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Caulobacter crescentus β-Xylosidase II Is Highly Tolerant to Inhibitors Present in Fermentative Processes Involving Lignocellulosic Biomass

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Abstract

More stable enzymes with improved properties are in constant demand for various biotechnological processes. In this work, it was found that the activity of recombinant purified β-xylosidase II (CcXynB2) from the bacterium *Caulobacter crescentus* is increased 62% by 5 mM KCl, likely due to the presence of K^+ ions. CcXynB2 activity was measured in the presence of various compounds that have been described as inhibitors of lignocellulosic biomass hydrolysis and fermentation. CcXynB2 was found to be 61% more tolerant than 200 mM ethanol over a 48-h incubation at 37 °C. The specific activity of CcXynB2 was determined in the presence of phenol, hydroxymethylfurfural, ferulic, acetic, and coumaric acids; arabinose, glucose, xylose, and pectin. After 48 h, CcXynB2 activity in the presence of these compounds was found to be equal to 100% or higher than CcXynB2 activity in the absence of these compounds. When inhibitors were used in combination, CcXynB2 retained 67% of its initial activity over 48 h at 37 °C. Enzymatic hydrolysis of hemicellulose from corncobs was performed with CcXynB2 alone or with CcXynB2 in conjunction with recombinant xylanase and β-glucosidase-β-xylosidase-α-arabinosidase from *C. crescentus* producing reducing sugars. The immobilized CcXynB2 was more active than the soluble enzyme at both temperatures tested, 37 and 50 °C. In addition, immobilized CcXynB2 retained most of the enzyme activity incubated at 50 °C than at 37 °C, maintaining more than 70% of its initial activity after 1 h of incubation. In general, the CCXynB2 has potential to be applied in a bioprocess since it showed robust resistance in presence of many chemical compounds that may be generated during fermentation steps and physicochemical pre-treatment of biomass if considering a simultaneous saccharification fermentation process in a biorefinery.

Keywords Recombinant β-xylosidase · Cellulosic ethanol · Hydrolysis · Immobilization

Introduction

The enzyme system responsible for the degradation of hemicellulose is composed of xylanase (EC 3.2.1.8), β-xylosidases (EC 3.2.1.37), and other accessory enzymes that act on the xylan polymer. β-1, 4-Endoxylanase cleaves glycosidic linkages in the xylan main chain, releasing xylo-oligosaccharides and xylobiose. These products are then converted to xylose by β-D-xylosidases [1]. The use of these enzymes to hydrolyze hemicellulose is advantageous for the production of cellulosic ethanol for several reasons: after optimization, enzymatic hydrolysis occurs efficiently, can be conducted at mild temperature and pH conditions, and generates less pollution than conventional chemical processes while generating high levels of fermentable sugars [2, 3, 4].

Using enzymes that are compatible with fermentation processes is critical. In general, the most effective microorganisms for producing cellulosic ethanol are mesophilic and, usually, they ferment carbohydrates at temperatures between 28 and 37 °C [5, 6]. Enzymes that are stable and/or tolerant of the inhibitory compounds generated during the lignocellulosic biomass hydrolysis process [7–9], which liberates fermentable sugars, are required. In simultaneous saccharification and

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fermentation (SSF), sugars released by enzymatic hydrolysis are directly converted by microorganisms to value-added products [7]. This process is often conducted in a single reactor, allowing investment costs to be reduced [8].

During the biological pre-treatment, hemicellulose is broken down and compounds such as glucose, arabinose, xylose, mannose, galactose, and acetic acid sugars containing 5 and 6 carbons are generated by enzymes [9]. Cellulose is hydrolyzed to glucose. In the physical-chemical pre-treatment at high temperatures and pressure, arabinose and xylose are converted to furfural and hydroxymethylfurfural (HMF) is formed during the degradation of hexoses [10]. In addition to the levulinic acid that can be obtained from HMF, formic acid may be generated when furfural and HMF are degraded. Additional phenolic compounds may be released during the partial chemical decomposition of lignin [9–11]. The identification of inhibitors resulting from the hydrolysis of lignocellulosic residues allows the toxic effects of these compounds to be minimized via optimization of pre-treatment and hydrolysis conditions [12].

Caulobacter crescentus is a Gram-negative bacterium found in various soil and aquatic environments. The genome of *C. crescentus* NA1000 has been sequenced [13] and genome analysis revealed that the bacterium possesses at least 7 genes that are directly involved in xylan degradation, two genes encode xylanases and five genes encode β-xylosidases. The *xynB2* gene product, a recombinant GH39 β-xylosidase II (CCXynB2), was chosen in the present report because demonstrated more than 50% of its original activity (immediately after purification) after long periods (6 months) of incubation at pH 6.0 and 4 C [14]. So, the present study aimed to characterize CcXynB2 from *C. crescentus* in the presence of compounds that can act as enzyme cofactors or inhibitors of the fermentation processes. The study also aimed to determine the suitability of the purified CcXynB2 for future biotechnological processes. The ability of the enzyme to hydrolyze hemicellulose obtained from corn cobs was examined in the presence and absence of other enzymes. Finally, the effect of immobilization on the catalytic performance of CcXynB2 was evaluated.

Materials and Methods

Bacteria, Growth, and Culture Conditions

Escherichia coli (DH10B) carrying a pPROEX-HTa plasmid containing *C. crescentus* (NA1000 strain) *xynB2* fused to a N-terminal histidine tag was used [14]. The construct was stored at − 80 °C in 50% glycerol. Luria-Bertani medium (LB) containing ampicillin (1 mg mL^{-1}) at 37 °C was used for maintenance and growth of *E. coli*.

Purification of *C. crescentus* β-Xylosidase II (CcXynB2)

CcXynB2 was produced and purified according to the method described by Corrêa et al. [14] with the following modifications: *E. coli* cells (DH10B) containing the construct pPROEX-*xynB2* were incubated at 37 °C in LB medium containing ampicillin (1 mg mL^{-1}) in a rotating incubator at 120 rpm. Cells were grown to log phase (OD600nm = 0. 1–0.6), then induced for 1 h with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) to express the *C. crescentus xynB2* gene. A 20 mL aliquot of the induced recombinant cells was centrifuged for 20 min at 1 °C and 5000×*g*, and the cell pellet was resuspended in 1 mL of 1X FastBreakTM Cell Lysis Solution (Promega®) containing 10 μL of 40 mg mL^{-1} lysozyme (Sigma), 122 U μL^{-1} of DNAse A (Invitrogen), and 10 μL of protease inhibitor cocktail (GE Healthcare). The mixture was incubated for 30 min at 25 °C on a rotating incubator at 80 rpm. Lysed cells were centrifuged, and the supernatant was transferred to a pre-packed Ni-Sepharose column (Sigma). Following binding, wash steps were performed with binding buffer 20 mM phosphate buffer (pH 7. 1) containing 500 mM NaCl and 10 mM imidazole. The recombinant protein was then eluted using elution buffer 20 mM phosphate buffer (pH 7. 1) containing 500 mM NaCl and 500 mM imidazole. Following elution, the recombinant protein was dialyzed against 20 mM phosphate buffer pH 7. 1 and stored at 1 °C prior to further biochemical characterization.

