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Tailoring a cellulolytic enzyme cocktail for efficient hydrolysis of mildly pretreated lignocellulosic biomass

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ABSTRACT

Commercially available cellulase cocktails frequently demonstrate high efficiency in hydrolyzing easily digestible pretreated biomass, which often lacks hemicellulose and/or lignin fractions. However, the challenge arises with enzymatic hydrolysis of mildly pretreated lignocellulosic biomasses, which contain cellulose, hemicellulose and lignin in high proportions. This study aimed to address this question by evaluating the supplementation of a commercial cellulolytic cocktail with accessory hemicellulases and two additives (H_2O_2 and Tween® 80). Statistical optimization methods were employed to enhance the release of glucose and xylose from mildly pretreated sugarcane bagasse. The optimized supplement composition resulted in the production of 304 and 124 mg g^{-1} DM of glucose and xylose, respectively, significantly increasing glucose release by 84% and xylose release by 94% compared to using only the cellulolytic cocktail. This enhancement might be attributed to a coordinated hemicellulases action degrading hemicellulose, creating more space for cellulase activity, potentially boosted by the presence of H_2O_2 and Tween® 80. However, the addition of different concentrations of H_2O_2 in combination with hemicellulase and Tween® 80 did not result a significant difference on sugar release, which could be attributed to the limited range of concentrations studied (5 to 65 μM). The results obtained in this study using the mix of three supplements were also compared to the addition of only hemicellulase and only Tween® 80 to the cellulolytic cocktail. A significant increase in glucose release of 39% and 41%, respectively, was observed when using the optimized combination. For xylose, the increase was 38% and 41%, respectively. This study underscores the substantial potential in optimizing enzyme cocktails for the hydrolysis of mildly pretreated lignocellulosic biomass by using enzymes and additive combinations tailored to the specific biomass composition.

1. Introduction

Products derived from lignocellulosic biomass hold great potential in replacing fossil-derived products like fuels, chemicals, and materials, thereby playing a key role in the transition to a circular and bio-based economy [1]. However, developing an efficient, cost-effective, and environmentally friendly fractionation process that allows the complete utilization of biomass components while preventing the generation of undesirable byproducts, remains a challenge [2,3]. Additionally, a successful transition requires the adoption of green chemistry practices and the development of cost-competitive manufacturing alternatives [4]. Enzymes offer a promising avenue for biomass fractionation due to their high selectivity in hydrolyzing biomass polysaccharides (cellulose and hemicellulose) into sugars such as glucose and xylose [5]. Nevertheless, exploiting this potential is challenging due to several physicochemical, structural, and compositional factors that limit the digestibility of these polysaccharides [6]. To enhance enzyme accessibility, a pretreatment step is essential before enzymatic hydrolysis, as it helps to disrupt the rigid structure of the biomass.

A variety of pretreatment methods have been developed to date, ranging from chemical processes utilizing acids, alkalis, or hot water, to physicochemical processes such as ammonia fiber expansion (AFEX) or steam explosion, and even biological processes involving the use of enzymes or microorganisms [7]. Nonetheless, most conventional pretreatment methods are not economically viable due to several reasons. These include the need for strong chemicals, which are not only challenging and costly to remove and recover, but also contribute to substantial energy and water consumption [7]. As a more favorable alternative to these conventional pretreatment methods, the use of CO_2 for biomass pretreatment under mild conditions is increasingly gaining

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attention. Recognized as a green chemical, CO_2 is non-toxic, inexpensive, widely available, and easy to recover and recycle. Additionally, it exhibits a high diffusion rate and does not produce chemical waste [8]. Through the application of suitable process conditions, CO_2 can induce biomass swelling, thereby enhancing the accessibility of hydrolytic enzymes to the structure without causing degradation of biomass components. However, to maximize the effectiveness of this pretreatment, the enzyme cocktail employed for biomass saccharification must be tailored to the unique composition of the CO_2-pretreated material.

Cellulase cocktails currently available commercially contain a range of hydrolytic enzymes including cellulases, hemicellulases, endoglucanases, and lytic polysaccharide monooxygenases (LPMOs), to break down the diverse linkages present in the biomass structure [9]. These enzymes work synergistically, transferring positive characteristics to each other that can enhance biomass hydrolysis. However, such cellulase cocktails typically exhibit high efficiency in hydrolyzing biomass that had the hemicellulose and/or lignin structures degraded during pretreatment. These cocktails are not efficient to hydrolyze biomass pretreated under mild conditions, in which all the three main fractions (cellulose, hemicellulose, and lignin) are present. In addition, different enzyme cocktails with the same enzymatic activity may have different efficiencies depending on the specific composition of accessory enzymes present or biomass substrates to be hydrolyzed [10].

