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On-site produced and commercially available alkali-active xylanases compared for xylan extraction from sugarcane bagasse

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ABSTRACT

The purpose of this work was to investigate the enzymatic extraction of xylan using xylanases active in alkaline conditions. One on-site produced and three commercial xylanases designed to boost bleaching of pulped wood were characterized for their optimal pH, temperature and stability to compare their effects on xylan extraction from pretreated sugarcane bagasse. On-site produced xylanase was prepared from cultures of the alkaliphilic *Bacillus pumilus* CBMAI 0008 strain. The optimal temperatures and pH levels for all commercial xylanases were 60 °C and 6, whereas for *B. pumilus* xylanase, they were 50 °C and 8, respectively. *B. pumilus* was active up to pH 10, but it had low thermal stability. Five sugarcane substrates were prepared to contain decreasing lignin content based on alkaline-sulfite pretreatment or acid-chlorite delignification. Xylanase doses (5–100 IU/g substrate) were applied to these substrates at pH 8.0, 50 °C and a 24-h reaction. Xylan extraction yields were strongly dependent on the lignin content of the substrates. Maximal xylan extraction yields were obtained in acid-chlorite delignified substrates, reaching values of 64% and 45% for Luminase and *B. pumilus* xylanases, respectively. Residual (non-extractable) xylans seemed occluded by and/or complexed with residual lignin of the substrates.

1. Introduction

The growing global demand for bioenergy and bioproducts has led to the development of the biorefinery concept, which preconizes the efficient and integral use of various biomasses from agroindustries. Two commercially important lignocellulosic biomasses are sugarcane bagasse and corn stover (Anwar et al., 2014; Saini et al., 2015). Both are hemicellulose-rich materials (Templeton et al., 2009; Masarin et al., 2011) with potential application in the biorefinery context (Shah et al., 2017; Michailos; Webb, 2019). In both cases, the major hemicellulosic polymer is 4-O-methyl glucuronoarabinoxylan (Brienzo et al., 2009; Naran et al., 2017).

Autohydrolysis, steam-explosion and acid pretreatments of such hemicellulose-rich biomasses can yield xylose hydrolysates suitable for bioconversion processes. However, acidic pretreatments also produce biomass degradation products, which can limit full use of the hemicellulose fraction (Silveira et al., 2015; Kim et al., 2018). In contrast, alkaline pretreatments can reduce biomass recalcitrance mainly associated with lignin removal, leaving the cellulose- and hemicellulose-enriched substrate for subsequent processing (Mendes et al., 2011; Silveira et al., 2015). Direct enzymatic hydrolysis of alkali-pretreated biomasses produces a mixed glucose and xylose stream, which has some limitations for bioconversion with robust and industrially available microorganisms (Kricka et al., 2014). Hemicellulose extraction from alkali-pretreated substrates is a way to add value to the hemicellulosic fraction, avoiding production of a mixed glucose/xylose stream (Sporck et al., 2017). Recovery of this hemicellulose fraction from alkali-pretreated lignocelluloses is particularly important for potential uses of hemicelluloses in nutrition and food technology (Pitkänen et al., 2008), new materials for packing (Mikkonen; Tenkanen, 2012), coatings and adhesives (Ramos et al., 2017; Farhat et al., 2017), surfactants and cosmetics applications (Deutschmann; Dekker, 2012).

Hemicellulose extraction can be performed by several physico-chemical methods depending on the desired product (Carvalheiro et al., 2008; Brienzo et al., 2016). Hemicellulose extraction in acidic medium under mild conditions is efficient at producing oligomers of low molar mass, but the hydrolysates also contain a large amount of monosaccharides and undesirable byproducts (Kim et al., 2015) requiring several purification steps (Batalha et al., 2015). Alkaline extraction is the most adequate way to extract xylans of high molar mass in high yields (Oriez et al., 2018). Increasing loads of alkali provide increased yield of extracted hemicellulose (Jayapal et al., 2013; Wunna et al., 2017). However, simultaneous lignin extraction under high alkali loads requires hemicellulose purification steps (Hoije et al., 2005; Oriez et al.,

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2018).

In contrast with physicochemical methods of hemicellulose extraction, xylanases can boost extraction methods because the enzymes provide specific cleavage of internal 1,4-β-xylosidic linkages, facilitating partial xylan depolymerization and subsequent solubilization (Reddy and Krishnan, 2016). There are endoxylanases with different biochemical and structural characteristics, which have great potential for use in different industrial applications (Chen et al., 2015). Alkali-active xylanases are particularly useful for pulp bleaching processes (Kumar et al., 2017; Walia et al., 2017). Endoxylanases are also used in the production of high-quality dissolving pulp (Kumar and Christopher, 2017) and in the production of nanofibrillated cellulose (Hu et al., 2018; Poyraz, 2018). However, most of the previous work with xylanases used in hemicellulose extraction aimed to remove xylan to produce purified cellulose and not to recover xylan for subsequent use.

Enzymatic extraction of xylan assisted by endoxylanases has gained attention recently (Rémond et al., 2010; Hakala et al., 2013; Aguedo et al., 2014; Sporck et al., 2017). As an advantage over other extraction methods, the enzyme does not require high alkali loads, does not produce undesirable by-products and does not require specific equipment (Aachary and Prapulla, 2011). In addition, the products obtained are highly pure, saving chemical reagents.

In the particular case of sugarcane bagasse, previous work has shown that xylans can be extracted by xylanase from alkaline-sulfite pretreated material, producing high-purity xylans compared to other chemical methods (Sporck et al., 2017). However, the yield of xylan extraction using xylanase was relatively low, suggesting some limitations for currently available commercial xylanase preparations. For xylan extraction, alkali-active xylanases are particularly desired because high molar mass xylans are significantly more soluble in alkaline medium (Cheng et al., 2010). Currently, several commercially available xylanases can act at alkaline pHs. Most of the alkali-active xylanases are produced from bacteria, but some alkali-active fungal xylanases have been reported (Walia et al., 2017).