Assay of β-Xylosidase Activity and Total Protein Determination

The β-xylosidase activity of CcXynB2 was determined by measuring the amount of ρ-nitrophenol liberated from the substrate ρ-nitrophenyl-β-D-xylopyranoside (pNPX) (Sigma) as described by Corrêa et al. [14]. One unit (U) of β-xylosidase activity was defined as the amount of enzyme capable of releasing 1 μmol of ρ-nitrophenol per minute. Protein concentration was measured using the Bradford reagent from Bio-Rad with bovine serum albumin (BSA) as a standard.

Ion Effect on CcXynB2 Activity at Fermentation Process Temperatures

The effect of different ions on CcXynB2 activity was evaluated at 37 °C [15]. The enzyme was incubated in the presence of various compounds at 1 °C for 15 min: $(NH_4)_2SO_4$, HgCl, $MgSO_4$, $BaCl_2$, NH_4Cl, iodoacetamide, $Al_2(SO_4)_4$, $MnSO_4$, $FeSO_4$, $SnCl_2$, KCl, NaCl, $CaCl_2$, $CuSO_4$, $ZnSO_4$, EDTA, dithiothreitol (DTT), β-mercaptoethanol, $MgCl_2$, EGTA, $MgCl_2$+EDTA and $CaCl_2$+EGTA. All compounds were added at 2 mM. β-Xylosidase activity was then measured as described previously.

Influence of KCl in the CcXynB2 Activity

To determine the optimal salt concentration for enzyme activity, the catalytic behavior of the enzyme was evaluated at different KCl concentrations (0, 0.1, 0.5, 1, 2, 1, 4, 5, 7.5, and 10 mM). After incubation in different concentrations of KCl at 4 °C for 15 min, activities were measured by incubating the β-xylosidase II + KCl mixture with pNPX substrate according to the test conditions described by Corrêa et al. [14]. Enzymatic activity is expressed as U mL^{-1}. CcXynB2 incubated with the pNPX substrate in the absence of KCl was used as a control.

Influence of KCl on the Catalytic Efficiency of CcXynB2

To determine the catalytic efficiency of CcXynB2, we determined Km and Vmax using increasing concentrations of pNPX (0, 0.5, 1, 2, 1, 4, 5, 10, 15 mM) in sodium phosphate buffer 50 mM pH 6 in the absence and presence of KCl (0–10 mM). Reaction mixtures were incubated at 55 °C for 10 min [14]. Kinetic parameters were determined from dual reciprocal graphs. The catalytic efficiency is expressed as the ratio of K_{cat} (catalytic constant), which was calculated based on the experimental molecular mass of CcXynB2 determined via SDS-PAGE, to the observed K_M for pNPX under optimal assay conditions.

Effect of Different Compounds on β-Xylosidase Activity

To use CcXynB2 in a biotechnological process such as cellulosic ethanol production, the effects of compounds commonly known to inhibit fermentations were determined. CcXynB2 was incubated in the presence of these compounds (ethanol, phenol, hydroxymethylfurfural, acetic acid, formic acid, coumaric acid, ferulic acid, and pectin) at concentrations ranging from 1 to 200 mM. In parallel, CcXynB2 was also incubated in the presence of various saccharification products (arabinose, glucose, xylose at 1 to 200 mM). Enzyme activity was measured using pNPX as substrate at a typical fermentation temperature (37 °C) for 48 h. Two additional tests were performed for 48 h at 37 °C: the first test was performed with a mixture of compounds (ethanol, phenol, hydroxymethylfurfural, arabinose, glucose, xylose, pectin, acetic acid, formic acid, coumaric acid, and ferulic acid) at various concentrations (1 to 50 mM), while the second test was performed with the same set of compounds at 37 °C and concentrations in which CcXynB2 was most active, as follows: 200 mM ethanol, phenol, hydroxymethylfurfural, ferulic acid, arabinose, glucose, xylose, and pectin; 10 mM acetic acid; 30 mM coumaric acid; and 50 mM formic acid. Enzymatic activity is expressed as the percentage of specific activity (U mg^{-1}) compared with the specific activity of CcXynB2 in the absence of any inhibitor compounds.

Negative controls, which involved incubating the various compounds with pure enzyme with or without pNPX, were performed for each of the compounds to assess any interference that could generate false results. The effects of classically described enzyme inhibitors were evaluated in relation to a positive control (the enzyme in the total absence of inhibitor/enhancer compounds) and two negative controls (1: addition of compound and the pNPX substrate without enzyme; 2: addition of compound and enzyme without the pNPX substrate). These negative controls were performed to ensure that the various compounds investigated in this study were not able to generate positive chromogenic reactions in the absence of substrate or enzyme. Such positive reactions might be identified during spectrophotometric measurements of enzyme activity, leading to falsely positive results. All assays were performed as previously described and in biological duplicates. Experimental trials were performed in triplicate.

Hemicellulose Preparation from Corn Cobs

Corn cobs were dried at 70 °C for 16 h followed by trituration (SL30 slicer Willey) and sifting using a 20-mesh sieve and stored in glass vials at room temperature. Corn cob pretreatment was performed to extract hemicellulose for subsequent use in enzymatic hydrolysis assays. These assays used corncob hemicellulose obtained via the following auto-hydrolysis process: approximately 80 g of dried corn cob was ground to a homogeneous mixture with water at a 10:1 liquid/solid (g mL^{-1}) ratio. Specific glass tubes were used for auto-hydrolysis. Pre-treatment was performed for 1 h in a block digester heated to 200 °C. Following pre-treatment, samples were immediately cooled in an ice bath. The hemicellulose-containing liquid phase was separated via filtration from the solid fraction, which contained agro-residue remains. Three volumes of ethanol (PA) were added to the isolated liquid fraction to promote the precipitation of hemicellulose, and the liquid fraction was incubated at 25 °C for 16 h. The precipitate was recovered by filtration and dried at 37 °C for 16 h. The hemicellulose was stored in sterile tubes until use. A total of 80 g of dry corn cob was ground and subjected to auto-hydrolysis, generating 2.7 g of hemicellulose after precipitation and drying. Auto-hydrolysis was carried out at high temperature in the present work and this procedure allows the isolation of a fully soluble hemicellulose free of cellulose, lignin, and low weight molecules that could act as inhibitors of the microorganism growth [16].

Enzymatic Hydrolysis of Hemicellulose from Corn Cobs

Corn cob hemicellulose was diluted with water to a final concentration of 1% and incubated in sterile glass tubes with 2 U of the following enzyme mixtures: CcXynB2, BglX-V-Ara (recombinant β-glucosidase- β-xylosidase-α-arabinosidase from *C. crescentus* [17], and XynA1 (recombinant xylanase I from *C. crescentus* [18] in the following way: XynA1; XynA1 + CcXynB2; Xyn1 + BglX-V-Ara; and XynA1 + CcXynB2 + BglX-V-Ara. Mixtures were incubated in water bath at pH 6 either 37 °C and 50 °C for 48 h. At defined time, aliquots of each reaction mixture were collected and subjected to enzyme activity assays as described above. Reducing sugars were then quantified in each aliquot. The results obtained from hydrolysis testing were normalized to a negative control reaction performed without the addition of enzymes. The enzymatic hydrolysis was carried out in the presence of inhibitors using the combination of recombinant enzymes from *C. crescentus*, XynA1+ CcXynB2 + BglX-V-Ara, at 37 °C during 0 to 48 h. Enzymes were incubated with a combination of ethanol, phenol, hydroxymethylfurfural, ferulic acid, and pectin at 200 mM; acetic acid at 10 mM; coumaric acid at 30 mM; and formic acid at 50 mM. The results were expressed as mean data from two different experimental tests with triplicate measurements of each sample.

