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Application of In-House Xylanases as an Addition to a Commercial Cellulase Cocktail for the Sustainable Saccharification of Pretreated Blue Agave Bagasse Used for Bioethanol Production

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Abstract: The study involves the use of commercial cellulase Cellic CTec2 in combination with two in-house xylanases, *Px*Xyn10A (XynA), a recombinant purified enzyme from *Paenibacillus xylanivorans* A59, and a xylanase enzymatic extract from native *Moesziomyces aphidis* PYCC 5535T (MaPYCC 5535T), for the enzymatic hydrolysis of pretreated blue agave bagasse (BAB) at the high solids load of 20% (*w*/*v*). Three different combinations of cellulase and xylanases were evaluated. When Cellic® CTec2 was used at a dosage of 10 FPU/g oven-dried solids (ODS) supplemented with XynA or MaPYCC 5535T at an endo-xylanase dosage of 100 U/g ODS, increases in the xylose yield of 30% and 33%, respectively, were obtained. When applying in-house xylanases alone (at an endo-xylanase dosage of 100 U/g ODS), xylan in BAB was selectively hydrolyzed into xylose with 5% yield with MaPYCC 5535T, while no xylose was detected with XynA. Interestingly, a synergic effect of Cellic® CTec 2 with both xylanases was observed when using a low dosage of 1 FPU/g ODS (allowing for some liquefaction of the reaction mixture), promoting xylose and glucose release by either xylanase. A higher concentration of monomeric sugars was obtained with 10 FPU/g ODS of Cellic® Ctec 2 supplemented with 100 U/g ODS of MaPYCC 5535T, followed by XynA. The improvement in saccharification through the synergistic combination of in-house xylanases and commercial cellulases allows for the obtention of sugar-rich hydrolysates, which enhances the technical sustainability of the process. Hydrolysates were then fermented using recombinant Cellux 4^{TM} yeast to yield 45 g/L ethanol, representing an increase of about 30% with respect to the control obtained with only the commercial cellulase cocktail. The surface modification of agave biomass with the different combinations of enzymes was evidenced by scanning electron microscopy (SEM).

Keywords: in-house xylanases; blue agave bagasse; high solids load; sustainable enzymatic hydrolysis; fermentation

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1. Introduction

Tequila, one of the most commercially important Mexican alcoholic beverages in the world, is produced by the fermentation of fructans located in the stem (*piña*) of *Agave tequilana* Weber, better known as *Agave Azul*. In 2023, 598.7 million liters of tequila were

produced from 2288 mil tons of agave, according to the Regulatory Council of Tequila (2024). During tequila production, bagasse, a solid by-product of the process, is obtained after the sugar extraction from cooked agave, making up about 40% (*w*/*w*) of the agave plant [1–3]. This low-value material has various potential uses, including compost, animal feed, methane production, fertilizer, fuel, and active compounds like fructans [4–6]. However, the use of this by-product is insufficient compared to the large amounts generated annually, leading to an environmental problem. Bagasse represents an attractive feedstock that can be used for sustainable biofuel production as it contains, on average (*w*/*w*), 38.9% cellulose, 17.0% hemicellulose, and 18.4% lignin [7]. Nevertheless, the complex structure of such lignocellulosic material hinders the hydrolytic performance of enzymes on cellulose and hemicellulose to produce fermentable sugars [8]. Pretreatment is thus a critical step to convert lignocellulosic materials into less complex molecules, such as unstructured cellulose, with reduced crystallinity, and available hemicellulose. This process involves the removal of lignin and an important increase in the material porosity. One particularly effective method is thermo-chemo-mechanical extrusion pretreatment, which has demonstrated substantial improvements in both enzymatic hydrolysis and subsequent fermentation stages when applied to various substrates, such as corn stover, barley straw, blue agave bagasse, corn cob, corn straw, or rice straw [7,9–13]. This method consists of an extruder with a certain number of modules in which the chemical and mechanical deconstruction of the fibers is carried out, with the temperature being controlled in each module. As a result, cellulose and hemicellulose become more accessible to enzymatic hydrolysis, and increased yields of fermentable sugars are achieved, which can be converted into biofuels [10].

Depending on the substrate and the content of hemicellulose present in the fiber, the use of xylanases as accessory enzymes plays an important role in sustainably improving the hydrolysis of lignocellulosic biomass [14]. Xylanases, also known as 1,4-β-D-xylano hydrolases, are enzymes that can break down the complex molecule of xylan, the primary component of hemicellulose, to produce xylose. These enzymes, whether used alone or in combination with other lignocellulosic enzymes, such as cellulases, play a role in converting xylose into value-added products, such as bioethanol [15]. It has been reported that xylanases' ability to modify lignin–xylan interactions increase fiber porosity, representing a favorable strategy to improve the overall bioconversion process and to reduce the costs associated with biomass deconstruction [16,17].

The production and applications of xylanases, capable of degrading lignocellulosic biomass, had gained attention worldwide. These enzymes have been produced by bacteria [16,18], actinomycetes [19], yeasts [20], and fungi [21]. *Paenibacillus xylanivorans* A59 is a Gram-positive, facultative anaerobic, and mesophilic bacterium isolated from a forest soil consortium in southern Argentina. This bacterium has the capability to hydrolyze gelatin, starch, xylan, and other compounds [18]. Also, *Moesziomyces aphidis* PYCC 5535T is an anamorphic basidiomycetous yeast that produces relevant extracellular enzymes. Both microorganisms can thrive in xylan-rich media that can be obtained from D-xylose, beechwood xylan, or even pretreated agro-industrial residues, such as sugarcane residue, wheat straw, and brewery-spent grains. Xylanase *Px*Xyn10A (XynA) is the main extracellular xylanase produced by *P. xylanivorans* A59 [18], while *Moesziomyces aphidis* PYCC 5535T (MaPYCC 5535T) produces cellulase-free xylanases [19].