On-site production of enzymes is an alternative for several biorefinery designs. This tendency can be useful to reduce enzyme costs, taking advantage of substrate availability in many biorefineries (Silva and Filho, 2017). In this context, we evaluated on-site production of alkali-active xylanases from the alkaliphilic bacteria *Bacillus pumilus* aiming to compare their efficiency with commercial enzymes to extract xylan from pretreated sugarcane bagasse. The goal was to compare four different xylanase preparations based on their action profile at different pH levels and temperatures and to examine their efficiency to yield high amounts of xylan from sugarcane bagasse.

2. Material and methods

2.1. Bacillus pumilus strain and maintenance

Bacillus pumilus CBMAI 0008 was obtained from the culture collection of the Center for Biological and Agricultural Chemistry Research (CPQBA, Campinas-SP). The culture was grown on Mandel's agar media plates (Roncero et al., 2005) with the pH adjusted to 8.5 and incubated for 2 days at 45 °C. Stock cultures were maintained in the same medium and stored at 4 °C.

2.2. Xylanase production by B. pumilus and determination of enzymatic activities

Culture media used for xylanase production contained (in g/L) peptone (5.0), yeast extract (5.0), KNO_3 (5.0), KH_2PO_4 (1.0) and $MgSO_4{\cdot}7H_2O$ (0.10), plus varied carbon sources. Four different carbon sources were used separately at 30 g/L: raw sugarcane bagasse (milled to pass 20 mesh screen), sugarcane bagasse holocellulose (Siqueira et al., 2017), oat spelts xylan (Sigma X-0627), and commercial wheat bran. Erlenmeyer flasks (250 ml) containing 25 ml of culture medium were autoclaved at 121 °C for 15 min before inoculation with 10^8 cells/mL using active-growing *B. pumilus* (2-day-old cultures from Petri dishes). Inoculated flasks were incubated on a rotary shaker at 120 rpm at 45 °C for 24 h. The culture broth was centrifuged at 4750 g for 10 min, and the supernatants were used to recover xylanases. The supernatant was analyzed for protein content (Bradford et al., 1976) and xylanase, endoglucanase, β-xylosidase, β-glucosidase and arabinofuranosidase activities. The xylanase activity was determined according to Bailey et al. (1992), whereas endoglucanase activity was measured according to Ghose (1987) using carboxymethylcellulose as the substrate (CMC - Sigma). In both cases, reducing sugars were measured using the DNS assay (Miller, 1959). β-Glucosidase, β-xylosidase and arabinofuranosidase activities were determined according to Tan et al. (1987). All enzymatic assays were performed in 50 mM sodium phosphate buffer at a pH of 8.0 and 50 °C. The enzyme unit (IU) was defined as one micromole of reaction product per minute. Triplicate experiments were used in each case. Average values with corresponding standard deviations are reported in the text.

2.3. Commercial xylanases

The commercial xylanases used in this study were liquid formulations of Luminase PB-200 (BASF) and EnzAid (EDT). A powdered preparation corresponding to a recombinant xylanase (PRX) was obtained from Sigma-Aldrich (code X2753). In this case, powdered xylanase was dissolved in 50 mM phosphate buffer at a pH of 8. This liquid preparation was further used in the experiments. All commercial preparations were assayed for protein content and xylanase, endoglucanase, β-xylosidase, β-glucosidase and arabinofuranosidase activities as stated in item 2.2.

2.4. Determination of the optimal temperature and pH for xylan hydrolysis by xylanases

The optimal reaction temperature for the action of xylanases was determined by incubating 100 µL of properly diluted enzymes with 900 µL of 1% birchwood xylan (Sigma) at a pH of 8 for 5 min. The reaction temperature was set from 40 °C to 80 °C. For the pH effect on xylan hydrolysis, each xylanase preparation was assayed at 50 °C using 1% birchwood xylan solution as substrate. Reactions were equally performed for 5 min. Birchwood xylan was prepared in 50 mM buffer with varied pH levels from 5 (acetate buffer pH) to 6–8 (phosphate buffer) and 9–10 (glycine-NaOH buffer). The temperature and pH at which the enzyme presented maximal activity was considered 100% activity.

2.5. Temperature stability of the xylanases

The temperature stability of the xylanases was determined at 50 °C. For this process, enzyme solutions were maintained at 50 °C in 50 mM phosphate buffer at a pH of 8 for up to 150 min without any substrate. Samples from this solution were periodically removed, placed on ice and assayed for residual xylanase activity. *B. pumilus* xylanases were further evaluated for thermal stability at varied pH levels from all cases, relative activities of the xylanases were expressed as percentages of the initial activity.

2.6. Preparation of sugarcane bagasse substrates for subsequent enzymatic xylan extraction

Approximately 600 g of sugarcane bagasse was pretreated by an alkaline-sulfite chemothermomechanical process (Reinoso et al., 2018). Three different Na_2SO_3 loads were used: 2.5%, 5% and 10% Na_2SO_3 (g of chemical/100 g of sugarcane bagasse). In all cases, the NaOH load was half of the sulfite load. The bagasse to liquor ratio was 1:10 (w/v). The pretreatment reaction lasted 120 min at 120 °C. After chemical

cooking, solids were disk-refined at 2.4% (w/v) consistency using a 0.1-mm disk gap and 250 Wh of energy consumption.

Another set of substrates was prepared by selective delignification of raw sugarcane bagasse. Delignification was performed with sodium chlorite in acetic acid for 2 h and 4 h as previously described (Siqueira et al., 2017).

