UNIVERSIDADE DE SÃO PAULO
FACULDADE DE MEDICINA DE RIBEIRÃO PRETO
DEPARTAMENTO DE BIOQUÍMICA E IMUNOLOGIA

EMANUELLE NEIVERTH DE FREITAS

Biomassa lignocelulósica da gramínea forrageira *Panicum maximum* Jacq produzida em condições simuladas de clima futuro: potencial fonte de açúcares fermentescíveis para bioenergia

RIBEIRÃO PRETO
2021

EMANUELLE NEIVERTH DE FREITAS

Biomassa lignocelulósica da gramínea forrageira *Panicum maximum* Jacq produzida em condições simuladas de clima futuro: potencial fonte de açúcares fermentescíveis para bioenergia

Tese de Doutorado apresentada à Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo para obtenção do Título de Doutor em Ciências – Área de Concentração: Bioquímica. “Versão corrigida. A versão original encontra-se disponível tanto na Biblioteca da Unidade que aloja o Programa, quanto na Biblioteca Digital de Teses e Dissertações da USP (BDTD)”

Orientadora: Profa. Dra. Maria de Lourdes Teixeira de Moraes Polizeli

RIBEIRÃO PRETO
2021

Freitas, Emanuelle Neiverth de

Biomassa lignocelulósica da gramínea forrageira *Panicum maximum* Jacq produzida em condições simuladas de clima futuro: potencial fonte de açúcares fermentescíveis para bioenergia.

175 p.: il; 30 cm

Tese de Doutorado apresentada à Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo para obtenção do Título de Doutor em Ciências – Área de Concentração: Bioquímica.

Orientadora: Polizeli, Maria de Lourdes T.M.

1. Mudanças climáticas; 2. Bioenergia; 3. Biorrefinaria; 4. Biomassa lignocelulósica; 5. Hidrólise enzimática; 6. Pré-tratamento; 7. Módulos de ligação a carboidrato.

Freitas, E.N. de. **Biomassa lignocelulósica da gramínea forrageira *Panicum maximum* Jacq produzida em condições simuladas de clima futuro: potencial fonte de açúcares fermentescíveis para bioenergia.** Tese apresentada à Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo para obtenção do Título de Doutor em Ciências – Área de Concentração: Bioquímica. Ribeirão Preto, 2021.

Aprovada em: ___/___/____

Banca Examinadora

Prof. Dr. ____________________________________ Instituição: __________________________

Julgamento: _________________________________ Assinatura: __________________________

Prof. Dr. ____________________________________ Instituição: __________________________

Julgamento: _________________________________ Assinatura: __________________________

Prof. Dr. ____________________________________ Instituição: __________________________

Julgamento: _________________________________ Assinatura: __________________________

Prof. Dr. ____________________________________ Instituição: __________________________

Julgamento: _________________________________ Assinatura: __________________________

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) -Brasil, do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) – Brasil (Processo 141466/2017-0) e da Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) – Brasil (Processos 2017/23989-9 e 2019/07141-5).

AGRADECIMENTOS

Ao finalizar este trabalho e esta etapa da minha vida, olho para trás e vejo que o trabalho aqui apresentado possui muitos autores, os quais contribuíram para seu desenvolvimento. Cada um de sua forma, cada um dando o seu melhor, desde suporte técnico, teórico e emocional. Todos foram essenciais para realização desse projeto, e também, da profissional e pessoa que hoje me tornei. Com todo meu carinho e admiração, quero expressar meus sinceros agradecimentos:

A Deus, que é o Senhor do meu passado, presente e futuro. Para Ele toda honra e glória, pela orientação de cada dia, por ter me fortalecido em todos os momentos que achei que não conseguiria. Essa vitória é d'Ele.

Aos meus pais, Dirce e Waldir (*in memoriam*), minha mais profunda admiração e reconhecimento. Por todos os sacrifícios que vocês fizeram para que eu pudesse conquistar meus objetivos e por sempre acreditarem em mim. Pai, tudo que eu queria nesse mundo era que você estivesse comigo compartilhando essa conquista, obrigada pelo exemplo que sua vida foi para mim e por ter aprendido tanto com você. Mãe, obrigada por me incentivar em todos os meus sonhos, mesmo quando eles me levaram para longe de você. Obrigada por cada palavra de conforto, por todos os ensinamentos, pelas noites de conversa, saiba que cada uma delas me mostrava como eu era capaz.

Ao meu irmão João Alberto e minha cunhada Andrya, por viverem esse sonho comigo, por terem olhado pela mãe nos momentos que eu estive fora, pelo apoio e companheirismo durante todos esses anos.

Ao meu namorado Bruno, por todas as conversas, por ouvir meus desabafos, pelas palavras que me fizerem crescer imensamente durante esses anos e, também, pelos ensinamentos de estatística. Obrigada por topar minhas loucuras e por me ajudar a planejar a realização dos meus sonhos. Seu apoio e sua presença foi a base para essa conquista. Agradeço também por ter me presenteado com minha segunda família: Cristiana, Aparecido e Gabriel, que são muito importantes para mim.

À minha orientadora, Profa. Dra. Maria de Lourdes Polizeli, por todo suporte e ensinamentos a mim dedicados durante todos esses anos. Obrigada por ter acreditado em meu potencial e pelas oportunidades que me foram abertas nesse período; se hoje defendo este trabalho foi porque tive seu auxílio em todas as etapas.

Aos meus colegas de laboratório e aos técnicos Mariana e Maurício. Obrigada por terem tornado meus dias de trabalho melhores e por todos os aprendizados. Levo de cada um muitas lembranças carinhosas.

A todos os docentes do Programa de Pós-Graduação em Bioquímica da Faculdade de Medina de Ribeirão Preto (FMRP) da Universidade de São Paulo. Sabemos como é difícil produzir ciência no Brasil, onde a educação é tão desvalorizada. Acompanhei a luta diária de vocês para manter a qualidade do ensino e da pesquisa, realizadas neste programa. Assim, expresso minha sincera admiração e meu reconhecimento.

To my supervisor in Canada, Dr. Jack N. Saddler who welcomed me into the FPB/B group with open doors for six months. I know how valuable your time is; thus, I am grateful for our meetings and all the knowledge you shared with me. Also, thanks to all my colleagues from the University of British Columbia (UBC), especially to Adam, Masa, and Vinay for all the teachings, generosity, and empathy I have received from you.

À Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (Processos 2017/23989-9 e 2019/07141-5) pelo apoio financeiro no Brasil e no exterior. Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Processo 141466/2017-0) e à Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pelo apoio financeiro, imprescindível para a realização deste trabalho.

Por fim, a todos que me incentivaram, auxiliaram ou me inspiraram durante os anos de desenvolvimento do meu doutorado.

Agora estou preparada para novos desafios e, também, para todas as bênçãos que Deus tem preparado para minha vida!

GENERAL INDEX

RESUMO..........1
ABSTRACT..........3
I. INTRODUCTION..........7
  1. Deconstruction of lignocellulosic biomass for fuels and chemicals..........8
  2. Chemical Composition of lignocellulosic biomass..........10
  3. Energetic potential of grassy biomass..........12
  4. Pretreatment of lignocellulosic biomass..........14
  5. Enzymatic hydrolysis of lignocellulosic biomass..........16
  6. Plants responses to climate changes..........23
II. OBJECTIVES..........26
1) Main objective..........27
2) Specific objectives..........27
III. RESULTS..........28
CHAPTER I..........29
  Climate change affects cell wall structure and hydrolytic performance of a perennial grass as an energy crop..........30
CHAPTER II..........77
  Enzymatic pretreatment with laccases from *Lentinus sajor-caju* induces structural modification in lignin and enhances the digestibility of tropical forage grass (*Panicum maximum*) grown under future climate conditions..........78
CHAPTER III..........107
  Structural and compositional changes induced by hydrothermal and organosolv pretreatments impacts enzymatic hydrolysis of a tropical forage grass grown under future climate conditions..........108
IV. GENERAL CONCLUSIONS..........159
V. ADDITIONAL INFORMATION..........162
  1. Articles published in journals (related to this thesis)..........163
  2. Articles published in journals (Not related to this thesis)..........163
VI. ATTACHMENTS..........165
VII. REFERENCES..........168

RESUMO

Freitas, E.N.de. **Biomassa lignocelulósica da gramínea forrageira *Panicum maximum* Jacq produzida em condições simuladas de clima futuro: potencial fonte de açúcares fermentescíveis para bioenergia**. Tese de Doutorado. Faculdade de Medicina de Ribeirão Preto, Ribeirão Preto, 2021.

A biomassa produzida por gramíneas de crescimento rápido como o gênero *Panicum* representa uma fonte em potencial para produção de energia renovável em biorrefinarias. Diante do cenário de mudanças climáticas, esse trabalho teve como objetivo estudar o impacto de condições simuladas de clima futuro no potencial bioenergético da forrageira *Panicum maximum*. Para isso, foi utilizado um sistema de campo (Trop-T-FACE) que combina aumento controlado de temperatura e enriquecimento de dióxido de carbono a fim de investigar o efeito isolado e combinado da concentração elevada de CO_2 atmosférico (eC) (600 μmol.mol^{-1}) e da temperatura elevada (eT) em 2°C a mais que a temperatura ambiente. Tais efeitos foram observados sobre a estrutura, composição química e acessibilidade às enzimas, assim como sobre a performance hidrolítica da forrageira através da aplicação de diferentes metodologias de pré-tratamento e da otimização da hidrólise enzimática. Nosso trabalho mostra que modificações na composição química da parede celular podem ter um importante papel nos processos de aclimatação de *P. maximum* a estresses abióticos, sendo que os tratamentos de elevada de temperatura resultaram no aumento do conteúdo de celulose, da razão Xilose: Arabinose, Siringil: Guaiacil, e da acessibilidade à celulose por enzimas hidrolíticas. Além disso levaram a redução da cristalinidade da celulose. Esses fatores podem ter resultado no aumento do rendimento de hidrólise enzimática encontrado nesse estudo para a biomassa de *P. maximum* não pré-tratada e crescida sob elevada temperatura. Além disso, o pré-tratamento se mostrou um processo que possui grande influência sobre os efeitos das mudanças climáticas na hidrólise da forrageira. Através da otimização e aplicação de um pré-tratamento utilizando lacase bruta de *Lentinus sajor-caju,* foi possível aumentar significativamente o rendimento de hidrólise, e não foram encontradas diferenças entre as condições climáticas estudadas. Os pré-tratamentos organosolv e hidrotérmico (Água Quente Líquida) também resultaram no aumento expressivo da conversão de glucano. Entretanto, os resultados relacionados às condições climáticas foram diferentes entre os pré-tratamentos. Para o organosolv os grupos cultivados no tratamento isolado de elevado CO_2 (eC) exibiram maior conversão de glucano em todas as condições testadas, enquanto para o hidrotérmico esse aumento foi encontrado apenas em concentrações elevadas de sólido durante a hidrólise. Nossos resultados sugerem que as diferenças de performance hidrolítica encontradas entre as biomassas crescidas em diferentes condições climáticas, assim como a intensidade das mesmas é dependente do pré-tratamento aplicado. Além disso, observamos que o tratamento de elevada temperatura tem efeito dominante sob o elevado CO_2, uma vez que o tratamento combinado (eT+eC) tiveram comportamentos semelhantes aos de elevada temperatura (eT). Por fim, esse trabalho mostra que as futuras mudanças climáticas podem influenciar na estrutura e composição da parede celular, assim como na performance hidrolítica da gramínea forrageira *P. maximum*, principalmente com relação a biomassa não pré-tratada, além de permitir compreender as adaptações biológicas e auxiliar na tomada de decisão de seleção de gramíneas para aplicação em biorrefinarias.

Palavras-chave: mudanças climáticas, bioenergia, biorrefinaria, biomassa lignocelulósica, hidrólise enzimática, módulos de ligação de carboidratos, pré-tratamento.

