International Scientific Journal
Medical and Biological Sciences
http://bioscience.scientific-journal.com

ENZYMATIC CHARACTERIZATION OF THE RECOMBINANT BETA-XYLOSIDASE XynB2

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Abstract— **Enzyme research on thermophilic microorganisms has steadily increased given their high catalytic capabilities in extreme conditions, ideal for biotechnological as well industrial processes. XynB2, a β-xylosidase from *Geobacillus stearothermophilus*, and to date the only enzyme of this type displaying transglycosidase activity, offers attractive catalytic applications. Though previously biophysically and biochemically characterized, no detailed kinetics studies on the hydrolysis of various aryl-β-D-xyloside substrates have been reported. The goal of this study was to characterize XynB2 under maximal activity conditions (pH and temperature). XynB2 was expressed in *E. coli* C43 using the recombinant plasmid pJAVI91 which encodes the protein with a histidine tail (His-tag) at the stop codon, facilitating purification using Ni affinity chromatography, followed by size-exclusion chromatography. Purified XynB2 reached highest activity at 65 °C and pH 7.0, employing *4*-methylumbelliferyl xylopyranoside as substrate. Calculation of kinetic parameters gave as result a K_M =0.20 ± 0.04 mM, V_{max} =0.57 ± 0.06 nmol/s, k_{cat} =203 ± 21 (s^{-1}) and k_{cat}/K_M = 1017 ± 230 ($mM^{-1} \cdot s^{-1}$), under maximal activity conditions. The thermal stability of XynB2 was evaluated to be 69.0 ± 0.6 °C, with a $t_{1/2}$ of 116 ± 10 min, corresponding to a first order reaction. These results correlate with others reported in the literature from similar thermophilic microorganisms. Complete analysis employing other substrates is being developed. These results may be useful for other applications, like enzyme immobilization, in order to perform catalysis over other type of substrates, like those coming from wastewater, sewage, or industrial residues rich in xylans and/or cellulose.**

Keywords—aryl-β-D-xylosides, enzyme catalysis, kinetic parameters, xylanase.

I. INTRODUCTION

Lignocelullose is one of the most ubiquitous biomass available and with vast application possibilities, including alternative fuel production or chemical compound synthesis [1]. As the main structural component in plant cell walls, lignocellulose is primary composed by lignine, cellulose and hemicellulose [2]. Hemicellulose, unlike cellulose, is a heterogeneous polymer, composed by pentoses (xylose, arabinose), hexoses (glucose, galactose, manose) and sugar acid [3]. Xylan, made from xylose units, is a mayor type of hemicellulose, being the second most abundant polymer on Earth, after cellulose. Xylan degradation is accomplished by enzymes called xylanases (EC 3.2.1.8, endo-(1→4)-β-xylan 4-xylanohydrolase), which hydrolyses β-1,4-D-xylan into D-xylose, and also by β-xylosidase (EC 3.2.1.37, xylan 1,4-β-xylosidase), which hydrolyses β-1,4-D-xylan by removing successive D-xylose residues from the non-reducing termini [4].

Genetic engineering has enabled the development of recombinant enzymes from extremophiles. Research with these microorganisms has been promoted to the industrial field, given its unusual capacity to thrive at extreme conditions [5]. Though many microorganisms are able to express xylanases [6-8], *Geobacillus stearothermophilus* has received special attention. XynB2, a β-xylosidase from *G. stearothermophilus*, has been characterized, and the stereochemistry behind hydrolysis is known, temperature and pH values for maximal activity has been determined [9].

Heat is often used as a purification step for some recombinant thermophilic enzymes. However, this may diminish its stability and reduce its useful lifetime, even though the enzyme is thermostable [10], in comparison with other alternative techniques, like addition of a His-tale, allowing enzyme purification at lower temperatures as well as retaining its full activity [11].