All samples of raw sugarcane bagasse and pretreated materials were analyzed for lignin, glucan and hemicellulose contents as previously described (Ferraz et al., 2000). Details of the pretreatment conditions and compositional analysis can be obtained from previously mentioned studies.

2.7. Enzymatic extraction of xylan

Enzyme solutions corresponding to 5, 20, 40, 60 and 100 IU/g of substrate were added to pretreated sugarcane bagasse samples suspended at 5% (w/v) in 50 mM sodium phosphate buffer at pH 8. Control reactions used buffer solution without enzymes. The mixtures were shaken at 120 rpm in a water bath at 50 °C for 24 h. After reaction, the enzyme was inactivated by treatment in a boiling water bath for 5 min. Residual solids and supernatants were separated by centrifugation at 4750*g* for 25 min. Control reactions were performed in the absence of enzyme at the same conditions. Xylan amounts extracted under this control reaction were subtracted from total xylan detected in the enzymatic extraction procedures.

Supernatants were hydrolyzed with diluted sulfuric acid according to the method described in Sluitter et al. (2008). Released monosaccharides and acetic acid were determined by HPLC (Waters). For this process, a Bio-Rad HPX-87H column heated at 45 °C was eluted with 5 mM sulfuric acid at a flow rate of 0.6 ml/min. The refractive index detector (Waters 2414) was set to 35 °C and used to detect eluted monosaccharides. The contents of xylose, arabinose and acetic acid were used to calculate the total hemicellulose extracted in the supernatant. The yield of xylan extraction was determined by dividing the concentration of xylan in solution to the xylan in the pretreated samples (Sporck et al., 2017).

3. Results and discussion

3.1. Production of hydrolytic enzymes by B. pumilus

Extracellular xylanase activities were determined in cultures of *B. pumilus* grown in four different carbon sources: milled sugarcane bagasse (*in natura*), sugarcane bagasse holocellulose, wheat bran and xylan from oat spelts. Cultures were performed at pH levels of 8.5 and 9.5 to evaluate the alkaliphilic behavior of the *B. pumilus* CBMAI 0008 strain (Fig. 1). Xylanase activities were detected in all carbon sources at both alkaline pHs, confirming that the *B. pumilus* CBMAI 0008 strain

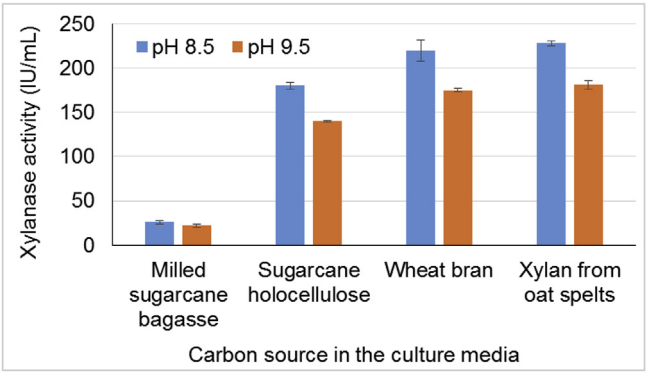

Fig. 1. Extracellular xylanase activities detected in *B. pumilus* cultures after 24 h of incubation in culture media containing different carbon sources.

produces xylanases at elevated pH as already described for other strains (Anuradha et al., 2007; Yasinok et al., 2010; Amorin et al., 2018). More xylanase was produced at pH 8.5, while the highest activities were detected in cultures with wheat bran and xylan from oat spelts as carbon sources. Sugarcane bagasse holocellulose was also a suitable carbon source, but *in natura* sugarcane bagasse provided very low xylanase levels due to its high recalcitrance. Although xylan from oat spelts provided the highest xylanase production, wheat bran has been reported to cost less as a carbon source compared to commercial xylan (Poorna and Prema, 2006). Other studies have suggested wheat bran as a suitable carbon source for production of xylanases on a commercial scale because it contains several nutrients necessary for microbial growth and arabinoxylan for induction of xylanases (Alazi and Ram, 2018; Taherzadeh-Ghahfarokhi et al., 2019).

The specific activity of *B. pumilus* xylanase was found to be 1.5 times higher than that observed in a commercial purified endo-β-(1 → 4)-xylanase from *Thermomyces lanuginosus* (PRX) and approximately 8 times higher than the xylanases Luminase PB-200 and EDT available on the market (Fig. 2A).

In addition to xylanase activity, the enzyme preparations were assayed for β-xylosidases, β-glucosidases, endoglucanases and arabinofuranosidases (Fig. 2B). Except for the PRX extract that had high endoglucanase, β-xylosidase, and β-glucosidase activities, the other enzyme preparations contained predominantly endo-xylanase activity (Fig. 2A and B). In the crude extract of *B. pumilus*, arabinofuranosidase activity was not detected, even though wheat bran substrate is an arabinose-rich carbon source. The activities of β-xylosidase and β-glucosidase were lower than 0.1 IU/ml, and endoglucanase activities reached maximum levels of 0.7 IU/ml. These data corroborate previous studies reporting that *B. pumilus* cultures produce extracellular endoxylanases almost free of cellulases and xylosidases (Chakdar et al., 2016). Low β-xylosidase and β-glucosidase extracellular activities have been reported for the *Bacillus* and *Geobacillus* genera. These genera produce extracellular endoxylanases to hydrolyze xylan into oligosaccharides, which are then transported to the intracellular environment through specialized permeases (Shulami et al. 2007, 2014; Teplitsky et al., 2000). This mechanism has been associated with a specialized strategy for growth of these microorganisms (Teplitsky et al., 2000). In fact, β-xylosidase and β-glucosidase are found to be intracellular in several bacteria. To date, genome sequencing of *B. pumilus* permitted identification of genes encoding three endo-xylanases belonging to families GH 10, GH 11, and GH 30, one manannase (GH 26), one β-xylosidase (GH 43) and one arabinofuranosidase (GH 51). A number of other putative xylanases were also similarly predicted by sequence (Cantarel, 2009). Furthermore, a search for signal peptides on the SignalP 4.0 server indicated that only the GH 11 and GH 30 xylanases and GH 26 mannanase are secreted into the extracellular medium. Efficient secretion of highly specific xylanases is an interesting characteristic of *B. pumilus*. However, other properties such as stability at high temperatures and alkaline pH are desirable for many applications (Chen et al., 2015).

