Production and biochemical characterization of α -glucosidase from *Aspergillus niger* ITV-01 isolated from sugar cane bagasse

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Abstract

Aspergillus niger ITV-01 presents amylolytic activity, identified as α -glucosidase, an enzyme that only produces α -D-glucose from soluble starch and that presents transglucosylase activity on α -D-glucopyranosyl-(1-4)- α -D-glucopyranose (maltose) (200 gL⁻¹). Biochemical characterization was performed on *A. niger* ITV-01 α -glucosidase; its optimum parameters were pH 4.3, temperature 80 °C but stable at 40 °C, with an energy of activation (E_a) 176.25 kJ mol⁻¹. Using soluble starch as the substrate, K_m and V_{max} were 5 mg mL⁻¹ and 1000 U mg⁻¹, respectively. As α -glucosidase is not a metalloenzyme, calcium and EDTA did not have any effect on its activity. The molecular weight was estimated by SDS-PAGE to be about 75 kDa. It was also active in methanol and ethanol. When ammonium sulfate (AS) and yeast extract (YE) nitrogen sources and calcium effect were evaluated, the greatest activity occurred using YE and calcium, as opposed to AS media where no activity was detected. The results obtained showed that this enzyme has industrial application potential in the processes to produce either ethanol or malto-oligosaccharides from α -D-glucopyranosyl-(1-4)- α -D-glucopyranose (maltose).

Keywords α -glucosidase · *Aspergillus niger* · Soluble starch · Calcium · Yeast extract

Introduction

Exo-enzyme α -glucosidase (EC 3.2.1.20, α -D-glucoside glucohydrolase) catalyzes the liberation of α -glucose from nonreducing ends of α -glucosides or from complex polymers with α -(1-4) bonds, such as malto-oligosaccharides, soluble starch, amylose and glycogen, although it cannot act on raw starch (van der Maarel et al. 2002a; Okuyama et al. 2016).

Some α -glucosidases have transglucosylase activity and produce isomalto- and malto-oligosaccharides (IMOs) such as isomaltose, maltotriose, panose and nigerose (Duan et al. 1994; Kato et al. 2002). Therefore, α -glucosidases could be used to produce IMOs with prebiotic activity (Chiba 1997; Chen et al. 2010), glucose from starchy sources in industrial brewing, also for syrup and biofuel production and recently in medical biosensors (Mohiuddin et al. 2016).

Alpha-glucosidases are produced by all kinds of organisms (bacteria, molds, animals, and plants), and due to this wide distribution, its substrate specificity is broader, basically depending on the enzyme origin. These enzymes are conventionally classified according to their substrate specificity. Type I α -glucosidases hydrolyze aryl glucosides, such as p-nitrophenyl α -D-glucopyranoside (pNPG) faster than short malto-oligosaccharides. Type II enzymes are more active on maltose and have low activity towards aryl glucosides. Type III enzymes resemble type II, but hydrolyze oligosaccharides and starch at similar rates (Marín et al. 2006).

For industrial applications, the main producing organisms are bacteria and fungi (*Lactobacillus*, *Bacillus* and *Aspergillus*) (van der Maarel et al. 2002b; Gupta et al. 2003). Meso-philic bacteria with α -glucosidase activity are active at neutral and alkaline pHs values, and at temperatures between 20

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and 40 °C (Ojima et al. 2012; Delgado et al. 2017; Kim et al. 2017; Wang et al. 2017). Specificity characteristics vary according to the enzyme. *Bifidobacterium* α -glucosidase exhibits activity on maltose derivatives releasing glucose, but no activity on polymers such as amylose, amylopectin and starch (Kim et al. 2017). On the other hand, *Halomonas* sp. H11 α -glucosidase displayed a strong transglycosylation activity with alcohols and glycerol, but was negative for starch hydrolysis (Ojima et al. 2012). *Bacillus pseudofirmus* 703 α -glucosidase exhibited activity on both α -(1-4) and α -(1-6) linkages; however, no transglycosylation activity was detected; also, 50% of its activity in methanol and ethanol was lost (Wang et al. 2017).