Reducing Sugar Dosages

After hydrolysis tests, reducing sugars were quantified according to the DNS method (4, 5-dinitro-salicylic acid) described by Miller [19]. A 100 μL aliquot of the mixture obtained from enzymatic hydrolysis was added to 100 μL of DNS reagent. Samples were boiled for 5 min, cooled to 4 °C and diluted with 1 mL of distilled water. Absorbances were read at $\lambda = 540$ nm using water and DNS as controls. Reducing sugar content in the various samples was estimated using a previously generated xylose standard curve. The data are expressed in μmol mL^{-1} as the average of results obtained from two independent experiments, with three replicates for each test.

Enzyme Immobilization

CcXynB2 was immobilized on Ni-Sepharose Excel resin (GE Sigma) following expression and lysis as described above (see Purification of β-xylosidase II). A total of 2 U of immobilized and soluble CcXynB2 were incubated at 37 or 50 °C for up to 5 h. At defined times, aliquots of both soluble and immobilized enzyme were incubated with pNPX to determine β-xylosidase activity. Enzymatic activities were also measured after the soluble and immobilized enzymes were frozen at − 20 °C for a period of 1 to 30 days. The data are expressed as relative percentages of residual enzymatic activity compared with the activities of soluble and immobilized pure enzyme stored at 4 °C after purification.

Results

Purification of CcXynB2

The amount of fusion protein expressed and the quality and purity of the purified recombinant enzyme was visualized by SDS-PAGE (Fig. S1). As demonstrated by Corrêa et al. [14], a considerable amount of recombinant protein can be observed by gel analysis. The recombinant protein appears as a single band of approximately 60 kDa. The CcXynB2 enzyme displayed an activity of 2484 U mL^{-1} and a specific activity of 76.7 U mg^{-1}.

Effect of Ions

Table 1 shows the effect of different compounds on enzyme activity at 37 and 50 °C. Activities were measured at different

Table 1 Effect of ions and different compounds on the enzymatic activity of CcXynB2

Relative enzymatic activity (%)		
Substance (2 mM)	50 °C	37 °C
Control	100 ± 0.10	100 ± 0.01
$(NH_4)_2SO_4$	63 ± 0.14	71 ± 0.02
HgCl	48 ± 0.90	39 ± 0.03
$MgSO_4$	24 ± 0.40	16 ± 0.01
$BaCl_2$	26 ± 0.10	20 ± 0.03
NH_4Cl	23 ± 0.30	17 ± 0.03
Iodoacetamide	23 ± 0.20	36 ± 0.04
CuCl	41 ± 0.30	nd
$Al_2(SO_4)_3$	36 ± 0.40	57 ± 0.11
$MnSO_4$	77 ± 0.04	74 ± 0.02
$FeSO_4$	47 ± 0.30	65 ± 0.01
$SnCl_2$	44 ± 0.02	41 ± 0.07
KCl	132 ± 0.19	135 ± 0.01
NaCl	25 ± 0.20	82 ± 0.01
$CaCl_2$	31 ± 0.70	14 ±0.02
$CuSO_4$	23 ± 0.03	27 ±0.01
$ZnSO_4$	47 ± 0.06	48 ± 0.03
EDTA	23 ± 0.03	19 ± 0.03
DTT	23 ± 0.03	18 ± 0.01
β-Mercaptoethanol	65 ± 0.07	52 ± 0.07
Source	[15]	This work
$MgCl_2$	90 ± 0.10	84 ± 0.05
EGTA	133 ± 0.01	106 ± 0.05
$MgCl_2$ + EDTA	106 ± 0.12	101 ± 0.01
$CaCl_2$ + EGTA	116 ± 0.01	102 ± 0.04
Source	This work	This work

nd not determined

temperatures and results were not different from those obtained by Corrêa et al. [14].

Activity increases were observed in the presence of KCl at both 37 and 50 °C. Practically all other compounds had a negative effect on CcXynB2 activity. To date, no studies have satisfactorily determined whether this activity increase is due to the presence of K^+ or Cl^-. However, an interesting result was obtained when enzyme assays were conducted in the presence of $CaCl_2$ and $MgCl_2$. Assays were performed in the presence of either EDTA (to chelate Mg^{2+} ions) or EGTA (to chelate Ca^{2+} ions), allowing the effect of the Cl^- ion alone to be analyzed. These assays demonstrated that Cl^- ions alone were not able to inhibit CcXynB2 at 37 or 50 °C, suggesting that the enhancement of β-xylosidase II activity in the presence of KCl is due to the K^+ ion.

Influence of KCl on CcXynB2 Activity

Although KCl enhances CcXynB2 activity by 21% under optimal conditions (50 °C) [14], activity enhancement by KCl varied only slightly at 37 °C, between 21 and 35% (Table 1). To determine the optimal KCl concentration for CcXynB2 activity enhancement, CcXynB2 was incubated with different concentrations of KCl (Fig. 1). From Fig. 1, it is clear that 5 mM KCl results in the most activity enhancement: without KCl, CcXynB2 activity was 26 U mL^{-1}; with 5 mM KCl, CcXynB2 activity was 42 U mL^{-1}, a 62% improvement in activity. KCl functions as an activity enhancer up to a concentration of 5 mM. Further increases in salt concentration resulted in decreased β-xylosidase activity (Fig. 1).

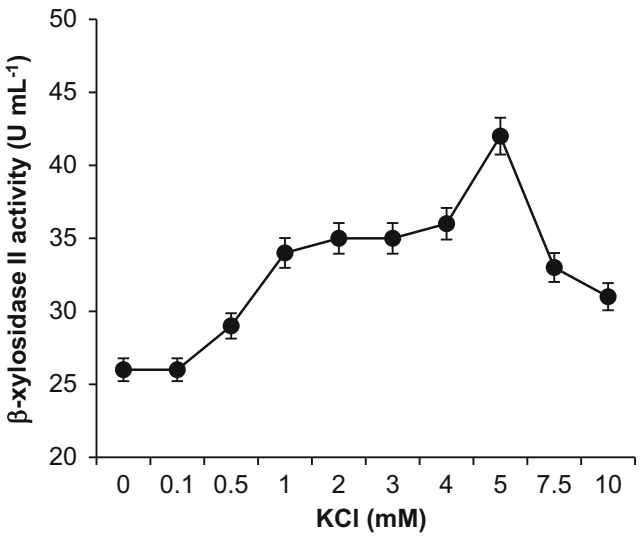

Fig. 1 Influence of various KCl concentrations on CcXynB2 activity. Vertical bars indicate standard deviations

Determination of CcXynB2 Catalytic Efficiency in the Presence of KCl

To assess the catalytic efficiency of CcXynB2 in the presence of KCl, the V_{Max} and K_M of the enzyme were determined in the presence and absence of KCl. The results were compared with data previously reported by Corrêa et al. [14]. The kinetic data allowed the specificity constant (K_{cat}/K_M) of CcXynB2 in the presence of KCl to be determined. Although enzymatic activity is increased approximately 62% in the presence of 5 mM KCl (Fig. 1), catalytic efficiency is unchanged: the catalytic efficiency of CcXynB2 in the presence of KCl is nearly equivalent to the catalytic efficiency of CcXynB2 in the absence of KCl (Table 2).

