Enzymatic hydrolysis cocktail optimization for the intensification of sugar extraction from sugarcane bagasse

Moya, Eva Balaguer; Syhler, Berta; Manso, Julen Ordeñana; Dragone, Giuliano; Mussatto, Solange I.

Published in:
International Journal of Biological Macromolecules

Link to article, DOI:
10.1016/j.ijbiomac.2023.125051

Publication date:
2023

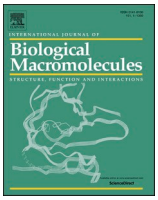
Document Version
Publisher's PDF, also known as Version of record

International Journal of Biological Macromolecules 242 (2023) 125051

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac



Enzymatic hydrolysis cocktail optimization for the intensification of sugar extraction from sugarcane bagasse

Eva Balaguer Moya, Berta Syhler, Julen Ordeñana Manso, Giuliano Dragone, Solange I. Mussatto *

Department of Biotechnology and Biomedicine, Technical University of Denmark, Søltofts Plads, Building 223, 2800 Kongens Lyngby, Denmark

ARTICLE INFO

Keywords:
Enzymatic hydrolysis
Lytic polysaccharide monooxygenases
Cellulases
Lignocellulosic biomass
Biorefinery

ABSTRACT

Lignocellulosic biomasses have a very important role as a raw material to produce biofuels and biochemicals. However, a sustainable, efficient, and economically competitive process for the release of sugars from such materials has still not been achieved. In this work, the optimization of the enzymatic hydrolysis cocktail was evaluated as an approach to maximize sugar extraction from mildly pretreated sugarcane bagasse. Different additives and enzymes, including hydrogen peroxide (H_2O_2), laccase, hemicellulase and the surfactants Tween 80 and PEG4000 were added to a cellulolytic cocktail with the aim of improving biomass hydrolysis. An increase of 39 % and 46 % of glucose and xylose concentrations, respectively, compared to the control (when only the cellulolytic cocktail (20 or 35 FPU g^{-1} dry mass), was obtained when H_2O_2 (0.24 mM) was added at the beginning of the hydrolysis. On the other hand, the addition of hemicellulase (81–162 μL g^{-1} DM) increased the production of glucose up to 38 % and xylose up to 50 %. The findings of this study reveal that it is possible to increase the extraction of sugars from mildly pretreated lignocellulosic biomass by using an appropriate enzymatic cocktail supplemented with additives. This opens up new opportunities for the development of a more sustainable, efficient, and economically competitive process for biomass fractionation.

1. Introduction

Biorefinery processes based on lignocellulosic biomass play a key role in the production of biofuels and bioproducts, as they help to achieve the sustainable development goals that are targeted for the transition to a circular and biobased economy [1,2]. Lignocellulosic biomass is a low-cost, widely available and renewable material, which potentially balances the carbon footprint of bioproducts by CO_2 fixation through photosynthesis [3]. It presents a complex and recalcitrant structure, difficult to depolymerize and break down into simpler units that can be used as a product or as raw material for conversion into other products [4]. To overcome this constraint, an efficient fractionation process should be used, where the biomass is separated into its main components (cellulose, hemicellulose, and lignin). This part constitutes one of the most important processes of a biorefinery because of the high costs involved due to the high energy, water and chemical demand [5,6].

Several strategies for biomass pretreatment have been developed to enhance the accessibility of enzymes to cellulose and hemicellulose. These methods include steam explosion or ammonia fiber expansion (AFEX), which aim to cause the swelling of biomass, increasing surface area and pore size; chemical processes that use acids, alkaline solutions or hot water, which degrade the biomass structure removing hemicellulose and lignin; and biological processes that degrade cell wall components through the action of enzymes or microorganisms [7]. However, most of conventional pretreatment methods are economically unviable to be implemented industrially because they present disadvantages, such as the need of strong chemicals that are difficult and expensive to remove in subsequent steps [5]. Thus, it is still needed to develop an economical and environmentally friendly method that allows a complete biomass fractionation [8,9]. As an alternative to conventional fractionation methods, the use of CO_2 for pretreatment under mild conditions could be a promising pretreatment. CO_2 is non-toxic, low cost, extensively available, easy to recover and recycle, has a high diffusion rate and does not generate chemical waste [10]. In presence of water and critical conditions, CO_2 can form carbonic acid, which could disrupt the chemical bonds of lignocellulose, and subsequently removing hemicellulose from the biomass. Thus, the effect of this pretreatment could be comparable to the one of diluted acid pretreatment

* Corresponding author.
E-mail addresses: smussatto@dtu.dk, solangemussatto@hotmail.com (S.I. Mussatto).

https://doi.org/10.1016/j.ijbiomac.2023.125051
Received 21 February 2023; Received in revised form 6 May 2023; Accepted 18 May 2023
Available online 26 May 2023

[11]. In this work, the purpose of using a mild CO_2 pretreatment using conditions below the critical point was to cause biomass swelling and facilitate the access of hydrolytic enzymes in the subsequent stage, while avoiding the degradation of biomass components. This would not only avoid the expensive steps related to removal of inhibitors and chemicals before enzymatic hydrolysis, but the full utilization of the lignocellulosic fractions for sugar monomers production would be achieved.

The hydrolysis of the hemicellulosic fraction into fermentable sugars by enzymes is, along with biomass pretreatment, an important contributor to the economic and technological constraints of a biorefinery process. Due to the number of factors that are responsible for low biomass hydrolysis (high recalcitrance, low access of enzymes [12,13], unproductive enzyme-lignin bonding [14], competitive and feedback inhibition [12], etc.), considerable amounts of expensive enzymes are commonly required to achieve high hydrolysis yields [15]. Finding the optimum combination of enzymes and enzyme stabilizers is therefore required to achieve the highest yield while using the minimum enzyme dosage [16]. Enzymes can act in cooperation, providing each other positive features that can enhance biomass hydrolysis. In addition, different enzyme cocktails, but with the same enzymatic activity, may have a different efficiency depending on the accessory enzymes or substrates that are added [17].

The discovery of lytic polysaccharide monooxygenases (LPMOs) demonstrated the idea that oxidative processes also contribute to the conversion of cellulose [18]. For this reason, their use as accessory enzymes has received increased attention in academia and industry [19]. LPMOs are monocopper [20] enzymes that bind to the crystalline portion of cellulose [21]. These enzymes catalyse the hydroxylation of either the C1 or C4 carbon of the glycosidic bond and, by the oxidation of the chain ends of cellulose, they generate new cavities for the access of enzymes [22]. Some LPMOs from the AA9 family may also participate in the degradation of hemicellulose as they can cleave some hemicellulose polysaccharides [23–25]. They require to reduce their metal ion from Cu (II) to Cu (I) to become catalytically active [26]. Moreover, LPMOs can use H_2O_2 as co-substrate [19,20,27], and, with a controlled addition of it, the reaction can arrive to higher rates than the ones observed in reactions driven by O_2 [28]. Some studies reported increased activity of LPMOs with the addition of H_2O_2 [19,26,29,30], which may positively affect the industrial use of biomass. On the other hand, LPMOs are sensitive to inactivation by oxidative damage [22], fact that increases the complexity of the process and depends on the amount of substrate and H_2O_2 used [19,26,31]. Therefore, the addition of exogenous reductants needs to be carefully regulated in biomass systems to maintain the catalytically active LPMO-Cu(I) state, while avoiding adverse side reactions among the LPMOs, reductant and components from the substrate.

Reducing agents can originate from the biomass itself, such as those derived from lignin or phenolic compounds [32,33], from other redox enzymes [34–36], and from light-activated photosynthetic pigments [37]. Importantly, lignin and low-molecular-weight lignin-derived compounds (LMWLDC), which can be obtained from the plant cell wall with the use of lignin active enzymes, can deliver electrons to LPMOs and activate them, alleviating the need to add an external reducing agent. However, there are still few studies focused on the interplay between different oxidases in relation to lignocellulose degradation [38]. Laccases are multicopper-containing oxidases which have phenol oxidase activity, they catalyse the oxidation of phenolic compounds using one electron from molecular oxygen [39]. When using substrates that contain lignin, these enzymes can act by oxidation, via an electron transfer process where LMWLDC act as shuttles or mediators between the enzyme and the polymer [40]. While this happen, lignin is removed, creating micropores in the biomass where the other enzymes can access to hydrolyse hemicellulose and cellulose [41]. However, their efficiency has shown to be different according to the type of pretreatment used: while laccase showed to have a positive effect in diluted acid pretreated wheat straw [12], it had no effect when this biomass was pretreated using steam explosion [15].

The addition of hemicellulases to cellulolytic enzyme cocktails has also been suggested as an strategy to improve the production of fermentable sugars [42–44], as they boost the saccharification efficiency of both cellulose and hemicellulose, by improving the cellulose accessibility with the removal of xylan coating [17,44]. The use of surfactants such as Tween 80 and polyethylene glycol (PEG) has also shown beneficial effects on the hydrolysis of cellulose by enzymes [16,45,46]. When using a surfactant in a cellulosic solution, its hydrophobic part adheres to lignin, helping to remove the hydrophobic molecules (that will be prove to cellulase adhesion) and to the hydrophobic parts of cellulases, also hindering hydrophobic sites where enzymes could bind to lignin [13]. Thus, these additives act by lowering the non-productive adsorption of cellulase on lignin (which is considered as one of the main obstacles during enzymatic hydrolysis) and form a network at the liquid-air interface that reduces the surface available for the enzymes preventing their deactivation [47].

