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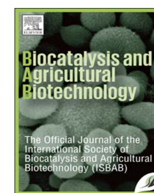

Biochemical characterization of an isolated 50 kDa beta-glucosidase from the thermophilic fungus *Myceliophthora thermophila* M.7.7

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ABSTRACT

This study characterized a 50 kDa β-glucosidase (BGL50) produced by the thermophilic fungus Myceliophthora thermophila M.7.7 in solid state cultivation using a mixture of (1:1) sugarcane bagasse and wheat bran. The crude extract zymogram showed two isoforms of β-glucosidase with approximately 50 and 200 kDa, which were separated by gel filtration chromatography. The characterization of BGL50 showed optimum activity at 60 °C and pH 5.0 when 4-nitrophenyl β-D-glucopyranoside (pNPG) was used as the substrate, whereas when using cellobiose, the highest activity was observed at 50 °C and pH 4.5. Several ions and reagents produced different effects on the enzyme activity depending on the substrate and there was complete inhibition with Cu^{2+} and Fe^{3+} for both substrates. In addition, nine phenolic compounds showed no inhibitory effects on the enzyme, a significant feature since β-glucosidase is used for the saccharification of lignocellulosic biomass that generates several phenolic compounds. Kinetic studies revealed competitive inhibition by glucose when pNPG was used, with a K_i value of 1.5 mM and a significantly lower K_m (0.52 mM) than for cellobiose (8.50 mM). The thermodynamic parameters showed that BGL50 is very stable at 60 °C displaying a half-life of 855.6 min but it is easily denatured above this temperature. The results emphasize the importance of investigating potential β-glucosidases based on cellobiose instead of using only pNPG since, in the industrial process, the enzyme will act on this natural substrate. In addition, understanding the thermostability of the enzyme is an important contribution to enzyme technology.

1. Introduction

The World Energy Council considers that oil, natural gas and coal (non-renewable energy sources) contribute with more than 82% of global energy needs and 20% of CO_2 emissions are mostly due to oil-based fossil fuels (Shaheen et al., 2013). Therefore, sustainable and renewable alternatives are urgently needed to reduce the dependence on these non-renewable resources. Biomass can be considered as an alternative energy source with increasing importance in the future. First generation ethanol has been an alternative energy source in Brazil, this biofuel being produced by fermenting glucose from sugarcane juice (Kang et al., 2014). However, both agricultural and the food industry generate a significant amount of residues that are a source for fermentable sugars that can then sustain the production of second generation ethanol.

Sugar cane bagasse and sugar cane straw are the residues from sugar and ethanol industries plants which contain around 75% sugar in the form of cellulose and hemicellulose polymers. Enzymatic saccharification of crystalline cellulose by enzymes from fungi and bacteria to obtain fermentable sugars requires the action of several highly specific enzymes, the final product being mainly free glucose. The cellulolytic complex consists of a variety of hydrolytic and redox enzymes acting synergistically. The hydrolases that can degrade cellulose comprise endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.176), exoglucohydrolases (EC 3.2.1.74) and β-glucosidases (EC 3.2.1.21) but they act in combination with hemicellulases and other enzymes (Maitan-Alfenas et al., 2015). Taking into account their substrate specificity, β-glucosidases (BGLs) have been classified into three groups: cellobiases (high specificity for cellobiose, a β−1,4-linked glucose dimer), aryl-β-glucosidases (high specificity for substrates such as p-nitrophenyl-β-D-glucopyranoside pNPG), or broad specificity BGLs, the last being the prevailing ones (Sørensen et al., 2013).

It is known that microbial cellulolytic enzymes are inhibited by cello-oligosaccharides, cellobiose or the final product of hydrolysis, glucose, and by other monosaccharides as well (Hsieh et al., 2014). β-glucosidases release glucose from the non-reducing terminus of

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cellobiose and cello-oligosaccharides (Sawant et al., 2016). Many commercial enzymes lack good BGL activity, so characterizing new enzymes is essential (Xia et al., 2016) and that is the reason for this study where the biochemical properties of an isolated BGL from *Myceliophthora thermophila* M.7.7 were analyzed and the inhibitory mechanisms of glucose and other putative inhibitors were also investigated. Beta-glucosidases hydrolyze soluble cellodextrins and cellobiose to D-glucose, avoiding cellulase inhibition by cellobiose (Karnaouri et al., 2013).

2. Materials and methods

2.1. The microorganism

The thermophilic fungus *Myceliophthora thermophila* M.7.7 was isolated from a compost pile of sugarcane bagasse in a previous work (Moretti et al., 2012) and is part of the working fungal collection at the Laboratory of Biochemistry and Applied Microbiology, IBILCE/UNESP, São José do Rio Preto, SP, Brazil.

2.2. Solid state fermentation and enzyme obtainment

To obtain the inoculum, the fungus was incubated at 45 °C for 72 h in 250 mL Erlenmeyer flasks containing 50 mL of a slanted PDA (potato-dextrose-agar) medium. After this period the spores suspension was done with addition of sterile 100 mL of nutrient solution composed of: (g L^{-1}) KH_2PO_4, 3.0; $MgSO_4$ $7H_2O$ 0.5; $CaCl_2$ 0.5, Tween 80 (1.0% v/v), and (1%), yeast extract as a nitrogen source, and pH was adjusted to 5.0.