Effect of Different Compounds on the Specific Activity of CcXynB2

The influence of different compounds on CcXynB2 activity was determined by measuring specific enzyme activity values relative to the specific activity of a pure enzyme in the absence of any interfering compounds. The specific activity of the pure enzyme in the absence of interfering compounds, which represented the positive control for all experiments, was set at 100%, and other activities were calculated as percentages below or above this reference value.

Effect of Ethanol, Phenol, and Hydroxymethylfurfural

In these tests, it was found that the addition of ethanol, phenol, and HMF (Fig. 2a–c) separately did not affect the relative specific activity of CcXynB2 after 48 h of incubation. In all concentrations of ethanol used, the specific activity of CcXynB2 was higher than that observed in the control tubes during the 48 h of incubation (Fig. 2a). The enzyme remained active in the concentrations of 5 to 10 mM of phenol and lost 20% of its activity after 48 h of testing at concentrations other concentrations (50 to 200 mM) used. So, CcXynB2 was tolerant to phenol only at low concentrations (5 to 10 mM, Fig. 2b). Phenol concentrations exceeding 50 mM caused a

Table 2 Effect of KCl on the kinetic parameters of CcXynB2 at 55 °C

	β-Xylosidase II	
Kinetic parameters	Control	KCl 5 mM
V_{Max} (μmoles min^{-1} mg^{-1})	402	479
K_M (mM)	9.3	11.3
K_{cat} (s^{-1})	402	479
K_{cat}/K_M (mM^{-1} s^{-1})	43.3	42.4
Source	[15]	This work

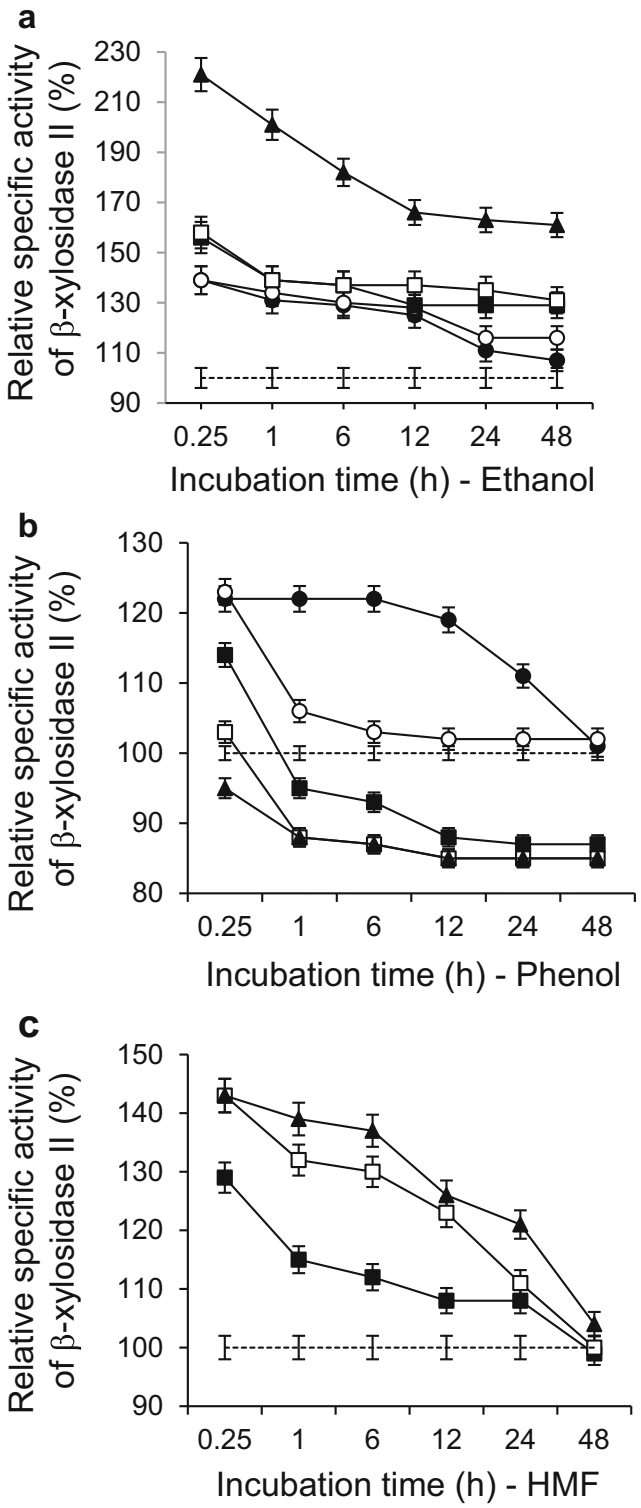

Fig. 2 Effect of ethanol, phenol, and HMF on β-xylosidase II activity. Specific activity of CcXynB2 in the presence of different concentrations of ethanol (**a**), phenol (**b**), and HMF (**c**). Compounds were added at concentrations of 5 mM (closed circle), 10 mM (open circle), 50 mM (closed square), 100 mM (open square), and 200 mM (closed triangle). The pure enzyme in the absence of inhibitor is shown as a dashed line. Enzymatic activity was expressed as the percentage of specific activity (U mg^{-1}) compared with the specific activity of CcXynB2 in the absence of inhibitor

reduction in the specific activity compared with the control, reducing CcXynB2 activity to 85% of the control (Fig. 2b). CCxynB2 maintained activity higher than that observed in the control at concentrations of 50 to 200 mM of HMF. At the highest concentration of HMF tested, the best catalytic performance of β-xylosidase II occurred in the first 15 min of incubation (Fig. 2c).

Arabinose, Glucose, Xylose, and Pectin

Activity assays carried out in the presence of sugar and pectin revealed that arabinose (Fig. 3a) had a inhibitory effect at 5, 10, 100, and 200 mM during the first 15 min of incubation. Activity was only 72% of the control at the highest arabinose concentration. However, over time, the enzyme seems to acquire tolerance to the compound, with activities equal to or higher than the control.

The specific activity of CcXynB2 in the presence of glucose and xylose was higher than the control: CcXynB2 was 114% more active (Fig. 3b) in 50 mM glucose and 67% more active in 200 mM glucose compared with the control. CcXynB2 was 44% more active in 200 mM compared with the control after 48 h (Fig. 3c).

In the presence of pectin, CcXynB2 retained 63% of its initial activity after 15 min of incubation in 200 mM pectin. Enzyme activity was highest after 48 h of incubation when pectin was added at 10 mM. At this concentration of pectin, CcXynB2 activity was 141% higher than the control (Fig. 3d). It should be noted that CcXynB2 demonstrated good pectin tolerance during the first 24 h of incubation in pectin concentrations between 5 and 50 mM (Fig. 3d).