Several strategies can be used to increase the release of sugars during enzymatic hydrolysis, and they are highly dependent on the specific composition of the biomass, the pretreatment technology applied, and the compounds generated during pretreatment [11]. An interesting approach is to increase the activity of the LPMOs present in the enzyme cocktail. LPMOs are monocopper enzymes [12] that bind to the crystalline regions of cellulose [13], and in some cases, to hemicellulose [14–16]. These enzymes can cleave cellulose and hemicellulose polysaccharides via oxidation, creating new cavities for other enzymes to access [17]. Some studies have reported increased activity of LPMOs with hydrogen peroxide (H_2O_2) supplementation [18–22], which may have a positive impact on the industrial use of biomass. Another potential strategy involves the use of hemicellulases [23–25], which can enhance accessibility to cellulose by hydrolyzing hemicellulose, thereby removing it as a physical barrier [17], [44]. This is especially relevant when hydrolyzing substrates with a high content of hemicellulose. The use of nonionic surfactants, such as Tween® 80, has also been reported to improve the enzymatic digestibility of cellulose [11,23,26]. These surfactants have a critical role in minimizing the non-productive adsorption of cellulase on lignin, a recognized significant obstacle during enzymatic hydrolysis. In addition, they facilitate the formation of a network at the liquid-air interface, resulting in a reduction in the surface area that is accessible to enzymes. As a result, surfactants help prevent enzyme inactivation [27].

The objective of this study was to maximize the efficiency of enzymatic hydrolysis of CO_2-pretreated sugarcane bagasse by supplementing a commercial cellulolytic enzyme cocktail with accessory hemicellulases and additives (H_2O_2 and Tween® 80). The selection of these enzymes and additives was based on a previous study in which several supplementation alternatives to the cellulolytic enzyme cocktail were evaluated [22]. The hypothesis of the effect of these supplements was based on the structure and composition of CO_2-pretreated sugarcane bagasse, containing the full polymeric composition of cellulose, hemicellulose, and lignin. The addition of hemicellulases would degrade hemicellulose and would create space for the access of cellulase enzymes. At the same time, the additive H_2O_2 would boost the activity of LPMOs, leading to an enhanced degradation of cellulose and hemicellulose. Finally, the addition of Tween® 80 would prevent the non-productive binding of cellulases to the lignin structure and to the air/liquid interface, enhancing the efficiency of the cellulolytic cocktail. Results from the former study showed that hemicellulases and the two additives tested increased the glucose and xylose release when they were independently supplemented to the commercial cellulolytic enzyme cocktail. The purpose of the present work was then to evaluate the combination of hemicellulases, H_2O_2 and Tween® 80 supplementation to the commercial cellulolytic enzyme cocktail, assess the optimal dose for each of them and to compare the results with the independent supplementation approach. Results were evaluated and optimized using statistical tools.

2. Materials and methods

2.1. Biomass composition and pretreatment

The sugarcane bagasse used in this study was provided by the company Raízen (São Paulo, Brazil). To be used in the experiments, the material was finely ground to a particle size of 2 mm using a hammer mill (Polymix, PX-MFC 90 D, Kinematica AG, Switzerland), then rehydrated to achieve a moisture content of 50% (w/w), and finally subjected to a mild subcritical CO_2 pretreatment using a SFE Lab 500 mL supercritical CO_2 extraction unit (SFE Process, France). The contents of cellulose, hemicellulose, lignin, acetyl group, ash and extractives in the raw and pretreated material were determined according to the NREL protocols [28,29].

2.2. Enzymatic hydrolysis

The enzymatic hydrolysis of pretreated sugarcane bagasse was performed in 24 deep-well plates with a volume of 10 mL (Enzyscreen, The Netherlands). Prior to the reactions, the moisture content of the biomass samples was measured using a Touch moisture analyzer (VWR International bvba, Belgium). Following this, in the enzymatic hydrolysis process, a 0.05 M sodium acetate buffer with a pH of 4.8 was added until the dry mass content reached 10% (w/w). A reaction volume of 2 mL was used. The volume or weight of enzymes and additives was added on top of the buffer needed to reach the desired solid loading. The cellulolytic cocktail Cellic® CTec3 HS (CC3, Novozymes, Denmark) was used at an enzyme load of 35 FPU g^{-1} dry mass (DM). The hydrolysis was conducted at 150 rpm, 50 °C for 72 h. Samples were taken at the end of the process and heated at 100 °C for 10 min to inactivate the enzymes. The remaining solids were separated by centrifugation using a centrifugal force of 1957g for 6 min and filtered through a 0.45 μm syringe filter (Millipore, MA, USA).

