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Purification and biochemical characterization of a novel thermostable lichenase from *Aspergillus niger* US368

Fatma Elgharbi[a], Aïda Hmida-Sayari[a,*], Mouna Sahnoun[a], Radhouane Kammoun[a], Lobna Jlaeil[b], Hajer Hassairi[b], Samir Bejar[a]

[a] *Laboratoire de Microorganismes et de Biomolécules (LMB), Centre de Biotechnologie de Sfax (CBS), Université de Sfax, Route de Sidi Mansour Km 6, BP "1177" 3018 Sfax, Tunisia*

[b] *Laboratoire d'analyse, Centre de Biotechnologie de Sfax (CBS), Université de Sfax, Route de Sidi Mansour Km 6, BP "1177" 3018 Sfax, Tunisia*

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ABSTRACT

New β-1,3;1,4-glucanase was purified from *Aspergillus niger* US368. The pure glucanase has a molecular mass of about 32 kDa. The N-terminal sequence of the purified enzyme (A-G-T-N-P-P-I-G-V) was determined. The optimum pH and temperature recorded for enzyme activity were 5 and 60 °C, respectively. It also displayed marked thermostability with a half-life of 30 min at 70 °C. At 37 °C, the enzyme showed 100% stability from pH 3 to 10. The K_m and V_{max} values exhibited by the enzyme on barley β-glucan were 0.62 mg ml^{-1} and 34.46 U ml^{-1}, respectively. The enzyme is a retaining-one and was only active toward glucan containing β-1,3;1,4-linkages. The production of β-glucanase with barley flour as the sole carbon source was optimized. This is the first report on the purification and characterization of a β-1,3;1,4-glucanase from *A. niger*. This lichenase could be considered as a candidate for future application particularly in the animal feed industry.

1. Introduction

β-Glucans are major cell wall components in cereals consisting of linear β-D-glucosyl residues linked through β-1,3 and/or β-1,4 glycosidic bonds (Tang et al., 2012). Variations have, however, often been reported in the proportions of β-1,3- (25–30%) and β-1,4-linkages as well as the length of mixed-linked segments (Planas, 2000). The degradation of β-glucans in nature is catalyzed by β-glucanases, which, based on the type of glycosidic linkage that they cleave, can be grouped into four main categories, namely β-1,3;1,4-glucanases (lichenases, EC 3.2.1.73), β-1,4-glucanases (cellulases, EC 3.2.1.4), β-1,3-glucanases (laminarinases, EC 3.2.1.39), and β-1,3(4)-glucanases (EC 3.2.1.6) (Boyce & Walsh, 2007; McCarthy, Hanniffy, Savage, & Tuohy, 2003; Yang, Yan, Jiang, Fan, & Wang, 2008).

Due to their suitability for a wide range of industrial applications, β-1,3;1,4-glucanases (lichenases) that belong to the glycoside hydrolase (GH) family 16 have attracted special attention in recent research. These enzymes have been reported to constitute important biotechnological aids in the brewing industry since they increase extraction yields and lead to high-quality brewer malt by reducing mash viscosity and turbidity (Celestino, Cunha, & Felix, 2006). They have also been described to offer attractive opportunities in the animal feed industry, particularly the increase of β-glucan digestibility in feed stuffs, the improvement of feed conversion efficiency, and the alleviation of sanitary problems, such as sticky droppings (Beckmann, Simon, & Vahjen, 2006; Mathlouthi, Mallet, Saulnier, Quemener, & Larbier, 2002).

Most of the microbial β-1,3;1,4-glucanases so far reported in the literature have been produced by bacteria, especially the genus *Bacillus* (Ekinci, McCrae, & Flint, 1997; Huang et al., 2008; Planas, 2000; Teng et al., 2006). Several extracellular β-1,3;1,4-glucanases have also been purified from various fungal sources, such as *Orpinomyces* sp. PC-2 (Chen, Li, & Ljungdahl, 1997), *Talaromyces emersonii* (Murray et al., 2001), and *Rhizopus microsporus var. microsporus* (Celestino et al., 2006). Overall, most of the β-1,3;1,4-glucanases known to date are, however, optimally active at a temperature range of 40–60 °C and in acidic or neutral pH (Planas, 2000).

Due to their limited properties, the currently marketed commercial β-1,3;1,4-glucanases may not be ideally suitable for brewing and poultry industries. Usually, malting (50–70 °C) or feed pelleting processes (65–90 °C) require enzymes with good thermostability. Therefore, thermostable β-1,3-1,4-glucanases will be highly desirable owing to their stability at high operation temperatures (Tang et al., 2012). Accordingly, there are continuing efforts to search for new sources for the production of glucanases with interesting properties that can be produced in high yields.

Aspergillus niger, an important microorganism of the genus *Aspergillus*, is known to produce a variety of enzymes that attack different types of β-glucosidic linkages (Manners, Stark, Wilson, &

* Corresponding author. Tel.: +216 74 440 451; fax: +216 74 440 451.
E-mail address: aidahmida@yahoo.fr (A. Hmida-Sayari).

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Brodie, 1976). Although this organism has been extensively used to produce large quantities of desired enzymes, no previous studies have investigated its potential with regards to the production of β-1,3;1,4-glucanases.

The present study was undertaken to optimize the production conditions for a relatively thermoactive and thermostable β-1,3;1,4-glucanase from a new *A. niger* US368 strain. It also reports on the purification and physico-chemical characterization of this interesting β-glucanase that could be used in various industrial applications and processes, particularly in the animal feed industry.

2. Materials and methods

2.1. Materials

The strain of *A. niger* US368 was already described (Hmida-Sayari, Taktek, Elgharbi, & Bejar, 2012).

Barley β-glucan, laminarin, lichenan, and CM-cellulose were purchased from Sigma Chemical Co., Ltd.

