

Article

Evaluation of Enzymatic Hydrolysis of Sugarcane Bagasse Using Combination of Enzymes or Co-Substrate to Boost Lytic Polysaccharide Monooxygenases Action

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Abstract: This study evaluated innovative approaches for the enzymatic hydrolysis of lignocellulosic biomass. More specifically, assays were performed to evaluate the supplementation of the commercial cellulolytic cocktail Cellic[®] CTec2 (CC2) with LPMO (GcLPMO9B), H₂O₂, or cello-oligosaccharide dehydrogenase (CelDH) FgCelDH7C in order to boost the LPMO action and improve the saccharification efficiency of biomass into monosaccharides. The enzymatic hydrolysis was carried out using sugarcane bagasse pretreated by hydrodynamic cavitation-assisted oxidative process, 10% (*w/w*) solid loading, and 30 FPU CC2/g dry biomass. The results were compared in terms of sugars release and revealed an important influence of the supplementations at the initial 6 h of hydrolysis. While the addition of CelDH led to a steady increase in glucose production to reach 101.1 mg of glucose/g DM, accounting for the highest value achieved after 72 h of hydrolysis, boosting the LPMOs activity by the supplementation of pure LPMOs or the LPMO co-substrate H₂O₂ were also effective to improve the cellulose conversion, increasing the initial reaction rate of the hydrolysis. These results revealed that LPMOs play an important role on enzymatic hydrolysis of cellulose and boosting their action can help to improve the reaction rate and increase the hydrolysis yield. LPMOs-CelDH oxidative pairs represent a novel potent combination for use in the enzymatic hydrolysis of lignocellulosic biomass. Finally, the strategies presented in this study are promising approaches for application in lignocellulosic biorefineries, especially using sugarcane bagasse as a feedstock.

Keywords: enzymatic hydrolysis; LPMO; hydrogen peroxide; cello-oligosaccharide dehydrogenase; lignocellulosic biomass; sugarcane bagasse



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1. Introduction

With the aim to prevent the devastating consequences of reaching limit global temperatures, a drastic transition to net zero greenhouse gases (GHG) emission is crucial. During these times, when increased products consumption have met with materials scarcity, industrial practices and technological processes need a reformulation to meet the demands while being more sustainable, using renewable sources, and targeting the mitigation of GHG emission [1]. An attractive solution is lignocellulosic biomass as it can provide a renewable source of carbon that can be used for fuel as well as to produce a significant variety of renewable products, at a low cost and being extensively available. The global production of sugarcane is of approximately 1.6 billion tons, generating during its processing about 279 million metric tons of sugarcane bagasse (SCB), which could be an environmental concern if not treated [2]. Therefore, SCB is an important source of lignocellulose that can be

used to produce biofuels, biochemicals, and lignin for value-added applications. However, to achieve this goal at an industrial scale, and to contribute significantly to a bioeconomy scenario where biobased solutions compete with their fossil-based counterparts, there are still some technology advances to be achieved [3].

Lignocellulosic biomass is composed of a complex structure of cellulose, hemicellulose, and lignin. Due to its recalcitrance, its fractionation and conversion to the product of interest is challenging. An efficient pretreatment step is required to open the structure and enable the access of enzymes for the subsequent enzymatic conversion step [4]. Different pretreatment methods have been developed over the years, using physical, chemical, biological, and physicochemical processes. However, these processes usually imply the utilization of strong chemicals or/and high amounts of water and energy, also resulting in the generation of inhibitors that are difficult and expensive to remove, which cause inhibition in the next steps of the process. One example of a promising pretreatment is hydrodynamic cavitation (HC), a technology that has shown good results for pretreatment of sugarcane bagasse and that allows combinations of other methods, such as utilization of sodium hydroxide [5], hydrogen peroxide [6], and oxidative processes [7]. Terán Hilares et al. (2018) [8], for example, reported an increase in biomass digestibility, exhibiting carbohydrate hydrolysis yields higher than 90% for sugarcane bagasse pretreated by an HC-assisted process.

