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ORIGINAL ARTICLE

Catalytic and thermodynamic properties of an acidic α-amylase produced by the fungus *Paecilomyces variotii* ATHUM 8891

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Abstract

An extracellular acid stable α-amylase from *Paecilomyces variotii* ATHUM 8891 (PV8891 α-amylase) was purified to homogeneity applying ammonium sulfate fractionation, ion exchange and gel filtration chromatography and exhibited a reduced molecular weight of 75 kDa. The purified enzyme was optimally active at pH 5.0 and 60 °C and stable in acidic pH (3.0–6.0). K_m, v_{max} and k_{cat} for starch hydrolysis were found 1.1 g L^{-1}, 58.5 μmole min^{-1} (mg protein)$^{-1}$, and 73.1 s^{-1}, respectively. Amylase activity was marginally enhanced by Ca^{2+} and Fe^{2+} ions while Cu^{2+} ions strongly inhibited it. Thermodynamic parameters determined for starch hydrolysis (E_α, ΔH^*, ΔG^*, ΔS^*, ΔG^*_{E-S} and ΔG^*_{E-T}) suggests an effective capacity of PV8891 α-amylase towards starch hydrolysis. Thermal stability of PV8891 α-amylase was assessed at different temperatures (30–80 °C). Thermodynamic parameters ($E_{(a)d}$, ΔH^*, ΔG^*, ΔS^*) as well as the integral activity of a continuous system for starch hydrolysis by the PV8891 α-amylase revealed satisfactory thermostability up to 60 °C. The acidic nature and its satisfactory performance at temperatures lower than the industrially used amylases may represent potential applications of PV8891 α-amylase in starch processing industry.

Keywords Acidic α-amylase · *Paecilomyces variotii* · Kinetic parameters · Thermodynamic parameters

Introduction

Starch is, after cellulose, the most abundant heterogeneous polysaccharide produced by plants in the form of water-insoluble granules and is the primary source of energy for most organisms on Earth. It is composed of amylose and amylopectin, exclusively composed of D-glucose with α-(1→4) linkages in a linear amylose and α-(1→4) linkages and ~5% α-(1→6) branch linkages in amylopectin (Robyt 2008). Amylases are the enzymes that hydrolyze starch and based on their mode of action are classified as endoamylases, exoamylases, debranching enzymes and transferases (Xian et al. 2015). α-Amylase (endo (1→4)-α-D-glucan glucohydrolase: E.C.3.2.1.1) randomly cleaves the internal α-(1→4)-glycosidic linkages in starch resulting in low molecular weight products of α-configuration, which are further hydrolyzed to glucose by exo-acting amylases and other amylolytic enzymes (Wu et al. 2018). α-Amylases can be derived from plants, animals and a wide variety of microorganisms (Gupta et al. 2003). They are produced commercially in bulk from microorganisms (*Bacillus*, *Aspergillus* sp.) and represent about 30% of the world enzyme market (Wu et al. 2018). α-Amylases have the widest range of industrial applications, including starch saccharification, baking, brewing, textile desizing, bioethanol production, and laundry and dish-washing detergents (Gupta et al. 2003; Rana et al. 2013).

α-Amylases based on their optimal pH for activity, which ranges from 2 to 12, can be classified as acidic, neutral or alkaline, with the majority of them being active on neutral pH (Gupta et al. 2003; Sharma and Satyanarayana 2013).

Liquefaction and saccharification are the two main steps in starch processing. The pH range of native starch homogenate is 3.2–4.5. α-Amylases used in liquefaction step are

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active at near neutral pH, whereas the saccharification step is performed at pH 4.3–4.8. Therefore, acidic amylases are preferred for the liquefaction and saccharification steps of starch processing since they do not require the pH adjustment step which is time consuming and increases the cost of the products and limiting the formation of by-products (Goyal et al. 2005; Sharma and Satyanarayana 2013).

The activity and stability of enzymes, determined by their catalytic and thermodynamic properties, are parameters that affect the efficiency of an industrial bioprocess (da Silva et al. 2018). According to Gummadi (2003) thermodynamic studies can be used as a tool to predict how the protein molecule changes its conformation under various environmental conditions. Activation energy (E_a), thermal inactivation energy ($E_{(a)d}$) and the changes in Gibbs free energy (ΔG^*), enthalpy (ΔH^*) and entropy (ΔS^*) are the parameters used to describe thermodynamics of enzyme activity and stability (da Silva et al. 2018; Kikani and Singh 2012; Samanta et al. 2014; Shukla and Singh 2015; Karam et al. 2017; de Oliveira et al. 2018; Ademakinwa et al. 2019; Porto et al. 2006).

The present work focuses in the determination of the kinetic and thermodynamic properties of the activity and thermostability of an extracellular acidic α-amylase produced by an indigenous wild type *Paecilomyces variotii* ATHUM 8891 strain (designated as PV8891 α-amylase). These parameters are an excellent tool that will help to optimize the operating range of α-amylase under study and to fully exploit its possible industrial applications.

Materials and methods

Microorganism and culture conditions

An indigenous wild-type *Paecilomyces variotii* strain, isolated from decaying biomass, was used throughout the study. The strain was molecularly and phenotypically characterized and deposited in the ATHUM culture collection of fungi https://en.mycetotheca.biol.uoa.gr/ under the accession number ATHUM 8891 (Zerva et al. 2014). *P. variotii* ATHUM 8891 was maintained in Potato Dextrose Agar plates at 4 °C.

PV8891 α-amylase was produced under submerged fermentation conditions in 1 L Erlenmeyer flasks (400 mL working volume) containing the basal growth medium (BGM) described elsewhere (Zerva et al. 2014), using wheat bran (20 g L^{-1}) and NH_4NO_3 (6 g L^{-1}) as carbon and nitrogen sources, respectively. The pH of the medium was adjusted to 6.0. Flasks were sterilized by autoclaving for 20 min at 121 °C. Cultures were inoculated at a final concentration of 5×10^6 spores mL^{-1}, with a spore suspension prepared by adding 12 mL of a sterile 1‰ Tween 80 solution into a 5- to 7-day-old PDA Petri culture. Incubation was carried out at 30 °C and 180 rpm for 7 days.

Enzyme purification

At the end of fermentation, mycelium was separated from the culture broth by filtration (Whatman no. 4 filter paper). The filtrate was centrifuged at 7000×*g* for 15 min at 4 °C, to remove the suspended particles. The supernatant was used as the source of crude extracellular α-amylase.

The crude enzyme was concentrated with ammonium sulfate precipitation (90% saturation levels) prior to chromatographic separations. The precipitate was collected by centrifugation at 12,000×*g* (4 °C) for 20 min, dissolved in a minimum volume of 20 mM piperazine/HCl buffer pH 7.0 and dialyzed overnight against the same buffer.