Effect of Different Acids on the Activity of CcXynB2

The effects of different acids on CcXynB2 activity were evaluated at various acid concentrations. CcXynB2 was incubated at 37 °C for 48 h in 1-10 mM acetic acid (Fig. 4a), 1-50 mM formic acid (Fig. 4b), 1 to 50 mM coumaric acid (Fig. 4c), and 5-200 mM ferulic acid (Fig. 4d). In the presence of 5 to 10 mM acetic acid, CcXynB2 activity dropped below that of the control after only 30 min of incubation (Fig. 4a). However, tolerance to low concentrations of acetic acid (1 mM) is evident, as enzyme activity was 20% higher in 1 mM acetic acid compared with the control. In the presence of 5 to 10 mM formic acid, CcXynB2 was 50% more active than the control after 48 h of incubation (Fig. 4b). However, activity dropped dramatically after 1 h of incubation with higher concentrations of formic acid (30 to 50 mM). Similarly, CcXynB2 activity dropped below that of the control after 40 min of incubation in the presence of 1-10 mM coumaric acid. Coumaric acid concentrations greater than 30 mM sharply reduced CcXynB2 activity after 1 h of incubation to less than 50% of its original activity (Fig. 4c).

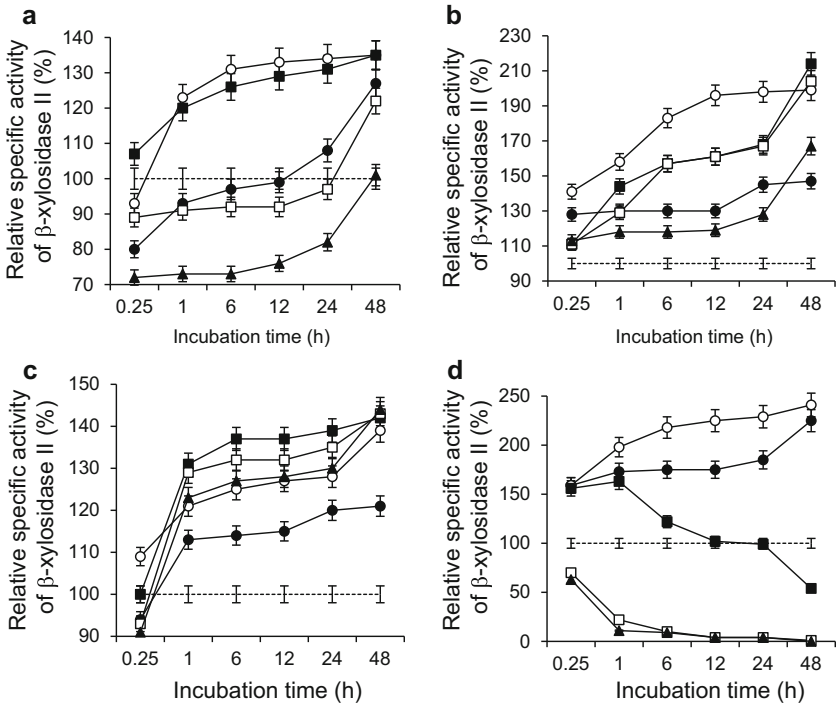

Fig. 3 Effect of arabinose, glucose, xylose, and pectin on CcXynB2 activity. Specific activity of CcXynB2 in the presence of various concentrations of arabinose (**a**), glucose (**b**), xylose (**c**), and pectin (**d**). In all graphs, the positive control is shown as a dashed line. Compounds were added at concentrations of 5 mM (closed circle), 10 mM (open circle), 50 mM (closed square), 100 mM (open square), and 200 mM (closed triangle)

In terms of acid tolerance, better results were obtained with 5 to 200 mM ferulic acid (Fig. 4d). During 48 h of incubation with 5 to 200 mM ferulic acid, β-xylosidase II specific activities were close to or higher than that of the control. In fact, CcXynB2 retained nearly 100% of its original activity after 48 h of incubation with 50 to 200 mM ferulic acid. For incubation times below 6 h, the enzyme was extremely tolerant to the presence of ferulic acid at concentrations ranging from 5 to 200 mM. In terms of incubation time and acid concentration, CcXynB2 activity was highest during the first hour of

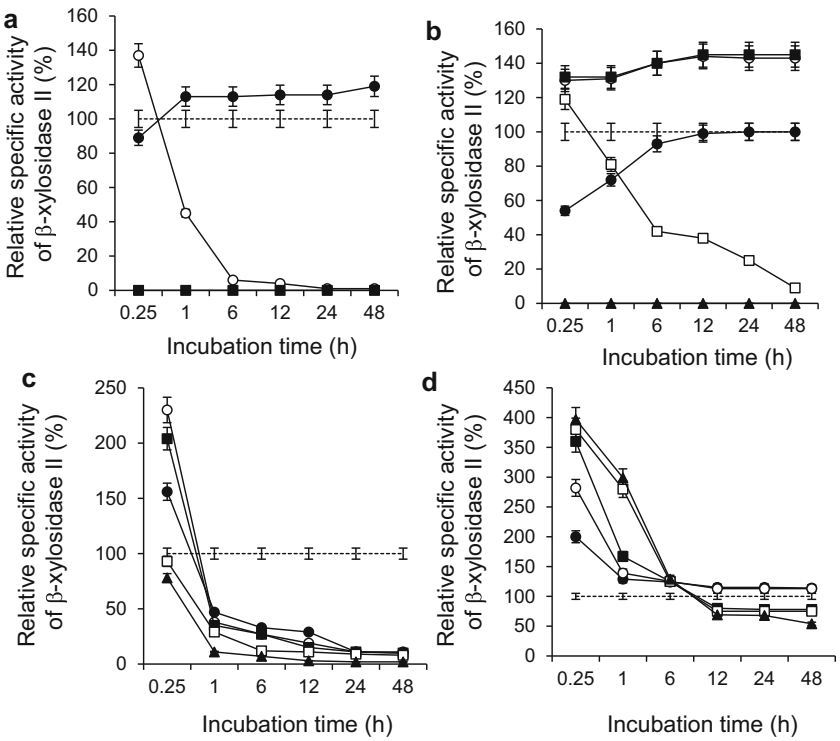

Fig. 4 Effect of acetic, formic, coumaric, and ferulic acids on CcXynB2 activity. Specific activity of CcXynB2 in the presence of various concentrations of acetic acid (**a**), formic acid (**b**), coumaric acid (**c**), and ferulic acid (**d**). In all graphs, the positive control is shown as a dashed line. In (**a**)–(**c**), compounds were added at concentrations of 1 mM (closed circle), 5 mM; open circle, 10 mM (closed square), 30 mM (open square), and 50 mM (closed triangle). In (**d**), compounds were added at concentrations of 5 mM (closed circle), 10 mM (open circle), 50 mM (closed square), 100 mM (open square), and 200 mM (closed triangle)

incubation in the presence of up to 200 mM ferulic acid. Under these conditions, the specific activity of CcXynB2 was three times higher than that of the control (Fig. 4d).