Together with pretreatment, the conversion of polysaccharides to fermentable sugars by enzymatic hydrolysis is a key contributor to the technological and economical constraints of the process. This is due to the complexity of the system and the high cost of enzymes [1]. To maximize final sugar yields while minimizing enzyme dosage, enzyme cocktails must be optimized based on the biomass composition and process conditions [9,10]. The discovery and industrial implementation of lytic polysaccharide monooxygenases (LPMOs) have been a significant step towards the development of improved enzyme cocktails [11]. Contrary to typical cellulases, which are hydrolases, LPMOs are mono-copper oxidoreductases [12] that catalyze the hydroxylation of the C1 or/and C4 carbons in the glucosyl units of cellulose, disrupting its crystalline structure and improving the accessibility for cellulases by generating new access points [13]. Therefore, the activity of LPMOs can potentially reduce the enzyme loading in the hydrolysis step of biorefineries, improving the process' economic viability [14]. To be activated, this enzyme must have its metal ion reduced from Cu(II) to Cu(I), which can occur by two different ways: O_2 - and H_2O_2 -based, as shown in Figure 1. It has been suggested that it binds to the flat, solid, and well-structured surface of crystalline cellulose and, by a mechanism that ends up with the oxidation of one of the new chain ends, it breaks the chain [12]. The boosting effect of LPMOs on the activity of hydrolytic cocktails can vary extensively depending on different factors, such as the structure and composition of the biomass [15], the co-substrate used, or the enzymatic formulation. Detailed understanding of the factors that contribute to the efficacy of oxidative enzymes in biomass processing and the interplay of these enzymes with hydrolytic counterparts is needed to unlock the full potential of these enzymes to achieve a successful implementation [16].

Hydrogen peroxide has been recently identified as a preferred co-substrate for LPMOs, as compared to the initially proposed mechanism that involves molecular oxygen (O_2) as the supplier of protons, electrons, and reactive oxygen species for the reaction [12,17,18]. This finding not only alleviates the need for an efficient aeration but also significantly reduces the need of a reductant, as it has shown to only be needed to reduce the catalytic resting state LPMO-Cu(II) to the active Cu(I) state [11]. Amongst other electron sources for the LPMO reaction, lignin has been shown to act as a reductant, potentially avoiding the need for adding exogenous reducing power in complex lignin-containing substrates [19]. Recent studies have shown that the rates of LPMO reactions that are supplemented with H_2O_2 are far higher when compared to those driven by O_2 and an added reductant [11,19,20]. However, the sensitivity of LPMOs to inactivation by oxidative damage increases the complexity of the process and depends on the amount of substrate and H_2O_2 [21]. Thus, the addition of exogenous reductants, in complex biomass backgrounds, needs to be meticulously regulated

to maintain the catalytically competent reduced LPMO-Cu(I) form, while avoiding adverse side reactions between the reductant, LPMO, and substrate components.