ABSTRACT

Freitas, E.N. de. **Lignocellulosic biomass of *Panicum Maximum* Jacq forage grass produced in simulated future climate conditions: a potential source of fermentable sugars for bioenergy**. Doctoral Thesis. Ribeirão Preto Medical School, Ribeirão Preto, 2021.

Biomass produced by fast-growing grasses such as the genus *Panicum* represents a potential source for renewable energy production in biorefineries. Given the climate change scenario, this work aimed to study the impact of future climate conditions on the bioenergetic potential of the forage grass *Panicum maximum*. For this purpose, a field system (Trop-T-FACE) was used to combine temperature increase and carbon dioxide enrichment to investigate the isolated and combined effect of the elevate concentration of atmospheric CO_2 (eC) (600 µmol .mol^{-1}) and the elevate temperature (eT) at 2°C more than currently temperature. Such effects were studied on the structure, chemical composition, and accessibility to enzymes, as well as on the forage grass hydrolytic performance through the application of different pretreatment methodologies and the optimization of enzymatic hydrolysis. Our work shows that changes in the chemical composition of the cell wall may play an important role in the acclimatization process of *P. maximum* to abiotic stresses. The treatments of elevate temperature resulted in increasements in cellulose content, Xylose: Arabinose and Syringyl: Guaiacyl ratio, and on cellulose accessibility to enzymes, moreover they led to reduction in cellulose crystallinity. These factors caused an increase in the enzymatic hydrolysis yields found in the study for unpretreated *P. maximum* grown under elevate temperature. Furthermore, the pretreatment process showed to have a great influence on the effects of climate change on enzymatic hydrolysis. Through the optimization and application of an enzymatic pretreatment using crude laccase from *Lentinus sajor-caju,* the hydrolysis yield was significantly improved and differences between climatic conditions were not noted. The organosolv and hydrothermal pretreatments (Liquid Hot Water) also resulted in a significant increase in glucan conversion. However, the results concerning to climatic conditions were different between the pretreatments. For organosolv, the group that grew under isolated elevate CO_2 (eC) treatment exhibited the greatest conversion yields in all tested hydrolysis conditions, whereas for hydrothermal this increase was found just for hydrolysis in higher solid load. Therefore, our results suggest that the differences in hydrolytic performance found between biomasses grown under different climatic conditions, as well as their intensity, are dependent on the pretreatment method applied. Likewise, we observed that the elevate temperature treatment has a dominant effect over the elevate CO_2 since the combined treatment (eT+eC) had similar behaviors to the elevate temperature (eT). Finally, this work shows that future climate changes can influence the structure and composition of the cell wall and, therefore, in the hydrolytic performance of the forage grass *P. maximum*, especially concerning untreated biomass, going beyond, and allowing us to understand the biological adaptations and assist in the decision-making of grass selection for application in biorefineries.

Keywords: climate change, bioenergy, biorefinery, lignocellulosic biomass, enzymatic hydrolysis, carbohydrate-binding modules, pretreatment.

I. INTRODUCTION

1. Deconstruction of lignocellulosic biomass for fuels and chemicals

Since the industrial revolution, the world has applied a linear economic model of "take, make & dispose" based on the presumption of abundant and inexpensive non-renewable resources (1). However, total world energy consumption is predicted to increase 48% from 2012 to 2040, and the linear economic model needs to be replaced to attend economy demands while preserving environmental needs (2). The new bioeconomic economic model created in the last decades supports the reuse and recovery of natural resources as an alternative to fossil and non-renewable resources (1). For this purpose, global research programs have been encouraged to discover new and sustainable energy supply as the global economy can no longer depend on fossil fuels that release high amounts of greenhouse gases (3).

The main driving forces for the development of sustainable energy sources include (i) uncontrolled rise of global atmospheric CO_2 predicted to increase to 550 μmol mol^{-1} by 2050 and average global temperature rise by 0.85 °C compared to the pre-industrial era, (ii) global increment in the energy demand, (iii) depletion of fossil fuel, (iv) international energy security, and (v) development of rural economic (4–6).

The realization of the circular bioeconomic model is based on the biomass-based biorefinery, in which abundantly available and renewable lignocellulosic biomass is converted into fuels and chemicals (1). The biorefinery concept developed a path forward to a society less dependent on fossil fuels. In addition, it contributed to mitigating global warming, mainly because it is considered to maintain net-zero CO_2 emission into the atmosphere because the CO_2 generated through the use of the resources is used in biomass production via photosynthesis (7).

A biorefinery represents the renewable equivalent of a petroleum refinery, but with the possibility to use the renewable lignocellulosic feedstocks to produce novel value-added chemicals and fuels that are otherwise not obtainable from fossils (Figure 1) (8). The International Energy Agency Bioenergy Task 42 defines biorefining as "the sustainable processing of biomass into a spectrum of marketable bio-based products (chemicals, materials) and bioenergy (biofuels, power, heat)" (9). Nonetheless, achieving this goal requires the selection of microorganisms capable of utilizing biomass as substrate and a deep understanding of biomass chemistry to assist in developing conversion technologies for improved product yield. Along with this, in-depth knowledge of the production technology and the environmental effects are crucial for establishing biorefinery systems (7).

Considering the energy content ($/GJ), lignocellulosic materials have their cost estimated to be 50% lower than other feedstock (3,10). The high production and low cost of lignocellulosic biomass confirm its potential as an abundant source for bioenergy generation. In terms of economic valuation, fine chemicals and other commodities that are derived from lignocellulosic biomass is the one with the highest potential to maximize the value of the bioenergetic chain since the products could be applied in various economic sectors (*e.g.*, pharmaceutical, petrochemical, construction, automotive, cosmetics, agroindustry, and others.). At the second of valuation are the biofuels and materials, followed by energy commodities, fertilizers, and pesticides (11).

Second-generation or cellulosic ethanol is the biofuel with the highest potential to substitute fossil fuels while increasing productivity without altering the planted area. Considering the worldwide basis, the cellulosic ethanol market is predicted to achieve 27 billion gallons/year by 2022, translating the world's strong demand for second-generation ethanol, notwithstanding troubles related to conversion technologies (12). In addition, a couple of other products produced in biorefinery have significant interest due to their commercial users, such as succinic, fumaric, malic, and glutamic acid, glycerol, sorbitol, and xylitol/ arabinitol, which nowadays are formed through the high-value replacement products (8).

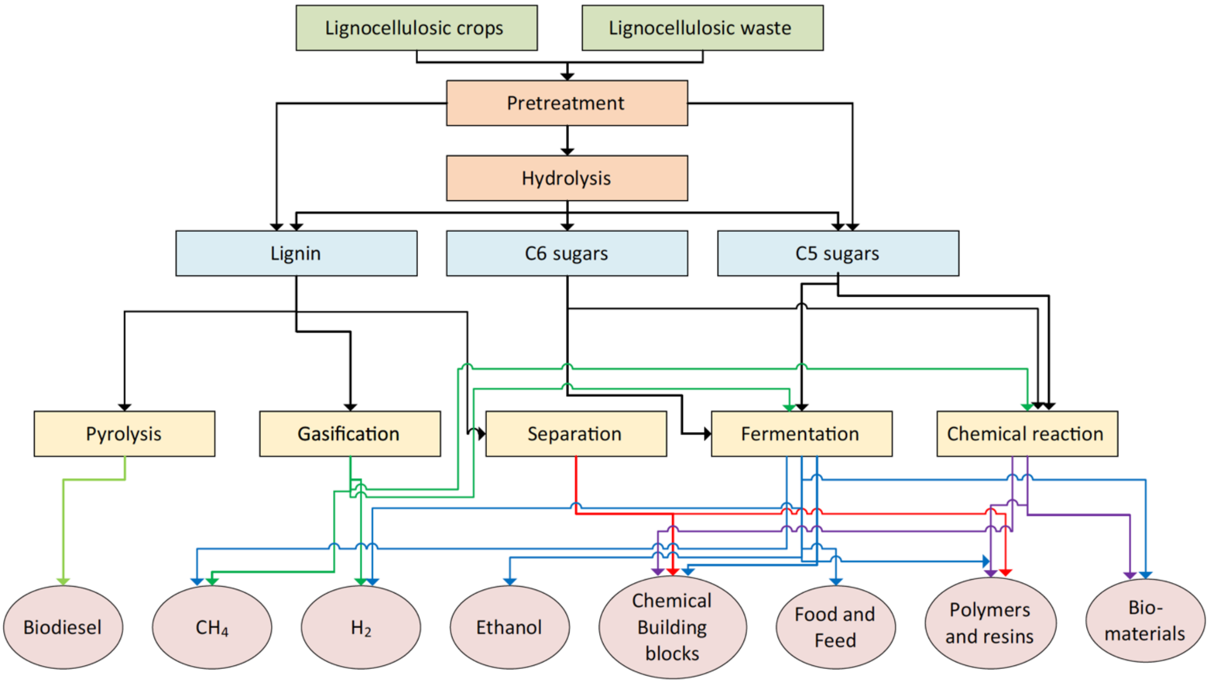


Figure 1: The biorefinery concept schematic illustration for the production of fuels and chemicals. Source: Galbe et al. (2019) (8).

2. Chemical Composition of lignocellulosic biomass

Lignocellulose is a material synthesized by plants to form a structure named cell wall, which is present in all cell types protecting against pathogens (3). Lignocellulosic biomass can be obtained from various sources such as agroforest residues, crops, trees, municipal waste, and microorganisms such as microalgae and fungi. Billions of tons of lignocellulosic materials are produced every year around the world; however, most of these residues are burned or discarded (1,7).

Polymeric carbohydrates (cellulose and hemicellulose) and lignin are the primary constituents of lignocellulosic biomass with minor amounts of proteins, pectin, extractives, and inorganic compounds. Nonetheless, the ratios among cell wall components and their structure will depend on several factors as age, species, and culture conditions (13).

Cellulose represents 25–50% of total lignocellulosic dry matter, while hemicellulose represents 20–40%, and lignin constitutes 15–25% of the entire content (14). The cellulosic component is composed of linear β-1,4-glucan chains that are tightly packaged into microfibrils and generally divided into two regions, one with low molecular order (called amorphous cellulose) and the other with high crystalline order (called crystalline cellulose). Likewise, the polymerization degree in cellulose is the highest among the lignocellulosic polymers and could range from 100 to 10,000 depending on its source, being responsible for cellulose's low flexibility (15). Hemicellulose polymers are branched heteropolysaccharides mainly composed of xylose, arabinose, glucose, and mannose, which hold some functional groups such as methyl, acetyl, glucuronic, and galacturonic cinnamic acids (16). Van der Waal interactions and hydrogen bonds are the main forces between cellulose microfibrils, whereas hemicelluloses bound cellulose fibrils surface through non-covalent linkages (13).

Lignin is produced to provide structural reinforcement to the plant tissue and consists of a phenylpropanoid polymer derived from three monolignols units: p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S), which may differ between species and cell tissue type. The differences in monolignols composition substantially affect the destruction of biomass and delignification (16). Lignin polymeric structures are assumed to arise from the polymerization reaction of phenoxy radicals formed by oxidative enzymes in the cell wall (17).

The cell wall constituents are intertwined with each other forming a complex and recalcitrant structure. Zhang (2008) found that only 0.0023–0.041% of the β-1,4-glycosidic bond of cellulose is accessible for enzymatic degradation (18). Hemicellulose is easier than cellulose to hydrolyze, whereas lignin polymer is resistant to hydrolysis and cannot be fermented to produce ethanol. However, it could be used in chemicals and resins production as part of the biorefinery process (3,14).

Furthermore, ferulic acid, generated through the phenylpropanoid pathway, can couple oxidatively with lignin and, due to its carboxylic group, has the ability to esterify arabinose residues from hemicelluloses establishing lignin carbohydrate complex (LCC) (Figure 2) (19). Thus, the formation of LCCs has an great contribution to cell wall recalcitrance against biological and chemical degradation. Other possible reasons for this resistance are considered to be (i) low accessibility of crystalline cellulose fiber that prevents cellulase attack and is also reported to have a role in cellulase adsorption and processivity and (ii) the presence of hemicellulose and lignin on the surface of cellulose blocking enzymatic to access the substrate, which is required for conversion (20,21).