The first chromatographic step involved anion exchange chromatography on a custom Q-Sepharose column (2.8 cm i.d., 20 cm length) previously equilibrated with 20 mM piperazine/HCl buffer pH 7.0. Following introduction of the crude enzyme sample, the column was washed with 150 mL of the equilibration buffer at a flow rate of 240 mL h^{-1}. A 300-mL linear NaCl gradient (0–1 M) in equilibration buffer was subsequently applied at the same flow rate. Fractions containing α-amylase activity were pooled, concentrated using an Amicon apparatus (Amicon chamber 8400 with membrane Diaflo PM10, exclusion size 10 kDa) and rebuffered into 100 mM phosphate buffer pH 7.0 plus 150 mM sodium chloride.

This sample was applied to a HiPrep 26/60 Sephacryl S-100 High-Resolution Column (GE Healthcare Life Sciences) equilibrated and eluted with the same buffer at a flow rate of 78 mL h^{-1}. Fractions with α-amylase activity were pooled and dialyzed overnight against 50 mM phosphate buffer pH 7.0.

Amylase activity and protein determination

Amylase activity was determined by measuring the reducing sugar production rate from starch using 3,5-dinitrosalicylic acid (DNS) (Miller 1959) as described elsewhere (Wu et al. 2018). Briefly, the reaction system consisted of 450 µL of 1.0% (*w*/*v*) starch in 50 mM citrate–phosphate buffer, pH 5.0, and 50 µL of enzyme sample. After 15 min-incubation at 60 °C, 500 µL of DNS solution was added and the mixture was boiled for 5 min. The release of reducing sugars was determined spectrophotometrically at 540 nm.

One unit of amylase activity was defined as the amount of enzyme that produced 1 µmol of reducing sugar per minute, determined as glucose equivalents.

For quantitative protein estimation the dye-binding procedure of Bradford (1976) was employed, using bovine serum albumin as standard. A_{280} was used to monitor protein in column effluents.

TLC analysis of starch hydrolysis products

The purified PV8891 α-amylase was incubated with 1% starch in 50 mM citrate–phosphate buffer, pH 5.0 at 60 °C. Samples were removed at different time intervals, and hydrolysis products were detected by thin-layer chromatography (silica gel 60 F_{254}, Merck), as described by Allala et al. (2019).

Determination of kinetics parameters

The kinetic constants (v_{max}, K_m, k_{cat}, and k_{cat}/K_m) were determined by incubating a fixed amount of purified PV8891 α-amylase with varied concentrations of starch at a substrate range from 0 to 20 g L^{-1} at 60 °C, pH 5.0. In all cases the amount of purified enzyme used was equal to 1.8 ng of protein. All experiments were performed in triplicate, and mean values obtained were fitted to a standard Michaelis–Menten model.

Effect of pH on enzyme activity and stability

The activity response of the purified α-amylase to pH was determined using the standard assay described above, with the appropriate buffers. The following buffer systems (100 mM each) were used: citrate–phosphate (pH 2.5–7.5) and Tris–HCl (pH 7.5–9.0). Stability was tested after incubation of the enzyme at the above buffers for 24 h at 4 °C and determination of the residual activity. In all cases the amount of purified enzyme used was equal to 1.8 ng of protein. All experiments were performed in triplicate.

Effect of metal ions and EDTA

The effect of various metal ions, namely Ca^{2+}, K^{+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Fe^{3+}, Fe^{2+}, Cu^{2+}, Co^{2+} and a chelator (EDTA) at concentrations 1 and 5 mM on amylase activity was studied. The purified amylase was pre-incubated with the metal ions and EDTA at 25 °C for 30 min (Wu et al. 2018), and then amylase activity was measured using the standard assay described above. In all cases the amount of purified enzyme used was equal to 1.8 ng of protein. All experiments were performed in triplicate. Enzyme activity in the absence of any metal ions or EDTA was considered to be 100%.

Optimum temperature, activation energy and temperature quotient (Q_{10})

Optimum temperature and activation energy (E_a) of α-amylase were determined using the standard assay described above, at temperatures ranging from 30 to 80 °C. In all cases the amount of purified enzyme used was equal to 1.8 ng of protein. All experiments were performed in triplicate. Activation energy (E_a) was calculated using the Arrhenius plot.

The effect of temperature on the reaction rate was expressed in terms of temperature quotient (Q_{10}). Q_{10} is the factor by which the reaction rate is increased by raising the temperature by 10 °C, and it was calculated by Eq. (1) (Dixon and Webb 1979):

$$Q_{10} = \text{antilog}_{\varepsilon}\left(\frac{E_a \cdot 10}{R \cdot T}\right), \tag{1}$$

where E_a is the activation energy (kJ mol^{-1}), T is the absolute temperature (K) and R is the universal gas constant (8.314 J mol^{-1} K^{-1}).

Thermodynamics of starch hydrolysis

The thermodynamic parameters for starch hydrolysis were calculated by rearranging the Eyring's absolute rate equation derived from the transition state theory (Eyring and Stearn 1939):

$$k_{cat} = \left(\frac{k \cdot T}{h}\right) \cdot e^{(-\Delta H^*/RT)} \cdot e^{(\Delta S^*/R)}, \tag{2}$$

where k_{cat} is the turnover number (s^{-1}), k is the Boltzmann's constant (1.38×10^{-23} J K^{-1}), T the absolute temperature (K), h is the Planck's constant (6.626×10^{-34} J s), ΔH^* is the enthalpy of activation (J mol^{-1}), R is the universal gas constant (8.314 J mol^{-1} K^{-1}) and ΔS^* is the entropy of activation (J mol^{-1} K^{-1}).

For ΔH^*, ΔG^* and ΔS^*, the following equations apply:

$$\Delta H^* = E_\alpha - R \cdot T, \tag{3}$$

$$\Delta G^* (\text{free energy of activation}) = -R \cdot T \cdot \ln\left(\frac{k_{cat} \cdot h}{k \cdot T}\right), \tag{4}$$

$$\Delta S^* = \frac{\Delta H^* - \Delta G^*}{T}, \tag{5}$$

The free energy of substrate binding and transition state formation was calculated using the following equations (Riaz et al. 2007):

$$\Delta G^*_{E-S} (\text{free energy of substrate binding}) = -R \cdot T \cdot \ln K_\alpha, \tag{6}$$

where $K_\alpha = \frac{1}{K_m}$

$$\Delta G^*_{E-T} (\text{free energy for transition state formation}) = -R \cdot T \cdot \ln\left(\frac{k_{cat}}{K_m}\right), \tag{7}$$

where K_m is the Michaelis–Menten constant.

Thermodynamics of enzyme stability

Isothermal deactivation treatment was performed at temperatures ranging from 30 to 80 °C at pH 5.0 (50 mM citrate–phosphate buffer). Aliquots were withdrawn at different time intervals, cooled immediately on ice and assayed for α-amylase activity under standard assay conditions. All experiments were performed in triplicate.