Control samples without enzymes were prepared and analyzed for released sugar content to verify whether spontaneous degradation of biomass occurred over time.

2.3. Experimental design and data analysis

A 3-factor Box-Behnken design (BBD) with 3 levels and 3 replicates at the center point was used to evaluate the influence of CC3 enrichment with hemicellulase (NS22244, Novozymes, Denmark) (x_1), H_2O_2 (x_2), and Tween® 80 (x_3) on the enzymatic release of glucose from CO_2-pretreated sugarcane bagasse.

The model obtained from the BBD included the quadratic and linear terms as well as the linear relation between the different independent factors. Lack-of-fit and analysis of variance (ANOVA) were used for model validation to assess the accuracy and reliability of the developed model. Lack-of-fit is a method used to check how well a model fits the experimental data. It compares the variability of the model's residuals to the variability of the pure error. If the lack-of-fit is found to be significant, it indicates that the model may not accurately represent the true relationship between the factors and the response variable [30]. In such cases, further adjustments or improvements to the model may be necessary[31]. ANOVA was used to determine the significance of the model terms and their contributions to the overall variability of the response. It helps to identify which terms are statistically significant and should be retained in the model.

The desirability tool was used to define and apply a desirability function to optimize glucose and xylose release in combination. A single

desirability score, which shows the overall preference for a particular combination of input factors, was obtained. This score can range from 0 to 1, where 0 represents the least desirable outcome and 1 the most desirable.

Building upon previous findings [22], which investigated 52 different approaches of single supplementation to CC3, it was found that H_2O_2, hemicellulase, and Tween® 80 exhibited the best performance as additives for the cocktail. These results were used to determine the working ranges to be studied during the subsequent statistical optimization phase, employing the Box-Behnken Design (BBD). Also, the results obtained in this study were assessed in comparison with the addition of only hemicellulase and only Tween® 80 to CC3 obtained in [22].

StatisticaTM 14.0.1 (TIBCO Software Inc., Palo Alto, California, USA) was the software used to compute the model and perform the statistical analysis.

2.4. Analytical methods

The quantification of soluble sugars in the hydrolysates after enzymatic hydrolysis was carried out by High-Performance Liquid Chromatography (HPLC) using a Dionex Ultimate 3000 high-performance liquid chromatography UHPLC+ Focused system (Dionex Softron GmbH, Germany) with a Bio-Rad Aminex column HPX-87 H (300 mm × 7.8 mm) at 60 °C, a Shodex RI-101 refractive index detector, 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL min^{-1}, and injection volume of 20 μL.

The production of glucose and xylose was calculated as follows, where $C_{glucose}$ and C_{xylose} are the concentration of glucose and xylose, respectively (g L^{-1}), $V_{hydrolysis}$ is the hydrolysis working volume (L), and DM is the amount (g) of dry mass added.

$$Glucose\ \ production\ \ \left(mg\ \ g^{-1}DM\right) = \frac{C_{glucose}\ \ \bullet V_{hydrolysis} \bullet 1000}{DM} \quad (1)$$

$$Xylose\ \ production\ \ \left(mg\ \ g^{-1}DM\right) = \frac{C_{xylose}\ \ \bullet V_{hydrolysis} \bullet 1000}{DM} \quad (2)$$

3. Results and discussion

3.1. Effect of cellulolytic enzymatic cocktail enrichment on biomass hydrolysis

The chemical composition of raw and CO_2 pretreated sugarcane bagasse used in this study is shown in Table 1. As can be seen, there is not much change in biomass composition after pretreatment. This can be explained by the fact that the CO_2 pretreatment do not promote the solubilization of hemicellulose or lignin fractions, but instead, it promotes a disorganization in the biomass fibers and increases the porosity of the material, then favoring the access of the enzymes during enzymatic hydrolysis, in the subsequent step. Then, both hemicellulose and cellulose sugars are released during enzymatic hydrolysis.