The thermophilic β-xylanase XynB2 from *G. stearothermophilus* has been well characterized [9,11-13], nevertheless all assays have been performed at 40 °C. This work focuses in the enzymatic activity analysis of XynB2 at higher temperatures (~75 °C) employing *4*-methylumbelliferyl xylopyranoside as substrate. Temperature and pH dependence of the enzyme were profiled and kinetic parameters were determined at the maximal activity conditions.

After this biochemical characterization, XynB2 has now better chances for further studies concerning chemical modification and immobilization, aspects relevant for the industrial field [14]. As reported [10], biocatalyzers reduces manufacture costs, by reducing production times, but also is a more ecological alternative. Xylanases are of great interest for the industrial agriculture, e.g. by maximizing nutrient extraction in feed [15], for the paper industry, in the pulp bleaching [16] and in the production of alternative fuels [2].

II. MATERIALS AND METHODS

A. Materials

All materials used were of high purity. *4*-methylumbelliferyl xylopyranoside (4MUX),

2-mercaptoethnaol (2ME), phenylmethylsulfonyl fluoride (PMSF), imidazole, LB-medium and xylose were purchased from Sigma-Aldrich. Acrylamide, bromophenol blue and Coomassie brilliant blue R-250 (CBB) were from Fisher. Bis-acrylamide, sodium dodecyl sulfate (SDS), glycerol, isopropyl β-D-1-thiogalactopyranoside (IPTG), molecular weight marker V849A, Tris-Base and Triton X-100 were from Promega.

B. XynB2 expression

Plasmid pJAVI91 was used to transform competent *E. coli* C43 cells [4], thus introducing the sequence corresponding to the XynB2 gene from *G. stearothermophilus*, besides including a gene for ampicillin resistance and a His marker just before the ending codon [11]. Competent *E. coli* C43 cells kept at -80 °C were thawed and 1% 2ME was added. Cells were incubated for 10 min over ice, with gentle agitation every 2 min. Following, 4.5 µL pJAVI91 plasmid was added and left undisturbed over ice for 30 min. After incubation, thermal shock was induced by heating for 45 s at 42 °C. Cells were placed again over ice for 2 min and 1 mL LB medium was added and incubated at 37 °C for 1 h at 200 rpm [11].

Culture was then briefly centrifuged and the resulting pellet was resuspended in LB medium and seeded in a LB-ampicillin-agar medium, incubated overnight at 37 °C. The resulting colonies were seeded in liquid LB medium with ampicillin, left overnight at 37 °C, under agitation at 200 rpm. A 25 mL aliquot from this pre-inoculum was used to inoculate 600 mL LB-ampicillin medium (0.05 g ampicillin/L). Bacterial growth was achieved under constant agitation at 200 rpm at 37 °C and monitored by measuring its absorbance at 600 nm. Once optical density reached values in the range 0.4-0.6, 1 mM IPTG was added. Bacterial growth was left overnight (~15 h) at 37 °C and 200 rpm. Cells were collected by centrifugation at 6000 rpm for 10 min and preserved at -20 °C [11]. Protein expression was visualized by SDS-PAGE (10%).

C. Cellular lysis

The preserved cells were thawed and resuspended in 50 mL lysis buffer [50 mM NaCl, 5 mM imidazole, 20 mM Tris (pH 8.0), 0.1% Triton X-100, 1 mM 2ME, 1 mM PMSF], avoiding bubble formation. Sample was sonicated by 10 cycles, each of 45 s with 15 s pause over ice. The lysate was centrifuged at 12000 × *g* for 60 min, the resulting supernatant was kept over ice until purification procedure.

D. XynB2 Purification

The supernatant was applied to a Ni-Sepharose High Performance (Pharmacia) column, equilibrated with washing buffer [100 mM NaCl, 30 mM imidazole, 20 mM Tris, 1 mM 2ME]. XynB2 was eluted with elution buffer [500 mM NaCl, 500 mM imidazole, 20 mM Tris, 1 mM 2ME]. Absorbance at 280 nm was measured and fractions with values between 0.3 and 1 A.U. were pooled. The pooled fraction was analyzed by SDS-PAGE (10%) and submitted to gel filtration chromatography (Sephacryl S-300). Fractions were eluted with 50 mM Tris buffer pH 7.0, 150 mM NaCl and collected at 1 mL/min [11].