Aspergillus α -glucosidases in general are more robust, due mainly to post-translational modifications, exhibiting operational conditions varying from pH 4.5–6, and optimum temperature from 50 to 65 °C. As well, *A. niger* has a high acid tolerance so bacterial contamination can be easily avoided (Yuan et al. 2008; Monteiro de Souza and de Oliveira e Magalhães 2010); it is a GRAS (Generally Recognized As Safe) organism and, therefore, can be used in foods (Monteiro de Souza and de Oliveira e Magalhães 2010). Da Silva et al. reported a α -glucosidase from *A. niveus* that exhibited little hydrolytic activity towards amylopectin, but its enzyme activity was lower with other substrates (glycogen, starch and amylose). *A. niger* α -glucosidase expressed in *P. pastoris* displays strong transglycosylation activity; its optimum temperature is 60 °C, but it does not hydrolyze starch (Chen et al. 2010). It is, therefore, necessary to characterize the enzyme in order to determine its possible industrial application.

Aspergillus niger ITV-01 was isolated in South East Mexico from sugarcane bagasse, presenting amylase activity with diverse uncharacterized starchy sources. The objective of this research was to produce, identify and characterize *A. niger* ITV-01 amylase in order to determine its potential application with soluble starch in the biofuel industry.

Materials and methods

Microorganism

Aspergillus niger ITV-01 was isolated from sugarcane bagasse in the Bioengineering Laboratory of the Veracruz Institute of Technology, Veracruz, Mexico (Ortega Platas 2011), using the following medium: 5 gL⁻¹ xylan, 5 gL⁻¹ KH₂PO₄, 2 gL⁻¹ (NH₄)₂SO₄, 0.4 gL⁻¹ MgSO₄·7H₂O, 15 gL⁻¹ agar and 1 gL⁻¹ yeast extract.

Activation of *Aspergillus niger* ITV-01

Spores of *Aspergillus niger* ITV-01 were inoculated on activation Petri dish agar containing yeast extract (10 gL⁻¹), peptone (20 gL⁻¹), glucose (20 gL⁻¹), and incubated 120 h at 30 °C. Spores were recovered by adding a solution of 5 mL 0.1% (v/v) Tween 80 in the Petri dishes, then used to inoculate the fermentation medium with an inoculum concentration of 1 × 10⁶ spores mL⁻¹.

Effect of nitrogen sources and calcium on the α -glucosidase production from *Aspergillus niger* ITV-01

The effect of nitrogen source on enzymatic activity was evaluated using a medium containing starch (80 gL⁻¹) and potassium phosphate dibasic (2 gL⁻¹). The source and concentration of nitrogen (ammonium sulfate: AS; yeast extract: YE) and calcium chloride (CaCl₂) varied according to the following nomenclature: **M1**: 25 gL⁻¹ AS, **M1-Ca**: 25 gL⁻¹ AS + 0.3 gL⁻¹ CaCl₂, **M2**: 25 gL⁻¹ AS + 5 gL⁻¹ YE, **M2-Ca**: 25 gL⁻¹ AS + 5 gL⁻¹ YE + 0.3 gL⁻¹ CaCl₂, **M3**: 10 gL⁻¹ YE, **M3-Ca**: 10 gL⁻¹ YE + 0.3 gL⁻¹ CaCl₂. The medium was inoculated with 1 × 10⁶ spores mL⁻¹ and incubated at 34 °C and 250 rpm. During the kinetics, every 4 h, samples were withdrawn and centrifuged (Eppendorf Centrifuge 5424, Germany) at 10000 rpm for 10 min. The samples were analyzed for reducing sugars with the DNS technique (Miller 1959) using a D-glucose standard curve (sigma), and for amylolytic activity in Petri dishes with soluble starch agar.

Amylolytic activity in Petri dishes

Amylolytic activity was measured by adding 80 μ L supernatant obtained from the fermentation medium to Petri dishes containing 1% (w/v) soluble starch agar. The dishes were incubated at 30 °C for 12 h, stained with Lugol's solution, and clear halo diameters were measured. All measurements were made in duplicate.

Amylolytic activity assay on soluble starch

Soluble starch was prepared autoclaving 1% (w/v) starch solution at 121 °C for 15 min. Amylolytic activity was evaluated in 50 mM acetate buffer (pH 4.3) containing 1% (w/v) soluble starch at 50 °C, by measuring the release of reducing sugars in 800 μ L volume reactions containing 190 μ g purified protein. Samples of 100 μ L were withdrawn at 2 min intervals and reducing sugars quantified by the DNS method (Miller 1959) using a D-glucose standard curve (sigma). One

unit (U) of enzyme activity was defined as the amount of enzyme that releases 1 μmol glucose equivalents per minute. These reaction conditions were used to evaluate residual activity when thermal and pH stability were performed. All measurements were made in duplicate.