Thermal deactivation of enzymes can often be described by the first-order reaction can be described by the following equation (Eq. 8):

$$\frac{A_t}{A_0} = e^{-k_d \cdot t}, \tag{8}$$

where A_t/A_0 is the residual α-amylase activity at treatment time t (min), and k_d (min^{-1}) is the thermal deactivation constant at the specific temperature.

The deactivation rate constants (k_d values) can be estimated by non-linear regression analysis. Thermal deactivation energy ($E_{(a)d}$) was estimated by the slope of the straight line resulted from the Arrhenius plot, that is the plot of $\ln k_d$ vs 1/T.

Half-life ($t_{1/2}$) (min) value of inactivation, which is defined as the time needed to reduce to 50% the initial enzyme activity at a given temperature, is calculated using the following expression (Eq. 9):

$$t_{1/2} = \frac{ln(2)}{k_d}. \tag{9}$$

The D value is the time (min) needed to reduce the initial activity 90% and it can be calculated using the following equation (Eq. 10):

$$D = \frac{ln(10)}{k_d}. \tag{10}$$

The z value is the temperature interval to vary D value one log unit, and it was obtained by plotting the log(D values) as a function of the corresponding temperatures. The slope of the curve represents the $-\frac{1}{z}$ value (Gouzi et al. 2012).

Thermodynamics of α-amylase deactivation was determined by rearranging the Eyring's absolute rate equation derived from the transition state theory (Eyring and Stearn 1939):

$$k_d = \left(\frac{k \cdot T}{h}\right) \cdot e^{(-\Delta H^*/RT)} \cdot e^{(\Delta S^*/R)}, \tag{11}$$

ΔH^*, ΔG^*, and ΔS^* of deactivation were calculated by applying Eqs. (3)–(5) with the modifications that in Eq. (3) $E_{(a)d}$ was used instead of E_a and in Eq. (4) k_d was used in place of k_{cat} (Kikani and Singh 2015).

Enzyme is deactivated and thus its activity becomes a function of the operating time. The integral activity can be estimated for a hypothetical continuous process by integrating the product of A_0 to the activity coefficient according to the following equation (Eq. 12) (Porto et al. 2006; Hasmann et al. 2007):

$$P(t) = \int A_0 e^{-k_d t} dt = \frac{A_0}{k_d}\left(1 - e^{-k_d t}\right). \tag{12}$$

Data analysis

All data analyses were performed using linear and nonlinear regression fitting by application of the program SigmaPlot, version 12.5 (Copyright 2003–2013, Systat Software, Inc.) for Windows.

Results and discussion

Purification of the extracellular PV8891 α-amylase

Extracellular α-amylase from *P. variotii* ATHUM 8891 was purified to homogeneity, as confirmed by the single band on SDS-PAGE (Fig. 1), applying ammonium sulfate fractionation, anion exchange chromatography followed by gel filtration chromatography. Results of the purification procedure

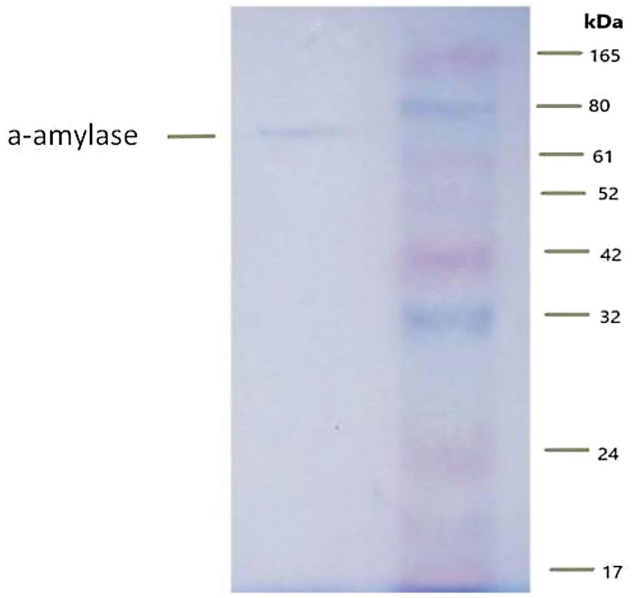

Fig. 1 SDS-PAGE electrophoresis of PV8891 α-amylase. The gel was stained with Coomassie Brilliant Blue. Left lane, purified enzyme; Right lane, MW markers

Table 1 Summary of the purification procedure for PV8891 α-amylase

Purification step	Total activity (Units)	Total Protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification factor (fold)
Crude extract	14,607.8	125.5	116.4	100.0	1.0
$(NH_4)_2SO_4$	11,289.8	38.2	295.5	77.3	2.5
Q-Sepharose	5243.2	10.0	522.1	35.9	4.5
Sephacryl S-100	2952.7	5.6	530.2	20.2	4.6

are summarized in Table 1. The complete purification procedure yielded 20% of the total activity, with approximately a fivefold increase in specific activity compared to the crude extract.

The reduced molecular mass of PV8891 α-amylase was estimated at 75 kDa (Fig. 1) and it is in agreement with the previous reports on the molecular mass of amylases from different *Paecilomyces* species (Zenin and Park 1983; Michelin et al. 2010). In general, molecular mass of α-amylases varies from about 10 to 210 kDa (Gupta et al. 2003; Sharma and Satyanarayana 2013). The lowest molecular mass of 10 kDa has been reported for a *B. caldolyticus* α-amylase (Grootegoed et al. 1973), while the highest of 210 kDa for *Chloroflexus aurantiacus* (Ratanakhanokchai et al. 1992).

The classification of *P. variotii* ATHUM 8891 enzyme as α-amylase was based mainly on the products of starch hydrolysis. Enzyme was incubated in the presence of starch and samples were withdrawn at 8 and 24 h. TLC of the hydrolysis products indicated that the main products (as identified by the R_f values) against starch were mainly maltose and maltotriose. These results indicated the endo-amylolytic character of the enzyme, which was classified as an α-amylase (data not shown).

Effect of pH on the enzyme activity and stability

The effect of pH on starch hydrolysis by the purified PV8891 α-amylase was determined in the range of pH 2.5–9.0 using different buffer systems, at 60 °C. PV8891 α-amylase exhibited highest activity at pH 5.0 with citrate–phosphate buffer system (Fig. 2a). The enzyme retains more than 50% of its optimum activity at pH range 3.0–6.0, while at higher pHs a considerable drop of activity was detected. In general, α-amylases display activity over a broad pH range from 2.0 to 12.0. The optimal pH of most of the α-amylases falls in the acidic and neutral range (Sharma and Satyanarayana 2013). Examples of α-amylases that act on acidic pHs are those that have been isolated from *Paecilomyces* sp., *Bacillus* sp. DR90, *B. licheniformis* B4-423, *A. awamori* KT-11 (pH optimum 4.0) (Wu et al. 2018; Anindyawati et al. 1998; Asoodeh et al. 2013; Zenin and Park 1983; Michelin et al. 2010),