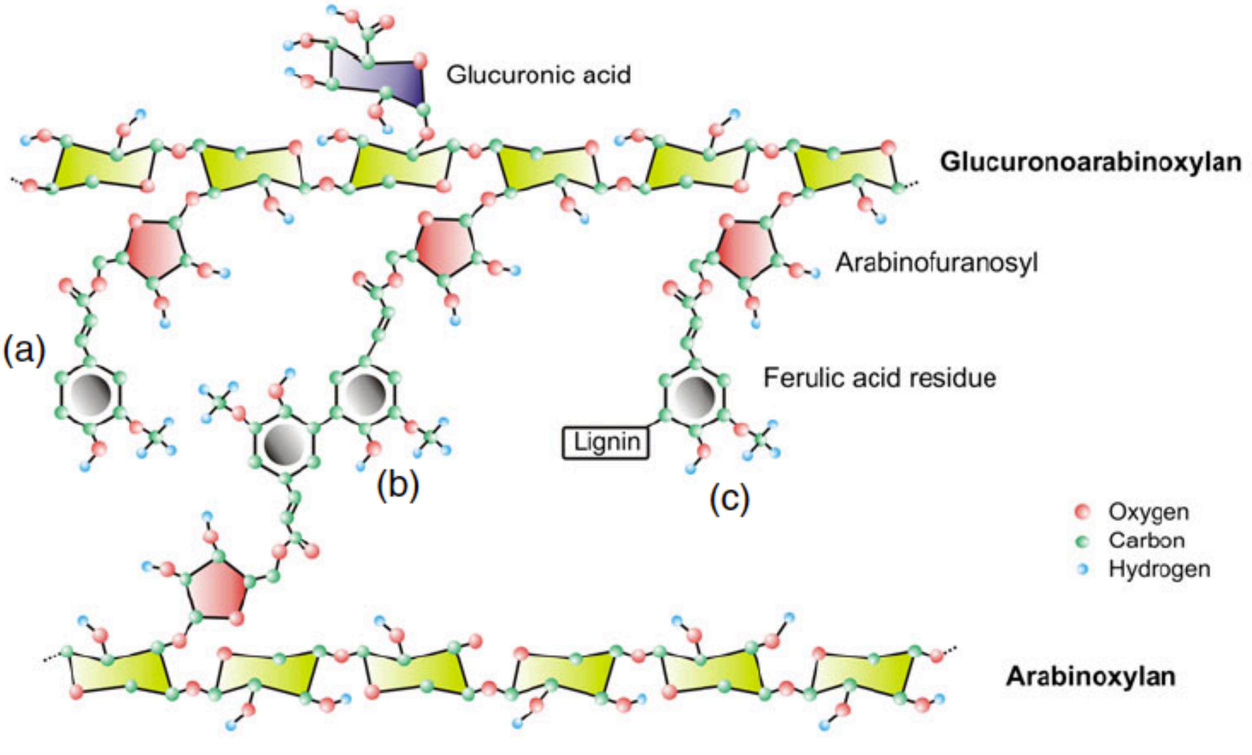


Figure 2: Ferulic Acid esterified with arabinofuranosyl residue of glucuronoarabinoxylan (GAX) (a); cross-linking involving diferulic acid (b); ferulic acid residue attaching lignin to GAX forming lignin carbohydrate complex (LCCs) (c). Source: Adapted from Oliveira et al. (2014) (19).

3. The energetic potential of grassy biomass

Currently, maize, sugarcane, soybean, and oil palm are the major crops used worldwide for biofuel production, including bioethanol and biodiesel. Sugarcane and maize are the main crops applied for bioethanol production in Brazil and the USA, respectively, although, in Europe, wheat and sugar beet are the primary crops (11,22). However, the fuel vs. food conflict coupled with the insufficient production of these crops for attending first and second-generation fuels imply the need to look for other crops that can be used for cellulosic ethanol to be cost-competitive with their sucrose and starch-based equivalents.

Despite the traditional crops, grassy crops are commonly used for dedicated bioenergy production. In this context, fast-growing C4 grasses have unique characteristics that provide great potential as lignocellulosic feedstock for biofuel production, especially for temperate and cold climate, such as high biomass yield, broad geographic adaption, and low mineral-nutrient inputs making it possible to grow in lands abandoned for agriculture uses (23). Furthermore, these raw materials are particularly sustainable since if the harvest is for bioenergy purposes, the feedstock can leave nutrient reserves in the soil that can be used in the next harvest (22).

These characteristics contribute to the use of the feedstock for biofuel and biorefinery purposes since it does not require extensive capital-intensive processes, making the final product economically feasible (24). *Miscanthus* spp. and switchgrass are fast-growing C4 grasses widely used in Europe and United States as dedicated bioenergy crops due to their high biomass yield (23). *Miscanthus* is harvested in several countries, including the United Kingdom (17,000 ha), Germany (15,000 ha), France (3,500 ha), and Ireland (2,200 ha) (11). Although commercial production of C4 grasses is limited nowadays, it has been considered promising for the near future.

Panicum maximum (Jacq. Cv. Mombaça) is one of the essential C4 grasses used as pasture in South America, mainly in Brazil. It has several advantages to be considered as a dedicated energy crop, such as greater efficiency in the conversion of solar energy into biomass through photosynthesis, tolerance in low/medium fertility soils, in addition to similar potential with sugarcane bagasse to cellulosic bioethanol production, 8571.0 L / ha for *P. maximum* compared to 8,478.6 L/ ha for sugarcane bagasse (25,26).

Although perennial grasses, like *P. maximum*, Miscanthus, and switchgrass, have higher are described to be more recalcitrant than annual grasses (e.g., sugarcane bagasse, wheat/rice straw, and corn stalks), they are deconstructed without considerable efforts. Glucuronoarabinoxylan (GAX) is the primary hemicellulose sugar found in grasses and is composed of xylose backbone with arabinose and glucuronic acid side chains that can be easily removed during pretreatment. Moreover, lignin is mainly composed of syringil (S) units, considered labile towards hydrolysis reaction, thereby being easily extracted (27). Ultra-structure studies suggest that grasses have great potential as lignocellulosic biomass for bioenergy purposes due to their easy hemicelluloses degradation and low lignin content (3).

4. Pretreatment of lignocellulosic biomass

The low saccharification rate of cellulose is mainly promoted by the steric hindrance imposed by cellulose by lignin, hemicellulose, and cellulose crystalline morphology. Usually, in the native form, less than 2% of the polysaccharides from lignocellulosic biomass undergo enzymatic digestion. Therefore, the pretreatment process is an utmost important part of the lignocellulosic-based industry to reach conversion of cell wall polysaccharides into a significant number of fermentable sugars (3).

Furthermore, pretreatment is the most energy-demanding step in biomass conversion and can contribute to more than 40% of the total processing (28). Thus, this challenge encouraged the investigation for technologies to achieve scalable, efficient, and greener pretreatments. The key goals for biomass pretreatment are; (a) to reduce crystallinity index of cellulose and increase the cellulases accessibility, (b) to decrease cellulose and hemicelluloses association, (c) solubilization of hemicelluloses and lignin, and (d)) lignin structure modification and redistribution (29).

Among all the constraints that impact hydrolysis of lignocellulosic biomass by cellulolytic and hemicellulolytic enzymes, lignin is described as the main one (30). This polymer has several effects that decrease the hydrolysis rate, acting as a physical barrier to cellulose and hemicellulose, inhibiting enzymes due to lignin by-products, and also because cellulase enzymes non-specifically bind to lignin, which leads to losses on catalytic activity (30,31).

It is difficult to define an ideal pretreatment to be applied for all raw materials and situations. The type and composition of particular biomass represent crucial selection factors in choosing a pretreatment method since different physical and chemical properties demand different approaches (3). One technique might be effective for some types of biomass but may not be for others. However, some important features must be fulfilled for a method to be a potential pretreatment technology, such as high carbohydrates recovery, low formation of inhibitory compounds as furfurals and organic acid, low energy requirement, value all compounds present in lignocellulosic materials not only cellulose, and minimal effluent generation (32,33).

Generally, the pretreatment methods are divided into four categories: physical (e.g., milling, grinding, irradiation, sonication); chemical (e.g., acid, alkali, acid, organic solvents, oxidizing agents, and ionic liquids); physicochemical methods (e.g., hydrothermal pretreatments w/wo catalyst,); or biological methods (e.g., enzymatic or microorganisms) (Table 1) (34). Combinations of two or more methods are also possible to found. Typically, on chemical and physical methods, temperatures range from 140 to 200 °C and a residence time from minutes to hours (8).

Among the factors that could influence pretreatments effects on biomass (temperature, residence time, severity factor, and others), the pH significantly affects a pretreatment step. At low pH, most hemicelluloses are removed from the solid material and released as monomeric sugars, while cellulose is kept almost intact. Working at neutral or around neutral pH leads to partial hemicelluloses hydrolysis due to organic acid formation resulting in the autohydrolysis process; still, most hemicellulose stays in oligomeric form since the conditions are not severe enough. In contrast, lignin is dissolved at alkaline pH, while most cellulose and hemicellulose are retained in the solid fraction (8). Consequently, the selection of the pretreatment method depends on the final application.

The employment of ligninolytic enzymes in biological pretreatment is a potential alternative to the pretreatment step since it does not require high energy demand and no chemical is applied in the process; however, their production costs need to be considerably reduced (35). The on-site production of ligninolytic enzymes is an alternative to reduce production and operation costs significantly (36). Metabolic engineering of microbial strains of industrial interest constitutes a tool that modifies the protein expression and regulation, increasing the yield of ligninolytic enzymes. Some examples of successful engineered strains to improve ligninolytic enzyme production are discussed in Li et al. (2016a-b) (37,38).

Table 1: Commonly utilized pretreatment methods.

Pretreatment method	Active agent	Mode of action
Dilute-acid	H_2SO_4, H_3PO_4, and other strong acids	Hydrolysis of hemicelluloses
Alkali	NaOH, Na_2CO_3, and similar alkaline compounds	Extraction of lignin
Hydrothermal	High-temperature and pressure; w/wo catalyst	Hydrolysis of hemicelluloses
Ionic liquids	Large organic cation and a small inorganic anion	Fractionation of polymers
Organosolv	Organic solvents, e.g., ethanol, butanol; w/wo catalyst	Extraction of lignin
Milling/grinding	Particle size reduction	Surface increase and improved access
Biological	Degradation of the material	Brown-rot: hemicelluloses and cellulose White-rot: lignin Soft-rot: cellulose

Source: Adapted from Galbe and Wallberg (2019) (8).

5. Enzymatic hydrolysis of lignocellulosic biomass

Subsequently to the pretreatment step, the pretreated biomass needs to be broken down into monomeric (C5 and C6) sugars that can be converted into ethanol or other chemicals through the fermentation step. The degradation of lignocellulosic material into simple sugars can occur either enzymatically or chemically. However, enzymatic hydrolysis is a better choice since the energy input is lower, conducted in mild environmental conditions, and fewer fermentation inhibitors are generated (30).

Due to the plant cell wall complexity and heterogeneity, it is necessary to use a mixture of different types of enzymes to efficiently hydrolysis of cellulose and hemicellulose compounds.

Microorganisms play a crucial role in the production of enzymes for biomass saccharification. Any bacterial or fungal strain that grows on lignocellulosic biomass as a carbon source presents all required enzymes to convert polysaccharides into monomeric sugars (30).

Nonetheless, the hydrolysis efficiency of their enzymatic preparations is low; the reason is that although the microbial strains secrete all cellulolytic enzymes, the production levels are limited and not in an optimum proportion. Hence, to get maximum hydrolysis, an enzymatic cocktail in high protein titer and balanced ratio are required (39).