This study evaluated the saccharification of BAB pretreated by thermo-chemo-mechanical extrusion with both in-house xylanases, alone or combined with commercial Cellic® CTec 2, and their influence on the sustainable production of ethanol by fermentation.

2. Materials and Methods

2.1. Microorganism and Xylanase Production

Moesziomyces aphidis PYCC 5535T was obtained from the Portuguese Yeast Culture Collection (PYCC), Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Portugal. Yeast was cultivated in shake flasks as described by Faria et al. [19,22]. Stock cultures were prepared by the propagation of yeast

cells in a liquid medium for the inoculum, as described below, and stored in 20% (*v*/*v*) glycerol aliquots at −70 °C for later use. The inoculum was prepared by incubating stock cultures in a liquid medium for 48 h at 28 °C and 140 rpm in an orbital shaker. The liquid medium contained glucose (40 g/L), $NaNO_3$ (3 g/L), $MgSO_4$ (0.3 g/L), KH_2PO_4 (0.3 g/L), and yeast extract (1 g/L). To produce xylanases, pretreated BAB replaced glucose as a carbon source at a concentration of 10 g/L in terms of xylose equivalents (corresponding to 48 g ODS/L). This culture medium was inoculated with 10% (*v*/*v*) of working culture and incubated for 10 days at 28 °C and 140 rpm in an orbital shaker. One mL culture samples were centrifuged at 13,000× *g* rpm, 4 °C, and the supernatants were stored at −20 °C for later analysis. All experiments were carried out in duplicates, at least.

*Px*Xyn10A from *P. xylanivorans* A59 (XynaA) was expressed in *Escherichia coli* Rossetta and purified according to Ghio et al. [23]. The recombinant enzyme expression was obtained in shake flasks (final volume 1 L), in Luria–Bertani media supplemented with 50 µg/mL of Kanamycin (Km). The culture was induced when it reached an optical density of Abs_{600nm} = 0.8, with 1 mM IPTG for 16 h at 37 °C. The cells were harvested by centrifugation (4000× *g*, 20 min, 4 °C). After cell disruption assisted by ultrasound, the recombinant protein was purified from the soluble fraction by IMAC with Ni-NTA agarose resin (Qiagen, Hilden, Germany), using 50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole, at pH 8, as the elution buffer. The total yield was approximately 0.8 g of purified recombinant protein (more than 80% purity) per L of induced culture.

2.2. Xylanase Activity Assay

The endo-xylanase activity was measured using 1% (*w*/*v*) beechwood xylan from Megazyme in 50 mM potassium phthalate buffer (pH 5.5). The reaction was carried out in a final volume of 0.2 mL at 50 °C and 400 rpm in an Eppendorf Thermomixer for 30 min. Following the reaction, the samples were centrifuged, and the released reducing sugars were measured using the 3,5-dinitrosalicylic acid (DNS) method described by Miller [24]. Upon the completion of the reaction, 0.6 mL of DNS was added, and the solution was heated for 5 min. After cooling the samples in ice, the absorbance was measured at 550 nm using xylose as the standard. Each reaction and its control were performed four times. A total of 1 unit (U) of xylanase activity was defined as the amount of enzyme needed to release 1 µmol of reducing sugar (D-xylose) equivalents per minute.

2.3. Cellulase Activity Assay

The activity of Cellic Ctec2 was measured in filter paper units (FPUs), as it has been described since 1987 [25]. A 1 × 6 cm strip of Whatman no. 1 filter paper (50 mg) was hydrolyzed in 1.5 mL of the enzyme solution 0.05 M of citrate buffer (pH 5.5). The samples were then incubated for 1 h at 50 °C in an orbital shaker. After stopping the reaction, 200 µL of the sample was obtained, and the reducing sugars were measured using the DNS method. Glucose was used as the standard. A total of 1 FPU is defined as the amount of enzyme that produces 2.0 mg of reducing sugar from 50 mg of filter paper within 1 h.

2.4. Enzymatic Saccharification of PBAB

The enzymatic hydrolysis of pretreated BAB (PBAB) was undertaken at 20% (*m*/*v*) solids concentration in 50 mM sodium acetate buffer (pH 5.5) at 50 °C in 50 mL Erlenmeyer flasks agitated at 200 rpm in an orbital shaker with temperature control. Three different configurations for the xylanase (XynA or MaPYCC 5535T) application were tested. In the first one, Cellic® CTec2 (at a dosage of 10 FPU/g ODS) was supplemented with XynA or MaPYCC 5535T at an endo-xylanase dosage of 100 U/g ODS, and the reaction was carried out for 24 h. In the second configuration, each xylanase was applied alone (at an endo-xylanase dosage of 100 U/g ODS) for 72 h as a selective pre-hydrolysis step, followed by the addition of Cellic® CTec2 (at a dosage of 10 FPU/g ODS). In the third configuration, the selective pre-hydrolysis step was started with each xylanase (at an endo-xylanase dosage of 100 U/g ODS) supplemented with 1 FPU/g ODS of Cellic® CTec2 (to promote

some liquefaction), followed by the addition of 9 FPU/g ODS of Cellic® CTec2 after 72 h reaction time (to make up the 10 FPU/g ODS dosage). The saccharification degree for both glucose and xylose was determined using high-performance liquid chromatography (HPLC) quantification, as outlined in Section 2.7. The results are expressed as a percentage of conversion relative to the carbohydrate content (potential glucose and xylose) in the initial substrate. All experiments were conducted in triplicate.