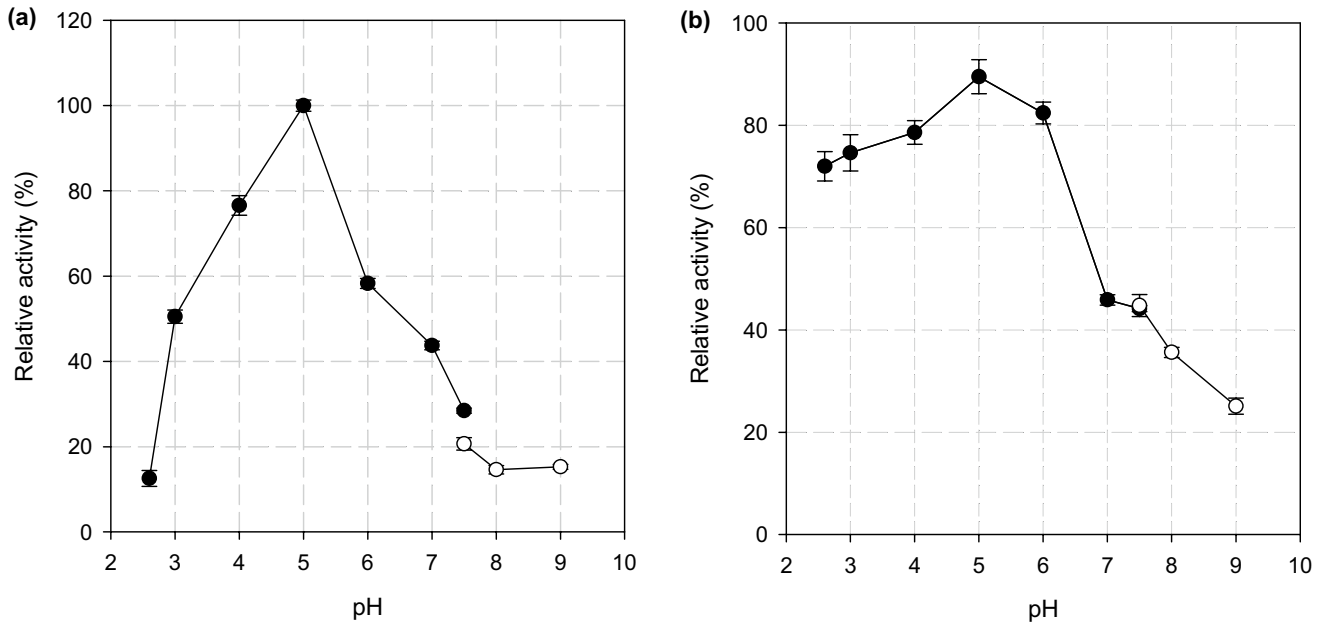

Fig. 2 Effect of pH on **a** activity and **b** stability of PV8891 α-amylase. Symbols: (filled circle) citrate–phosphate buffer (2.5–7.5), (opend circle) Tris–HCl buffer (7.5–9.0)

Talaromyces pinophilus strain 1–95, *A. oryzae* IFO-30103, *Aureobasidium pullulans*, *T. lanuginosus* ATCC 34,626 (pH optimum 4.6–6.6) (Xian et al. 2015; Ademakinwa et al. 2019; Dey and Banerjee 2015; Nguyen et al. 2002).

The stability of PV8891 α-amylase was studied at different pHvalues and the results are presented in Fig. 2b. The enzyme retained more than 70% of its initial activity at acidic pHs, i.e. citrate–phosphate buffer system of pH 2.5–6.0 after 24 h incubation at 4 °C, while at pH 7.0–9.0 the residual activity was in the range of 25–45%. Amylases in general are stable within a wide pH range. For example, *B. licheniformis* B4-423 amylase was stable at pH range of 5.0–10.0 (Wu et al. 2018), *T. pinophilus* strain 1–95 amylase was reported stable at pH 5.0–9.5 (Xian et al. 2015) while *T. lanuginosus* ATCC 34,626 and *A. awamori* KT-11 amylases were stable at pH range 4.5–8.5 (Anindyawati et al. 1998; Nguyen et al. 2002). On the other hand, amylases with stability in the acidic to neutral pH range have also been reported, such as the *Bacillus* sp. DR90 amylase which was found stable at a pH range 3.0–5.5 (Asoodeh et al. 2013) and the *A. oryzae* IFO-30103 amylase in the 4.5–7.0 range (Dey and Banerjee 2015).

The pH-stability results of PV8891 α-amylase indicated the acidic nature of the enzyme, a feature that makes it a favorable choice for the starch-based industries. The enzymatic hydrolysis of starch consists of three steps: gelatinization, liquefaction and saccharification. Starch gelatinization is the disruption of molecular orderliness within the starch granule and it is required to increase the accessibility of the substrate and to enhance the hydrolysis rate (Baks et al. 2008). Liquefaction and saccharification steps in industrial starch processes are performed by amylolytic enzymes with different pH optima. Liquefaction step is performed at pH 5.8–6.2 while saccharification at pH 4.2–4.5. Both of these steps are time-consuming and increase the total production cost (Sharma and Satyanarayana 2013). Using an amylase active and stable at low pH values is of great significance in the starch processing since the pH adjustment steps could be omitted. Furthermore, according to Goyal et al. (2005) the use of an acidic α-amylase could prevent the formation of some by-products, such as maltulose which is usually produced at higher operation pH values.

Effects of metal ions and chelator on PV8891 α-amylase activity

The effect of metal ions (Ca^{2+}, K^{+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Fe^{3+}, Fe^{2+}, Cu^{2+} and Co^{2+}) and of a chelator (EDTA) on the enzyme activity was studied at two different concentrations (1 and 5 mM) (Table 2). PV8891 α-amylase activity was slightly elevated in the presence of Ca^{2+} and Fe^{2+} at 5 mM and 1 mM concentration, respectively. Similarly, the presence of Ca^{2+} ions marginally affected the α-amylase activity from *Bacillus* sp. DR90 (Asoodeh et al. 2013), *B. licheniformis* B4-423 (Wu et al. 2018), *T. pinophilus* 1–95 (Xian et al. 2015), while in two of them Fe^{2+} enhanced enzyme activity (Xian et al. 2015; Asoodeh et al. 2013).