It is already known that none of single enzymes is capable of promoting biomass hydrolysis efficiently. Therefore, enzymatic cocktails with different enzymes promote synergism among hydrolytic and accessory enzymes and play an important role in boosting hydrolysis. Furthermore, the cocktail should speed up the saccharification rate and minimize the amount of enzymes required in the process; thus, it could reduce the cost of sugar generation (29).

In most cases, enzymatic extracts from a single microorganism are not enough to degrade cellulosic materials in an optimum manner, considered to be the conversion rate higher than 70% within 48 h hydrolysis with more than 10% solid loading at economic enzyme amount. Consequently, adding extracts from diverse microbial sources is essential to make it satisfactory (39). The fungi strains with the most industrial interest for the cellulolytic enzyme are *Trichoderma* spp., *Rasamsonia* spp., *Penicillium* spp., *Aspergillus* spp., etc. They are described to produce the core cellulase enzymes as cellobiohydrolases, endoglucanases, and β-glucosidases, and other essential enzymes to biomass deconstruction (Figure 3). *Trichoderma reesei* is the leader of cellulase production because of its availability, high production ability, and popularity (40,41).

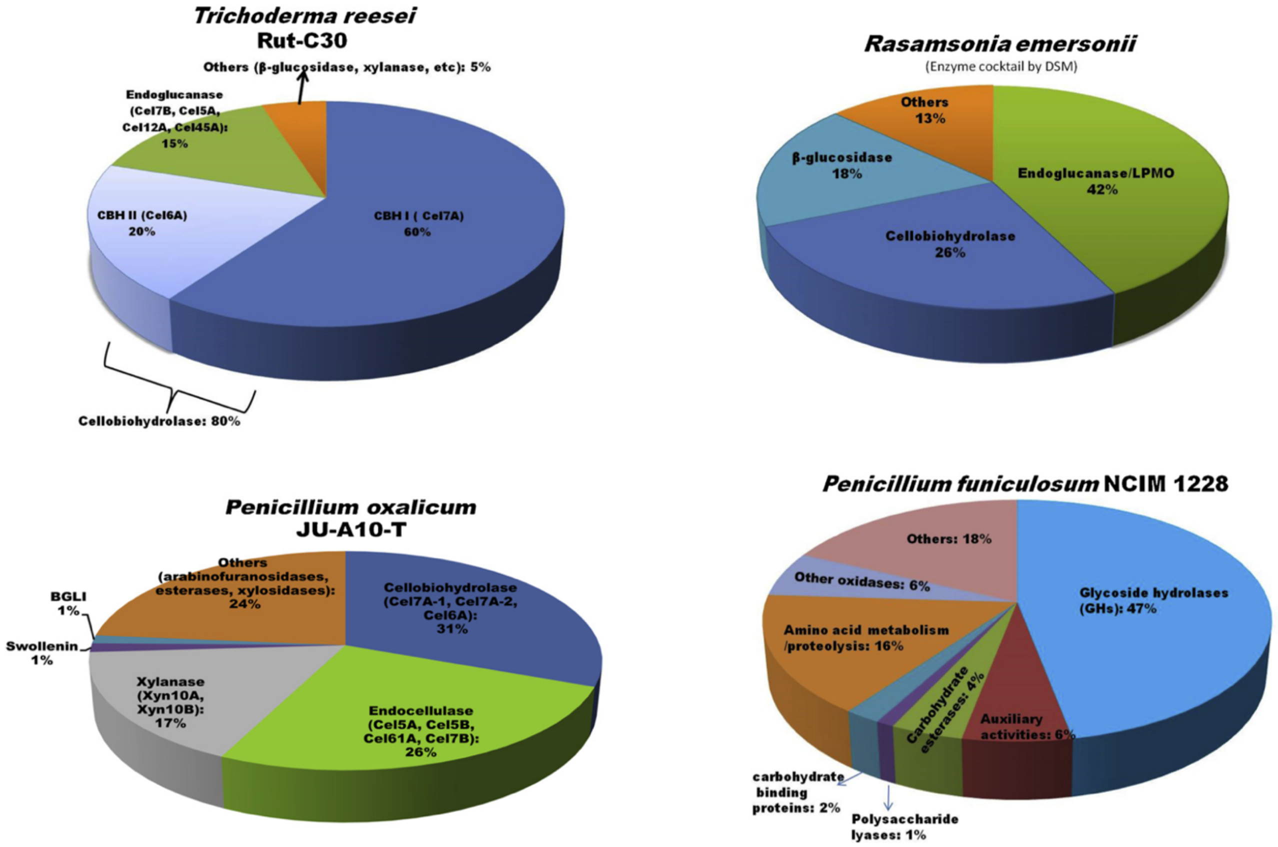


Figure 3: Carbohydrate-active enzymes/proteins (CAZy) extracellular secretion by fungal strains of industrial relevance. Source Adsul et al. (2020) (29).

Improving enzymatic bioconversion of lignocelluloses to bioethanol involves some challenges that might be overcome to commercial applications, such as (I) selection of proper enzyme sets with the ideal amount of each enzyme to optimize the synergistic effects; (II) enzymes that can work effectively at high solid loadings of biomass; (III) reduce unspecific adsorption and improve catalytic efficiency of enzymes; and (IV) reduce end-product inhibition of β-glucosidases (30,36). In addition, biomass composition and structure have a significant influence on enzymatic hydrolysis. Therefore, the category of pretreatment and the biomass applied are essential parameters to be considered for enzymatic cocktail development (29). Cocktails enzymes that can overcome the aforementioned challenges and fit in different post-pretreatment scenarios are under development using different research approaches.

The enzymes that should be composing enzymatic cocktails necessary for the complete hydrolysis of lignocellulose feedstock are core cellulolytic, hemicellulolytic, and accessory enzymes. These enzymes are included in different CAZy families and are discussed above.

5.1 Core cellulase enzymes

At least three classes of cellulolytic enzymes are essential for enzymatic hydrolysis of cellulose: endo-β-1,4-glucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21), commonly termed the "core cellulase mixture" (Figure 4). The cellobiohydrolases group is composed of two glycosyl hydrolase families (GH): GH6 (EC 3.2.1.91) and GH7 (EC 3.2.1.176), which act on β-1,4-glycoside bonds in nonreducing and reducing ends of cellulose, respectively, releasing cellobiose. Endoglucanase (EC 3.2.1.4) hydrolyze the β-1,4-glycoside bonds in amorphous regions of cellulose, while β-glucosidases (EC 3.2.1.21) acts on cellobiose and cellodextrins to release glucose. β-glucosidases play an important role as catalysts in lignocellulosic degradation since this enzyme release glucose, used *by Saccharomyces cerevisiae* in the fermentation process to produce ethanol(42).

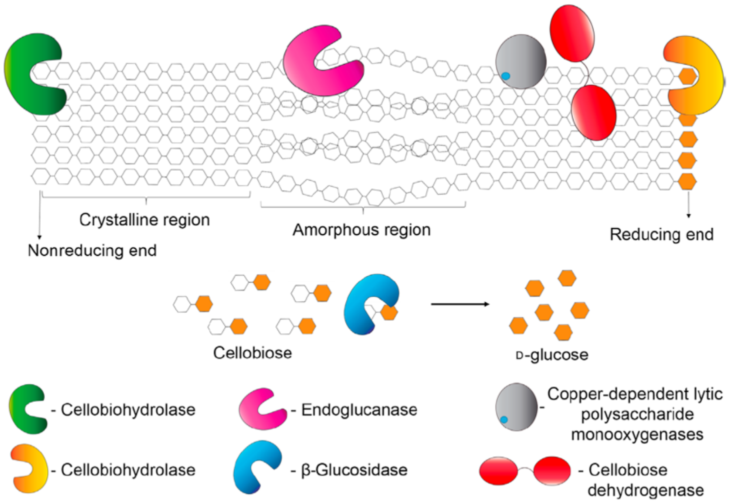


Figure 4: Representation of enzymes from core cellulase mixture and accessory enzymes involved in cellulose degradation. Source: Polizeli et al. (2016) (42).

5.2 Hemicellulolytic enzymes

Since hemicelluloses is a hetero and branched polymer composed of different monomeric units and functional groups, its debranch and degradation to C6 and C5 sugar need the cooperation action of numerous enzymes (Figure 5). These enzymes assist in hemicellulose removal and increase the effectiveness of cellulase's attack by exposing the cellulose microfibrils and increasing the incidence of paracrystalline sites, which favor cellulase binding (43).

The enzymes endo-β-1,4-xylanase and (EC 3.2.1.8) and β-D-xylosidase (EC 3.2.1.37) are the most important xylan-degrading enzymes, especially for arabinoxylan (AX) from grasses. While endo-β-1,4-xylanase cleaves glycosidic linkages in the internal part of the xylan backbone, releasing xylose and xylooligosaccharides, the β-D-xylosidase enzyme hydrolyzes β-1,4-D-xylans, xylooligosaccharides, and xylobiose from nonreducing ends, releasing xylose as the product (29,44).

The hydrolyze of L-Arabinoses units from heteropolysaccharides requires the action of arabinanases (EC 3.2.1.99) and arabinofuranosidases (EC 3.2.1.55). Arabinanases acts in the α-1,5-arabinofuranosidic bonds between arabinose residues present on arabinan to release arabinose in mono or oligomeric form (45). Arabinofuranosidases hydrolyze, from the nonreducing end, the α-1,2, α-1,3, and α-1,5- glycosidic linkages in arabinan, arabinoxylan, and arabinogalactan to arabinofuranosidic residues (46).

As previously discussed, glucuronic acid residuals are also part of hemicelluloses, as glucuronans and glucuronoglycan. β-glucuronidases (EC 3.2.1.31) and xylan α-1,2-glucuronidases (EC 3.2.1.131) hydrolyze glucuronic acid.

Xyloglucan endo-β-1,4-glucanases (EC 3.2.1.151) involve the hydrolyze of xyloglucan hemicellulases, consisting of a β-1,4-glucan backbone with xylosyl side chains linked at O-6 position of glycosyl residue (42).

Finally, for the deconstruction of mannan hemicelluloses, which are formed by a linear backbone of D-mannose/glucose with galactosyl side groups, endo-1,4-β-mannosidase (EC 3.2.1.78) and β-mannosidases (EC 3.2.1.25) are needed. The former hydrolyzes linkages in the mannans' backbone, whereas the latter hydrolyze mannans from the nonreducing end (47).

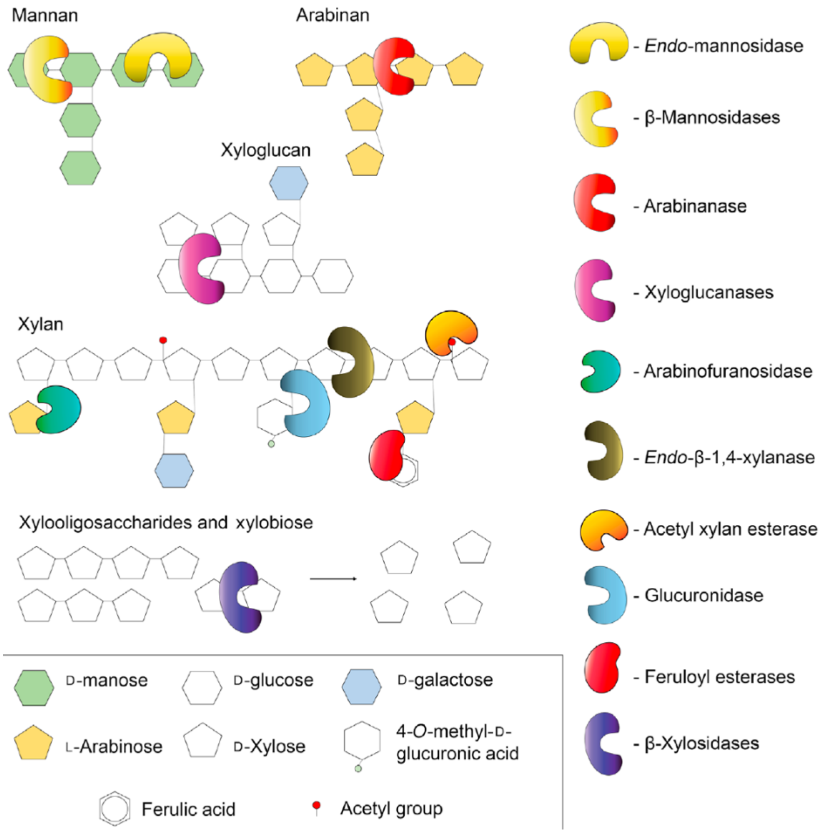


Figure 5: Representation of hemicellulolytic enzymes and accessory enzymes involved in hemicellulases debranching and degradation. Source: Polizeli et al. (2016) (42).