2.5. Raw Material

Tequila Patron, a renowned tequila production facility located in Guadalajara, Jalisco, Mexico, generously provided the blue agave bagasse (BAB). BAB was dried at room temperature and ground to particles smaller than 3 mm. The resulting material was subsequently dried at 60 °C in a dry heat oven and stored for future use.

2.6. Extrusion Pretreatment of BAB

The pretreatment stage was conducted using a Clextral Processing Platform Evolum EV25 twin-screw extruder from Clextral, France, comprising ten 100 mm long modules with electrical heating. The raw BAB, obtained as detailed in Section 2.5, was introduced in the first module of the extruder at a flow rate of 1 kg/h. Along the extruder, a sequential addition of NaOH (3.75% (*w*/*v*)), heating at 150 °C, and neutralization with H_2SO_4 (0.5% (*w*/*v*)) was performed. In Module 9, a stainless-steel filter removed the liquid part from the solids. In Module 10, there were two reverse screws that acted as a compression zone. The extruder was set to a speed of 100 rpm and the operation conditions were adjusted to achieve a NaOH/dry matter (DM) ratio of 4.1% (*w*/*w*). After extrusion, the pretreated BAB was recovered, dried, and analyzed for further use.

2.7. Glucose and Xylose Determination

The xylose and glucose were separated and quantified using the HPLC-PR-ELSD equipment with a Unison UK-Amino column (3 µm, 250 Å × 3.0 mm, Imtakt Corp., Kyoto, Japan). The mobile phase consisted of acetonitrile and water in a ratio of 80:20 at a flow rate of 0.2 mL/min. The column temperature was maintained at 37 °C, while the evaporator and nebulizer temperatures were set to 80 °C and 60 °C, respectively, with a 1 mL/min nitrogen flow rate at the light scattering detector. The concentration of each component was determined by using standard curves.

2.8. Scanning Electronic Microscopy

Scanning electron microscopy images of the raw BAB, PBAB, and PBAB remaining after an enzymatic reaction were obtained using a JEOL Company scanning microscope (model JSM-5900, Peabody, MA, USA). The samples were analyzed in low vacuum and at various magnifications (×90, ×150, and ×500) to observe any morphological surface changes.

2.9. Fermentation of PBAB Hydrolysate

Cellux 4™ yeast cells (a GMO strain of *Saccharomyces cerevisiae* developed to ferment both xylose and glucose) were kindly provided by Lessafre (Marcq-en-Barœul, France). The strain (20 mg) was activated with 5 times its weight of deionized water for 30 min and then added to the hydrolysate supplemented with 0.2% (*w*/*v*) yeast extract. Fermentations were carried out in 50 mL Erlenmeyer flasks (30 mL volume working volume) and incubated in an orbital shaker at 200 rpm for 72 h at 30 °C. Ethanol concentrations were determined using HPLC analytics.

2.10. Ethanol Determination

A total of 500 µL samples was obtained from the fermentation flasks to monitor the ethanol production. All samples were centrifuged to remove yeast cells, and the remaining liquid was filtered through 0.22 µm membrane filters. The ethanol produced was quantified

using an HPLC equipped with a refraction index detector and a Carbomix H-NP10 column; the mobile phase was water at a flow rate of 0.6 mL/min. The analysis was carried out at 60 °C for 20 min. Ethanol concentrations were determined by using a standard curve.

2.11. Statistical Analysis

All the experimental values are presented as the mean and standard error of the means. A one-way ANOVA followed by Dunnett's multiple comparison was performed using GraphPad Prism version 9.0 for Mac, GraphPad Software, San Diego, CA, USA.

3. Results and Discussion

3.1. Xylanase Activity

To assess the efficacy of *Px*Xyn10A (XynA) recombinant xylanase, the enzyme was purified entirely (>80% purity). The crude extracellular extract was utilized in the case of xylanase from Moesziomyces aphidis PYCC 5535T (MaPYCC 5535T). The enzyme activity was measured using beechwood xylan as the substrate under the conditions applied for the enzymatic hydrolysis of PBAB. The enzyme activity for XynA was found to be 250 U/mL, while that of MaPYCC 5535T was 50 U/mL. Xylanases are mostly extracted from *Bacillus* sp. and typically have activities ranging from 14 to 500 units using xylan as a substrate [26]. Since Xyna and MaPYCC 5535T are enzymes from different microorganisms, they exhibit a similar activity to those reported for *Bacillus* sp.