3.2. Temperature and pH profile of the evaluated xylanases

B. pumilus xylanase produced at pH 8.5 using wheat bran as the carbon source was compared with commercial xylanases as a function of reaction temperature and pH (Figs. 3 and 4, respectively).

Luminase PB-200 and EDT enzymes presented maximal activities at 60 °C. However, EDT promptly inactivated at 65 °C, presenting less than 50% of original activity at this temperature, whereas Luminase maintained at least 65% of the original activity up to 80 °C. PRX presented an optimal temperature at 55 °C and promptly deactivated at 65 °C. *B. pumilus* xylanase presented maximal activity at 50 °C maintaining over 90% of the original activity up to 60 °C, with prompt inactivation over 65 °C (Fig. 3). Previous work also found that 55 °C was the optimal temperature for *B. pumilus* xylanases at pH 8.0 (Duarte et al., 2003).

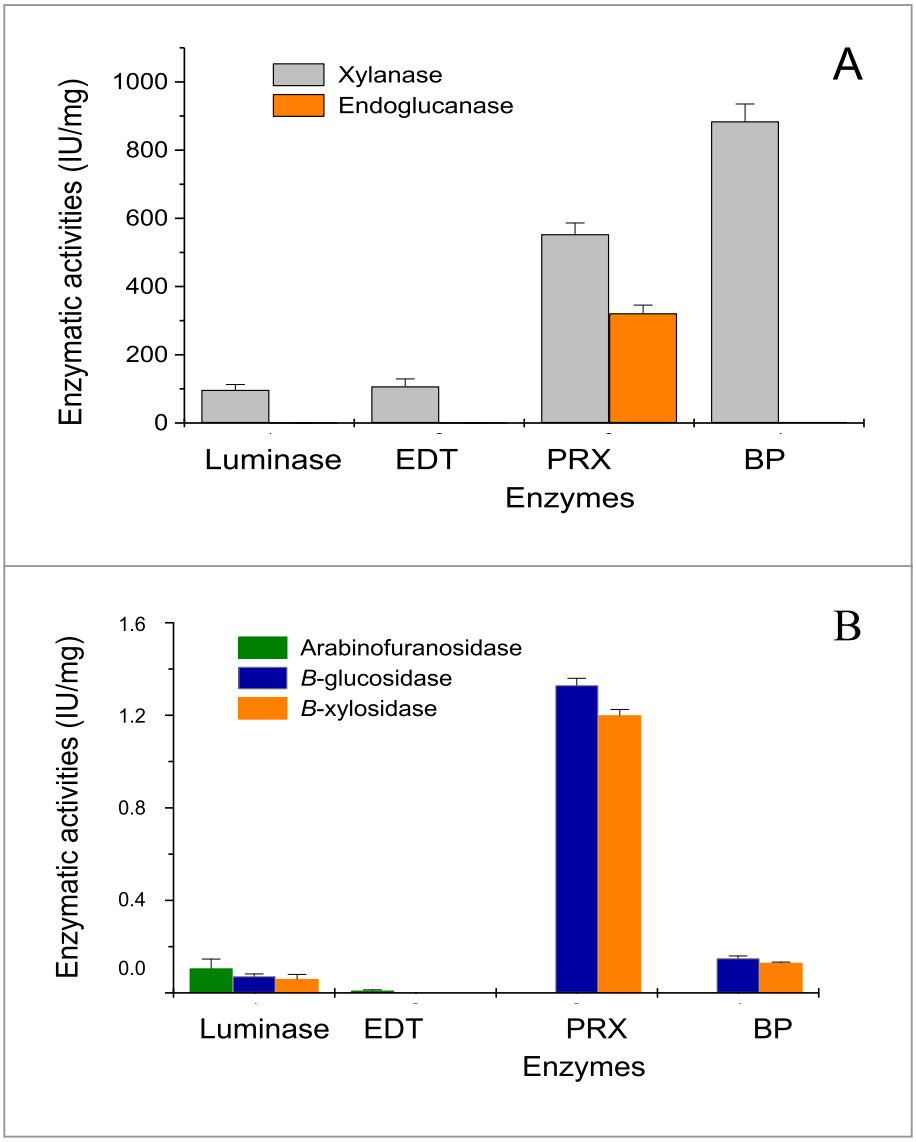

Fig. 2. Enzymatic activities present in commercial xylanase preparations (Luminase, TRX and EDT) and in *Bacillus pumilus* (BP) crude extract.

Data in Fig. 3 corresponds to 5 min reactions in which the positive effect of temperature on the reaction rates tends to overestimate optimal temperatures as compared with longer residence time used in enzymatic bioprocesses. At longer reaction times, heat inactivation of the enzymes tends to overcome the positive effect of temperature on the reaction rate.

The reaction pH affected enzymatic activities with varied behavior (Fig. 4). *B. pumilus* xylanases presented maximal activities at pH 7.0–8.0 with 80% of the maximal activity still detected at pH 9.0. At pH 10, *B. pumilus* xylanase was still active at 50% of the maximal activity. Commercial enzymes presented optimal pH in the range of 6.0–7.0 but were still active at pH 8.0 (60%–81% of the maximal activity, depending on the commercial preparation) (Fig. 4). Several alkali-active xylanases have been reported for *Bacillus* spp (Duarte et al., 2003; Thomas et al., 2014).

