Development and Integration of Sustainable Processes for Biomass Fractionation

Balaguer Moya, Eva

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PhD thesis by Eva Balaguer Moya

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DTU Bioengineering

Department of Biotechnology and Biomedicine

Technical University of Denmark (DTU)

Development and Integration of Sustainable Processes for Biomass Fractionation

PhD thesis

August 2023

This PhD thesis was prepared by

Eva Balaguer Moya

Principle supervisor: Prof. Solange Inês Mussatto, DTU Bioengineering

Co-supervisor: Dr. Giuliano Marcelo Dragone, DTU Bioengineering

Preface

This thesis was prepared to fulfil the requirements for the completion of a PhD programme at the Technical University of Denmark (DTU). The work presented was performed at the DTU Bioengineering Department of Biotechnology and Biomedicine and Novozymes in the period from February 2021 to August 2023. The work was funded by the Novo Nordisk Foundation (NNF), Denmark.

Eva Balaguer Moya

Lyngby, August 2023

Abstract

Industrial practices and technological processes require a fast reformulation to meet the demands while preventing the horrible consequences of reaching limit global temperatures. Lignocellulose biomass is widely produced worldwide and holds a great potential to substitute fossil-based fuels and materials. Its implementation into biorefinery processes can be a key contributor to the transition to a circular and biobased economy, in which waste fractions are considered a resource rather than a burden. A major constraint of biomass conversion processes is the high recalcitrance of its structure, that is difficult to depolymerize and partition into simpler units which can subsequently be used as a product or as a raw material for conversion. This issue could be solved by using an efficient and sustainable fractionation method, in which the three main components of the biomass (cellulose, hemicellulose and lignin) could be obtained for conversion, however it has still not been achieved.

This thesis addresses the topic with the utilization of sugarcane bagasse and the introduction of a novel and mild pretreatment approach using carbon dioxide. When applying it to low moisture sugarcane bagasse, it has shown to increase the release of sugar monomers in the subsequent hydrolysis step compared to the untreated biomass. Results also showed that the chemical composition of the structure remained almost constant after pretreatment, which proves the potential of this pretreatment to use cellulose, hemicellulose, and lignin for the conversion of valuable compounds.

The optimization of the enzyme cocktail formulation was also covered with the supplementation of enzymes and additives to the commercial enzyme cocktails Cellic®Ctec 2

and Cellic®Ctec 3 HS. The addition of hydrogen peroxide, lytic polysaccharide monooxygenases, cello-oligosaccharide dehydrogenase, surfactants and hemicellulases were some of the approaches that showed a great potential for the enhancement of sugar monomers production during hydrolysis of the mildly pretreated sugarcane bagasse.

The sustainability of two systems for bioethanol production, one using the CO_2 pretreatment method and other diluted acid pretreatment, were evaluated and compared using life cycle assessment. Results showed that the CO_2 emissions of the production of bioethanol using one tonne of bagasse were substantially lower for the system that used CO_2 pretreatment method.

The results obtained through this thesis provide a profound knowledge of the potential that the utilization of a mild and sustainable fractionation has and paves the way for future research studies focused on reducing the environmental impact of biomass conversion processes.

Dansk resumé

Industriel praksis og teknologiske processer kræver en hurtig omstilling for at imødekomme kravene og samtidig forhindre de alvorlige konsekvenser ved at ramme de globale grænsetemperaturer. Lignocellulose biomasse produceres bredt på verdensplan og har potentialet til at erstatte fossile brændstoffer og materialer. Dens implementering i bioraffinaderiprocesser kan være et nøglebidrag til overgangen til en cyklisk og biobaseret økonomi, hvor affaldsprodukter betragtes som en ressource snarere end en byrde. En væsentlig begrænsning ved biomasseomdannelsesprocesser er den høje genstridighed i dens struktur, som er svær at depolymerisere og nedbryde til enklere enheder, som efterfølgende kan bruges som et produkt eller som et råmateriale til konvertering. Dette problem kunne løses ved at bruge en effektiv og bæredygtig fraktioneringsmetode, hvor de tre hovedkomponenter i biomassen (cellulose, hemicellulose og lignin) frigives til omdannelse, men dette er stadig ikke opnået.

Denne afhandling behandler emnet, hvor sukkerrørsbagasse og introduktionen af en ny og mild forbehandling, ved brug af kuldioxid, vil blive undersøgt. Når forbehandling med kuldioxid udføres på bagasse af sukkerrør med lav fugtighed, har det vist sig at øge frigivelsen af sukkermonomerer i det efterfølgende hydrolysetrin sammenlignet med den ubehandlede biomasse. Resultaterne viste også, at den kemiske sammensætning af strukturen forblev næsten uændret efter forbehandling, hvilket beviser potentialet, ved denne forbehandling, til at udvinde cellulose, hemicellulose og lignin til videre omdannelse til værdifulde forbindelser.

Optimeringen af enzymcocktailformuleringen blev også undersøgt ved tilsætning af enzymer og additiver til de kommercielle enzymcocktails Cellic®Ctec 2 og Cellic®Ctec 3 HS. Tilsætning af hydrogenperoxid, lytiske polysaccharid-monooxygenaser, cello-oligosaccharid-dehydrogenase, overfladeaktive stoffer og hemicellulaser var nogle af de tilgange, der viste et stort potentiale for at forbedre produktionen af sukkermonomerer under hydrolyse af den mildt forbehandlede sukkerrørsbagasse.

Bæredygtigheden af to systemer til bioethanolproduktion, det ene ved hjælp af CO_2-forbehandlingsmetoden og en anden forbehandling med fortyndet syre, blev evalueret og sammenlignet ved hjælp af livscyklusvurdering. Resultaterne viste, at CO_2-emissionerne ved produktion af bioethanol, ved brug af et ton bagasse, var væsentligt lavere for systemet, der brugte CO_2-forbehandlingsmetoden.

Resultaterne opnået gennem denne afhandling giver en dybtgående viden om det potentiale introduktionen af en mild og bæredygtig fraktionering har, og baner vejen for fremtidige forskningsstudier med fokus på at reducere miljøpåvirkningen af biomasseomdannelsesprocesser.

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Mil gracias a los míos. Papá, por ser como eres y por tu amor y apoyo incondicional. A Javi, mi amor, por estar siempre.

List of publications

Paper 1. Balaguer Moya, E.; Cunha, M.L.S.; Prado, C.A.; Turella, S.; da Silva, S.S.; Abou-Hachem, M.; Dragone, G.; dos Santos, J.C.; Mussatto, S.I. Evaluation of Enzymatic Hydrolysis of Sugarcane Bagasse Using Combination of Enzymes or Co-Substrate to Boost Lytic Polysaccharide Monooxygenases Action. Catalysts 2022, 12, 1158. https://doi.org/10.3390/catal12101158 **(Chapter 4)**

Paper 2. Eva Balaguer Moya, Berta Syhler, Julen Ordeñana Manso, Giuliano Dragone, Solange I. Mussatto. Enzymatic hydrolysis cocktail optimization for the intensification of sugar extraction from sugarcane bagasse. International Journal of Biological Macromolecules. Volume 242, Part 3, 2023, 125051, ISSN 0141-8130. https://doi.org/10.1016/j.ijbiomac.2023.125051. **(Chapter 5)**

Paper 3. Eva Balaguer Moya, Berta Syhler, Giuliano Dragone, Solange I. Mussatto. Tailoring a cellulolytic enzyme cocktail for efficient hydrolysis of mildly pretreated lignocellulosic biomass. Enzyme and Microbial Technology Journal. Manuscript submitted and peer-reviewed. **(Chapter 6)**

List of papers pendent of submission

Paper 4. Eva Balaguer Moya, Giuliano Dragone, Solange I. Mussatto. Advancing the Sustainable use of Biomass through CO_2 Pretreatment.

Paper 5. Eva Balaguer Moya, Giuliano Dragone, Solange I. Mussatto. Comparative life cycle assessment of ethanol production from sugarcane bagasse using diluted acid pretreatment and CO_2 pretreatment.

List of abbreviations

µL	Microlitre
µm	Micrometre
µM	Micromolar
µmol	Micromole
°C	Degree Celsius
1G	First generation
2G	Second generation
5-HMF	Hydroxymethyl furfural
AA7	Auxiliary Activity 7
AA9	Auxiliary Activity 9
AFEX	Ammonia fiber explosion
ANOVA	Analysis of variance
BBD	Box-Behnken design
BR	Brazil
CAZy	Carbohydrate Active enzyme
CBM	Carbohydrate binding module
CC2	Cellic® Ctec2
CC3	Cellic® Ctec3 HS

CD	Catalytic domain
CDH	Cellobiose dehydrogenase
CelDH	Cello-oligosaccharide dehydrogenase
$C_{glucose}$	Concentration of glucose
CH_4	Methane
CHP	Combined heat and power
CO_2 eq	CO_2 equivalent
CO_2	Carbon dioxide
COS	Cello-oligosaccharides
CT	Control temperature
Cu	Copper
C_{xylose}	Concentration of xylose
DA	Diluted acid
DM	Dry matter/biomass
FPU	Filter Paper Units
g	Gram
GHG	Greenhouse gas
glu	Glucose
GWP	Global warming potential
h	Hours

H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
HC	Hydrodynamic cavitation
HPLC	High Performance Liquid Chromatography
HSL	High-solids loadings
HT	High temperature
kg	Kilogram
kV	Kilovolt
kWh	Kilowatt-hour
L	Litre
LCA	Life Cycle Assessment
LCB	Lignocellulosic biomass
LCI	Life Cycle Inventory
LCIA	Life Cycle Impact Assessment
LMWLDC	Low-molecular-weight lignin-derived compounds
LPMOs	Lytic polysaccharide monnoxygenases
M	Molar
mg	Milligram
min	Minute
MJ	Megajoule

mL	Millilitre
mm	Millimetre
mM	Millimolar
mmol	Millimole
n.a.	Not applicable
NMVOC	Non-methane volatile organic compounds
O_2	Oxygen
P	Pressure
PEG	Polyethylene glycol
ppm	Parts per million
RED	Renewable Energy Directive
rpm	Revolutions per minute
s	Second
SCB	Sugarcane bagasse
Sc-CO_2	Supercritical CO_2
SEM	Scanning Electron Microscopy
sub	Subcritical
sup	Supercritical
t	Tonne
T	Temperature

U	Enzyme units
v/v	Volume per volume
$V_{hydrolysis}$	Hydrolysis working volume (L)
w/w	Weight per weight
wt	Weight
WWT	Wastewater treatment
xg	Relative centrifugal force
xyl	Xylose

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Chapter 1: Introduction

Despite the short-term benefits from the current energy crisis, renewable energy sources, along with nuclear power, are continuously getting stronger. The reliability on fossil fuels is declining gradually since the mid-2020s. However, there is still a big gap between our aspirations to fulfill the climate change commitments and the goal of stabilizing the temperature increase at 1.5ºC [1]. Aggravating this challenge is the projection that global energy consumption is expected to increase by 28% within a period of 25 years [2]. In this situation, it is imperative to transition more rapidly towards renewable and sustainable energy options.

Employing lignocellulosic biomass (LCB) as raw material for the generation of various products such as energy, fuels, materials, and chemicals holds significant importance to shift from an economy reliant on fossil fuels to a circular bioeconomy. The integration of biomass residues into a biorefinery together with the utilization of green chemistry will enable the upgrade of biomass valorization approaches [3]. Among the biomasses that are available worldwide, sugarcane bagasse is one of the most abundant ones, with a worldwide annual generation of approximately 540 million metric tons. Brazil stands out as the biggest producer with an annual yield of around 181 million metric tons [4]. It presents a big potential for the production of bioethanol and other chemicals such as xylitol [5].

A key bottleneck in the process of conversion of LCB is its structural recalcitrance, which can be mitigated through the effective application of a suitable pretreatment method [6]. Different methods have been developed, which can be classified into four groups: physical, chemical, physicochemical, and biological. However, they present some constraints as the insufficient separation of cellulose and lignin, the production of inhibitory compounds,

high energy and/or water demands, the utilization of strong chemicals, and a significant amount of waste generation [7].

Another key step in the valorization process chain of lignocellulosic biomass is the enzymatic saccharification of pretreated biomass. The enzyme cocktail of cellulases and hemicellulases works synergistically to convert the cellulose and hemicellulose fibers into sugar monomers. Also, lytic polysaccharide monooxygenases (LPMOs) and laccases have been used recently as they improve the efficiency of the process by increasing glucose yield and lowering the amount of cellulases. However, the high cost of enzymes, their high sensitivity, low stability, and the high dose needed limit significantly the economic feasibility of the process [6].

It is usually considered that production processes with biomass as a raw material will result in lower greenhouse gas emissions (GHG) than petroleum-based ones. However, there exist several potential impacts associated with biomass conversion processes [7]. In this context, it becomes essential to incorporate Life Cycle Assessment (LCA) modeling to evaluate the environmental hot spots of the system and to determine if the bioproduct presents a lower impact compared to fossil fuel-based one [8].

This thesis brings together two approaches for the development of a more sustainable and efficient method for biomass fractionation. The first involves the utilization of carbon dioxide (CO_2) for pretreatment. CO_2 is a green chemical that has shown to be able to increase the enzymatic hydrolysis of biomass [9]–[11]. The CO_2-based pretreatment is performed without the addition of extra water to the biomass and using mild conditions. The second approach involves the optimization of a commercial enzyme cocktail adapted to the pretreated biomass composition.

The work has been organized into different chapters which contain separate tasks conducted to increase the understanding of the effectiveness of the novel pretreatment on sugarcane biomass, of how the different supplements interact with the enzyme cocktail, and the evaluation of the process of pretreatment for integration into bioethanol production.

Chapter 2 contains background information about the potential utilization of LCB, specifically addressing sugarcane bagasse, the different pretreatment methods available, and the enzymatic hydrolysis with a focus on the enzyme cocktail formulation.

In **Chapter 3** the novel process for CO_2 pretreatment is introduced. The evaluation of different process conditions is carried out, providing a comparison between different CO_2 states. The enzymatic hydrolysis of the pretreated biomass is performed to evaluate the sugar production of pretreated samples compared to the raw biomass. This work was written as a research article; however, it has not been submitted yet as an application for a patent is on course.

Chapter 4 contains the first published paper (Paper 1). In this one, the focus was to boost LPMOs contained in a commercial enzyme cocktail with an extra addition of LPMO (GcLPMO9B), H_2O_2, or cello-oligosaccharide dehydrogenase (CelDH) FgCelDH7C. The study showed promising strategies to boost LPMOs and to enhance the efficiency of hydrolysis and demonstrated the new potent combination of using LPMO together with CelDH.

In **Chapter 5** the expansion to more supplementation alternatives takes place. In this second published paper (Paper 2), apart from the strategy of using H_2O_2, the investigation of laccases as another enzyme relevant to boost LPMOs is included. Also, the addition of hemicellulases and the surfactants PEG4000 and Tween® 80 was studied. While adding

laccases did not show to be beneficial for hydrolysis, H_2O_2, hemicellulases, and the surfactants showed to increase the sugar monomer production from sugarcane biomass.

Chapter 6 contains the last study regarding enzyme cocktail formulation. The best alternatives studied for supplementation were combined to evaluate their synergistic behavior. These were H_2O_2, hemicellulases, and Tween® 80. The optimization of the enzyme and additives dose was performed through statistical tools. The results showed an increased sugar production compared to using a single additive or enzyme supplementation; however, the question whether the high dose required would compensate for the increased sugar production was raised. This study was structured as a research article and has been peer-reviewed already for publication in a scientific journal (Paper 3).

Chapter 7, a comparative LCA of bioethanol production using CO_2 pretreatment and the conventional pretreatment method using diluted acid is presented. This showed a great potential of CO_2 pretreatment, as the emissions of CO_2 per tonne of bagasse when using the novel pretreatment process were significantly lower than when using dilute acid pretreatment. A very interesting discussion of the utilization of sugarcane bagasse for different scenarios was done. This work was done in collaboration with Novozymes and Raízen. The included study also follows a scientific article structure, and it is still not submitted as it should be carefully evaluated together with the two companies for not disclosing any confidential information.

Finally, **Chapter 8** includes a final discussion, conclusion and future perspectives on the work contained in this thesis.

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Chapter 2: Background

2.1. Lignocellulosic biomass as a renewable source

In the effort to shift towards a bio-based economy, different renewable energy sources, such as solar, wind, hydrothermal, and biomass, have been investigated so far. Among these, biomass emerges as a particularly promising option as it has the potential to replace petroleum-based fuels for transportation and chemical production.

With approximately 75% of its composition consisting of carbohydrates from cellulose and hemicellulose, lignocellulosic biomass (LCB) possesses significant chemical energy, rendering it highly suitable for various bioenergy uses [1], [2]. Furthermore, its relevance extends to numerous additional industries, including those related to food, feed, pharmaceuticals, and materials, as they can obtain benefits from obtaining valuable substances like protein, amino acids, oligosaccharides, phenolic compounds, or sugars for fermentation purposes [3]. Importantly, it is characterized for being in abundance, biodegradable, and biocompatible [4]. Thus, the transformation of waste LCB into energy results in a logical transformation, turning an environmental burden into an economic benefit [5].

2.1.1. Structure of lignocellulosic biomass

LCB presents and inherent recalcitrance to degradation by microbial and enzymatic processes, posing a significant challenge for its effective valorization in the production of bioenergy and biomaterials on an industrial scale. It is key to understand and overcome the chemical and structural attributes that contribute to the recalcitrance of lignocellulose within plant cell walls to optimize deconstruction processes [6].

The composition of LCB consists mainly of biopolymers of cellulose, hemicellulose, and lignin, making up around 90% of its dry mass [7]. The abundance of each of them is specific to each plant species, and it is very important to assess the optimal energy conversion route for each kind of LCB [8].

Cellulose is the most abundant component of lignocellulose, typically present in the range of 35-50 wt% [7]. It is a highly stable polysaccharide that consists of molecules comprising over 10,000 glucose subunits linked by (β–1,4)-glycosidic bonds, contributing to the linear structure of the molecule, and with hydroxyl groups situated at the equatorial position [9]. The disaccharide cellobiose is the basic repeating unit [10]. Because of the high molecular weight and the low flexibility of cellulose polymer chains, it is insoluble in water, while the glucose monomer and short oligomers are soluble [7].

Cellulose chains are arranged jointly to form microfibrils, which at the same time are packed together to form cellulose fibers. The structure of cellulose presents covalent bonds, hydrogen bonds, and Van der Waals forces. Hydrogen bonding within a cellulose microfibril plays a pivotal role in establishing the chain's "straightness", hydrogen bonds between different chains can either introduce a sense of organization (crystallinity) or lack of it (amorphousness) into the cellulose structure [11]. The amorphous part of cellulose is 3-30 times more easily digestible by enzymes than the crystalline part [12], [13]. Cellulose fibers are surrounded by a cross-linked matrix consisting of hemicellulose and lignin [10].

The second most predominant polymer is hemicellulose, which is usually 20-35% wt of LCB [13]. In contrast to cellulose, hemicellulose is made of short branched chain sugars and it is chemically heterogeneous [11], [14]. It is composed of biopolymers with 500-3000 sugar monosaccharides [15] of pentoses (such as xylose and arabinose), hexoses (such as mannose, glucose, and galactose), and acetylated sugars that are linked to each other by

glycosidic and ether linkages [16]. The most abundant monomer found in hemicelluloses is usually xylose, but in softwoods, mannose can be the richest [15]. The main groups of hemicelluloses are xyloglucans, arabinans, mannans, and xylans, which are based on the sugar monosaccharides that are linked by (β–1,4)-glycosidic bonds. It interacts with cellulose and lignin molecules providing strength to the cell wall [17]. As hemicellulose is amorphous, it has low physical strength, being able to be hydrolyzed by diluted acids or bases and by hemicellulase enzymes [13].

Lignin is a complex aromatic polymer that is highly branched, amorphous, heterogeneous, and lacks primary structure [15]. It contributes to 10-25% wt. of LCB structure [14]. It forms a network of repetitive phenyl propane units (*p*-coumaryl alcohol, sinapyl alcohol, and coniferyl alcohol) that are linked together by different kinds of linkages [18]. The chemical structure of phenylpropane units only differ in the degree of substitution of the functional groups in the aromatic rings and the abundance of each different unit depends on the type of biomass [19].

Lignin provides the plant with structural stability, impermeability, and resistance against microorganisms and oxidative stress. All these mentioned traits, combined with their hydrophobicity and lack of optical activity, make the degradation of lignin very difficult [18].

The recalcitrance of lignocellulose biomass is enhanced by the interactions between the three polymers. While cellulose and hemicellulose are closely linked together by hydrogen bonds, lignin is covalently associated with hemicelluloses to form the lignin-carbohydrate complex [13]. Also, lignin acts as a link between cellulose fibrils and hemicellulose structure and holds the polymers together [14]. Lignin has been identified as the major impediment to the degradation of cellulose, as it is closely attached to it. This does not only happen because of the action of lignin as a physical barrier but also because of the specific adsorption of

hydrolytic enzymes to lignin´s structure and the toxicity of byproducts resulting from lignin derivatives [11].

Other components in biomass include extractives and ash. Extractives consist of compounds that are soluble in water (such as proteins and non-structural sugars), and components that are soluble in ethanol (such as chlorophyll and waxes). Ash is an inorganic material that can be found in the biomass structure such as calcium and potassium ions, or have an anthropogenic precedence, such as silica obtained during harvest [20].

2.1.2. Sugarcane bagasse as raw material for biofuels and chemicals

Sugarcane is one of the most extensively produced biomass in the world, reaching 1.6 billion tons per year. Bagasse makes up approximately 35% of the total weight of sugarcane, with only a minimal portion of about 50% being used to produce power and heat for the thermochemical treatment of biomass [17]. The combustion of sugarcane bagasse to obtain energy still emits significant amounts of gaseous pollutants [21].

Brazil stands as the globe's leading sugarcane producer, contributing to 40% of the world's total output. Approximately 46% of the sugarcane crop is dedicated to sugar refining, while the remaining portion is allocated to produce first-generation bioethanol (1G) [22]. However, the generation of 1G bioethanol leads to the food versus fuel competition together with an increase in land utilization, and thus, it cannot be established as a sustainable and long-term fuel alternative [23], [24].

In line with what is mentioned above, sugarcane bagasse stands as a very attractive raw material to produce second-generation (2G) bioethanol. This option enables to decrease the competition of fuel versus food and reduces the environmental impact [25]. Furthermore, from bioethanol, commodity chemicals and biopolymers can also be produced through

different chemical routes [22]. Sugarcane bagasse has also been used as a raw material for microbial conversion to different products such as xylitol [26], [27], citric acid [28], or succinic acid [29].

2.2. Lignocellulosic biomass fractionation

It is crucial to perform an efficient fractionation of LCB into its main components (cellulose, hemicellulose, and lignin) to ensure the optimal use of lignocellulosic materials and the creation of cost-effective, strong, and reliable processes for the development of a biorefinery [30]. This task poses a great challenge because of the lignocellulosic matrix recalcitrance [31].

To obtain monosaccharides that can be used for bioconversion, cellulose and hemicelluloses need to be hydrolyzed using acids or enzymes, to fermentable sugars. However, as cellulose is closely attached to hemicellulose and lignin, the accessibility to the structure is very challenging. Also, the crystalline structure of cellulose represents an obstacle to hydrolysis [32]. Other physical factors that impede the hydrolysis of biomass are the particle size (by reducing it, the increase of the specific surface area and accessibility to enzymes is achieved), the porosity (higher porosity enables higher penetrability of enzymes to the biomass structure), and cell wall thickness [33].

Conventional fractionation methods are often focused on the removal of hemicellulose and lignin barrier to obtain cellulose that can be hydrolyzed to glucose and fermented to ethanol [32]. However, biomass has a higher potential than just cellulose conversion. Hemicellulose polysaccharides can be used to obtain multiple biochemicals (such as xylitol, edible coatings, hydrogels, and binders for drug delivery and functional composites for heavy metals) and biofuels [34]. On the other hand, lignin also offers a wide range of possibilities to

develop eco-friendly and biodegradable value-added products such as polymers and resins, adhesives and binders, and carbon materials for water or air purification, as well as for biofuel production [35]. The utilization of every constituent of LCB to generate several economically valuable goods presents a promising option for achieving a 'zero-waste LCB biorefinery.' This approach aims to maximize the value obtained from biomass and to foster the development of innovative bio-based industries [36].