3.2. BAB Pretreatment and Composition

The chemical–mechanical extrusion method reported by Montiel et al. [7] was used to pretreat the bagasse with some modifications. Specifically, the concentration of NaOH was decreased from 5 to 3.75% (*w*/*v*). Instead of using phosphoric acid (5% (*w*/*v*)) to neutralize the solution, a more diluted sulfuric acid (0.5% *v*/*v*) was used to reduce the amount of Na_2SO_4 produced as a potential fermentation inhibitor. Also, the temperature was increased from 50 to 150 ºC to favor fiber deconstruction. The percentage of cellulose recovered from the pretreated BAB was enhanced with these new conditions (Table 1), while the hemicellulose content was unaffected. The results are compared to values previously reported [7]. Although the acid and base concentrations were reduced, the effectiveness of the process to deconstruct the BAB fiber was maintained, with an increase in the hydrolysis yield of approximately 50% with respect to the raw BAB. The study by Wang et al. reported that using a twin-screw extruder with NaOH and glycerol led to a 35% increase in the hydrolysis of pretreated corn stalks [27]. Additionally, Fasheun et al. employed a twin-screw extruder to pretreat a mixture of Cassava starch with sugarcane bagasse and achieved a 330% higher glucose yield than the raw material [28]. These findings indicate that extrusion, whether mechanical or chemical–mechanical, can improve the enzymatic hydrolysis yield compared to raw materials. This improvement can be attributed to factors such as the rupture or weakening of hydrogen bonds, reduction in the particle size, increased surface area, changes in the structure of lignocellulosic material, solubilization of lignin, and minimal production of inhibitory compounds that could otherwise affect enzymatic hydrolysis or fermentation [7,29,30]. It can be challenging to compare the results in the literature because this technology has numerous variables that may include the residence time; temperature; whether it is only a mechanical pretreatment or supplemented with compounds, such as NaOH, H_3PO_4, H_2SO_4, glycerol, enzymes, etc.; screw speed; and screw profile [29]. Even the type of raw material plays an important role in this kind of pretreatment.

Table 1. Composition of raw and extruded BAB (% (*w*/*w*)).

Composition	Raw BAB	Modified Extruded BAB	Extruded BAB [7]
Cellulose	38.9 ± 0.9	50.8 ± 1.0	47.2 ± 0.9
Hemicellulose	16.9 ± 1.3	16.1 ± 0.7	18.5 ± 0.4
Lignin	22.2 ± 0.9	18.3 ± 0.9	16.4 ± 0.2
Mineral matter	5.9 ± 0.7	3.9 ± 0.0	--
Inert compounds	16.1 ± 1.8	10.7 ± 0.7	8.6 ± 0.1

3.3. Effect of In-House Hemicellulases on the Hydrolysis of PBAB

Initially, the performance of both xylanases (XynA or MaPYCC 5535T) on the enzymatic hydrolysis of the pretreated BAB (PBAB) was evaluated at the endo-xylanase dosage of 100 U/g ODS, as a supplement to the commercial cellulase Cellic® CTec2 (10 FPU/g ODS). The hydrolysis yields for conversion into glucose and xylose are presented in Figure 1, relative to the control assay (in the absence of in-house xylanase). It is verified that this supplementation increases the yield of cellulose and xylan hydrolysis by 10% and 12%, respectively, compared to the sample treated with 10 FPU. This suggests that the addition of xylanase not only enhances the hydrolysis of hemicellulose but also that of cellulose. It is believed that this result is due to the action of hemicellulase that, by removing either the hemicellulose backbone and/or the hemicellulosic side chains, increases the exposure of the cellulose microfibrils array [31,32].