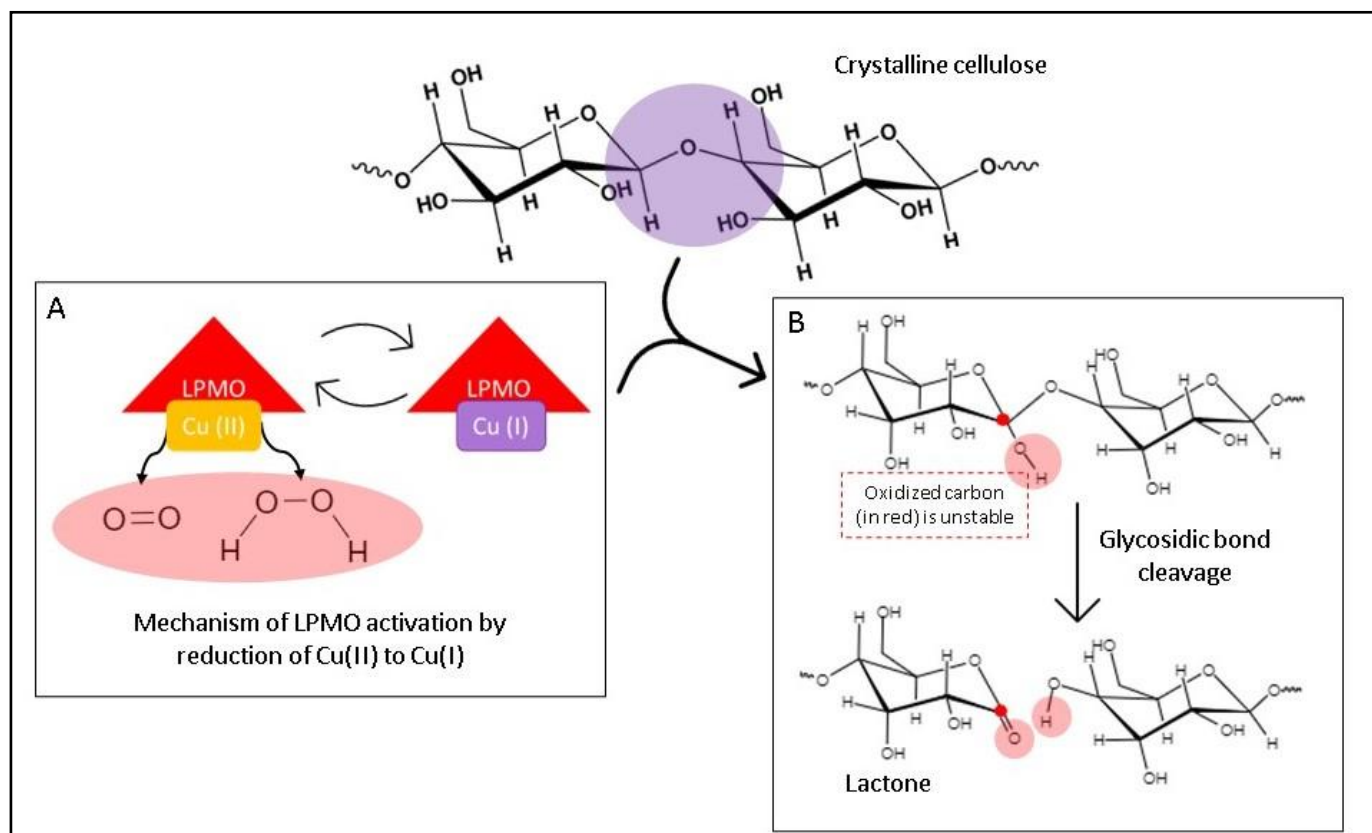


Figure 1. Simplified scheme of LPMO action in the hydrolysis of crystalline cellulose. (A) represents the transition from a resting to an active catalytic state of LPMO (reduction of Cu(II) to Cu(I) in the active site) in presence of O_2 and/or H_2O_2 . (B) shows the cleavage of the glycosidic bond of crystalline cellulose by the insertion of O_2 .

Recently, the cello-oligosaccharide dehydrogenase (CelDH) from *Fusarium graminearum* (FgCelDH7C), from the auxiliary activity family 7 (AA7) in the Carbohydrate Active enZyme (CAZy) database (<http://www.cazy.org/> accessed on 19 August 2022), was shown to fuel LPMO activity towards crystalline cellulose (Avicel) in the absence of an added reductant [22]. Putative AA7 enzymes, reported to be co-secreted with LPMOs in fungal secretomes during growth on complex biomasses, including lignocellulose [23], may offer a simple and efficient AA7-LPMO system that could be applied to commercial cellulase cocktails.

Based on the above, the present work aimed to evaluate three different strategies to boost LPMO activity to maximize the action of the commercial cellulolytic cocktail Cellic[®] CTec2 (CC2) for saccharification of lignocellulosic biomass into monosaccharides. One approach was the supplementation with the LPMO from *Geotrichum candidum* (GcLPMO9B), which displays activity towards cellulose and xyloglucan [24]. The other two included the independent addition of H_2O_2 , as a co-substrate, and the enzyme CelDH FgCelDH7C. The experiments were carried out using sugarcane bagasse pretreated by an HC-assisted oxidative process as a feedstock, and the results were compared in terms of glucose and xylose accumulation during hydrolysis.