An economically viable industrial fractionation process for lignocellulosic materials has yet to materialize. These processes present limitations such as the generation of inhibitor compounds, high use of energy or chemicals, generation of waste, or expensive equipment [32]. For instance, in the context of producing cellulosic ethanol, a substantial portion of the expenses (close to 40%) is linked to biomass fractionation, which encompasses pretreatment, enzyme generation, and enzymatic hydrolysis, with pretreatment alone accounting for nearly half of this overall proportion (approximately 18%) [30]. Because of this, it is key to develop effective and low-cost pretreatment methods to overcome the cost-related challenges associated with biomass conversion [30].

In this thesis, the emphasis was placed on adopting a fractionation approach that enables the recovery of the three components of the biomass. With special focus on the obtention of sugar monomers from cellulose and hemicellulose to be utilized for fermentation purposes. For this purpose, pretreatment and hydrolysis steps will have key impacts. Pretreatment will be used to open the structure of biomass and ease the access of enzymes, with strong focus on avoiding the degradation of any of the components of biomass. Hydrolysis enzyme cocktail will be optimized to separate lignin from the biomass and convert cellulose and hemicellulose into sugar monomers that can be used for fermentation.

2.2.1. Pretreatment

Pretreatment is key to reduce the recalcitrance of biomass and to ensure accessibility of enzymes to LCB structure. It is challenging to define the best pretreatment alternative for all raw materials, as this decision strongly depends on the specific composition and structure of the raw biomass [37]. Nevertheless, there are some features that should be fulfilled in every pretreatment method [30], [38]:

1) Maintain the native structure of lignin to enable its subsequent valorization.
2) Low energy, water, and chemicals consumption and low-cost operation.
3) Reduce cellulose crystallinity and modify the structural matrix of biomass.
4) Reduce particle size of biomass and porosity to increase the surface area available for the attack of enzymes.
5) Avoid excessive degradation of the chemical components of LCB and the formation of inhibitor compounds (particularly acetic acid from hemicellulose, furfural and 5-hydroxymethyl furfural, from sugar decomposition, and phenolic compounds from lignin degradation).
6) Use green chemicals that are easily removed from biomass and recycled and are non-toxic.
7) Require minimal and simple post pretreatment operations.

In general, the pretreatment should ensure high sugar hydrolysis yields from cellulose and hemicellulose. Yields are usually 20% without pretreatment, while with it the yields can increase to over 90% [39].

An extended number of pretreatment technologies have been suggested, and they can be categorized into physical, chemical, physicochemical, and biological processes [30].

2.2.1.1. Physical pretreatments

These methods are often used as a first pretreatment step to reduce the particle size of biomass, resulting in the increase of surface area, decrease of polymerization and crystallinity [40]. They involve the use of tools such as grinders and screws, or techniques such as ultraviolet or microwave radiations [38]. These methods are eco-friendly and rarely release any toxic substances. However, these processes present the big disadvantage of consuming a high amount of energy, which usually depends on the type of LCB used [40]. Another disadvantage is that they do not remove the lignin fraction from biomass [41].

Milling is a commonly used pretreatment method which aim is to reduce the size of biomass particles. This can be achieved using different techniques such as grinding, milling, shredding, and chipping. Each technique leads to a different biomass particle size, for example, grinding or milling results in a particle size of 0.2-2 mm while chipping produces particles of 10-30 mm [38].

Extrusion is another widely employed physical pretreatment which consists of breaking down LCBs under intense shearing forces by bringing them into contact with one or two rotating screws enclosed within a barrel, called extruder [42]. It requires short residence times, and it can be run at different temperatures from mild temperatures up to 300 ºC [40], [42].

Subjecting lignocellulosic biomass to irradiation, such as γ-rays, electron beams, and microwaves, induces cellulose degradation, transforming it into oligosaccharides and cellobiose [43]. Microwave irradiation consists on the application of an electromagnetic field in LCB. Within this approach, dielectric polarization leads to molecular collisions and the consequent generation of thermal energy. This thermal energy then brings about the disruption of the intricate lignocellulosic structure [40].

Utilizing ultrasonic waves for sonochemical treatment of plant biomass presents an alternative, efficient, cost-effective, and environmentally friendly approach. Numerous recent investigations have explored the impact of ultrasonic waves on lignocellulosic biomass, aiming to enhance lignin dissolution and removal, and the improved reaction of chemical compounds with the biomass components [43]. Acoustic waves are transmitted through the medium, giving rise to acoustic cavitations, such as microscopic bubbles, that expand to an optimal size and subsequently collapse violently [43]. Another way to generate cavitation is by using the geometry of the system, such as orifice plates and venturi; this technology is called hydrodynamic cavitation and it is relatively new [44]. Small perforations caused by the high-speed micro-jets contribute to an increase in the surface area, making the carbohydrate fraction more susceptible to enzyme attack [45].

2.2.1.2. Chemical pretreatments

Chemical pretreatments are fast methods that enable a high level of separation of components of LCB [43]. Acid pretreatment can be performed using concentrated acid (such as 30-70%) and low temperature (<100 ºC) or diluted acid (0.1-10%) and high temperature (100-250 ºC) [40]. Both inorganic and organic acids, such as sulfuric acid (H_2SO_4), hydrochloric acid (HCl), nitric acid (HNO_3), and acetic acid, have been utilized, with H_2SO_4 being the most common. Despite the efficiency of concentrated acid on cellulose hydrolysis, its toxicity, corrosiveness, and costly reactor construction limit its use. Dilute acid is preferred over concentrated acid, and it is one of the most applied chemical pretreatment methods for lignocellulosic biomass due to safety, recovery challenges, and cost considerations [46].

The acid pretreatment method is highly effective at disrupting the hemicellulose fraction of LCB, by the cleavage of glycosidic bonds, which results in the conversion of

polysaccharides into oligomeric and monomeric sugars [47]. As a result, a high recovery of the hemicellulose sugars is achieved in the pretreated liquid while a cellulose fraction with enhanced enzymatic degradability is recovered in the solid fraction [48]. However, the main limitations of this pretreatment are the low concentration of reducing sugars in the pretreatment liquid due to the high liquid-to-solid ratio and the formation of inhibitory compounds (such as furfural and 5-HMF), that require additional steps for the concentration of sugars and removal of inhibitors [49].

Utilizing alkalis like NaOH, KOH, $Ca(OH)_2$, hydrazine, and anhydrous ammonia in alkaline pretreatment induces biomass swelling, increasing its internal surface area and reducing the degree of polymerization and cellulose crystallinity. Alkaline pretreatment causes lignin structure disruption and breaks the linkages between lignin and other carbohydrate components in lignocellulosic biomass, thereby enhancing the accessibility of enzymes to the carbohydrates present in LCB [50]. The main disadvantages of alkaline pretreatment are the high cost associated to the post pretreatment neutralization of the slurry, the long residence time, and their inefficiency for application in biomasses containing high amount of lignin- [51].

2.2.1.3. Physicochemical pretreatments

Physicochemical techniques act in a hybrid mode, affecting the physical structure of biomass as well as its chemical structure and intermolecular interactions. In these, chemicals are used together with pressure and/or temperature. Some of the methods included under this category are steam and CO_2 explosion, ammonia fiber explosion, and liquid hot water [52].

Steam explosion is one of the most extensively explored methods [37] that is very promising because of its limited energy consumption and low environmental impact [53]. It involves the utilization of high-pressure saturated steam, typically within the range of 7–48

bar, coupled with temperatures ranging from 160 to 260 °C, for short durations ranging from seconds to minutes. The steam penetrates the subjected biomass, leading to a dilation of the cell walls within the fibers before a subsequent explosion and partial hydrolysis occur. Subsequently, the pressure is released fast until it reaches atmospheric conditions. During the pretreatment, acetyl residues originating from xylan hemicellulose are released as acetic acid and catalyze the chemical reaction [54]. A significant drawback of this approach is its inability to break down xylan and lignin, which results in the generation of inhibitory compounds. Thus, it requires an additional washing step to eliminate them introducing extra cost and effort [25].

Ammonia fiber explosion often uses lower temperatures than steam explosion (60 to 170 °C) for 5-60 min at pressures ranging from 15 to 30 bar and applies to the LCB anhydrous ammonia (1:1 w/w). It improves the surface area of LCB and the accessibility of enzymes for hydrolysis while producing negligible amounts of inhibitors and highly retains cellulose and hemicellulose. However, the expensive equipment needed to use the high pressure required for the process, the high cost of ammonia and increased energy requirement to recycle ammonia are some challenges associated to the commercialization of the technology [38].

Alternative, sustainable and promising technologies for the pretreatment of LCB are supercritical fluids (SCF). SCF show an intermediate behavior between liquid and gases, which enable them to present a gas-like diffusivity and liquid-like viscosity easing their penetration through solid materials. Their use for different industrial applications has gained interest because of the low operational costs and high energy efficiency [38]. Supercritical CO_2 (Sc-CO_2) explosion consists of applying pressure to LCB with CO_2 above its critical temperature and pressure (31 ºC and 73.8 bar) during some tens of minutes or hours, and then releasing the pressure rapidly to obtain an “explosion” effect. In contrast, to dilute acid

pretreatment, this approach has nearly zero water requirements and produces a sugar mixture that is low in inhibitory compounds. The main drawback of this technology is the high cost associated with the compression of CO_2 and thus, the success in the commercialization of the Sc-CO_2 process will rely on the sugar yield optimization while minimizing pretreatment time, pressure, and temperature [55].

2.2.1.4. Biological pretreatments

Biological processes usually use wood-degrading fungi (soft, brown, and white rot) to modify the chemical composition of the LCB. Typically, soft and brown rot fungi predominantly break down hemicellulose while making slight modifications to lignin. In contrast, white-rot fungi exhibit a greater ability to target the lignin component [41].

Biological pretreatment provides a milder and eco-friendly alternative to remove lignin and hemicellulose from biomass, in contrast to chemical, physical, and physicochemical pretreatment methods. Furthermore, this technique offers economic advantages compared to the previous mentioned alternatives. However, it has a significant drawback of exhibiting low degradation rates and efficiency [43]. It also requires careful control of the growth conditions of the microorganisms and large amounts of space and time. For these reasons, these types of pretreatments are less attractive on a commercial scale [41].

2.2.1.5. Utilization of Carbon dioxide as a resource for pretreatment

Various anthropogenic activities generate CO_2, which is recognized as the primary contributor to global warming because of its greenhouse properties. The actual annual emission volume of CO_2 is approximately 35 billion tonnes, which are primarily originated

from the burning of fossil fuels, the conversion of biomass into energy, and the breakdown of carbonates (mainly within the steel and cement sectors) [56]. The concentration of CO_2 in the atmosphere is currently 421 ppm (measured in May 2022), increasing more than 50% since the beginning of the industrial revolution (when it was 280 ppm) [57]. This increase in emissions raises serious concerns, and within this context, technologies that use CO_2 are worthy of investigation [55].

CO_2 is considered a green chemical because is it nonflammable, nontoxic, widely available, renewable, easy to recover and recycle, and low cost [58], [59]. Because of its attributes, it can access the LCB structure and lead to a reduction in its crystallinity by disrupting the linkages between cellulose and hemicellulose [55]. A more detailed description of its properties and mode of action during pretreatment is provided in Chapter 3, together with the evaluation and comparison of different process conditions.

CO_2 is released from various industrial production processes such as brewing, ethanol fermentation and cement production; thus, it can be obtained in large amounts at a relative affordable prize [3]. During glucose fermentation to produce bioethanol, each mole of ethanol comes alongside with 1 mol of CO_2 as a by-product, which could be used for biomass pretreatment [55]. Ethanol production from fermentation generates a high purity (99%) stream of CO_2, which contains only CO_2, H_2O and little quantities of sulfur. Thus, CO_2 can be captured at a relatively low cost, only demanding dehydration and compression afterward [60]. The analysis of Dees et al. [60] is an example of the technical and economic potential demonstration of this alternative and was used in Chapter 8 to integrate the CO_2 capture from fermentation with CO_2 pretreatment in one of the scenarios studied in a Life Cycle Assessment (LCA) for bioethanol production.

2.2.2. Enzymatic hydrolysis

Enzymatic hydrolysis has become the most suitable way to convert LCB polysaccharides into fermentable sugars because of the low energy requirement as it is operated in a temperature range from 40 to 50 ºC [61]. Usually, the solid content present in the enzymatic hydrolysis media is an important factor that affects the economic feasibility of the process. In the traditional conversion process, there is a low loading of solids, leading to reduced product concentrations, inefficiency in equipment utilization, substantial wastewater discharge, and elevated capital expenses [62]. Thus, the industrial production of sugar liquors from LCB requires the performance of the enzymatic hydrolysis step using high-solids loadings (HSL), which involves using reaction mixtures containing more than 15% solids (w/w). With this, more concentrated sugar liquors are obtained, improving both capital and operational costs. However, this approach presents technical difficulties that affect the process efficiency, known as the "high-solids effect", which means that the glucose conversion yield lowers as the solid load increases [63].

Because of the pretreatment applied in this work, the composition of LCB before hydrolysis contains almost entirely the full composition of cellulose, lignocellulose, and lignin. For this reason, the approach used to increase the efficiency of hydrolysis is the optimization of the enzyme cocktail through the supplementation with extra enzymes and additives. The resulting sugar liquids, potentially containing the produced sugar monomers from cellulose and hemicellulose, will contain higher sugar concentration that those pretreatments that degrade and remove the hemicellulose fraction before hydrolysis. Although not having reached the 15% solids (w/w) loading requirement for industrial feasibility, a relatively high solid loading of 10% (w/w) was used to perform the experiments at lab scale, which has the potential to be increased if a reactor containing an efficient mixing system is used.

2.2.2.1. Enzymes for the degradation of lignocellulosic biomass

The hydrolysis of LCB requires multiple enzymes with different specificities to decompose the complex lignocellulosic structure. These are cellulases, hemicellulases, ligninolytic enzymes and, most recently lytic polysaccharide mono-oxygenases (LPMOs) that act in synergy [64].

Cellulases are a class of widely used enzymes that include endoglucosidases, cellobiohydrolases or exoglucanases and β-glucosidases or cellobiases and work in collaboration to degrade cellulose into shorter chain polysaccharides such as cellodextrin, cellobiose or glucose [65]. They usually have a catalytic domain (CD), that cleaves the glycosidic linkage, a carbohydrate-binding module (CBM), that directs the CD to the polysaccharide substrate, and often, extra types of ancillary modules [64]. Cellulases can be inhibited by the low accessibility to cellulose surface, mainly due to hemicellulose and lignin, or due to the low availability of binding sites, because of the hydroxyl groups of lignin, that can form hydrogen bonds with the aminoacid residue of cellulases, avoiding their action on cellulose. Another type of inhibition are due to external factors such as temperature, pH or mixing [66] or because the accumulation of products or toxic compounds [67].

Hemicellulases can be obtained from a broad range of microorganisms found in nature, but the most commercial ones are produced from fungi and thermophilic bacteria. Their primary role involves the hydrolysis of hemicellulose polysaccharides. Some examples of hemicellulases include xylanases, endoxylanases, xylosidases, and arabinofuranosidases [66]. They are mostly modular proteins that as cellulases, contain CDs and CMBs, and other functional groups that ease the cleavage of either glycosidic or esterified acid side groups [64].

Lignin-degrading enzymes have usually been divided into two main groups: lignin-degrading auxiliary enzymes and lignin-modifying enzymes. The first are not able to degrade lignin on their own and need additional enzymes to be involved to achieve complete degradation. Through the sequential action of different proteins, they facilitate the process of lignin degradation. This group includes cellobiose dehydrogenase, aryl alcohol oxidase, glucose oxidase, etc. Lignin-modifying enzymes are also called ligninolytic enzymes and are produced by various microorganisms. They are grouped as laccase and heme-containing peroxidase [68].

Other important enzymes in the degradation of LCB are LPMOs, which were first discovered in 2010 by Vaaje-Kolstad et al. [69]. These enzymes are classified as the enzyme families AA9, AA10, AA11, and AA13 of the carbohydrate-active enzyme (CAZy) database. Numerous fungal species are now recognized as producers of AA9 LPMOs (formerly known as GH 61 enzymes). They are characterized as type II copper enzymes with a mononuclear center. They carry out oxidation of glucose units and the cleavage of cellulose chains, resulting in the formation of aldonic acids at the C1 position and/or 4-ketoaldoses (gemdiols) at the C4 position [67], [70]. The activity of LPMOs often needs electron donors, such as small-molecule reductants like ascorbate, compounds containing sulfur, pyrogallol, or gallic acid. Alternatively, more intricate systems like cellobiose dehydrogenases (CDH), glucose-methanol-choline oxidoreductases, phenols derived from plants and fungi, or photosynthetic pigments like chlorophylls, can serve as electron donors [67].

There are different commercial cocktails for the enzymatic hydrolysis of lignocellulosic biomass. They are formulated to target different LCB containing diverse cellulose, hemicellulose, and lignin composition. Although their exact composition is often not disclosed, they use a mix of the different most important lignocellulosic biomass-degrading enzymes that act in cooperation. Examples of commercial enzyme cocktails are

Cellic® Ctec 2 (CC2) and the newer version Cellic® Ctec3 HS (CC3) [71], these cocktails contain a blend of cellulases that are boosted using advanced AA9 molecules and improved β-glucosidases. It also contains some hemicellulase activities. Both enzyme cocktails have been used in this thesis, CC2 was used during Chapter 4 and CC3 in Chapters 5 and 6.

2.2.2.2. Enzyme cocktail optimization for CO_2 pretreated biomass

Although commercial cellulolytic enzyme cocktails offer process and substrate versatility and high conversion yields. They usually work well in substrates that have undergone conventional pretreatments [72]–[74]. In these, usually the hemicellulose and/or lignin fraction is degraded and solubilized in a liquid fraction prior to enzyme hydrolysis. As outlined in Chapter 3, the chemical composition of biomass remained nearly unchanged after pretreatment, in comparison to its state prior to undergoing pretreatment. Throughout Chapters 5 and 6, the optimization of the formulation of the enzyme cocktail for the specific biomass composition after CO_2 pretreatment is sough. Chapter 4 also served as a preliminary study of the effect of boosting LPMOs in sugarcane bagasse that was also pretreated using the mild pretreatment method hydrodynamic cavitation (HC).

The approaches used for the optimization of the enzyme cocktail are included in **Table 1**, together with a short description of their potential effect on mildly pretreated biomass. A more detailed discussion can be found in Chapters 4-6.

Table 1. Enzymes and additives used in the attempt to optimize enzyme cocktail formulation for mildly pretreated sugarcane biomass.

Enzyme/additive name	Effect	References
LPMO from Geotrichum candidum (GcLPMO9B)	Disrupt the crystalline structure of cellulose and increase the access of cellulases to LCB structure. By increasing the LPMOs load the reduction of the cellulase enzyme load could be achieved, improving the process economic viability of the process	[75]
H_2O_2	Reduce the catalytic resting state of LPMOs to the active state. Increases the rate of LPMOs action compared to using O_2 or another reductant.	[76]–[80]
Cello-oligosaccharide dehydrogenase (CelDH) from *Fusarium graminearum* (*Fg*CelDH7C)	They might present a straightforward and effective AA7-LPMO combination suitable for incorporation into cellulase blends for commercial purposes as they have shown to fuel activity towards crystalline cellulose.	[81]
Laccase Novozym® 51003	They catalyze the oxidation of phenolic compounds, creating micropores in the biomass where cellulases or hemicellulases can access. They also generate low-molecular-weight lignin derived compounds (LMWLDC) that can deliver electrons to LPMOs and activate them.	[82]–[84]

Hemicellulase NS22244	Improve cellulose accessibility through the removal of xylan coating.	[85] [86]
PEG4000	Lower the non-productive adsorption of cellulases to lignin and form a network at the liquid-air interface that limits the space available for enzymes preventing their inactivation	[86]
Tween® 80		[87]

2.3. Sustainability evaluation using Life Cycle Assessment

Life Cycle Assessment (LCA) is an environmental management tool used to evaluate the life cycle of a product based on the framework given by the guidelines ISO 14040 and 14044 [88]. It holds significant importance in assessing the impact of any production process and involves the evaluation of the environmental impacts of a process on the ecosystem through its entire lifecycle, from the raw material to each process step, energy utilization and generation, as well as the waste produced at the different stages of the process. LCA involves four main steps: goal and scope definition, inventory analysis, impact assessment and interpretation of results [89].

The goal and scope describe the most relevant choices made, and include the reason for performing the LCA, a definition of the product and its life cycle, and the definition of the system boundaries. The system boundaries depict what the assessments includes and what is not included, for example the stages of the process or compounds used that contribute little to the overall impact of the process. In the inventory analysis, the mass and energy inputs and outputs as well as the emissions are collected to create a life cycle inventory (LCI). Then, in the life cycle impact assessment (LCIA), the environmental impacts of every process within

the boundaries are collected and modeled in the LCI and translated into environmental topics such as global warming [90]. Finally, during the interpretation step, the results obtained, data, and procedures used in LCI and LCIA are evaluated. During interpretation, the first three stages can be modified in an iterative way until the analysis is considered complete, sensitive, and consistent [91].

To evaluate the suitability of the fractionation approach suggested in this thesis in terms of GHG emissions, a LCA was conducted. The efficient utilization of LCB involves its integration into a biorefinery setup where different products can be obtained [3]. Sugarcane bagasse is a cost-effective and desirable substrate to produce bioethanol [25]. For these reasons, the study involved the implementation of the novel fractionation approach in a bioethanol production plant to evaluate the environmental impacts of the process and to compare it with a conventional pretreatment technique as diluted acid pretreatment.

2.4. References

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Chapter 3: Advancing the Sustainable use of Biomass through CO_2 Pretreatment

This chapter involves the study of the effect of CO_2 pretreatment in sugarcane bagasse biomass. The manuscript is intended to be published, however, the application for a patent is still on course and the information contained in it is confidential. This chapter is authored by Eva Balaguer Moya, Giuliano Dragone and Solange I. Mussatto.

Abstract

Biomass pretreatment is essential to unlock the full potential of lignocellulosic biomass. However, a key challenge lies in achieving an efficient breakdown of complex biomass structures while minimizing the formation of inhibitory compounds, loss of polysaccharides, and process costs. An innovative pretreatment solution that could fulfill the specified requirements is suggested in this work, which consists of using CO_2 in low-moisture biomass. Experiments were carried out to evaluate the effect of different moisture contents, pressures, times, and temperatures for CO_2 pretreatment of sugarcane bagasse. The best results were obtained when the process was carried out under subcritical conditions at a temperature of 40^{o}C and a pressure of 50 bar. Under these conditions, the chemical composition of pretreated biomass was almost unaltered. However, differences in terms of pore size and restructuration of the biomass were clear when comparing raw and pretreated sugarcane bagasse, suggesting a physical effect of pretreatment rather than chemical. In addition, after enzymatic hydrolysis, 260 mg g^{-1} of glucose and 115 mg g^{-1} of xylose were obtained, representing an increase of about 27 and 36% in the contents of glucose and xylose, respectively, compared to the raw biomass. Finally, this study demonstrated that CO_2 pretreatment under subcritical conditions is able to improve the enzymatic hydrolysis of biomass, with little degradation of cellulose, hemicellulose, and lignin, showcasing an environmentally friendly approach to produce glucose and xylose from sugarcane bagasse.