The effect of enriching the commercial cellulolytic enzyme cocktail CC3 with accessory hemicellulases, H_2O_2, and Tween® 80 on the hydrolysis of CO_2-pretreated sugarcane bagasse was studied using a 3-factor Box-Behnken design. The levels of additives and accessory enzyme were selected according to the results obtained in a previous screening study [22]; a high cellulase activity (35 FPU/g) was used to eliminate the possibility of limitations due to the amount of cellulase added, ensuring that the effects of the supplements could be clearly observed. The different experimental conditions used for the 3-factor Box-Behnken design and the results obtained for glucose and xylose production are shown in Table 2. As it can be seen, there was a significant variation in the responses of glucose production (171.71–247.71 mg g^{-1}DM) and xylose production (67.73–104.86 mg g^{-1}DM) according to the conditions used for hydrolysis. The highest glucose production (247.71 mg g^{-1}DM) was achieved when the enzyme mix was enriched with 553 μL g^{-1}DM of hemicellulase, 65 μM of H_2O_2, and 350 mg g^{-1}DM of Tween® 80 (assay 4).

The statistical significance of the experimental data of glucose production was evaluated by analysis of variance, ANOVA (Table 3). The goodness of fit of the model was assessed using the coefficient of determination (R^2), which was 0.92. This high R^2 value suggests that the model accounts for 92% of the total variation observed in glucose release. Furthermore, the lack-of-fit analysis was not statistically significant ($p > 0.05$), revealing that the model adequately fits the experimental data.

When considering the effect of the factors on the response, the ANOVA showed that for hemicellulase (x_1), only the linear term was significant at a 95% confidence level. For Tween® 80 (x_3), both the linear and quadratic terms were statistically significant ($p < 0.05$), while no significant terms were found for H_2O_2 (x_2). Overall, these findings provide further insights into the interplay of the factors and suggest that optimizing the amount of hemicellulase and Tween® 80 can lead to improved outcomes, while varying the addition of H_2O_2 from 5 to 65 μM in combination with hemicellulase and Tween® 80 () may not yield significant benefits to glucose production.

Our previous study [22] showed that the single addition of H_2O_2 using a concentration of 20 μM resulted in 22% and 27% increase in glucose and xylose production, respectively. The optimal concentration tested was 240 μM, in which the increase was 31% and 38%, for glucose and xylose production, respectively. However, when using concentrations higher than 240 μM, the effect of H_2O_2 was less prominent, until observing inhibition when using a concentration of 23.50 mM. Based on this observation, a different Box-Behnken design using higher H_2O_2 concentration ranges was studied (data not shown). However, the results did not show that this term is significant in combination with hemicellulase and Tween® 80. To the best of our knowledge, this marks the initial study employing a combination of H_2O_2 with extra hemicellulases and Tween® 80. Actually, the interaction between H_2O_2 and LPMOs has also received limited prior investigation. Thus, it seems that the effect of H_2O_2 in this particular combination is complex and the non-significance of this term could be regarded as not providing significant benefits to glucose production. However, another hypothesis could be that a limited addition of H_2O_2 is enough to boost LPMOs present in the cocktail under these conditions, and extra doses do not show benefit.

A plot of the observed versus predicted values for glucose production (Fig. 1a) indicated that the model accurately represents the experimental data, as the data points are quite close to the regression line. Upon examination of the response surface (Fig. 1b), it can be noted that optimal conditions can be identified for both hemicellulase and Tween® 80.

Eq. 3represents the model equation describing the glucose release as a function of the variables used for hydrolysis. Terms not statistically significant according to the ANOVA were excluded from the model.

Table 1
Chemical composition of raw and CO_2 pretreated sugarcane bagasse.

Sugarcane bagasse	Composition (wt%)					
	Cellulose	Hemicellulose	Lignin	Acetyl group	Ash	Extractives
Raw	44.87 ± 0.35	22.20 ± 0.39	24.83 ± 0.40	2.60 ± 0.02	1.80 ± 0.12	3.69
Pretreated	46.10 ± 1.37	21.73 ± 0.83	23.76 ± 0.30	2.93 ± 0.29	1.80 ± 0.03	3.68

Table 2
Experimental conditions used for enzymatic hydrolysis of CO_2-pretreated sugarcane bagasse according to the 3-factor Box-Behnken design and responses.