5.3 Accessory enzymes

Besides the enzymes mentioned above, the cocktail should also cover accessory enzymes such as feruloyl and acetyl esterases and lytic polysaccharides monooxygenases, and proteins, such as expansins-like, to completely degrade lignocellulose substrates (Figure 4 and 5). These enzymes have been described to assist biomass deconstruction by opening up the lignocellulosic matrix and acting synergistically with the canonical hydrolytic enzymes, allowing to extract more bioenergy power from lignocellulosic biomass and helping to reduce the cost of its production.

Among auxiliary activity enzymes, AA9 (GH61 family), a lytic polysaccharide monooxygenase (LPMO), have been reported mainly in the fungal system. These enzymes are copper-dependent that cleave cellulose chains by an oxidative mechanism either in C1 or C4 carbon of glucose in the cellulose chain, requiring an electron donor and molecular oxygen (48). Furthermore, the combined action of AA9 with hydrolytic enzymes has been demonstrated to result in higher levels of sugar release since AA9 acts through the creation of reactive sites for cellulases on the recalcitrant crystalline regions, with low levels of monomeric sugars released (49).

Acetyl xylan esterase (EC 3.1.1.72) catalyzes the deacetylation of xylans and xylo-oligosaccharides by removing *O*-acetyl substitutes from the C-2 and C-3 positions (50). Feruloyl esterases (EC 3.1.1.73) act on carboxylic ester linkages between arabinose and ferulic acid side groups formed the lignin-carbohydrate complexes (LCCs). As previously described, LCCs significantly reduce biomass recalcitrance by exposing cellulose microfibrils to the hydrolytic attack of CAZy enzymes (47).

Another class of accessory enzymes is the non-hydrolytic/non-oxidative proteins, known for their amorphogenesis-inducing action. These enzymes disturb the plant cell wall structural matrix, facilitating the deconstruction of the polysaccharides by hydrolytic and oxidative enzymes (51). Swollenin is an example of such protein that targets the amorphous regions of cellulose weaken hydrogen bonding with hemicellulose rather than directly disrupting the crystalline regions (52). Therefore, by promoting substrate amorphogenesis, Swollenin increases plant cell wall porosity and provides the catalytic enzymes enhanced access to the glycosidic linkages within the sugar polymers.

Laccases, lignin peroxidases, and manganese peroxidases are also accessory enzymes responsible for lignin depolymerization and chemical modification (53). Generally, it is preferred to be added before enzymatic hydrolysis during biological pretreatment of biomass. These enzymes are not typically part of hemicellulolytic cocktail development mainly because if lignin-degrading enzymes release many lignin-derived compounds, that could affect the enzymatic hydrolysis of cellulose microorganism fermentation of ethanol (54). Furthermore, the enzymatic pretreatment of lignin preserves a significant amount of lignin chemical structure until distillation, which is an advantage since then lignin could be converted into the monomer or other value-added products (53).

6. Plants responses to climate changes

Anthropogenic activities have impacted environmental conditions in the last decades leading to changes in global climate conditions. The human effect on climate is altogether changing the carbon cycle by releasing more carbon into the atmosphere via the emission of greenhouse gases (GHGs). Hence, the global temperature and atmospheric CO_2 concentration ([CO_2]) and have significantly increased over the past few decades (55). The changes in climate conditions are projected to impact the growth, development, and yield of lignocellulosic biomass. Since they represent a promising alternative to generate clean energy, their acclimation reactions to climate change should be explored (56).

The C3 and C4 photosynthetic types of plants respond differently to higher carbon dioxide concentrations ([CO_2]). Concerning C3 species, the higher [CO_2] positively affects photosynthesis since in this condition D-ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) speeds up carboxylation reaction and suppresses the oxygenation reaction. Consequently, the energy costs related to CO_2 losses are reduced, leading to the accumulation of carbohydrates in leaves (57). Nevertheless, as photosynthesis in C4 species already occurs in saturated conditions of CO_2 at the Rubisco active site, C4 crops would not benefit much from atmospheric CO_2 increment (58).

Regarding higher temperatures effects, the photosynthetic response of species depends on their optimum growth temperature, commonly improved in well-watered C4 plants (59). However, as plants are susceptible to heat, the global temperature increase might reduce photosynthetic efficiency due to stomatal closure resulting in minor CO_2 flux into leaves the stomate and decreased Rubisco affinity for CO_2 with rising temperature (60).

Moreover, climate changes compromise the joint effect of different factors, i.e., temperature and atmospheric [CO_2] are predicted to increase simultaneously. It is known that plants' vulnerability and behavior to isolated stresses differ from those resulting from a stress combination and depend on their tolerance conditions of combined stress (61). The correlation between plant physiological processes and their influence on crop yields has been extensively explored (62–64). However, despite the yield, several factors must be considered regarding climate change since the composition of the plant's organic matter is affected by climate, directly affecting the quality of crops as food and the lignocellulosic biomass composition for bioenergy applications (65).

Besides the photosynthetic response, plant cell wall biosynthesis and composition are dynamically regulated as a response to environmental stresses induced by climate change (66). Upon biotic or abiotic stress (e.g., heat or water stress), plants employ specific mechanisms of tolerance to maintain productivity and growth. However, the extension of the stress influence on physiological processes depends on plant genotype, species, age, and stress intensity (67).

Several studies reported that cell walls could be affected at different levels under biotic or abiotic stress for C3 and C4 photosynthetic systems. For example, transcriptome studies have found that genes of several cell-wall-related proteins such as those that participate in cellulose, hemicellulose, and pectin biosynthesis showed transcript levels impacted by elevated CO_2 and temperature (68–70). In addition, cellulose, hemicellulose, and lignin have their contents increased or decreased as stress tolerance mechanisms (71–73). Thus, cell wall adjustment under abiotic stress is essential in plant adaptation to environmental stresses, which directly affect the lignocellulosic potential for biorefinery.

However, most studies on cell wall remodeling induced by abiotic stress have concentrated on proteins and genes involved in cell wall metabolism. As a result, much less is known about the structural and chemical alterations of the cell wall polymers and hydrolytic performance induced by abiotic stress. Hence, since C4 fast-growing grasses represent an essential alternative as an energy crop, the impact of climate changes on its bioenergetic potential should be investigated.

II. OBJECTIVES

1. General objective

This work aims to evaluate the effect of future climate conditions, atmospheric temperature, and CO_2 concentration increment on the lignocellulosic biomass of C4 *Panicum Maximum* cv Mombaça grass its ability to provide for a sustainable and cost-effective feedstock for biofuel production.

2. Specific objectives

- Evaluate the biochemical characteristics, cell wall-bound phenolics, cellulose crystallinity, and lignocellulosic component accessibility to enzymes, as well as the enzymatic hydrolysis yields of non-pretreated *P. maximum* to study the impact of elevated CO_2 and temperature on potential production of bioethanol;
- Optimize an enzymatic pretreatment with crude laccase from white-rot fungus applying a response surface methodology to achieve maximum fermentable sugar yield in the saccharification step;
- Study chemical reactions and physical rearrangements caused by laccase pretreatment and further correlate it with modifications in *P. maximum* biomass induced by climate change;
- Formulate an optimized enzyme cocktail to efficiently degrade *P. maximum* by defining the amounts of cellulolytic and accessory enzymes in the cocktail as a way to obtain the significant yield of reducing sugar among the climate conditions tested;
- Understand how climate change and pretreatment processes affect synergistic associations between cellulolytic and accessory enzymes during saccharification and, therefore, the sugar yields of *P. maximum* forage grass.

III. RESULTS

CHAPTER I

Climate change affects cell-wall structure and hydrolytic performance of a perennial grass as an energy crop

This chapter was published as:

Freitas, E.N.d.; Khatri, V.; Contin, D.R.; Oliveira, T.B. de; Contato, A.G.; Peralta, R.M.; Santos, W.D. dos; Martinez, C.A.; Saddler, J.N.; Polizeli, M.L.T.M. **Climate change affects cell-wall structure and hydrolytic performance of a perennial grass as an energy crop.** Biofuels, Bioproducts & Biorefining, 2021. https://doi.org/10.1002/bbb.2312.

Climate change affects cell wall structure and hydrolytic performance of a perennial grass as an energy crop

Emanuelle N. de Freitas[a,c], Vinay Khatri[c], Daniele R. Contin[a], Tássio B. de Oliveira[a], Alex G. Contato[a], Rosane M. Peralta[b], Wanderley D. dos Santos[b], Carlos Alberto Martinez[a], Jack N. Saddler[c], Maria de Lourdes T. M. Polizeli[a]*

[a]*Department of Biology, FFCLRP, University of São Paulo, Ribeirão Preto, São Paulo, Brazil*

[b] *Department of Biochemistry, Maringá State University, Maringá, Paraná, Brazil*

[c] *Department of Wood Science, Faculty of Forestry, University of British Columbia, Vancouver, British Columbia, Canada*

**Corresponding author: * Dr. Maria de Lourdes Teixeira de Moraes Polizeli, Biology Department,*
Faculty of Phylosophy, Science and letters of Ribeirão Preto, University of São Paulo
Bandeirantes Av., 3.900. CEP: 14050-901.
Ribeirão Preto, São Paulo, Brazil
Tel.: +55-16-3315 4680, FAX: +55-16-3315 4886
E-mail: polizeli@ffclrp.usp.br

Abstract

Perennial grasses, such as *Panicum maximum*, are important alternatives of dedicated energy crops for bioethanol production. Thus, this study investigates whether future climate conditions could influence *P. maximum* cell wall structure and hydrolytic performance. In order to analyze the interactions with environmental factors in field conditions, a combined temperature-free air controlled and a free-air carbon dioxide enrichment (Trop-T-FACE) facility was used to investigate the isolated and combined effect of elevated atmospheric CO_2 concentration (eC) (600 μmol.mol^{-1}) and elevated temperature (eT) by 2˚C more than the ambient temperature, on cell wall composition, cellulose crystallinity, accessibility, and hydrolysis yields. The elevated temperature treatments (eT and eT+eC) exhibited the most pronounced effects on *P. maximum* cell wall. Warming reduced starch content and crystallinity index (CI) of cellulose while increased cellulose content. The fluorescent protein-tagged carbohydrate-binding modules analysis demonstrated that warming led to an improvement in the total cellulose surface exposure/accessibility in eT and eT+eC by 181% and 132%, respectively. Consequently, glucan conversion yields were improved by 7.07 and 5.37%, showing that warming led to lower recalcitrance in *P. maximum* biomass, which positively affects its use in biorefineries. Therefore, this work provides important information from an ecological and economic point of view, allowing us to understand the mitigation process applied by this forage grass under future climate conditions, and might assist in selecting tropical forage grasses that efficiently adapted to climate changes with a positive effect on bioenergy production.

Keywords: climate change, bioenergy, biorefinery, lignocellulosic biomass, enzymatic saccharification, carbohydrate-binding modules.