A sterile mixture of sugarcane bagasse and wheat bran (1:1 m/m) was used for solid state fementation (SSF). The choice of these substrates was based on previous results (Moretti et al., 2012) in which higher β-glucosidase activity was observed. Polypropylene bags were used containing 5 g of a substrate (2.5 g wheat bran plus 2.5 g of bagasse) and 20 mL of a nutrient solution containing spores (10^7 spores g^{-1}), resulting in a final moisture content of 80%. This material was incubated at 45 °C and, after 96 h, 50 mL of distilled water (1:10 m/v) was added to the fermented material. The mixture was stirred for 30 min in a shaker at 100 rpm, filtered and centrifuged at 10,000 × *g* for 15 min at 10 °C for clarification. The supernatant was used as a crude enzyme solution.

Total protein was quantified by a classic method (Lowry et al., 1951), using a standard curve of bovine serum albumin (Sigma-Aldrich, St. Louis, USA).

2.3. Enzyme assay

For the β-glucosidase activity assay using a chromogenic substrate, 50 mL of enzyme solution were added to a mixture of 250 mL of sodium acetate buffer (0.1 mol L^{-1} pH 5.0) and 250 mL of 4 mmol L^{-1} pNPG (Sigma-Aldrich, St. Louis, USA). The reaction was maintained at 60 °C for 5 min and halted with 2 mL of a 2 mol L^{-1} solution of Na_2CO_3. The released nitrophenol was quantified by absorbance readings at 410 nm using a standard curve.

To determine the β-glucosidase activity using cellobiose as a substrate, a sequence of two reactions was performed. In the first reaction, 20 mL of enzyme solution were added to 10 mL of 4 mmol L^{-1} cellobiose in a 0.1 mol L^{-1} sodium acetate buffer (pH 5.0). The reaction was maintained at 50 °C for 20 min and stopped by immersing the tubes in boiling water for 2 min. Then the concentration of glucose released by the enzyme was determined by a glucose kit (Katal, Belo Horizonte, Brazil), adding 1 mL of the kit solution to 10 mL of the mixture from reaction 1, maintaining at 37 °C for 15 min. The absorbance readings were performed at 505 nm and the amount of released glucose was determined from a standard curve.

In order to determine the optimum incubation time of the enzymatic assays, enzymatic activities were measured according to the methods described above for pNPG and for cellobiose by only changing the reaction time (2–20 min) for each substrate. The maximum incubation time adopted was that which ensured the linear release of the product in relation to the time. The initial reaction rate V_o, was calculated as μmol min^{-1} (John, 2002).

2.4. Separation and purification of the enzyme isoforms

The crude enzyme solution, maintaining its high activity prior to gel filtration chromatography was concentrated using salting-out precipitation with 70% ammonium sulfate. The solution was stirred for 30 min at room temperature and subsequently centrifuged at 3000 × *g* for 40 min at 4 °C. The precipitate was re-suspended and filtered using a 0.45 μm membrane and subsequently subjected to gel filtration chromatography using a 16/100 column filled with Sephacryl S-100 h (GE HealthCare, Amersham, UK) attached to a GE Äkta Purifier 10 FPLC System (GE HealthCare, Amersham, UK) equilibrated with 20 mmol L^{-1} acetate buffer pH 5.0 containing 0.3 mol L^{-1} NaCl. The linear flow was 6 cm h^{-1}, and 1 mL fractions were collected as the absorbance at 280 nm started to increase. The separation of the isoforms BGL200 and BGL50 was checked by zymography using esculin.

The BGL50 isoform was further purified by applying 10 mL of the pooled fractions obtained after gel filtration in an XK16 ion-exchange chromatography column packed with a Q-Sepharose resin and connected to the Äkta equipment described above. Initially, the proteins were eluted with Tris buffer pH 7.5 (20 mmol L^{-1}) at the same linear flow as before (6 cm h^{-1}). After washing with 5 column volumes, a linear salt gradient was started using a similar buffer but also containing 0.5 mol L^{-1} NaCl, collecting 1 mL per tube. Beta-glucosidase activity was determined using pNPG.

2.5. Enzyme characterization

The protein profile and molecular mass estimation was assessed by SDS-PAGE (See et al., 1990) and zymograms using esculin (Kwon et al., 1994). For the estimation of the molecular mass '*m*' of the isoforms (expressed in kDa) under denaturing conditions, a polyacrylamide gel electrophoresis was performed in the presence of sodium dodecyl sulfate (SDS-PAGE) using 10% running gel and 5% stacking gel. The gel was divided into two parts, one named "A" stained with Coomassie Blue and the other labeled "B" stained by the esculin/ferric chloride method after being washed twice in 2.5% Triton X-100. The estimation of the molecular mass of the isoforms was done by comparing the zymogram bands with the migration of standard globular proteins and the logarithm of their *m*.

With the purpose of assessing the effect of pH and temperature on the activity and stability of BGL50, the enzyme characterization tests were performed using the same experimental conditions for both substrates, only varying the final enzymatic assay used for each one.

The behavior of the enzyme activity as a function of pH was studied by incubating the enzyme and substrate in several suitable buffers: citrate (pH 3.0), acetate (pH 3.5–5.5), MES (pH 5.5–6.5), HEPES (pH 7.0–8.0) and glycine (pH 8.5–10.5), and measuring the activity at 60 °C.

The effect of temperature on the enzymatic activity was evaluated by incubating the reaction mixture at temperatures in the range from 30° to 80°C, and the activity was measured at the optimum pH.

The thermostability of the enzyme in the absence of substrate was evaluated maintaining the enzyme solution for one hour at temperatures from 30° to 80°C. After this period, samples were taken for an enzymatic activity assay performed under optimum pH and temperature conditions. The effect of pH on enzyme stability was achieved by keeping the enzyme solution for 24 h at 25 °C in buffers between pH 3 and 11, subsequently assaying the enzymatic activity under optimum pH and temperature conditions.