E. SDS-PAGE

The Mini-Protean III (Bio-Rad Laboratories, USA) system was used to run 10% SDS-PAGE according to Laemmli [17]. Gels were run at 120 V, and stained with CBB. A molecular weight marker (Promega V849A) was employed.

F. Protein quantification

The Bradford method was used to quantify total protein concentration [18]. A 100 µg/mL BSA solution was used as standard. Parallel, direct absorbance measurements at 280 nm of the purified XynB2 were done and the molar extinction coefficient (133,850 $M^{-1}\cdot cm^{-1}$) [11] used to calculate protein concentration.

G. XynB2 enzymatic activity

XynB2 activity was measured by the release of *4*-methylumbelliferone from *4*-methylumbelliferyl-β-D-xylopyranoside (4MUX). Assays were performed at an excitation/emission λ of 365/450 nm, with an extinction coefficient of 3.4 $mM^{-1}\cdot cm^{-1}$. Measurements were done each second with a total reaction time of 5 min, enough to obtain constant velocity and a typical hyperbolic curve corresponding to a Michaelis-Menten kinetic. Relative activity percentage was defined as [enzyme activity (U)/highest enzyme activity (U)]×100, where U was in µmol/min.

H. Kinetic parameters determination

Prior to the velocity and kinetic constants determination, the temperature and pH dependence of the enzyme activity were first measured. Activity was assayed between 40 and 85 °C, at 5 °C intervals, and from pH 2.00 until 11.00 at 0.50 units intervals. The following 0.05 M buffers were employed: H_3PO_4 / KH_2PO_4 (pH 2.00; 2.50 and 3.00); glycine-HCl (pH 3.50); acetic acid (pH 4.00, 4.50, 5.00, 5.50); KH_2PO_4 / K_2HPO_4 (pH 6.00, 6.50, 7.00); Tris-HCl (pH 7.50, 8.00, 8.50, 9.00); and $NaHCO_3$ / Na_2CO_3 (pH 9.50, 10.00, 10.50, 11.00).

Afterwards, initial velocity of the reactions were measured at several substrate concentration [0.010; 0.0125; 0.050, 0.075; 0.100; 0.150; 0.250; 0.300; 0.40 and 0.500 mM], using 0.0028 nmol XynB2, at 65 °C, pH 6.50, for 5 min. K_M and V_{max} were determined by different approaches (Michaelis-Menten hyperbolic curve, Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstee). OriginPro 8 software was used for the hyperbolic adjustment, proper of a Michaelis-Menten first order kinetic.

I. XynB2 Thermostability determination

Thermal stability profile was evaluated by incubating XynB2 in the range from 40 until 85 °C, at 5 °C intervals) for 30 min before adding 0.0028 nmol XynB2 to the reaction mixture. After 5 min at 65 °C and pH 6.5, activity was measured by fluorescence employing 0.075 mM 4MUX. Thermal inactivation kinetic was done by preincubating XynB2 for 5 h at 65 °C and pippeting each 30 min, 0.0028 nmol enzyme, to 0.075 mM 4MUX to measure activity as described before.

III. RESULTS AND DISCUSSION

A. *XynB2 overexpresion and purification*

Cell replication time was slow, taking ~5 h to reach ~0.4 absorbance units at 600 nm. Enzyme overexpression was visualized by SDS-PAGE (Fig. 1). Lane 5 shows the overexpressed protein with an apparent molecular weight of 75 kDa, corresponding to reported values [11]. Xyn B2 is a 150 kDa protein composed of two 75 kDa homodimers, which separate under denaturing conditions, inactivating the enzyme irreversibly. Given the few amount of contaminant bands present at the final stage of purification, it was possible to assume that XynB2 was purified appropriately and ideally for the nature of the kinetic assays to be performed.