Effect of a Combination of Compounds

In a simultaneous saccharification and fermentation process for cellulosic ethanol production, various inhibitory compounds capable of inhibiting hemicellulose-degrading enzymes are produced. Therefore, an assay was performed using a mixture of all of the previously used compounds (ethanol, phenol, hydroxymethylfurfural, galacturonic acid in the pectin, acetic acid, formic acid, coumaric acid, and ferulic acid) at concentrations ranging from 1 to 50 mM, the average concentration range tolerated by CcXynB2. The enzyme retained 100% of its specific activity at concentrations of 1–10 mM (Fig. 5a) after 48 h. At concentrations ranging from 30 to 50 mM, CcXynB2 exhibited decreased specific activity after the first 6 h of incubation at 37 °C. At 30 mM, the enzyme retained 48% of its activity for 6 h (Fig. 5a). When inhibitors were combined and added at concentrations at which the enzyme showed higher performance than the control (Fig. 5b), CcXynB2 retained approximately 67% of its initial activity after 48 h.

Enzymatic Hydrolysis

The performance of CcXynB2 alone and in combination with one or more recombinant enzymes for hemicellulose degradation (e.g., xylanase [17] and a multifunctional GH3-BglX β-glucosidase-β-xylosidase-α-arabinosidase member in *C. crescentus* [18]) was examined by incubating these enzymes at 37 °C with hemicellulose obtained from corn cobs. After 0, 6, 12, 24, and 48 h of incubation, the amount of reducing sugars released by the enzymes was measured. For the various incubation times analyzed, it was evident that the synergistic action of the three enzymes was most efficient for hemicellulose hydrolysis at the two incubation temperatures investigated (Fig. 6a). For all of the incubation times analyzed, the combinations of XynA1 + CcXynB2 + BglX-V-Ara were more efficient than the combination of CcXynB2 + xylanase (Fig. 6a). Over 48 h of incubation, the XynA1 + CcXynB2 combination was 120–130% more efficient on average than the XynA1+ BglX-V-Ara to hydrolyze the hemicellulose at both 37 °C and 50 °C (Fig. 6a).

A positive correlation was observed between incubation time and the amount of sugar produced by each enzyme alone and by a combination of all three enzymes: the longer the enzymes were allowed to act individually or in concert, the greater amount of reducing sugar that was formed (Fig. 6). The enzymatic hydrolysis was carried out by the three recombinant enzymes from *C. crescentus* in the presence of various inhibitory compounds in the enzymes maintained 70% of the hydrolysis capacity after 48 h of incubation at 37 °C. These data suggest that, in addition to exhibiting high tolerance and catalytic ability in the presence of inhibitory compounds, CcXynB2 efficiently produces reducing sugars that can be then converted to commercially valuable compounds, even at temperatures far away from the enzyme's optimal temperature (Fig. 6b). Away from the optimal temperature, enzyme activity (in U mL^{-1}) is significantly lower, but the enzyme remains functionally active during the 48-h-long hydrolysis of hemicellulose from corn cobs (Fig. 6c).

Test of Immobilized CcXynB2

Immobilized CcXynB2 was more active than soluble enzyme at both of the temperatures tested (Fig. 7a). Interestingly,

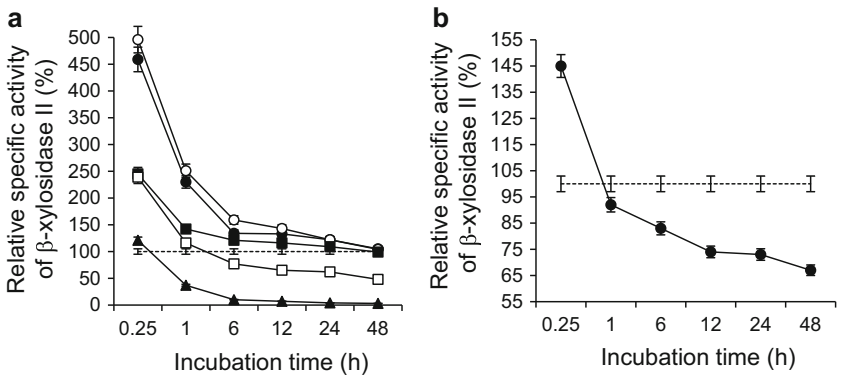

Fig. 5 Effect of a combination of compounds on CcXynB2 activity. **a** Specific activity of CcXynB2 in the presence of ethanol, phenol, HMF, arabinose, glucose, xylose, pectin, acetic acid, formic acid, coumaric acid, and ferulic acid at concentrations of 1–50 mM. Symbols correspond to concentrations of 1 mM (closed circle), 5 mM (open circle), 10 mM (closed square), 30 mM (open square), and 50 mM (closed triangle). **b** Specific activity of CcXynB2 during incubation with compounds at concentrations that enhanced enzyme activity in pre-trials (closed circle). CcXynB2 was incubated with a combination of ethanol, phenol, hydroxymethylfurfural, ferulic acid, arabinose, glucose, xylose, and pectin at 200 mM; acetic acid at 10 mM; coumaric acid at 30 mM; and formic acid at 50 mM. In both graphs, pure enzyme in the absence of inhibitors is shown as a dashed line