The effect of temperature on the conservation of the enzyme was

also evaluated at temperatures of −80, −10, 10 and 25 °C. Enzyme assays were done as described after 6 h, 1 day, 1 and 2 months.

The influence of cations and reagents in the enzyme assay on BGL50 was evaluated by measuring the enzyme activity in the presence of KCl, $MgCl_2$, NaCl, $MnCl_2$, $FeCl_3$, $CuCl_2$, $CoCl_2$, NiCl, $AlCl_3$, $BaCl_2$, $ZnCl_2$, $LiCl_2$, PVA, DMSO, Triton-X-100, Isopropanol, PEG, Ethanol, Acetone, EDTA, DTT, SDS and glucose in a final concentration of 2.5 mmol L^{-1}. The results were compared with those of the control sample not exposed to those chemicals (with a reference of 100%) using Student's *t*-test for two independent samples (Zar, 2010), adopting as significant values of $p < .05$, performed by QtiPlot software version 0.9.9.11 (© Ion Vasilief 2004–2017).

The influence of phenolic compounds representative of those that can be released during the pretreatment of lignocellulosic biomass was evaluated for both substrates by the measurement of enzymatic activity in the presence of tannic acid, p-coumaric acid, syringic acid, gallic acid, ferulic acid, 4-hydroxybenzoic acid, vanillin, vanillic acid and syringaldehyde. The phenolics final concentration was 2.5 mmol L^{-1} in the reaction mixture using cellobiose as substrate. However, when used pNPG, due to the coloration of the tannic acid, ferulic acid and syringaldehyde reagents, their concentrations were changed to 0.062, 0.5 and 0.25 mmol L^{-1} respectively, while keeping the other phenolics at 2.5 mmol L^{-1}. The effects were measured by combining the phenols with the enzyme and the substrate immediately at the beginning of the assay. The results were compared with those of the control sample (reference of 100% using Student's *t*-test, adopting as significant values $p < .05$). The tests were performed using QtiPlot.

The kinetic parameters of BGL50 were determined by varying the concentration of the substrate pNPG (0.2–20 mmol L^{-1}) in the absence and presence of glucose (8 mmol L^{-1}) with a 5-min incubation. The values of the parameters K_m and V_{max} were obtained by nonlinear regression performed by QtiPlot using the Michaelis-Menten equation, allowing the identification of competitive inhibition. The value of the dissociation constant for the enzyme-inhibitor complex (K_i) for glucose was calculated from the equation $K_m^{app} = K_m (1 + [I]/K_i)$, where K_m^{app} is the apparent K_m for competitive inhibition and [I] is the molar concentration of the inhibitor (Wilson and Walker, 2010).

The kinetic parameters using the natural substrate (cellobiose) were determined by varying the concentration of cellobiose (0.5–80 mmol L^{-1}) with a 3-min incubation and performing the enzyme assay. The values of K_m and V_{max} were calculated as previously described.

The thermodynamic analysis of BGL50 thermal denaturation was done using pNPG. The calculation of the activation energy E_a, the temperature coefficient Q_{10}, half-life $T_{1/2}$ parameters of the enzyme, as well as those related to the thermal denaturation (the activation energy $E_{a(D)}$, $\Delta H_{(D)}$, $\Delta G_{(D)}$ and $\Delta S_{(D)}$) followed the method proposed in the literature (Saqib et al., 2012, 2010) and done in a previous study (Trindade et al., 2016). It is assumed that the irreversible denatured “I” state is evaluated using $N \leftrightarrow D \rightarrow I$, where "N" represents the native conformation and "D" the reversible denatured conformation.

3. Results and discussion

Two liters of crude enzyme extract were obtained after solid-state cultivation. The protein concentration in the sample determined by the Lowry assay was 2.8 mg mL^{-1} and β-glucosidase activity 17 U mL^{-1}, a total of approximately 34,000 U, with a specific activity of 6 U mg^{-1}. A zymogram showed distinct bands displaying β-glucosidase activity (Fig. 1) corresponding to two isoforms named according to their apparent molecular mass as BGL200 and BGL50 (near 200 and 50 kDa respectively, as estimated from a plot of migration vs. the logarithm of the *m* of the protein markers). Several filamentous fungi have the property of expressing different β-glucosidase isoforms, depending on the culture conditions or carbon sources (Singhania et al., 2011). The regulation mechanism of the expression of multiple cellulase isoforms is not yet fully elucidated and requires further research on the sequences and different expressions of the isoforms (Badhan et al., 2007). Induction of different isoforms may be related to the metabolites present in the culture media and understanding this regulation would be important in designing culture conditions for the desired overincreased production of isoforms or secondary metabolites. The type of submerged or solid state cultivation also influences the expression of the distinct isoforms (Gomes et al., 2009; Nazir et al., 2010; Silva et al., 2007; Willick and Seligy, 1985).

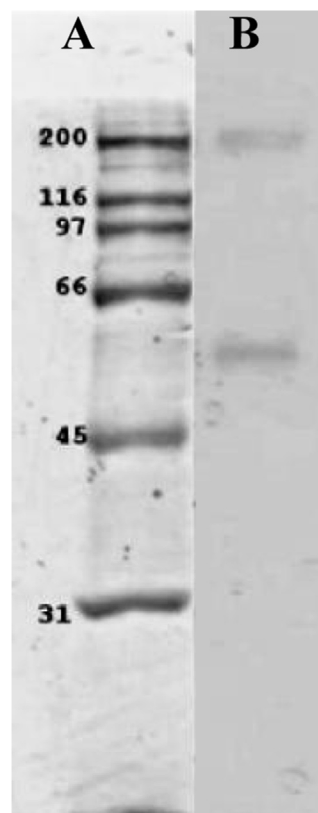

Fig. 1. 10% polyacrylamide gel under denaturing conditions. (A) part of the gel stained for proteins with Coomassie Blue showing the molecular mass protein standards and (B) another portion of the same gel stained with the esculin / ferric chloride method (zymogram) showing two bands with β-glucosidase activity for a sample of crude extract of *M. thermophila* M.7.7.