Some studies have assigned the alkali-active behavior of selected xylanases to the frequent occurrence of Arg and His in their structures (Bharadwaj et al., 2008; Mamo et al., 2009; Bai et al., 2016). In agreement with this observation, two of the 3 xylanases coded in the *B. pumilus* genome (GH 11 and GH 30) are Arg and Lys rich proteins (Cantarel et al., 2009).

The thermal stability of the xylanases was evaluated in experimental conditions without the presence of the substrate (Fig. 5). Assays with Luminase PB-200 demonstrated that the enzyme keeps 100% of its activity at 50 °C for 150 min (Fig. 5) and remained active for 6 h, retaining 79% residual activity (data not shown). The EDT and PRX xylanases were less stable than Luminase PB-200, decreasing their activities especially at long exposures at 50 °C. *B. pumilus* xylanase presented the lowest thermal stability, with activity decreasing below 50% after 30 min of incubation at 50 °C. These data indicate that although *B. pumilus* xylanase acted at the highest pH (Fig. 4), the enzyme presented poor stability at 50 °C compared to commercial xylanase preparations.

The low thermal stability of the *B. pumilus* xylanase was further investigated at varied incubation pHs (Fig. 6). Data obtained with incubation pHs from 6.0 to 9.0 corroborated that the low stability of *B. pumilus* xylanase at 50 °C was detectable in a broad range of pHs, suggesting that the enzymes unfolded irreversibly at 50 °C when the enzyme is not attached to substrate. However, several reports indicate that thermal stability of the xylanases is increased in presence of their substrates (Anbarasan et al., 2010; López, Estrada, 2014).

Studies related to the pH and temperature profile of the evaluated xylanases suggest that *B. pumilus* is suitable for application in biomass conversion processes under scenarios requiring alkaliphilic conditions but was unstable at 50 °C over long reaction periods. In contrast,

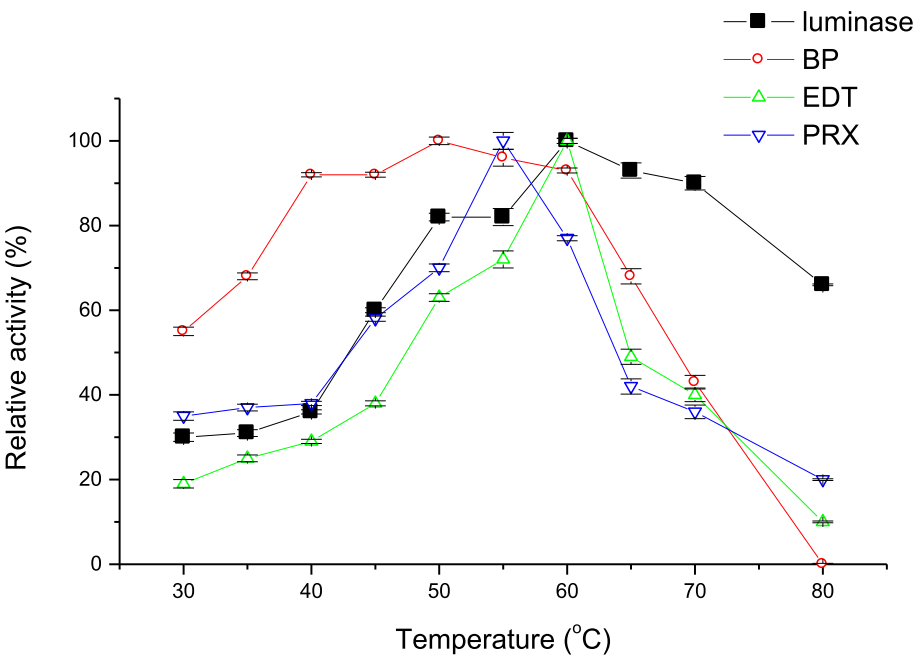

Fig. 3. Effect of reaction temperature on the activities of xylanases (Luminase, EDT, recombinant PRX and from *Bacillus pumilus*) at a fixed reaction with pH 8.0.

Luminase PB-200 and the other commercial preparations seemed to be more suitable for high temperature applications restricted to lower pHs. Industrial applications for these enzymes have been reported for direct enzymatic pulp bleaching under alkaline conditions (Nagar et al., 2013; Bai et al., 2016), enzymatic deinking (Thomas et al., 2014), and textile processing (Battan et al., 2012). It is also possible to use these xylanases to prepare dissolving pulp from hardwood kraft pulp (Aachary and Prapulla, 2011). Another potential application for these enzymes is for xylan extraction using biorefinery concepts (Sporck et al., 2017; Lu et al., 2016). Xylan extraction boosted by alkali-active xylanases is particularly relevant because partially hydrolyzed xylans are promptly dissolved at alkaline pHs (Kumar and Satyanarayna, 2011).

3.3. Use of alkali-active xylanases for xylan extraction from pretreated sugarcane bagasse

One of the goals of the present study was to use alkali-active xylanases as boosting agents for xylan extraction from sugarcane bagasse. Previous work showed a limited ability of xylanases to act on untreated sugarcane bagasse, even in the presence of cellulases (Mendes et al., 2011). This study showed that xylan hydrolysis rates are significantly increased after alkaline-sulfite pretreatment of the sugarcane bagasse. Accessibility to xylan backbones depends on lignin removal during pretreatment. Therefore, the current work evaluated xylan extraction by alkali-active xylanases acting on sugarcane bagasse progressively depleted in lignin either by alkaline-sulfite pretreatment or by selective acid-chlorite delignification. Five different sugarcane substrates with