Keywords: Lignocellulosic biomass; CO_2 pretreatment; Biorefinery

3.1.Introduction

Harnessing agricultural residues as feedstock in a biorefinery presents a promising solution to replace fossil resources in the production of energy carriers and chemicals. This approach not only contributes to mitigating climate change but also strengthens energy security by reducing reliance on non-renewable sources [1]. Lignocellulosic biomass is a widely available, sustainable, and cost-effective resource [2]. An example of a globally abundant residue is sugarcane bagasse, which causes an environmental concern if not managed appropriately [3]. Its structure consists of 40-45% cellulose, 30-35% hemicellulose, and 20-30% lignin [4] forming a complex matrix in which cellulose is intricately linked with hemicellulose and lignin [5]. Cellulose and hemicellulose polymers can be hydrolyzed to sugar monomers which subsequently can be used for fermentation [1] while lignin presents an immense potential to produce value-added compounds and fuels [6]. Enzymatic hydrolysis is a promising method to obtain sugars from this type of biomass, but the limited accessibility of enzymes to native cellulose presents a major challenge [7]. To overcome this issue, a pretreatment step is needed before the hydrolysis process to make the structure more susceptible to the attack of enzymes.

Different pretreatment methods have been studied for different lignocellulosic materials [8], which efficiency highly depends on the structure and composition of the biomass. These include physical (such as milling and grinding), physico-chemical (such as steam explosion and grinding), chemical (such as alkali, dilute acid, and organic solvents), and biological methods [9]. An efficient pretreatment enables the recovery of the highest amount of solids [10] while avoiding the formation of unwanted by-products, demands low energy, and requires low investment and operational costs [10]–[12]. However, conventional approaches are harsh, destructive, and not sufficiently effective [13]. Thus, it is key to explore

and develop milder and greener pretreatment technologies that enable the selective depolymerization of lignocellulose [10].

A novel pretreatment approach is introduced in this work, in which carbon dioxide (CO_2) is applied to the biomass using mild conditions. CO_2 is considered an ideal green solvent as it is nonflammable, nontoxic, abundant, renewable, easy to recover and recycle, and low cost [14], [15]. Its critical temperature is 31ºC, well below that of common chemicals such as water (374.2ºC) and ethanol (243.1ºC). On the other hand, its critical pressure is 73.8 bar higher than the one for ethanol (63.8 bar), but considerably lower than that of water (221.2 bar) [16]. Depending on the pressure and temperature, CO_2 can be present in three different phases. Supercritical CO_2 (SC-CO_2) occurs above its critical point of temperature and pressure, while below any of these critical points, the conditions will be subcritical, and the CO_2 will be either in the gas or liquid phase [17]. Because of these mentioned properties, it can be easily adjusted to give a high diffusion coefficient, high solvation power, high diffusivity, and high selectivity [18]. And thus, the presence of CO_2 leads to swelling and increases the malleability of many polymers, allowing them to be efficiently processed at low temperatures [15]. Also, when combined with water, it can form carbonic acid which can degrade hemicellulose, easing the access to enzymes to the substrate [19], [20]. Despite all the benefits of this pretreatment, there is a significant challenge related to the cost of compressing the CO_2 (often exceeding 100 bar), which poses a major challenge to the commercial viability of the SC-CO_2 process. Therefore, the success of this process in the market will rely upon finding the optimal balance between maximizing sugar yield in a subsequent hydrolysis stage and minimizing the duration, temperature, and pressure required for pretreatment [16].

Subcritical CO_2 presents advantages such as the utilization of mild conditions and the formation of lower amounts of inhibitory products [21]. Although this process needs higher processing time and is less efficient than supercritical CO_2, it retains and protects fragile

constituents, thus it is more suitable when the purpose is to utilize the full composition of biomass [22].

Numerous investigations have employed supercritical CO_2 either independently [23], [24], in conjunction with co-solvents [25], [26] or other pretreatments such as alkali [23] or ultrasound [27]. However, only a limited number of studies have examined subcritical CO_2 [28]–[31]. To achieve an increased production of glucose and xylose compared to the raw biomass during enzymatic hydrolysis, they frequently work with high moisture content and temperatures, while maintaining a low pressure to reach subcritical conditions. Consequently, this process leads to the degradation of sugar polymers that are solubilized in the liquid fraction, needing to handle big amount of water to recover and use them. Additionally, the density of CO_2 plays a key role in the process, as higher densities result in increased CO_2 uptake in the biomass. While higher temperatures require higher pressures to attain adequate CO_2 densities for CO_2 uptake, lower temperatures allow for operation at reduced pressures to achieve the necessary CO_2 density for biomass absorption. However, this approach requires longer durations [18], [32]. Thus, the problems mentioned associated with subcritical pretreatment (low efficiency, high water requirements, and long reaction times) make this pretreatment less frequently applied and studied.

The purpose of the present study was to evaluate the effect of the CO_2 pretreatment in sugarcane bagasse using low moisture content and subcritical conditions. The optimal pretreatment conditions were sought to allow the separation of the three main components of biomass and facilitate its enzymatic hydrolysis while minimizing chemical modifications to hemicellulose and lignin. The impact of subcritical conditions at both low and high temperatures and supercritical conditions was studied and compared to the non-treated sugarcane bagasse. Also, the study assessed the influence of distinct contents of moisture in

the biomass under the mentioned pretreatment conditions. Subsequently, the effect of two different exposure times was evaluated in the best performing pretreatment conditions found.

3.2.Materials and methods

3.2.1. *Raw material*

Sugarcane bagasse was 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 and rehydrated to the required moisture for the different experimental runs (50% and 80% (w/w) moisture content). Cellulose, hemicellulose, lignin, and ash composition of the raw and pretreated samples was determined using the NREL protocols [33], [34].

The 99.995% purity CO_2 cylinder was purchased from Air Liquide S.A.

3.2.2. CO_2 pretreatment

CO_2 pretreatment was performed in a SFE Lab 500 mL supercritical CO_2 extraction equipment (SFE Process, France). A total of 7.5g (dry mass) of sugarcane bagasse was added to the vessel. Then, the reactor was heated up to the desired temperature and loaded with CO_2 until the required pressure was attained. The biomass inside the reactor was mechanically mixed at a rate of 50 rpm. Different temperature and pressure conditions were tested at two different biomass moisture contents for 1 h. The best performing conditions were then selected based on monomeric sugar release during subsequent enzymatic hydrolysis and were run for 2 h to evaluate if time would affect the performance of the pretreatment. The 12 different experimental conditions studied are shown in **Table 1**. Once the biomass had been subjected to the designed pretreatment procedure, the pressure was released rapidly to create an explosion effect caused by the fast depressurization. As the moisture content used for pretreatment was 50 and 80% the resulting pretreated biomass was absorbing the water and

thus, a liquid fraction could not be separated by filtration. For analysis, 3 g of biomass sample were dispersed in 27 mL of distilled water and filtered. The pH was measured in the liquid fraction while the solid was dried and stored for characterization. The rest of the pretreated biomass was stored in plastic bags for the subsequent enzyme hydrolysis step.

Table 1. Water content, temperature (T), pressure (P), and time used in the different evaluated test runs for CO_2 pretreatment.

Run nr	Sample name	Water content (%)	T (oC)	P (bar)	Time (h)	Fluid state
1	Sub50	50	40	50	1	Subcritical
2	Sub50-2h	50	40	50	2	Subcritical
3	Sup50	50	40	300	1	Supercritical
4	CT50	50	100	0	1	n.a.
5	Sub50-HT	50	100	50	1	Subcritical
6	Sup50-HT	50	100	300	1	Supercritical
7	Sub80	80	40	50	1	Subcritical
8	Sup80	80	40	300	1	Supercritical
9	CT80	80	100	0	1	n.a.
10	Sub80-HT	80	100	50	1	Subcritical
11	Sub80-HT-2h	80	100	50	2	Subcritical
12	Sup80-HT	80	100	300	1	Supercritical

Sub: subcritical; Sup:supercritical; CT: control temperature; HT:high temperature; n.a.:not applicable.

3.2.3. Enzymatic hydrolysis of biomass

The purpose of the analytical saccharification was to show differences in the susceptibility of pretreated samples to enzymatic hydrolysis rather than to achieve high sugar yields [35].

The enzymatic hydrolysis of pretreated sugarcane bagasse was carried out using 10% (w/w) of dry mass loading and a total volume of 10 mL in 50 mL Erlenmeyer flasks. 0.05 M sodium acetate buffer at pH 4.8 was used as the reaction medium. An enzyme load of 40 FPU g^{-1} dry matter (DM) of the cellulolytic cocktail Cellic® CTec3 HS (CC3, provided by Novozymes, Denmark) was used. The hydrolysis was conducted at 150 rpm, 50°C, for 72 h. Control samples without enzymes were prepared and analyzed regarding the sugar content released to verify whether the spontaneous degradation of biomass occurred over time. Samples were withdrawn after 6, 24, 48, and 72 h of hydrolysis and boiled for 10 min to inactivate the enzymes. Then, the remaining solids were separated by centrifugation at 5000 rpm for 6 min and filtered through 0.20 μm syringe filter (Millipore, MA, USA) to measure sugar monomers concentration using HPLC as described in section 2.4. All hydrolysis experiments were performed in duplicate; mean values and average deviations are shown.

3.2.4. Analytical methods

The soluble sugars present in the hydrolysates after enzymatic hydrolysis were quantified using HPLC, with a Dionex Ultimate 3000 high-performance liquid chromatography UHPLC+ Focused system (Dionex Softron GmbH, Germany) and a Bio-Rad Aminex column HPX-87H (300 mm x 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.

3.2.5. Scanning Electron Microscopy (SEM)

To investigate the effect of CO_2 pretreatment on the biomass fibers morphology, the untreated and some of the CO_2 pretreated samples were visualized by using a QFEG 200 Cryo ESEM. Samples were secured on a carbon adhesive tip (Agar Scientific Ltd, Stansted, United Kingdom) attached to a SEM specimen stub (Agar Scientific Ltd, Stansted, United Kingdom) and coated with gold. The electron microscope was operated at an accelerating voltage of 10 kV in a low-vacuum mode. Secondary electron detector was used for imaging.

3.3.Results and discussion

3.3.1. Chemical composition of raw and pretreated biomass

The determination of the chemical composition of sugarcane bagasse before and after pretreatment was carried out to assess the effects of different CO_2 pretreatment conditions on the cellulose, hemicellulose, and lignin contents. The results are shown in **Table 2**.

Table 2. Chemical composition of raw and CO_2-pretreated sugarcane bagasse under different experimental conditions.

Sample	Composition (wt%)				pH
	Cellulose	Hemicellulose	Lignin	Acetyl groups	
Raw biomass	44.9 ± 0.4	22.2 ± 0.4	24.8 ± 0.4	2.6 ± 0.02	6.27 ± 0.01
Sub50	45.3 ± 1.4	22.0 ± 0.8	23.7 ± 0.4	2.7 ± 0.02	5.95 ± 0.00
Sub50-2h	45.6 ± 1.1	22.6 ± 0.4	23.5 ± 0.4	2.8 ± 0.08	5.91 ± 0.01
Sup50	46.9 ± 1.4	20.6 ± 0.7	24.1 ± 1.1	2.9 ± 0.09	5.82 ± 0.00
CT50	45.4 ± 0.4	22.7 ± 0.6	23.9 ± 1.2	2.5 ± 0.03	6.16 ± 0.00

Sub50-HT	46.1 ± 1.4	21.7 ± 0.8	23.8 ± 0.3	2.9 ± 0.29	6.04 ± 0.00
Sup50-HT	46.2 ± 1.7	21 ± 0.2	25.2 ± 0.6	2.1 ± 0.02	5.76 ± 0.02
Sub80	45.8 ± 0.9	22.7 ± 0.6	23.7 ± 1.3	2.3 ± 0.07	5.85 ± 0.00
Sup80	46.7 ± 0.5	21.3 ± 0.7	24.6 ± 1.1	2 ± 0.05	5.55 ± 0.00
CT80	45.9 ± 0.8	22.5 ± 0.7	23.9 ± 0.5	2.2 ± 0.01	6.34 ± 0.00
Sub80-HT	46.0 ± 1.3	22.7 ± 0.1	23.5 ± 0.7	2.3 ± 0.02	5.76 ± 0.01
Sub80-HT-2h	46.5 ± 0.7	21.1 ± 0.3	24.2 ± 0.2	2.7 ± 0.04	5.65 ± 0.00
Sup80-HT	46.8 ± 0.8	21.1 ± 0.5	24.3 ± 0.1	2.3 ± 0.04	5.48 ± 0.00

Sub: subcritical; Sup:supercritical; CT: control temperature; HT:high temperature.

Putrino et al. [26] and Gao et al, [36] also showed that the chemical composition of green coconut fiber and wheat straw, respectively, remained largely unchanged after pretreatment using supercritical CO_2. Thus, the findings obtained from this study, together with the ones obtained in other works, strongly support the notion that the impact of CO_2 is predominantly physical [18].

The determination of the pH of raw and pretreated biomass samples was carried out to ensure that the subsequent hydrolysis step would not have an extreme pH that would prevent the enzyme from working under its optimal conditions because of the possible presence of carbonic acid formed during the pretreatment. Samples pretreated without CO_2 (CT50 and CT80) showed a similar pH to the raw biomass while the ones treated with CO_2 presented a lower pH. The minimum pH observed was in the 80% moisture sample pretreated using supercritical conditions, which are the harsher ones used in this study and probably led to a higher production of carbonic acid.

3.3.2. Glucose and xylose production from raw and pretreated biomass

Enzyme hydrolysis of the raw and pretreated sugarcane bagasse was carried out and the results are depicted in **Figure 1**. To discern whether the glucose and xylose released from samples pretreated at 100°C were influenced by CO_2 or solely by the temperature, a control experiment only involving pretreatment at 100°C was conducted.

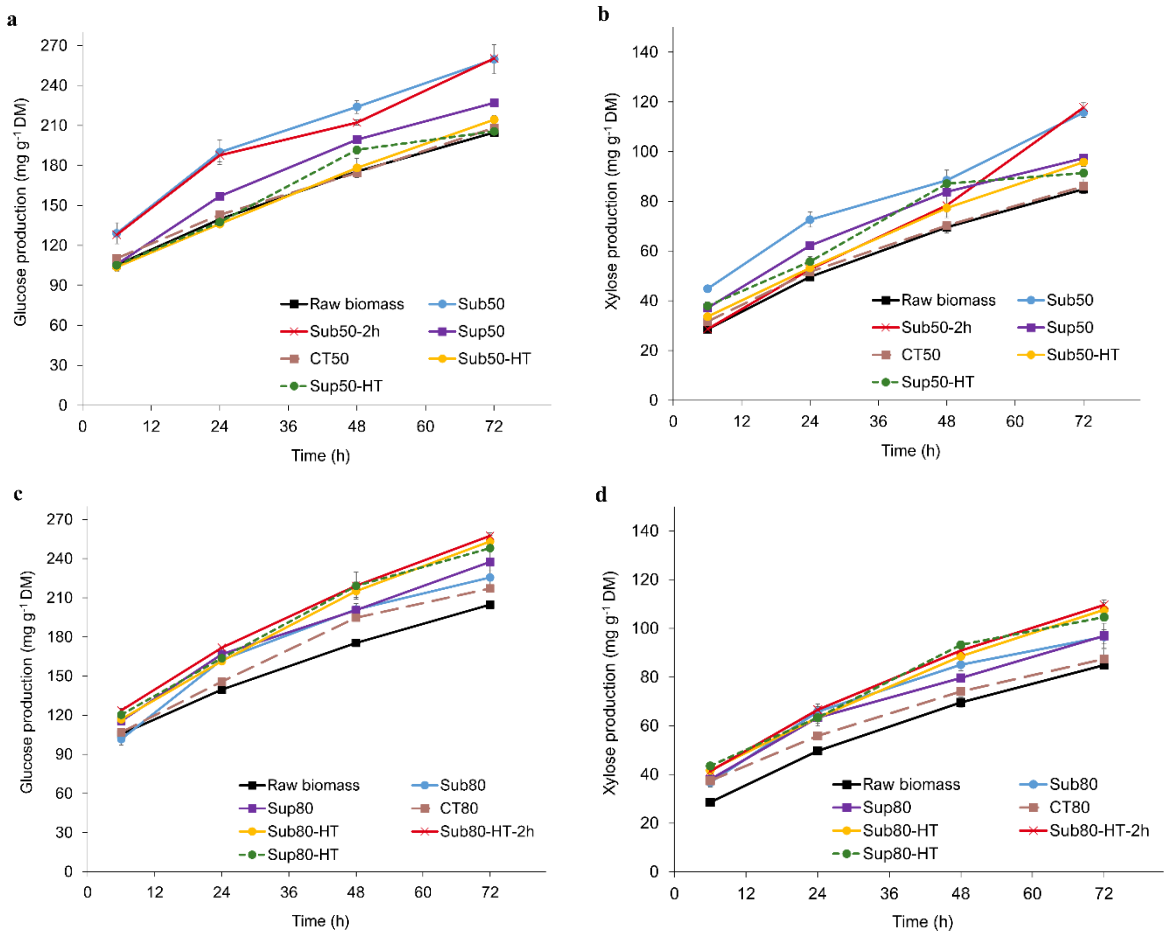

Figure 1. Glucose (a,c) and xylose (b,d) production during enzymatic hydrolysis of 50% moisture (a-b) and 80% (c,d) moisture pretreated and raw sugarcane bagasse. The data shown is the average of two replicates of the same experiments. Error bars indicate the average deviation.

For the pretreated samples with 50% moisture (CT50), the glucose and xylose release resembled that of the raw biomass. However, for the pretreated biomass with 80% moisture content (CT80), this difference was more significant, resulting in a 6% increase in glucose production and a 5% increase in xylose production compared to the raw biomass. The higher moisture content and the elevated temperature could have exhibited hydrothermal pretreatment effects and have increased to a certain extent the degradability of the sample compared to the raw biomass.

When conducting pretreatments with biomass containing 50% moisture (**Figure 1**a-b), it was observed that the highest glucose and xylose release after enzymatic hydrolysis occurred when the process was carried out under subcritical conditions at a temperature of 40ºC and a pressure of 50 bar, in which 260 mg g^{-1} of glucose and 115 mg g^{-1} of xylose production was obtained. In the raw biomass, the amount of glucose and xylose obtained was 205 and 85 mg g^{-1}, respectively. Thus, an increase of about 27% in the content of glucose and 36% in the content of xylose, resulted after pretreatment with subcritical CO_2. This clearly shows that this method of pretreatment can be used in sugarcane bagasse to enhance the release of sugars during enzyme hydrolysis. Conversely, when using subcritical conditions at 100ºC (Sub50-HT) the production of glucose and xylose was lower, confirming that a higher swelling effect due to CO_2 uptake occurs when the CO_2 density is greater, achieved when low pressures and low temperatures are used.

During the pretreatment process using supercritical CO_2 conditions in 50% moisture samples 227 and 97 mg g^{-1} of glucose and xylose, respectively, were released when using 40ºC (Sup50), which was 11 and 14% higher than the respective glucose and xylose released with the raw biomass. However, when using 100ºC (Sup50-HT) no relevant increase in glucose and xylose production was observed.

In the pretreatment of biomass with 80% moisture content (**Figure 1**c-d), the results obtained varied extensively compared to when using a lower moisture content. In this case, the highest glucose production was 258 mg g^{-1} and for xylose, it was 110 mg g^{-1} when using a condition of 50 bar at 100ºC for 2 h (Sub80-HT-2h). Interestingly, the results closely resembled those when the biomass was pretreated under the same conditions but for only one hour (Sub80-HT). In this case, the glucose release was 253 mg g^{-1}, and the xylose release was 107 mg g^{-1}, which is a 24 and 29% increase in glucose and xylose production, respectively, compared to the raw biomass. Promising results were also achieved when using supercritical conditions at 100ºC (Sup80-HT), showing a 21% increase in glucose production and 26% increase in xylose production. Unlike the case with 50% moisture content biomass, where the glucose and xylose production were lower when using lower temperatures when using 80% moisture content, higher temperature was required to achieve the best performing conditions. This observed outcome could be attributed to the high content of water in the biomass, which likely have been saturated it and hindered the access of CO_2 into its structure. However, when a temperature of 100ºC was applied, some of the water contained in the biomass might have evaporated, creating a more favorable environment for CO_2 to access and interact with the structure of biomass. As a result, this increased access to the biomass facilitated the intended goal.

Kim et al. and Narayanaswamy et al. demonstrated that dry biomass does not exhibit a significant response to supercritical CO_2 pretreatment [24], [37]. However, the presence of moisture seems to play a crucial role in biomass swelling caused by CO_2 uptake, enhancing the effect of disorganization of the structure with the pressure release, and thus making the process more effective. Some studies [24], [38] have shown that increasing the water content has a positive effect on sugar yield. However, there is an optimal condition in which the

moisture content reaches an optimal level, beyond which further increases in water content can lead to a decrease in the sugar yield.

The best performing conditions in terms of glucose and xylose release were tested using an extended period to assess the possibility of further enhancement in sugar production. However, the increase in pretreatment time to 2 h did not yield a significant effect. For example, the samples pretreated with 50% moisture at 50 bar and 40ºC for 1 h showed a glucose release of 260 mg g^{-1} and a xylose release of 116 mg g^{-1} while the samples submitted to 2 h of pretreatment resulted in the same glucose production and 118 mg g^{-1} of xylose production after enzymatic hydrolysis.

Benazzi et al. [39] obtained a glucose production of 287 mg g^{-1} of fermentable sugars from sugarcane bagasse with a moisture range from 45-65% pretreated using supercritical CO_2. In this work, the maximum obtained (375 mg g^{-1} of fermentable sugars) was under subcritical conditions when using 50% moisture biomass. This result is very promising, as with the use of a non-toxic and reusable chemical as CO_2, low moisture content and under mild conditions of pressure and temperature, a higher production of fermentable sugars compared to the raw biomass can be achieved without the generation of inhibitor compounds. Furthermore, because of the low water content used, the process yields a product that consists only of a solid fraction, making unnecessary a step of filtration and liquid concentration.

3.3.3. SEM analysis

Morphological characteristics of raw and pretreated (50 bar, 40ºC for 1 h) solid samples were analyzed using SEM to evaluate the effect of CO_2 pretreatment on the surface of sugarcane bagasse biomass. Physical changes can be noticed on the surface of the pretreated sugarcane bagasse compared to the untreated sugarcane bagasse. The untreated (**Figure 2**a) showed a flat and intact surface, which provides a lower enzyme accessibility to

hemicellulose and cellulose. On the other side, for the pretreated sample (**Figure 2**b), a rougher surface can be observed, containing a higher number of pores probably resulting from the swelling of the biomass. The impregnation of CO_2 in the biomass likely resulted in a rougher surface and removal of the outer layer of the cell wall, distinguishing it from the raw biomass sample. Similar observations were also reported in a previous study where subcritical CO_2 was used [20].

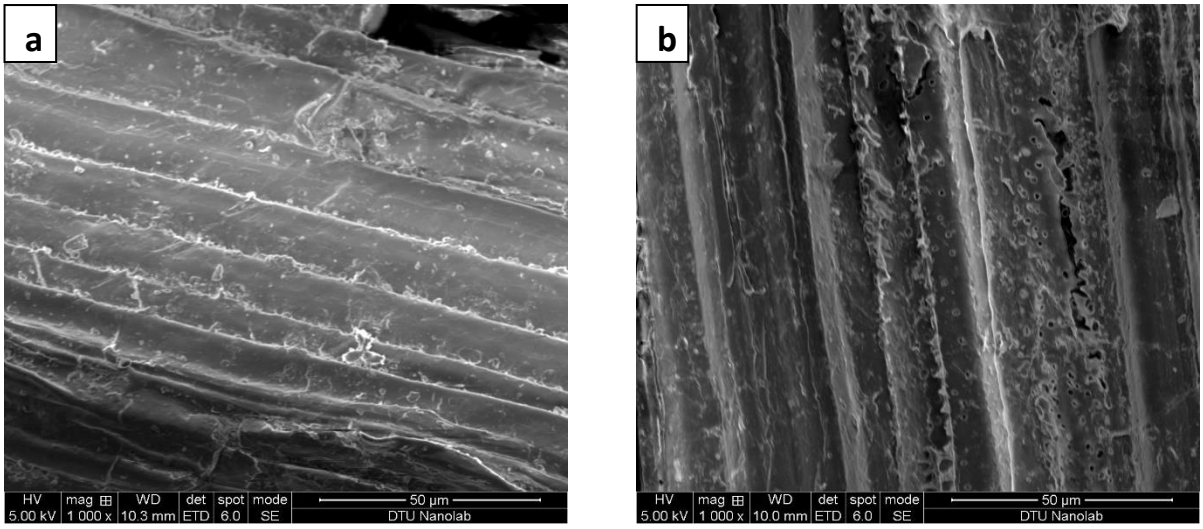

Figure 2. SEM images of sugarcane bagasse before and after CO_2 pretreatment at 50 bar, 40ºC for 1 h. a) Transverse section of sugarcane bagasse before pretreatment. b) Transverse section of sugarcane bagasse after pretreatment.