Assay	Real (and coded) values of independent factors			Predicted response	Responses obtained experimentally	
	Hemicellulase (x_1, μL g^{-1} ^{11}DM)	H_2O_2 (x_2, μM)	Tween® 80 (x_3, mg g^{-1}DM)	Glucose production (mg g^{-1}DM)	Glucose production (mg g^{-1}DM)	Xylose production (mg g^{-1}DM)
1	5 (−1)	5 (−1)	350 (0)	201.44	189.94 ± 4.59	71.26 ± 6.43
2	5 (−1)	65 (1)	350 (0)	218.36	216.64 ± 5.24	90.27 ± 8.15
3	553 (1)	5 (−1)	350 (0)	230.89	232.62 ± 5.62	84.43 ± 7.62
4	553 (1)	65 (1)	350 (0)	236.21	247.71 ± 5.99	104.86 ± 9.47
5	5 (−1)	35 (0)	10 (−1)	162.45	174.46 ± 4.22	72.01 ± 6.50
6	5 (−1)	35 (0)	690 (1)	216.59	217.80 ± 5.27	86.82 ± 7.84
7	553 (1)	35 (0)	10 (−1)	183.98	182.77 ± 4.42	67.73 ± 6.12
8	553 (1)	35 (0)	690 (1)	242.38	230.37 ± 5.57	83.31 ± 7.52
9	279 (0)	5 (−1)	10 (−1)	172.22	171.71 ± 4.15	69.01 ± 6.23
10	279 (0)	5 (−1)	690 (1)	230.27	240.56 ± 5.82	81.04 ± 7.32
11	279 (0)	65 (1)	10 (−1)	185.12	174.84 ± 4.23	72.46 ± 6.54
12	279 (0)	65 (1)	690 (1)	239.6	240.11 ± 5.81	97.84 ± 8.83
13	279 (0)	35 (0)	350 (0)	229.79	225.46 ± 5.45	82.77 ± 7.47
14	279 (0)	35 (0)	350 (0)	229.79	236.06 ± 5.71	98.47 ± 8.89
15	279 (0)	35 (0)	350 (0)	229.79	227.87 ± 5.51	95.43 ± 8.62

Table 3
Analysis of variance (ANOVA) for glucose production from the hydrolysis of CO_2-pretreated sugarcane bagasse using a cellulolytic cocktail supplemented with hemicellulase (x_1), H_2O_2 (x_2), and Tween® 80 (x_3), according to the 3-factor Box-Behnken design.

Source	Sum of squares	df	Mean square	F value	*p*-value
x_1	1119.36	1	1119.36	36.26	0.03*
x_1^2	168.74	1	168.74	5.47	0.14
x_2	247.25	1	247.25	8.01	0.11
x_2^2	6.32	1	6.32	0.20	0.70
x_3	6332.07	1	6332.07	205.14	0.005*
x_3^2	1735.86	1	1735.86	56.24	0.02*
$x_1 \cdot x_2$	33.63	1	33.63	1.09	0.41
$x_1 \cdot x_3$	4.54	1	4.54	0.15	0.74
$x_2 \cdot x_3$	3.19	1	3.19	0.10	0.78
Lack-of-fit	773.53	3	257.84	8.35	0.11
Pure Error	61.73	2	30.87		
Total SS	10409.44	14			

$R^2 = 0.92$. df = degree of freedom.
* = Values significant at 95% confidence level

According to this model, the highest predicted glucose production within the experimental range was 252 mg g^{-1}DM, achievable by adding 465 μL g^{-1}DM of hemicellulase and 568 mg g^{-1}DM of Tween® 80.

$$\text{Glucose (mg g}^{-1}\text{DM)} = 147{\cdot}14 + 0{\cdot}10{\cdot}x_1 + 0{\cdot}21{\cdot}x_3 - 1{\cdot}88 \times 10{-}4{\cdot}x_3^2 \quad (3)$$

The desirability function was then utilized to evaluate the overall effectiveness of the combined glucose and xylose release (Fig. 2). This tool considers multiple response variables (glucose and xylose release in this case) and assigns a desirability value to each combination. The integrated analysis suggested a combination of 417 μL g^{-1} DM of hemicellulase, 65 μM of H_2O_2, and 521 mg g^{-1} of Tween® 80. The desirability value of 0.95 obtained indicates a favorable combination of these two response variables that produces an outcome closely approximating the optimal result. Indeed, the glucose release derived from the desirability function was 245 mg g^{-1} DM, only 3% lower than the optimal result achieved from the Box-Behnken design, which solely considered glucose release as a response. It may be noted that the doses of hemicellulases, H_2O_2 and Tween ® 80 predicted using the desirability function are 10%, 6% and 8% lower, respectively, compared to the doses requirement for the optimal result.