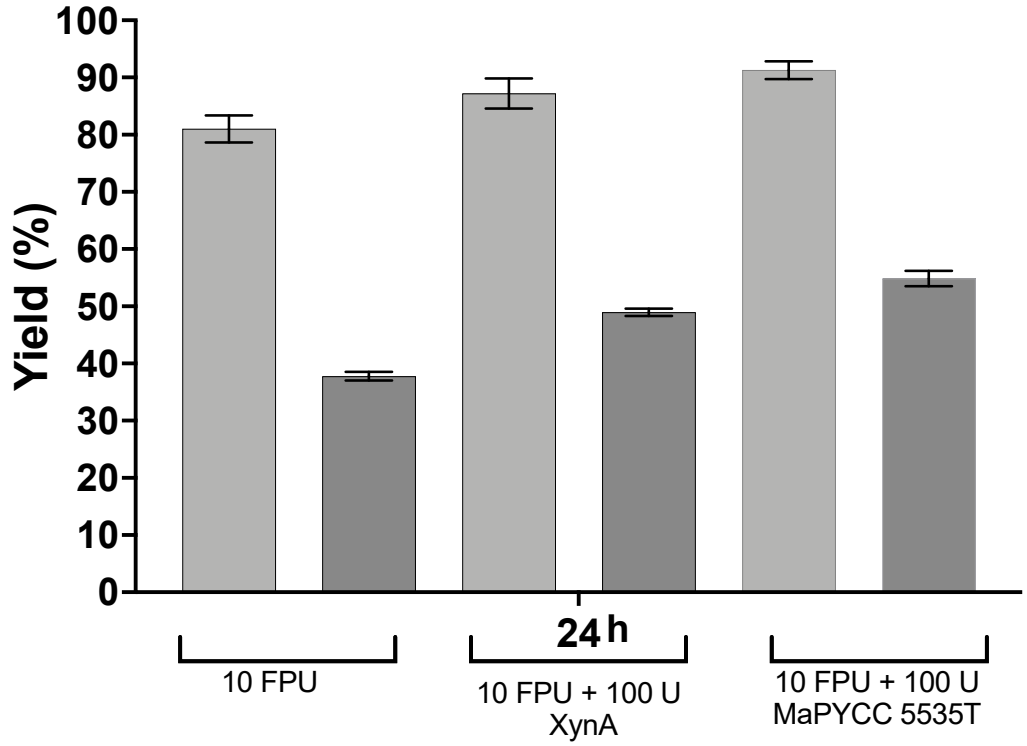

Figure 1. Yield of hydrolysis of PBAB into glucose and xylose using a different combination of in-house xylanase for 24 h. The reaction was performed at 50 °C, pH 5, and 200 rpm for the entire duration, with a 20% (*w*/*v*) solids load. Cellic® CTec2 (10 FPU/g ODS) was used along with 100 U/g ODS of in-house xylanases (XynA or MaPYCC 5535T), compared to the control group (without in-house xylanase). The conversion yields into glucose are shown as light gray bars and into xylose as dark gray bars.

As an alternative strategy, in-house xylanases were evaluated separately (at 100 U/g ODS dosage) as a selective pre-hydrolysis step, followed by hydrolysis with the commercial enzyme cocktail to complete the hydrolysis. Under this strategy, when XynA was added

in the absence of cellulases, no hemicellulose hydrolysis was observed after the 72 h (Figure 2A). The addition of sole MaPYCC5535T to PBAB yielded 4.8% hydrolysis into xylose after 72 h (Figure 2B). After this 72 h period of pre-hydrolysis, Cellic® CTec2 (at a dosage of 10 FPU/g ODS) was added to the reaction flask, and the reaction was carried out for an additional 48 h. The hydrolysis yield at the specified times is shown in Figure 2. Interestingly, the final glucose yield was similar to that obtained previously (see Figure 1), but the xylose yield was increased by about 10% (GH10XynA) and 13% (MaPYCC5535T). It appears that cellulase activity and some degree of depolymerization are required to activate or expose the xylan chains that are not available to the action of xylanases as previously reported [33].

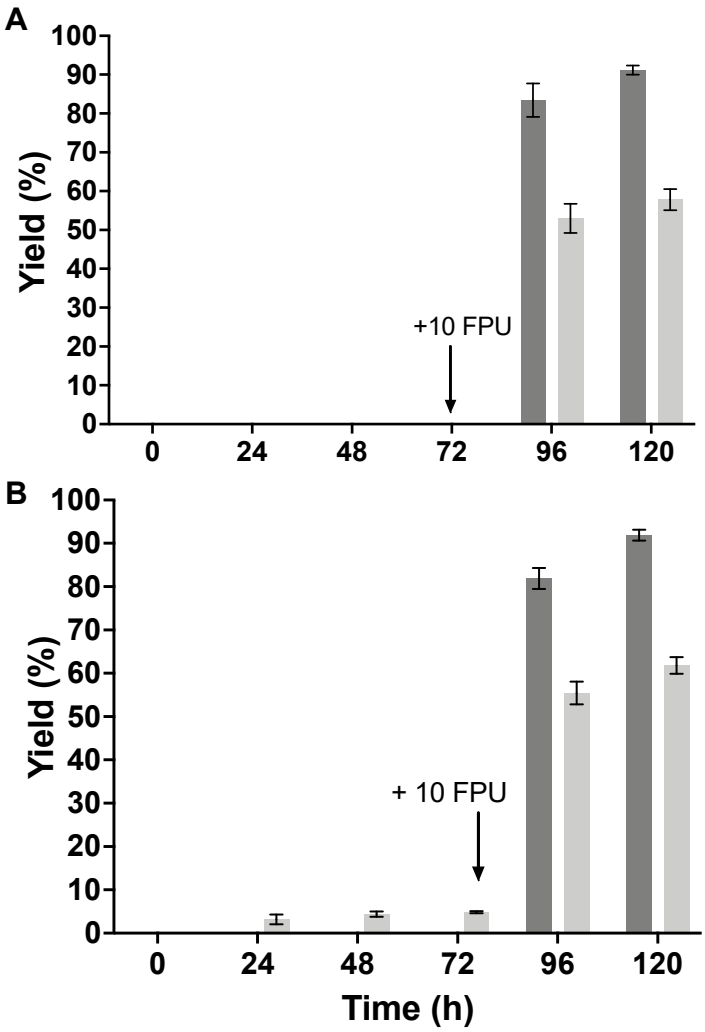

Figure 2. Yield of hydrolysis of PBAB into glucose and xylose using in-house xylanases alone for 72 h. The reaction was performed at 50 °C, pH 5, 200 rpm, for 48 h, PBAB at 20% (*w*/*v*) solids load with 100 U/g ODS of in-house xylanases. After 72 h, 10 FPUs were added: (**A**) XynA; (**B**) MaPYCC5535T. Yields of conversion into glucose, dark gray bars; and xylose, light gray bars.

In the study by Han et al. in 2020, a bifunctional enzyme called CtCel7 was used that exhibited both xylanase and cellobiohydrolase activities, in conjunction with a commercial enzyme acting on pretreated wheat straw. They discovered that using CtCel7 alone resulted in a lower concentration of reducing sugar compared to using the commercial enzyme (Cellic Ctec3). However, when CtCel7 was combined with Cellic Ctec3, it produced a higher reducing sugar concentration than the commercial enzyme alone; this behavior is similar

our observation. This suggests that xylanases have a synergistic effect with cellulase on the lignocellulosic material [34].