α -Glucosidase activity

A chromogenic substrate (p-nitrophenyl- α -D-glucopyranoside, α -PNPG, Sigma) was used to evaluate α -glucosidase activity. An aliquot of 200 μL enzyme (80 U mL^{-1}) was added to 200 μL α -PNPG (25 mM) in 50 mM acetate buffer (pH 4.3) and incubated for 2 h at 50 °C. The reaction was stopped with a 25-mM sodium hydroxide solution and the absorbance was measured at 400 nm compared to an appropriate control (blank). All measurements were made in duplicate.

α -Glucosidase purification

At the beginning of the stationary phase (36 h), the supernatant was recovered by centrifugation (20 min, 5500 rpm, 4 °C), then filtered with filter paper (Whatman No. 5) and 0.2 μm membranes (Millipore). Filtered supernatant was concentrated by ultrafiltration (tenfold) using 10 kDa membranes (Millipore); afterwards, it was dialyzed against 50 mM acetate buffer (pH 4.3) and used for biochemical characterization.

SDS-PAGE analysis and zymography

The enzyme preparations were analyzed by electrophoresis in denaturing conditions (10% SDS-PAGE). To determine the molecular weight, the gel was stained with 0.2% (w/v) Coomassie brilliant blue (Bio-Rad) and the molecular weight was estimated comparing the protein bands with the molecular marker. For amylase activity staining, the resolving gel was supplemented with 0.1% (w/v) soluble starch (final concentration) and after electrophoresis, SDS was removed by washing the gel three times with 2.5% (v/v) Triton X-100 (Sigma) solution at 4 °C, 20 min each wash. Triton X-100 excess was rinsed with water for 5 min. The gel was soaked in 1% (w/v) soluble starch in acetate buffer (pH 4.3) and incubated overnight at 30 °C. The gel was washed with water three times to eliminate starch excess and stained with Lugol's solution [0.33% (w/v) iodine, 0.66% (w/v) potassium iodide]. Clear bands on the gel indicated amylase activity.

Mass spectrometry

Spots from 10% SDS-PAGE stained with Coomassie brilliant blue were analyzed in the Proteomic Unit of

the National Genomic Institute (Instituto Nacional de Genómica- INMEGEN-Mexico). Spots were destained with 50 mM ACN: NH_4HCO_3 (50:50 v/v), digested with trypsin and analyzed in MALDI/TOF/TOF 4800. Analysis and identification of the resulting peptides were carried out by ProteinPilot software.

Biochemical characterization

Effect of pH on enzymatic activity and pH stability

The effect of pH on amylase activity was determined at 50 °C in the range of 4.0–9.0 in 50 mM acetate buffer (pH 4.0–5.0), phosphate buffer (pH 6.0–8.0), and glycine–NaOH buffer (pH 9.0) using the activity assay while pH stability was measured from enzyme solutions incubated at 4 °C for 24 h in 50 mM buffer at pH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. Residual activity was measured as described in the activity assay. All measurements were made in duplicate.

Effect of temperature on enzymatic activity and thermal stability

The effect of temperature was evaluated at pH 4.3 in the range of 40–100 °C using the activity assay for amylase. Thermal stability was measured from enzyme solutions incubated at 50, 56, 60 and 70 °C in 50 mM acetate buffer (pH 4.3). The residual activity was measured using the activity assay with enzyme samples withdrawn at different intervals. All the measurements were performed twice. Energy activation (E_a) and k values were determined by semi-logarithmic regression.

Kinetic properties

Reaction rates were measured at different concentrations of soluble starch in the range of 1–16 mg mL^{-1} . Reaction conditions were 50 °C and pH 4.3. Kinetic parameters K_m and V_{max} were calculated by nonlinear regression of the data to the Michaelis–Menten equation, using the OriginPro 8 program (version 8.0724). All measurements were performed twice.

Effect of metal ions, inhibitors, alcohol and surfactants on purified α -glucosidase

The enzyme was incubated in NaCl (5 mM), MgSO_4 (5 mM), CaCl_2 (1 mM), FeCl_3 (1 mM), $(\text{NH}_4)_2\text{SO}_4$ (1 mM), EDTA (1 mM), Tween 80 (Hycel) (0.5%), methanol (10%) (JT Baker) and ethanol (10%) for 1 h at 4 °C and residual enzymatic activity was determined. All measurements were made in duplicate.