B. *pH and temperature dependence over XynB2*

XynB2 behave typically (Fig. 2) as compared with other glycosyl hydrolases, when enzyme activity is tested varying the pH [19-21]. Though the highest activity was reached at pH 7, pH 6.5 was chosen for further experiments. It has been reported that at pH <4.0, the enzyme is insoluble (10), justifying the complete absence of activity. Others XynB2 from different thermophilic microorganisms (*Thermoanaerobacter ethanolicus*, *Thermoanaerobacterium saccharolyticum*, *Thermotoga maritima*) were reported to have slightly lower pH values (5.00-6.00) at maximal activity [22-25].

XynB2 presented maximal activity at 65 °C and descended abruptly over 70 °C, indicating progressive thermal denaturation of the enzyme during the reaction time [11,23]. This value resulted slightly lower than those reported for other thermophiles (*Thermoanaerobacter ethanolicus*, 75 °C; *Bacillus stearothermophilus*, 70 °C; *Thermoanaerobacterium saccharolyticum*, 70 °C, *Thermotoga maritima*, 90 °C) [22-25]. From the assays, 65 °C was chosen for all further experiments. Noticeably is the adequacy of such high value, if the enzyme is meant to have any industrial application, e.g. pulp bleaching, which is performed up to 70 °C [1].

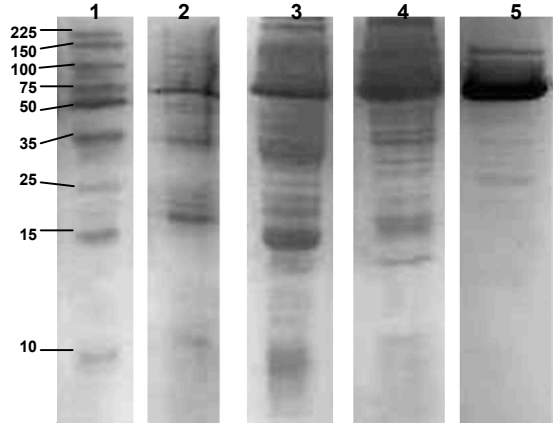
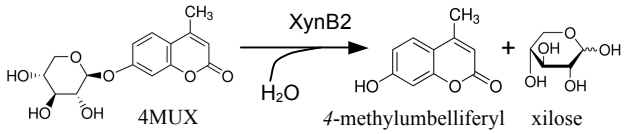


Fig. 1. Electropherograms of the protein profile from the XynB2 purification using SDS-PAGE (10%). Lanes: (1) Molecular weight standard (values shown in kDa). (2) Bacterial growth at 3 h 30 min (3) Cell lysate. (4) XynB2 fraction eluted from Ni^{2+} column. (5) Pooled fraction from Sephadex S-300. Gel was stained with CBB.

C. *Kinetic parameters*

K_M and k_{cat} for XynB2 had been determined for numerous substrates at 40 °C and pH 7.0 [12]. However, being XynB2 an enzyme belonging to a thermophilic microorganism, it was considered more appropriate to update the catalytical data at a more realistic temperature, like 65 °C, which coincides better with its biological niche. The overall reaction studied is depicted in (1).



(1)

Four different approaches were employed to determine the kinetic parameters, including the Lineweaver-Burk, Eadie-Hofstee y Hanes-Wolf plots as well as the hyperbolic adjustment of velocity vs. substrate concentration (Fig. 3).

Table 1 condenses the results obtained for the kinetic parameter determination. The lowest sd values are observed when using the Eadie-Hofstee plot. However, all parameters determined by the different approaches gave essentially the same values.