3.4.Conclusion

The effects of various pretreatment conditions applied to sugarcane bagasse were evaluated. Samples containing 50% moisture content subjected to subcritical conditions at 40ºC and 50 bar showed negligible chemical modifications in the lignocellulose structure. Interestingly, the production of glucose and xylose after enzymatic hydrolysis increased 27 and 36% compared to the untreated sugarcane bagasse. Thus, these conditions of pretreatment

not only increased the release of sugar monomers but also possessed the capability to facilitate the complete utilization of the polysaccharides present in sugarcane bagasse. Notably, no breakdown to undesired products was detected, suggesting the potential for efficient utilization of the biomass. This study highlighted the potential to use CO_2 pretreatment under conditions that have not been extensively studied in the past. The introduction of mild subcritical conditions together with low moisture content presents a novel and promising pretreatment that holds potential for various biorefinery applications. It could be used to increase the production of fermentable sugars through enzymatic hydrolysis compared to the raw biomass and also to harness the residual lignin fraction obtained after enzymatic hydrolysis. Enzymatically hydrolyzed sugars enable biofuel production and value-added products, reducing reliance on non-renewable fuels. Lignin fraction can create high-value chemicals, enhancing circular economy principles in biorefineries. This multi-purpose approach enables the sustainable utilization of biomass, easing the transformation toward greener and more efficient industrial processes.

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Chapter 4: Evaluation of Enzymatic Hydrolysis of Sugarcane Bagasse Using Combination of Enzymes or Co-Substrate to Boost Lytic Polysaccharide Monooxygenases Action

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

Eva Balaguer Moya [1,†], Maria Laura Silva Cunha [2,†], Carina Aline Prado [2], Simone Turella [1], Silvio Silvério da Silva [2], Maher Abou-Hachem [1], Giuliano Dragone [1], Júlio César dos Santos [2] and Solange Inês Mussatto [1,*]

[1] Department of Biotechnology and Biomedicine, Technical University of Denmark, Søltofts Plads, Building 223, 2800 Kongens Lyngby, Denmark
[2] Department of Biotechnology, Engineering School of Lorena, University of São Paulo, Lorena 12602-810, SP, Brazil
* Correspondence: smussatto@dtu.dk or solangemussatto@hotmail.com
† These authors contributed equally to this work.

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), H_2O_2, or cello-oligosaccharide dehydrogenase (CelDH) *Fg*CelDH7C 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 H_2O_2 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 lignocellulose 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.

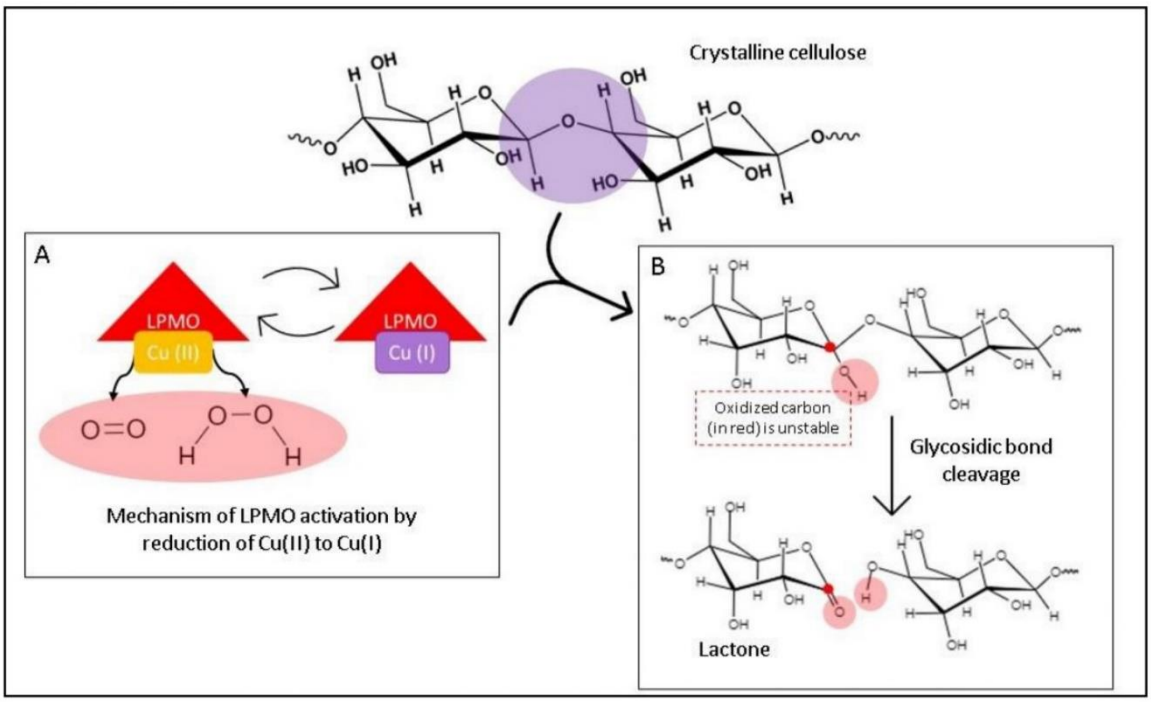

Figure 1. Simplified scheme of LPMO action in the hydrolysis of crystalline cellulose. (**A**) represents the transition from a resting to an active catalytic state of LPMO (reduction of Cu(II) to Cu(I) in the active site) in presence of O_2 and/or H_2O_2. (**B**) shows the cleavage of the glycosidic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

Recently, the cello-oligosaccharide dehydrogenase (CelDH) from *Fusarium graminearum* (*Fg*CelDH7C), 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 *Fg*CelDH7C. 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 *Fg*CelDH7C 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.

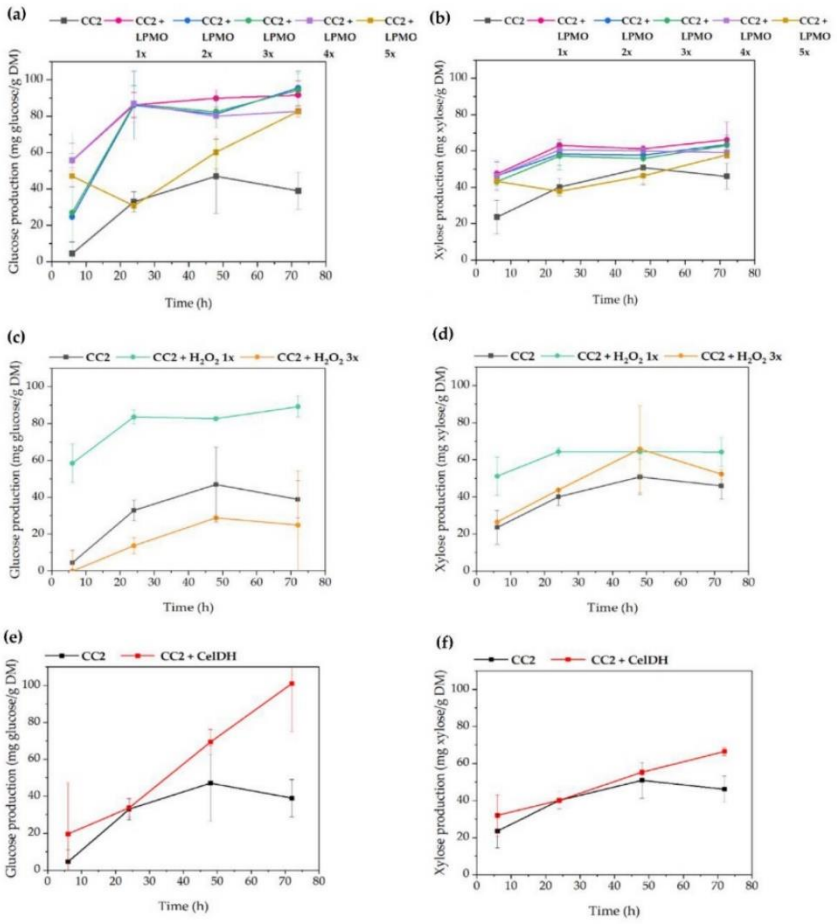

Figure 2. Glucose (**a**,**c**,**e**) and xylose (**b**,**d**,**f**) release in enzymatic hydrolysis of sugarcane bagasse with Cellic® CTec 2 and different supplementation approaches: supplementation with the LPMO GcLPMO9B (**a**,**b**), H_2O_2 (**c**,**d**), or with the *Fg*CelDH7C dehydrogenase (**e**,**f**) (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 μM 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 (*Fg*CelDH7C) 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	*p*-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 µL, 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 µmol/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 µL of a 5 mmol/L solution was added in order to obtain a final H_2O_2 concentration of 14 µmol/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) *Fg*CelDH7C. *Fg*CelDH7C 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 µmol/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 mm × 7.8 mm) at 60 °C, using a Shodex RI-101 refractive index detector and 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min, and a sample injection of 20 µL.

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 (pH4.8) and 50 mmol/L HEPES (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.

Author Contributions: Conceptualization, S.I.M.; methodology, E.B.M., M.L.S.C. and C.A.P.; validation, E.B.M. and M.L.S.C.; formal analysis, E.B.M. and M.L.S.C.; investigation, E.B.M. and M.L.S.C.; resources, S.I.M., J.C.d.S., S.S.d.S., S.T. and M.A.-H.; data curation, E.B.M. and M.L.S.C.; writing—original draft preparation, M.L.S.C., E.B.M. and C.A.P.; writing—review and editing, S.I.M., J.C.d.S., G.D. and M.A.-H.; visualization, E.B.M. and M.L.S.C.; supervision, S.I.M., J.C.d.S. and G.D.; project administration, S.I.M.; funding acquisition, S.I.M. and J.C.d.S. All authors have read and agreed to the published version of the manuscript.

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

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Chapter 5: Enzymatic hydrolysis cocktail optimization for the intensification of sugar extraction from sugarcane bagasse

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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

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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).

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[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

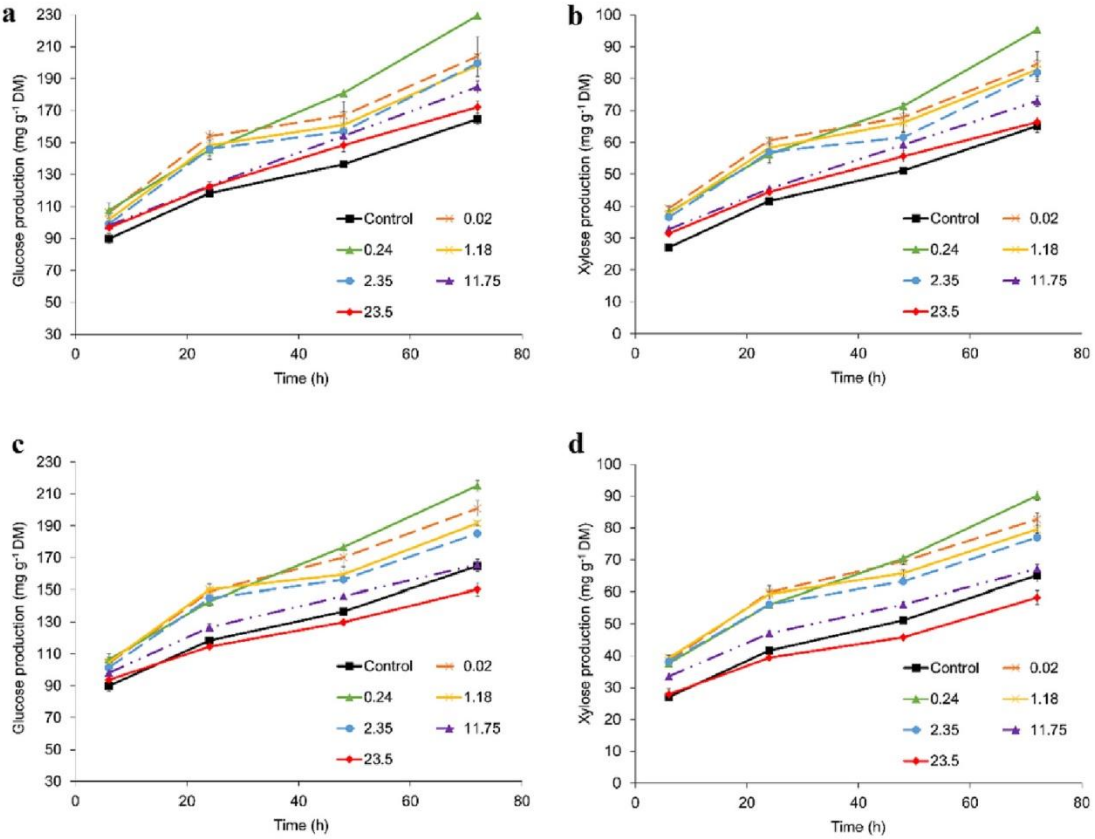

Fig. 1. Glucose (a,c) and xylose (b,d) release during the enzymatic hydrolysis of sugarcane bagasse using the cellulolytic cocktail and adding H_2O_2 at the beginning of the process (0 h) (a,b) or adding three doses of H_2O_2 during times 0, 24 and 48 h (c,d). H_2O_2 was supplemented adding dosages of 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.

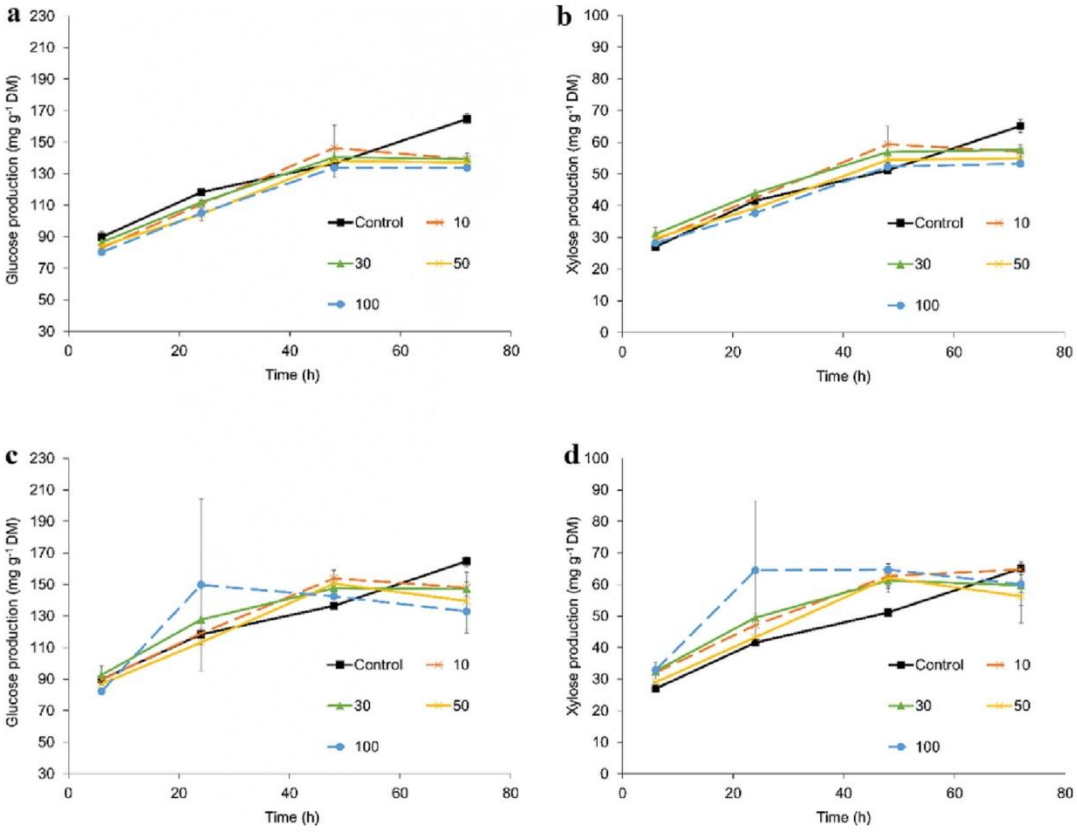

Fig. 2. Glucose (a,c) and xylose (b,d) release in enzymatic hydrolysis of sugarcane bagasse with different loads of laccase and 20 FPU of cellulase g^{-1} DM (a,b) or 35 FPU cellulase g^{-1} DM (c,d). Laccase doses were 10, 30, 50 or 100 μL g^{-1} DM. The data shown is the average of two replicates of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

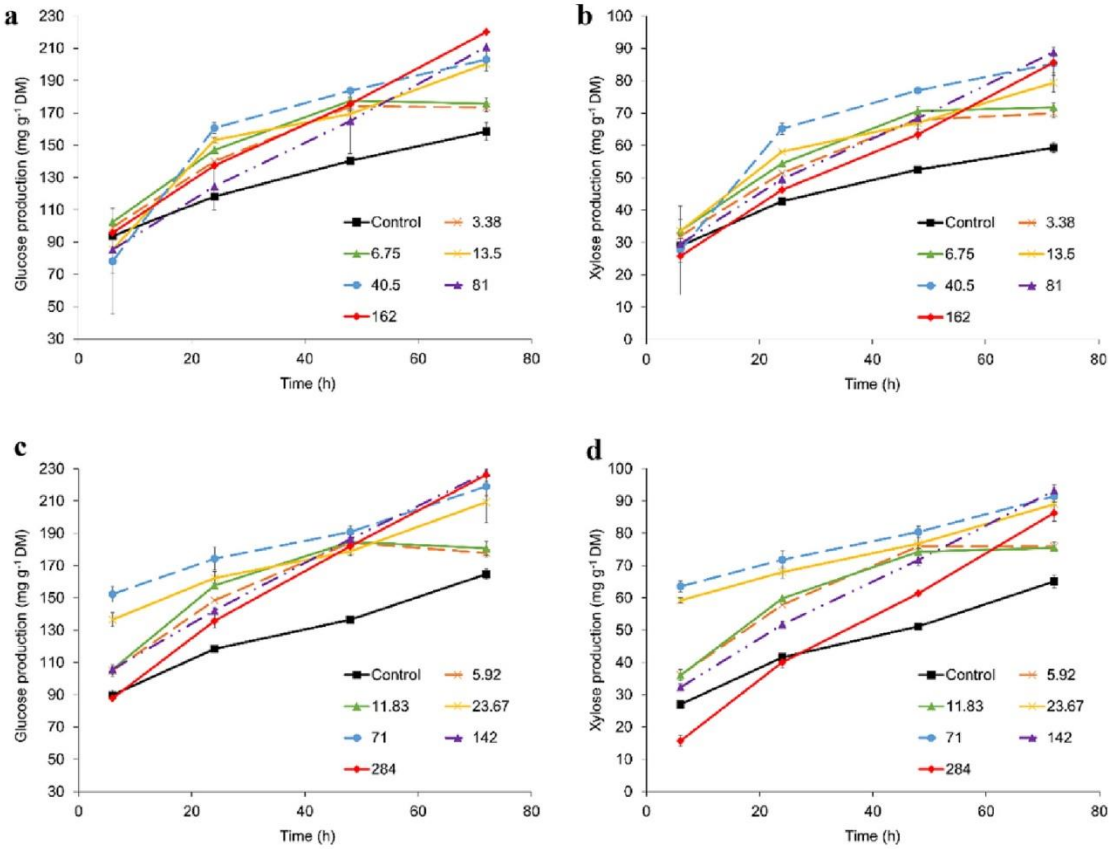

Fig. 3. Glucose (a,c) and xylose (b,d) release during the enzymatic hydrolysis of sugarcane bagasse with different loads of hemicellulase: 3.38, 6.75, 13.5, 40.5, 81 and 162 μL g^{-1} DM for 20 FPU cellulase g^{-1} DM (a,b); and 5.92, 11.83, 23.67, 71, 142 and 284 μL g^{-1} DM for 35 FPU cellulase g^{-1}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

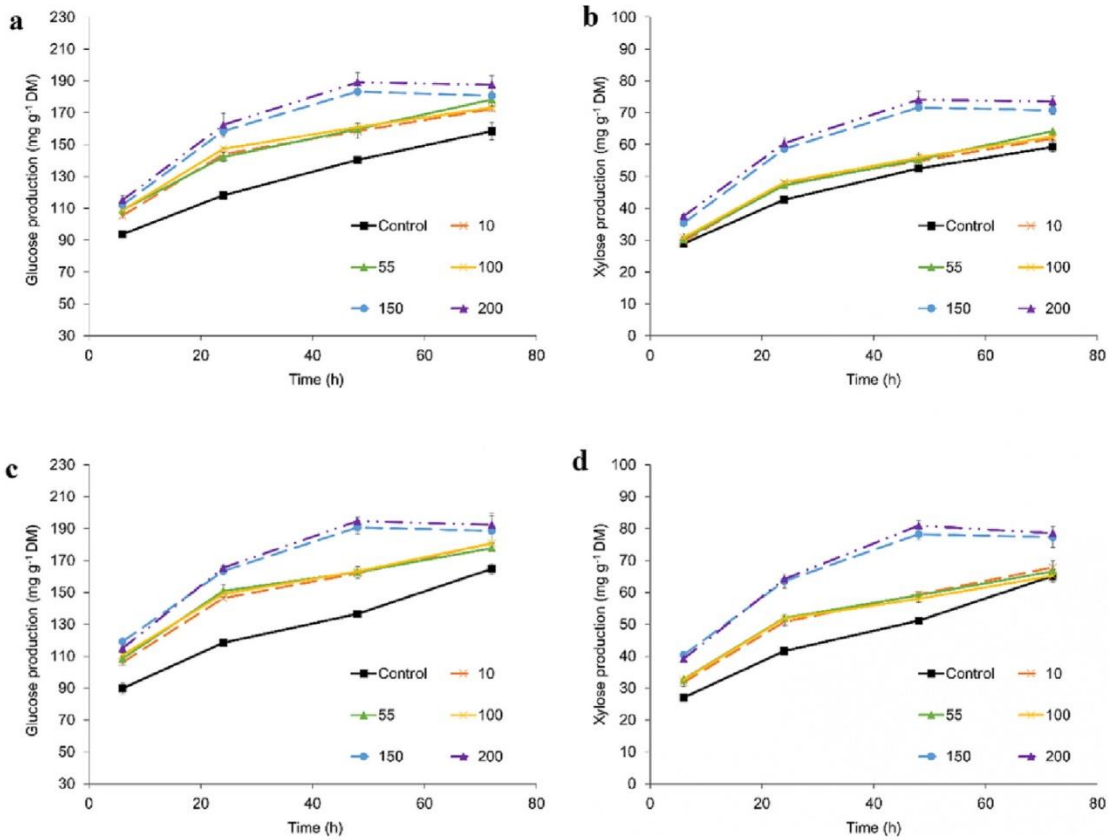

Fig. 4. Glucose (a,c) and xylose (b,d) release during the enzymatic hydrolysis of sugarcane bagasse using different concentrations of PEG4000 (10, 55, 100, 150 or 200 mg g^{-1} DM) and 20 FPU cellulase g^{-1} DM (a,b) or 35 FPU cellulase g^{-1} DM (c,d). The data shown is the average of two replicates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

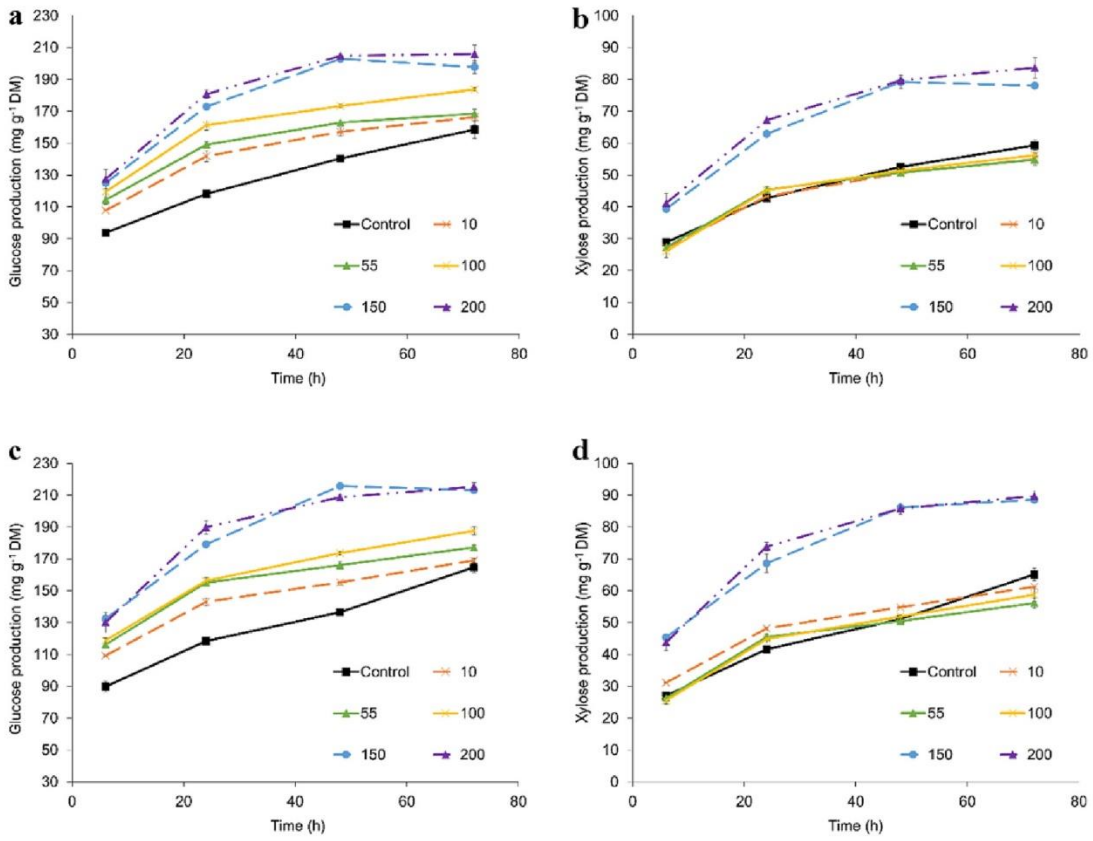

Fig. 5. Glucose (a,c) and xylose (b,d) release during the enzymatic hydrolysis of sugarcane bagasse using different concentrations of Tween 80 (10, 55, 100, 150 or 200 mg g^{-1} DM) and 20 FPU cellulase g^{-1} DM (a,b) or 35 FPU cellulase g^{-1} DM (c,d). The data shown is the average of two replicates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

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Chapter 6: 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. 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 techniques were employed to enhance the release of glucose and xylose from mildly pretreated sugarcane bagasse. The optimized supplement composition resulted in a 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. The addition of H_2O_2 in combination with hemicellulase and Tween® 80 had no significant effect on sugar release, which could have happened because of the short concentration range studied. 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.