2. Results and Discussion

2.1. Enzymatic Hydrolysis in Deep-Well Plates

Three different approaches were studied to increase the total LPMO activity of the commercial cocktail Cellic[®] CTec 2 (CC2), namely the supplementation with the LPMO

GcLPMO9B (Figure 2a,b), H_2O_2 (Figure 2c,d), or with the FgCelDH7C dehydrogenase (Figure 2e,f) (see Section 3.3). Figure 2 shows the monomeric sugar release in terms of mg glucose and mg xylose per g of pretreated dry biomass (DM). Overall, the supplementation approaches tested for CC2 were effective to increase the release of sugars, especially glucose, and to a less extent xylose, during enzymatic hydrolysis. The final xylose release from hemicellulose hydrolysis was similar for all the experiments with supplementation, and the improvement when compared to the standard CC2 hydrolysis was clear, raising the pentose production by 33% when adding one pulse of H_2O_2 (Figure 2d). The initial reaction rates presented a small difference among the experiments with the LPMO addition (Figure 2b) and the supplementation with CelDH (Figure 2f). The hydrolysis of hemicellulose may not be as influenced by the activity of the LPMOs present in CC2 as the hydrolysis of cellulose was.

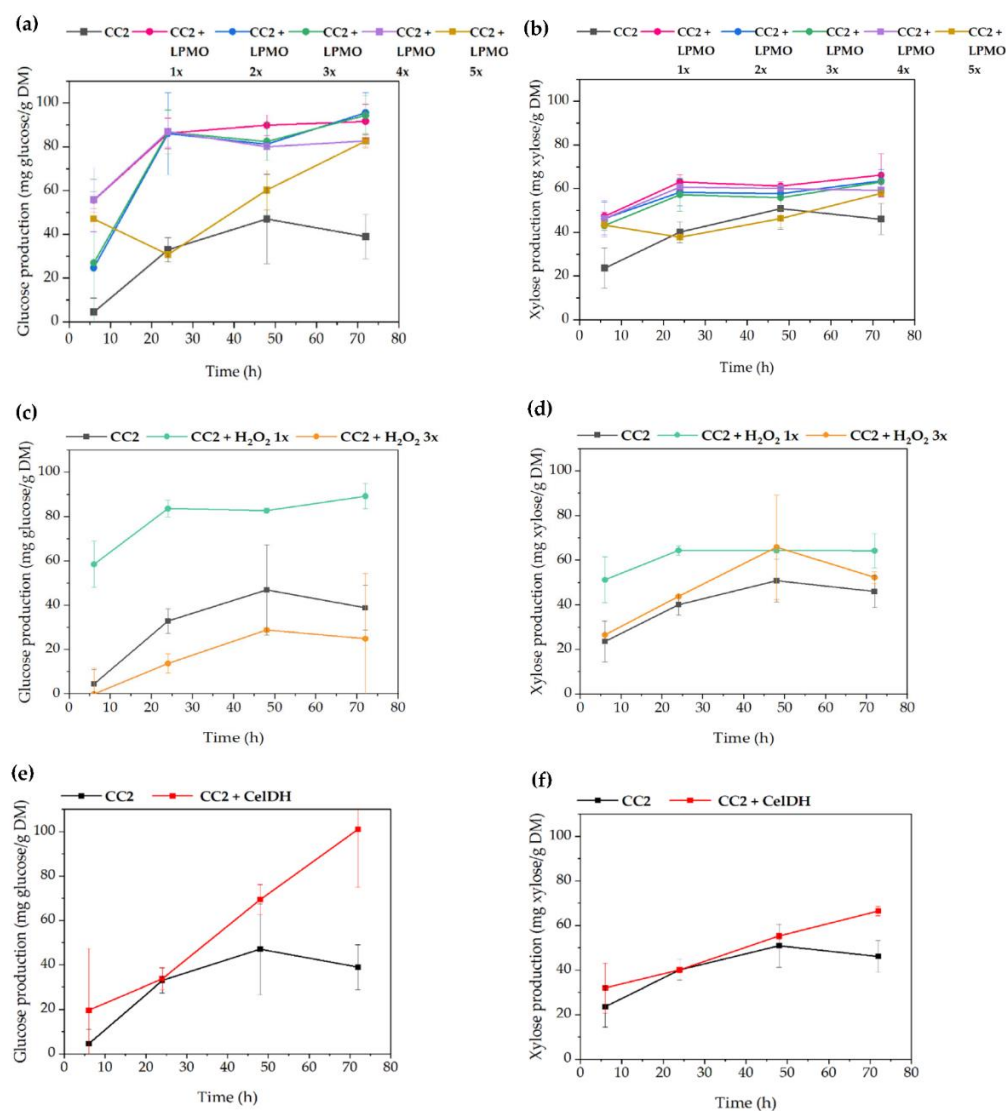


Figure 2. Glucose (a,c,e) and xylose (b,d,f) release in enzymatic hydrolysis of sugarcane bagasse with Cellic® CTec 2 and different supplementation approaches: supplementation with the LPMO GcLPMO9B (a,b), H_2O_2 (c,d), or with the FgCelDH7C dehydrogenase (e,f) (as Section 3.3). Error bars for glucose and xylose production represent average deviations for two replicates.

Earlier studies have shown that LPMOs improve the accessibility of cellulases to celluloses, enhancing their efficiency and increasing lignocellulose hydrolysis [14,25]. Thus, it could be a good strategy to reduce the loading of cellulases in the cocktail. The supplementation with GcLPMO9B (Figure 2a,b), in general, improved the efficiency of glucose

and xylose release when compared to standard CC2. However, inhibition of the glucose production at a five-fold-higher added LPMO concentration (5x) was observed during the first 24 h compared to experiments with a lower load of the oxidative enzyme. This indicates possible saturation of LPMOs during this time, with limitations of available binding sites for LPMOs. Another plausible explanation for the decreased activity at high LPMO concentration is that the reduced LPMO (LPMO-Cu(I)) in free form (not bound to substrate), exhibits oxidase activity resulting in the accumulation of H_2O_2 and oxidative damage [16,26]. On that account, it is important to prevent unproductive active LPMO by assuring substrate availability. Furthermore, it is noticeable that a small supplementation was enough to improve the yield of the process effectively. Among the LPMO supplementation experiments, CC2 + LPMO 2x obtained the highest glucose release, accounting for 95.5 ± 9.3 mg glucose/g DM, an increase of 145% compared to standard CC2, while the best xylose release accounted for 61.2 ± 1.9 for the experiment CC2 + LPMO 1x, corresponding to a 44% growth in xylose generation.