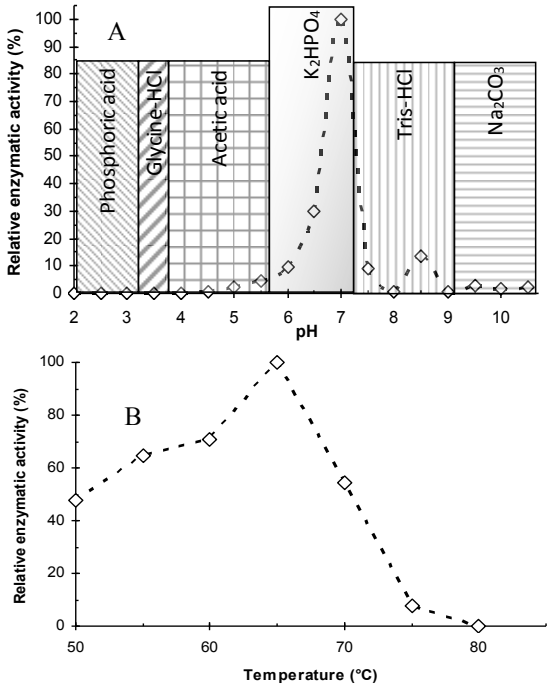


Fig. 2. pH and temperature dependence of XynB2. (A) Variation of the enzymatic activity of XynB2 vs pH at 65 °C. Buffer type are defined inside each box. (B) Variation of the enzymatic activity of XynB2 vs temperature at pH 6.5.

This work was sponsored by FONACIT N° PEII2012-925. Participation at the ICBMB-2014 was sponsored by the Etta Becker-Donner Program.