Hydrolysis and transglycosylation products of α -glucosidase on soluble starch and α -D-glucopyranosyl-(1-4)- α -D-glucopyranose (maltose)

In order to evaluate transglycosylation activity, α -glucosidase (0.15 U mL^{-1}) was incubated (50°C , pH 4.3) on soluble starch (14 g L^{-1}) and on α -D-glucopyranosyl-(1-4)- α -D-glucopyranose (maltose, 100 and 200 g L^{-1}). Aliquots ($200 \mu\text{L}$) were withdrawn each hour for 24 h, centrifuged and boiled for 5 min to inactivate the enzyme. All samples were observed by thin-layer chromatography (TLC) on silica gel ($15 \times 10 \text{ cm}$, Merck) with the mobile phase composed of butanol:ethanol:water (30:50:20 v/v/v). The spots were visualized by a α -naphthol solution (JT Baker) composed of ethanol: sulfuric acid: water (171:22:13 v/v/v) on silica gel and the TLC plates were dried at 110°C with hot air for 10 min.

Results and discussion

Nitrogen source on the α -glucosidase production of *A. niger* ITV-01

Different concentrations of two nitrogen sources, YE ($5, 10 \text{ g L}^{-1}$) and AS (25 g L^{-1}), were used to evaluate their effect on *A. niger* ITV-01 extracellular amylolytic activity. Also, calcium chloride (0.3 g L^{-1}) and six media were tested in this study.

Amylolytic activity in M3-Ca was the highest of the media tested. In the YE medium (10 g L^{-1}) without calcium, (M3), activity decreased 45% (Fig. 1a) whereas in the media with AS (25 g L^{-1}) and YE (5 g L^{-1}) without and with calcium, (M2 and M2-Ca), amylolytic activity decreased 75 and 55%, respectively. Finally, amylolytic activity in M1 (AS, 25 g L^{-1}) and M1-Ca (AS, 25 g L^{-1} , $0.3 \text{ CaCl}_2 \text{ g L}^{-1}$) diminished 100 and 50%, respectively. Reducing sugars were the highest (Fig. 1b) in the media M3-Ca, 24 g L^{-1} , following M3, M2-Ca (21 and 15 g L^{-1} , respectively) (Fig. 1b). In the remaining media reducing sugars were not observed, probably having been consumed by microorganism immediately after their production, because amylolytic activity was observed in almost all media tested (except M1). The medium containing YE and calcium exhibited almost two-fold enhanced amylolytic expression compared to the media with AS and YE, so the former medium was used to produce the enzyme.

Nitrogen source generally affects α -glucoamylase and α -amylase production in the *Aspergillus* genus; the excess or lack of nitrogen content in the media may be a limiting factor for substrate degradation (Rajaratnam and Bano 1989), which probably accounts for the M1, M1-Ca (lack) and M2, M2-Ca (excess) media results. Generally, organic

Fig. 1 Effect of nitrogen source and calcium on amylolytic activity and reducing sugar production. Hydrolysis haloes (a) and reducing sugars (b) produced according to culture medium

sources (peptone, yeast extract, casein) result in almost twice the specific activity compared to inorganic sources (sodium nitrate, ammonium sulfate) (Morita et al. 2004; Varalakshmi et al. 2009), because they can be assimilated more easily than inorganic sources. Increased production of α -glucosidase and α -amylase could not be due to increased transcription of the related genes, but could be caused by a faster secretion of these enzymes or by their lower binding to the cells. Calcium is an important ion in the *Aspergillus* genus in glucoamylase transportation to the extracellular medium via ER, and it is required by chaperones and foldases (Kwon et al. 2012). In this case, calcium probably improved the translocation of enzymes to extracellular medium, which increased the amylolytic activity in all media with calcium.

Identification and purification of α -glucosidase from *A. niger* ITV-01

To purify the target enzyme, AS was used at several concentrations (20–80%) on the supernatant, but with a negative effect on amylolytic activity. The method was, therefore, discarded and the amylase was ultra-filtered instead.

The protein purified with Laemmli buffer (β -mercaptoethanol, 4% (w/v) SDS and boiled) migrated as a simple polypeptide on 10% SDS-PAGE (Fig. 2) and showed a molecular weight of 75 kDa. The molecular weight of *Aspergillus* genus α -glucosidases ranges from 50 to 145 kDa (Brízová et al. 1992; Kato et al. 2002; Da Silva et al. 2009; Chen et al. 2010; Zhang et al. 2011).