The supplementation of H_2O_2 at the start of the process (CC2 + H_2O_2 1x) had a remarkable improvement in the glucose and xylose release (Figure 2c,d). The higher reaction rate is already noticed after 6 h for the supply of H_2O_2 at the beginning, when compared to the standard CC2 (1191% increase for glucose release and 117% for xylose). The addition of H_2O_2 led to a faster activation of LPMOs present in the commercial enzyme blend. The boost of LPMO activity at the beginning of the hydrolysis process is interesting because it enhances cellulose digestibility by oxidizing insoluble crystalline regions of the polymer, which are less accessible to cellulases [27]. At the end of 72 h, the monomeric sugar production in CC2 + H_2O_2 1x accounted for almost two times the one obtained without supplementations. Costa et al. (2019) [18] reported that the addition of peroxide increased the concentration of oxidized products in the reaction, which consequently improved the glucose conversion in the hydrolysis of sulfite-pulped Norway spruce in a bioreactor employing Cellic[®] CTec3 (12% (*w/w*) of the substrate and 4% (*w* liquid/*w* DM of substrate of the enzyme load)) and continuous pumping of H_2O_2 , starting after 20 h. Additionally, Bissaro et al. (2017) [17] reported enhancements in the reaction rate when hydrogen peroxide was supplied, reaching up to 4.2 min^{-1} (standard aerobic conditions reached around 0.3 min^{-1}) in the hydrolysis of Avicel with CC2 and ascorbic acid (10% *w/v* of substrate, 4 mg of protein/g of substrate, and 1 mM, respectively). These experiments employed varied feeding rates (from 30 to $60 \mu\text{M h}^{-1}$), evidencing that the presence of a reductant (such as ascorbic acid) is not required when using lignocellulosic biomass (such as sugarcane bagasse), since the reducing power can be sufficiently provided by the lignin present in the biomass to boost LPMO action. Even though high lignin content usually has been considered inhibitory of cellulase activity, this study, together with other works [11,14,15], demonstrated a positive aspect of high lignin content, which has shown to be beneficial for LPMO activity and, in general, for the overall hydrolysis performance. Thus, the utilization of mild pretreatments that do not remove lignin from the biomass have the potential to be a better option than conventional ones, not only in terms of reducing inhibitory products' formation and water and energy consumption, but also could increase the efficiency of hydrolysis if LPMOs are used.