Table 2 Effect of metal ions and chelator on PV8891 α-amylase activity

Cations/chelator	Relative activity (%)	
	1 mM	5 mM
None	100.0	100.0
Ca^{2+}	100.0 ± 4.7	107.7 ± 0.8
K^{+}	89.2 ± 1.1	62.2 ± 0.9
Zn^{2+}	69.1 ± 1.1	52.7 ± 1.2
Mn^{2+}	95.8 ± 1.2	74.5 ± 0.3
Mg^{2+}	87.0 ± 1.0	71.5 ± 0.3
Fe^{3+}	51.5 ± 0.6	43.2 ± 1.5
Fe^{2+}	103.0 ± 0.7	99.0 ± 0.4
Cu^{2+}	28.9 ± 1.1	7.6 ± 1.1
Co^{2+}	81.2 ± 0.4	76.5 ± 1.0
EDTA	75.5 ± 1.4	50.2 ± 0.4

The enzyme was incubated with the indicated concentrations of the various modulators for 30 min at room temperature and the remaining activity was determined at pH 5.0 and 60 °C. Enzyme activity of control sample without any addictives was taken as 100%

± indicates standard deviation among three independent readings

PV8891 α-amylase activity is moderately inhibited by the presence of K^{+}, Mn^{2+} and Mg^{2+} ions. The enzyme retains more than 87% of its initial activity in the presence of 1 mM of the above ions. Loss of activity in the range of 23–30% was observed in the presence of 1 mM Co^{2+} and Zn^{2+} ions, while Fe^{3+} ions reduced enzyme activity by 49%. Cu^{2+} ions strongly inhibited amylase activity resulting in 92% loss of activity at 5 mM concentration. Finally, incubation of PV8891 α-amylase with EDTA resulted in 25 and 50% loss of activity at 1 and 5 mM, respectively. In general the enzyme was inhibited by metal ions studied to a different extent and in a concentration-dependent manner.

Similar to the present study, Cu^{2+} ions inhibited α-amylases from *T. fonticaldi* (Allala et al. 2019), *T. pseudokoningii* (Abdulaal 2018) and from two different strains of *B. licheniformis* (Wu et al. 2018; Samanta et al. 2014). Zinc and cobalt ions inhibited the enzymatic activity of *T. pseudokoningii* (Abdulaal 2018), *T. fonticaldi* (Allala et al. 2019), *T. lanuginosus* ATCC 34,626 (Nguyen et al. 2002) as well as of different *Bacillus* species (Wu et al. 2018; Samanta et al. 2014; Asoodeh et al. 2013). Ferric ions reduced enzymatic activity of α-amylase from different strains of *B. licheniformis* (Wu et al. 2018; Samanta et al.

2014) as well as from *Laceyella sacchari* TSI-2 (Shukla and Singh 2015).

Temperature optima, activation energy and temperature quotient (Q_{10})

The optimum temperature of PV8891 α-amylase for starch hydrolysis was found 60 °C. Enzyme activity increases up to 60 °C, whereas an opposite trend was observed over this threshold value, as the enzyme gradually lost activity with increasing temperatures (Fig. 3a). The temperature optimum for the activity of α-amylases produced from mesophilic fungi ranges from 40 to 60 °C. α-amylases of bacterial origin exhibited higher temperature optima for activity (up to 100 °C) (Gupta et al. 2003; Sharma and Satyanarayana 2013). The α-amylase isolated from a Brazilian *P. variotti* strain had the same temperature optimum as the α-amylase under study (Michelin et al. 2010), while the α-amylase of a *Paecilomyces* sp. showed a temperature optimum of 45 °C (Zenin and Park 1983).

Arrhenius plot (Fig. 3b) was used to calculate activation energy (E_a) of the enzyme for starch hydrolysis, which was 10.7 kJ mol^{-1}. The low E_a value estimated for the α-amylase under study indicates that less energy is required to form the activated complex of starch hydrolysis, thus highlighting an effective hydrolytic capacity.

The temperature quotient (Q_{10}) for the enzyme was found to be 1.1. Q_{10} value suggests whether or not the metabolic reaction is mainly controlled by temperature or by other factors. In general for enzymatic reactions, Q_{10} values range between 1 and 2 and any deviation from this value indicates the influence of other factors (Ademakinwa et al. 2019). A comparison of E_a and Q_{10} for starch hydrolysis of PV8891 α-amylase with others reported in literature is presenting in Table 3.

Kinetic constants and thermodynamics of starch hydrolysis

Classical Michaelis–Menten kinetics was observed with starch as the substrate for PV8891 α-amylase (Fig. 4). The K_m and v_{max} values determined through non-linear regression fitting of initial reaction velocity *vs* substrate concentration data on the Michaelis–Menten equation at 60 °C were 1.1 g/L and 58.5 µmole min^{-1}(mg protein)$^{-1}$, respectively. The kinetic values of different amylases cannot be easily compared due to variation in substrates and assay conditions. The K_m value of PV8891 α-amylase for starch is within the reported range for amylases of bacterial or fungal origin (0.19–11.66 g L^{-1}) (Xian et al. 2015; Sharma and Satyanarayana 2013; Samanta et al. 2014; Shukla and Singh 2015; Kikani and Singh 2015; Abdulaal 2018; Allala et al. 2017; Dey and Banerjee 2015; Gangadharan et al. 2009; Michelin et al. 2010; Nguyen et al. 2002). The low K_m value of PV8891 α-amylase indicates high affinity for its substrate and thus requires a lower concentration of substrate to achieve v_{max}. The turn over number (k_{cat}), which is the second-order rate constant for the conversion of the enzyme–substrate complex to the product, was calculated

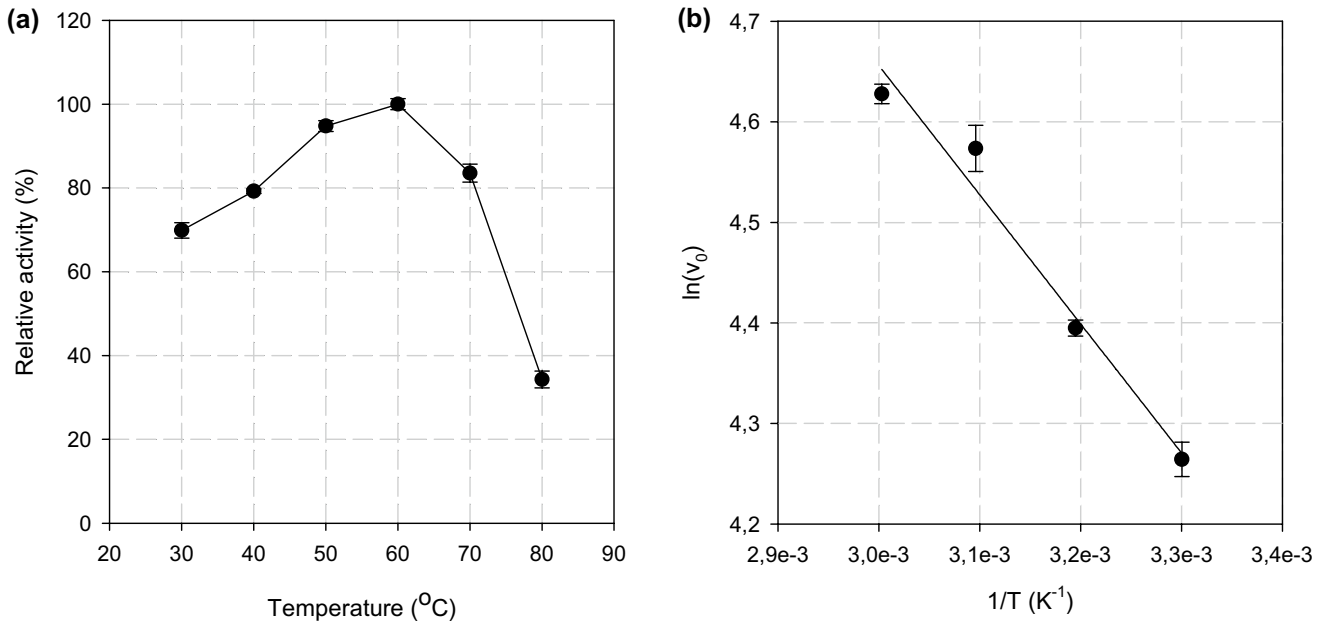

Fig. 3 **a** Effect of temperature on PV8891 α-amylase activity. **b** Arrhenius-type plot for determination of the activation energy (E_a) for starch hydrolysis catalyzed by PV8891 α-amylase