Amylases, a broad group of enzymes that hydrolyze starch, are classified according to type of product generated. Generally, α -amylases produce malto-oligosaccharides (DP1-DP5, degree polymerization); glucoamylases and α -glucosidases only produce glucose (DP1) with α and β conformation, respectively (Chiba 1997; van der Maarel et al. 2002a). Besides, α -PNPG is a substrate specific to α -glucosidases and glucoamylases and when hydrolyzed, a

yellowish product (p-nitrophenol) is released. As opposed to glucoamylases, α -glucosidases present transglycosylase activity on acceptor molecules such as α -D-glucopyranosyl-(1-4)- α -D-glucopyranose (maltose) and they cannot act on raw starch (Duan et al. 1994; Kato et al. 2002). To determine what type of amylolytic enzyme *A. niger* ITV-01 produces, three substrates were tested: soluble and raw starch (14 gL⁻¹), α -D-glucopyranosyl-(1-4)- α -D-glucopyranose (maltose, 100 and 200 gL⁻¹) and α -PNPG (25 mM) (Fig. 3). The enzyme did not hydrolyze raw starch at all, and α -D-glucose was only produced from soluble starch (Fig. 3b) and 100 gL⁻¹ α -D-glucopyranosyl-(1-4)- α -D-glucopyranose (data not shown); however, the enzyme produced malto-oligosaccharides (not identified) on α -D-glucopyranosyl-(1-4)- α -D-glucopyranose (maltose) at the higher concentration (200 gL⁻¹) (Fig. 3a), exhibiting activity as an α -glucosidase. Besides, the purified enzyme hydrolyzed α -PNPG, producing p-nitrophenol (distinctive yellow color) in the reaction (Fig. 3c). Therefore, taken as a whole, these results indicate that the purified enzyme had only α -glucosidase activity and not α -amylase activity. These assays correlate with mass spectrometry analysis of the 75-kDa band sequence, where the peptides obtained had a 20% coverage with *A. fumigatus* α -glucosidase: DLTWSYAAFLTANMR, KESDGSIVWESDPR, SVYAINSGIPQGA AVSAGR. Based on the specificity classification used for α -glucosidases, this α -glucosidase belongs to group III, since it hydrolyzes oligosaccharides and starch.

Biochemical characterization of α -glucosidase from *A. niger* ITV-01

As α -glucosidase had no activity on raw starch, the experiments were carried out on soluble starch.

Fig. 2 Electrophoresis analysis of purified α -glucosidase (lane 1) and zymogram (lane 2) on 10% SDS-PAGE with 0.1% soluble starch. M, molecular weight marker

Fig. 3 Hydrolysis products of α -glucosidase on 200 gL⁻¹ maltose (**a**) and 14 gL⁻¹ soluble starch (**b**). Hydrolysis of α -PNPG (**c**). G: glucose, M: maltose, 1–24 h: starch soluble and maltose hydrolysis products at different times

Effect of temperature and calcium on enzymatic activity, thermal stability and E_a of *A. niger* ITV-01 α -glucosidase

Temperature effect on activity was evaluated in the range of 30–100 °C (Fig. 4a). Activity of α -glucosidase was observed in the temperature range 30–90 °C and maximum temperature was observed at 80 °C. Activity doubles for each 10 °C temperature increase up until 60 °C; later, in the range 70–80 °C, activity only increases slowly. It is possible that the protein slightly unfolded due to increased temperature. At 100 °C, α -glucosidase was not active. Calcium was also analyzed to evaluate its effect on activity at lower and higher temperatures; however, no effect was observed (data not shown).

Optimum temperature of *Aspergillus* α -glucosidase varies between 50 and 65 °C. The results generated in this research are different to those shown by the α -glucosidase from *A. niveus*, *A. nidulans* and *A. niger* M1 whose optimum temperature were 65, 60 and 50 °C, respectively (Da Silva et al. 2009; Chen et al. 2010; Zhang et al. 2011),

Fig. 4 Effect of temperature on enzymatic activity (a) and thermal stability of *A. niger* ITV-01 α -glucosidase at 50–60 °C (b)

whereas for the *A. niger* ITV-01 in this study, the optimum temperature was shown to be 80 °C.