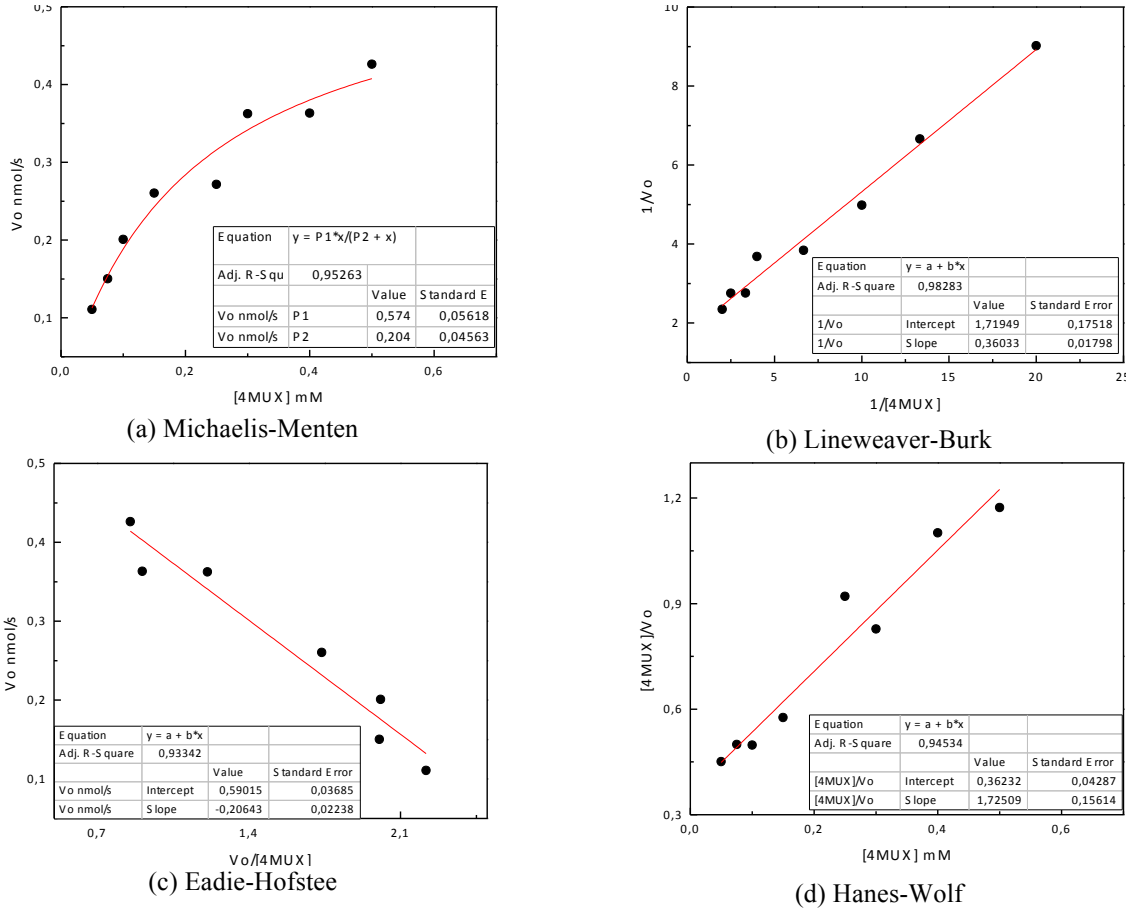


Fig. 3. Kinetic parameter determination of the hydrolysis reaction of 4MUX catalyzed by XynB2 employing (a) Michaelis-Menten hyperbolic adjustment, (b) Lineweaver-Burk plot, (c) Eadie-Hofstee plot and (d) Hanes-Wolf plot.

TABLE I. KINETIC PARAMETERS OF THE HYDROLYSIS OF 4MUX BY XYNB2.

Method /Formula	**K_M ± Δsd (mM)**	**V_{max} ± Δsd (nmol·s^{-1})**	**k_{cat} ± Δsd (s^{-1})**	**k_{cat}/K_M ± Δsd ($mM^{-1}·s^{-1}$)**
Hyperbolic $Vo = V_{max}[S]/(K_M + [S])$	0.20 ± 0.04	0.57 ± 0.06	203 ± 21	1017 ± 230
Lineweaver-Burk $1/Vo = K_M/V_{max}(1/[S])+1/ V_{max}$	0.21 ± 0.01	0.58 ± 0.06	208 ± 21	992 ± 113
Eadie-Hofstee $Vo = - K_M(Vo/[S]) + V_{max}$	0.21 ± 0.02	0.59 ± 0.03	211 ± 11	1003 ± 108
Hanes-Wolf $[S]/Vo = (1/V_{máx})[S] + (K_M/V_{max})$	0.21 ± 0.03	0.58 ± 0.05	207 ± 19	986 ± 172

Here, the results obtained by the Eadie-Hofstee were taken as final values, i.e. K_M= 0.21 mM; V_{max}= 0.59 nmol·s^{-1} and k_{cat} = 211 s^{-1} at pH 6.5 and 65 °C. For XynB2 at 40 °C, other values has been reported though [K_M= 0.35 mM; k_{cat} = 80 s^{-1}] [12]. The 25 °C difference evidently influences the kinetic energy associated to the molecules during the hydrolysis, making that the product releases faster from the ES intermediate complex, consequently increasing the k_{cat}. No other values have been reported though for higher temperatures.

D. Thermal stability profile of XynB2

To calculate the mean deactivation temperature, a Boltzmann adjustment was done, that corresponded to the tendency of the results shown (Fig. 4). XynB2 is a homodimer, with two monomers making a total molecular weight of 150,000 Da, and its thermal denaturation is irreversible [11] and leads to an associated activity loss, because once the enzyme is exposed to higher temperatures, an unfolding process occurs, the protein is decompressed, consequently

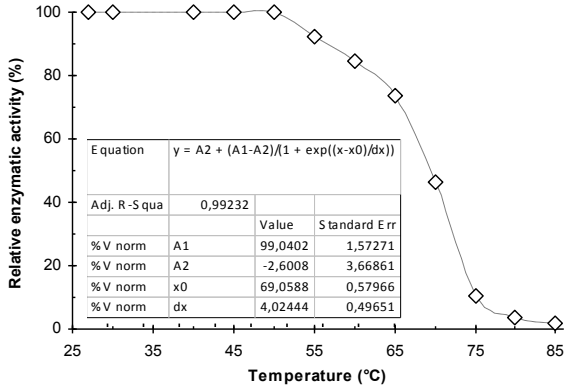


Fig. 4. Boltzmann adjustment for the thermal stability profile study of XynB2 employing 0.075 mM 4MUX as substrate

damaging irreversibly the active site, formed by amino acids residues from both monomers [11,12].