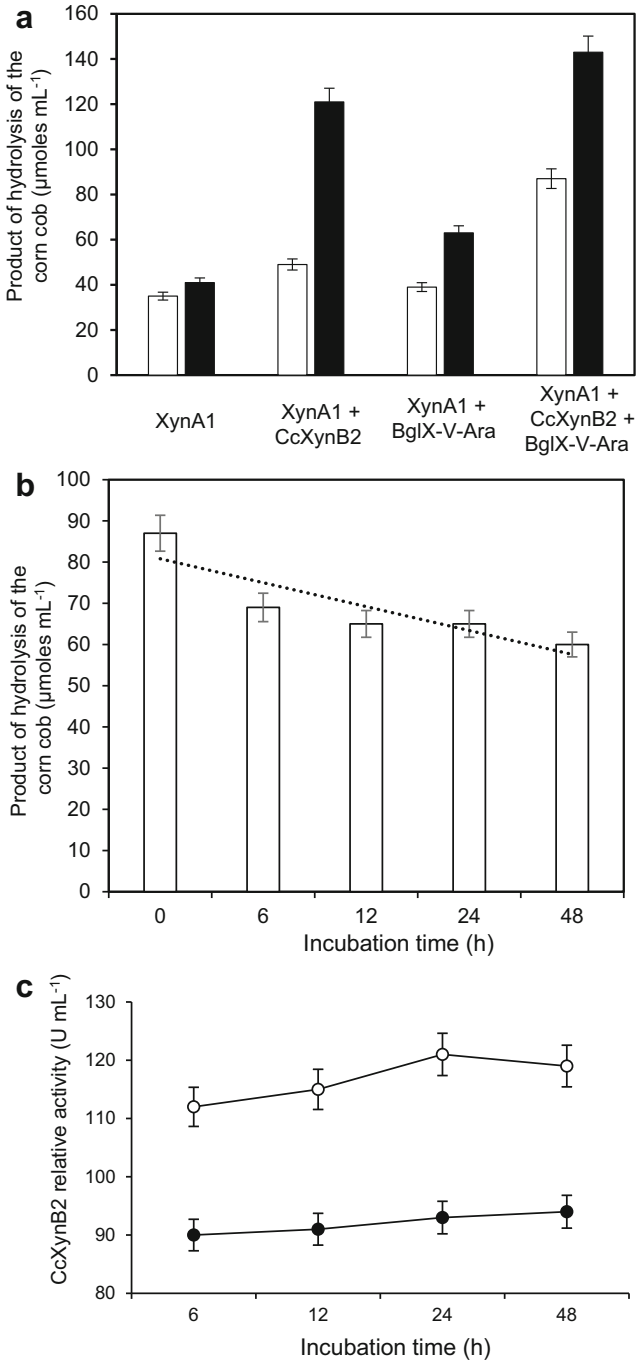

Fig. 6 **a** Reducing sugar content obtained after enzymatic hydrolysis of hemicellulose from corn cobs incubated with different recombinant enzymes from *C. crescentus* at 37 °C (white bars) and 50 °C (black bars). **b** Enzymatic hydrolysis performed with XynA1 + CcXynB2 + BglX-V-Ara in the presence of different inhibitors at 37 °C (combination of ethanol, phenol, hydroxymethylfurfural, ferulic acid, and pectin at 200 mM; acetic acid at 10 mM; coumaric acid at 30 mM; and formic acid at 50 mM). **c** β-Xylosidase activity after enzymatic hydrolysis of hemicellulose from pre-treated corn cobs at 37 °C (closed circle) and 50 °C (open circle)

immobilized CcXynB2 retained most of its enzymatic activity when incubated at 50 °C compared with 37 °C, retaining more than 70% of its initial activity after 1 h of incubation. At 50 °C,

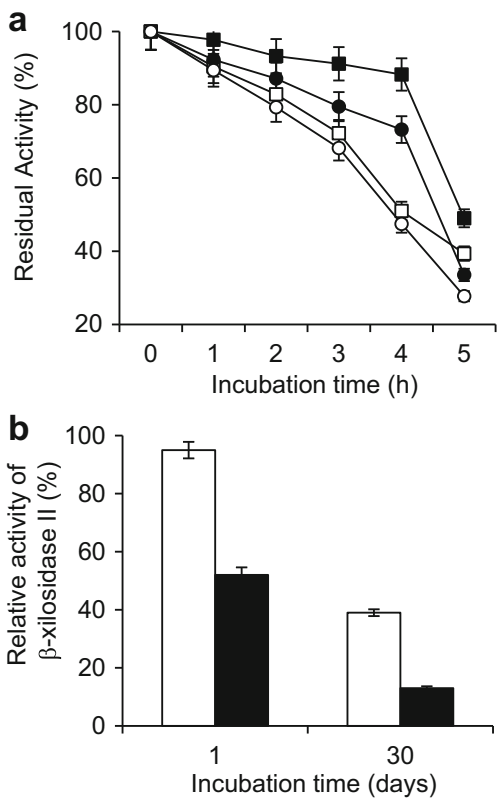

Fig. 7 Thermal stabilities of immobilized and free CcXynB2. **a** Activities of immobilized CcXynB2 (square and closed circles) and free CcXynB2 (square and open circles) were evaluated at 37 °C (circle) and 50 °C (square) over a 5-h incubation. **b** Stability of immobilized CcXynB2 after freezing for 30 days. Activities of immobilized CcXynB2 (open square) and free CcXynB2 (closed square) after one and 30 days of freezing

CcXynB2 retained 88% of its initial activity. At 37 °C, CcXynB2 retained 73% of its initial activity. In comparison, soluble CcXynB2 retained 51% of its initial enzyme activity at 50 °C and only 47% of its initial activity at 37 °C. These data show that the enzyme can be used at both 50 °C and 37 °C; however, the enzyme has a greater activity at 50 °C. This behavior is consistent with preliminary data indicating that the soluble pure enzyme has a temperature optimum at 55 °C but greater stability at 50 °C [14].

Previous enzyme characterization experiments indicated that CcXynB2 was able to maintain more than 50% of its catalytic activity even after 6 months of incubation at 1 °C [14]. However, this stability was not observed after freezing (data not shown). Thus, to verify that immobilization facilitates the maintenance of catalytic capacity, the immobilized enzyme was frozen at − 20 °C for 30 days, then thawed. For comparison, soluble enzyme was also subjected to the same test conditions. The immobilized enzyme was able to retain 39% of its specific activity after 30 days of frozen storage (Fig. 7b), while the soluble enzyme retained 4-fold less activity (13%).

Discussion

C. crescentus genes encoding β-xylosidases *xynB1*, *xynB2*, and *xynB5* [2, 14, 16–18, 21] and a gene coding endoxylanase (*xynA1*), a family 10 of glycosyl-hydrolase (GH-10) [17] have been expressed, purified, and analyzed. CcXynB2 is encoded by *xynB2* and has the ability to hydrolyze xylooligosaccharides, has an optimum pH of 6, an optimum temperature of 55 °C, exhibits stability at 50 °C, indicating a certain degree of thermotolerance and suggesting that the enzyme may be robust enough for biotechnology applications at non-physiological temperatures [14, 21]. It has been reported that the activity of this enzyme is increased by 21% when incubated in the presence of 2 mM KCl [2, 14, 21]. Despite the obvious biotechnological potential of different *C. crescentus* enzymes, the removal of enzyme inhibitory effects from fermentation processes is still an important step towards improving hemicellulose hydrolysis [22]. In this context, the data presented in this study are highly relevant.

In this study, the final yield of CcXynB2 was 71 U mg^{-1}, allowing the enzyme to be fully characterized for future biotechnological applications. Although the purification process produced a good yield and a single protein band of 60 Da (Fig. S1), the purification procedure used in this study resulted in a lower yield than that obtained by Correa et al. [14] (215 U mg^{-1}). This result suggests that the use of mobile phase Ni-Sepharose resin is more advantageous for CcXynB2 purification compared with solid phase Ni-Sepharose. Regarding the effects of different compounds (Table 1), CcXynB2 inhibition by divalent ions was similar to that observed for β-xylosidases from other bacteria [23, 24]. EDTA and DTT also strongly inhibited CcXynB2: the enzyme lost over 70% of its initial activity in the presence of these compounds, similar to β-xylosidases from other microorganisms [24, 25]. Inhibition by zinc and a greater tolerance to manganese have been observed in bi-functional enzymes such as β-xylosidase-α-arabinofuranosidase [24].