Table 3 Thermodynamic properties of starch hydrolysis

Microorgnism	E_α (kJ mol^{-1})	Q_{10}	ΔH^* (kJ mol^{-1})	ΔG^* (kJ mol^{-1})	ΔS^* (J mol^{-1} K^{-1})	ΔG^*_{E-S}(kJ mol^{-1})	ΔG^*_{E-T}(kJ mol^{-1})	References
B. altitudinis	37.32		30.09	30.442	− 115.9			Singh et al. (2018)
B. licheniformis SKB4	31.53	1.0				5.53	− 17.4	Samanta et al. (2014)
B. cereus MTCC 1305	22.806		20.119	92.149	− 210			Sugumaran et al. (2012)
A. pullulans	14.56	1.2						Ademakinwa et al. (2019)
A. awamori	5.82							Karam et al. (2017)
P. variotii ATHUM 8891*	10.7 ± 0.8	1.1 ± 0.1	7.9 ± 0.6	68.4 ± 1.5	− 181.2 ± 1.2	1.0 ± 0.1	− 12.5 ± 0.7	Present study

Comparison of the results obtained for PV8891 α-amylase with literature reports

*Thermodynamic parameters of PV8891 α-amylase were calculated at optimum temperature for activity, 60 °C, ± indicates standard deviation among three independent readings

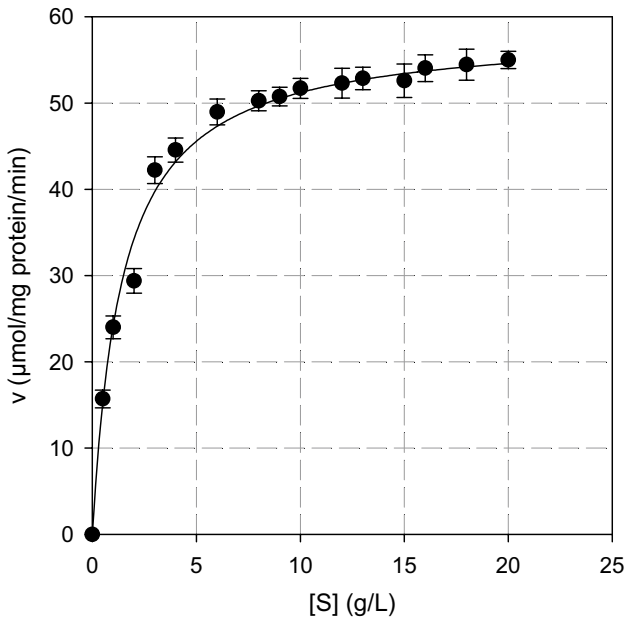

Fig. 4 Michaelis–Meneten plot for the determination of the kinetic constants (K_m and v_{max}) of starch hydrolysis by PV8891 α-amylase

as 73.1 s^{-1} and the catalytic efficiency constant (k_{cat}/K_M) as 51.1.

Thermodynamic parameters provide a detailed portrait of inside of many chemical and biological reactions. The enthalpy of activation (ΔH^*), Gibbs free energy (ΔG^*) and entropy of activation (ΔS^*) for starch hydrolysis by *P. variotii* ATHUM 8891 amylase were calculated as 7.9 kJ mol^{-1}, 68.4 kJ mol^{-1} and -181.2 kJ mol^{-1} K^{-1}, respectively. A comparison of thermodynamic parameters for starch hydrolysis of PV8891 α-amylase with others reported in literature is presented in Table 3.

The lower ΔH^* recorded for PV8891 α-amylase and negative values of ΔS^* may suggest formation of a more efficient and ordered transition state complex between enzyme and substrate. The feasibility and extent of an enzyme-catalyzed reaction is best determined by measuring the change in ΔG^* for substrate hydrolysis, i.e. the conversion of an enzyme–substrate complex into a product. Low ΔG^* values suggest that the conversion of a transition state complex into a product was more spontaneous. The lower ΔG^* value for PV8891 α-amylase indicated that the conversion of its transition complex into products was more spontaneous compared to the α-amylase of *B. cereus* (Sugumaran et al. 2012).

The free energy of substrate binding (ΔG^*_{E-S}) and transition state formation (ΔG^*_{E-T}).

for PV8891 α-amylase were calculated as 1.0 and − 12.5 kJ mol^{-1}, respectively (Table 3). Low values of free energy for substrate binding (ΔG^*_{E-S}) and transition state formation (ΔG^*_{E-T}) suggest high affinity of PV8891 α-amylase towards starch hydrolysis.

Thermodynamics of PV8891 α-amylase stability

Thermal stability represents the capacity of an enzyme molecule to resist thermal unfolding in the absence of substrate. The temperature effect on PV8891 α-amylase stability was determined by measuring the residual activity after incubation (in the absence of substrate) at temperatures of 30, 40, 50, 60, 70 and 80 °C. The enzyme was found quite stable at 40 °C as it retains 84% of its initial activity after 60 min of incubation. There was a reduction of 30% and 40% in enzyme activity at temperatures 50 and 60 °C, respectively,