Only the lower molecular weight β-glucosidase (BGL50) was partially purified and characterized since the enzyme similar to isoform BGL200 has already been studied (Brognaro, 2014).

A salting out precipitation of the crude extract using ammonium sulfate concentrated the enzymes to 72.7 U mL^{-1} for gel filtration chromatography. The separation of β-glucosidase isoforms (Fig. 2) was compatible with a profile for monomeric forms with the previously estimated values of 50 and 200 kDa and was verified by a 10% polyacrylamide gel zymogram using esculin as substrate and ferric chloride (Fig. 3).

For the characterization of BGL50, fractions 35–49 were pooled, since BGL200 was not detected in those samples. The gel filtration was performed several times in order to obtain sufficient activity, always checking the purity by zymography, reaching a purification factor of 11.2 but a low yield of 25.5%, which can be explained in part because the other isoform (BGL200) was discarded and and this activity is compared to the initial activity of the crude extract containing both isoformsthe comparison considers the initial activity of the crude extract. Additional purification steps, namely ion-exchange and hydrophobic interaction chromatography, were tried but the enzyme yield decreased substantially.

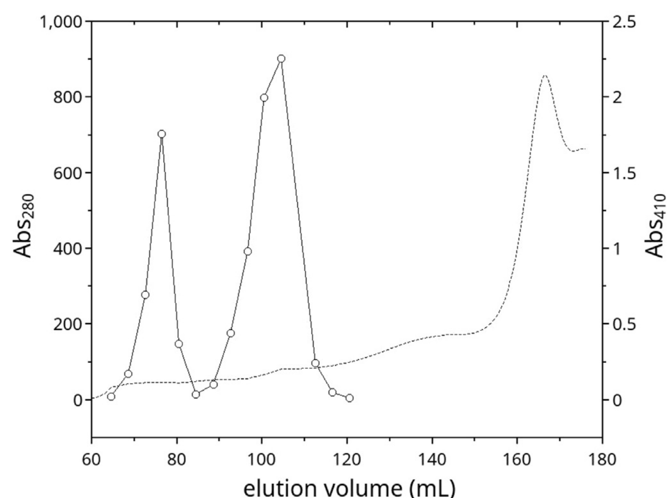

Fig. 2. Chromatographic profile of the enzyme solution produced by the fungus *M. thermophila* M.7.7 applied to a C16/100 column filled with Sephacryl S-100 h. Protein absorbance values at 280 nm are shown as a dashed line and enzymatic activity of β-glucosidase as a continuous line, expressed as the absorbance at 410 nm of the product p-nitrophenol.

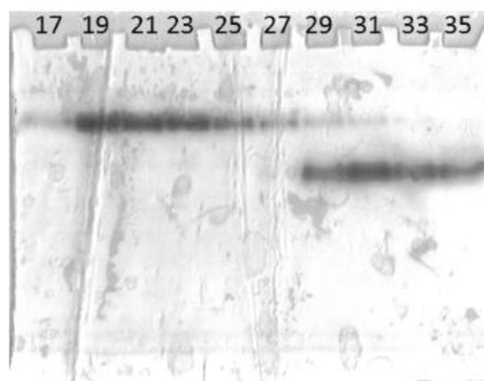

Fig. 3. Zymogram of the partially purified β-glucosidase isoforms by gel filtration on Sephacryl S-100. The numbers represent the fraction numbers, and the figure does not show the fractions beyond tube 35, which contained only the BGL50 isoform.

For the enzyme assays, the release of the product was linear up to five minutes for pNPG and three minutes for cellobiose, these being adopted as the incubation times. All the assays were performed in triplicate.

Regarding the effect of temperature, most β-glucosidases from mesophilic fungi exhibit optimum temperatures for activity between 40 and 50 °C (Bhatia et al., 2002). The enzymatic activity profile for BGL50 when varying the assay temperature resulted in optimum temperatures of 60 and 50 °C using pNPG and cellobiose, respectively (Fig. 4A), that can be explained by the different incubation times, being shorter for pNPG. As for thermostability, BGL50 remained stable up to one hour of incubation at 60 °C in the absence of substrates (Fig. 4B). The BGL200 isoform studied by Brognaro (2014) presented maximal activity at 65 °C on pNPG.

The high rigidity of thermophilic enzymes may result from the interplay of various putative factors: hydrogen-bonds, hydrophobic interactions, internal packing, salt-bridges and secondary structural features (Shiraki et al., 2001). So, the determination of the definitive factor for this property depends on molecular and structural studies.

With regard to the pH effect, the literature reports that fungal β-glucosidases exhibit optimum pH values between 4.0 and 6.0 (Bhatia et al., 2002). The optimal pH of BGL50 was 4.5 for cellobiose and pH 5.0 for pNPG (Fig. 5A). For comparison, BGL200 presented optimum pH of 4.5 using pNPG (Brognaro, 2014).

