-acetylgalactosamine. The aqueous phase was subsequently extracted thrice with *n*-butanol. The total yield of 4MU-α-GalNAc extracted into *n*-butanol was 90%.

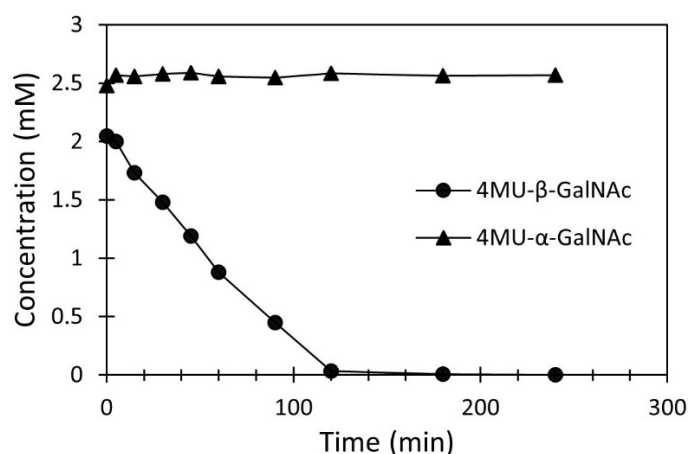


Figure 6. Enzymatic hydrolysis of 4MU-β-GalNAc in the chemically synthesized mixture of 4MU-α/β-GalNAc.

As for further simplification of the process, the buffer in the reaction medium was replaced with distilled water. The 4MU-α/β-GalNAc anomer mixture was thus dissolved in distilled water, and the pH of the mixture was adjusted to pH 4.5 at the beginning of the reaction and was then controlled during hydrolysis. The enzymatic activity of Hex and the product yield remained the same. The improved procedure for the preparation of α-anomeric derivatives of glycosides enables the effective production of 4MU-α-GalNAc in a buffer-free environment, thus, minimizing the impurity content in the final product.

β-*N*-acetylhexosaminidases from *Penicillium crustosum* presented in this work have proved a great potential in the hydrolytic resolution of glycoside anomeric mixtures. More-

over, the acceptance of novel synthetic substrates by these enzymes additionally opens up opportunities for research in the biosynthesis of non-natural oligosaccharides.

4. Conclusions

In conclusion, β -N-acetylhexosaminidases from *P. crustosum* were isolated and characterized for the first time in terms of their biochemical properties, kinetic parameters, and substrate specificity toward several synthetic substrates. The relatively broad substrate promiscuity and easy handling of β -N-acetylhexosaminidases were promising properties for their use in the hydrolysis and synthesis of various glycosides. In this research, PcHex1 was successfully applied to prepare anomerically pure 4MU- α -GalNAc from a chemically synthesized anomeric mixture of 4MU- α / β -GalNAc through total enzymatic hydrolysis of the corresponding β -anomer. The findings of this research will aid in the preparation of α -anomers of substituted glycosides as the synthetic substrates for rapid enzyme-based diagnosis.

5. Patents

The above process is patented under Slovak Patent Application no. 50042–2018 filed on 9 September 2018.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/app13063399/s1>, Figure S1: SDS-PAGE electrophoresis of PcHex1 enzyme preparation after purification with cation exchange chromatography.

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