Although CcXynB2 displays an improvement in enzymatic activity in the presence of 5 mM KCl (Fig. 1), our data indicated that the catalytic efficiency of the enzyme was not significantly altered by KCl (Table 2): the catalytic efficiency of CcXynB2 in the absence or presence of KCl was substantially equivalent. In fact, KCl, which acts as a cofactor in CcXynB2, enhanced enzyme activity but also led to a proportional increase in V_{Max} and K_M for both of the conditions tested (Table 2). As with other β-xylosidases in *C. crescentus*, catalytic efficiency (K_{cat}/K_M) is more reliable than enzyme activity for evaluating enzyme catalytic capacity [2, 21]. The results obtained when investigating the effect of ions suggest that K^+ acts at both of the temperatures tested (37 °C and 50 °C); however, the exact causes of this ion effect would be better understood by conducting crystallographic studies in the presence or absence of KCl, which may reveal possible structural alterations in CcXynB2 in the presence of KCl. Valuable work has been performed with CcXynB2, which is part of glycoside-hydrolase family 39 (GH39). This work suggested that CcXynB2 acts as a monomer in the bacterial cell. Unlike GH39-β-xylosidases, other bacteria exert their catalytic activities in tetrameric form [26]. However, these crystallographic data were not obtained in the presence of KCl, so better structural-level explanations cannot be suggested here. In some enzymes, stabilization can be induced by ions, which stabilize the tertiary structure of the protein at low ion concentrations and promote cross-linking, giving the protein greater stability and increased activity [26].

In a manner relevant to fermentation processes involving cellulosic ethanol production, CcXynB2 activity was enhanced not only in the presence of phenol but also at high concentrations of HMF and ethanol, substances that usually inhibit the fermentation processes used to produce cellulosic ethanol [9, 11, 12] (Fig. 2). Michelin et al. [22] showed that the phenolic compounds present in pre-treated sugarcane bagasse inactivate cellulolytic enzymes such as hemicellulases. A rapid loss of the catalytic ability of a commercial β-xylosidase was observed upon incubation with a sugarcane bagasse hydrolysate containing phenolic derivatives obtained using acetone.

Interestingly, arabinose monosaccharides (Fig. 3a) significantly inhibited CcXynB2 activity at the different concentrations tested. As mentioned above, the genome of *C. crescentus* contains 5 genes that encode β-xylosidases [13], one of which (*xynB5*) has been characterized as a tri-functional β-glucosidase-β-xylosidase and α-L-arabinofuranosidase [18]. The enzyme mainly functions as a β-glucosidase. Before employing the enzymatic hemicellulose-debranching arsenal of *C. crescentus*, it must be understood that CcXynB2, which essentially operates with β-xylosidase activity, is repressed by a monosaccharide that the microorganism is unable to metabolize [14]. In addition, sugars considered easy to metabolize by most microorganisms such as glucose (Fig. 3b) and xylose (Fig. 3c) typically negatively regulate the activity of hemicellulose-degrading enzymes such as β-xylosidases [2]. However, these sugars were not found to repress CcXynB2 activity: enzyme activity was 67% higher in the presence of glucose compared with the control and 44% higher in the presence of xylose (200 mM) compared with the control after 48 h of incubation. In agreement with these data, experiments using agro-industrial residues suggest that CcXynB2 can be used for the pre-degradation of hemicellulose fibers, releasing xylose that can be used in downstream fermentation processes [14]. The advantage of CcXynB2 is its enzymatic activity and the fact that gene expression is not regulated by low concentrations of xylose (0.2%) [14]. We have further demonstrated in this study that the specific activity of CcXynB2 is elevated even at high xylose concentrations.

In the present report, CCXynB2 activity increases in response at high concentrations of monosaccharides because it is supposed that these sugars may have a positive effect on the protein activity and the enzyme can act stimulating the activity of other β-xylosidases or its genes in the bacterium by an unknown mechanism [27] (Fig. 3).

The data reported in Figs. 3, 4, 5, and 6 are considered extremely important in this work: to our knowledge, this is the first time that such promising findings have been described for a bacterial β-xylosidase in the literature. Generally, the enzyme is not only tolerant to most of the compounds tested but actually activated in the presence of a combination of these compounds, some of which are classically described as inhibitors of fermentation processes (e.g., ethanol and glucose). These compounds have been reported to be causative factors in the inhibition of cellulolytic enzymes as well as hemicellulases [28]. The use of enzymes tolerant to ethanol, glucose, and other compounds is extremely important for the efficient conversion of lignocellulosic biomass. Thus, the application of CcXynB2 to simultaneous saccharification and other fermentation processes is possible. In combination with other enzymes, CcXynB2 can be optimized to release fermentable sugars, increasing the production rate of chemicals and fuels from agroindustry waste or the rate of waste saccharification.

Enzymatic hydrolysis assays (Fig. 6) indicate that CcXynB2 can be used to degrade hemicellulose fibers derived from corn cobs, releasing sugars that can be used in fermentation processes. It is noteworthy that the benefits of CcXynB2 are more evident when used in combination with other enzymes that can act synergistically: because β-xylosidase cannot efficiently hydrolyze xylan alone, bioconversion of lignocellulosic biomass and hemicellulose deconstruction is much more efficient via the combined action of various enzymes [29–32].

Immobilization of CcXynB2 was able to stabilize enzymatic activity. Furthermore, the immobilized enzyme performed remarkably better at high temperatures (50 °C, Fig. 7) as soon as show to soluble CcXynB2 by Correa et al. [14]. Although it was evident that CcXynB2 is equally efficient in its soluble or immobilized forms, the results presented here indicate that the thermal stability of CcXynB2 is improved by the immobilization process. In addition, soluble and immobilized enzyme activities measured at 37 °C suggest that both form of the enzyme can be used in fermentation processes with high catalytic capacity. In general, the immobilized enzyme is more resistant than the free form at various temperatures and in the presence of denaturing agents [2]. Similar to previous studies, a Nickel-Sepharose column was used to purify the recombinant protein and as a tool for CcXynB2 immobilization. The mobile phase resin was used for immobilization assays in this work. The advantage of using Nickel-sepharose resin is its high binding capacity for recombinant enzymes, which helps maintain enzyme stability and increases enzyme activity [, 20]. In the future, further investigation will be necessary to obtain a more economically viable process in terms of a cheap immobilization matrix to CCxynB2 for industrial application.

Conclusion

CcXynB2 of *C. crescentus* is an enzyme with high potential for use in simultaneous saccharification and fermentation processes due to its ability to function at typical fermentation temperatures (37 °C). This enzyme exhibited high specific activity even in the presence of potential inhibitors commonly produced during pre-treatment, hydrolysis, and fermentation, to name a few, ethanol, phenol, hydroxymethylfurfural, glucose, xylose, pectin, and ferulic acid. CcXynB2 was not only tolerant to different inhibitory compounds but also exhibited considerable increases in activity in the presence of many of these compounds. When inhibitors were combined and added at concentrations at which the enzyme showed higher performance than the control, β-xylosidase II retained approximately 67% of its initial activity after 48 h. In addition to exhibiting high tolerance and catalytic ability in the presence of inhibitory compounds, CcXynB2 efficiently produces reducing sugars that can be then converted to commercially valuable compounds, even at temperatures distant from the enzyme's optimal temperature. The results highlighted here provide fundamental information for further improving β-CcXynB2 and suggest possible applications of the enzyme in SSF processes aimed at producing cellulosic ethanol or in enzyme cocktails for biomass degradation in various industrial processes. In addition to being far more stable when immobilized, CcXynB2 also showed potential for use in the bioconversion of hemicellulose as a component of an enzyme cocktail, facilitating the saccharification of lignocellulosic biomass. To our knowledge, this is the first literature report of a bacterial β-xylosidase resistant to high concentrations of several inhibitory compounds.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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