With the aim of obtaining a further insight on how the enzymatic hydrolysis of lignocellulosic biomass could be optimized, this work evaluated four different strategies to improve sugar extraction when using a commercial cellulolytic enzyme cocktail. This cocktail was supplemented with different enzymes or additives to enhance the hydrolysis yield when processing mildly pretreated sugarcane bagasse.

2. Materials and methods

2.1. Biomass pretreatment and composition

Sugarcane bagasse was kindly supplied by Raízen (São Paulo, Brazil). It was ground with a hammer mill (Polymix, PX-MFC 90 D, Kinematica AG, Switzerland) into particles of size 2 mm, remoisturized to 50 % (*w*/w) moisture, and pretreated with CO_2 under mild subcritical conditions using a SFE Lab 500 mL supercritical CO_2 extraction equipment (SFE Process, France). Cellulose, hemicellulose, lignin, and ash composition was determined using the NREL protocols [48,49].

2.2. Enzymatic hydrolysis

The enzymatic hydrolysis of pretreated sugarcane bagasse was performed in 24 deep-well plates (Enzyscreen, Netherlands). For the reactions, moisture of samples was initially measured using a Touch moisture analyser (VWR International bvba, Belgium). Then, 0.05 M sodium acetate buffer at pH 4.8 was added until the amount of dry mass in the enzymatic hydrolysis was 10 % (*w*/w). Enzyme loads of 20 and 35 FPU g^{-1} dry matter (DM) of the cellulolytic cocktail Cellic® CTec3 HS (CC3, provided by Novozymes, Denmark) were used. The hydrolysis was carried out at 150 rpm, 50 °C, for 72 h. Control samples without enzyme were prepared and analysed regarding the sugar content released to verify whether the spontaneous degradation of biomass occurred over time. Samples were taken after 6, 24, 48 and 72 h of hydrolysis and heated at 100 °C for 10 min to deactivate the enzymes. Then, the remaining solids were separated by centrifugation at 5000 rpm for 6 min and filtered through 0.45 μm syringe filter (Millipore, MA, USA). All hydrolysis experiments were performed in duplicate; mean values and average deviations are shown.

2.3. Enzyme and additive supplementation

The study included 52 different approaches of supplementation to the cellulolytic cocktail CC3. This involved testing different scenarios using (for most of them) different CC3 loads (20 and 35 FPU g^{-1} DM) together with H_2O_2, laccase, hemicellulase or two different surfactants (Table 1). A 30 % (*w*/w) H_2O_2 solution (Sigma Aldrich) was diluted in Milli-Q® water to the desired concentrations and added at the beginning of hydrolysis (0 h) or three times along the process (0, 24 and 48 h of hydrolysis) to boost the LPMO activity present in CC3 (Strategy 1). The

Table 1
Load of cellulolytic cocktail (CC3), concentration of H_2O_2 and number of H_2O_2 doses used, load of laccase and hemicellulase, and dosages of PEG 4000 and Tween 80 used in the different strategies evaluated for biomass hydrolysis.

Experiment	1	2	3	4	5	6	7	8	9	10	11	12
Strategy 1. Addition of H_2O_2												
CC3 (FPU g^{-1} DM)	35	35	35	35	35	35	35	35	35	35	35	35
H_2O_2 (mM)	0.02	0.24	1.18	2.35	11.75	23.5	0.02	0.24	1.18	2.35	11.75	23.5
Number of H_2O_2 doses	1	1	1	1	1	1	3	3	3	3	3	3
Strategy 2. Addition of laccase												
Experiment	13	14	15	16	17	18	19	20				
CC3 (FPU g^{-1} DM)	20	20	20	20	35	35	35	35				
Laccase (μL g^{-1} DM)	10	30	50	100	10	30	50	100				
Strategy 3. Addition of hemicellulase												
Experiment	21	22	23	24	25	26	27	28	29	30	31	32
CC3 (FPU g^{-1} DM)	20	20	20	20	20	20	35	35	35	35	35	35
Hemicellulase (μL g^{-1} DM)	3.38	6.75	13.5	40.5	81	162	1.92	11.83	23.67	71	142	284
NS:CC3 ratio	24:1	12:1	6:1	2:1	1:1	2:1	24:1	12:1	6:1	2:1	1:1	2:1
Strategy 4a. Addition of PEG 4000												
Experiment	33	34	35	36	37	38	39	40	41	42		
CC3 (FPU g^{-1} DM)	20	20	20	20	20	35	35	35	35	35		
PEG 4000 (mg^{-1} g^{-1} DM)	10	55	100	150	200	10	55	100	150	200		
Strategy 4b. Addition of Tween 80												
Experiment	43	44	45	46	47	48	49	50	51	52		
CC3 (FPU g^{-1} DM)	20	20	20	20	20	35	35	35	35	35		
Tween 80 (mg^{-1} g^{-1} DM)	10	55	100	150	200	10	55	100	150	200		

commercial laccase Novozym 51,003 (Sigma-Aldrich, USA) was employed using different loads (Strategy 2). Hemicellulase NS22244 (Novozymes, Denmark) was also supplemented using different loads (Strategy 3). The surfactants PEG4000 (Cas number 25322–68-3 Sigma-Aldrich, USA) and Tween 80 (CAS number 9005-65-6, Sigma-Aldrich, USA) were also added to enzymatic hydrolysis using 20 and 35 FPU g^{-1} DM of CC3 (Strategies 4a and b). Control experiments with only CC3 were performed by using 20 or 35 FPU g^{-1} DM for the enzymatic hydrolysis.

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-87H (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.

3. Results and discussion

3.1. Composition of raw and pretreated biomass

The purpose of the mild pretreatment of biomass with CO_2 was not to release sugars but recover as much of the original biomass fraction as possible, while increasing as much as possible the surface area available for the adsorption of enzymes in the next step. Thus, the aim of this work was to avoid the degradation of cellulose and hemicellulose fractions during pretreatment. As can be seen in Table 2, pretreated sugarcane bagasse showed a very similar composition to the raw material. The recovery of hemicellulose and lignin was around 98 and 97 %, respectively, after the process. On the other hand, the contents of cellulose and acetyl groups were slightly increased due to the little reduction of hemicellulose and lignin contents after pretreatment. This decrease in hemicellulose content and increase in acetyl groups can be attributed to the hydrolysis of hemicellulose during pretreatment, which released the acetyl groups present in the hemicellulose side chains.

3.2. Effect of H_2O_2 supplementation on biomass hydrolysis by cellulolytic cocktail containing LPMOs

The recent discovery that H_2O_2 can be used to activate LPMOs rather than O_2 brings an essential element toward the development of optimized enzymatic cocktails for the hydrolysis of lignocellulosic biomass: the reductant will only be needed to activate the enzyme, in contrast to the previous thinking that it should be added following the amount of generated products [20]. Fig. 1a-d shows the production of glucose and xylose obtained after 72 h of hydrolysis when feeding H_2O_2 at the beginning of the reaction (Fig. 1a,b) or with a stepwise addition during times 0, 24 and 48 h of reaction (Fig. 1c,d).

In both approaches for adding H_2O_2 (just at the beginning and three times addition), and up to 0.24 mM there was a linear relationship between the amount of H_2O_2 dosed and the sugar production, suggesting that low concentrations of H_2O_2 were able to boost the efficiency of the LPMOs present in the cellulolytic cocktail. The maximum sugar extraction was obtained when adding 0.24 mM of H_2O_2 in one or three pulses,

Table 2
Chemical composition of raw sugarcane bagasse 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