Keywords: Biorefinery; Cellulases; Enzymatic hydrolysis; Hemicellulase; Lignocellulosic biomass

6.1. 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 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 through pretreatment. These cocktails are not efficient in 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 this work is to evaluate the combination of hemicellulases, H_2O_2 and Tween® 80 supplementation to the commercial

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.

6.2.Materials and methods

6.2.1. 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 were determined according to the NREL protocols [28,29], and are shown in **Table 1**.

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

6.2.2. 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 1957 × g 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.

6.2.3. 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 (x_1), H_2O_2 (x_2), and Tween® 80 (x_3) on the enzymatic release of glucose from CO_2-pretreated sugarcane bagasse. An overview of the experimental setup is shown in **Table 2**.

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. In such cases, further adjustments or improvements to the model may be necessary [30,31]. 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.

6.2.4. 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 x 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.

$$Glucose\ production\ (mg\ g^{-1}DM) = \frac{C_{glucose} \cdot V_{hydrolysis} \cdot 1000}{DM} \quad \text{(Eq. 1)}$$

$$Xylose\ production\ (mg\ g^{-1}DM) = \frac{C_{xylose} \cdot V_{hydrolysis} \cdot 1000}{DM} \quad \text{(Eq. 2)}$$

6.3.Results and discussion

6.3.1. Effect of cellulolytic enzymatic cocktail enrichment on biomass 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]. 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).

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}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	71.26
2	5 (-1)	65 (1)	350 (0)	218.36	216.64	90.27
3	553 (1)	5 (-1)	350 (0)	230.89	232.62	84.43
4	553 (1)	65 (1)	350 (0)	236.21	247.71	104.86
5	5 (-1)	35 (0)	10 (-1)	162.45	174.46	72.01
6	5 (-1)	35 (0)	690 (1)	216.59	217.80	86.82
7	553 (1)	35 (0)	10 (-1)	183.98	182.77	67.73
8	553 (1)	35 (0)	690 (1)	242.38	230.37	83.31
9	279 (0)	5 (-1)	10 (-1)	172.22	171.71	69.01
10	279 (0)	5 (-1)	690 (1)	230.27	240.56	81.04
11	279 (0)	65 (1)	10 (-1)	185.12	174.84	72.46
12	279 (0)	65 (1)	690 (1)	239.6	240.11	97.84
13	279 (0)	35 (0)	350 (0)	229.79	225.46	82.77
14	279 (0)	35 (0)	350 (0)	229.79	236.06	98.47
15	279 (0)	35 (0)	350 (0)	229.79	227.87	95.43

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.

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

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 the addition of H_2O_2 in combination with hemicellulase and Tween® 80 (in the range of 5 and 65 μM) may not yield significant benefits to glucose production.

Our previous study 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 BBD 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 this conditions, and extra doses do not show benefit.

A plot of the observed versus predicted values for glucose production (**Figure 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 (**Figure 1b**), it can be noted that optimal conditions can be identified for both hemicellulase and Tween® 80.

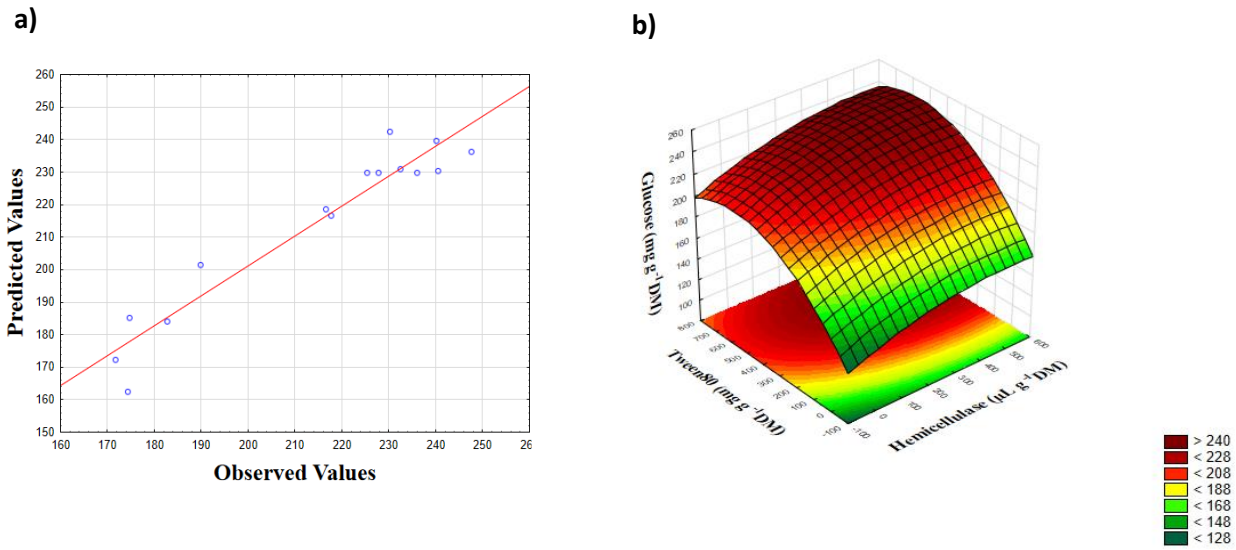

Figure 1. Observed (data determined experimentally) versus predicted values (**a**) and response surface graphs for glucose release from the hydrolysis of CO_2-pretreated sugarcane bagasse according to the 3-factor Box-Behnken design (**b**).

$$\text{Glucose (mg } g^{-1DM}) = 147.14 + 0.10 \cdot x1 + 0.21 \cdot x3 - 1.88 \times 10{-}4 \cdot x32$$

(Eq. *3* represents 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. 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.

$$\text{Glucose (mg } g^{-1}\text{DM}) = 147.14 + 0.10 \cdot x_1 + 0.21 \cdot x_3 - 1.88 \times 10{-}4 \cdot x_3^2 \quad \text{(Eq. 3)}$$

The desirability function was then utilized to evaluate the overall effectiveness of the combined glucose and xylose release (**Figure 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.

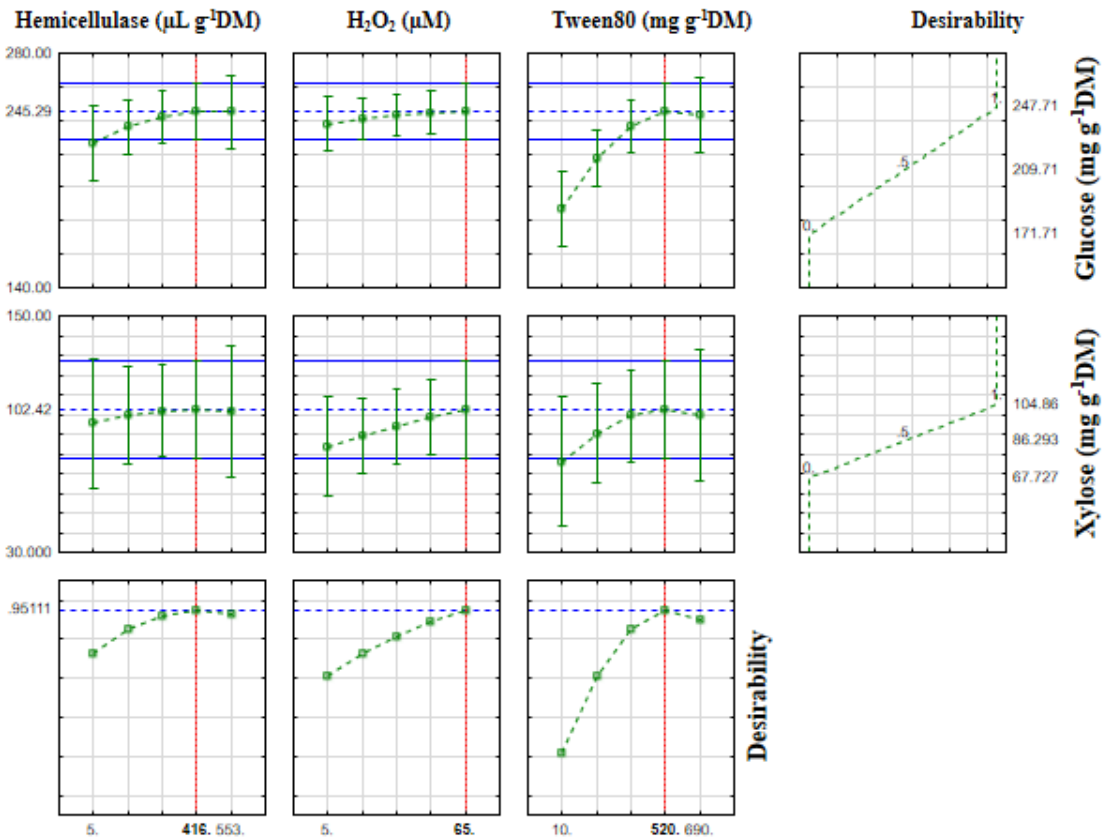

Figure 2. Desirability plot for glucose and xylose release from the hydrolysis of CO_2-pretreated sugarcane bagasse based on the Box-Behnken design.

To validate the model, the optimal combination of additives and 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. On the other hand, Tween® 80

avoids the unproductive binding of cellulases to lignin and avoids their inactivation caused in the liquid-air interface, allowing an increased glucose production.

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

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 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.

6.3.2. 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.

6.4.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 for only 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.

Acknowledgments

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Chapter 7: Comparative life cycle assessment of ethanol production from sugarcane bagasse using diluted acid pretreatment and CO_2 pretreatment

This chapter contains a LCA study of bioethanol production and was performed in collaboration with Novozymes and Raízen. The manuscript presented is intended to be published. However, it needs to be revised by all the parts involved to avoid disclosing confidential information. This chapter is authored by Eva Balaguer Moya, Giuliano Dragone and Solange I. Mussatto.

Abstract

This study presents an investigation into the promising potential of using sugarcane bagasse for second-generation ethanol production, with a specific focus on evaluating the environmental implications of two distinct pretreatment methodologies through life cycle assessment. The comparative analysis involved a conventional approach utilizing dilute acid and a novel CO_2-based pretreatment. Both scenarios exhibited a negative environmental impact, quantified as -102 kg CO_2 eq/tonne of bagasse for the dilute acid method and -228 kg CO_2 eq/tonne for the CO2 approach. This difference in emissions was due to the intrinsically linked gasoline displacement by the ethanol produced in each system: for the diluted acid was 45 kg while for the CO2 was 100 kg. However, when studying the carbon intensity of ethanol for both systems, the results were similar, being 9 g CO_2 eq/MJ of ethanol. This means that the impact associated to ethanol production in both scenarios is equal; however, on a per tonne of bagasse basis, as more ethanol is obtained in the CO2 system, the emission savings are higher. This finding is key when feedstock scarcity is a concern and when the highest climate benefit per unit of biomass is sought. The study served to identify several critical aspects of the process, such as the enzyme dosage and the marginal supplier of electricity used when modeling sugarcane bagasse, which were evaluated through sensitivity analysis. The insights derived from this life cycle assessment served as an initial step in assessing the viability of the novel CO_2 pretreatment technology. This study highlights the potential of the CO_2-based pretreatment as a more sustainable approach for implementation into the second-generation bioethanol process.

7.1.Introduction

In the present day, the primary challenge confronting humanity primarily stems from the scarcity of traditional energy sources and the subsequent implications for environmental sustainability [1]. Driven by these considerations, numerous global initiatives have been undertaken to discover clean and sustainable energy alternatives capable of replacing fossil fuels [2], [3]. The utilization of first-generation (1G) bioethanol has been extensive in vehicle fuels, resulting in reduced net carbon dioxide emissions. However, the growing need for this type of bioethanol led to elevated feedstock production, leading to the food versus fuel competition, along with an increase in land utilization [2]. Thus, it cannot be established as a sustainable and long-term fuel option [4]. Second-generation (2G) bioethanol production is derived from non-food crops or crop residues and can significantly reduce greenhouse gas (GHG) emissions and result in positive environmental impacts [4].

Sugarcane bagasse is an agriculture-based lignocellulosic residue that is widely abundant and attracts attention worldwide as a potential substitute for fossil fuels [3]. Most of it is primarily used as fuel for boilers found in the sugar and ethanol industries [5]. Nevertheless, thanks to biotechnology advancements, sugarcane bagasse has been transformed into a cost-effective and desirable substrate for producing ethanol on a large scale [3]. However, its use as feedstock for 2G ethanol production is challenging because of the recalcitrance of lignocellulosic biomass structure [6]. In fact, the primary economic obstacle that hinders the cost-effective production of lignocellulosic biofuels is the expenses related to fractionation (pretreatment and enzymatic hydrolysis) [7].

Different pretreatments have been developed to make cellulose more accessible to the attack of enzymes, such as steam explosion, alkaline, diluted acid, and ammonia [8]. However, several factors make pretreatment technologies still a technological challenge, such as the severe operational conditions applied, which require the utilization of expensive

equipment and high energy consumption, the elevated amounts of water used, the excessive degradation of biomass, and the formation of process inhibitors [1]. There exists a need to develop pretreatment processes that strike a balance between breaking down the cell wall structure of plant biomass while avoiding excessive chemical degradation of biomass constituents. Novel pretreatment methods should use chemicals that are cost-effective and easy to recover, along with affordable equipment, and at the same time give priority to environmentally friendly and energy-efficient approaches [9].

Acid pretreatment is one of the most common conventional pretreatments for lignocellulosic biomass. Acid degrades polysaccharides (especially hemicellulose) present in the biomass into soluble monomers [10]. It increases the enzymatic hydrolysis efficiency of biomass as the process leads to a solid fraction containing easily digestible cellulose together with lignin. However, the process leads to the formation of inhibitors, such as furfural and 5-hydroxymethyl furfural (HMF), that are inhibitory for the growth of microorganisms [11]. Also, the concentration of reducing sugars is relatively low because of the elevated liquid-to-solid ratio used. Because of these challenges, the costs of this kind of pretreatment are still high [12]. On the other hand, a novel and sustainable pretreatment involves the use of CO_2 using mild operating conditions. CO_2 is a low-cost green chemical that is non-toxic, abundant, renewable, and easy to recover and recycle [13], [14]. In combination with water, CO_2 can generate carbonic acid, capable of breaking down hemicellulose, thus facilitating enzyme access to the substrate [15], [16].

To address the obstacles associated with sustainable bioethanol production processes, there is an interest in adopting a circular economy approach to enhance environmental sustainability and maximize the efficient utilization of resources and byproducts. Within this framework, integrating the simulation of bioethanol production with life cycle assessment (LCA) modeling becomes imperative. By doing this, technical bottlenecks of the processes

that require optimization can be tackled while assessing the critical environmental hot spots of the system [17].

Different LCA studies have previously evaluated the environmental impacts of bioethanol production; while some were focused on 1G ethanol [18], [19], 2G ethanol[20], [21], or 1G2G hybrid production [22], [23], others compared the implementation of 1G versus 2G or 1G2G [24], [25]. On the other hand, Maga et al. [26] performed a comparative LCA of the complete sugarcane plant for 1G ethanol production, 2G ethanol production, and integrated 1G2G ethanol production in Brazil. Parsad et al. [27] provided an LCA of some emerging pretreatment technologies (using dilute acid, steam explosion, liquid hot water, and organosolv). Until now, there is no available study comparing the environmental impact of conventional pretreatment and CO_2-based pretreatment. Both pretreatments yield biomasses that have a very different chemical composition and subsequently, require different operational strategies to obtain the maximum extraction of sugar monomers that will be converted to ethanol. For this reason, in this study, LCA was used to evaluate the suitability of the novel CO_2 pretreatment method in comparison to the well-established pretreatment method using diluted acid.

7.2.Materials and methods

A methodological structure based on ISO 14040 [28] and ISO 14044[29] was followed to conduct the LCA.

7.2.1. Methodological assumptions

7.2.1.1.Goal

This LCA aims to get an insight of the sustainability and suitability of an optimized pretreatment technology compared to the conventional and widely used pretreatment technology using diluted acid. This will enable an understanding of the existing greenhouse

gas (GHG) emissions of each part of the process. Also, the identification of potential changes in GHG emissions arising from the introduction of the new pretreatment method.

7.2.1.2.Scope

The functional unit chosen for this study was the processing of 1 tonne of waste sugarcane bagasse from a 1G ethanol processing plant in Brazil. This amount of sugarcane bagasse was partially allocated to ethanol production, as it was also used to produce energy to fuel the different production stages. In addition, the study included results expressed per MJ of cellulosic ethanol.

7.2.1.3.Sugarcane bagasse modelling

Sugarcane bagasse is a by-product from sugar production (used for human consumption or for '1st generation' bioethanol production). Hence, sugarcane bagasse is not produced due to a specific demand for bagasse. It is generated because of demand for other products. In other words, the bagasse is there regardless of its subsequent use. Therefore, no emissions are assigned to bagasse in this LCA study. This is also in line with the principles outlined in the European Renewable Energy Directive (RED) [30]. While bagasse can be considered a 'zero emission feedstock', it may have alternative uses. To explore this, the present study also considered an alternative case where the bagasse is used for electricity production rather than cellulosic ethanol. This is to allow for a comparison and a discussion of optimal use of bagasse, today and in the future.

7.2.1.4.Included systems and boundary definition

Two different ethanol scenarios with different pretreatments were evaluated. The base case included one of the most widely used commercial pretreatments, dilute acid (DA) pretreatment. In the alternative system, the novel CO_2-based pretreatment technology was studied and compared to the base case.

Both systems are closely aligned and include very similar processes for ethanol production, the only steps that were different were the pretreatment process and the steps associated with the adaptation of the pretreated biomass to the required conditions of enzymatic hydrolysis. The manufacturing of inputs and the production of heat and electricity were included together with the by-products obtained during the different process steps.

Infrastructure elements, such as the construction of the plant, were not included. The bagasse was assumed to be obtained from an annexed 1G ethanol plant (hence no transport of feedstock).

The DA system (**Figure 1**) starts with the preparation of bagasse, where it undergoes a cleaning process to remove stones and significant impurities. Following this, the bagasse is subjected to pretreatment, involving the use of sulfuric acid and water. As a result, two outputs are obtained: a liquid fraction containing soluble sugars and inhibitors, which is concentrated through evaporation while simultaneously removing inhibitors, and a solid fraction predominantly comprising cellulose and lignin. This solid fraction is then directed to enzymatic hydrolysis, where the cellulose is broken down through the action of enzymes. The concentrated liquid fraction obtained from pretreatment and the hydrolysate obtained from enzymatic hydrolysis are fermented to ethanol using yeast able to ferment C5 and C6 sugars. Finally, a distillation step is carried out to obtain dehydrated ethanol. A wastewater treatment (WWT) step is included to handle the wastewater resulting from distillation, yeast propagation, and steam and power generation unit (red lines in **Figure 1**). In this, biogas is produced and sent to the steam and power generation unit. This unit will burn the solid waste from hydrolysis and fermentation, together with the biogas obtained from the WWT unit and sugarcane bagasse, to produce the electricity and steam required by the entire system.

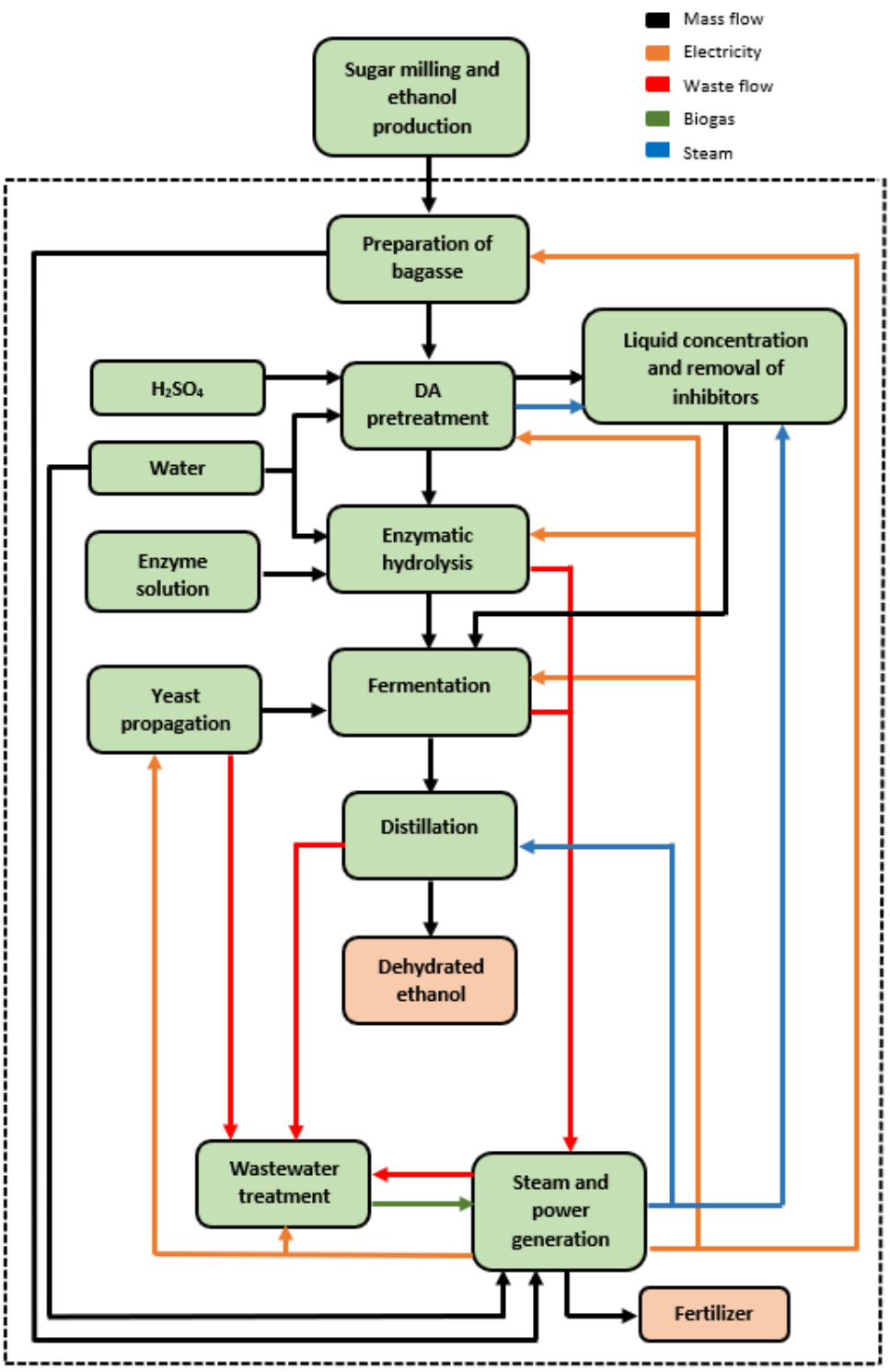

Figure 1. System boundary (dotted line) for DA pretreatment system, including the unit process considered (reference system).