To further investigate the effectiveness of in-house xylanases for the pre-hydrolysis of PBAB in synergy with cellulases, each xylanase, added at 100 U/g ODS, was supplemented with a very low cellulase dosage of Cellic® CTec2 (1 FPU/gODS) to assure some liquefaction. After a 72 h period of pre-hydrolysis, 9 FPU/g ODS of Cellic® CTec2 was added to make up the 10 FPU/g ODS dosage. Incubation was maintained for an additional time of 48 h. The progression of hydrolysis for both stages is shown in Figure 3. It can be seen that after 24 h of pre-hydrolysis, the conversion surpassed the yield obtained with Cellic® CTec2 alone by 22% and 43% (XynA and MaPYCC5535T), respectively. After the 72 h period of pre-hydrolysis with in-house xylanases supplemented with the low dosage of 1 FPU/g ODS of Cellic® CTec2, the glucose and xylose conversion reached yields close to 60% and 39% for XynA and MaPYCC5535T, respectively. By the end of the hydrolysis period, i.e., 48 h after making up the dosage of 10 FPU/g ODS of Cellic® CTec2, a higher yield was obtained with this experimental setup, with both glucose and xylose reaching 96% and 67% of the theoretical yields, respectively.

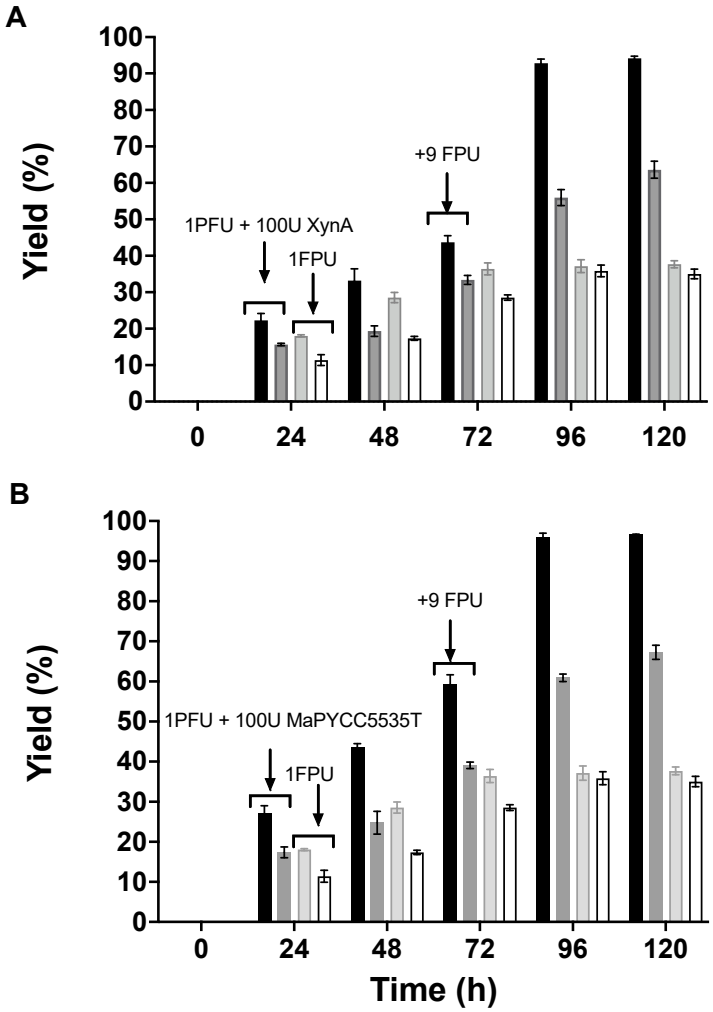

Figure 3. Yield of hydrolysis of PBAB into glucose and xylose using 1 FPU and in-house xylanases. The reaction was performed at 50 °C, pH 5, 200 rpm, for 48 h, PBAB at 20% (*w*/*v*) solids load with Cellic® CTec2 (9 FPU/g ODS). Initial phase of pre-hydrolysis for 72 h with 1 FPU/g ODS of Cellic® CTec2 supplemented with 100 U/g ODS of in-house xylanases. An extra dose of 9 FPU/g ODS was added at 72 h: (**A**) XynA; (**B**) MaPYCC5535T. Control, 1 FPU in the absence of in-house xylanase. Yields of conversion into glucose, dark gray bars; and xylose, light gray bars.

Some authors suggest that xylan surrounds each cellulose microfibril and becomes trapped within or between the microfibrils during crystallization after cellulose synthesis [33,35]. The context of our experiments with in-house xylanases could indicate that the partial (but minor) hydrolysis of PBAB by a low dose of cellulase could expose xylan chains entangled between the cellulose, allowing the hemicellulases to exert their enzymatic action on the xylan fraction. This is in agreement with the increased glucose and xylose yields observed after the initial 24 h period, in comparison to the single effect of the cellulase or the xylanases acting individually.