To validate the model, the optimal combination of additives and

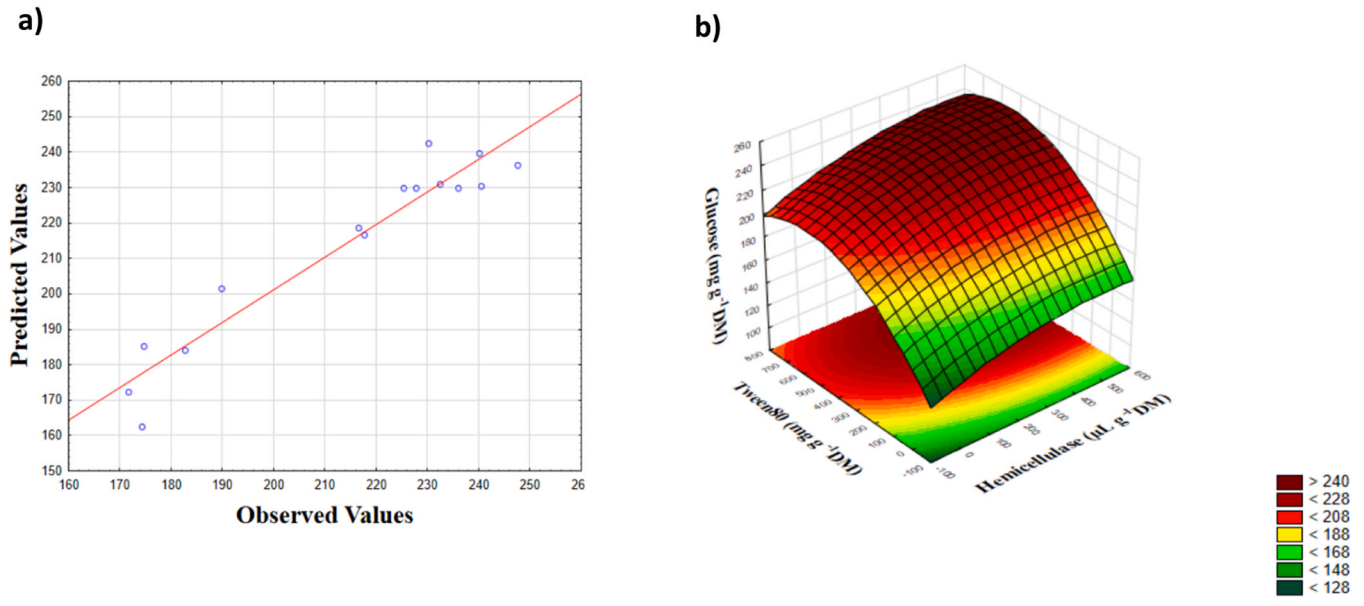

Fig. 1. Observed (data determined experimentally) versus predicted values (**a**) and response surface graphs for glucose release from the hydrolysis of CO_2-pretreated sugarcane bagasse according to the 3-factor Box-Behnken design (**b**).