Contrary to what resulted when adding H_2O_2 at the start of the hydrolysis, the sugar release in the case of CC2 + H_2O_2 3x (the supplementation of H_2O_2 at three time points, triplicating the total amount) was significantly lower than when no supplementation of CC2 was used (Figure 2c,d). This could be due to the accumulation of H_2O_2 , which leads to self-inactivation of LPMOs by oxidative damage at the active site [17]. Müller et al. (2018) [11] studied a constant hydrogen peroxide supplementation on the enzymatic hydrolysis of steam-exploded birch at 10% *w/w* with Cellic[®] CTec 2 (2 mg protein/g DM), and also observed a depletion in LPMO activity when employing the highest feeding rates of H_2O_2 , which can be explained by the inactivation of LPMOs. This highlights the importance of a balanced supply of H_2O_2 to have a sustained LPMO activity.

The supplementation with CelDH was studied, as it has the potential to simplify the process by eliminating the needs for an added reductant and by supplying low levels of H_2O_2 and possibly other reactive oxygen species (Figure 2e,f). Even though the reaction happened faster using H_2O_2 , the addition of CelDH resulted in the highest conversion after 72 h of hydrolysis, with a steady increase of the released sugars during the process. There was an increase of 160% and 44% of glucose and xylose release, respectively, in 72 h of hydrolysis when compared to the process without supplementation with CelDH. In contrast to the other supplementation approaches, a slower sugar release rate during the first hours of reaction was observed, which could be due to the low oxidase activity (rate of generation of H_2O_2) of the enzyme that uses cello-oligosaccharides (COS) as substrates. This is the first study describing the beneficial impact of the addition of the newly discovered CelDH activity on the breakdown of a complex recalcitrant biomass substrate by a hydrolytic-LPMO-containing cocktail. Therefore, further experiments are warranted to investigate the novel strategy and optimize the LPMO-redox partner combination. However, this initial result already indicates this enzyme as a promising component for modern enzyme cocktails.

The oxidative system of cellobiose dehydrogenase (CDH) NcCDHIIa from *Neurospora crassa* OR74B with LPMOs and endoglucanases has been studied by Barbosa et al. (2020) [28] to obtain COS by hydrolysis of hydrothermally pretreated sugarcane straw. The LPMO activity was analyzed with respect to the presence of the chosen COS, concluding that there was a significant increase in their production in the presence of NcCDHIIa and demonstrating the beneficial interplay between these enzymes. The single-domain cello-oligosaccharide dehydrogenase employed (*FgCelDH7C*) offers the simplest enzymatic redox-partner that is able to provide the priming electrons to convert LPMOs to their active LPMO-Cu(I) form and to fuel their activity in pure model cellulose substrates [22] and apparently against a complex pre-treated sugarcane bagasse biomass substrate, which is shown in this study.

2.2. Statistical Analysis

At the beginning of the process, the various supplementation strategies to CC2 led to different responses in terms of the mechanism of enzyme activation or the availability of enzymes, which explains the different values for glucose release. While the material is being hydrolyzed, the proportion of crystalline and recalcitrant biomass increases in the solid fraction, which impedes hydrolytic breakdown. Thus, after the accessibility of the biomass to hydrolases becomes limited, the sugar concentration stabilizes, and the hydrolytic process proceeds very slowly or stagnates. In this case, after 24 h, this maximum is reached and the conversion stabilizes at the same level, with few fluctuations.

The statistical test *F* with a significance level of 5% was performed to compare the results of experimental conditions for each time point, considering cellulose conversion. Table 1 shows the ANOVA single-factor analysis for this timepoint. As it can be observed, the values obtained for 6 h of the process were statistically different (*p*-value < *F* crit).

Table 1. ANOVA: single factor for glucose production in 6 h.