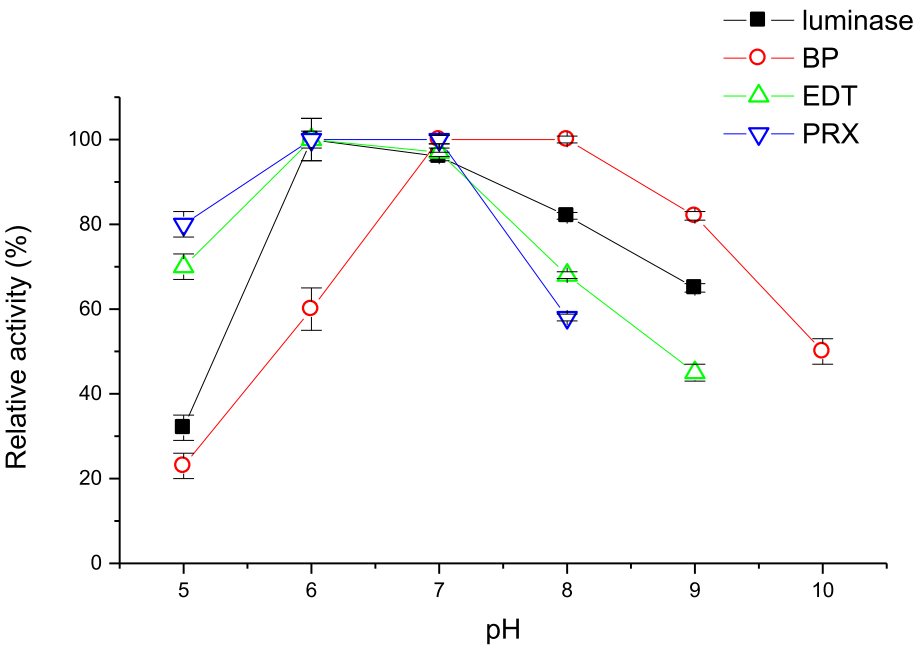

Fig. 4. Effect of reaction pH on the activities of xylanases (Luminase, EDT, recombinant PRX and from *Bacillus pumilus*) at a fixed reaction temperature of 50 °C.

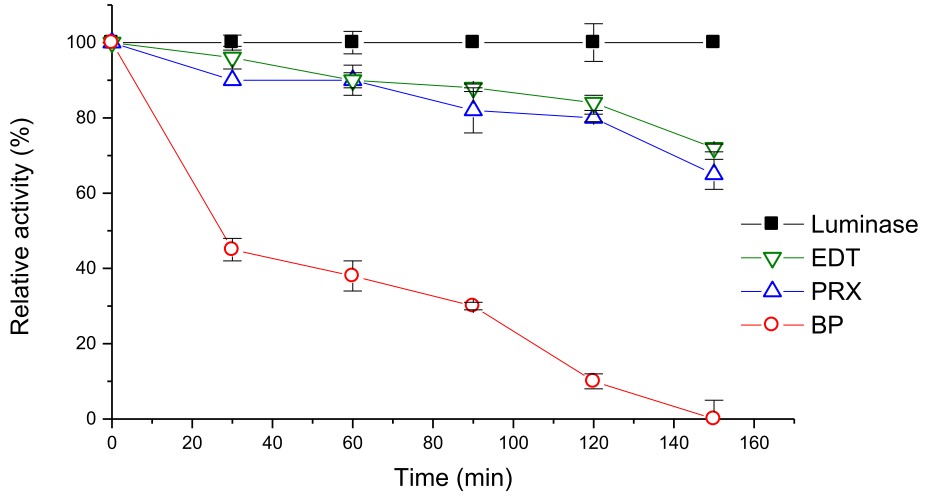

Fig. 5. Xylanase activities after enzyme preincubation up to 150 min at 50 °C and pH 8. Enzyme activities were determined at 50 °C and pH 8.0 for a 5-min reaction after preincubation of the enzyme preparation at defined time intervals.

decreasing lignin contents were prepared according to previously determined conditions (Siqueira et al. 2013, 2017; Reinoso et al., 2018). The processing yields and chemical compositions of the prepared substrates are shown in Table S1 (Supplemental file). Lignin contents of the five prepared substrates ranged from 24.8% to 6.2%, whereas glucan contents ranged from 40.0% to 48.3% depending on the alkaline-sulfite loads or the reaction time in the acid-chlorite delignification. Xylan contents of prepared substrates were high, ranging from 19.6% to 25.1%. Arabinose (1.8%–2.8%) and glucuronic acid (2.7–3.8%) contents detected in prepared sugarcane substrates permitted determination of the molar ratios of side groups in the xylan backbone. The Ara/Xyl and Uro/Xyl ratios were very similar in all substrates, ranging from 0.08 to 0.11 for Ara/Xyl and 0.11 to 0.13 for Uro/Xyl. In contrast, acetyl groups that are abundant in untreated sugarcane bagasse (3.6%) were almost completely removed after alkaline-sulfite pretreatment, mainly when the highest alkali-sulfite load was used in the pretreatment.

Previously described xylanases were used for xylan extraction from the five sugarcane bagasse substrates (Fig. 7). As mentioned before, the chosen enzymes did not present significant activities for endoglucanase,

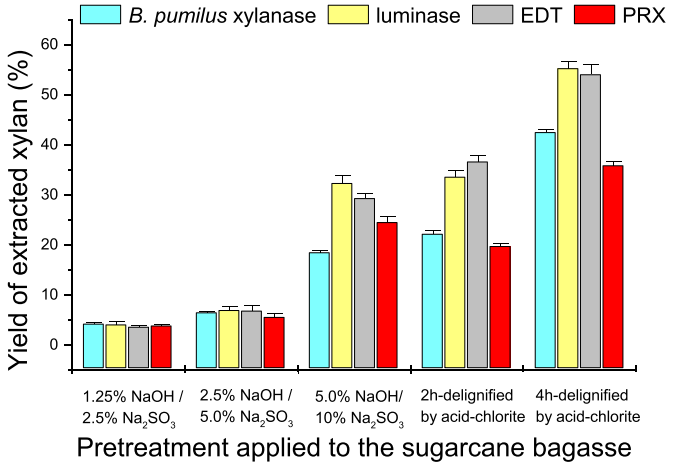

Fig. 7. Yields of xylan extraction from pretreated sugarcane substrates by four different xylanases. Extraction reactions lasted 24 h at 50 °C and pH 8.0. The enzyme dosage was 20 IU/g of substrate.