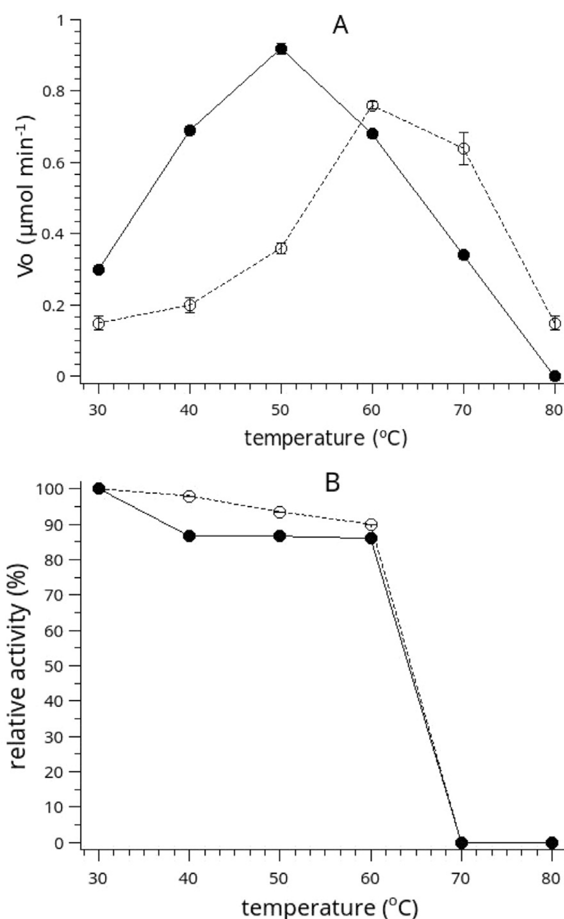

Fig. 4. Effect of temperature on BGL50 using cellobiose (filled circles) and pNPG (open circles) as substrate: (A) Variation of BGL50 activity at different temperatures; (B) Residual activity of BGL50 when incubated at different temperatures for 1 h in the absence of substrate. The graphs represent the means (symbols) and standard deviations (bars).

Using cellobiose, the activity decreases more significantly around the maximum value when compared to the curve produced using pNPG, probably due to various residues side chains that interact differently with each substrate, as verified in a study of a bacterial β-glucosidase (Rajoka et al., 2015). Those authors demonstrated the different interactions of a β-glucosidase (BGLA) from the bacteria *Thermotoga maritima* with cellobiose and pNPG. In the BGLA-cellobiose complex, three hydrogen-bond interactions were obtained involving residues Asn223, Ser229, and His298 while the BGLA-pNPG interacts with active site residues: Glu166, Tyr295, and Asn223. Obviously, the comparison of enzymes from Fungi and Archaea may not be the best approach due to the phylogenetic distance and primary sequence differences but a similar explanation could be considered.

In terms of the effect of pH on the BGL50 enzyme, it exhibited the highest activity around pH 4.0–4.5 and decreased below 50% only at pH values above 9.5 (Fig. 5B).

When the effect of ions and other chemicals on the enzymatic activity was tested, no ion caused any significant increase in enzymatic activity and it was completely inhibited by Cu^{2+} and Fe^{2+} ions when using either substrate (Table 1). Different effects on beta-glucosidases have been reported for those metals, mostly decreasing the enzyme activity by several degrees (Pereira et al., 2017) supposing that at least part of the iron oxidizes to Fe^{3+}, a favorable reaction. Heavy metals, such as Cu^{2+}, exhibit high affinity for thiol groups; usually, these heavy metal ions oxidize the functional groups of the cysteine residues and may inhibit the enzymatic activity of certain proteins (Hayashi et al., 1999). Another possibility could be the redox effect of copper and iron

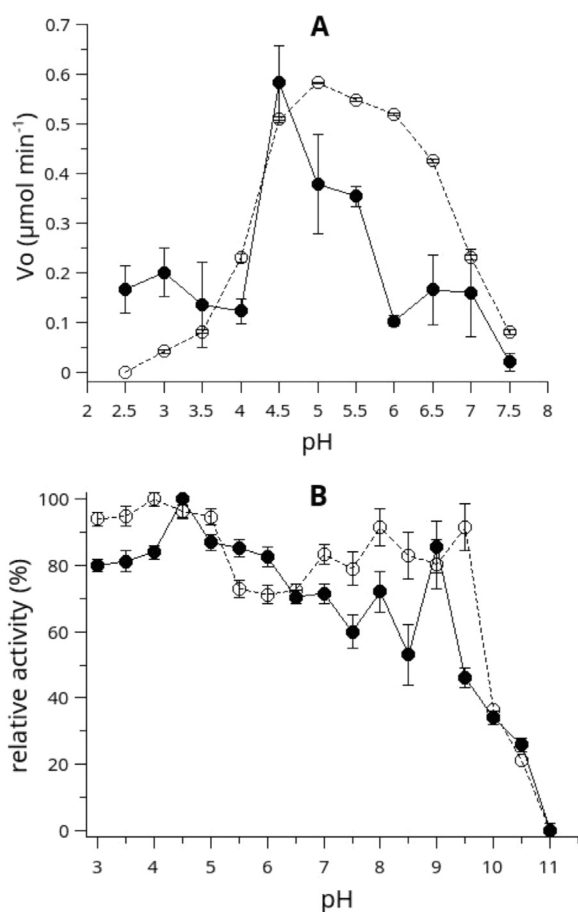

Fig. 5. Effect of pH on BGL50 using cellobiose (filled circles) and pNPG (open circles) as substrate: (A) Variation of BGL50 activity at different pH values; (B) Residual activity of BGL50 when incubated at different pHs for 24 h at room temperature in the absence of substrate. The graphs represent the means (symbols) and standard deviations (bars).

Table 1
Effect of ions in a final concentration of 2.5 mmol L^{-1} on the enzymatic activity of BGL50.