Source of Variation	SQ	gl	MQ	F	<i>p</i> -Value	F Crit
Between groups	8121.72	8	1015.22	2.39	0.11	3.23
Within groups	3826.22	9	425.14			
Total	11,947.94	17				

In Figure 2, it is possible to observe that the average deviations of some timepoints and conditions overlap, showing similar values, while, for other conditions in which the average deviations do not overlap, a difference can be seen and discussed on the effect of each supplementation. There was difference in the values of the experiments with LPMO supplementation. The main differences were between the standard CC2 and CC2 + H_2O_2 1x, CC2 + LPMO 1x, CC2 + LPMO 4x, or CC2 + LPMO 5x. These four conditions represent the highest initial conversions, which may be from a greater LPMO activity. Further analysis

can be performed to detect the presence of C1 and C4-oxidized carbons and better compare the activity of these enzymes and to what extent the supplementations are interfering in it. It is also necessary to optimize the load of LPMO, H_2O_2 , or CelDH to maximize the hydrolysis yields employing the minimum load of the enzyme.

Another important aspect is the distinction between CC2 + H_2O_2 1x and CC2 + CelDH. According to Momeni et al. (2021) [22], CelDH activates LPMOs and potentiates its activity, being able to supply the electrons needed for maintaining the LPMO in active form and for supplying reaction oxygen species, e.g., H_2O_2 , at a low rate for the hydroxylation of the glycosidic bonds in cellulose. On the other hand, H_2O_2 can also be used as the co-substrate to activate LPMOs at a faster rate. This compound could potentially be a cheaper option when compared to an enzyme addition; however, the problems associated with an excessive addition of H_2O_2 and subsequent loss in activity, the environmental impact of H_2O_2 , or its removal if it may cause a threat during the fermentation step, would balance the economic and environmental impacts of the production of other accessory enzymes. Therefore, an evaluation of economic and sustainability aspects of the addition of hydrogen peroxide or CelDH to the process is crucial to determine the best option.

3. Materials and Methods

3.1. Biomass Pretreatment and Composition

Sugarcane bagasse was pretreated in a hydrodynamic cavitation system made as reported by Terán Hilares et al. (2017) [29]. After pretreatment, the biomass composition, analyzed as reported by Mesquita et al. (2016) [30], was (dry weight values): 45.24% cellulose, 26.45% hemicellulose (24.45% xylan, 1% arabinan, 1% acetyl groups), 20.60% lignin, 1.5% ashes, and 2.4% extractives.

3.2. Enzymatic Hydrolysis

The enzymatic hydrolysis of pretreated sugarcane bagasse was performed in 24-deep-well plates (EnzyScreen, The Netherlands) using 2 mL of the reaction volume, 10% (*w/v*) of initial solid loading in 0.05 mol/L sodium acetate buffer (pH 4.8), and 30 FPU/g DM of enzyme loading (Cellic[®] CTec2, Novozymes, Denmark). The experiments were carried out at 50 °C, 150 rpm for 72 h. Samples were taken after 6, 24, 48, and 72 h of hydrolysis. The inactivation of enzymes was done at 100 °C for 10 min, followed by their separation by centrifugation at 5000 rpm for 6 min. All hydrolysis experiments were performed in duplicate; mean values and average deviations are presented.

3.3. Enzyme and Additives Supplementation

Three different approaches were tested for supplementation of the commercial enzyme cocktail Cellic[®] CTec2 (CC2). The first was based on increasing the total amount of LPMOs present in the cocktail. For this, the LPMO GcLpmo9B (NZYTech, Lisbon, Portugal) was added in increased doses, until reaching a maximum of 51.7 μ L, which corresponds to an increase in 8 times in the LPMO activity of CC2, according to Table 2.

The second approach consisted of adding H_2O_2 to the reaction medium. H_2O_2 solution was added to boost the LPMO activity present in CC2; the amount added was the corresponding amount to have a stoichiometric ratio of 1:1 of LPMO and H_2O_2 . The concentration of LPMO in the hydrolysis media was assumed to be 14 μ mol/L, considering that CC2 has 154.13 g of protein/L (measured following the ninhydrin assay [31] according to Haven et al., 2013), 165 FPU/mL (measured following the filter paper activity assay [32]), 15% (*w/w*) of LPMO [19], and 30,000 g/mol as the mean molecular mass [11]. Then, 5.6 μ L of a 5 mmol/L solution was added in order to obtain a final H_2O_2 concentration of 14 μ mol/L. The addition of H_2O_2 was studied using two different strategies: the first one (CC2 + H_2O_2 1x) consisted of adding this content at 30 min after the start of the process, while in the second one (CC2 + H_2O_2 3x), the total amount added was triplicated, but divided in 3 steps after 30 min, 24 h, and 48 h. The reason for starting the addition after

30 min was in order to avoid high concentrations of H_2O_2 in the reaction medium, enabling a good initial mixing.