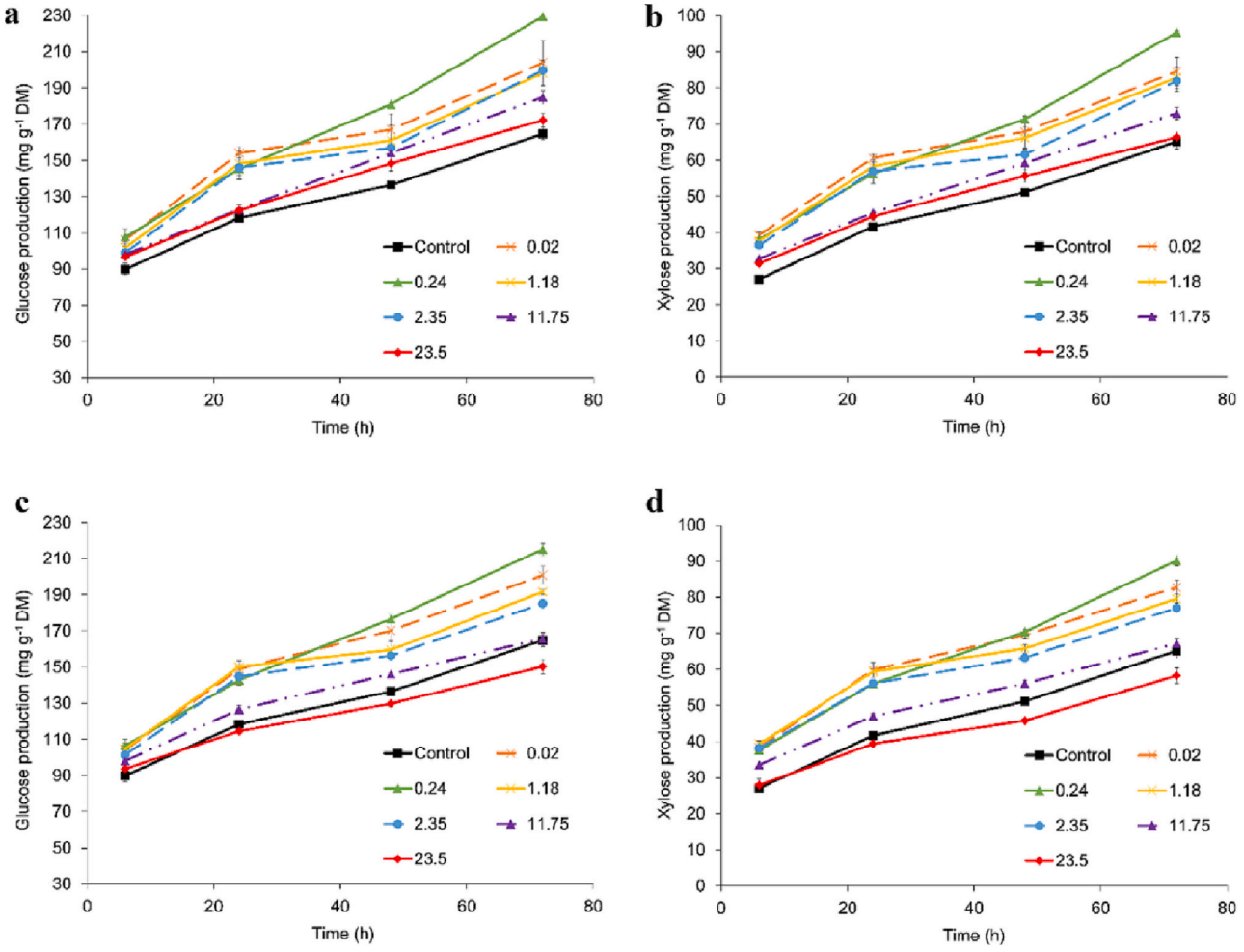


Fig. 1. Glucose (a,c) and xylose (b,d) release during the enzymatic hydrolysis of sugarcane bagasse using the cellulolytic cocktail and adding H_2O_2 at the beginning of the process (0 h) (a,b) or adding three doses of H_2O_2 during times 0, 24 and 48 h (c,d). H_2O_2 was supplemented adding dosages of 0.02, 0.24, 1.18, 2.35, 11.75 or 23.5 mM. The data shown is the average of two replicates of the same experiment. Error bars indicate the average deviation.

where 229.4 and 215 mg glucose g^{-1}DM were released, corresponding to an increase of 39 and 30 %, respectively, compared to the control (where no H_2O_2 was added). Interestingly, the increase in xylose production was more prominent, being 46 and 38 % for one and three pulses, and releasing 95.3 and 90.1 mg xylose g^{-1} DM, respectively. It was also observed that the boosting effect of H_2O_2 on sugars release was reduced when it was added in concentrations higher than 0.24 mM. Furthermore, three doses of 23.5 mM H_2O_2 resulted in a production of glucose and xylose even lower than the control, revealing that an excess of H_2O_2 is detrimental to the performance of LPMOs as the damage of their active site by self-inactivation occurs.

Recent studies have also reported that H_2O_2 supplementation to enzymatic cocktails containing LPMOs improved the extraction of sugars from lignocellulosic biomasses other than that used in the present study. Costa et al. (2019) [50] studied the hydrolysis of sulfite-pulped Norway spruce using Cellic® Ctec 3 and obtained 33 % higher glucan conversion when H_2O_2 was added (a continuous pumping of 200 μMh^{-1} starting after 20 h of hydrolysis) than the reaction carried out without H_2O_2, at 60 h. In addition, their maximum conversion was observed at 100 h of hydrolysis, while this maximum was not achieved when H_2O_2 was not added, even after 165 h of hydrolysis. Müller et al. (2018) also studied the hydrolysis of sulfite-pulped Norway spruce as well as steam-exploded birchwood using the cellulolytic cocktail Cellic® Ctec 2 and observed a 10 % increase in the glucan conversion from sulfite-pulped Norway spruce compared to the control when using a constant feeding rate of 300 μMh^{-1} for 48 h. Steam-exploded birchwood hydrolysis showed a maximum glucan conversion around 15 % higher than the control when using a feeding rate of 90 μMh^{-1}; however, when the feeding rate was increased, the glucan conversion reduced being even lower than the control. It is worth highlighting that in both studies presented by Costa et al. (2019) and Müller et al. (2018), an extra reducing agent (such as ascorbic acid) was added to the hydrolysis. When using an external reducing agent, more H_2O_2 can be supplemented until reaching an inhibitory concentration. Müller et al. (2018) also stated that the efficiency of the conversion of H_2O_2 by LPMOs decreases when increasing the lignin content in the substrate. Thus, the effect of accumulation and inhibition of H_2O_2 is higher when using substrates with higher lignin content. It is demonstrated then the positive effect of lignin to reduce the amount of reducing agent needed and to increase the sugars yield during enzymatic hydrolysis. Thus, it is pointed out another reason for believing that mild pretreatments (as that used in our work), in which lignin is not removed from the substrate, could be a better option than conventional ones.

3.3. Effect of laccase supplementation on biomass hydrolysis by cellulolytic cocktail containing LPMOs

Previous studies have shown that laccases have the potential to boost LPMOs contained in cellulase cocktails. Laccases can depolymerize lignin and the released LMWLDC can deliver electrons to activate LPMOs [51]. In contrast, when used together with cellulases, they can compete for the oxygen present in the media and a negative effect may be

observed [38]. Furthermore, their inhibitory effect toward B-glucosidase activity has also been studied [52]. Some works have reported that laccase treatment increased the efficiency of biomass hydrolysis. For instance, Gutiérrez et al. (2012) [53] observed 61 % and 12 % increased glucose yield during the enzymatic hydrolysis of Eucalyptus and Pennisetum compared to the cases without laccase treatment. Also, Moilanen U et al. (2011) [54] reported a 12 % increase in hydrolysis of steam pretreated spruce; however, they reported a 17 % decrease in hydrolysis of steam exploded giant reed when laccase was added. Thus, it can be expected that different substrates with different composition and chemical structures may respond in a different way to the supplementation with laccase.

In this work, *Myceliophthora thermophila* laccase was added to the cellulolytic cocktail at different doses ranging from 10 to 100 μL g^{-1} DM (equivalent to 10 to 100 U g^{-1} DM). It was observed (Fig. 2) that during the first 48 h of hydrolysis, the production of glucose and xylose took place at higher rates when using laccase. The maximum glucose extraction of 153 mg g^{-1} DM was obtained when adding 35 FPU g^{-1} DM and a laccase dosage of 10 μL g^{-1} DM; while for xylose, 64 mg were extracted per g DM when using the same cellulase load and 100 μL of laccase per g DM, which corresponded to a 12.9 % and 26.5 % of increase compared to the control, respectively. However, the release of sugars stopped after 48 h. Although during this study the structure and composition of lignin has not been evaluated after enzymatic hydrolysis, it is hypothesized that a reason for this fact could be related to the changes on the structural characteristics of lignin during the process. With the laccase action, lignin should have been oxidized (increasing its ramification degree) and subsequently converted into smaller molecules. With this, the probability and space available for cellulases to bind non-productively to the resulting lignin structure would be enhanced. Also, a depletion of O_2 during the process could have occurred causing a competition between laccases and cellulases for it. When comparing the experiments containing different cellulase loads, 20 and 35 FPU g^{-1} DM, while both supplemented with the same laccase load, the decreasing production effect was higher when using lower cellulase load (Fig. 2a,b vs Fig. 2c,d). As an example, when using 100 μL g^{-1} DM of laccase, the production of glucose was 15.5 % lower than the control when 20 FPU g^{-1} was used (Fig. 2a), while it decreased 10.1 % when the cellulase load was 35 FPU g^{-1}. When the proportion of cellulases to ramified lignin is higher, it could result in a higher amount of active cellulases that could be degrading cellulose. In general, it seems that the previously explained effect in hemicellulases was lower because of two reasons, the first is that results for xylose are generally more promising than for glucose production - the enhanced production of xylose (Fig. 2b,d) was more prominent than for glucose (Fig. 2a,c) after 48 h, compared to the control. And the second, when using 35 FPU g^{-1} DM, the maximum production after 48 h for xylose was obtained when dosing the highest load of laccase, while for the glucose, the maximum was obtained when using the lowest dose.