1. Introduction

Climate change, resulting from the increasing emission of greenhouse gases, significantly impacts all living organisms. The CO_2 atmospheric concentration ($[CO_2]$) is predicted to increase to 550 μmol mol^{-1} by 2050 while the average global temperature has risen by 0.85 °C comparing to the pre-industrial era.[1] Due to the growing of environmental concerns the research aiming the production of fuel from renewable sources has been encouraged. In this way, lignocellulosic biomass, as a renewable and abundant material, become a promising and sustainable method to reduce our carbon footprint while improving energy production efficiency to meet our ever-increasing energy demand.[2]

Due to the composition of cell wall polysaccharides, lignocellulosic biomass (LCB) can be used, via fermentation, for second-generation ethanol and other value-added commodities production thought the biorefinery concept.[3] However, agroecosystems growth, productivity, and development might be strongly impacted by the increased $[CO_2]$ and temperature expected for the next decades. [4–6] Several studies described plant cell wall as a dynamic structure, which can be remodeled during plant development and also in response to abiotic and biotic stresses as an important stress tolerance mechanism.[7,8] Nevertheless, much less is known about their effect on structural alterations of the cell wall polymers and on the hydrolytic performance, and since LCB is one of the most potential alternatives to obtain clean energy, their acclimation responses to climate change must be investigated.

Moreover, the changes in climate conditions are not expected to be isolated events, i.e., both temperature and atmospheric $[CO_2]$ are anticipated to increase simultaneously; however, studies interacting climate variables have not been adequately explored under field conditions. Plants response and vulnerability to isolated stresses are different from those resulting from combined stresses and depend on their tolerance and mitigation ability to combined stress situations.[9]

Among diverse lignocellulosic feedstock classification, fast-growing perennial C4 grasses have remarkable potential as a dedicated energy crop for cellulosic ethanol production due to their high biomass yield, broad geographic adaption, and low mineral-nutrient and pesticides inputs.[10] Feedstocks with these characteristics contribute to the production of second-generation ethanol because they do not compete with our food resources, do not require extensive capital investment, and can be used to avoid currently intermittent biofuel production, which makes the final product more feasible, especially during idle periods.[11] *P. maximum* (Jacq. cv. Mombaça) is one of the most important C4 grass used as pasture in South America, especially Brazil, and also has significantly high efficiency in converting solar energy into biomass through photosynthesis.[12] Moreover, *P. maximum* grass represents an significant alternative to sugarcane cellulosic ethanol, since it has a lignocellulosic composition similar to sugarcane bagasse and a bioethanol yield of 8571,0 L/ha compared to 8478,6 L/ha for sugarcane bagasse.[13]

In this study, *P. maximum* perennial C4 grass grew under expected isolated and combined future climate conditions (i.e., elevated CO_2 and temperature) under field conditions through a Trop-T-FACE system and characterized to study the change in their chemical composition, cell wall-bound phenolics, cellulose crystallinity, and lignocellulosic component accessibility to enzymes using carbohydrate-binding modules (CBMs) approach. Following this, enzymatic hydrolysis was performed to study the impact of elevated CO_2 and temperature on potential production of bioethanol. Since the composition of the plant's organic matter, transpiration, photosynthesis, and metabolism can be influenced by climate temperature and [CO_2], we hypothesize that the climate change expected in the next years will impact the set of characteristics studied here, and thus affect *P. maximum* use for bioenergy production. We strongly believe that this work will enable more accurate projections of the responses of this important C4 crop species regarding climate and productivity models and contribute to future decision-making in efficient production of bioenergy.

2. Materials and methods

2.1 Growth study site and treatments

We assessed the effect of two factors involved in climate change expected to impact on the environment in the upcoming decades (i.e., elevate CO_2 atmospheric concentrations [CO_2] and global average temperature increase) in the tropical grassland *Panicum maximum* cv. Mombaça by using a Trop-T-FACE facility, located at the campus of the University of São Paulo, Ribeirão Preto, SP, Brazil. The following treatments were used: (a) ambient atmospheric [CO_2] and temperature (named here as Control: C) (b) ambient atmospheric [CO_2] and +2 °C above ambient temperature (annotated as elevate temperature: eT), (c) 600 ppm atmospheric [CO_2] and ambient temperature (termed as elevate CO_2: eC) and (d) 600 ppm atmospheric [CO_2] and +2 °C above ambient temperature (designate as eT+eC).

2.2. The Trop-T-Face facility

The Trop-T-FACE facility combines a miniFACE (free-air CO_2 enrichment) and T-FACE (temperature free-air controlled enhancement) technologies to simulate future elevated atmospheric carbon dioxide concentration [CO_2] and elevated temperatures, respectively, as described in detail elsewhere.[14,15] Briefly, in elevated [CO_2] plots, pure CO_2 is fumigated into the plant canopy using two-meter-diameter PVC rings with laser perforated micro-holes. A proportional integral derivative (PID) algorithm regulates the amount of CO_2 required to elevate the [CO_2] to the set point of 600 ppm (eC). The [CO_2] of each plot was measured by a carbon dioxide transmitter sensor, model GMT222 (Vaisala, Helsinki, Finland). The daily average [CO_2] in control plots was 400 ± 20 ppm, while FACE fumigation increased average diurnal [CO2] to 600 ± 20 ppm in eC and eT+eC plots during the experiment.

To increase the canopy temperature to +2˚C more than the ambient canopy temperature (eT) we used a T-FACE system as described by Kimball (2005).[16] In each eT plot, the plant canopy was warmed by six infrared TFE 750–240 heaters mounted on aluminum ALEX-F reflectors (Mor Electric Heating, Comstock Park, MI, USA) in a 2-m-diameter hexagonal design. The heaters were suspended 0.8 m above the canopy with aluminum bars and had their angle and height adjusted according to plant growth. The canopy temperature of each plot was measured by SI-1H1-L20 infrared radiometers (Apogee Instruments, USA). The T-FACE control temperature was performed by a PID algorithm installed in a CR1000 datalogger with AM25T multiplexors (Campbell Scientific, Logan, UT, USA). A setpoint of 2°C above the ambient canopy temperature was used in the present study. The control system integrates the canopy temperature of non-warmed (Control and eC) plots, and warmed (eT and eT+eC) plots and automatically regulates the canopy temperature to 2˚C more than the ambient canopy temperature in warmed plots. Trop-T-FACE data was monitored and collected using Loggernet software (Campbell Scientific, Logan, UT, USA). The canopy temperature of the plants under eC was on average 0.25°C higher than those under Control, possibly because of the effects of eC on stomatal closure. Under eT, the average canopy temperature was 1.8±0.2°C higher than of Control. These discrepancies in the setpoint are explained by diurnal variations resulting from wind and transpiration. Under eT + eC, the temperature was 2.0±0.2°C higher than Control, presumably because of the independent additive effects of eC and infrared heating.[26]

After 2 months, plants were cut at 30 cm above the ground, followed by [CO_2] and temperature treatments. After 24 days of experiment, at 12 P.M., *P. maximum* leaves were collected, milled at 20 mesh, and stored in a free humidity environment until analyses. Meteorological data of total solar radiation (Rad), relative air humidity (Rh), and air temperature (Tair), throughout the growing season is discussed elsewhere.[15]

2.3. Lignocellulosic preparation and chemical composition

Lignocellulosic substrate preparation and the chemical composition of *P. maximum* under different climate conditions were determined using a modified Klason lignin method derived from the TAPPI standard method T222 om-88, as described elsewhere.[17] The *P. maximum* extractives quantification was performed following the protocol proposed by National Renewable Energy Laboratory (NREL).[18]

2.4. Profile cell wall-bound phenolics

The cell wall bound phenolics analysis was done as described elsewhere.[19] Cell wall-bound phenolics analysis was accomplished by an HPLC system (Shimadzu® Liquid Chromatograph, Tokyo, Japan).

2.5. Quantification of lignin and composition of monomers

To accurately quantify the lignin content in its monomer composition, it is crucial to obtain a protein-free cell wall to exclude protein and other UV-absorbing materials. The dry matter contained protein-free cell wall fraction obtained according to a defined protocol.[20] Lignin was quantified by the acetyl bromide assay described elsewhere,[19] and alkaline nitrobenzene oxidation was used to determine monomeric lignin composition.[21]

2.6. Cellulose crystallinity

XRD analyses were performed on a Bruker D8-Advance X-ray diffractometer (Bruker, Germany), with Cobalt Kα radiation (λ= 0.178892 nm) as described elsewhere.[22] Individual crystalline peaks were extracted by a curve-fitting method using the diffraction intensity profiles.

2.7. Non-structural carbohydrate content

Non-structural carbohydrates were quantified on leaves at 24 days of experiment collected at 12 P.M. of the respective day. The procedure used for reducing sugar content was based on,[23] and starch quantification was carried out employing an enzymatic assay.[24]

2.8. Enzymatic Hydrolysis

P. maximum samples were washed by immersion in 80 ºGL ethanol six times and then washed with water until the soluble sugars in the material were removed entirely to avoid soluble sugar interference during hydrolysis experiments. All samples were then dried in an oven at 50ºC. The enzymatic hydrolysis of *P. maximum* (non-treated) was carried out applying enzyme cocktails Cellic CTec2 and Cellic HTec (Novozymes®) in the proportion of 9:1 (Ctec2:Htec ratio)[25] with a total amount of 20 mg of protein per gram of cellulose. The hydrolysis experiments were carried out at 2, 4, and 6 % (w/v) solids loading in sodium acetate buffer (50 mM, pH 4.8) at 50°C and 150 rpm. Subsequently to 72 hours of hydrolysis, the enzymes were inactivated by heating the hydrolysis mixture for 10 minutes at 100 °C. After centrifugation at (16,000 *x g*/10 minutes), the supernatants were collected and stored at -20 °C for further analyses.

The influence of starch to glucan conversion yields was evaluated by removing starch with 25 mL of 90% DMSO (dimethylsulfoxide) for 12 hours. The starch extraction was repeated three times (3 h) and finally washed with distilled water.[26] The remaining biomass was composed of structural carbohydrates and was submitted the enzymatic hydrolysis as previously described.

2.9. Sugar and protein assay

The quantitative analysis of glucose and xylose content in the hydrolysate was performed using a high-performance anion-exchange chromatography (Dionex DX-3000, Sunnyvale, CA). The total glucan or xylan conversion of the substrate was calculated as a percentage of the theoretical glucan/xylan available in the substrate.

2.10. Holocellulose (cellulose + hemicellulose) accessibility using FTCM-depletion assay

The FTCM (**F**luorescent protein-**T**agged **C**arbohydrate-binding modules **M**ethod) - depletion assay was used to evaluate the cellulose and hemicellulose surface accessibility in untreated and un-hydrolyzed *P. maximum* fibers grown in four different climate conditions (C, eT, eC, and eT+eC). The assay has proceeded as described elsewhere[27–33] with four different FTCM probes: eGFP-CBM3a (GC3a) (fluorescent protein eGFP genetically linked with CBM3a), mCherry-CBM17 (CC17) (construct of the fluorescent protein mCherry and CBM17), mOrange2-CBM15 (OC15) (a chimera made of mOrange2 fluorescent protein and CBM15) and eCFP-CBM27 (CC27) (composed of eCFP and CBM27), targeting crystalline cellulose, amorphous cellulose (non-crystalline), xylan and mannan hemicellulose, respectively. The specificities and affinities of the FP-CBM probes had been previously characterized by isothermal titration calorimetry (ITC), a solid-state depletion assay (SSDA), and affinity gel electrophoresis (AGE). [27,28,33] Prior to FTCM-depletion assay, all substrates were incubated with bovine serum albumin (BSA) to block the lignin to prevent the non-specific binding of CBM probes to lignin.[27] The difference between the binding isotherm of substrates without BSA blocking and with BSA blocking is summarized in Figure S1.

2.11. Statistics and numerical analysis

All tests were performed in triplicates, and the means and standard deviations were analyzed and indicated as the mean of three replicates ± standard error of the means (SD). A principal component analysis (PCA) was done to detect patterns in the dataset. The study was carried out in Past (version 2.14). Analysis of variance (ANOVA) was performed to test the significance of the observed differences using the Graph-Pad Prism 8.0 software (San Diego, EUA). Differences among the studied factors were assessed by the Tukey test, and p values ≤ 0.05 were considered statistically significant.