The CO_2 system (**Figure 2**) shares the same processes for sugarcane bagasse preparation, hydrolysis, fermentation, distillation, wastewater treatment (WWT), and

combined heat and power (CHP) generation as the DA system. The key distinction between the CO_2 and DA systems is the pretreatment process. In the CO_2-based system, water is not utilized during biomass pretreatment. Instead, the biomass maintains for the pretreatment the same moisture content as that obtained from the preparation of bagasse. Consequently, only a solid fraction is obtained after pretreatment, with a very similar chemical composition than before pretreatment. It is then directed to enzymatic hydrolysis, continuing through the same process sequence as the DA system. As no liquid fraction is obtained and mild pretreatment conditions are used for pretreatment (more details found in section 3.2.), the need for concentrating sugars and removing inhibitors is avoided.

In addition to the mentioned systems where bagasse is used to produce ethanol, it is assumed that bagasse would be used for electricity production if not used for ethanol (counterfactual scenario). Hence, there is an 'opportunity cost'. Here, this is modeled by assuming the lost electricity production to be compensated by the current average grid electricity in Brazil (Electricity, high voltage {BR} market for electricity, high voltage | Cut-off, U). The electricity generated per kg of sugarcane bagasse ranges from 0.2 to 0.46 kWh/kg bagasse [31], [32]. To select the more cautious approach, the upper limit was chosen.

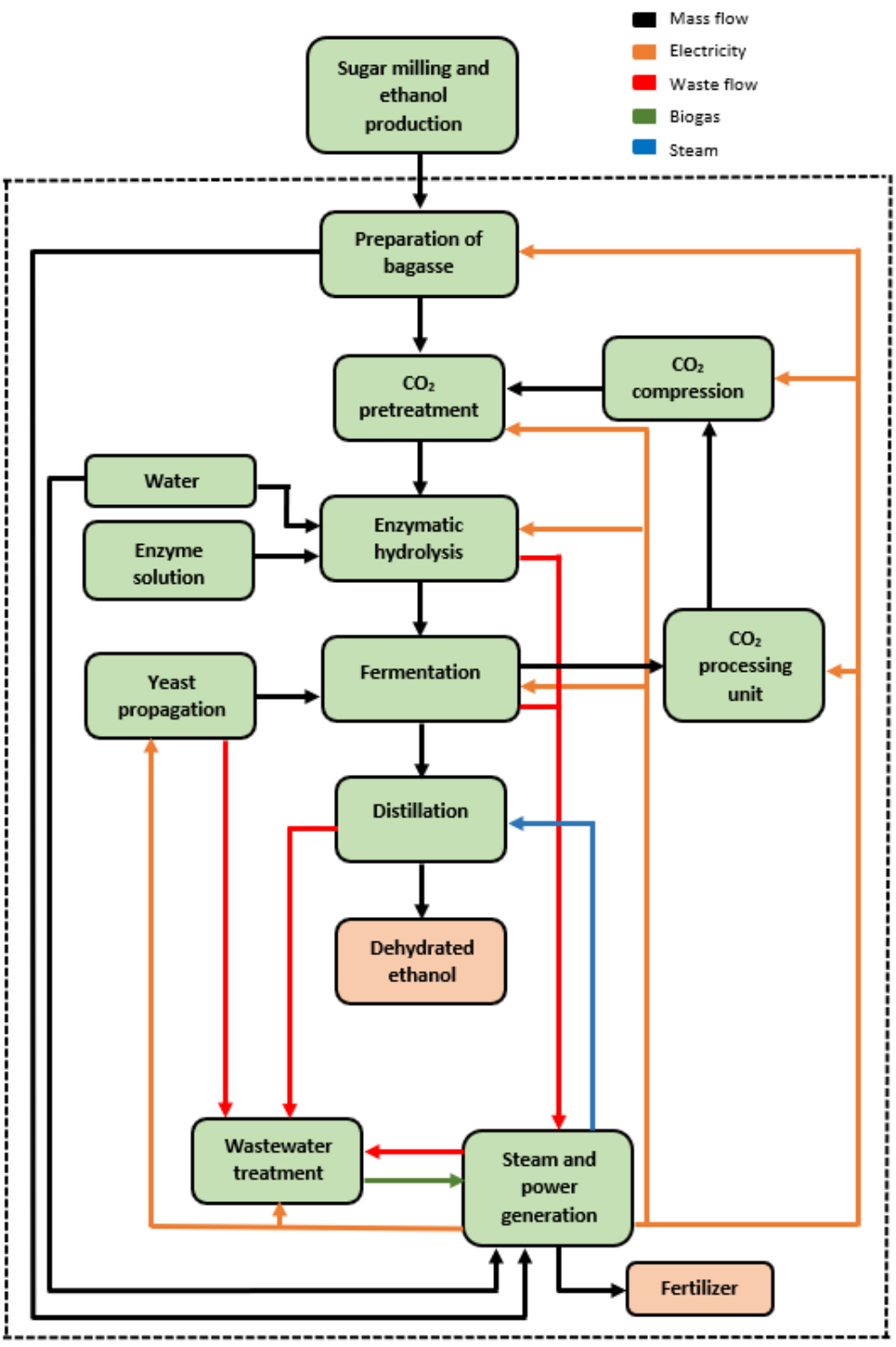

Figure 2. System boundary (dotted line) for CO_2 pretreatment system, including the unit process considered.

7.2.1.5.Environmental impact category

The impact category climate change was evaluated. It is measured in kg CO_2 eq. The life cycle impact assessment (LCIA) method used is ReCiPe 20216 Midpoint (H) V1.08 / World (2010) H.

7.2.1.6.Sources of data

Foreground data associated to the mass balances of the pretreatment steps associated with the DA and CO_2 systems was obtained from experiments performed in lab scale. Energy consumption was obtained using process simulation software Aspen Plus.

Electricity consumption from other processes was extracted and adapted from literature references as Maga et al. [26] and John Dees et al.[33] and will be specified throughout the explanation of each process subunit.

Background data from electricity generation, chemicals, and emissions was obtained from Ecoinvent v.3 database. The calculations were performed using SimaPro 9.5 software (PRé Sustainability, Netherlands).

Some assumptions regarding enzyme utilization and emissions were performed based on expert advice [A. B. Nielsen, Novozymes, personal communication May 23, 2023].

7.2.2. System description and inventory

An overview of the product systems for DA and CO_2 pretreatment systems covers 8 processes and are shown in **Figure 3** and **Figure 4**, respectively: (1) Sugarcane bagasse cleaning and pressing, (2) Pretreatment and (2.a) Evaporation (only included in DA system), (3) Enzymatic hydrolysis and filtration, (4) Yeast propagation, (5) C5/C6 sugar fermentation and filtration, (6) Distillation and dehydration, (7) Wastewater treatment and (8) Steam and power generation. The emissions related to enzyme production are included in the analysis although the process and input-output data table was not disclosed.

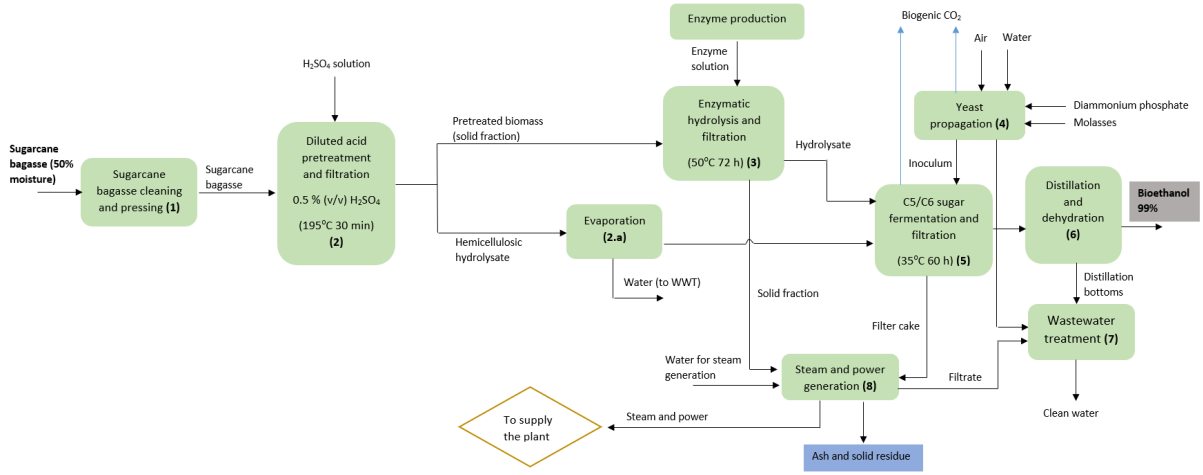

Figure 3. Flow diagram for DA pretreatment system (higher size image found in Appendix Figure S1).

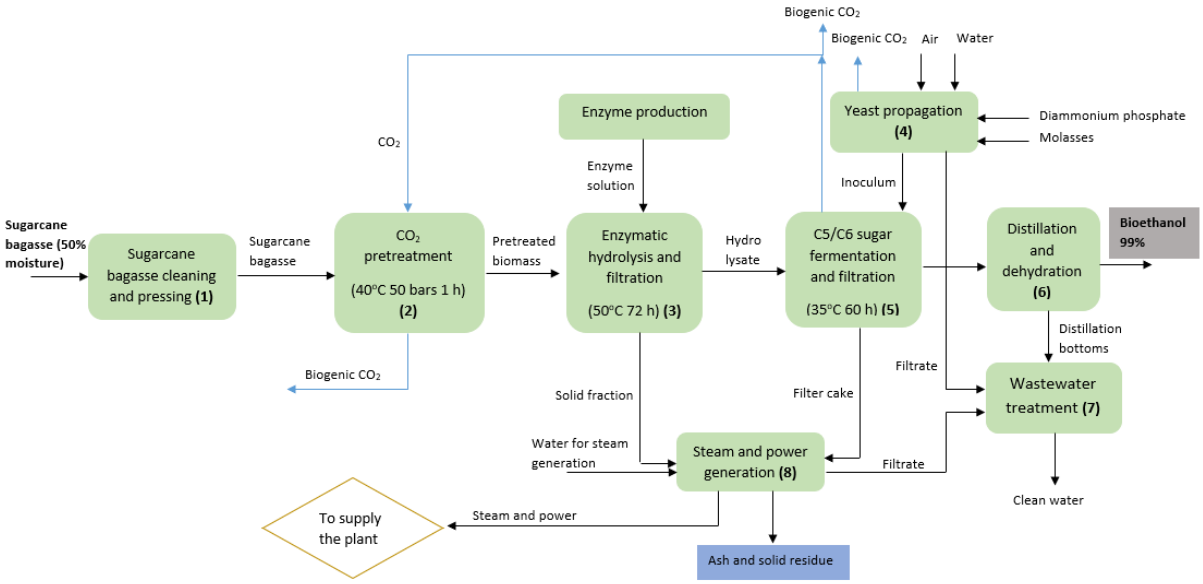

Figure 4. Flow diagram for CO_2 pretreatment system (higher size image found in Appendix **Figure S1**).

The main inputs and outputs of the DA and CO_2 systems are shown in **Table 1**. A detailed explanation of each process subunit is explained in this section. The inventory containing a detailed overview of the mass and energy flows of each process step is given in Supplementary material in **Tables S1-S8**.

Table 1. Input-output material flow for complete DA and CO_2 systems.

	Flow	Unit	DA system	CO2 system
Input	Sugarcane bagasse	t	1	1
	Sulfuric acid	t	0.013	-
	Enzymes	t	0.0061	0.024
	Water	t	3.2	1.7
	Molasses	t	0.013	0.017
	Diammonium phosphate	t	0.0018	0.0024
Output	Dehydrated ethanol	t	0.045	0.10
	Ash (recycle to the field)	t	6.4x10-3	4.5x10-3
	Solid after combustion	t	0.022	0.021
Emissions	Carbon dioxide into the air (biogenic)	t	0.65	0.49
	Carbon monoxide into the air (biogenic)	kg	0.005	0.007
	Dinitrogen monoxide into the air	kg	0.8x10-5	1.3x10-5
	Methane into the air	kg	2.4x10-3	2.4x10-3
	Non-methane volatile organic compounds (NMVOC), unspecified origin	kg	0.05	0.078
	Particulates, < 2.5 um into the air	kg	0.8x10-5	1.3x10-5
	Particulates, > 10 um into the air	kg	0.33	0.51
	Particulates, > 2.5 um into the air	kg	0.05	0.08
	Particulates < 10 um into the air	kg	0.35	0.29
	Nitrogen oxides into the air	kg	0.37	0.26

	Propanal into the air	kg	0.8x10-6	1.3x10-6
	Polycyclic hydrocarbons into the air	kg	3.2x10-4	2.2x10-4
	Sulfur dioxide into the air	kg	$1.8x10^{-3}$	0.003

7.2.2.1.Provision and preparation of sugarcane bagasse (1)

Sugarcane bagasse initially contains 50% moisture and undergoes a cleaning process to remove stones and other impurities. Then, it is pressed for further refinement and compaction.

The operation of provision and preparation at this point is equal for both scenarios and the electricity consumption was adapted to the results shown by Maga et al. [26]. The amount of sugarcane processed is lower than 1 tonne of bagasse as the remaining part is used for the generation of energy for sustaining the process.

*7.2.2.2.*Pretreatment (2)

7.2.2.2.1. System 1. Dilute acid (DA) pretreatment

This pretreatment was carried out at the lab using a 1 L stainless steel batch cylindrical reactor, which was placed in a silicone oil bath at a temperature of 195ºC for 30 min. A 10% solid load (w/w) and 0.5% (v/v) of sulfuric acid were used. The sugarcane bagasse used was provided by the company Raízen (São Paulo, Brazil). Prior to pretreatment, it 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). The composition of bagasse (cellulose, hemicellulose, lignin, and ash) was analyzed before and after pretreatment according to the NREL protocols [34], [35] and it is shown in **Table 2**.

It was assumed that the steam produced during the DA pretreatment process because of the high temperature is recovered and reused.

Table 2. Chemical characterization of sugarcane bagasse before and after DA pretreatment performed at the lab.

	Sugarcane bagasse	
Composition (wt%)	**Raw**	**Pretreated**
Mass yield	100	57 ± 1.6
Tot.lignin	25 ± 0.40	44 ± 0.87
Cellulose	45 ± 0.35	48 ± 1.4
Hemicellulose	22 ± 0.39	0.08 ± 0.70
Acetyl group	2.6 ± 0.02	0.45 ± 0.11
Ash	1.8 ± 0.12	6.7 ± 0.08
Extractives	3.7	0

After pretreatment, the solid and liquid fractions are separated by gravity using a filter. Thus, no energy is contemplated for this part. The solid fraction, mainly containing cellulose and lignin, is sent to enzyme hydrolysis, and the liquid fraction, containing the soluble C5 sugars and inhibitors (**Table 3**), is assumed to be evaporated using low pressure steam, obtained from the DA pretreatment and from the combined heat and power (CHP) unit, to concentrate the sugars and remove the inhibitors.

Table 3. Composition of liquid fraction obtained after diluted acid pretreatment of sugarcane bagasse performed at the lab.

Composition (g/L)	**Liquid fraction**
Glucose	6.2 ± 0.33
Xylose	1.2 ± 0.02
Acetic acid	29 ± 3.4
Arabinose	1.0 ± 0.12
Cellobiose	0.088 ± 0.048
HMF	14 ± 1.0
Furfural	15 ± 0.87

The determination of soluble sugars in the liquid fraction obtained after DA pretreatment was conducted using High-Performance Liquid Chromatography (HPLC). This analysis was performed employing a Dionex Ultimate 3000 high-performance liquid chromatography UHPLC+ Focused system, manufactured by Dionex Softron GmbH in Germany. The chromatographic separation was achieved using a Bio-Rad Aminex HPX-87H column (300 mm x 7.8 mm) operated at a temperature of 60 °C. A Shodex RI-101 refractive index detector was used in conjunction with a mobile phase consisting of 5 mM H2SO4, flowing at a rate of 0.6 mL min-1. The injection volume for each sample was 20 µL.
The process was modeled using the process software tool Aspen Plus (Aspen Technology, United States), where the electricity requirement was extracted for each sub-unit.

7.2.2.2.2. System 2. CO_2 pretreatment

Sugarcane bagasse from the same origin as in the DA system was finely ground as described before and then rehydrated to achieve a moisture content of 50% (w/w). It was then

pretreated at the lab using a SFE Lab 500 mL supercritical CO_2 extraction unit (SFE Process, France) for 1 h using a pressure of 50 bar at 40^{o}C. The composition of the biomass before and after pretreatment was determined as described before and it is shown in **Table 4**.

Table 4. Composition of sugarcane bagasse before and after CO_2 pretreatment performed at the lab.

	Sugarcane bagasse	
Composition (wt%)	**Raw**	**Pretreated**
Tot.lignin	25 ± 0.40	24 ± 0.30
Cellulose	45 ± 0.35	45 ± 1.37
Hemicellulose	22 ± 0.39	23 ± 0.83
Acetyl group	2.6 ± 0.02	2.7 ± 0.29
Ash	1.8 ± 0.12	1.8 ± 0.03
Extractives	3.7	3.6
Moisture	50 ± 0.25	44 ± 0.53

To measure the quantity of CO_2 required for the pretreatment, the CO_2 cylinder was located on top of a balance, and the weight of the cylinder was measured before and after pretreatment. It is assumed that the CO_2 is recirculated and reused, considering a 1% wt loss of CO_2 during the pre-treatment process. To compensate for these losses and supply the system with the necessary CO_2, it is assumed that it will be captured from fermentation. For this, a CO_2 capture and purification unit is needed, which data has been adapted from John Dees et al. [33].

The electricity requirement of the CO_2 pretreatment process and CO_2 compression was determined using Aspen Plus.

7.2.2.3.Enzymatic hydrolysis (3)

The enzymatic hydrolysis is assumed to be carried out at 50 ºC and 150 rpm for 72 h using a solid loading of 20%. It was assumed to reach 90% of glucose and xylose yield (w/w). The enzyme load to achieve the mentioned yield was assumed to be 77 and 134 g/kg cellulose for DA and CO_2 pretreatment, respectively. This choice was done taking into account two reasonings: the DA-pretreated biomass would be less recalcitrant and more exposed to the action of enzymes than the CO2-pretreated biomass, and thus, a lower enzyme load would be required to achieve the same yield; and that the CO_2-pretreated biomass contains a higher amount of polysaccharides to be degraded (cellulose, hemicellulose and lignin) while the DA-pretreated biomass only contains cellulose and lignin . Enzyme cocktail DA would be the enzyme cocktail used for DA system, which is optimized for DA pretreatment, and an enzyme cocktail adapted to CO_2 pretreated biomass composition (Enzyme cocktail CO_2) will be used for the CO_2 system. Based on this, an extra 75% dose was assumed for CO_2 pretreatment system. Sodium citrate buffer is assumed to be used to arrive to the required solid loading, and ampicillin to avoid the growth of bacteria (1 µl/mL). No enzyme recycling was considered.

Enzyme production was assumed to be taking place off-site, in Brazil, around 370 km from the ethanol plant. The analysis includes the emissions associated with enzyme production (cradle to gate) and transportation in Brazil. The environmental impacts of the production of Enzyme cocktail DA and Enzyme cocktail CO_2 were assumed to be equal, although the composition of both cocktails would be different.

After hydrolysis, a centrifugation step is assumed to be used to separate the soluble C5/C6 sugars contained in the liquid fraction from the solid fraction, which mainly contains lignin. This solid fraction is assumed to be dewatered and sent to the combined heat and power (CHP) unit.

7.2.2.4.Yeast propagation (4)

This step was extracted from Maga et al. [26] and adapted to the amount of inoculum required to reach the same yeast load for the fermentation of both scenarios under study. In this, it was assumed a recombinant yeast propagation from 0.5 to 20 g DW/L at a yield of 0.44 g biomass/g sugar. It was assumed to be carried out at 30 ºC for 30 h and using an aeration rate of 1 vvm (volume of air per volume of media per minute). The medium was assumed to be prepared with 60 wt% of sugarcane molasses and the stoichiometric supplementation of ammonium phosphate. The yeast (inoculum) was assumed to be separated from the media. Then, this liquid media is assumed to be sent to wastewater treatment (WWT). Due to a lack of the product sugarcane molasses in Ecoinvent 3.0 database, the available process of "molasses from sugar beet, at sugar refinery" was modified for using Brazilian energy mix and using sugarcane as raw material (Sugarcane {BR}| market for sugarcane | Cut-off,U).

7.2.2.5.C5/C6 fermentation (5)

An industrial recombinant yeast able to ferment both C6 and C5 sugars (i.e., glucose and xylose, respectively) is selected. The fermentation is carried out at 35 ºC for 60 h. The yeast load was 7.5 g DW/L [26]. The considered yields were 0.45 and 0.35 g of ethanol /g from glucose and xylose, respectively [26]. Glucose and xylose are assumed to be consumed entirely (100%). As mentioned above, the generated CO_2 is expected to be captured and reused during pretreatment.

*7.2.2.6.*Distillation and dehydration (6)

This part was adapted from Maga et al. [26], where a first step of concentration takes place until alcoholic content is between 92.6 and 93.8 (wt%). Then, these authors suggest the use of molecular sieves in an adsorption process to obtain anhydrous ethanol (99.3 wt%) from its hydrated form.

7.2.2.7.Wastewater treatment (7)

The liquid residue containing organic matter is sent to wastewater treatment (WWT), and it is composed of the filtrate after filter cake concentration obtained during the different filtration processes, filtrate from inoculum preparation and the water residue from the distillation unit.

The organic material present in these flows undergoes anaerobic digestion in the wastewater treatment unit, achieving an 81% efficiency in converting it into biogas. This biogas is used for steam and energy generation after being subjected to boiling, to remove impurities and moisture.

7.2.2.8.Cogeneration unit (CHP) (8)

To achieve on-site energy generation, steam and power generation is considered as in Maga et al [26]. In Brazilian sugarcane mills, cogeneration systems (CHP) are commonly used, which are founded on the Rankine cycle. The system assumes the utilization of efficient boilers and turbines designed to handle high-pressure steam (1000 bar and 530 ºC) and condensing steam turbines at 0.12 bar. The solid residue obtained after enzymatic hydrolysis, which is mainly composed of lignin, and the filter cake after fermentation are sent to CHP. Biogas produced in the wastewater treatment is co-fired with solid residues and sugarcane

bagasse to produce the steam and electricity required to feed the plant. The objective of incorporation of a portion of the sugarcane bagasse within the functional unit is to establish the integration of electricity and steam. With the utilization of 1 tonne of bagasse, the system accomplishes two outcomes simultaneously: ethanol production and the generation of energy required to feed the plant's demands. This integrated approach simplifies the assessment process by avoiding the need to account for excess energy generation or external energy requirement.