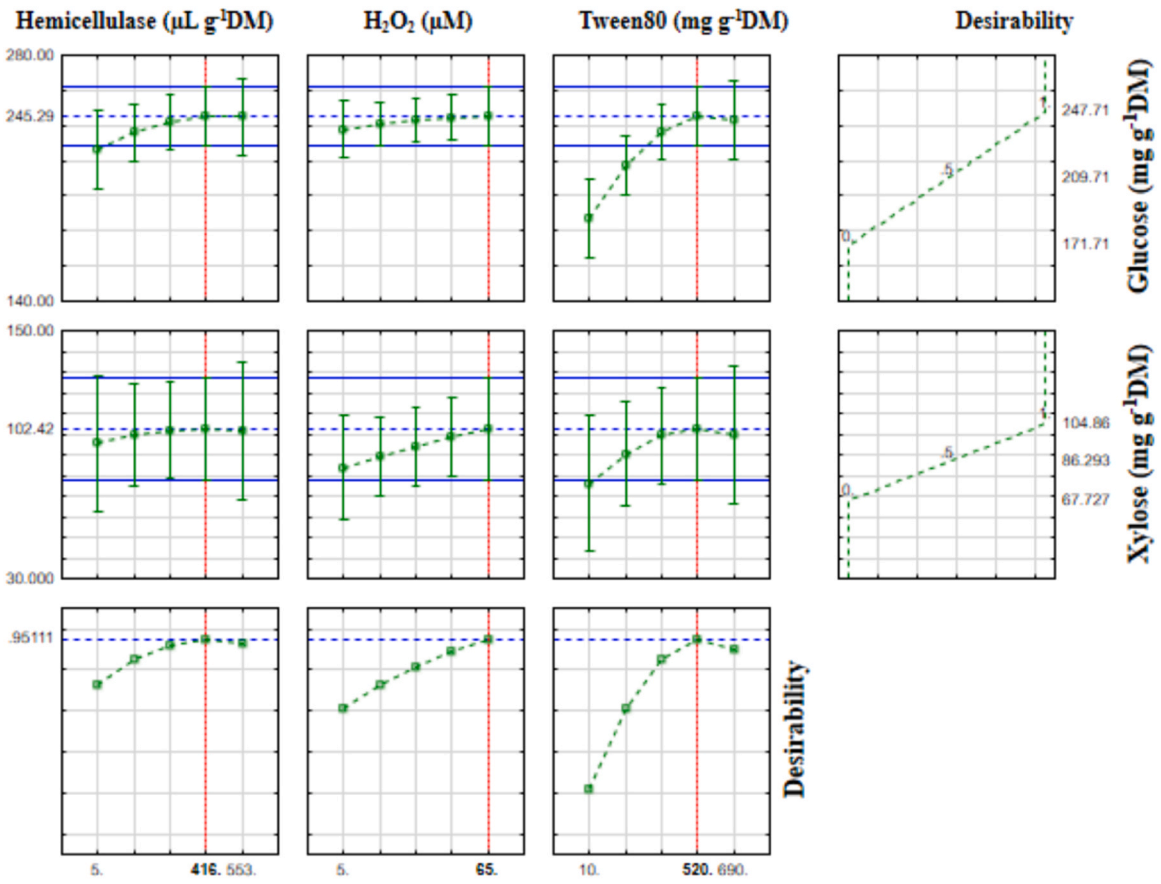

Fig. 2. Desirability plot for glucose and xylose release from the hydrolysis of CO_2-pretreated sugarcane bagasse based on the Box-Behnken design.

accessory enzymes as predicted by the Box-Behnken design, as well as the optimal combination derived from the desirability tool were tested experimentally. The results for the obtained and predicted glucose and xylose release are presented in Table 4. For the optimal combination resulting from the BBD (based solely on glucose release), the predicted values for glucose and xylose release were 252 and 109 mg g^{-1}DM, respectively. However, for the lab-scale tests, glucose and xylose production reached 304 and 124 mg g^{-1}DM, respectively, which were 20% and 14% higher than the predicted values for glucose and xylose. For the results obtained using the desirability function, the experimental glucose released was 24% higher than the predicted amount, and for xylose release, the increase was 18%. This disparity between the model's prediction and the actual experimental result indicates that the model may not accurately capture the true relationship between the factors studied and the response variable. This discrepancy could be due to experimental errors or model limitations.

Compared to the hydrolysis process relying solely on the addition of CC3, the combinations of additives and accessory hemicellulase suggested by both the model's optimum and the desirability function resulted in an increase of 84% for glucose and 94% for xylose production. These results confirm that the sugar release from mildly pretreated sugarcane bagasse containing high amounts of hemicellulose and lignin (compared to conventional pretreated biomasses) can be enhanced using extra dosage of hemicellulases and Tween® 80. Hemicellulases play a pivotal role in breaking down hemicellulose, leading to a direct impact by enhancing the liberation of xylose. Additionally, they expose a greater surface area of cellulose to the influence of cellulases found in the enzyme mixture, resulting in an indirect effect that boosts the production of glucose [17], [44]. On the other hand, Tween® 80 avoids the unproductive binding of cellulases to lignin and avoids their inactivation caused in the liquid-air interface [27], allowing an increased glucose production.