Finally, CC2 was supplemented with the cello-oligosaccharide dehydrogenase (CelDH) *FgCelDH7C*. *FgCelDH7C* was produced using a *Pichia pastoris* expression system in 2 L shake flasks as previously described [22], and a two-step purification protocol was employed, the first involving immobilized metal ion affinity chromatography, followed by size-exclusion chromatography using a Superdex G75 high-load gel filtration column installed on an Äkta Avant chromatograph. The amount of CelDH added to CC2 was calculated according to Momeni et al. (2021) [22] and corresponded to 10% of the LPMO loading ($1.4 \mu\text{mol/L}$ at the start of the reaction).

Table 2. Conditions for LPMO supplementation experiments. Different LPMO loads were supplemented to a standard Cellic® CTec 2 load and added at the start of the reaction.

Conditions	LPMO Activity ** (U/mL)	Added Volume of LPMO (μL)
CC2 *	1.47×10^{-5}	0.0
CC2 * + 1x LPMO	3.56×10^{-5}	10.3
CC2 * + 2x LPMO	5.64×10^{-5}	20.6
CC2 * + 3x LPMO	7.72×10^{-5}	31.0
CC2 * + 4x LPMO	9.80×10^{-5}	41.3
CC2 * + 5x LPMO	11.89×10^{-5}	51.7

* CC2: Cellic® CTec 2 ** LPMO activity was measured using the coerolignone method as described by Breslmayr et al. (2019) [33].

The results from the hydrolysis experiments were compared using the statistical test F for a 95% confidence level to check the statistical similarities among the data.

3.4. Analytical Methods

The quantification of sugars was carried out by high-performance liquid chromatography (HPLC) using a Dionex Ultimate 3000 high-performance liquid chromatograph UHPLC+ focused system (Dionex Softron GmbH, Germering, Germany) with a Bio-Rad Aminex column HPX-87H ($300 \text{ mm} \times 7.8 \text{ mm}$) at 60°C , using a Shodex RI-101 refractive index detector and $5 \text{ mM H}_2\text{SO}_4$ as the mobile phase at a flow rate of 0.6 mL/min , and a sample injection of $20 \mu\text{L}$.

The LPMO activities of Cellic® CTec 2 and GcLpmo9B were measured by the coerolignone method as described by Breslmayr et al. (2019) [33], using a microplate for the reaction. Amounts of 50 mmol/L citrate ($\text{pH} 4.8$) and 50 mmol/L HEPES ($\text{pH} 7.5$) buffers were employed for Cellic® CTec2 and GcLpmo9B, respectively, and the reaction time was 300 s.

4. Conclusions

The supplementation of the cellulolytic cocktail Cellic® CTec2 with a LPMO, a cello-oligosaccharide dehydrogenase (CelDH), or with the LPMO co-substrate H_2O_2 improved the enzymatic hydrolysis of sugarcane bagasse. The supplementation of CelDH lead to the highest glucose release in 72 h, with an increase in the glucose and xylose contents of almost four and two times, respectively, compared to the amount obtained without supplementation. The addition of H_2O_2 and the LPMO GcLPMO9B contributed to enhancing the efficiency of the enzymatic cocktail and, consequently, to obtaining a higher saccharification reaction rate at the initial points of the hydrolysis. The supplementation with the lowest amount of LPMO already exhibited an important impact in 6 h of reaction, being a great step towards shortening the hydrolysis process time. Moreover, boosting LPMO activity promotes better cellulose conversion, since this enzyme can oxidize crystalline parts of the polymer, improving its digestibility. These results indicate a basis for further development of modern enzyme blends and the processing of lignocellulosic biomass, upgrading the hydrolysis step in biorefineries.

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Conflicts of Interest: The authors declare no known conflict of interest.

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