3.4. Analysis of the BAB Surface

Surface changes in the raw BAB and PBAB, caused by pretreatment and enzyme action on hydrolysis, were observed using scanning electron microscopy (SEM). The SEM analysis revealed that the extrusion pretreatment of BAB led to some modifications in the material surface. The SEM images of all samples are shown in Figure 4. The alterations that occurred in the pretreated material can be observed in Figure 4B. Lignin was found to be severely stripped by the extrusion and NaOH action, which promoted changes in BAB composition when compared to the untreated raw BAB (Figure 4A). This enhanced the exposition of the cellulosic region to the enzymes (cellulases and hemicellulases). The SEM image of PBAB after hydrolysis with 10 FPU/g ODS of Cellic® CTec2 showed that the PBAB structure broke down into individual fibers of varying sizes. This suggests that cellulases could penetrate the cellulose network and initiate the enzymatic reaction (Figure 4C). The effects of both in-house xylanases on the surface of the PBAB fibers are shown in Figure 4E,F when 1 FPU/g ODS of Cellic® CTec2 was supplemented. The structures contained multiple pores that are not visible after pretreatment (Figure 4B) or even barely noticeable with only Cellic Ctec2 (Figure 4D). The presence of these small pores persists even with a low dosage of xylanase. It is well established that xylanases are employed as auxiliary agents in the bleaching of cellulosic pulps, and they cause certain alterations in the fibers, increasing their porosity [35]. The PBAB subjected to the action of in-house xylanases alone did not show the presence of pores on the surface of the material, which indicates that the action of hemicellulases requires the presence of cellulase (even supplemented at a low dosage) to initiate the fiber fracture.

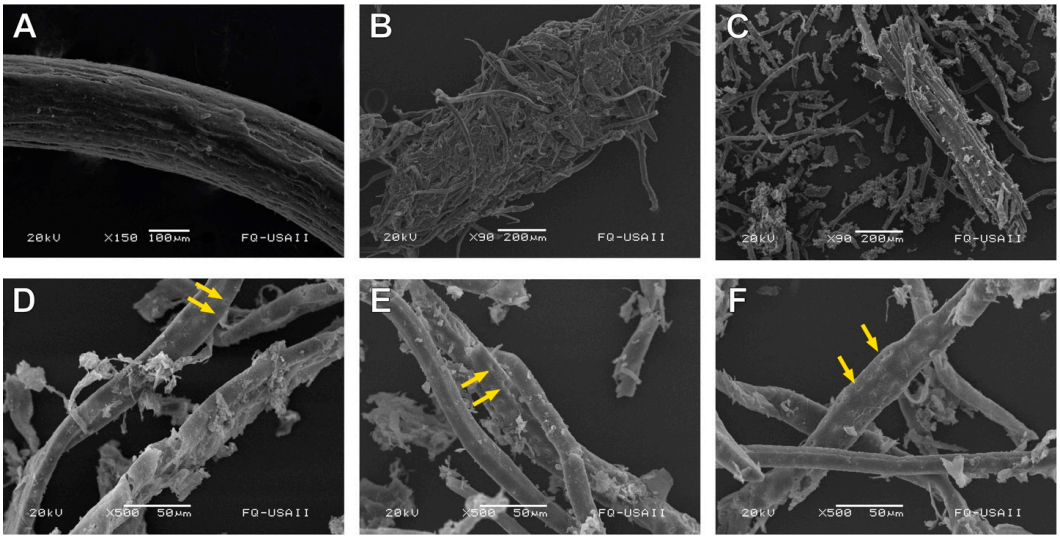

Figure 4. SEM images of BAB before and after enzymatic hydrolysis. (**A**) Untreated (raw) BAB; (**B**) BAB pretreated by thermo-chemical extrusion; (**C**) PBAB after enzymatic hydrolysis with 10 FPU/g ODS of Cellic® CTec2; (**D**) PBAB after enzymatic hydrolysis with 1 FPU/g ODS of Cellic® CTec2; (**E**) PBAB after enzymatic hydrolysis with 100 U/g ODS of XynA supplemented with 1 FPU/g ODS of Cellic® CTec2; (**F**) PBAB after enzymatic hydrolysis with 100 U/g ODS of MaPYCC5535. All reactions were performed with 20% (*w*/*v*) PBAB, at 50 °C, pH 5.5, 200 rpm for 24 h.

3.5. Fermentation of Hydrolysates from PBAB

The hydrolysates with fermentable sugar content, after the 120 h period, as shown in Figure 3, were used without filtration for the following fermentation assays. Hydrolysates of PBAB obtained with only 10 FPU/g ODS of Cellic® CTec2 were also subjected to fermentation as a control. When MaPYCC553 xylanase or XynA was applied for the pre-hydrolysis of PBAB in combination with 1 FPU/g ODS of Cellic® CTec2, followed by the addition of Cellic® CTec2 to make up the dosage of 10 FPU/g ODS, the glucose and xylose concentrations obtained were approximately 85 and 20 g/L, respectively. As shown in Figure 5, Cellux 4 yeast was able to promptly consume both glucose and xylose during the first 48 h of fermentation, producing a maximum ethanol concentration of 45 g/L. Compared to the control, a 30% increase was observed in ethanol production when in-house xylanases were used. The ethanol production achieved in this study was similar to the results reported by Delfin Ruiz in 2020 (47.3 g/L), who used optimized pretreated BAB with H_2O_2 [36]. However, other studies have shown a range of ethanol production from different agave bagasse, varying between 16.5 and 40 g/L [1,37–40]. The variation in ethanol concentration observed may be attributed to the process of obtaining the bagasse, the specific variables in each experimental process, the type of pretreatment, composition of hydrolysates, enzymatic reaction conditions, and the strain used for sugar fermentation.