The results obtained in this study are in agreement with other studies reported in the literature that have also explored the combination of surfactants and hemicellulases for biomass hydrolysis and have shown a significant increase in glucose and xylose yields. For instance, Li et al. [32] used Celluclast 1.5 L (10 FPU g^{-1}DM) in combination with various hemicellulases and surfactants on different types of bamboo materials with similar cellulose and hemicellulose content. Their results showed that combining Celluclast 1.5 L with both hemicellulase and surfactants resulted in higher glucose and xylose release compared to using Celluclast 1.5 L alone or in combination with either hemicellulase or surfactants separately. Similarly, Yang et al. [33] achieved enhanced glucose and xylose yields from dilute sulfuric acid-pretreated barley straw (33.0–36.6% glucan, 2.0–5.2% xylan) by adding 20 mg xylanase and PEG4000 g^{-1}DM alongside 10 FPU g^{-1}DM of Celluclast 1.5 L. As a

Table 4
Effect of optimal supplementation combinations of accessory enzyme and additives on glucose (glu) and xylose (xyl) production both predicted by the Box-Behnken design and the desirability function as well as obtained experimentally.

Prediction tool	Hemicellulase (µL g^{-1}DM)	H_2O_2 (µM)	Tween® 80 (mg g^{-1}DM)	glu/xyl predicted (mg g^{-1}DM)	glu/xyl released (mg g^{-1}DM)
Control (solely 35 FPU g^{-1}DM CC3)	-	-	-	-	165/65
Model optimum	465	69	568	252/109	304/124
Desirability function	417	65	520	245/102	303/124

result, the glucose and xylose yields increased from 53.2% to 86.9% and from 36.2% to 70.2%, respectively. Despite variations in biomass, solid content, hemicellulose and cellulose content, enzyme loading, and additive concentrations across different studies, these examples demonstrate that the combination of different enzymes and additives positively impacts glucose and xylose release, which aligns with the findings of the present study.

3.2. Comparison with single supplementation experiments

A noticeable increase in both glucose and xylose release was noted when comparing the optimal results obtained by combining hemicellulase, H_2O_2, and Tween® 80 with single supplementations. For instance, when comparing the best results for 35 FPU g^{-1}DM using single supplementation of hemicellulase and Tween® 80 (71 μL g^{-1}DM of hemicellulase and 200 mg g^{-1}DM of Tween® 80) with the best result obtained from the statistical optimization through BBD (35 FPU g^{-1}DM, 465 μL g^{-1}DM of hemicellulase, 69 μM of H_2O_2, and 568 mg g^{-1}DM of Tween® 80), a significant increase in glucose release of 39% and 41%, respectively was observed when using the statistically optimized combination. Similarly, for xylose, the increase was 38% and 41%, respectively. It is worth noting that the amount of hemicellulase and Tween® 80 needed for the optimal glucose and xylose release resulting from the statistical optimization was respectively 555% and 184% higher than the amounts used when single additions were carried out. Thus, it should be considered whether this approach is economically feasible and sustainable, especially considering the large amounts of hemicellulase and Tween® 80 used in the results obtained through statistical optimization.

4. Conclusions

This study demonstrated that it is possible to maximize the release of glucose and xylose during the hydrolysis of mildly pretreated lignocellulosic biomass by optimizing the enzyme cocktail to be used for hydrolysis. Compared to the results obtained using only cellulase for hydrolysis, the supplementation of additives and accessory hemicellulase resulted in an increase of 84% and 94% for glucose and xylose production, respectively. Such increase was achieved both with the dose suggested by the model optimized only for glucose as a response and with the desirability function. This last suggested a combination of 417 μL g^{-1} DM of hemicellulase, 65 μM of H_2O_2, and 521 mg g^{-1} of Tween® 80. These results allow concluding that it is possible to develop a more sustainable approach for biomass fractionation using a mild pretreatment followed by hydrolysis using an optimized enzyme cocktail. Although the concept is promising, attention should be given to potential costs associated with the use of higher enzyme loadings for hydrolysis, which can be significant in biorefineries, for example, due to their scale and high enzyme loadings often used. A potential alternative to alleviate costs associated with the use of high amount of additives and accessory enzymes would be the development of tailor-made enzyme cocktails using the mildly pretreated biomass as substrate for cultivation of the enzyme producer microorganism.

Author Agreement

We certify that all authors have seen and approved the final version of the manuscript being submitted. We also confirm that this is an original work that has not been published previously and that is not under consideration for publication elsewhere. All the authors agreed that this manuscript should be submitted to Enzyme and Microbial Technology. If accepted for publication, it will not be published elsewhere in the same form, in English or any other language, including electronically, without the consent of the copyright holder.

CRediT authorship contribution statement

Balaguer Moya Eva: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft. **Syhler Berta:** Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization. **Mussatto Solange I.:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. **Dragone Giuliano:** Supervision, Writing – review & editing.

Declaration of Competing Interest

none.

Data availability

Data will be made available on request.

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