Residual activity % (mean ± standard deviation)				
	pNPG	± SD	Cellobiose	± SD
Controle	100.0	13.3	100.0	0.3
NaCl	70.6*	6.7	57.1*	8.4
$MnCl_2$	49.6*	9.9	67.1*	8.8
KCl	76.2*	5.0	81.9	15.6
$FeCl_3$	0.0*	0.0	0.0*	0.0
$BaCl_2$	105.4	6.9	76.7	11.1
$MgCl_2$	100.0	7.8	62.7*	9.1
$AlCl_3$	83.5	6.9	43.7*	7.2
$ZnCl_2$	63.0	10.6	ND*	0.0
$CuCl_2$	ND*	0.0	ND*	0.0
LiCl	102.6	2.7	128.4	37.3

* Significant differences ($p < .05$) in Student's *t*-test. ND: not detected.

oxidizing the hemiacetal ends and leading to inhibition, as pointed out for cellulases (Tejirian and Xu, 2010).

The Zn^{2+} ion and dithiothreitol (DTT) showed large differences in enzyme behavior when assayed on both substrates. Using cellobiose a total inhibition of the enzyme was observed, whereas, when using pNPG, the average inhibitions were 37% and 17.5%, respectively (Tables 1 and 2). The effects of zinc and DTT in the cellobiose assay are worth noting because they cause interference in the assay itself.

The independence of cofactors and a wide tolerance to the presence of ions observed for *M. thermophila* BGL50 show a similarity to a ß-glucosidase produced by the fungus *Aureobasidium pullulans* (Saha et al., 1994).

Table 2
Effect of reagents in a final concentration of 2.5 mmol L^{-1} on the enzymatic activity of BGL50.

Residual activity % (mean ± standard deviation)				
	pNPG	± SD	Cellobiose	± SD
Control	100.0	13.3	100.0	0.3
DMSO	92.9	5.6	57.0*	1.3
PVA	71.2	3.0	56.8	19.0
Triton	45.0	10.6	57.5*	8.9
Isopropanol	70.1	7.9	66.5	14.9
PEG 8000	67.6	6.2	58.5*	8.3
Ethanol	70.6	15.9	55.6*	5.7
Acetone	76.5	8.9	52.8*	1.3
EDTA	68.3	17.0	62.0*	8.5
DTT	82.5	6.9	ND*	0.0
SDS	82.4	9.2	60.6	27.1

* Significant differences ($p < .05$) in Student's *t*-test. ND: not detected.

Although none of the tested cations significantly increased BGL50 activity (Table 1), the decrease in activity induced by ethylenediaminetetraacetic acid (EDTA) (Table 2) suggests the need to look for some divalent metal ion not tested here.

Thermal and chemical pretreatments of sugar cane bagasse release a series of compounds that can act as potential inhibitors of both the enzymatic hydrolysis and subsequent fermentation. The types of toxic compounds and their concentrations in lignocellulosic hydrolysates depend on both the raw material and the pretreatment operating conditions. One class of degradation products, which are potential fermentation inhibitors are phenolic derivatives (Palmqvist and Hahn-Hägerdal, 2000). Among the phenols released in the hydrolysis of lignocellulosic material, vanillin, syringaldehyde and hydroxybenzoic acid significantly inhibit cellulases, especially β-glucosidases (Ximenes et al., 2010).

The results of the present study (Table 3) show a wide resistance of BGL50 to several phenolic compounds in a short preincubation time, showing that the enzyme has a potential application in the hydrolysis of lignocellulosic materials that release these phenolic derivatives. Most of the activities were higher with cellobiose than when tested using pNPG and, due to the high standard deviation, the observed average increase or decrease of the activity in the presence of phenolics was not significant, the only exceptions being gallic acid using cellobiose and tannic acid using pNPG. Nevertheless, the effects of phenols on β-glucosidases present in commercial enzymes from *Trichoderma reesei* (Spezyme CP®) and *Aspergillus niger* (Novozyme 188®) showed a significant inhibition by tannic acid that was evident in short or especially after long (24 h) preincubation times, while other phenols induced different degrees of inhibition (Ximenes et al., 2011), exerting higher inhibition when the enzymes were exposed for longer periods.

The kinetic parameters, maximum reaction rate (V_{max}) and Michaelis constant (K_m), were calculated by nonlinear regression, a more adequate method than the popular Lineweaver-Burk linearization (Helfgott and Seier, 2007; Mason and Lai, 2000); the latter assumes linearity, constant variance and errors with normal distribution, which often do not occur or the operator is unaware of the validity of these assumptions (Helfgott and Moore, 2011).

V_{max} and K_m values for BGL50 using pNPG as substrate (Fig. 6) for the reaction without glucose were 0.42 ± 0.01 µmol min^{-1} and 0.52 ± 0.07 mmol L^{-1} respectively. In the presence of the inhibitor, the values were 0.45 ± 0.02 µmol min^{-1} and 3.27 ± 0.49 mmol L^{-1}, confirming, within the margin of error, that the kinetic parameter affected by the presence of glucose is the K_m constant, a typical competitive inhibition when the ligand has a chemical structure similar to the substrate, being able to bind at the active site but not in a productive

Table 3
Effect of phenolic compounds on the enzymatic activity of BGL50 with pNPG and cellobiose substrates, measured as residual activity (%). The values represent the arithmetic mean for each substrate ± standard deviation of triplicates.

	mmol L^{-1}	pNPG	± SD	mmol L^{-1}	cellobiose	± SD
Control	2.5	100.0	13.7	2.5	100.0	8.1
4-hydroxybenzoic acid	2.5	97.5	6.0	2.5	109.7	1.4
ferulic acid	0.5	97.4	3.1	2.5	99.1	4.2
gallic acid	2.5	91.1	14.4	2.5	75.3*	2.3
p-coumaric acid	2.5	70.5	17.4	2.5	118.0	0.9
syringaldehyde	0.25	92.8	36.9	2.5	96.4	2.3
syringic acid	2.5	95.2	18.9	2.5	111.4	2.3
tannic acid	0.0625	ND*	ND*	2.5	99.5	12.3
vanillic acid	2.5	87.6	0.1	2.5	125.1	4.7
vanillin	2.5	94.0	1.3	2.5	118.0	4.7

* Significant differences ($p < .05$) in Student's *t*-test ND: not detected.