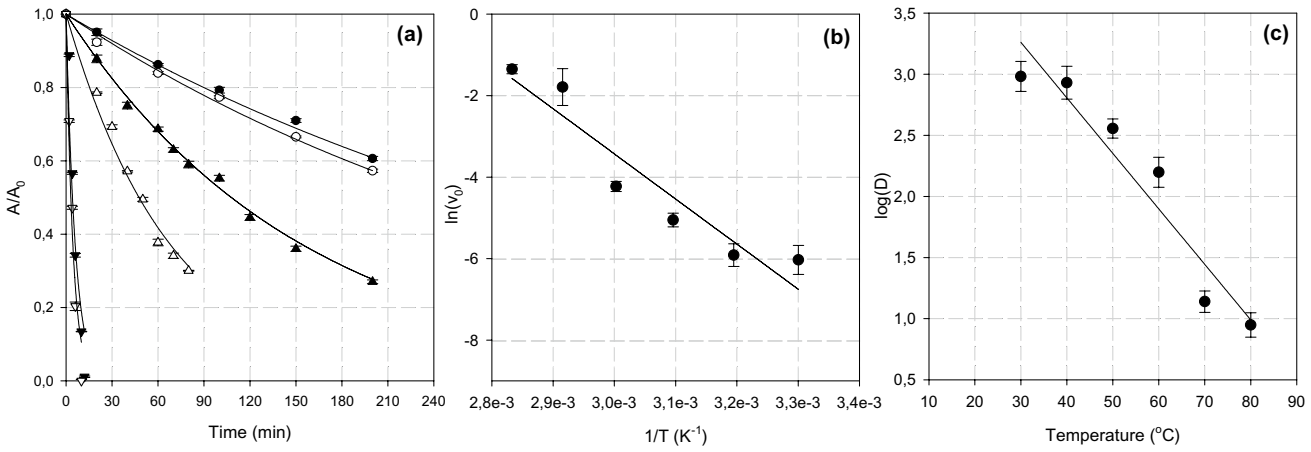

Fig. 5 **a** Thermal stability of PV8891 α-amylase, **b** Arrhenius plot for the determination of thermal inactivation energy ($E_{(a)d}$), **c** Variation of decimal reduction times with temperature for the PV8891 α-amylase (determination of z-value)

after 40-min incubation. Rapid loss of activity was observed at 70 and 80 °C where after 6 min of incubation the enzyme retains only 34% and 20% of its initial activity, respectively (Fig. 5a). Asoodeh et al. (2013) reported that the α-amylase produced by *Bacillus* sp. DR90 retained more than 50% of its initial activity after 1 h of incubation at temperatures 30 to 50 °C. *T. pinophilus* 1–95 α-amylase was found stable at temperatures below 45 °C (Xian et al. 2015) and the α-amylase of *Bacillus amyloliquefaciens* was stable up to 25 min at 60 °C (Gangadharan et al. 2009).

The values of thermal inactivation rate constant (k_d), calculated as in "Materials and Methods" are given on Table 4. The gradual inactivation of PV8891 α-amylase occurred with an increase in temperature as it was observed by the increase in the values of k_d. The lower the value of k_d at higher temperatures, the more stable the enzyme. Half-life ($t_{1/2}$) and decimal reduction time (D value) are important economic parameters in many industrial applications, because the higher its value, the higher the enzyme thermostability. The $t_{1/2}$ and D values for PV8891 α-amylase over the temperature range tested are presented on Table 4. The enzyme under study exhibits $t_{1/2}$ and D value of 108.3 min and 359.8 min, respectively, at 50 °C. Asoodeh et al. (2013) reported $t_{1/2}$ of 138 min at 45 °C for the amylase produced by *Bacillus* sp. DR90. α-amylase of *A. awamori* had $t_{1/2}$ and D value of 289 min and 959 min, respectively, at 50 °C, while much higher values were reported for the *A. pullulans* α-amylase ($t_{1/2}$ 15 h, D value 51.6 h) at the same temperature (Karam et al. 2017; Ademakinwa et al. 2019).

PV8891 α-amylase presented a z value of 22.1 °C, which indicates that to reduce 90% of decimal reduction time, it is necessary to increase temperature by 22.1 °C (Fig. 5c).

Thermal inactivation energy $E_{(a)d}$ is the minimum energy that must be acquired before protein unfolding takes place. It was estimated by the semi-log plot of $\ln k_d$ vs 1/T (Arrhenius plot) at 92.1 kJ mol^{-1} (Fig. 5b). This value is higher than those reported for the amylases of *A. awamori* (Karam et al. 2017), *A. pullulans* (Ademakinwa et al. 2019) and *Anoxybacillus beppuensis* TSSC-1 (Kikani and Singh 2012), indicating that PV8891 α-amylase needed excess energy for thermal inactivation. The latter is a good sign of thermal stability.

Table 4 Thermodynamic parameters for thermal inactivation of PV8891 α-amylase

Temperature (K)	k_d (min^{-1})	$t_{1/2}$ (min)	D (min)	$E_{(a)d}$ (kJ mole^{-1})	Z (°C)	ΔG^* (kJ mole^{-1})	ΔS^* (J mole^{-1} K^{-1})	ΔH^* (kJ mole^{-1})
303	2.4×10^{-3}	288.8 ± 2.9	959.6 ± 9.6	92.1 ± 2.7	22.1 ± 1.0	99.8 ± 1.0	− 33.6 ± 0.3	89.6 ± 0.9
313	2.7×10^{-3}	256.7 ± 3.9	853.0 ± 12.8			102.8 ± 1.5	− 42.6 ± 0.6	89.5 ± 1.3
323	6.4×10^{-3}	108.3 ± 3.4	359.8 ± 11.2			103.9 ± 3.2	− 44.8 ± 1.4	89.4 ± 2.9
333	1.5×10^{-2}	47.5 ± 1.1	157.7 ± 3.8			104.9 ± 2.5	− 46.8 ± 1.3	89.3 ± 2.1
343	1.7×10^{-1}	4.2 ± 0.2	13.8 ± 0.3			102.9 ± 2.4	− 39.0 ± 0.9	89.2 ± 2.1
353	2.6×10^{-1}	2.7 ± 0.0	8.9 ± 0.1			101.2 ± 1.0	− 34.8 ± 0.3	89.2 ± 0.9

k_d thermal inactivation rate constant; ± indicates standard deviation among three independent readings. Their values under column k_d were too small and have not been presented, $t_{1/2}$ half-life, D decimal reduction, $E_{(a)d}$ thermal inactivation energy, z thermal resistance constant, ΔG^* Gibbs free energy of inactivation, ΔH^* enthalpy change of inactivation

The thermal inactivation of enzymes is due to the disruption of non-covalent linkages, including hydrophobic interactions. Gibbs free energy (ΔG^*) is considered as the energy barrier for enzyme inactivation, enthalpy change (ΔH^*) is a measure of the number of non-covalent bounds broken in forming a transition state for enzyme inactivation and entropy change (ΔS^*) indicates the enzyme disorder due to disruptions of enzyme structure (Gummadi 2003).

The values of the thermodynamic parameters of PV8891 α-amylase were calculated in the temperature range of 30–80 °C and presented in Table 4. ΔH^* values in the temperature range studied were slightly decreased (89.6–89.2 kJ mole^{-1}) when temperature increased indicating progressively less energy was required to denature the enzyme. PV8891 α-amylase ΔH^* values are in the range of values reported for thermal reversible unfolding (56–150 kJ mole^{-1}) and are much lower than those reported for the irreversible denaturation of different enzymes (220–235 kJ mole^{-1}) (Porto et al. 2006). PV8891 α-amylase had a ΔH^* value of 89.4 kJ mole^{-1} at 50 °C, which is much higher than the values reported for the amylases of *A. awamori* at 50 °C, *L. sacchari* TSI-2R at temperature range 50–100 °C, *A. beppuensis* TSSC-1 at temperature range 50–90 °C and *A. pullulans* at temperature range 50–80 °C and lower than that of *Bacillus* sp. TSSC-3 at temperature range 50–90 °C (Kikani and Singh 2012; Shukla and Singh 2015; Karam et al. 2017; Ademakinwa et al. 2019; Hasmann et al. 2007). According to Pace (1992) the energy required to remove a -CH_2 moiety from a hydrophobic bond is approximately 5.4 kJ mole^{-1} and thus the formation of transition state leading to inactivation of PV8891 α-amylase implied the disruption, as an average, of 16.6 non-covalent bonds.