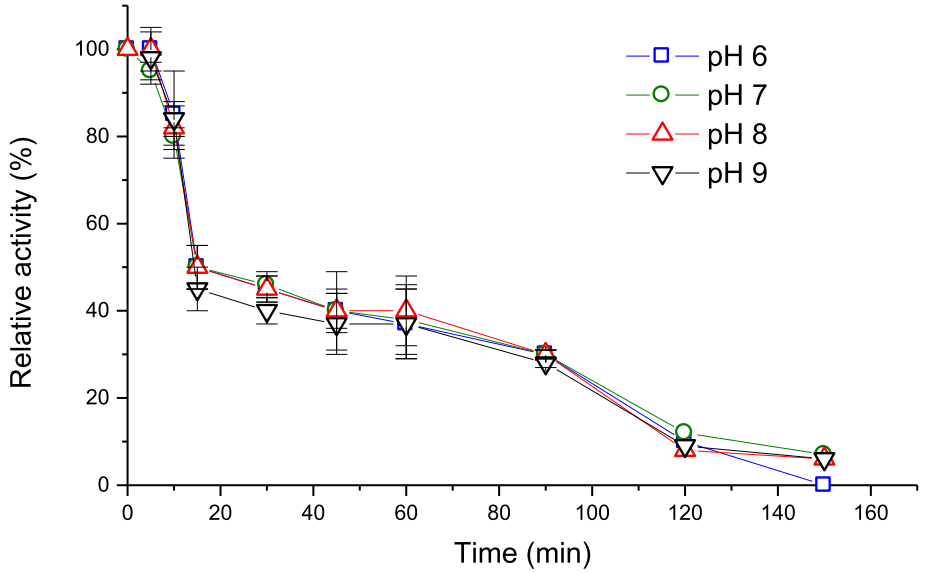

Fig. 6. Xylanase activities of *B. pumilus* enzymes after preincubation up to 150 min at 50 °C under varied pH levels. Enzyme activities were determined at 50 °C and pH 8.0 for a 5-min reaction after preincubation of the enzyme preparation at defined time intervals.

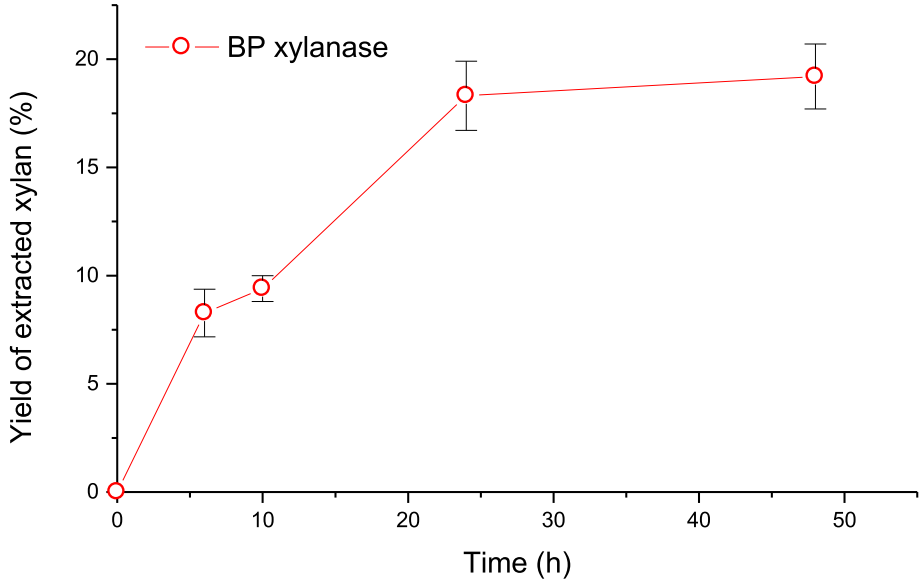

Fig. 8. Time course of xylan extraction from alkaline sulfite pretreated sugarcane bagasse (5% NaOH:10% Na_2SO_3) by *B. pumilus* xylanases (20 IU/g) at 50 °C. Control was performed in the absence of enzyme.

β-glucosidase or β-xylosidase, providing specific cleavages of the internal linkages present in the xylan backbone. Varied performances of the enzymes were more evident only when low lignin substrates were evaluated (substrates delignified with acid-chlorite and pretreated with the highest alkaline-sulfite load). As shown in Fig. 7, Luminase and EDT performed very similarly, providing up to 55% xylan extraction. *B. pumilus* xylanases provided higher xylan extraction yields than PRX in the low lignin substrates but were less efficient than Luminase and EDT.

Despite *B. pumilus* xylanases presented low thermal stability in absence of substrate (Fig. 3), xylan extraction yields after 24-h reactions at 50 °C were high, suggesting that the enzyme remains stable for longer periods in presence of the substrate. The time course of xylan extraction by *B. pumilus* xylanase shown in Fig. 8 indicates increasingly yields up to 24-h reaction corroborating that enzyme remained active under this reaction condition.

To determine if the xylanase loads had a significant role in the xylan extraction efficiency, Luminase and *B. pumilus* xylanases were further assayed at increasing enzyme loads using the sugarcane bagasse substrates with low lignin contents (Fig. 9). For the 4 h-delignified sugarcane substrate containing only 6.2% of residual lignin, the xylanase loads increasing from 20 IU/g to 100 IU/g of substrate resulted in limited increases in xylan extraction efficiencies: 55%–64% for Luminase and 41%–45% for *B. pumilus* xylanases. Similar improvements in the xylan extraction efficiencies were also observed for the alkaline-sulfite pretreated substrate (Fig. 9).