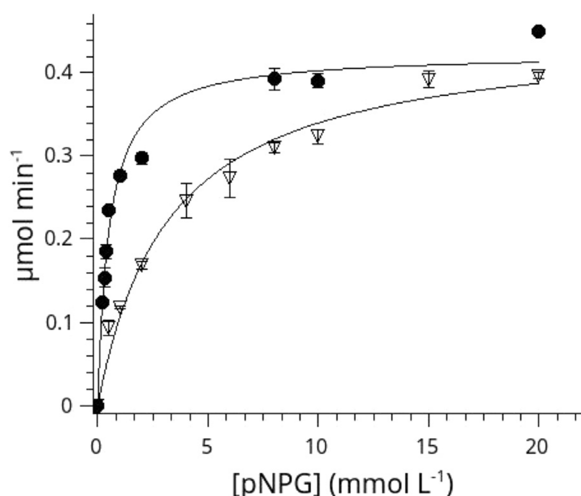

Fig. 6. Michaelis-Menten curve for BGL50 using pNPG as substrate in the presence (empty triangles) and absence (filled circles) of glucose (8 mmol L^{-1}). The graphs represent the means (symbols) and standard deviations (bars).

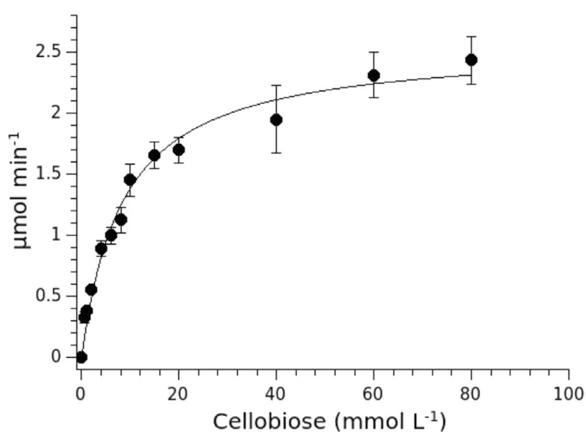

Fig. 7. Michaelis-Menten curve for BGL50 using cellobiose as substrate. The graph represents the means (symbols) and standard deviations (bars).

way. The rate K_m/K_m^{app} using pNPG was 0.16, lower than the value of 0.21 reported for a commercial preparation (Novozymes SP188) from *A. niger* (Chauve et al., 2010), suggesting a stronger inhibition for BGL50.

From these data it was also possible to estimate the inhibition constant K_i = 1.5 mmol L^{-1}; accordingly, glucose has a strong competitive inhibition effect on *M. thermophila* BGL50. For the yeast *Clavispora* NRRL Y-50464, K_i values were approximately 38 and 62 mmol L^{-1} for two isoforms (Wang et al., 2016), but K_i values for β-glucosidase inhibition from other fungi can reach more than 100 mmol L^{-1} (Riou et al., 1998). For comparison, kinetic values of an *M. thermophila* β-glucosidase, expressed when the BGL3a gene was cloned in *Pichia pastoris* (Karnaouri et al., 2013) were K_m = 0.39 ± 0.12 mmol L^{-1} (close to our estimated value) but K_i was 0.28 mmol L^{-1}, lower than for BGL50. Since V_{max} depends on enzyme concentration E_t ($V_{max} = k_{cat}.[E_t]$), usually it is not a reliable parameter for comparing to values in the literature.

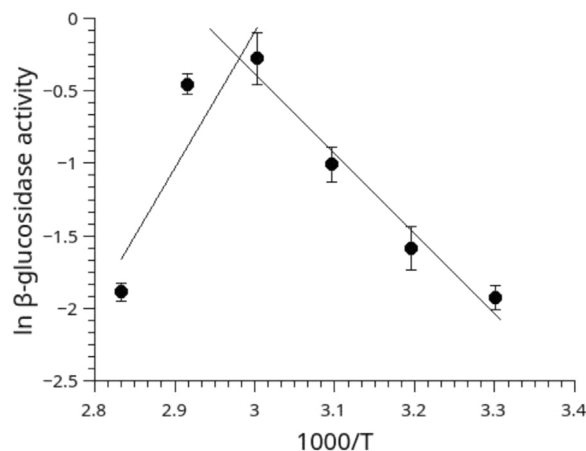

Fig. 8. Arrhenius plot for the calculation of the activation energy (Ea) and optimum temperature of the BGL50. The graph represents the means (symbols) and standard deviations (bars).

Table 4
Temperature coefficient (Q10) estimate based on the Arrhenius Graph.