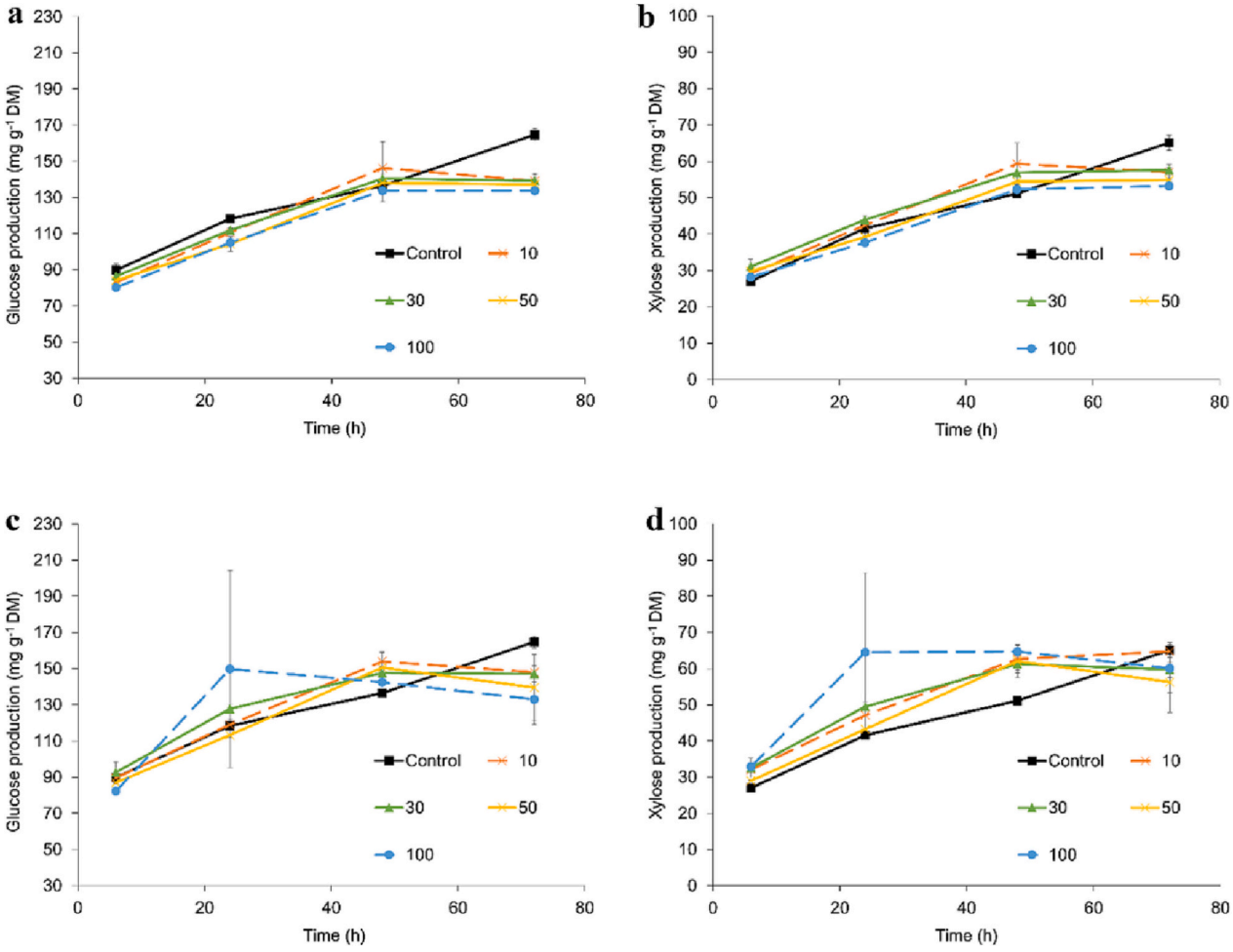


Fig. 2. Glucose (a,c) and xylose (b,d) release in enzymatic hydrolysis of sugarcane bagasse with different loads of laccase and 20 FPU of cellulase g^{-1} DM (a,b) or 35 FPU cellulase g^{-1} DM (c,d). Laccase doses were 10, 30, 50 or 100 μL g^{-1} DM. The data shown is the average of two replicates of the same experiment. Error bars indicate the average deviation.

3.4. Effect of hemicellulase supplementation on biomass hydrolysis by cellulolytic enzymes

As during mild CO_2-pretreatment the hemicellulose content remained almost intact, it was hypothesized that adding extra hemicellulase to the cellulolytic cocktail would be important to increase the xylose release. In addition, as a higher degradation of hemicellulose would occur in that case, a higher glucose release would also be expected, as the hemicellulose degradation would expose more the cellulose fibers to the attack of cellulases [55]. To confirm whether this strategy would work, the addition of different hemicellulase loads to the cellulolytic cocktail were tested: 3.38 to 162 μL hemicellulase g^{-1} DM for 20 FPU cellulase g^{-1} DM, and 5.92 to 284 μL hemicellulase g^{-1} DM for 35 FPU cellulase g^{-1} DM (Fig. 3a-d).

For 20 FPU cellulase g^{-1} DM the highest glucose production was 220 mg g^{-1} DM after 72 h when adding 162 μL hemicellulase g^{-1} DM (Fig. 3a). That glucose production was 38 % higher in comparison to the highest glucose production obtained without hemicellulase addition (control). On the other hand, a lower dosage of hemicellulase (81 μL g^{-1} DM) was enough to reach the highest xylose production (89 mg g^{-1} DM), representing a 50 % increase of xylose in relation to the hydrolysis using cellulase only.

For 35 FPU cellulase g^{-1} DM, the effect of adding 142 and 284 μL hemicellulase g^{-1} DM was similar to that observed for 20 FPU cellulase g^{-1} DM, obtaining around 227 mg of glucose g^{-1} DM, while in the case of xylose, a 43 % increased release was observed, resulting in 93 mg of xylose g^{-1} DM. Although during the first 24 h of hydrolysis the rate of sugar production was generally faster when using 35 FPU g^{-1} DM, the maximum values of glucose and xylose released were very similar comparing both cellulase loads (20 and 35 FPU g^{-1} DM), which suggests that the addition of hemicellulase can reduce the amount of cellulase needed for hydrolysis to achieve the same final sugars release.

Also, with lower loads of hemicellulase (40.5 and 71.00 μL g^{-1} DM, for 20 and 35 FPU cellulase g^{-1} DM, respectively) an increase in the sugar release of around 30 % was observed. This proves the idea that the addition of hemicellulases, even in little amounts, helped to increase the degradation of hemicellulose and consequently, lead to a better accessibility of the enzymes to the cellulose fibers [17,56]. However, an economic analysis would be useful to assess if the outcome of the increased sugar production compensates the higher expenses related to the use of a higher enzyme load.

Xu et al. [42] tested different loads of hemicellulase during the hydrolysis of alkali pretreated sugarcane bagasse and obtained an increase in glucose release of 7.6 % after 48 h when using 150 U hemicellulase g^{-1} DM (in the present study, around 25 μL hemicellulase g^{-1} DM was needed to reach this dosage) and 4 FPU g^{-1} DM of Cellic® Ctec3. This little increase compared to only using the cellulase cocktail was probably due to the harsh conditions used during pretreatment, as it recovered only 63 % of the hemicellulose and removed 78.1 % of lignin, making the addition of hemicellulase less relevant than when higher