Thermal inactivation of α -glucosidase was evaluated at 50, 56, 60 and 70 °C (Fig. 4b). Calcium effect on thermal stability was evaluated, but with no outcome (data not shown). Residual activity by α -glucosidase was observed at 50, 56 and 60 °C during the assay; however, when it was incubated at 70 °C residual activity ceased at 40 min of incubation.

Inactivation in all cases was of the first order (data not shown) and the half-life of α -glucosidase at 50, 56 and 60 °C was 433, 99 and 63 min, respectively, in comparison with the half-life of *A. niveus* α -glucosidase at 60 °C of 104 min (Da Silva et al. 2009) (Table 1). *A. niger* amylases present high values of inactivation (k): 0.245 min⁻¹ (60 °C) free and 0.029 min⁻¹ (60 °C) immobilized (Raviyan et al. 2003). In contrast, *A. niger* ITV-01 α -glucosidase k value at 60 °C in buffer was 0.011 min⁻¹ and, therefore, almost three times more stable than *A. niger* amylase. The E_a value of α -glucosidase was 176.25 kJ mol⁻¹ comparable to the E_a value of *A. niger* amylase immobilized on bis-acrylamide and Novo Nordisk amyloglucosidase immobilized on cassava starch (Zanin and De Moraes 1998; Raviyan et al. 2003). Thus, *A. niger* ITV-01 α -glucosidase is more stable than other free α -glucosidases and almost as stable as immobilized α -glucosidases. Besides, this behavior could be improved by chemical modifications or immobilization for its use as a biocatalyst to produce ethanol.

Effect of pH on activity and stability of *A. niger* ITV-01 α -glucosidase

Effect of pH on α -glucosidase activity was evaluated in the 4–10 pH range (Fig. 5). Between pH 4.3 and 8, α -glucosidase was active, inactive at pH 4 and 9, and its optimum was at pH 4.3. At pH 6, α -glucosidase only showed 37% relative activity, at pH 5 and 7, it showed nearly 50% relative activity, and at pH 8, it only showed 20% relative activity. With respect to stability on pH, α -glucosidase presented the same profile as the effect of pH on activity, with slight changes in residual activity (data not shown). *A. niger* recombinant α -glucosidase (Aglu) presented a similar optimum pH of 4.5 (Chen et al. 2010); however, *A. niveus*, *A. niger* M1 and *A. nidulans* (AgdB) α -glucosidases presented their optimum pH at 6, 6 and 5.5, respectively (Da Silva et al. 2009; Chen et al. 2010; Zhang et al. 2011). Enzyme activity at pH 4.3 is desirable for glucose production from amylaceous sources and fermentation by *S. cerevisiae*, since the yeast has its best yield at this pH value.

Table 1 Inactivation parameters of amylases

Enzyme (source)	T (°C)	K (min ⁻¹)	Half-life (min)	E_a (kJ mol ⁻¹)	References
α -Glucosidase (<i>A. niger</i> ITV-01)	50	0.0016	433	178	This work
	56	0.007	99		
	60	0.011	63		
α -Amylase free (<i>A. niger</i>)	55	0.0910	25	156	Raviyan et al. (2003)
	60	0.245	9.4		
	65	0.591	3.9		
α -Amylase immobilized (<i>A. niger</i>)	55	0.036	63	198	
	60	0.144	16		
	65	0.357	6.45		
Amyloglucosidase immobilized in casava (Novo Nordisk)	55	0.0287	24.1	210	Zanin and De Moraes (1998)
	60	0.1297	5.34		
	65	0.3764	1.84

Fig. 5 pH effect on *A. niger* ITV-01 α -glucosidase activity at 50 °C using different buffers: pH 4–5 acetate buffer, pH 6–8 phosphate buffer, pH 9 glycine–NaOH buffer **Fig. 6** Effect of substrate (soluble starch) on *A. niger* ITV-01 α -glucosidase activity at 50 °C, on pH 4.3 acetate buffer