The quantities of ash and other solid residues produced in CHP will not be considered, as they are minimal and nearly identical in both scenarios, making their impact negligible for comparison.

7.3.Results and discussion

7.3.1. Lifecycle impact assessment (LCIA) and interpretation

The analysis included two scenarios in which sugarcane bagasse is used for ethanol production, DA and CO2 systems, which differ in the pretreatment process used (**Figure 5**). In these, the emissions associated with the replacement of gasoline for the ethanol produced in each system were considered (**Figure 5**. Gasoline label in horizontal axis). The emissions for average gasoline were considered 94 g CO2 eq/MJ following RED guidelines [30].

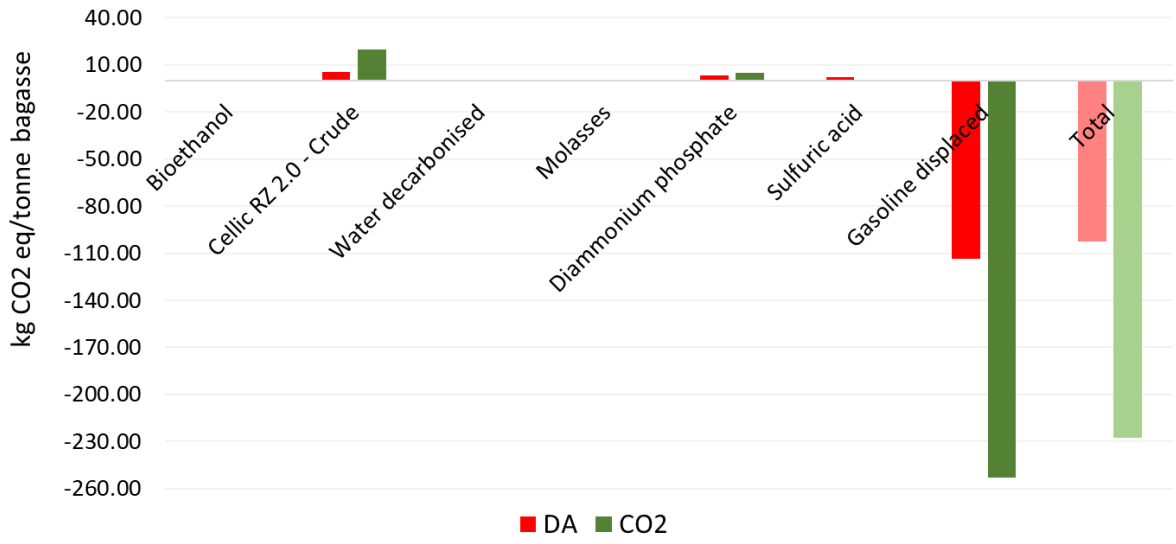

Figure 5. Climate change impact of using one tonne of bagasse for cellulosic ethanol with respectively diluted acid (DA) and CO_2 pretreatment.

In terms of climate change and considering gasoline replacement, the impact of the two evaluated scenarios for ethanol production is negative, being -102 and -228 kg CO_2 eq/tonne of bagasse for DA and CO2 systems, respectively. The CO2 system stands as the most favorable scenario. Considering ethanol production, the DA system yielded 45 kg of ethanol per tonne of bagasse, whereas the CO2 system produced 100 kg, which is reflected in the difference of impact regarding gasoline displacement. In the DA scenario, the hydrolysis process involves converting 80 kg of cellulose to ethanol per tonne of processed bagasse. On the other hand, the CO2 scenario involves the hydrolysis of 160 kg of cellulose per tonne of processed bagasse. This disparity arises from two facts, one being that in the DA scenario, a smaller quantity of sugarcane bagasse is allocated for ethanol production (576 kg) compared to the CO2 scenario (704 kg). The reason behind this difference is attributed to the higher energy demand in the DA scenario, which requires more allocation of biomass (bagasse) to the production of energy. The other fact is that during diluted acid pretreatment, 43% of the

solid content is lost in the form of inhibitors and soluble sugar monomers. Consequently, due to the extra 75% load required in the CO2 scenario and the hydrolysis of twice the amount of cellulose, the CO2 scenario's overall enzyme load is more than three times higher than that of the DA scenario. For this reason, the impact associated with enzymes is 6 kg CO2 eq/tonne of bagasse for DA system and 20 kg CO2 eq/tonne of bagasse for CO2 system, which is a 50 and 78% of the total emissions associated to the production of ethanol, respectively.

Diammonium phosphate emissions are also noticeable, being 3 kg CO_2/tonne of bagasse for the DA system and 5 kg CO_2/tonne of bagasse for the CO_2 system. This component is a nitrogen-based fertilizer used for yeast propagation prior to fermentation. The primary source of this emission arises from the chemical reaction used to synthesize the fertilizer [36].

The emissions of CO_2 per MJ of ethanol produced are depicted in **Figure 6**. The total ethanol emissions for both DA and CO_2 systems are similar and around 9 g CO_2 eq/MJ of ethanol. In both cases, the greenhouse gas (GHG) emissions reduction compared to average gasoline is 85 g CO_2 eq/MJ of ethanol. Considering the ethanol yield in each case, 45 kg for DA system and 100 kg for the CO_2 system, these savings translate to 102 and 227 kg CO_2 eq per tonne of bagasse, respectively (**Figure 5**). Although the CO_2 system presents a similar carbon intensity as in the DA system on a per MJ of ethanol basis, the disparity becomes evident when assessing emissions on a per tonne of bagasse basis. In this context, the GHG emission savings would be 122% higher compared to the DA system. This distinction is significantly relevant in scenarios where feedstock scarcity is a concern and when the highest climate benefit from one tonne of bagasse is sought.

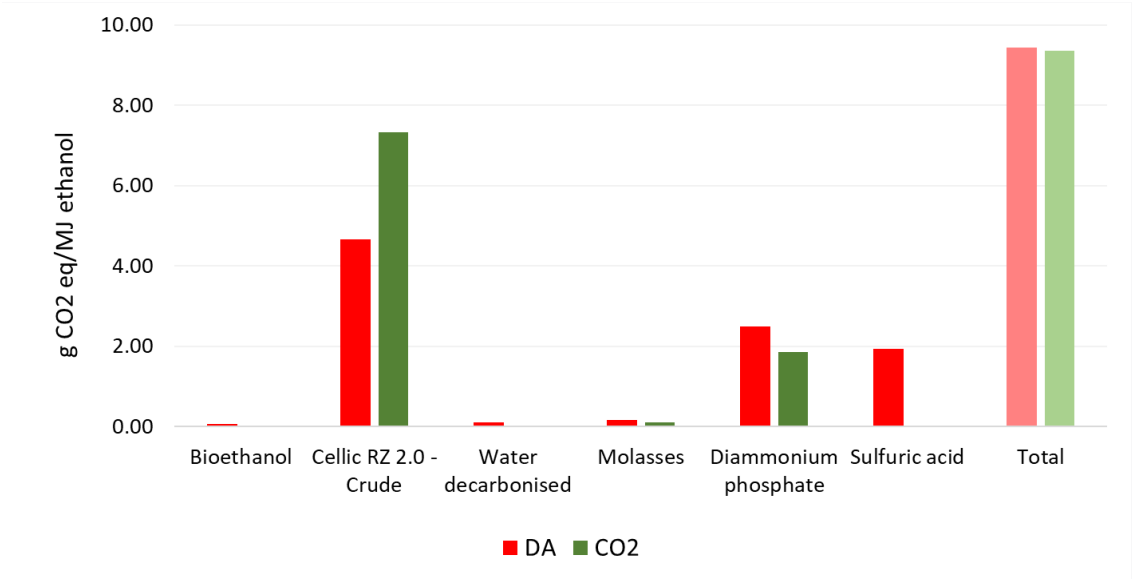

Figure 6. Climate change impact of diluted acid (DA) and CO_2 pretreatment scenarios per MJ of ethanol produced.

In addition, an 'electricity system' has been included where 1 tonne of bagasse is burned to produce electricity, which in turn is assumed to displace average Brazilian grid electricity on the grid. This scenario resulted in an impact of -122 kg CO_2 eq/tonne of bagasse. The DA system results in a higher climate change impact (-102 kg CO_2/tonne of bagasse) than the electricity production scenario. If the focus is primarily on reducing carbon emissions, the lower impact electricity from sugarcane bagasse might be more advantageous. However, it is necessary to consider the broader energy landscape and the specific limitations and opportunities of each energy source. Biofuels might still play a very important role in sectors where electrification is challenging, such as aviation, heavy-duty transportation, and remote areas that lack a robust electricity infrastructure. While low-carbon electricity is more readily accessible due to established renewable technologies, obtaining low-carbon biofuels presents complexities in terms of feedstock and process challenges. Thus, a careful assessment should be made, aligning both options with energy and sustainability goals.

7.3.2. Sensitivity analysis

Throughout the sensitivity analysis, various aspects were identified as critical and are examined in the sections below.

7.3.2.1.Lower enzyme impact emission

In this section, the reduction of enzyme production emissions was examined. Results are shown in **Figure 7** and **8**, where the baseline systems are compared to the lower emissions rate enzyme production. Considering the substantial contribution of the enzyme-related emissions in the overall climate change impact, implementing measures to decrease their impact (such as the improvement of enzyme production strains [37] or by increasing the production energy efficiency) can yield substantial reductions in CO_2 emissions. The emissions related to enzyme production for both DA and CO_2 systems were cut by 43%.

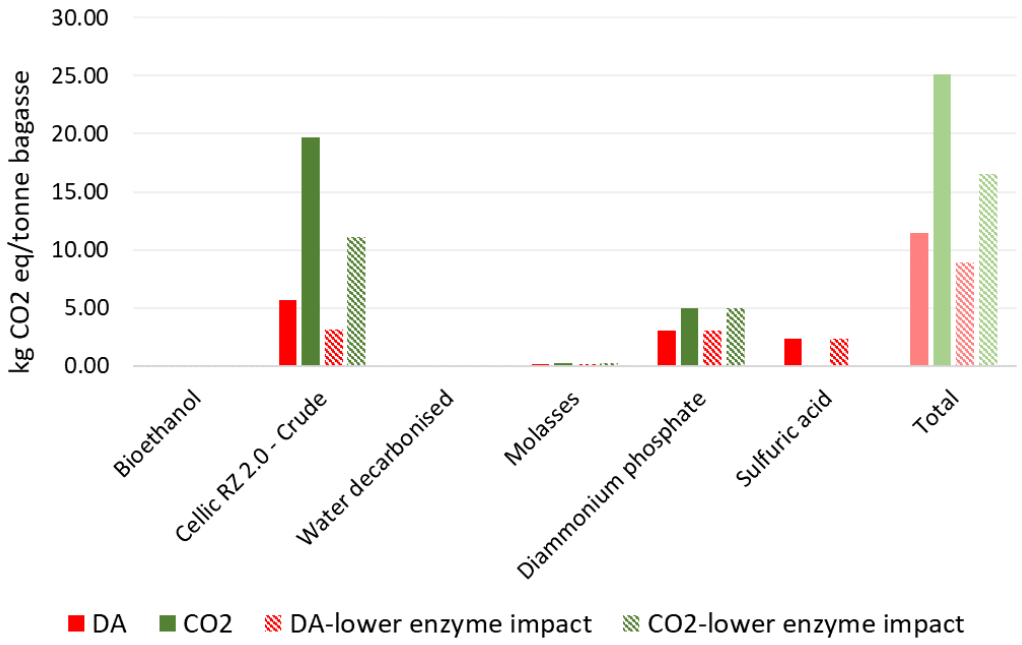

Figure 7. Implementing a 40% lower enzyme impact production process.

In contrast to the baseline scenarios, when considering a lower enzyme production impact, the CO_2 emissions per MJ of ethanol (**Figure 8**) were notably different for DA and CO_2 systems. The variation is attributed to the distinct enzyme impact contribution within each system. For CO_2 system, the reliance on a higher load of enzymes results in a higher contribution of enzyme emissions than for DA system. Consequently, the CO_2 system results in a more pronounced reduction in the overall emitted CO_2.

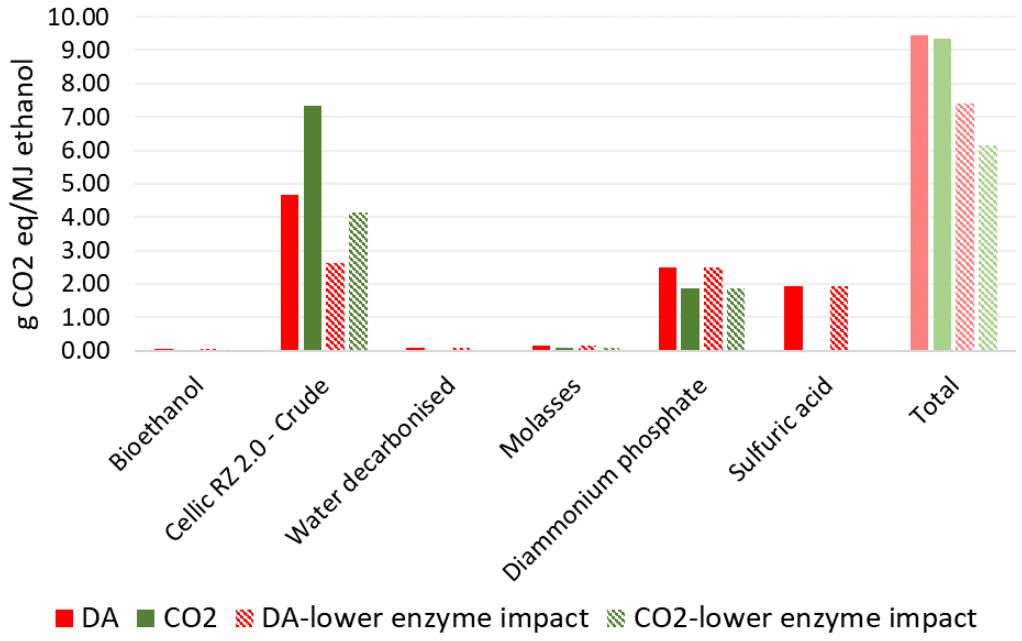

Figure 8. Implementing a 40% lower enzyme impact production process. Impact per MJ of ethanol produced.

7.3.2.2.Different enzyme load

Given the significant influence linked to the production of enzyme in both systems under examination, it is important to carefully evaluate the difference of enzyme load in each one. While the DA system considers a conventional pretreatment which results in a biomass product that is easily degradable, the CO_2 system considers a novel pretreatment

which obtained biomass is expected to be more recalcitrant and contains high amounts of hemicellulose. While an optimized enzyme cocktail exists for the conventional pretreatment, the formulation of the cocktail for CO_2 pretreatment has not been studied to the same extent as in the DA system. For this reason, it remains to be an unknown, and an enzyme load was estimated within a range guided by expert input [A. B. Nielsen, Novozymes, personal communication May 23, 2023].

Figure 9 and **Figure 10** show the sensitivity evaluation of different enzyme loads for the CO_2 system compared to the base DA system. The different employed enzyme loads have a substantial influence on the CO_2 emissions. When considering the carbon intensity of ethanol, it is important to note that only in the extreme scenario where a 100% extra enzyme load is applied to the CO_2 system, it results in higher emissions of CO_2 per MJ of ethanol than in the DA systems. Also, when using a lower enzyme dose, equal to DA system or 50% extra, the impact per MJ of ethanol is significantly lower in CO_2 than in DA system.

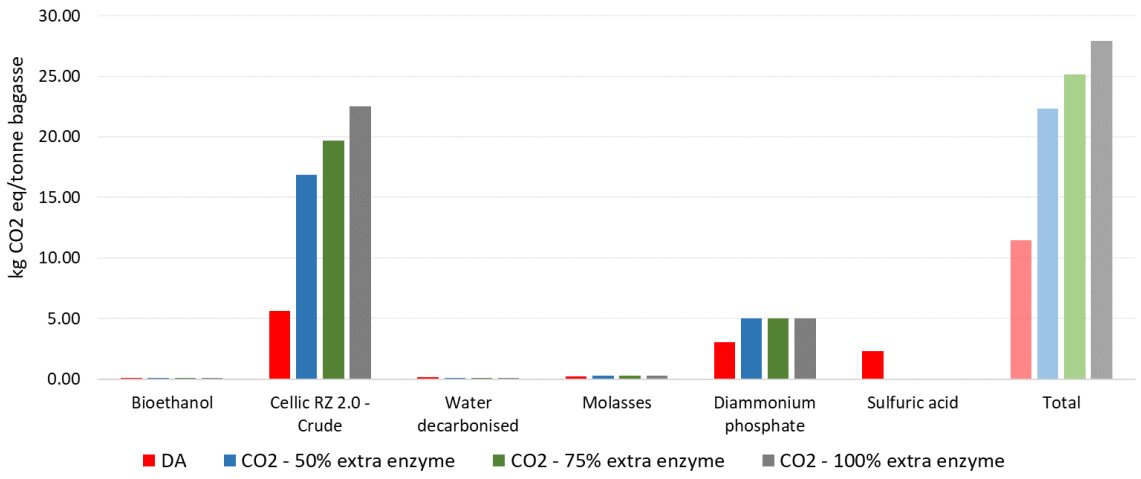

Figure 9. Using different enzyme load for CO_2 pretreatment scenario, CO2 – 50% extra enzyme: using 50% higher enzyme load for CO2 system than for DA system; CO2 – 75 % extra enzyme: using 75% higher enzyme load for CO2 system than for DA system (basis for the impact analysis); and CO2 –100 % extra enzyme: using 100% higher enzyme load for CO2 system than for DA system.

The impact of DA and CO_2 per MJ of ethanol with the same enzyme load is different, as the amount of enzyme/kg ethanol produced is lower for the CO_2 scenario. The reason for this is that the enzyme dose is calculated based on kg of cellulose in the pretreated solid. While for both scenarios the yield for cellulose degradation is equal, leading to the same portion of glucose that will be fermented, the hemicellulose content is higher in the CO2 system, leading to a higher portion of xylose that will be fermented to ethanol.

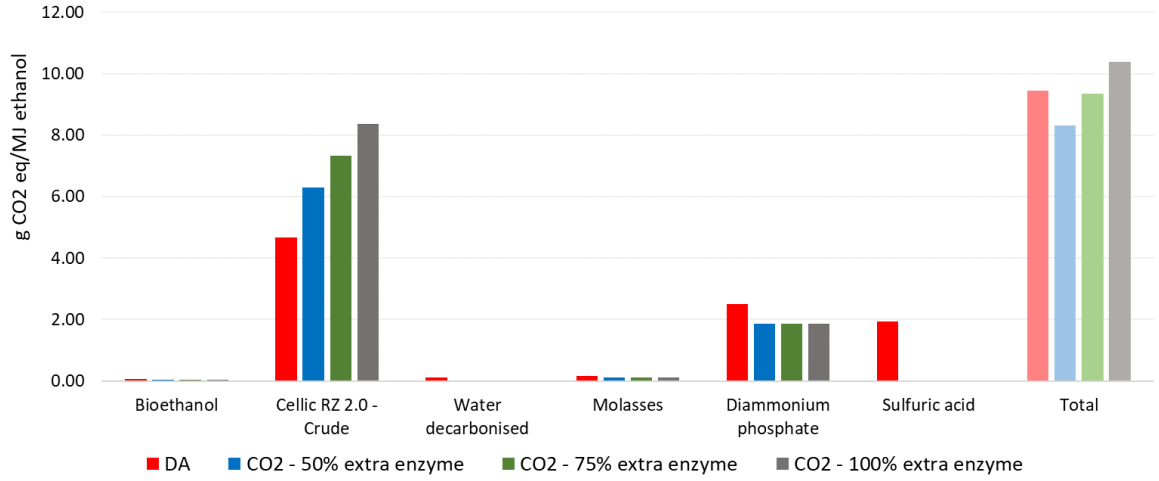

Figure 10. Using different enzyme load for CO2 pretreatment scenario: CO2 – 50% extra enzyme: using 50% higher enzyme load for CO2 system than for DA system; CO2 – 75 % extra enzyme: using 75% higher enzyme load for CO2 system than for DA system (basis for the impact analysis); and CO2 –100 % extra enzyme: using 100% higher enzyme load for CO2 system than for DA system. Impact per MJ of ethanol produced.

7.3.2.3.Modelling bagasse using system expansion with different marginal electricity sources

In this sensitivity analysis, three different scenarios using different assumptions about the electricity displaced if bagasse was used as fuel for electricity production were studied:

- Displacement of Brazilian electricity mix, as in base case scenario (Electricity, high voltage {BR} market for electricity, high voltage | Cut-off, U).
- Displacement of fully renewable energy (Electricity, high voltage {BR} electricity production, hydro, reservoir, tropical region | Cut-off, U).
- Displacement of coal-based electricity (Electricity, high voltage {BR} electricity production, hard coal | Cut-off, U).

The results of the analysis are depicted in **Figure 11**, showing the Brazilian electricity mix to be an intermediate between hard coal and renewable electricity displacement. The emissions of hard coal-based marginal electricity are much lower than both ethanol scenarios studied (DA and CO_2 systems). However, the situation changes when using more up-to-date electricity sources. When the displacement takes place with the Brazilian electricity mix, the emissions are 19% lower than the DA system and 46% higher than the CO_2 system. Finally, when analyzing the assumption of 100% renewable energy, the emission related to the displacement is 67 and 65% higher than the production of ethanol with the DA system and CO_2 system, respectively.

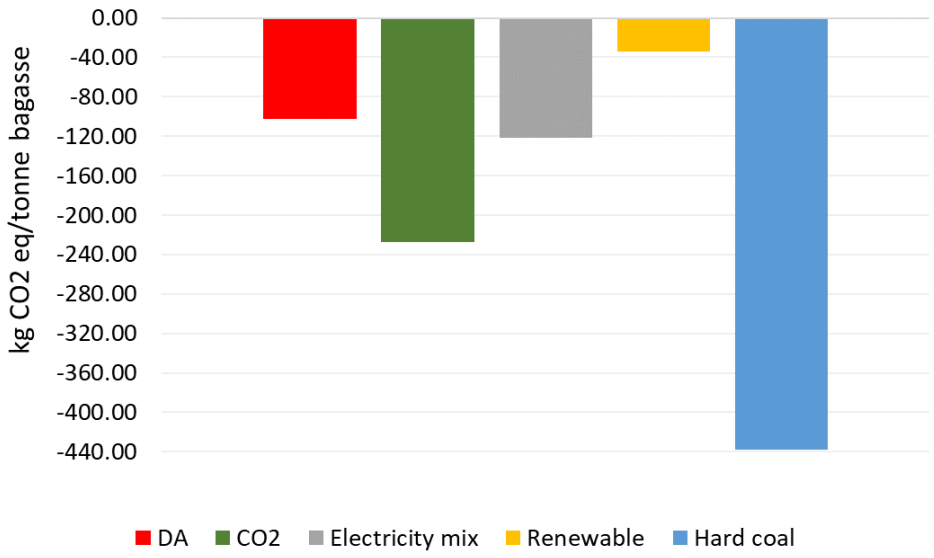

Figure 11. Total GWP impact of DA and CO_2 systems for the conversion of 1 tonne of bagasse to ethanol considering gasoline displacement (in red and green). Total GWP impact of the Brazilian electricity mix, renewable electricity, and hard coal-based electricity (in grey, yellow, and blue) displacement by the electricity produced by 1 tonne of bagasse.