Broad-range (2–212 kDa) molecular weight marker proteins were from BioLabs.

2.2. Growth condition

Fermentations were carried out in 1 l flasks that were autoclaved at 121 °C for 20 min and then cooled and inoculated with fungal spores grown on PDA slants. Inocula were prepared by washing conidiospores from the surface of PDA slants with 25 ml of sterile distilled water. Spore densities were determined using a Thoma cell counting chamber.

2.3. Basal medium

Before optimization, the strain was cultivated in a medium consisting of 1% (w/v) barley flour, 0.5% (w/v) yeast extract and 0.5% (w/v) casein peptone.

2.4. Production medium

At optimized conditions, cultures were performed in 1 l flask containing 100 ml of barley flour (8%, w/v), yeast extract (1.5%, w/v), casein peptone (0.5%, w/v), and oligoelements solution (0.2%, v/v) consisting of $ZnSO_4$ (1.4 g/l), $FeSO_4{\cdot}7H_2O$ (5 g/l), $MnSO_4$ (1.6 g/l), and $CaCl_2$ (2 g/l).

2.5. Enzyme assay

β-Glucanase activity was determined at 60 °C using barley β-glucan (0.2%, w/v) as a substrate in 100 mM sodium acetate buffer (pH 5). The reaction was interrupted after 30 min of incubation by adding 3,5-dinitrosalicylic acid, and the reducing sugars released were then quantified (Miller, 1959).

One unit (U) of enzyme activity was defined as the amount of enzyme capable of releasing 1 μmol of reducing sugars/min, under the defined assay conditions.

2.6. Optimization of carbon source

Based on the literature, and aiming to improve enzyme production while minimizing production costs, different economical and inexpensive carbon sources were assayed for their effects on the expression of β-glucanase. *A. niger* US368 was cultivated in the basal medium mentioned above where different carbon sources (wheat bran, starch, corn steep liquor, glucose, soybean meal and gruel) were assayed instead of barley flour at 30 °C with an agitation speed of 150 rpm. Glucanase activity was determined after 4 days of cultivation.

2.7. Optimization of inoculum size

The optimization of inoculum size for β-glucanase production was carried out by assaying various sizes of inocula ranging from 10^2 to 10^7 spores/ml of sterile media. The cultures were incubated for 4 days at 30 °C with an agitation speed of 150 rpm.

2.8. Identification of critical culture variables using Plackett–Burman design

Various medium components and culture parameters were assayed for screening purposes using a Plackett–Burman (PB) factorial design (Yinghua, Xu, Zhijing, Qingbiao, & Gang, 2007). Each factor was examined in two levels coded as −1 for low level and +1 for high level. The screened factors were temperature, pH, agitation, oligoelements, yeast extract, casein peptone, soybean meal hydrolysate, and $MgSO_4$ (12 trials). All experiments were carried out in triplicate and the average of glucanase activity was taken as a response. The effect of each parameter was determined as the difference between the average of measurements made at the high (+1) and low (−1) levels using the following equation:

$$E_{(X_i)} = \frac{\sum Y_{i+} - \sum Y_{i-}}{N}$$

where $E_{(X_i)}$ refers to the factor effect or the contrast coefficient; Y_{i+} and Y_{i-} to the glucanase activities of the trials where the variable (X_i) measured was present at high and low levels, respectively, and N to the number of these experiments.

2.9. L18C Chakravarty design

An L18C Chakravarty experimental design was applied to identify the optimum conditions for enzyme production. This experimental design consisted of a matrix of 18 trials (Mintong, Gunjan, Wendy, Yun, & Larry, 2002) aiming to study the 5 major variables among the ones screened by the PB design to have the most significant effects on β-glucanase activity. Temperature and oligoelements were studied at low and high levels coded as −1 and +1, whereas pH, yeast extract, and casein peptone were studied at low, middle, and high levels coded as −1, 0, and +1, respectively. The experiments were performed in triplicate, and data given represent average values.

2.10. Software tools

The experimental data were analyzed using the statistical software package "SPSS" (Version 11.0.1 2001, LEAD Technologies, Inc., USA). The EXCEL software (Version 2003, Microsoft Office, Inc., USA) was employed to generate the response surface that allows to find out the levels of the variables for maximal β-glucanase production.

2.11. Enzyme purification

A. niger β-glucanase was purified from a culture grown in the production medium for 4 days at 37 °C and 150 rpm, all purification steps were carried out at 4 °C. The supernatant was concentrated 10-fold by rotavaporation and ultrafiltration using a 10 kDa cut-off membrane. Purification to homogeneity was performed by HPLC using a gel filtration column (PL aquagel OH 40 column/300 mm × 250 mm) pre-equilibrated with sodium acetate buffer pH 5.5 (25 mM). The active fractions pooled from the gel-filtration step were re-injected into the same column OH 40 to achieve purification.

2.12. Electrophoresis and zymogram

SDS–PAGE was performed using a 5% stacking gel and 10% resolving gel under reducing conditions as described by Laemmli (1970). Protein concentration was determined using Bio-Rad protein assay kit, based on the method of Bradford using bovine serum albumin as the standard (Bradford, 1976).

The molecular mass estimated for the native purified glucanase was determined by PAGE under denaturating and non-denaturating conditions. Protein bands were visualized by Coomassie brilliant blue R-250 (Bio-Rad) staining. Enzyme activity was detected in situ in SDS/−polyacrylamide gels containing 0.2% β-glucan. After electrophoresis, proteins were re-natured by soaking the gel for 2 h in 50 mM sodium acetate buffer (pH 5) at room temperature using horizontal shaker. The gel was then incubated for 1 h at 60 °C in the same buffer and stained with 0.1% Congo Red (Teather & Wood, 1982).