Kinetic parameters on soluble starch

The effect of the soluble starch substrate on α -glucosidase activity was evaluated in the range of 1–16 mg mL⁻¹ of the substrate and the kinetic parameters were obtained (Fig. 6). The kinetic behavior of α -glucosidase was adjusted to the Michaelis–Menten model and the kinetic parameters K_m and V_{max} were determined by Origin Lab software (v 9.0) as 5.7 mg mL⁻¹ and 1000 U mg⁻¹, respectively. K_m value was not similar to the α -glucosidase from *A. niveus* (0.07 mg mL⁻¹) or from *A. niger* (31.3 mg mL⁻¹; (Brízová et al. 1992) but was similar to *Xanthophyllomyces dendrorhous* α -glucosidase (5.7 mg mL⁻¹); V_{max} value was not similar to *A. niveus* (310 U mg⁻¹), but was similar to *X. dendrorhous* α -glucosidase (940 U mg⁻¹) (Marín et al. 2006; Da Silva et al. 2009). *A. niger* ITV-01 α -glucosidase k_{cat} value and catalytic efficiency were 1282 s⁻¹ and 224.91 (g L⁻¹)⁻¹ s⁻¹, respectively. This catalytic efficiency was similar to *A. niveus* α -glucosidase (354.7 s⁻¹ mg⁻¹ mL⁻¹)

(Da Silva et al. 2009) and 500 times higher than *X. dendrorhous* α -glucosidase (Marín et al. 2006). *A. niger* ITV-01 α -glucosidase has a low affinity for soluble starch; however, its catalytic efficiency is compensated due to a high turnover number (k_{cat}). Therefore, the enzyme could be used efficiently to breakdown soluble starch in industrial processes and also for modulation of postprandial hyperglycemia research studies (McCue et al. 2005).

Effect of metal ions, inhibitors, alcohol and surfactants on purified α -glucosidase

Some amylases are dependent on ions such as calcium, cobalt, sodium, where their function is to stabilize the enzyme structure (van der Maarel et al. 2002a). According to statistical analysis (Tukey test, $p < 0.05$), *A. niger* ITV-01 α -glucosidase was not affected by any of the substances tested (Table 2). On the contrary, Tween 80 improved the activity by 10% and EDTA decreased it by only 10%,

Table 2 Effect of ions and alcohols in α -glucosidase activity

Reagent	Relative activity (%)
Control	100 $\pm$ 2 ^a
NaCl (5 mM)	100 $\pm$ 2 ^a
MgSO ₄ (5 mM)	91 $\pm$ 5 ^a
CaCl ₂ (1 mM)	104 $\pm$ 5 ^a
FeCl ₃ (1 mM)	94.88 $\pm$ 4 ^a
(NH ₄) ₂ SO ₄ (1 mM)	105 $\pm$ 6 ^a
EDTA (1 mM)	90 $\pm$ 3 ^a
Tween (0.5%)	111 $\pm$ 1.5 ^a
Methanol (10%)	103 $\pm$ 1 ^a
Ethanol (10%)	98 $\pm$ 6.5 ^a

^a represent significant differences ($p < 0.05$) between ions and alcohols on the alpha-glucosidase activity determined by Tukey's comparison of averages

indicating that α -glucosidase is not a metalloenzyme. Therefore, α -glucosidase could be used in processes with chelating agents such as syrup production. Similarly, *A. niveus* α -glucosidase activity was not affected by calcium, barium, sodium, magnesium and EDTA (Da Silva et al. 2009). Also, *A. niger* ITV-01 α -glucosidase activity was not affected by methanol and ethanol, and thus it can be used in any process where organic solvents are utilized. This behavior has been reported for amylase from *A. oryzae* IFO-30103 (Bhanja Dey and Banerjee 2014).

According to these results, *A. niger* ITV-01 α -glucosidase has potential as a biocatalyst to produce α -D-glucose in acid media at 50 °C, due to its thermal stability and high turnover number, besides being stable in ethanol and only producing α -D-glucose which is metabolized directly by the fermentative yeast. So it could be used to produce glucose syrup or malto-oligosaccharide in water miscible-organic solvents, or cloned to produce ethanol.

Conclusion

A. niger ITV-01 α -glucosidase was produced in liquid medium with YE and calcium, with better activity in extra-cellular media, and whose activity efficiently degraded soluble starch to α -D-glucose and produced malto-oligosaccharides on α -D-glucopyranosyl-(1-4)- α -D-glucopyranose (maltose). It also presented the highest optimum temperature reported for *Aspergillus* genus α -glucosidases and preferably showed an acidic character with good stability in this condition. The addition of ions, EDTA or alcohols did not have any effect on activity and thermal stability, so it could be cloned and expressed on *S. cerevisiae* to produce ethanol from starch sources.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interests.

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