7.3.2.4.Marginal gasoline emissions

In the base case scenario, the comparison of ethanol with the fossil fuel it replaces (gasoline) is done. The emission factor for average gasoline was considered. However, in practice, biofuels do not replace the average fossil fuels available on the market, but rather the marginal ones. These are the ones that would otherwise not be produced due to a sustained lower demand resulting from the adoption of biofuels. Over time, the share of high carbon footprint unconventional fossil fuels gradually increases in the total fuel supply. Thus, the greenhouse gas emissions of the average fuel consumption also increase. Also, even in the case of traditional oil production areas, as the larger existing fields are depleted, the effort needed for extraction grows, and smaller fields are brought into operation. Both factors contribute to a greater carbon footprint for conventional fossil oil. Thus, it becomes necessary to raise the emission factor to accurately account for these changes [38]. The analysis performed in the base case scenario was done considering a higher impact factor for marginal electricity of 115 g CO_2 eq/MJ. In **Figure 12**, it can be observed the comparison of GWP impacts of DA and CO_2 systems with the average and marginal impact factors considered.

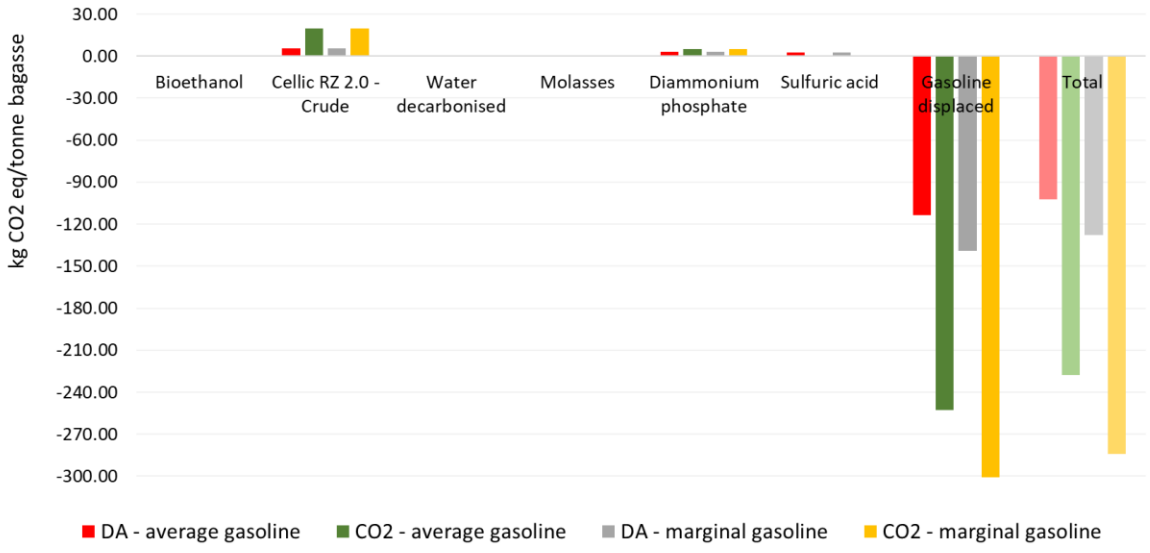

Figure 12. GWP impact of DA and CO_2 systems including average and marginal gasoline displacement.

As the impact factor for gasoline increases, the total emissions for both ethanol production systems are decreased when gasoline displacement is considered. The difference is increased in the CO_2 system as in this the ethanol yield per tonne of bagasse is higher.

7.5.Conclusions

Based on the data available in the literature, the data obtained in the lab, and several assumptions applied during the performance of this LCA, the proposed CO_2-based pretreatment system, showed clear advantages over the conventional diluted acid system. This is linked to the avoidance of extra water addition during pretreatment, which eliminates the necessity for the evaporation step required to concentrate sugar monomers before fermentation. In addition, the implementation of mild pretreatment conditions avoids the excessive biomass degradation and undesired conversion of polysaccharides into unwanted products, and it also eliminates the need for detoxification of inhibitors. It can be concluded

that the CO_2 impact per tonne of bagasse for the CO_2 pretreatment system was considerably lower than for the DA system. The sensitivity analysis showing alternative scenarios using lower enzyme production impact and lower enzyme dose showed that the CO_2 emission savings associated to CO_2 system can be further enhanced, and the difference when comparing to the DA scenario would be even greater. Thus, this novel approach enables the utilization of the full structure of the biomass for the extraction of fermentable sugar monomers.

Acknowledgments

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Chapter 8: Final discussion, conclusion and future perspectives

The work presented in this thesis contributes with useful knowledge for the discovery of a more sustainable and efficient fractionation approach for lignocellulosic biomass. It included the investigation of a novel pretreatment using CO_2 and the improvement of the subsequent hydrolysis of the pretreated biomass by the optimization of the enzyme cocktail formulation. In addition, the suitability of the CO_2-based pretreatment was evaluated in terms of its global warming impact potential and compared to a conventional pretreatment approach using diluted acid. For this, the implementation of each of the pretreatment approaches in a second-generation bioethanol production plant was evaluated using life cycle assessment.

In particular, the first part of the thesis involved the study of the novel pretreatment approach in sugarcane bagasse. As presented in Chapter 3, the best result was obtained when the pretreatment was performed under mild subcritical CO_2 conditions. In this case, the pretreated biomass showed an increase in glucose and xylose production of 27 and 36%, respectively, compared to the untreated sugarcane bagasse, after a subsequent enzymatic hydrolysis step. Furthermore, the biomass composition before and after pretreatment remained largely unaffected, demonstrating the potential of this pretreatment to fully utilize the polysaccharides contained in the biomass. The moisture content of the biomass was maintained at 50%, aligning with the moisture level found in sugarcane bagasse sourced from the sugarcane processing plant, and thus no extra water was used for the process. The use of

mild conditions prevented the degradation of polysaccharides and formation of inhibitory compounds, while the low moisture used avoided the obtention of a liquid liquor that conventionally presents low sugar concentration. Thus, one of the main conclusions in this chapter was that the utilization of the CO_2-based pretreatment has the potential to cut down an energy and cost-intensive step to concentrate the sugar monomers and eliminate inhibitor compounds of this liquid fraction prior to fermentation.

Following the thinking that the unique composition of the CO_2 or other mildly pretreated sugarcane bagasse (such as the one pretreated using hydrodynamic cavitation), containing almost the full composition of cellulose, hemicellulose, and lignin would require a unique enzyme cocktail formulation, the optimization and adaptation of a commercial cellulolytic enzyme cocktail was investigated.

The first step was the study of how to increase the efficiency of the enzyme cocktail by tackling LPMOs. Enhancing LPMO activity facilitates improved conversion of cellulose, as this enzyme has the capability to oxidize the crystalline structural parts of the polymer, thereby enhancing its digestibility. In the work presented in Chapter 4, different approaches were used with the aim of boosting the activity of LPMOs to obtain a higher production of sugar monomers during enzyme hydrolysis. Enriching the cellulolytic cocktail Cellic® CTec2 with extra lytic polysaccharide monooxygenases (LPMOs), a cellooligosaccharide dehydrogenase (CelDH), or the LPMOs co-substrate H_2O_2, resulted in enhanced sugar production during enzymatic hydrolysis of pretreated sugarcane bagasse, compared to the sole utilization of the cellulolytic cocktail. Particularly noteworthy was the impact of CelDH supplementation, which resulted in the most substantial glucose release over a period of 72 h, resulting in a four-fold increase in glucose content and a two-fold increase in xylose content when compared to the non-supplemented counterpart. Furthermore, the introduction of H_2O_2

and the use of extra LPMOs, also showed a positive impact on sugar production during the initial stages of hydrolysis.

The study about the optimization of commercial enzyme cocktails was continued in Chapter 5 but using a more recent generation of cellulolytic enzyme cocktail for the degradation of biomass, namely Cellic® Ctec3 HS, and the CO_2 pretreated sugarcane bagasse. This time the supplementation was not only addressed to boost LPMOs, but also to enhance the degradation of hemicellulose and reduce the unproductive binding of cellulases to lignin and their deactivation in the air-liquid interface. The cellulolytic cocktail was supplemented with H_2O_2, laccase, hemicellulase, and the surfactants Tween® 80 and PEG4000. The best result was obtained when using a concentration of 0.24 mM of H_2O_2, which resulted in an increase of 39% and 46% of glucose and xylose concentrations, respectively, compared to only using Cellic® Ctec3 HS.

The last work performed regarding enzyme optimization was presented in Chapter 6. With the objective of maximizing the hydrolysis efficiency of CO_2-pretreated sugarcane bagasse, the supplements showing the best performance in Chapter 5 were carefully selected with the purpose of boosting LPMO activity, enhancing hemicellulose degradation to enable space for the action of cellulases on cellulose and lower the non-productive binding of these to lignin. The combination of hemicellulases, H_2O_2, and Tween® 80 supplementation to the commercial enzyme cocktail was studied and the optimal dose for each of them was determined using statistical methods. Compared to only using Cellic® Ctec3 HS, the optimal formulation enabled an 84% increase in glucose release and 94% increase in xylose release.

The outcomes of the investigation presented in Chapters 4, 5, and 6 shed light on the feasibility of enhancing sugar extraction from lignocellulosic biomass subjected to mild

pretreatment, achieved through the strategic integration of a tailored enzymatic cocktail enriched with supplementary additives.

The last part of the thesis is presented in Chapter 7 and involves the investigation of using sugarcane bagasse for second-generation bioethanol production. The focus of this study was the evaluation of the environmental implications of the CO_2 pretreatment method and comparison with the diluted acid pretreatment method using Life Cycle Assessment. Results revealed that although the carbon intensity of both studied approaches is similar, the CO_2-based pretreatment allows a more efficient biomass utilization than the conventional diluted acid pretreatment, resulting in a lower CO_2 impact per unit of sugarcane bagasse.

Overall, the results obtained through this thesis pave the way for the exploration of new avenues in designing processes that are not only environmentally sustainable but enable the utilization of the complete biomass structure. This thesis holds a promising trajectory toward revolutionizing biomass fractionation practices on the fronts of sustainability and efficiency. Furthermore, the insights obtained from the Life Cycle Assessment could serve as an initial step in assessing the viability of the novel CO_2 pretreatment technology.

This thesis provides the lignocellulosic biomass conversion research area with a substantial knowledge foundation. It is the first time that CO_2 pretreatment conditions are applied using low water content, and thus, there has been little chance of finding enzyme cocktail formulations that are highly efficient for its hydrolysis. The main goal was to find sustainable and efficient solutions that could enhance the degradability of the lignocellulosic biomass. With every step performed through this journey, an increase in sugar production was observed. However, there is still room for improvement until this new fractionation approach can arrive at industrial implementation. The first one requires the obtention of a more efficient commercial enzyme cocktail adapted to the biomass composition pretreated by CO_2.

The use of accessory enzymes that facilitate a more complete utilization of the biomass would be key to developing processes that are less energy-intensive and do not rely on strong chemicals. It would also allow to increase the fermentable sugar recovery. Another approach will entail the implementation of CO_2 pretreatment on a larger scale and improving the effect of explosion created by the system.

Supplementary Materials: Chapter 7

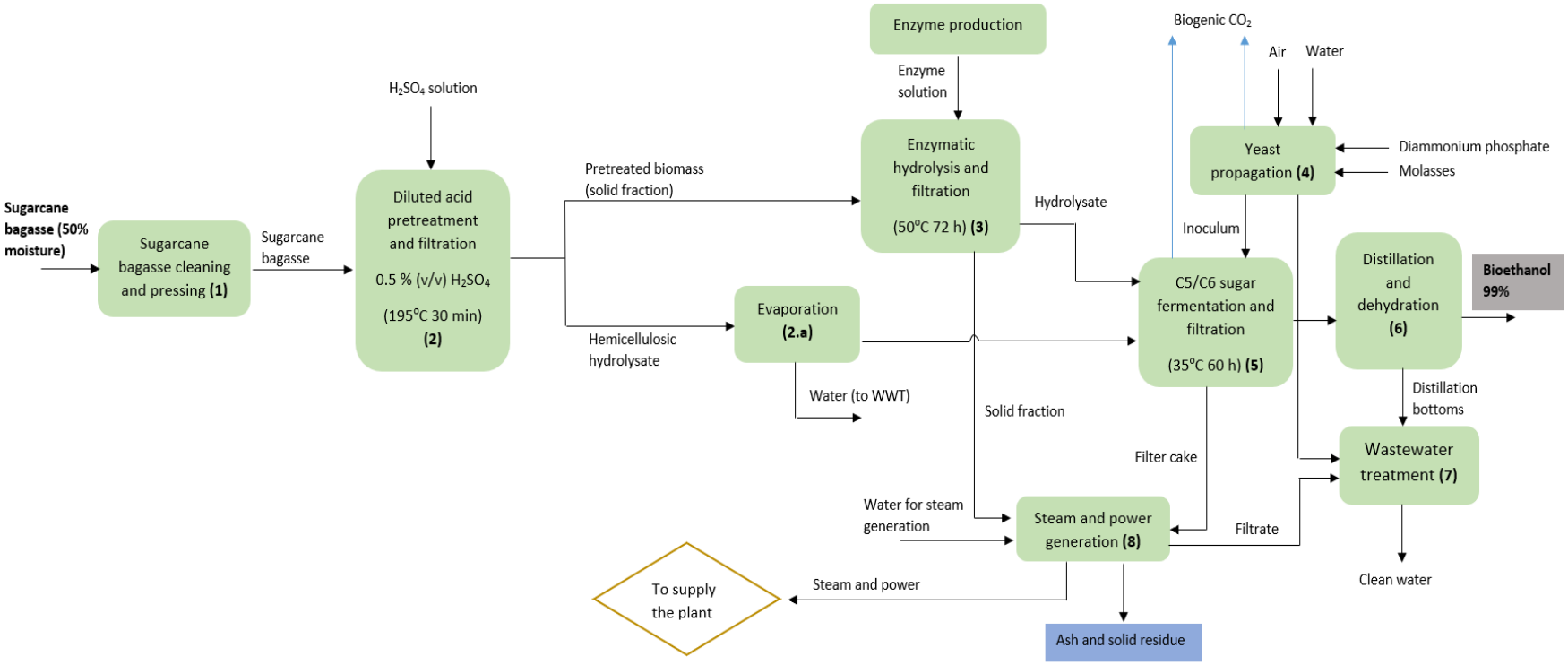

Figure S1. Flow diagram for DA pretreatment system

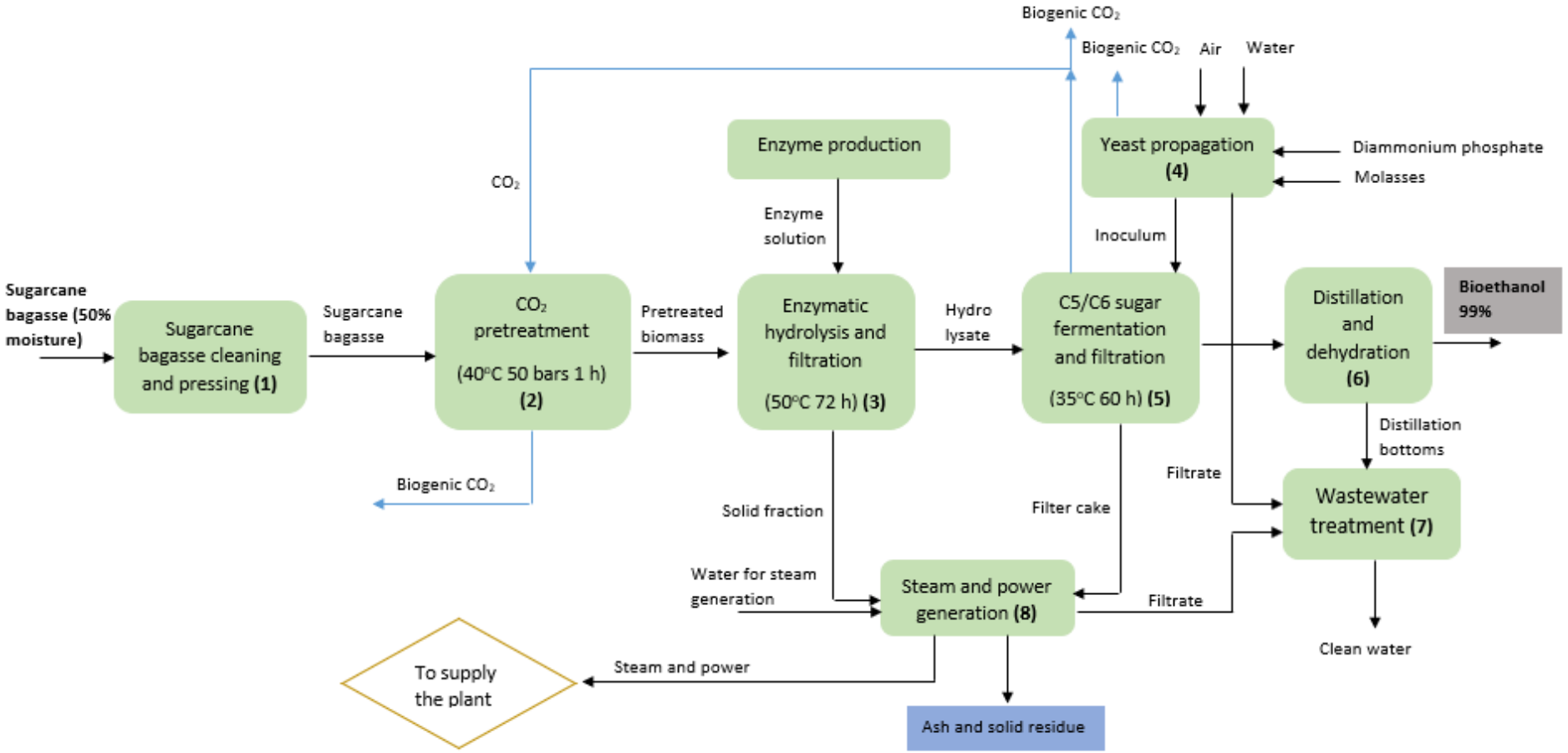

Figure S2. Flow diagram for CO_2 pretreatment system

Table S1. Input-output material flow for the provision and preparation of sugarcane bagasse.

	Flow	Unit	DA system	CO_2 system
Input	Sugarcane bagasse (50% moisture)	t	0.58	0.70
	Electricity	kWh	11	13
Output	Sugarcane bagasse (50% moisture)	t	0.58	0.70

Table S2. Input-output material flow for DA and CO_2 pretreatment system.

	Flow	Unit	DA system	CO_2 system
Input	Sugarcane bagasse (50% moisture)	t	0.58	0.70
	Electricity	kWh	125	63
	CO_2	t	-	0.039
	H_2SO_4	t	0.013	-
	Water	t	2.6	-
Output	Pretreated sugarcane bagasse for hydrolysis (44 % moisture)	t	0.32	0.66
	Liquid hemicellulose hydrolysate	t	2.8	-
	Steam recovered for evaporation	t	0.2	-

Table S2.a. Input-output material flow for evaporation needed for DA system.

	Flow	Unit	DA system
Input	Liquid hemicellulose hydrolysate	t	2.8

	LP steam	t	0.60
Output	Concentrate hemicellulose hydrolysate	t	0.29
	Water (to WWT)	t	2.5

Table S3. Input-output material flow for enzymatic hydrolysis of DA and CO_2 systems.

	Flow	Unit	DA system	CO_2 system
Input	Pretreated biomass	t	0.32	0.66
	Buffer + Amp	t	0.64	1.5
	Enzyme cocktail DA	t	0.0061	-
	Enzyme cocktail CO_2	t	-	0.021
	Electricity	kWh	26	56
	Cooling water (25-30 °C)	kWh	3.55	7.7
Output	Liquid hydrolysate for fermentation	t	0.81	1.46
	Solid fraction for energy cogeneration (50% DM)	t	0.18	0.30
	Enzyme cocktail DA	t	0.0061	-
	Enzyme cocktail CO_2	t	-	0.021

Table S4. Input-output material flow for yeast propagation process of DA and CO_2 systems.

	Flow	Unit	DA system	CO_2 system
Input	Diammonium phosphate	t	0.0018	0.0024
	Molasses	t	0.013	0.017
	Water	t	0.16	0.21

	Air	t	0.018	0.24
	Chilled water (5-10 °C)	kWh	0.27	0.37
	Electricity	kWh	2.9	3.8
Output	Carbon dioxide into the air (biogenic)	t	0.005	0.0069
	Inoculum	t	0.008	0.011
	Filtrate to be treated in WWT	t	0.17	0.22

Table S5. Input-output material flow for C5/C6 fermentation process of DA and CO_2 systems.

	Flow	Unit	DA system	CO_2 system
Input	Liquid hydrolysate for fermentation	t	0.81	1.5
	Concentrate hemicellulose hydrolysate	t	0.29	-
	Inoculum	t	0.008	0.011
	Chilled water (5-10 °C)	kWh	8	10
	Electricity fermentation	kWh	18	24
	Electricity CCS	kWh	-	8.7
Output	Fermentation broth for distillation	t	0.9	1.24
	Filter cake for energy cogeneration	t	0.07	0.09
	Carbon dioxide to carbon capture	t	-	0.04
Emissions	Carbon dioxide (biogenic, to the air)	t	0.09	0.08

Table S6. Input-output material flow for the distillation process of DA and CO_2 systems.

	Flow	Unit	DA system	CO_2 system

Input	Fermentation broth for distillation	t	0.9	1.2
	Low pressure steam	t	0.4	0.57
	Chilled water (5-10 °C)	kWh	5	6.3
	Cooling water (25-30 °C)	kWh	47	62
Output	Dehydrated bioethanol	t	0.045	0.10
	First distillation column bottom	t	0.8	1.0
	Second distillation column bottom	t	0.14	0.17

Table S7. Input-output material flow for wastewater treatment of DA and CO_2 systems (7)

	Flow	Unit	DA system	CO_2 system
Input	First and second distillation column bottoms	t	0.9	1.17
	Filtrate from yeast propagation	t	0.17	0.22
	Filtrate after residue concentration from energy cogeneration	t	0.54	0.28
	Water from evaporation	t	2.4	-
	Electricity	kWh	10	3.5
Output	Clean water	t	1.5	1.4
	Biogas (27% CH_4)	t	0.026	0.024
Emissions	Methane	t	7×10^{-5}	6.5×10^{-5}

Table S8. Input-output material flow for CHP of DA and CO_2 systems.

	Flow	Unit	DA system	CO_2 system
Input	Biogas (27% CH4)	t	0.026	0.024
	Residues to be dewatered	t	0.26	0.40
	Sugarcane bagasse (50% DM)	t	0.42	0.30
	Water for steam generation	t	1.09	0.86
	Air	t	2.29	1.82
	Cooling water (25-30 °C)	kWh	2.8	4.3
Output	Filtrate after residue concentration from energy cogeneration	t	0.54	0.28
	Ash (recycle to the field)	t	$6.4x10^{-3}$	$4.5x10^{-3}$
	Solid after combustion	t	0.022	0.021
	Condensed steam (after electricity generation)	t	0.013	0.021
	Steam at 13 bar for biomass treatment	t	0.015	0.023
	Low pressure steam (5 bar)	t	0.95	0.74
	Heat from flue gases to produce chilled water	kWh	13.31	20
	Electricity generated	kW	218	174
Emissions	Carbon dioxide biogenic	t	0.55	0.44
	Carbon monoxide into the air (biogenic)	kg	0.005	0.007
	Dinitrogen monoxide into the air	kg	$0.8x10^{-5}$	$1.3x10^{-5}$
	Methane into the air	kg	$2.4x10^{-3}$	$2.4x10^{-3}$

	Non-methane volatile organic compounds (NMVOC), unspecified origin	kg	0.05	0.078
	Particulates, < 2.5 um into the air	kg	$0.8x10^{-5}$	$1.3x10^{-5}$
	Particulates, > 10 um into the air	kg	0.33	0.51
	Particulates, > 2.5 um into the air	kg	0.05	0.08
	Particulates < 10 um into the air	kg	0.35	0.29
	Nitrogen oxides into the air	kg	0.37	0.26
	Propanal into the air	kg	$0.8x10^{-6}$	$1.3x10^{-6}$
	Polycyclic hydrocarbons into the air	kg	$3.2x10^{-4}$	$2.2x10^{-4}$
	Sulfur dioxide into the air	kg	0.0018	0.003