3. Results and Discussion

3.1 Biomass chemical composition

For all four studied energy crops (Figure S2), the biomass composition was analyzed for soluble extractives, ash, cellulose, hemicellulose, and lignin contents. The results are summarized in Table 1. As hemicellulose from grasses is mainly composed of Arabinoxylan (AXs) residues, the glucose from hemicellulose sources represents low quantities of the total glucan content; thus, the cellulose content was considered for further discussions as the total glucan content of each group. The highest glucan content was found for eT (30.2%) and eT+eC (29.7%), followed by eC (27%) and control (25.8%) (Table 1). Significant differences ($p \leq 0.05$) in glucan content were observed for eT and eT+eC compared to C and eC groups. There was no significant ($p \geq 0.05$) difference between C and eC substrates. Lignin contents (Table 1) were quite similar for all studied climate conditions, and no significant ($p \geq 0.05$) difference was found among them, showing that the expected futuristic climate condition seems not to affect total lignin content on the studied forage grass. The hemicellulose content (Table 1) was calculated based on the monomeric sugars (xylose, arabinose, galactose, and mannose) released after acid hydrolysis, and it was slightly ($p \leq 0.05$) higher for eC conditions when compared with C and eT (Table 1).

Lignocellulosic materials are mainly composed of cellulose, hemicelluloses, and lignin.[34] Cellulose is a linear homopolysaccharide composed of glucose monomers that can be converted into fermentable sugars through enzymatic hydrolysis,[35] which make feedstocks with higher glucan content likely to be favored in ethanol biorefinery industries from an economic point of view since the cellulose content of the feedstocks is directly proportional to the ethanol yields under optimal processing conditions.[36] Hence, as elevated temperature treatments (eT and eT+eC) led to an increase in glucan content, warming treatment could positively affect ethanol yields (Table 1).

Contrary to cellulose, the distribution of both lignin and hemicellulose in the cell wall is described as a physical barrier that contributes to biomass recalcitrance by encapsulating cellulose microfibrils obstructing cellulase domains to adsorb on cellulose and initiate enzymatic hydrolysis.[37] Therefore, the slightly higher hemicellulose content for eC conditions may lead to lower saccharification yields (Table 1). The findings for hemicellulose content agrees with a transcriptome study which found that genes of several enzymes involved in hemicellulose and pectin biosyntheses such as NDP-sugar epimerases, UDP-glucose pyrophosphorylase (UGPase), glycosyltransferase family 43 (GT43), and others, had increased transcript levels in response to growth under elevated CO_2. [38] The lignin content found in this work is consistent with previous studies and indicates that *P. maximum* has similar percent composition of lignin with those reported for sugarcane bagasse, about 27.79%.[13,19] Therefore, the lignin amount present in *P. maximum* does not represent a possible inconvenience (in terms of biomass deconstruction and modification) compared to sugarcane bagasse (Table 1).

Furthermore, grasses with different treatments exhibited somewhat similar hemicellulose monosaccharide profiles, primarily composed of xylose, arabinose, and galactose (Fig. 1). Moreover, mannose sugars were below detectable levels, which suggests that very low or no mannan was present in the hemicellulose and pectin fractions of the cell wall. A significant ($p \leq 0.05$) difference was found in the monosaccharide profiles for eC treatment, which exhibited more xylan (Fig. 1A) compared to C and more arabinose compared to other treatments (Fig. 1B). On the other hand, no significant difference was found for galactose sugar in all the studied conditions (Fig. 1C). Figure 1D illustrates the xylan/arabinose ratios that indicate the degree of arabinosyl substitution on grass's xylan backbone. In this work, we observed that the grasses in different climate conditions have a Xyl: Ara ratio of about 5:1, with a significant difference ($p \leq 0.05$). eT grasses exhibited the highest Xyl: Ara ratio and eC the lowest.

The results concerning monosaccharide profiles agree with the characteristics from type II hemicellulose cell wall from grasses is primarily composed of xylan with arabinofuranose (Ara*f)* substitution of the β-(1,4)-xylose backbone by α-linked, forming arabinoxylan (AXs).[39] Another critical parameter for biomass recalcitrance investigated in this work was the Xyl: Ara ratios. Substitution patterns on the xylan backbone (described here as Xyl: Ara ratio) has a robust relation with how strongly hemicellulose can form a hydrogen bond to other cell wall polysaccharides, mainly lignin and cellulose, affecting structural properties of the cell wall as well as enzymatic hydrolysis yields.[39] In this sense, hydrolysis yields of eT might be favored since this group exhibited higher Xyl: Ara, opposite to the eC group that had the highest degree of arabinosyl substitution (Fig. 1).

Also, the PCA analysis shows that warming is the main factor responsible for changes in *P. maximum* cell wall composition. It shows a well-defined separation of the warmed plots (eT and eT+eC) from the non-warmed plots (C and eC) along the PC1 axis, describing 62.31% of the variance in the cell wall composition and carbon storage (Fig. 2A). It also indicates that the glucan content was the main responsible for these differences. No pattern was observed for the PC2 axis, which explained 19.61% of the distribution. The residual variation observed can be explained by the [CO_2] (18.08%), as shown by the PC3 (Fig. 2B).

By analyzing the above-observed results for the chemical composition of *P. maximum* LCB, it is probable to suggest that abiotic stress induced by climate changes can influence the cell wall composition of the forage grasses especially, the heat stress (i.e., eT) as showed by PCA analysis (Fig. 2). Cell wall remodeling represents an important stress tolerance mechanism, some reports related that significant changes on cell wall might be driven by abiotic stress as a way to maintain growth and productivity.[8,40,41] However, how much the stress will influence the plant physiological process depends on the heating level and the plant's tolerance mechanisms.

In most cases, heat stress could result in water stress under modified environment situations (e.g., high temperatures and midday radiation).[42] Under this scenario, C4 photosynthetic pathway plants increase their evaporative demands resulting in temporary water stress. Evidence of water stress in *P. maximum* grown under warmer environment was found by Habermann et al.; the author described a 15% increase in transpiration rates for *P. maximum* cv. Mombaça under eT environment, a slight reduction on solo water content (SWC), and decreased bulliform cells size, which store water.[15] Furthermore, under eT conditions, *P. maximum* cv. Mombaça had an increment in amino acids content derived from pyruvate and oxaloacetate, reported to be related to the stress defenses.[43]

Lately, some studies have explored the effect of many abiotic stresses on plant metabolism, and some finds can help us understand the higher glucan content for eT and eT+eC (Table 1). Most plants make physiological adjustments that contribute to the acclimation and survival under heat and water stress. For example, genes encoding Cellulose Synthase-Like (CSL) proteins, a family of proteins that are similar to the Cellulose synthases (CesAs), seems to play some role in cellulose synthesis and appears to be responsive to osmotic stress and salt stress, which could be a consequence of water stress. Interestingly, CSLD1, CSLD2, and CSLD3 genes were induced by increased salt conditions, leading to a cell wall deposition in *Arabidopsis thaliana.*[44] Cellulose deposition was also reported to happen in *Zea mays* stems under salt stress.[45] Furthermore, an increased level of expression of sucrose synthase and UDP-glucose pyrophosphorylase genes were detected in cotton subject to drought stress, suggesting a possibly higher cellulose biosynthesis.[38] Therefore, these results showed that modifications in cell wall composition could play a role in the acclimation process of *P. maximum,* allowing the plant to survive to non-lethal temperature through thermotolerance.

3.2 Lignin profile

As the lignin content, the subunits ratio is highly variable between plant species, tissues, cell types, developmental stage, and stress conditions. It also has a role in the contribution of lignin to biomass recalcitrance.[46] Besides measuring the lignin content (Table 1), we also analyzed the guaiacyl (G), syringyl (S), and p -hydroxyphenyl (H) monomeric content for lignin for all climate conditions (Fig. 3). The results show that H and G content is similar among the treatments (Fig. 3A-B). However, we found higher ($p \leq 0.05$) syringyl (S) content for eT and lower content for eT+eC treatment when compared to the other groups (Fig. 3C). The S content observed was 5.12 and 3.5 µg/mg protein-free cell for eT and eT+eC, respectively, compared to about 4.5 µg/mg protein-free cell wall for C and eC treatments. Interestingly, differences were found in the S/G ratio (Fig. 3D) among the studied climate conditions, in which eT treatment presented higher S/G ratio (0.58) than C and eC conditions ($p \leq 0.05$).

The ratio of syringyl (S) and guaiacyl (G) units in lignin has been regarded as a factor that influences recalcitrance to sugar release from energy crops enzymatic hydrolysis.[47] This limit arises from the notion that during lignin biosynthesis coupling reactions in specific positions lead to polymers linked via C–O bonds and C–C bonds. The C-O bonds are the most abundant and the key for lignin depolymerization due to their labile nature. The S units are prone to the formation of C-O bond during lignin biosynthesis, which is more susceptible to cleavage.[48]

Therefore, biomass with higher S/G ratio, in this study represented by eT group, may represent a better choice for biorefinery purpose, since the lower G content allows that lignin structure to be easily modify increasing the accessibility to hydrolytic enzymes, and therefore, greater sugar release (Fig. 3). Plants with higher S/G ratios show that the negative influence of lignin on glucose release was less pronounced.[47] The data found for eT group agrees with previous results that observed an increase in sinapic acid abundance under eT treatment, which was pointed as an impact of the eT on lignin compositions by increasing S-type concentrations.[43] Also, *Zea mays* plants under salt stress also exhibited higher S/G ratios in roots and stem, which could be the case of eT treatment.[45]

3.3 Cell wall-bound phenolics

Produced in the phenylpropanoid pathway, the hydroxycinnamic acids, also known as ferulic acid (FA) and *p*-coumaric (*p*-CA), have a carboxylic group at the end of their propenyl group in contrast with the alcohol function of monolignols that are produced later.[49] Due to this fact, feruloyl and p-coumaroyl residues can further esterified to arabinose from the arabinoxylans (AXs) of the type II cell wall. The FA esterified with arabinosyl residue of AXs is able to connect with lignin by ether-linkage or dimerize with other FA-arabinoxylan, acting as a universal connector between cell wall polymers.[50] This reaction results in cross-linking among the cell wall polymers and represents an influencing factor in hydrolysis yields.[51]

Therefore, FA and *p*-CA content were analyzed to verify if it could interfere with the hydrolysis of the different climate conditions studied (Fig. 4). It was apparent that FA varied between 2.26 and 2.38 mg/g of biomass and *p*-CA between 3.04 and 3.41 mg/g of biomass, with no significant difference found among the groups for both FA and *p*-CA ($p \geq 0.05$). Hence, FA and *p*-CA should not be an aspect that leads to differences in hydrolysis yields among the climate conditions studied in this work.

3.4 Cellulose Crystallinity

Although many factors affect cellulose hydrolysis as available surface area and degree of polymerization, the cellulose crystallinity is a limiting step on cellulose conversion due to its recalcitrance. [52,53] In addition, many studies showed that enzyme adsorption, including the non-catalytic cellulose-binding module (CBM) and catalytic glycosyl hydrolase system, generally declined as cellulose crystallinity increased. [54]

Crystallinity index (CI) is a quantitative representation that symbolizes the relative amount of amorphous and crystalline cellulose regions. As cellulose crystallinity is believed to have a role in its biological conversion, we then evaluated the CI for all climate conditions studied. The XRD graphs with the analyzed peaks are represented in the supplementary material (Fig. S3). The CIs for C, eT, eC and eT+eC were 71.7; 53.2; 64.7 and 57.8%, respectively (Fig. S4). The elevated temperature treatments (eT and eT+eC) were the groups with lower crystallinity and should be the climate conditions that must result in higher cellulose conversions.