Temp (°C)	Temp (K)	Q_{10}
30	303	1.83
40	313	1.76
50	323	1.70
60	333	1.65
70	343	1.60

The values of V_{max} and K_m obtained for BGL50 using cellobiose as substrate (Fig. 7) were2.55 ± 0.09 µmol min^{-1} and 8.50 ± 1.03 mmol L^{-1}, respectively. The kinetic values obtained show that BGL50 has a much lower K_m for the synthetic substrate than for the natural one, suggesting that BGL50 is an aryl-β-glucosidase. In most cases, β-glucosidases show high catalytic activity and high affinity with the artificial substrate pNPG and MUG (methyl umbelliferyl β-D-glucoside), compared to cellobiose (Nam et al., 2010). According to those authors, the kinetics of β-glucosidase depends on the configuration of its substrate and cellobiose requires a conformational change for the catalysis. The enzyme has a very rigid structure at the S_1 subsite that

Table 5
Kinetic and thermodynamic parameters of irreversible thermoinactivation.

Temperature (°C)	Temperature (K)	k_d (min^{-1})	t ½ (min)	ΔH_d ($kJ\ mol^{-1}$)	ΔG_d ($kJ\ mol^{-1}$)	ΔS_d ($J\ mol.\ K^{-1}$)
60	328	0.00081	855.6	2.154	101.58	−311.5
63	333	0.01417	48.9	2.179	94.52	−287.8
65	338	0.01897	36.5	2.196	94.28	−285.4

will accommodate glucose from cellobiose but a second glucose will alter the rotation conformation to fit into the substrate binding site. This could be the reason behind the lower efficiency of β-glucosidases with cellobiose than with the synthetic substrate pNPG (Singhania et al., 2013). The released glucose, in turn, may also cause enzyme inhibition.

Analysis of irreversible thermal denaturation shows the optimum temperature as 62.6 °C and the activation energy (E_a) of BGL50 as 46.2 $kJ\ mol^{-1}$ from the Arrhenius curve shown in Fig. 8. The activation energy of BGL50 was three times higher than that reported for *A. fumigatus* β-glucosidase (14.8 $kJ\ mol^{-1}$) (Das et al., 2015) but lower than for *Fusarium solani* β-glucosidase (53.3 $kJ\ mol^{-1}$) (Bhatti et al., 2013). The temperature coefficient Q_{10}, which represents the increase in the reaction rate every 10 °C of temperature rise, was also estimated at different temperatures (Table 4). The Q_{10} values show a decrease as the temperature increases as a result of thermal denaturation. The best parameter to indicate the energy required to change enzyme conformation is the activation energy for denaturation $E_{a(d)}$, representing the energy barrier required to bring the enzyme from a native state to the denatured state (Saqib et al., 2010), and its value for BGL50 was calculated as 614.7 $kJ\ mol^{-1}$. Comparing to the β-glucosidases of the fungus *A. fumigatus* (48.80 $kJ\ mol^{-1}$) (Das et al., 2015), *Thermoascus aurantiacus* (414 $kJ\ mol^{-1}$) and *A. pullulans* (537 $kJ\ mol^{-1}$) (Leite et al., 2007) BGL50 has higher conformational stability.

The half-life of BGL50 at 60 °C is quite high compared to the other temperatures tested, representing a high structural stability when maintained at that temperature, but undergoes a significant denaturation before reaching 65 °C. Compared to the results found in the literature for other fungal enzymes, the half-life of BGL50 is much lower; for the β-glucosidase of the fungus *F. solani* at 65 °C the half-life is 159 min (Bhatti et al., 2013), for the β-glucosidase from *A. pullulans* $t_{1/2}$ is 90 min at 80 °C and for the enzyme from *Thermoascus aurantiacus* 30 min at 80 °C (Leite et al., 2007). Nevertheless, the experiments performed for up to 2 months for samples kept at −80, −10, 10 and 25 °C showed no significant decrease of the activity (results not shown).

The enthalpy variation (ΔH_d) indicates the amount of non-covalent bonds that are broken during the protein denaturation process. For BGL50, the values are lower than those reported for a beta-glucosidase from *F. solani* (50 $kJ\ mol^{-1}$) (Bhatti et al., 2013), *A. pullulans* (534 $kJ\ mol^{-1}$) and *T. aurantiacus* (411 $kJ\ mol^{-1}$) (Leite et al., 2007).

The value of ΔG_d is proportional to protein stability (Longo and Combes, 1999), and this will depend on the temperature. Therefore, BGL50 shows higher stability at 60 °C, as shown in Table 5. The results indicate that at 65 °C much of the structural organization has already been destroyed. In fact, the irreversible transition between the native and denatured state occurred in a narrow range of temperature, between 60 and 65 °C.

The variation of the denaturation entropy at 65 °C was −285.4 $J\ mol^{-1}\ K^{-1}$, lower than the result for *F. solani* β-glucosidase, in the range of −176 $J\ mol^{-1}\ K^{-1}$ (Bhatti et al., 2013). Low ΔS_d values suggest the exposure of nonpolar side chains, causing ordering of water molecules in the form of clathrates or "cages" (Siddiqui et al., 1997).

The thermodynamic parameters for the β-glucosidase produced by the mesophilic fungus *A. pullulans* (Leite et al., 2007) evaluated at 80 °C showed that the enzyme is more resistant to thermal inactivation than BGL50. Therefore, the complex understanding of enzymatic thermostability is not only associated with the thermophilicity of the organism.

4. Conclusion

The lower molecular mass beta-glucosidase from *M. thermophila* (around 50 kDa) was easily purified and the results suggest that it is a catalytically efficient enzyme for cellobiose hydrolysis. The differences in data about enzyme activity between the synthetic (p-PNPG) and the natural substrate (cellobiose) under different reaction conditions shows some important findings since they can be used to define the saccharification procedures. The kinetic and thermodynamic properties and its behavior with phenolic compounds properties suggest that the enzyme would be efficient for industrial purposes.

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