2.13. Amino acid sequencing

To determine the NH_2-terminal sequence, protein bands from SDS gels were blotted onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Automated Edman protein degradation was performed with a protein sequencer (Applied Biosystems Protein sequencer ABI Procise 492/610A) equipped with a 140C HPLC system (Hewick, Hunkapiller, Hood, & Dreyer, 1981).

2.14. Effects of temperature and pH

The effects of pH and temperature on the activity of β-glucanase were examined using barley β-glucan (0.2%) as a substrate. To determine the pH profile, reactions were performed at 65 °C in 100 mM glycine–HCl buffer (pH 3–4), sodium acetate buffer (pH 4–6), Na-phosphate buffer (pH 6–7) and Tris–HCl buffer (pH 7–9). The effect of temperature on the enzyme was investigated at temperature values ranging from 40 to 80 °C and at pH 5 (in 100 mM sodium acetate buffer).

2.15. pH and temperature stabilities

The thermal stability of the enzyme was assayed at 50, 60, 65, 70, and 80 °C. Incubation was performed in 100 mM sodium acetate buffer pH 5. Aliquots were drawn at regular time intervals and immediately cooled in ice-cold water. Residual activities, determined at 60 °C under the standard assay conditions, were expressed as percentage activity of zero-time control of untreated enzyme.

The stability of β-glucanase was investigated over a pH range of 3–10 in 100 mM buffers. Buffers used were glycine–HCl buffer (pH 3–4), sodium acetate buffer (pH 4–6), phosphate buffer (pH 6–7.5), Tris–HCl buffer (pH 7.5–9), and glycine–NaOH buffer (9–10). Aliquots were taken after 1 h of incubation at 37 °C and immediately assayed for residual β-glucanase activity.

2.16. Enzyme specificity

A. niger β-glucanase activity was determined under optimal conditions (60 °C, sodium acetate buffer pH 5), on barley β-glucan (0.2%, w/v), lichenan (0.2%, w/v), laminarin (0.2%, w/v), and CM–cellulose (0.2%, w/v) substrates prepared in Milli-Q water.

2.17. Hydrolysis properties of purified β-glucanase

For the analysis of barley β-glucan degradation products, 10 U of purified β-glucanase were incubated with 10 mg of substrate in 1 ml of 100 mM sodium acetate buffer (pH 5) at 50 °C. Aliquots were periodically withdrawn, and the reaction was stopped by placing the samples in boiling water for 5 min. Products of enzymatic hydrolyses were analyzed qualitatively by thin-layer chromatography (TLC) on silica gel 60 F254 plates (Merck 1.05554; 20 cm by 20 cm) with a butan-1-ol–acetic acid–water (2:1:1, v/v/v) solvent system (Yang et al., 2008). The plates were developed with one run followed by heating for a few minutes at 130 °C in an oven after spraying the plates with an ethanol–sulfuric acid mixture (95:5, v/v). Glucose and cellobiose were used as the standards. The end-products hydrolysis of the β-glucan were also analyzed by HPLC on an Agilent 1260 chromatograph (column: Sugar KS-802, rate 0.8 ml min^{-1}, elution: water) and the mass spectra are recorded, between 50 and 1500 atomic mass units (m/z), using a Q-TRAP mass spectrometer (Agilent, USA). In the electrospray ionization mass spectrometry (ESI-MS) experiments, the quadrupole scan mode was under an electrospray voltage of 3 kV at the tip of a stainless steel capillary needle, with a source block temperature of 350 °C.

2.18. Michaelis–Menten kinetics

β-Glucanase assays were performed using barley β-glucan at various concentrations ranging from 0.1% to 1% (w/v). Assays were performed at 60 °C using a 50 μg ml^{-1} enzyme concentration in the assay mixture. The reducing sugars generated were determined at regular time intervals and expressed in equivalent micromoles of glucose per ml of purified enzyme used for the reaction. The V_{max} and K_m values were calculated from a Lineweaver and Burk plot using the hyper-32 program.

2.19. Metal ion requirement

Several metallic ions (Co^{2+}, Zn^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, and Mg^{2+}) were assayed at 5 mM for their effects on β-glucanase activity under optimal conditions.

2.20. Configuration of the anomeric carbon of the released glucose

The method described in Genta, Dumont, Marana, Terra, and Ferreira (2007) and Saibi and Gargouri (2011) was used to identify the anomeric configuration of the glucose liberated following the action of the purified β-glucanase. At the end of the reaction, the liberated glucose can have α or β form depending on the action mechanism of the enzyme, qualified respectively as a retaining or inverting mode. The β-glucose might be converted into α form by a simple heating at 100 °C for 10 min. Moreover, the glucose oxidase can act only on the β-glucose. Consequently, the method described by Genta and his collaborators (2007) and adapted to β-glucanase assessment is based on the comparison of the amount of glucose liberated by the β-glucanase, determined by GOD (glucose oxidase kit) either directly or after a heat treatment and considering that glucose oxidase reacts exclusively with β-glucose form. In addition, the amount of β-glucan hydrolysis (for 4 h) is also measured spectrophotometrically (Miller, 1959), in order to compare it to the GODs determined amount of glucose, without heating. Hence, the enzyme was incubated with barley β-glucan (0.2%, w/v) as a substrate in 100 mM sodium acetate buffer (pH 5) for 4 h at 60 °C. The reaction was stopped by the addition of $MnSO_4$ (5 mM) which completely inhibits the β-glucanase activity. Two equivalent samples were removed, the first before and the second after boiling the reaction tube for 10 min. Heating insures the displacement of the equilibrium between the two anomeric forms of glucose toward the α-form. The GOD kit was used to measure the liberated glucose using two controls consisting in the fresh and boiled solution of glucose.