Calculations gave for XynB2 in the presence of 4MUX a T_m of 69.0 ± 0.6 °C, relating well with values reported in the range 60-80 °C [26,27]. There is the possibility as well, that the substrate might stabilize the enzyme. The tight bond E-S present in the native conformation may result in a reduced sensibility of the enzyme towards thermal denaturation, possibly as a consequence of a conformational change [28].

E. Inactivation kinetic of XynB2

An inactivation kinetic was performed to evaluate protein stability throughout time at maximal activity temperature (65 °C), quantifying residual activity. Fig. 5 shows the activity decay of XynB2 during constant heating for 5 h.

Half-life values were calculated with the slope of the equation from the plot ln(relative enzyme activity %) vs. time (Fig. 5B). Linear adjustments correspond to a first-order reaction, where $t_{1/2}$ describes more appropriately the behavior and adjusts to experimental values obtained. $t_{1/2}$ calculated was 116 min, and is ~300% higher than for other $t_{1/2}$ reported elsewhere, viz. $t_{1/2}$ 35 min at 80 °C (pH 9.0) for *Bacillus halodurans* TSEV1 [29]; $t_{1/2}$ 35 min at 57.5 °C (pH 5.0) for *Geobacillus thermoleovorans* IT-08 [30]. This leads to think that XynB2 is indeed an ideal enzyme candidate for industrial purposes.

IV. Conclusions

The purification strategy used was simple, obtaining ~10 mg XynB2 from a 750 mL culture. Using 4MUX as substrate, maximal activity was reached at pH 7 and 65 °C. However, pH 6.5 was chosen for assay purposes. Kinetic parameters were calculated to be K_M= 0.21 mM; V_{max}= 0.59 nmol·s^{-1} and k_{cat} = 211 s^{-1} at pH 6.5 and 65 °C. The relationship k_{cat} /K_M was 1003 (mM^{-1}·s^{-1}). XynB2 presented a $t_{1/2}$ of 116 min, which is about 300% higher in comparison to other values reported, though comparison is unsmooth, as differences in catalysis conditions are involved (temperature, pH, substrate

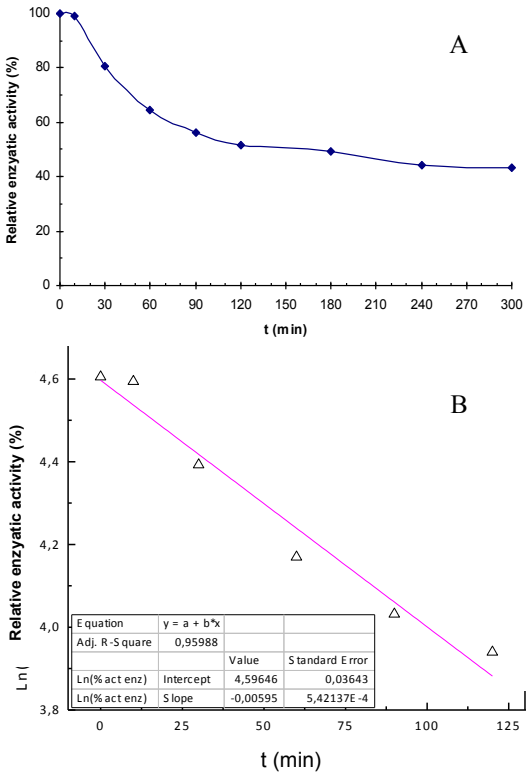


Fig. 5. Thermal inactivation of XynB2 (A) XynB2 relative enzymatic activity vs time. (B) Linear adjustment by plotting ln(realtive enzymatic activity %) vs time.

and microorganism), though all belong to a thermophilic origin. A progressive advance about the biochemistry of this enzyme was achieved by this study. Further work is yet to be done, involving other substrates and analyzing possible inhibitors during the catalytic reaction.

Acknowledgment

The authors thank the support of *Consejo de Desarrollo Científico y Humanístico*, Universidad de Carabobo and the Fondo Nacional de Ciencias y Tecnología, FONACIT-PEII2012-925, as well as the Etta Becker-Donner Program.

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