The occurrence of a residual (non-extractable) xylan in the pretreated substrates seems to be dependent on biomass structural characteristics such as the occurrence of covalent linkages between lignin and xylan or ultrastructural organization of the fiber cell wall components, which makes part of the xylans resistant to enzymatic solubilization (Peng et al., 2010; Busse-Wicher et al., 2016).

A general evaluation of the entire dataset indicates that substrates with less lignin permitted higher enzymatic solubilization of their residual xylan (Fig. 7). For instance, xylan extraction yields correlated linearly with the lignin contents of the substrates in all enzymatic extraction procedures (Fig. 10), suggesting that lignin can restrict enzyme accessibility to xylan and/or that part of the non-extracted xylan can occur as lignin-carbohydrate complexes.

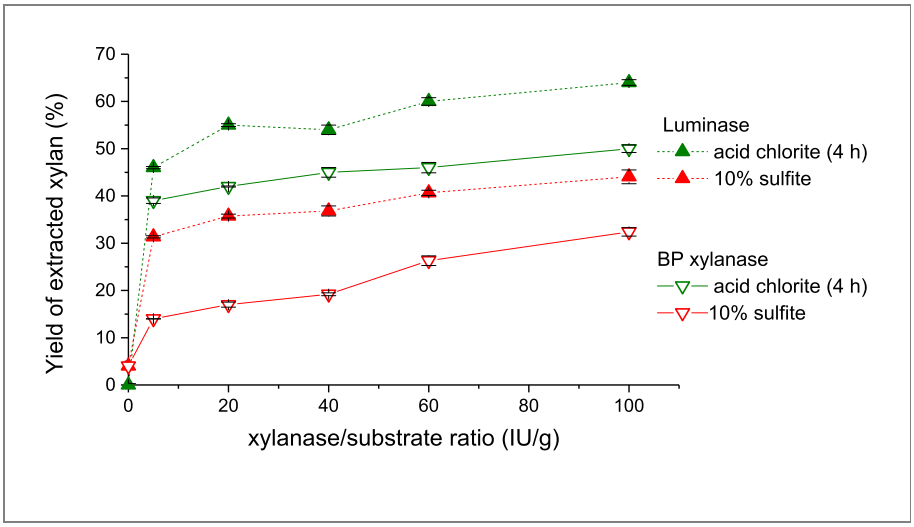

Fig. 9. Xylan extraction efficiency as a function of increasing xylanase dosages used in the extraction processes. Two different sugarcane bagasse substrates and two xylanases were used in the extraction process lasting 24 h at 50 °C. Dashed lines (Luminase), solid lines (*B. pumilus*) xylanases.

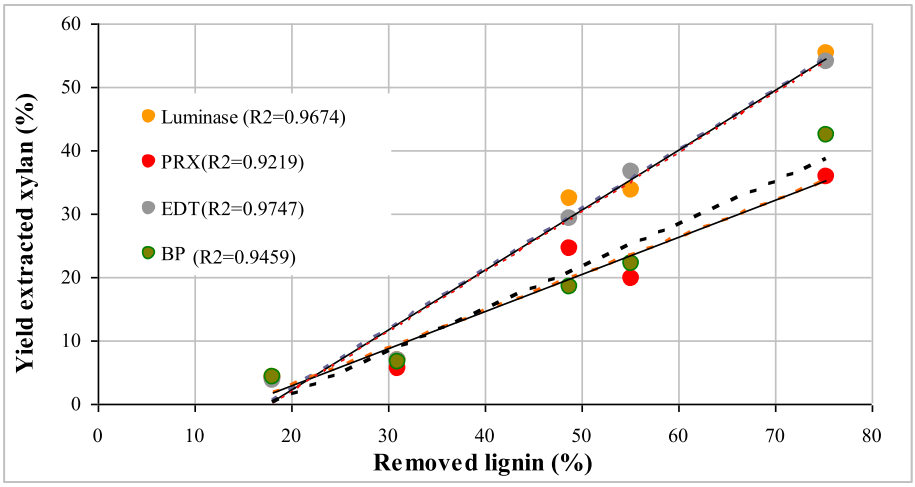

Fig. 10. Correlations between the lignin removed after bagasse pretreatment and xylan extraction efficiency by different xylanases.

In addition to xylan occlusion by lignin, xylan is naturally associated with cellulose microfibrils. Indeed, depolymerization of cellulose by the action of the cellulases within the fibers can expose hemicellulose chains, facilitating the access of xylanases to xylans (Busse-Wicher et al., 2016). This observation suggests that in addition to the negative effect of lignin on xylan accessibility, the close cellulose-hemicellulose interaction can affect access to hemicelluloses. This phenomenon was already documented in experiments of molecular dynamic simulations of lignocellulosic materials (Pereira et al., 2017).

4. Conclusions

The *B. pumilus* CBMAI 0008 alkaliphilic strain produced alkaline-xylanases when grown in different substrates containing xylan. This on-site prepared xylanase acted at alkaline pHs up to 10 and was useful for extracting xylan from pretreated sugarcane bagasse. On-site prepared xylanase had limited stability at 50 °C in the absence of substrate. However, the enzyme provided high yield for xylan extraction in reactions lasting 24 h at 50 °C, suggesting higher thermal stability in presence of the substrate. Maximal xylan extraction yields were obtained with lignin-depleted substrates, reaching 64% and 55% using high xylanase loads (100 IU/g of substrate) of Luminase and *B. pumilus*, respectively. Xylan extraction efficiency was strongly dependent on the lignin content of the substrates.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101081.

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