3. Results

3.1. Optimization of enzyme production

The traditional "one factor at a time" method was used to study the effects of various carbon sources and inoculum size on β-glucanase production by *A. niger* US368. Accordingly, β-glucanase production was assayed with different carbon sources, namely wheat bran, starch, barley flour, corn steep liquor, glucose, soybean meal, and gruel (a by-product of wheat milling; Kammoun, Naili, & Bejar, 2008), in the basal liquid media at 1%, 30 °C, pH 5.5, and 150 rpm (data not shown). The findings revealed that barley flour was the most appropriate carbon source for glucanase production, with maximum production of 0.6 U ml^{-1} within 4 days of growth. Fungal β-D-glucanase enzymes production is influenced by a number of factors including the type of strain used, the culture conditions and mainly the substrate type (Harman, Howell, Viterbo, Chet, & Lorito, 2004; Howell, 2003; Martin, McDougall, McIlroy, Chen, & Seviour, 2007; Pitson, Martin, & McDougall, 1993). Zymogram activity staining of the enzymatic extraction in the basal medium with 1% barley flour showed a single active band suggesting that our strain produces only one type of enzyme according to the culture conditions used. The substitution of barley flour in the culture medium by soybean meal, gave us different protein profiles (Fig. 1). The production of β-glucanase is dependent on carbon sources used (Vázquez-Garcidueñas, Carlos, Morales, & Estrella, 1998). We were interested on the glucanase produced in medium containing 1% barley flour since the activity was 0.6 U ml^{-1} against 0.4 U ml^{-1} in medium based on 1% soybean meal.

Accordingly, barley flour was further assayed at various concentrations ranging from 1 to 12% (w/v) to determine the optimal concentration required for maximal β-glucanase production. The results indicated that a maximal activity of 1.1 U ml^{-1} (1.6 U mg^{-1}) was obtained with barley flour used at a concentration of 8% (Fig. 2a).

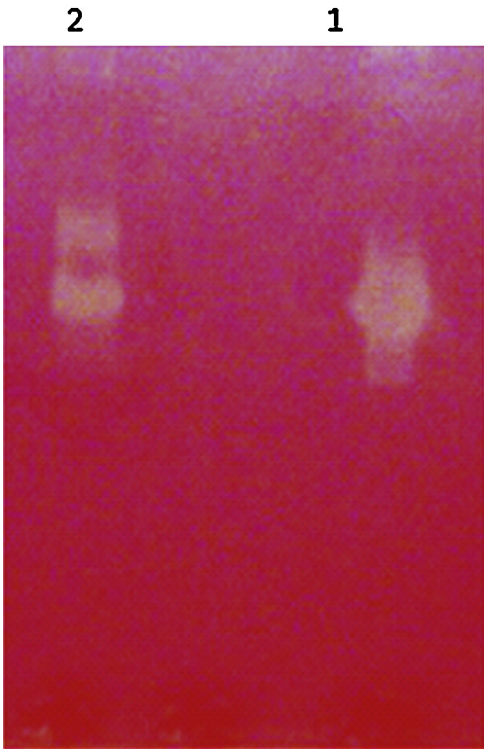

Fig. 1. Zymogram of β-1,3;1,4-glucanase activity in culture medium containing barley flour (lane 1) and culture medium containing soybean meal (lane 2).

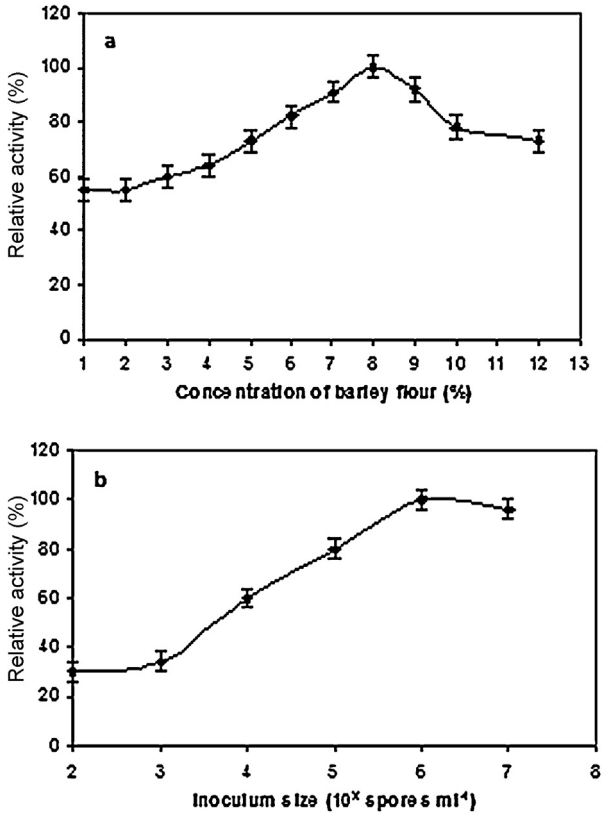

Fig. 2. (a) Optimization of barley flour concentration. Barley flour was assayed at various concentrations ranging from 1 to 12% (w/v) to determine the optimal concentration required for maximal β-glucanase production. (b) Optimization of inoculum size. β-Glucanase production activity was studied by assaying various sizes of inocula ranging from 10^2 to 10^7 spores/ml of sterile media.

The size of the inoculums is also reported to have significant effects on the growth of microorganisms and enzyme production (Pandey, 1992; Pandey, Soccol, & Mitchell, 2000). β-Glucanase production activity was, therefore, studied by assaying various sizes of inocula. The results revealed that optimal glucanase production was achieved at an initial inoculum size of 10^6 spores/ml of culture media. An important decrease in enzyme production was noted when the size of the inoculums increased (Fig. 2b).