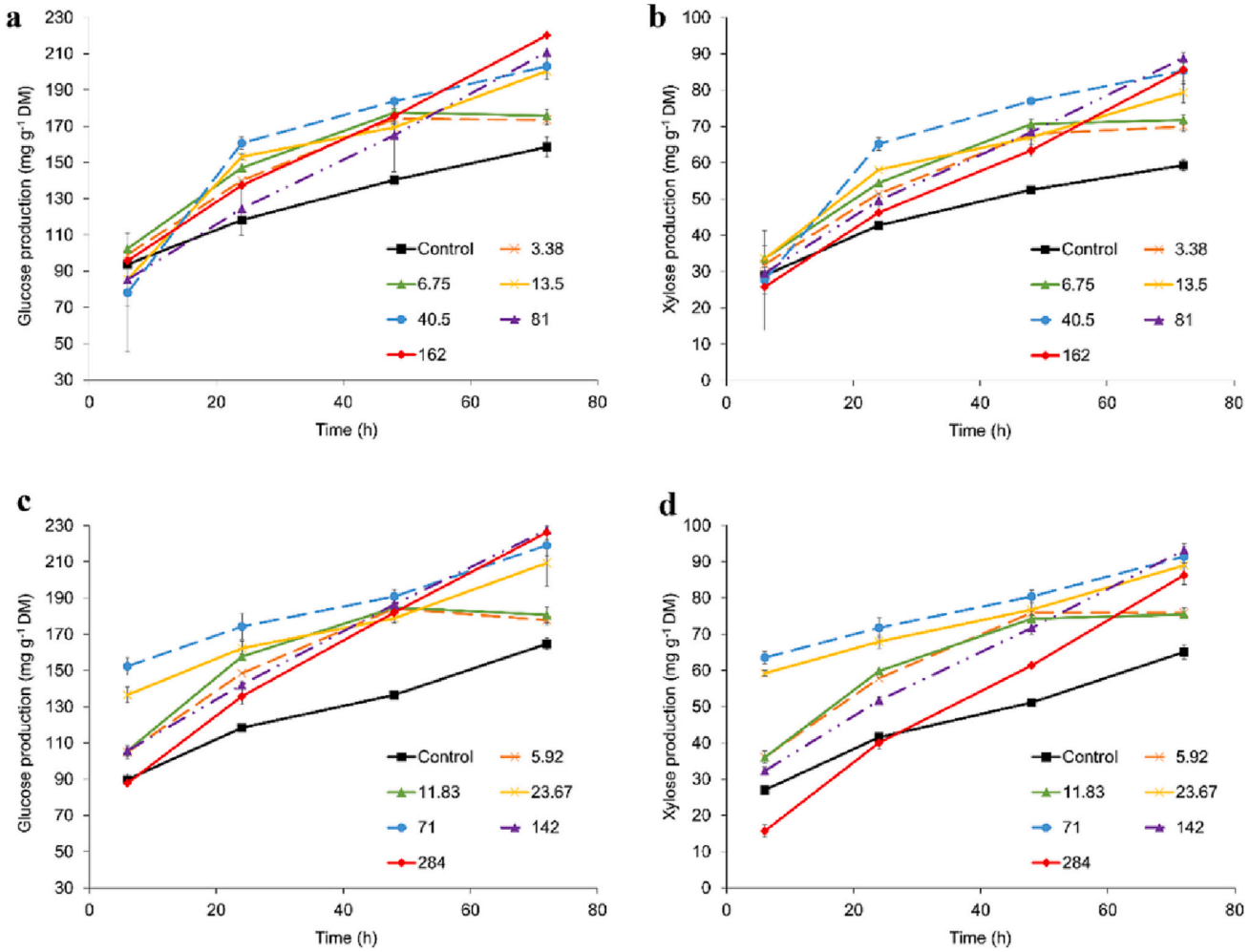


Fig. 3. Glucose (a,c) and xylose (b,d) release during the enzymatic hydrolysis of sugarcane bagasse with different loads of hemicellulase: 3.38, 6.75, 13.5, 40.5, 81 and 162 μL g^{-1} DM for 20 FPU cellulase g^{-1} DM (a,b); and 5.92, 11.83, 23.67, 71, 142 and 284 μL g^{-1} DM for 35 FPU cellulase g^{-1} DM (c,d). The data shown is the average of two replicates of the same experiment. Error bars indicate the average deviation.

proportion of hemicellulase compared to cellulase is present in the substrate, as in the present study.

It is also interesting to note the lower sugar production rate observed during the first 24 h of hydrolysis (present study), which occurred mainly in the case of supplementation with 35 FPU g^{-1} DM of cellulase and 142 and 284 μL hemicellulase g^{-1} DM (Fig. 3c,d), could be related to feedback inhibition. In these cases, the highest dosage of cellulase and hemicellulase could have led to a rapid conversion of cellulose and hemicellulose to sugar monomers and oligosaccharides during the first minutes of hydrolysis, which might have accumulated, causing stress conditions to the enzymes [57]. Similarly, Sun et al. (2018) [58] tried different loads of xylanase (Cellic® Htec between 150 and 300 U g^{-1} polysaccharide) for hydrolysis of atmospheric glycerol organosolv-pretreated wheat straw and found that loads higher than 75 U g^{-1} polysaccharide did not increase the enzymatic hydrolysis yield, which is in line with the results observed in the present study.

3.5. Effect of PEG4000 and Tween 80 supplementation on biomass hydrolysis by cellulolytic enzymes

The non-ionic surfactants PEG4000 and Tween 80 are examples of additives that have shown to improve the enzymatic hydrolysis of biomass and enable the reduction of enzyme loads for hydrolysis [16,59–61]. In this study, different concentrations of PEG4000 (10, 55, 100, 150 and 200 mg g^{-1} DM) and Tween 80 (10, 55, 100, 150 and 200 mg g^{-1} DM) were added to 20 and 35 FPU cellulase g^{-1} DM to verify their effect on the hydrolysis of mildly pretreated biomass.

As can be seen in Fig. 4a-c, increased concentrations of PEG4000 had a positive effect on the release of glucose for both 20 FPU g^{-1} DM and 35 FPU g^{-1} DM. The maximum glucose production obtained was 187 and 192 mg of glucose g^{-1} DM respectively, after 72 h of hydrolysis when using 200 mg PEG4000 g^{-1} DM, which were 18 % and 17 % higher when compared to the control. The trend for xylose release (Fig. 4b,d) was similar; however, the effect of the surfactant was higher in this case, since an increase in xylose production of 24 % and 20 % was observed with a production of 73 and 78 mg xylose g^{-1} DM, for 20 FPU g^{-1} DM and 35 FPU g^{-1} DM, respectively, when adding 200 mg PEG4000 g^{-1} DM. The effect of PEG addition was higher when using the lowest cellulase load (20 FPU g^{-1} DM). These results highlight the role that PEG4000 may have in reducing the cellulase load for biomass hydrolysis. Also, when comparing the different doses of surfactant added, and observing the glucose and xylose released in each case, the difference between adding 55 and 200 mg g^{-1} DM of surfactant was 5 % and 14 %, respectively. It can therefore be argued whether adding low concentrations of PEG4000 would be effective to reach a desired positive effect, or if a dosage increase of almost 4 times would be required to get a higher sugar extraction regardless of the surfactant cost.

Fig. 5a-d illustrate the results of the addition of Tween 80 when using 20 FPU cellulase g^{-1} DM and 35 FPU cellulase g^{-1} DM. As can be seen, an increased concentration of Tween 80 supplemented to the cellulolytic cocktail resulted in a higher glucose release for both enzyme loads. The addition of 200 mg Tween 80 g^{-1} DM gave the maximum final glucose

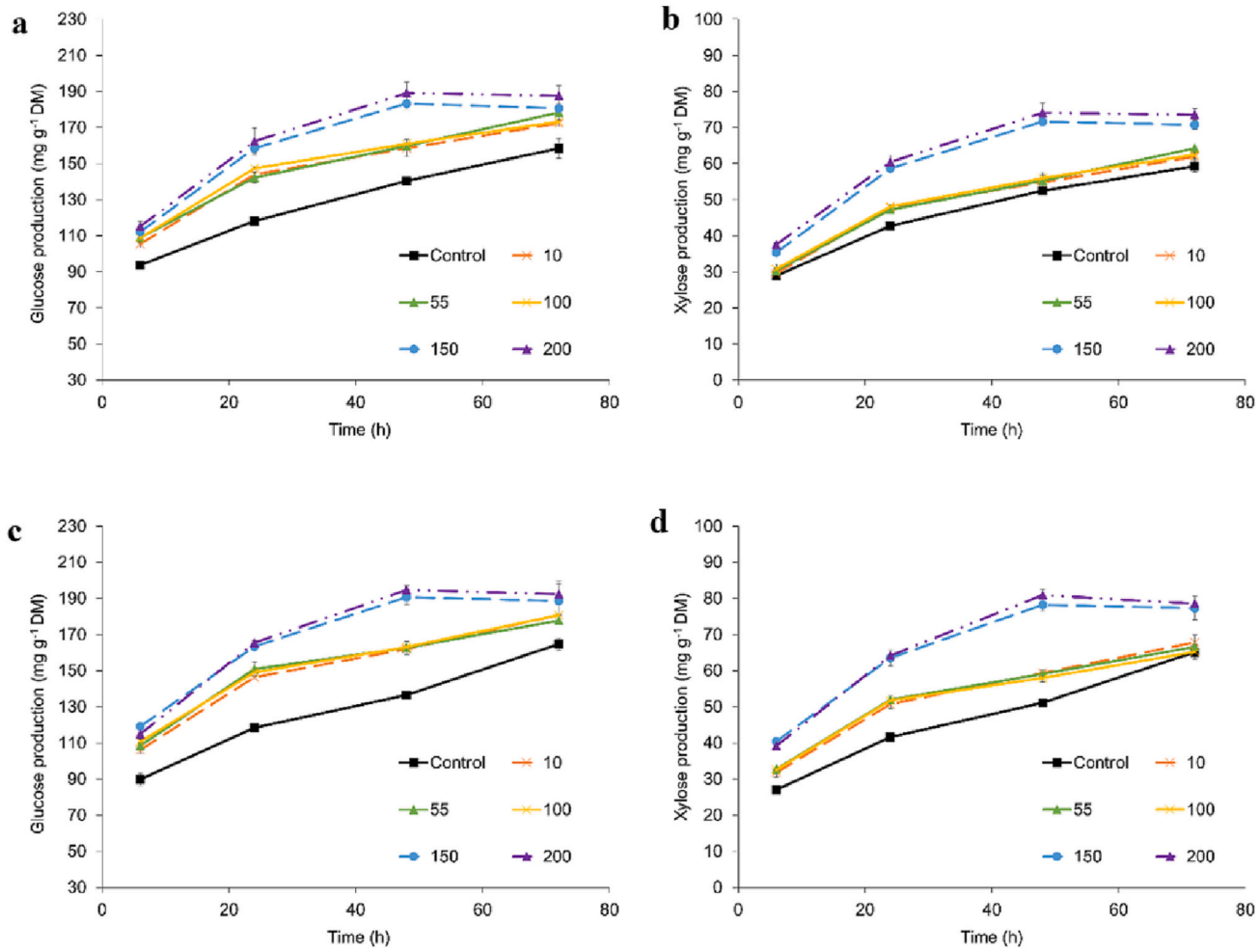


Fig. 4. Glucose (a,c) and xylose (b,d) release during the enzymatic hydrolysis of sugarcane bagasse using different concentrations of PEG4000 (10, 55, 100, 150 or 200 mg g^{-1} DM) and 20 FPU cellulase g^{-1} DM (a,b) or 35 FPU cellulase g^{-1} DM (c,d). The data shown is the average of two replicates of the same experiment. Error bars indicate the average deviation.