Disruption in enzyme structure is accompanied by an increase in its degree of disorder and randomness, indicated by large and positive values of entropy change (ΔS^*) (de Oliveira et al. 2018). In the present study ΔS^* values were negative (-46.8 to -33.6 J mol^{-1} K^{-1}) (Table 4) which suggests even higher orderliness of the transition state. Gummadi (2003) stated that negative values of entropy are often encountered in biological systems, especially in the case of proteins and that the negative entropy changes during inactivation are consistent with the compactation of the enzyme molecule, but equally such changes could arise from the formation of charged particles and the associated gain and the ordering of solvent molecules. For comparison ΔS^* values of amylases from *A. awamori*, *L. sacchari* TSI-2R, *A. beppuensis* TSSC-1, *A. pullulans* and *Bacillus* sp. TSSC-3 were reported in the range -249.7 J mol^{-1} K^{-1} $\leq \Delta S^* \leq$ - -118.9 J mol^{-1} K^{-1} (Kikani and Singh 2012, 2015; Shukla and Singh 2015; Karam et al. 2017; Ademakinwa et al. 2019).

The Gibbs free energy of inactivation (ΔG^*) which includes both enthalpic and entropic contributions, is a more accurate and reliable tool to evaluate enzyme stability (Ademakinwa et al. 2019). In the case of thermal inactivation of an enzyme, smaller or negative values of ΔG^* are associated with a more spontaneous process, meaning that the enzyme becomes less stable and more easily undergoes thermal inactivation, whereas an increase in ΔG^* reveals better resistance to thermal inactivation. ΔG^* values determined in the present study were in the range of 99.7–104.9 kJ mole^{-1}. More specifically Gibbs free energy values increased when the temperature increased from 30 to 60 °C, indicating that α-amylase thermal stabilization was due to the higher functional energy, which enabled the enzyme to resist the unfolding of its transition state (de Oliveira et al. 2018). Furthermore, the decrease in ΔG^* at 70 and 80 °C is in accordance with the reduction of $t_{1/2}$ and D-values (Table 4). The α-amylase produced by *Bacillus* sp. TSSC-3 had a much higher ΔG^* (930 kJ mole^{-1}) at 50 °C (Kikani and Singh 2015) compared to α-amylase under study. Comparable values of ΔG^* had been reported for the amylase isolated from *A. beppuensis* TSSC-1, while lower were the values of *L. sacchari* TSI-2R, *A. pullulans* and *A. awamori* amylases (Kikani and Singh 2012, 2015; Shukla and Singh 2015; Karam et al. 2017; Ademakinwa et al. 2019).

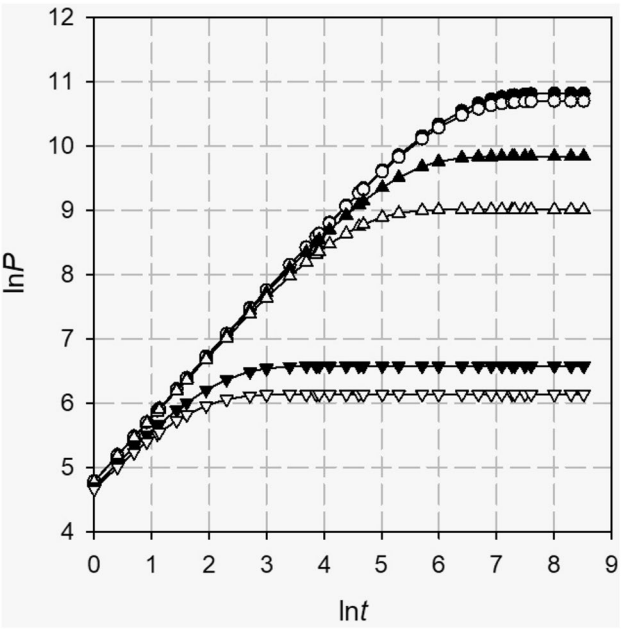

Fig. 6 Time (*t*, min) behavior at different temperatures of the integral activity (P, mM) of the purified PV8891 α-amylase. *Symbols*: (filled circle) 30 °C, (open circle) 40 °C, (filled triangle) 50 °C, (open triangle) 60 °C, (filled inverse triangle) 70 °C and (open inverse triangle) 80 °C

Integral enzyme activity

Potential industrial applications of PV8891 α-amylase demand the knowledge of long-term behavior of the integral activity, P(t) as defined in Eq. 12. P(t) represents the amount of starch hydrolyzed by 1 g of amylase under continuous feed conditions (de Oliveira et al. 2018; Porto et al. 2006). P(t) was estimated in the temperature range 30–80 °C and plotted versus time in the bi-log plot (Fig. 6). A linear increase in all curves was observed. Moreover, the higher the temperature, the lower the final integral activity due to higher k_d value, and the quicker its achievement, which confirms that enzyme inactivation became progressively more significant along the time. For instance, at temperatures 30–60 °C final integral activity was achieved at times ≥ 100 min, while the latter dramatically drops at temperatures 70 and 80 °C. Qualitatively similar behavior was observed in studies of the thermostability of enzymes such as ascorbate oxidase from *Cucurbita maxima*, protease from *A. tamarii* URM4634 and pectinase from *A. aculeatus* (da Silva et al. 2018; de Oliveira et al. 2018; Porto et al. 2006).

Conclusion

The work aimed at the evaluation of thermodynamic and kinetic properties of an extracellular α-amylase produced by an indigenous wild-type *Paecilomyces variotii* ATHUM 8891 strain. The purified enzyme was optimally active and stable at acidic pH. Kinetic and thermodynamic parameters of the enzyme on starch hydrolysis indicated stability and the spontaneity of the enzyme-catalyzed reaction. Thermal stability studies revealed an interesting thermostability of the purified enzyme for future applications and thus, the integral activity of a continuous system containing the enzyme has also been predicted. The properties of PV8891 α-amylase, e.g. the acidic nature, the effective hydrolytic capacity towards starch hydrolysis as well as its satisfactory performance at temperatures lower than the industrially used amylases may represent potential applications in starch processing industry.

Author contributions Conceptualization: DM, DH and DK; Methodology: DM, DH; Formal analysis and investigation: MEA, SK; Writing—original draft preparation: DM; Writing—review and editing: all authors.

Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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