To enhance glucanase production from *A. niger* US368, different culture conditions and nutrients were investigated in terms of their effects on enzyme production. Eight parameters were evaluated using a Plackett–Burman experimental design. The effects of the selected variables, namely temperature, oligoelements, pH, yeast extract, and casein peptone, were further investigated via an L18C-Chakravarty design to determine the optimal level of each variable for maximum glucanase production. The data were submitted to a Student's test, and an equation linking the measured response to the independent variables, was established (data not shown). The statistical model was validated with respect to β-glucanase production under the optimized conditions predicted by the model. An experimental response of 2.2 U ml^{-1} (1.85 U mg^{-1}) was obtained using 8% barley flour, 1.5% yeast extract, 0.5% casein peptone, 0.2% oligoelements solution, pH 5.5, 37 °C and 150 rpm.

3.2. Purification of β-glucanase and N-terminal amino acid sequence

A β-glucanase was purified from the supernatant of *A. niger* US368 grown in the optimized culture medium. The purification procedure, the purification degree, and production yield of each

Table 1
Summary of the purification protocol of the β-glucanase produced by *A. niger* US368.

Steps	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification (-fold)	Yield (%)
Culture supernatant	1080	2000	1.85	1	100
Concentrated crude extract	167.02	780	4.67	2.52	39
Gel filtration	6.2	207.8	33.33	14.24	10.39

step are summarized in Table 1. After purification, the β-glucanase was enriched about 14.24-fold, with a yield of 10.39% (Table 1).

The purified enzyme showed a specific activity of 33.33 U mg^{-1} and displayed a single protein band with a molecular mass of about 32 kDa on SDS–PAGE (Fig. 3a). Zymogram activity staining revealed one clear zone of glucanase activity (Fig. 3b), and the purified enzyme was also noted to exhibit a single protein band on native PAGE (data not shown), thus indicating that the β-glucanase extracted from *A. niger* US368 was a monomeric enzyme.

The sequence determined for the first 9 N-terminal amino acids of the purified β-glucanase, namely A-G-T-N-P-P-I-G-V, showed uniformity, strongly indicating that it was isolated in a pure form.

3.3. Optimum pH, temperature, and stability

The effects of pH and temperature on the activity of the purified β-1,3;1,4-glucanase are shown in Fig. 4a and b, respectively. The enzyme was noted to exhibit significant activity at 65 °C and in the pH range of 3–6.5, with maximal activity being recorded in the pH range of 4–5. No enzyme activity was detected at pH values higher than 6.5 (Fig. 4a).

At pH 5, the purified enzyme was substantially active in the temperature range of 40–80 °C, with maximal activity recorded at 60 °C (Fig. 4b).

The pH stability and thermostability profiles of the enzyme are shown in Fig. 4c and d, respectively. The glucanase from *A. niger* US368 showed high pH stability within the range of pH 3–10 after 1 h incubation at 37 °C (Fig. 4c). It also displayed a marked thermostability, retaining 100% and 70% of activity after incubation at 60 °C for 45 min and 90 min, respectively. The half-lives recorded for the purified enzyme at 50 °C, 60 °C, 65 °C, and 70 °C were 150 min, 135 min, 90 min, and 30 min, respectively. At 80 °C, the enzyme was noted to be inactive and unstable (Fig. 4d).

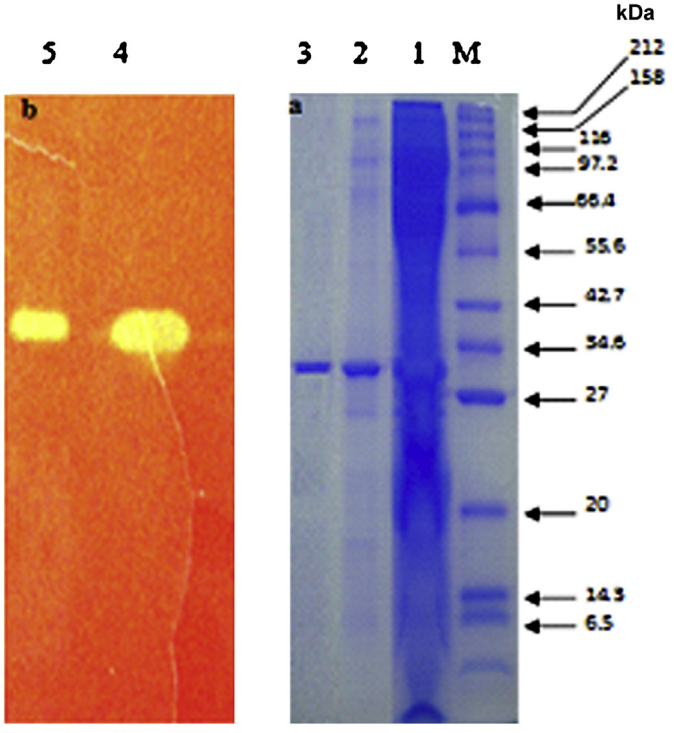

Fig. 3. (a) SDS–PAGE of proteins during the purification of β-1,3;1,4-glucanase. Lanes are designated as follows: lane M, molecular mass standard proteins: lane 1, crude culture supernatant; lanes 2 and 3, pooled fraction from the PL aquagel OH 40 column. (b) Zymogram of β-1,3;1,4-glucanase. Lane 4, crude culture supernatant; lane 5, purified enzyme.