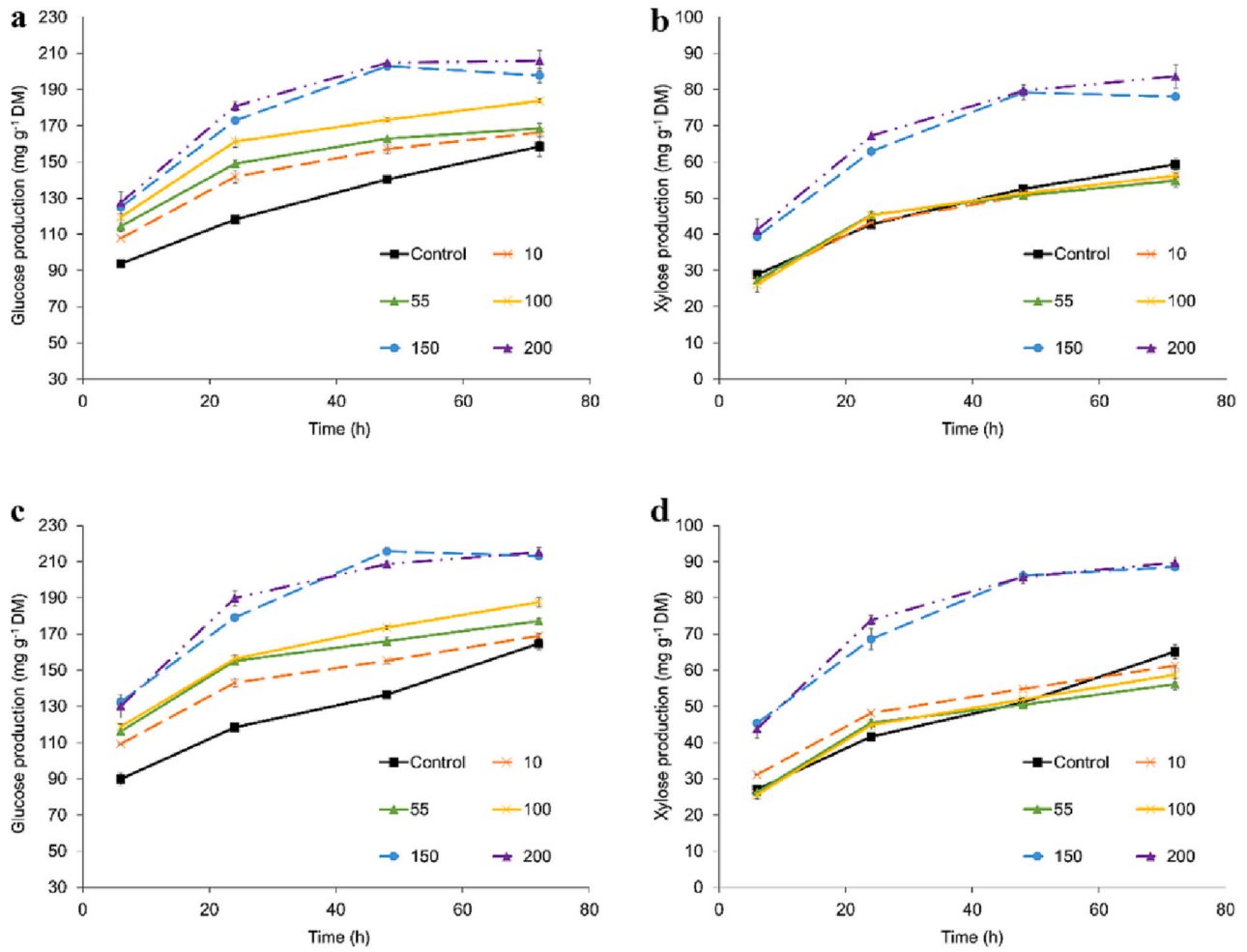


Fig. 5. Glucose (a,c) and xylose (b,d) release during the enzymatic hydrolysis of sugarcane bagasse using different concentrations of Tween 80 (10, 55, 100, 150 or 200 mg g^{-1} DM) and 20 FPU cellulase g^{-1} DM (a,b) or 35 FPU cellulase g^{-1} DM (c,d). The data shown is the average of two replicates of the same experiment. Error bars indicate the average deviation.

release of 205 and 215 mg g^{-1} DM after 72 h of hydrolysis, which were 30 % and 31 % higher than the situation where no surfactant was added, for 20 FPU g^{-1} DM and 35 FPU g^{-1} DM, respectively. The highest xylose release was also reached when adding 200 mg Tween 80 g^{-1} DM to 20 FPU cellulase g^{-1} DM and 35 FPU cellulase g^{-1} DM, being released 84 and 89 mg xylose g^{-1} DM, respectively, which were 41 % and 38 % higher than the control. In contrast, lower doses of Tween 80 (10, 55, and 100 mg g^{-1} DM) showed no effect for both cellulase loads (20 and 35 FPU g^{-1} DM). When comparing 150 mg Tween 80 g^{-1} DM and 200 mg Tween 80 g^{-1} DM for 35 FPU g^{-1} DM, no significant difference was observed for glucose or xylose production. It can also be noted that concentrations of Tween 80 higher than 150 mg g^{-1} DM did not lead to higher release of glucose or xylose. These results are in line with those reported by other authors [62].

In general, higher glucose and xylose release was observed when using Tween 80 in comparison to PEG4000. The same trend was also reported by Xu et al. (2019) [42] with only a small increase of 4 % when adding PEG4000 compared to 6.7 % when using Tween 80.

4. Conclusions

This study revealed that different approaches can be used to improve the performance of enzymatic hydrolysis from mildly pretreated lignocellulosic biomass. A controlled addition of H_2O_2 showed to be beneficial for the overall performance of enzymatic cocktails containing LPMOs. Furthermore, lignin present in the biomass had a positive impact on LPMOs action, being beneficial the fact that lignin was not removed from the substrate during the pretreatment. However, its presence adds complexity to the supplementation approaches using H_2O_2, as they should be adapted according to the lignin content present in the biomass. Also, hemicellulase and non-ionic surfactants supplementation had a positive effect on both glucose and xylose yield. The results obtained in this study highlights two facts that are key in the development of sustainable fractionation processes, and regarding the optimization of enzymatic cocktails: 1) a comprehensive knowledge of the chemical and structural composition of lignocellulosic biomass before hydrolysis is needed, as the cocktail should be carefully adapted to it; and 2) economic and environmental analysis would be crucial to evaluate if the increase in sugar release due to the supplementation of more enzymes or additives balances the costs and emissions associated with the production and acquisition of these extra components.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by the Novo Nordisk Foundation (NNF), Denmark (grant number: NNF20SA0066233). The authors thank Novozymes for supplying the enzyme Cellic® CTec3 HS and NS22244 used in the experiments.