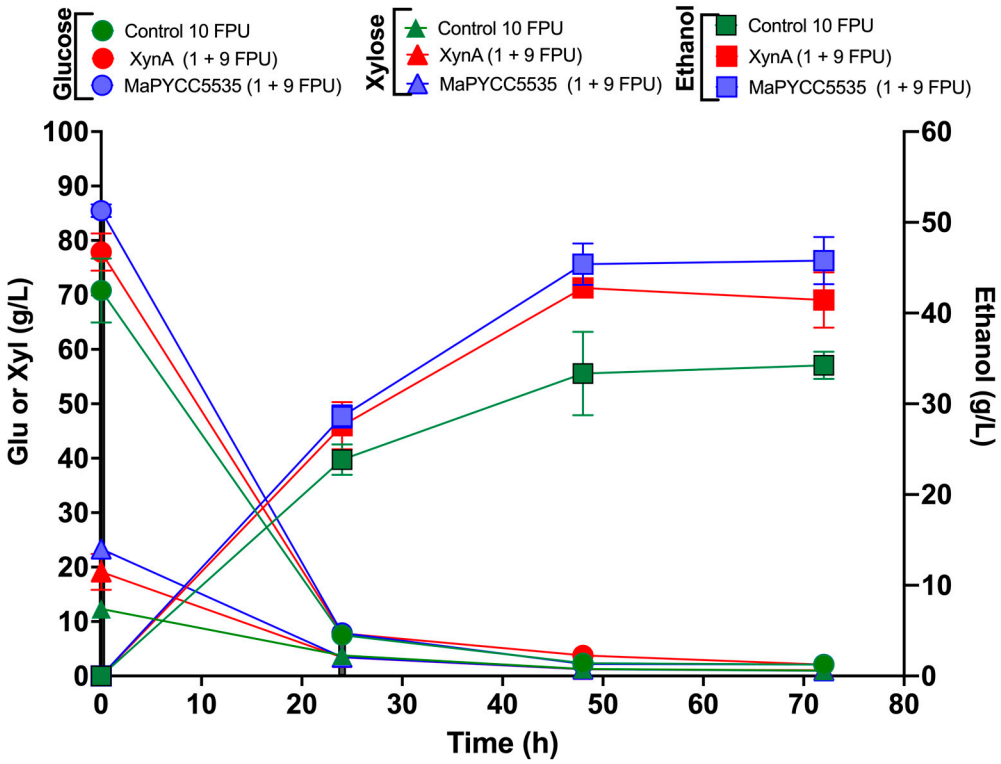

Figure 5. Changes in glucose, xylose, and ethanol concentrations during fermentation with Cellux 4 yeast on hydrolysates obtained during the reaction with different enzyme combinations. Fermentations were carried out at 30 °C with PBAB hydrolysates obtained after 120 h. Control (green symbols), 10 FPU/g ODS; XynA (at an endo-xylanase dosage of 100 U/g ODS supplemented with 1 FPU/g ODS of Cellic® CTec2 (during the first 72 h period) followed by the addition of 9 FPU/g ODS) (red symbols), or MaPYCC5535 (at an endo-xylanase dosage of 100 U/g ODS, supplemented with 1 FPU/g ODS of Cellic® CTec2 (during the first 72 h period) followed by the addition of 9 FPU/g ODS) (blue symbols).

4. Conclusions

The valuation of residual blue agave bagasse (BAB) from the tequila industry as a sustainable source of bioethanol has been studied by various groups. They have examined

the necessary hydrolysis of polysaccharides prior to fermentation using different enzymes, mainly cellulases from various sources. However, there are few reports on the use of mixtures of xylanases and cellulases. This report introduces a novel approach by integrating the complete process based on three sequential steps: extrusion, enzymatic hydrolysis with a combination of cellulase and xylanase, and alcoholic fermentation.

From our experimental data, it is observed that using lower concentrations of both NaOH and H_2SO_4 during the thermo-mechanical stage, in combination with increased temperature, resulted in improved enzymatic hydrolysis and better yields of glucose and xylose compared to the available data.

Two in-house xylanases were evaluated in combination with a commercial cellulase Cellic Ctec2. Of the two xylanases, only recombinant MaPYCC5535 showed minimal direct activity on the thermo-extruded BAB. However, this low activity notably enhanced the action of cellulase, increasing the yields of both glucose and xylose. Even at low doses of Cellic Ctec2 during a 72 h incubation, supplementing with an additional amount at 72 h resulted in increased yields of 58% for glucose and 30% for xylose within 24 h. A clear synergistic effect was observed between cellulases and xylanases, where a small dose of cellulase played a crucial role in breaking down the fibers and allowing xylanases to penetrate the matrix. This effect was validated by SEM images, showing that the xylanases generated pores on the surface and clear fractures on the cellulosic fibers, resulting in the more efficient and sustainable hydrolysis of both cellulose and hemicellulose. Furthermore, when the final hydrolysate was fermented with recombinant Cellux 4 yeast, a high concentration of 45 g/L ethanol was obtained in only 50 h, with almost all glucose and xylose consumed.

In summary, the pretreatment of BAB by thermo-extrusion, followed by saccharification using an optimized sequence of xylanase and cellulase, provides a medium rich in monosaccharides extracted in a high yield from the original lignocellulosic BAB material for an efficient and sustainable bioethanol production.

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