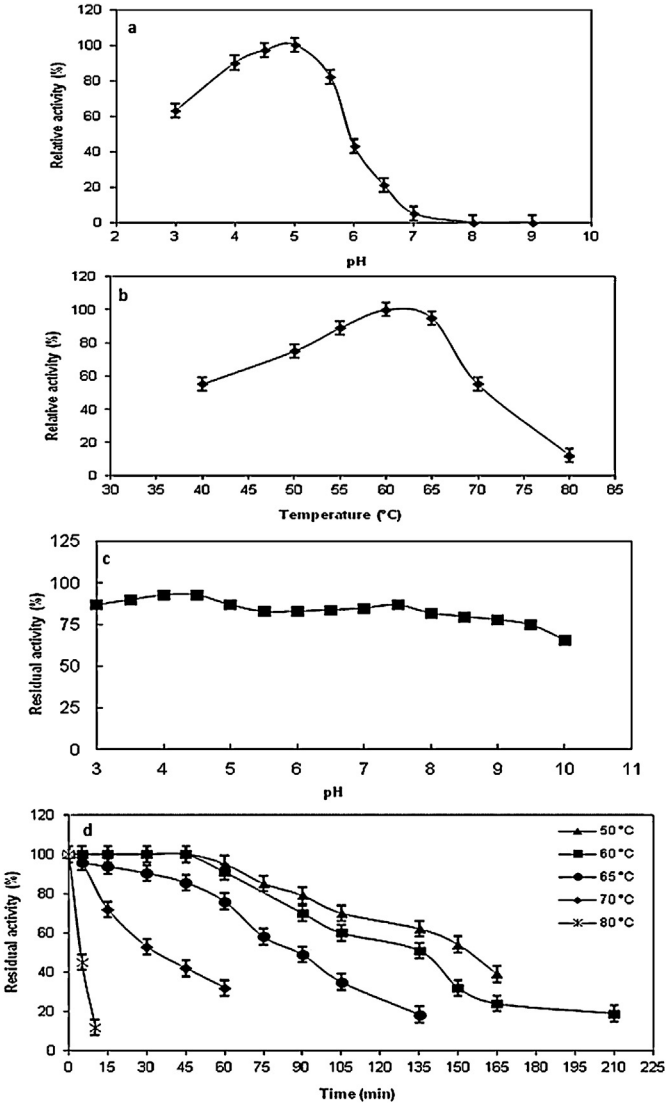

Fig. 4. (a) Effect of pH on the activity of the β-glucanase purified from *A. niger* US368. The relative activities were determined according to the standard assay with buffers solutions at 100 mM as follows: glycine–HCl buffer (pH 3–4), sodium acetate buffer (pH 4–6), phosphate buffer (pH 6–7) and Tris–HCl buffer (pH 7–9). (b) Effect of temperature on the activity of the β-glucanase purified from *A. niger* US368. The relative activities were determined according to the standard assay at pH 5 and temperatures varying from 40 to 80 °C. (c) pH stability of the β-glucanase purified from *A. niger* US368. The relative activities were determined according to the standard assay at pH values ranging from 3 to 10. Aliquots were taken after 1 h of incubation at 37 °C and immediately assayed for residual β-glucanase activity in standard assay conditions. (d) Thermostability of the purified *A. niger* US368 β-glucanase at pH 5.0 and temperatures of 50 °C (▲), 60 °C (■), 65 °C (●), 70 °C (♦), and 80 °C (*).

Table 2
Effects of metal ions on β-glucanase activity. The relative activities were measured at optimum of pH and temperature and enzyme activities without metal ions were taken as 100%.

Metal ions (5 mM)	Relative activity (%)	Specific activity (U mg^{-1})
None	100	1.85 ± 0.32
Mg^{2+}	98	1.22 ± 0.25
Cu^{2+}	0	0
Zn^{2+}	98	1.22 ± 0.25
Fe^{2+}	0	0
Co^{2+}	48	0.6 ± 0.22
Ca^{2+}	89	1.11 ± 0.31
Mn^{2+}	0	0

3.4. Effects of metal ions

No significant change in enzymatic activity was observed after extensive dialysis against EDTA, which suggested that divalent metal ions were not involved in the enzymatic catalysis process. The effects of cations on enzymatic activity were also investigated through the addition of 5 mM Mg^{2+}, Fe^{2+}, Zn^{+2}, Ca^{+2}, Cu^{2+}, Co^{2+}, and Mn^{2+} to the reaction buffer. The enzyme was noted to be completely inhibited by Fe^{2+}, Mn^{2+} and Cu^{2+}, and partially inhibited by Mg^{2+} and Zn^{2+} (2%), by Ca^{2+} (11%) and by Co^{2+} (52%) (Table 2).

3.5. Enzyme specificity

The *A. niger* US368 purified β-glucanase was assayed for its ability to hydrolyze several glucan substrates. Only barley β-glucan was efficiently hydrolyzed. No activity was detected for the enzyme against laminarin (1,3-β-glucan) and CM-cellulose (soluble 1,4-β-glucan), which strongly suggested that the enzyme was a 1,3 1,4-β-glucanase (or lichenase) that can presumably be considered as a member of the EC 3.2.1.73 enzyme category.

3.6. Hydrolysis properties of the purified β-glucanase

The hydrolysis products formed during the action of the purified enzyme on barley β-glucan were studied by TLC (Fig. 5a) and HPLC (Fig. 5b). When barley β-glucan was used as a substrate, oligosaccharides (mainly trisaccharide and tetrasaccharide) followed by disaccharide (cellobiose) and glucose were formed as the major products of hydrolysis after a prolonged incubation of 4 h with the enzyme. This was also confirmed by mass spectroscopic analysis. Indeed, the ESI-MS of the final hydrolysis products of β-glucan by the purified enzyme revealed the presence of small oligosaccharides and glucose (Fig. 5c). They are G4 (m/z = 664), G3 (m/z = 504; M+H$^+$), G2 (m/z = 343; M+H$^+$), and G1 (m/z = 162; M−H_2O + H$^+$, m/z = 180; M+H$^+$, m/z = 202; M+Na$^+$).

3.7. Kinetic parameters

The Michaelis–Menten constants were determined for barley β-glucan and lichenan (Table 3). The K_m and K_{cat} values were 0.62 mg ml^{-1} and 19.11 s^{-1} for barley β-glucan and 0.38 mg ml^{-1} and 13.32 s^{-1} for lichenan, respectively.

Table 3
Kinetic parameters for purified β-1,3;1,4-glucanase.