References

[1] "THE 17 GOALS | Sustainable Development." https://sdgs.un.org/goals (accessed Jan. 04, 2023).
[2] P. Manzanares, "The role of biorefinering research in the development of a modern bioeconomy," Acta Innovations, no. 37, pp. 47–56, Dec. 2020, doi:10.32933/ACTAINNOVATIONS.37.4.
[3] M. L. Lopes et al., "Ethanol production in Brazil: a bridge between science and industry," Braz. J. Microbiol., vol. 47, pp. 64–76, Dec. 2016, doi:https://doi.org/10.1016/J.BJM.2016.10.003.
[4] E. L. N. Escobar, T. A. da Silva, C. L. Pirich, M. L. Corazza, and L. Pereira Ramos, "Supercritical fluids: a promising technique for biomass pretreatment and fractionation," Front Bioeng Biotechnol, vol. 8, p. 252, Apr. 2020, doi:https://doi.org/10.3389/FBIOE.2020.00252/BIBTEX.
[5] S.I. Mussatto, C.K. Yamakawa, L. van der Maas, G. Dragone, New trends in bioprocesses for lignocellulosic biomass and CO2 utilization, Renew. Sust. Energ. Rev. 152 (2021), https://doi.org/10.1016/J.RSER.2021.111620.
[6] Y. Zhang, et al., Green fractionation approaches for isolation of biopolymers and the critical technical challenges, Ind. Crop. Prod. 177 (2022), https://doi.org/10.1016/J.INDCROP.2021.114451.
[7] M. N. Aftab *et al.*, "Different pretreatment methods of lignocellulosic biomass for use in biofuel production," Biomass for Bioenergy - Recent Trends and Future Challenges, Nov. 2019, doi:https://doi.org/10.5772/INTECHOPEN.84995.
[8] S.I. Mussatto, G.M. Dragone, Biomass pretreatment, biorefineries, and potential products for a bioeconomy development, Biomass Fractionation Technologies for a Lignocellulosic Feedstock Based Biorefinery (2016) 1–22, https://doi.org/10.1016/B978-0-12-802323-5.00001-3.
[9] A.R. Mankar, A. Pandey, A. Modak, K.K. Pant, Pretreatment of lignocellulosic biomass: a review on recent advances, Bioresour. Technol. 334 (2021), https://doi.org/10.1016/J.BIORTECH.2021.125235.
[10] M. Jiao Zhao *et al.*, "Pretreatment of agricultural residues by supercritical CO2 at 50–80 °C to enhance enzymatic hydrolysis," Journal of Energy Chemistry, vol. 31, pp. 39–45, Apr. 2019, doi:https://doi.org/10.1016/J.JECHEM.2018.05.003.
[11] C. Park, J. Lee, Recent achievements in CO2-assisted and CO2-catalyzed biomass conversion reactions, Green Chemistry 22 (9) (2020) 2628–2642, https://doi.org/10.1039/d0gc00095g. Royal Society of Chemistry.
[12] R. Agrawal *et al.*, "Improved saccharification of pilot-scale acid pretreated wheat straw by exploiting the synergistic behavior of lignocellulose degrading enzymes," RSC Adv., vol. 5, no. 87, pp. 71462–71471, Aug. 2015, doi:https://doi.org/10.1039/C5RA13360B.
[13] R. Agrawal, A. Satlewal, M. Kapoor, S. Mondal, and B. Basu, "Investigating the enzyme-lignin binding with surfactants for improved saccharification of pilot scale pretreated wheat straw," Bioresour. Technol., vol. 224, pp. 411–418, Jan. 2017, doi:https://doi.org/10.1016/J.BIORTECH.2016.11.026.
[14] C. Huang *et al.*, "Lignin-enzyme interaction: a roadblock for efficient enzymatic hydrolysis of lignocellulosics," Renew. Sust. Energ. Rev., vol. 154, p. 111822, Feb. 2022, doi:https://doi.org/10.1016/J.RSER.2021.111822.
[15] S. Kumar, et al., Synergistic enzyme cocktail to enhance hydrolysis of steam exploded wheat straw at pilot scale, Frontiers in Energy Research 6 (2018) 122, https://doi.org/10.3389/fenrg.2018.00122, www.frontiersin.org.
[16] S. Oladi and G. M. Aita, "Interactive effect of enzymes and surfactant on the cellulose digestibility of un-washed and washed dilute ammonia pretreated energy cane bagasse," Biomass Bioenergy, vol. 109, pp. 221–230, Feb. 2018, doi:https://doi.org/10.1016/j.biombioe.2017.12.005.
[17] F.F. Sun, et al., Accessory enzymes influence cellulase hydrolysis of the model substrate and the realistic lignocellulosic biomass, Enzym. Microb. Technol. 79–80 (2015) 42–48, https://doi.org/10.1016/j.enzmictec.2015.06.020.
[18] G. Vaaje-Kolstad, et al., An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides, Science 330 (6001) (1979) 219–222. Oct. 2010, https://doi.org/10.1126/SCIENCE.1192231/SUPPL_FILE/VAAJE-KOLSTAD.SOM.PDF.
[19] A.A. Stepnov, V.G.H. Eijsink, Z. Forsberg, Enhanced in situ H2O2 production explains synergy between an LPMO with a cellulose-binding domain and a single-domain LPMO, Scientific Reports 2022 12:1 12 (1) (Apr. 2022) 1–11, https://doi.org/10.1038/s41598-022-10096-0.
[20] B. Bissaro, et al., Oxidative cleavage of polysaccharides by monocopper enzymes depends on H2O2, Nature Chemical Biology 2017 13:10 13 (10) (Aug. 2017) 1123–1128, https://doi.org/10.1038/nchembio.2470.
[21] M. Eibinger, et al., Cellulose surface degradation by a lytic polysaccharide monooxygenase and its effect on cellulase hydrolytic efficiency, J. Biol. Chem. 289 (52) (2014) 35929–35938, https://doi.org/10.1074/JBC.M114.602227.
[22] P. Chylenski *et al.*, "Lytic polysaccharide monooxygenases in enzymatic processing of lignocellulosic biomass," 2019, doi:https://doi.org/10.1021/acscatal.9b00246.
[23] H. Østby, et al., Enzymatic processing of lignocellulosic biomass: principles, recent advances and perspectives, J. Ind. Microbiol. Biotechnol. 47 (3) (2020) 623–657, https://doi.org/10.1007/s10295-020-02301-8.
[24] J. W. Agger *et al.*, "Discovery of LPMO activity on hemicelluloses shows the importance of oxidative processes in plant cell wall degradation," Proc. Natl. Acad. Sci. U. S. A., vol. 111, no. 17, pp. 6287–6292, Apr. 2014, doi:https://doi.org/10.1073/PNAS.1323629111/-/DCSUPPLEMENTAL/PNAS.201323629SI.PDF.
[25] M. Frommhagen *et al.*, "Discovery of the combined oxidative cleavage of plant xylan and cellulose by a new fungal polysaccharide monooxygenase," Biotechnol Biofuels, vol. 8, no. 1, pp. 1–12, Jul. 2015, doi:https://doi.org/10.1186/S13068-015-0284-1/TABLES/1.
[26] G. Müller, P. Chylenski, B. Bissaro, V. G. H. Eijsink, and S. J. Horn, "The impact of hydrogen peroxide supply on LPMO activity and overall saccharification efficiency of a commercial cellulase cocktail," Biotechnol Biofuels, vol. 11, no. 1, p. 209, Jul. 2018, doi:https://doi.org/10.1186/s13068-018-1199-4.
[27] C. Rovira, P. H. Walton, B. Wang, Z. Wang, and G. J. Davies, "Activation of O2 and H2O2 by lytic polysaccharide monooxygenases," ACS Catal., vol. 10, no. 21, pp. 12760–12769, Nov. 2020, doi:https://doi.org/10.1021/ACSCATAL.0C02914/ASSET/IMAGES/LARGE/CS0C02914_0009.JPEG.
[28] B. Bissaro, et al., Fenton-type chemistry by a copper enzyme: molecular mechanism of polysaccharide oxidative cleavage, bioRxiv (2016), https://doi.org/10.1101/097022.
[29] E. Balaguer Moya, et al., Evaluation of enzymatic hydrolysis of sugarcane bagasse using combination of enzymes or co-substrate to boost lytic polysaccharide monooxygenases action, Catalysts 12 (10) (2022) 1158, https://doi.org/10.3390/CATAL12101158.
[30] G. Müller, A. Várnai, K.S. Johansen, V.G.H. Eijsink, S.J. Horn, Harnessing the potential of LPMO-containing cellulase cocktails poses new demands on processing conditions, Biotechnol Biofuels 8 (1) (2015), https://doi.org/10.1186/s13068-015-0376-y.
[31] A. Kadić, P. Chylenski, M.A.T. Hansen, O. Bengtsson, V.G.H. Eijsink, G. Lidén, Oxidation-reduction potential (ORP) as a tool for process monitoring of H2O2/LPMO assisted enzymatic hydrolysis of cellulose, Process Biochem. 86 (2019) 89–97, https://doi.org/10.1016/J.PROCBIO.2019.08.015.
[32] J. Hu, V. Arantes, A. Pribowo, K. Gourlay, and J. N. Saddler, "Substrate factors that influence the synergistic interaction of AA9 and cellulases during the enzymatic hydrolysis of biomass," Energy Environ. Sci., vol. 7, no. 7, pp. 2308–2315, Jun. 2014, doi:https://doi.org/10.1039/C4EE00891J.
[33] U. F. Rodríguez-Zúñiga, D. Cannella, R. D. C. Giordano, R. D. L. C. Giordano, H. Jørgensen, and C. Felby, "Lignocellulose pretreatment technologies affect the level of enzymatic cellulose oxidation by LPMO," Green Chem., vol. 17, no. 5, pp. 2896–2903, May 2015, doi:https://doi.org/10.1039/C4GC02179G.
[34] S. Garajova, et al., Single-domain flavoenzymes trigger lytic polysaccharide monooxygenases for oxidative degradation of cellulose, Scientific Reports 2016 6:1 6 (1) (Jun. 2016) 1–9, https://doi.org/10.1038/srep28276.
[35] D. Kracher, et al., Extracellular electron transfer systems fuel cellulose oxidative degradation, Science 352 (6289) (1979) 1098–1101. May 2016, https://doi.org/10.1126/SCIENCE.AAF3165/SUPPL_FILE/AAF3165-KRACHER-SM.PDF.
[36] M. Haddad Momeni, et al., Discovery of fungal oligosaccharide-oxidising flavo-enzymes with previously unknown substrates, redox-activity profiles and interplay with LPMOs, Nat. Commun. 12 (1) (2021), https://doi.org/10.1038/S41467-021-22372-0.
[37] D. Cannella, et al., Light-driven oxidation of polysaccharides by photosynthetic pigments and a metalloenzyme, Nature Communications 7 (1) (2016) 1–8, 7:1. Apr. 2016, https://doi.org/10.1038/ncomms11134.
[38] L. Brenelli, F.M. Squina, C. Felby, D. Cannella, Laccase-derived lignin compounds boost cellulose oxidative enzymes AA9, Biotechnol Biofuels 11 (1) (2018) 1–12, https://doi.org/10.1186/S13068-017-0985-8/FIGURES/6.
[39] Ú. Fillat, D. Ibarra, M.E. Eugenio, A.D. Moreno, E. Tomás-Pejó, R. Martín-Sampedro, Laccases as a potential tool for the efficient conversion of lignocellulosic biomass: a review, Fermentation 3 (2) (2017), https://doi.org/10.3390/FERMENTATION3020017. Page 17. May 2017.
[40] W. Agger, M. Larsen, and V. G. H, "General rights Enzymatic cellulose oxidation is linked to lignin by long-range electron transfer", doi:https://doi.org/10.1038/srep18561.
[41] Z. Deng, A. Xia, Q. Liao, X. Zhu, Y. Huang, and Q. Fu, "Laccase pretreatment of wheat straw: effects of the physicochemical characteristics and the kinetics of enzymatic hydrolysis," Biotechnol Biofuels, vol. 12, no. 1, pp. 1–12, Jun. 2019, doi:https://doi.org/10.1186/S13068-019-1499-3/FIGURES/8.
[42] C. Xu *et al.*, "Enhancement of high-solids enzymatic hydrolysis efficiency of alkali pretreated sugarcane bagasse at low cellulase dosage by fed-batch strategy based on optimized accessory enzymes and additives," Bioresour. Technol., vol. 292, p. 121993, Nov. 2019, doi:https://doi.org/10.1016/j.biortech.2019.121993.
[43] W. Gao, Z. Li, T. Liu, and Y. Wang, "Production of high-concentration fermentable sugars from lignocellulosic biomass by using high solids fed-batch enzymatic hydrolysis," Biochem. Eng. J., vol. 176, Dec. 2021, doi:https://doi.org/10.1016/J.BEJ.2021.108186.
[44] M. R. Mukasekuru, P. Kaneza, H. Sun, F. F. Sun, J. He, and P. Zheng, "Fed-batch high-solids enzymatic saccharification of lignocellulosic substrates with a combination of additives and accessory enzymes," Ind. Crop. Prod., vol. 146, Apr. 2020, doi:https://doi.org/10.1016/J.INDCROP.2020.112156.
[45] D. Cannella, H. Jørgensen, Do new cellulolytic enzyme preparations affect the industrial strategies for high solids lignocellulosic ethanol production? Biotechnol. Bioeng. 111 (2014) 59–68, https://doi.org/10.1002/bit.25098/abstract.
[46] C. Xu *et al.*, "Enhancement of high-solids enzymatic hydrolysis efficiency of alkali pretreated sugarcane bagasse at low cellulase dosage by fed-batch strategy based on optimized accessory enzymes and additives," Bioresour. Technol., vol. 292, Nov. 2019, doi:https://doi.org/10.1016/J.BIORTECH.2019.121993.