Substrate	V_{max} (U ml^{-1})	K_m (mg ml^{-1})	K_{cat} (s^{-1})	K_{cat}/K_m (ml mg^{-1} s^{-1})
Barley β-glucan	34.46 ± 7.315	0.62 ± 0.218	19.11	30.82
Lichenan	24.02 ± 3.9	0.38 ± 0.121	13.32	35.1

3.8. The β-glucanase is a retaining enzyme

The configuration of the anomeric carbon of glucose (end-product of the β-glucan hydrolysis) was determined according to the method described in Section 2 (Genta et al., 2007; Saibi & Gargouri, 2011). Finding showed that the amount of β-glucan hydrolysis measured spectrophotometrically (7.5 g ml^{-1}) and the amount of glucose determined using GOD method (6.8 g ml^{-1}) without heating (β form), are similar. Whereas, after boiling, the amount of β-glucose is roughly nil (0.054 g ml^{-1}). Consequently, β-glucanase could be considered as a retaining-enzyme.

4. Discussion

The strain of *A. niger* US368 was already described for producing an interesting xylanase (Hmida-Sayari et al., 2012). In the line to test whether this strain could produce a mixture of hydrolytic enzymes useful for degradation of water-soluble nonstarch polysaccharides of several feedstuffs, we demonstrated that it produce also an interesting β-glucanase.

In the present study, the production of β-glucanase by *A. niger* US368 was optimized using statistical experimental designs of Plackett–Burman and L18C Chakravarty by using barley flour as the sole carbon source. Hence the production level was substantially increased from 0.6 to 2.2 U ml^{-1}. This production was comparable to those from *Bacillus licheniformis* (Lloberas, Querol, & Bernués, 1988) and *Penicillium emersonii* (James, Fare, Sagar, Lucas, & DeGray, 1975). However, production was still lower than those attained from *Paecilomyces thermophila* (Yang et al., 2008) and *Rhizomucor miehei* (Tang et al., 2012).

The extracellular glucanase produced by *A. niger* US368 was purified and the molecular mass was estimated (32 kDa). β-1,3;1–4-glucanases with similar molecular mass values have previously been purified from *Rhizopus microsporus var. microsporus* (36.5 kDa) (Celestino et al., 2006) and *Orpinomyces strain* PC-2 (27 kDa) (Chen et al., 1997).The purified enzyme was demonstrated to be an β-1,3;1,4-glucanase (EC 3.2.1.73) or lichenase since it was able to hydrolyzed barley β-glucan (33.33 U mg^{-1}) and lichenan (16.8 U mg^{-1}) and did not attack laminarin and CM-cellulose. In fact, most endo-β-1,3;1,4-glucanases (lichenase) cleave β-1,4 linkages adjacent to β-1,3 bonds in glucans, yielding chiefly cellobiosyltriose and cellotriosyltetraose (Chen et al., 1997; Erfle, Teather, Wood, & Irvin, 1988; Hrmova & Fincher, 2001; Yoo, Lee, Chang, Lee, & Yoo, 2007). The hydrolysis patterns were similar to those previously described for β-1,3;1,4-glucanase from *Orpinomyces strain* PC-2 (Chen et al., 1997), *Talaromyces emersonii* (Murray et al., 2001), and *Aspergillus japonicus* (Grishutin, Gusakov, Dzedzyulya, & Sinitsyn, 2006). This enzyme represents the first lichenase studied from *A. niger*.

The analysis of the N-terminal sequence of β-glucanase using BLAST program (Gish & States, 1993) revealed no similarity with any of the sequence in the non-redundant databases. This was not, in fact, unexpected since there was a large heterogeneity on the lichenases already reported from fungi (Chen et al., 1997). Furthermore, this is the first time a lichenase was studied from *A. niger*.

The optimal pH and temperature values determined for the purified β-1,3;1,4-glucanase from *A. niger* US368 are in agreement with the ones previously reported for several other fungi and bacteria (Planas, 2000). They are also comparable to those described for the enzymes currently used in the brewing industry (McCleary & Nurthen, 1986).

The purified enzyme showed a manifest thermostability reaching 90 min and 30 min at 65 °C and 70 °C, respectively; better,

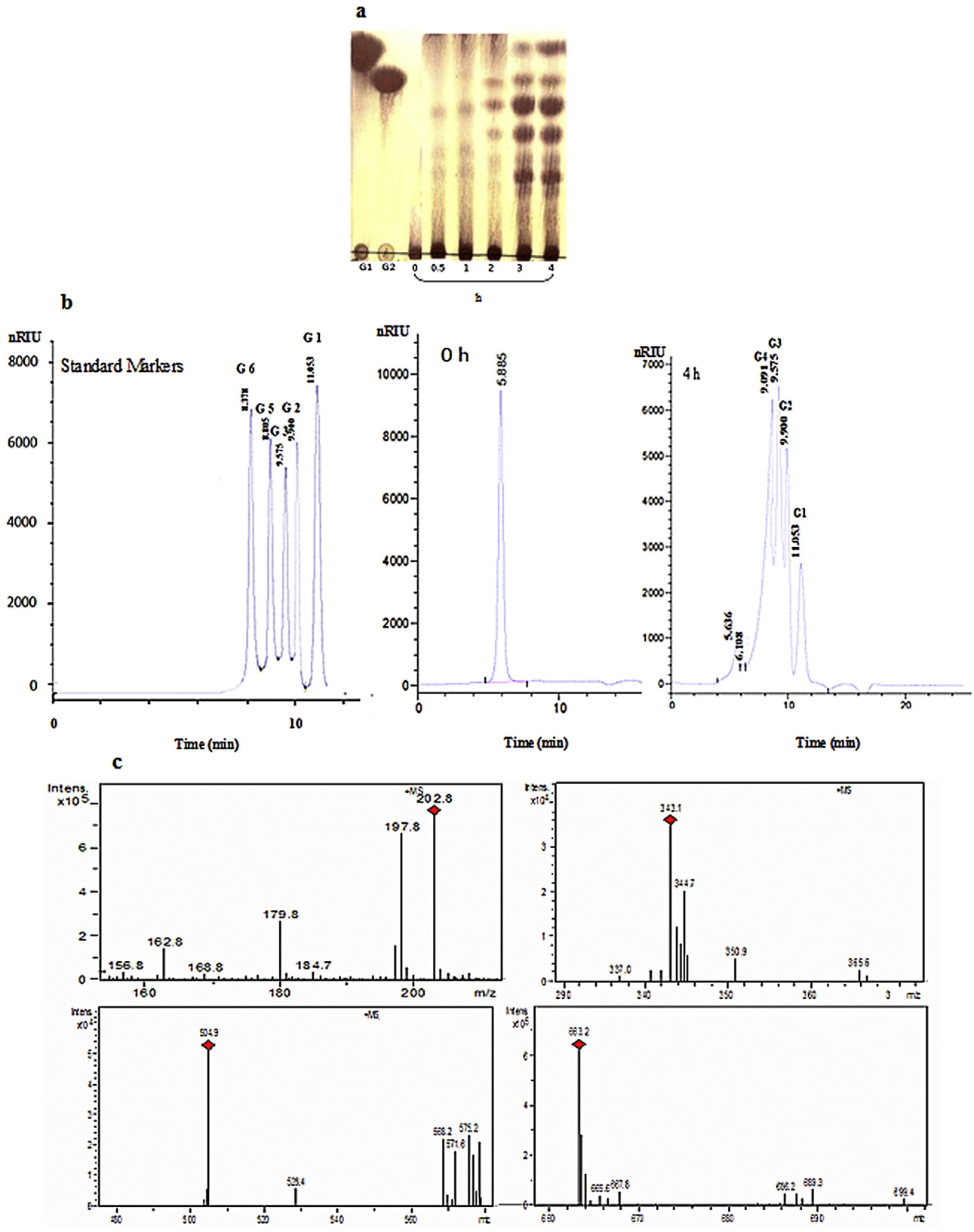

Fig. 5. (a) TLC analysis of final products of β-glucan hydrolysis by the purified β-1,3;1,4-glucanase. Incubation times (h) were indicated. Glucose (G1) and cellobiose (G2) were used as the standard markers. (b) HPLC elution profile of the final products of glucan hydrolysis by the purified β-1,3;1,4-glucanase. Glucose (G1), cellobiose (G2), cellotriose (G3), cellopentaose (G5) and cellohexaose (G6) were used as the standard markers. (c) Analysis of the end product of the enzymatic hydrolysis of β-glucan by ESI-MS/HPLC after incubation for 4 h. β-Glucan was sequentially cleaved into G4 (m/z = 664), G3 (m/z = 504; $M+H^+$), G2 (m/z = 343; $M+H^+$), and G1 (m/z = 162; $M-H_2O+H^+$, m/z = 180; $M+H^+$, m/z = 202; $M+Na^+$).

than those previously purified from several other strains, including *Rhizopus microsporus var. microsporus* (Celestino et al., 2006) and *Orpinomyces strain* PC-2 (Chen et al., 1997). Indeed, the half-lives reported for the β-glucanase of *Rhizopus microsporus* var. *microsporus* at the temperatures of 60 °C and 70 °C were 10 min and 1 min, respectively, while β-glucanase of *Orpinomyces strain* PC-2 was reported to retain only 30% of its activity at 55 °C after 1 h of incubation.

No significant change in enzymatic activity was observed after extensive dialysis against EDTA, which suggested that divalent metal ions were not involved in the enzymatic catalysis process. Similar insensitivity toward the divalent metal ions were previously reported for glucanases produced from *R. microsporus var. microsporus* (Celestino et al., 2006), *Rhizopus oryzae* (Murashima et al., 2002), and *Trichoderma harzianum* (Rana, Theodore, Naidu, & Panda, 2005).

The K_m and V_{max} values exhibited by the enzyme on barley β-glucan were 0.62 mg ml^{-1} and 34,46 U ml^{-1}, respectively. A broad range of K_m values have been previously reported for barley β-glucan from different sources (Celestino et al., 2006; Ekinci et al., 1997; Erfle et al., 1988; Planas, 2000). β-1,3;1,4-Glucanase from *Bacillus* species was, for instance, described to display K_m values in the range of 1.2–1.5 mg ml^{-1} on barley β glucan (Planas, 2000). In fact, the affinity constants described for the barley β-glucan presented in the current study were lower than those previously reported for *Talaromyces emersonii* (Murray et al., 2001) and *Rhizopus microsporus var. microsporus* (Celestino et al., 2006).

The purified β-glucanase is a retaining-enzyme. In fact, it is described that the glycoside hydrolases family 16 act with retention of configuration (Planas, 2000). According to these findings and the 3D structure of all known retaining β-glucanase, one can say that the distance between the two β-glucanase catalytic acids should be around 5.5° A (Thu & David, 2010).

Taken together, the findings presented in this work, particularly in terms of pH and thermal stability, indicate that the properties exhibited by glucanase US368 are comparable, and at times, superior to previously reported counterparts (Celestino et al., 2006; Chen et al., 1997; McCleary & Nurthen, 1986). These properties are of interest to various industrial applications and processes, particularly in the animal feed industry. In fact, the use of this lichenase can enhance the β-glucan digestibility in feed stuffs, improve feed conversion efficiency, and reduce sanitary problems. This enzyme could also be used in the brewing industry to reduce brewer mash viscosity and turbidity, increase extract yields, and produce high-quality brewer malt.

5. Conclusion

Our findings indicate that the lichenase from *A. niger* US368 could be considered as a potential strong candidate for future application particularly in the animal feed industry since it could be active in the stomach and intestine and could remain stable at high temperatures during feed pelleting processes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.07.009.

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