[47] A.S.A. da Silva, R.P. Espinheira, R.S.S. Teixeira, M.F. de Souza, V. Ferreira-Leitão, E.P.S. Bon, Constraints and advances in high-solids enzymatic hydrolysis of lignocellulosic biomass: A critical review, Biotechnology for Biofuels 13 (1) (2020), https://doi.org/10.1186/s13068-020-01697-w. BioMed Central Ltd., p. 58, Mar. 23.

[48] A. Sluiter *et al.*, "Determination of Structural Carbohydrates and Lignin in Biomass Laboratory Analytical Procedure (LAP) Issue Date: 7/17/2005," 2008, Accessed: Dec. 20, 2022. [Online]. Available: www.nrel.gov.

[49] A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, and D. Templeton, "Determination of Ash in Biomass: Laboratory Analytical Procedure (LAP); Issue Date: 7/17/2005," 2008, Accessed: Dec. 20, 2022. [Online]. Available: www.nrel.gov.

[50] T. H. F. Costa *et al.*, "Demonstration-scale enzymatic saccharification of sulfite-pulped spruce with addition of hydrogen peroxide for LPMO activation," Biofuels Bioprod. Biorefin., vol. 14, no. 4, pp. 734–745, Jul. 2020, doi:https://doi.org/10.1002/BBB.2103.

[51] B. Westereng, et al., Enzymatic cellulose oxidation is linked to lignin by long-range electron transfer, Scientific Reports 2015 5:1 5 (1) (Dec. 2015) 1–9, https://doi.org/10.1038/srep18561.

[52] A. Oliva-Taravilla *et al.*, "Unraveling the effects of laccase treatment on enzymatic hydrolysis of steam-exploded wheat straw," Bioresour. Technol., vol. 175, pp. 209–215, Jan. 2015, doi:https://doi.org/10.1016/J.BIORTECH.2014.10.086.

[53] A. Gutiérrez *et al.*, "Demonstration of laccase-based removal of lignin from wood and non-wood plant feedstocks," Bioresour. Technol., vol. 119, pp. 114–122, Sep. 2012, doi:https://doi.org/10.1016/J.BIORTECH.2012.05.112.

[54] U. Moilanen, M. Kellock, S. Galkin, and L. Viikari, "The laccase-catalyzed modification of lignin for enzymatic hydrolysis," Enzym. Microb. Technol., vol. 49, no. 6–7, pp. 492–498, Dec. 2011, doi:https://doi.org/10.1016/J.ENZMICTEC.2011.09.012.

[55] G.-G. Manuela del Rosario and M. L. Rita, "Cellulases, hemicellulases and ligninolytic enzymes: mechanism of action, optimal processing conditions and obtaining value-added compounds in plant matrices," MOJ Food Processing & Technology, vol. 10, no. 1, pp. 30–37, Dec. 2022, doi:10.15406/mojfpt.2022.10.00270.

[56] J. Hu, R. Chandra, V. Arantes, K. Gourlay, J. Susan van Dyk, and J. N. Saddler, "The addition of accessory enzymes enhances the hydrolytic performance of cellulase enzymes at high solid loadings," Bioresour. Technol., vol. 186, pp. 149–153, Jun. 2015, doi:https://doi.org/10.1016/j.biortech.2015.03.055.

[57] R. Agrawal, A. Verma, R. R. Singhania, S. Varjani, C. di Dong, and A. Kumar Patel, "Current understanding of the inhibition factors and their mechanism of action for the lignocellulosic biomass hydrolysis," Bioresour. Technol., vol. 332, Jul. 2021, doi:https://doi.org/10.1016/J.BIORTECH.2021.125042.

[58] F. Sun, et al., Optimization of on-site cellulase preparation for efficient hydrolysis of atmospheric glycerol organosolv pretreated wheat straw, J. Chem. Technol. Biotechnol. 93 (8) (2018) 2083–2092, https://doi.org/10.1002/jctb.5605.

[59] J. Rocha-Martín, C. Martinez-Bernal, Y. Pérez-Cobas, F. M. Reyes-Sosa, and B. D. García, "Additives enhancing enzymatic hydrolysis of lignocellulosic biomass," Bioresour. Technol., vol. 244, pp. 48–56, Nov. 2017, doi:https://doi.org/10.1016/J.BIORTECH.2017.06.132.

[60] J. Ouyang, Z. Dong, X. Song, X. Lee, M. Chen, Q. Yong, Improved enzymatic hydrolysis of microcrystalline cellulose (Avicel PH101) by polyethylene glycol addition, Bioresour. Technol. 101 (17) (2010) 6685–6691, https://doi.org/10.1016/j.biortech.2010.03.085.

[61] S. Cao, G.M. Aita, Enzymatic hydrolysis and ethanol yields of combined surfactant and dilute ammonia treated sugarcane bagasse, Bioresour. Technol. 131 (2013) 357–364, https://doi.org/10.1016/j.biortech.2012.12.170.

[62] W. Jin *et al.*, "Tween-80 is effective for enhancing steam-exploded biomass enzymatic saccharification and ethanol production by specifically lessening cellulase absorption with lignin in common reed," Appl. Energy, vol. 175, pp. 82–90, Aug. 2016, doi:https://doi.org/10.1016/J.APENERGY.2016.04.104.