The effect of temperature on reducing cellulose crystallinity for *P. maximum* could rely on the thermotolerances mechanisms employed under water stress experienced in warming treatments. Reactive oxygen species (ROS) are commonly observed as a plant response to abiotic stress. In general, they are used as a co-substrate by cell wall peroxidases, which lead to cross-linking increment between phenolic compounds and hemicellulose polymers.[63] However, if ROS production continues, and all cross-linkable substrates have already been used by peroxidase, this allows the production of OH-radical that is involved in cell wall loosing process, by cleaving sugar bonds in plant polysaccharides, action similar to enzymes like expansins that are reported to promote amorphogenesis in cellulose microfibrils.[8] This loosing process promoted by ROS is reported to be a tolerance mechanism employed by plants to overcome the growth arrest under abiotic stress and could also lead to enhancement in hydrolytic performance by, as described in this study, reducing the CI.

3.5 Non-structural carbohydrate content

Carbohydrates are molecules with an important role in signaling pathways and the tolerance of plant species to abiotic stresses.[56] The intercellular content of sugars regulates multiple functions in source and sink tissues, including photosynthetic performance, gene expression, and enzyme activities.[57] Due to the importance of non-structural carbohydrates to plant physiology, we estimated the total content of soluble reducing sugar and starch content after 24 DOE (Days of experiment), which are presented in Figure 5. Soluble reducing sugars reduced by 21.45% and 27.4% ($p \leq 0.05$) under eT and eT+eC, respectively, related to C (Fig. 5A). Regarding to the leaf starch content we found a 52.9 and 41.4% reduction ($p \leq 0.05$) for eT and eT+eC, respectively, when compared to C (Fig. 5B) and a non-significant increase (16%) in the leaf starch content for eC compared to C (Fig. 5B).

In this work, both alcohol-soluble reducing sugars and starch content were found to be reduced in warmed treatments (eT and eT+eC) (Fig. 5). These results agree with other *Panicum maximum* studies; the first one was a metabolome study of intracellular content that reported many carbohydrates (arabinose, frutose, galactose, maltose, ribose, xylose, and melobiose) with increased abundance under eT,[43] showing that these carbohydrates are being transported as signaling molecules, which could be a response for the abiotic stress when the plants are subjected to a warmer environment. The second one found larger quantities and sizes on starch grains in chloroplasts of bundle cells for eC treatment and minor starch grains for eT and eT+eC.[15,58]

The increased starch content in leaves for eC treatment responds to the higher carbon assimilation found in eC, showing that the *Panicum maximum* is not saturated under CO_2 atmospheric concentration.[58] Concerning eT and eT+eC, as previously discussed, warming treatments led to a stress statement in plants that demands more energy. In this case, starch is described to be involved in plant's reactions to abiotic stress, and it is the principal carbohydrate stored in source organs that is quickly remobilized to provide energy to sink tissues, consequently reducing its content in source tissues.[59] The outcomes found in this work suggest that starch was degraded and transported as sucrose to sinks organs; therefore, its content in leaves (source organs) is reduced under eT treatment.

3.6 Enzymatic saccharification

Further, the enzymatic saccharification of *P. maximum* grew under the four climate conditions (C, eT, eC, and eT+eC) was performed to identify whether there would be any impact of climatic conditions on sugars releases for ethanol production (Fig. 6). For this purpose, we used two commercial enzyme cocktails containing all the essential enzymes (cellulases and hemicellulases) for LCB deconstruction. We quantified the sugars released (glucose and xylose) by enzymatic hydrolysis in grasses without any pretreatment as a way to avoid interference in biomass recalcitrance among forage grasses grown under expected future climate conditions.

Glucan conversion (%) of hydrolysis from non-treated biomasses (2% solid loading and 72 hours) was significantly higher ($p \leq 0.05$) for warmed treatments (eT and eT+eC) with 43.9 and 43.2% of glucan conversion, while the control (41%) and eC (41.2%) groups showed lower values of glucan hydrolysis (Fig. 6A). Moreover, these values represent a relative increment of 7.07 and 5.37% in glucan conversion for eT and eT+eC treatments, respectively, compared to C group. Furthermore, the percentage of xylan conversion for eT treatment (11.05%) was also higher ($p \leq 0.05$) than C (9.4%), eC (9.6%) and eT+eC (9.5%) (Fig. 6B), which represent a relative increment of 16.31% for eT when compared to the control group. The glucan conversion was also evaluated 4 and 6% solid loading (Fig. 6C), and the conversion yields found in these conditions were similar to the ones reported at 2% solid loading with eT and eT+eC being the groups with higher glucan conversion as well ($p \leq 0.05$). Moreover, the glucose release (g/L) was greater when increasing the load of solids (Fig. 6D) for all climate conditions. The influence of starch for glucan conversion yields (Fig. S5) was studied and seems not to have great contribution to the saccharification yields when compared to total biomass hydrolysis (Fig. 6).

The efficiency of enzymatic saccharification, which is the critical step in the bioconversion of LCB to ethanol, can be influenced by several factors related to the biomass recalcitrance, such as the cellulose crystallinity, available surface area, degree of hemicellulose matrix polymerization, lignin distribution, etc.[53] As discussed earlier, elevated temperature treatments (eT and eT+eC) could play an important role in improving percent glucan content, reducing biomass recalcitrance by lowering cellulose crystallinity, and minimizing Xyl/Ara (Fig. 1) and S/G ratio (Fig. 3), which ultimately might result in a less polymerized matrix between hemicellulose elements and also between hemicellulose and lignin. All the results mentioned above led to improved accessibility to the hydrolytic enzymes for warming treatments, and thus, higher cellulose conversion into glucose during saccharification process (Fig. 6). Even though the differences in conversion rates were not much pronounced, minimal differences in sugar release could impact the biorefineries from an economic point of view.

Moreover, it is important to note that no pretreatment was applied in this study; that is why the conversion rates observed after enzymatic hydrolysis were relatively low. Thus, applying efficient pretreatment on *P. maximum* grasses, the glucan and xylan conversion rates could be further improved since it facilitates the access of glycoside hydrolases to polysaccharides.[37] Hydrolysis at higher solid loading is advantageous since the increment on substrate loading leads to increased sugar concentrations as observed in this work (Fig. 6D), and, therefore, higher ethanol yields after fermentation. This approach reduces costs and increases the efficiency of the bioconversion process.[60] However, solid loading more significant than 6% with non-treated substrates was challenging to achieve in this study due to rheological problems since is was not possible to achieve proper mixing during hydrolysis. We believe that pretreated fibers are likely to reduce these problems.

3.7 Surface carbohydrate accessibility using FTCM-depletion assay

Enzymatic hydrolysis depends on the cellulose accessibility to cellulase enzymes. Hence, it is utmost important to characterize lignocellulosic components' (i.e., cellulose and hemicellulose) accessibility and its impact on enzymatic hydrolysis. Carbohydrate-binding modules (CBMs) corresponds to a non-catalytic polysaccharide-recognition module of carbohydrate-active enzymes, such as glycoside hydrolases.[61] The high specificity of CBMs regarding to lignocellulosic polymers makes them attractive as probes to study the chemistry and structure variations on LCB, cellulose accessibility to cellulase enzymes and verify the effect of applied treatment.[62–64]

Therefore, to further study the change in the enzymatic hydrolysis data, we have evaluated *P. maximum* fibers accessibility using fluorescent protein-tagged carbohydrate-binding modules (FP-CBM). Via four specific FP-CBM molecular probes (i.e., GC3a, CC17, OC15, and CC27). The FTCM-depletion assay of *P. maximum* fibers was well approximated by a single-site binding model described elsewhere (Fig. 7).[27,29,32] The parameters derived from these fits (N_o (µmoles g^{-1}), K_a (μM^{-1}), ΔG (KJ mol^{-1}) are summarized in Table S1.

The total concentration of the available binding sites (No) also described as biding capacity on the control (C) fibers were 2.1 ± 0.3, 2.2 ± 0.7, and 1.3 ± 0.8 for GC3a (crystalline cellulose accessibility recognition probe), CC17 (amorphous cellulose accessibility recognition probe) and OC15 (xylan accessibility recognition probe) probes, respectively (Table S1; Fig. 7). It was apparent that there was no significant difference in the surface exposure/accessibility of crystalline cellulose, amorphous cellulose, and xylan components of the biomass. On the other hand, no binding of CC27 (mannan recognition probe) probes was observed, which complement the lack of mannan component in the *P. maximum* fibers. Furthermore, the total cellulose and hemicellulose accessibility could be derived by adding crystalline cellulose accessibility (i.e., No of GC3a) and amorphous cellulose accessibility (i.e., No of CC17), and xylan accessibility (i.e., No of OC15) with mannan accessibility (i.e., No of CC27), respectively (Table S1). The total cellulose accessibility was at least ~3-fold higher than total hemicellulose accessibility, suggesting that cellulose exposure on the surface of *P. maximum* fibers was overwhelmingly higher than hemicellulose.

In the case of eT fibers, crystalline cellulose, amorphous cellulose, and xylan accessibility increased by 267%, 100%, and 85%, respectively, when compared to C. This increment was also reflected in the 181% and 85% improvement in the total cellulose and total hemicellulose accessibility, respectively, compared to its control counterpart. It was apparent that growing *P. maximum* under elevated temperature increased the holocellulose accessibility (Fig. 7; Table S1) and, as a result, enzymatic hydrolysis of both glucan and xylan components (Fig. 6). This data also complement the above reported higher glucan chemical composition (Table 1), lower crystallinity (Figure S4), elevated xylan/arabinose (Fig. 1), and S/G ratio (Fig. 3) for eT condition. On the contrary, eC fibers did not exhibit any significant change in the holocellulose accessibility compared to C group. Therefore, eC did not demonstrate any significant improvement in the % glucan and % xylan conversion. This is likely due to the observed significantly lower xylan/arabinose (Fig. 1), and S/G ratio (Fig. 3) since both hemicellulose and lignin contribute to biomass recalcitrance. On the contrary, like eT, the combined impact of eT and eC improved the GC3a and CC17 bindings by 205% and 64%, respectively, and as a result, exhibited significantly higher % glucan conversion (Fig. 6A). However, there was no significant improvement observed in the xylan accessibility. Therefore, no hydrolytic change was observed in the % xylan conversion of the eT+eC substrate (Fig. 6B). It was apparent that eT+eC conditions followed similar trends as eT condition, which depicted that elevated temperature plays a dominant role over elevated CO_2 by suppressing the inherent changes that occurred via eC environmental condition. Furthermore, the lack of binding of mannan recognition probe (CC27) to eT, eC, and eT+eC biomass suggests that FTCM probes were highly specific towards their substrate, and there was negligible non-specific interaction.

Therefore, to examine the robustness of the relationship between bound probes and enzymatic hydrolysis observed here (*i.e.,* type of correlation and correlation coefficient), a statistical analysis using all the value pairs (bound probes (μmol/g of biomass) vs. glucan/xylan hydrolysis (%) generated in this study was performed. The results in Figure 8 indicate that the total cellulose and hemicellulose accessibility, as detected by FTCM probes, were positively correlated with percent glucan and xylan hydrolysis, respectively. The Pearson's correlation coefficients for glucan and xylan hydrolysis were R = 0.99, $p < .001$, and R = 0.99, $p < .001$, respectively, which supports the notion of a significant robust positive correlation.

4. Concluding remarks

Abiotic stress affects plant cell wall architecture, although the impact depends on the plant genotype, species, age, and the timing and intensity of the stress. Among all the studied climate conditions, eT exhibited higher percent glucan composition, S/G ratio, higher total cellulose accessibility, and hence elevated enzymatic hydrolysis. On the contrary, eC showed higher percent total hemicellulose composition (which was due to higher arabinose content), lower S/G ratio, significantly lower total cellulose accessibility, and therefore lower enzymatic hydrolysis yield. Furthermore, eT+eC conditions followed similar trends as eT conditions, which depicted that elevated temperature plays a dominant role over elevated CO_2. Moreover, state-of-the-art FTCM-depletion assay facilitated the study of surface exposure/accessibility in real-time to better understand the enzymatic hydrolysis. Our data provide clear evidences that *P. maximum* cell wall has several mechanisms of adjustment under abiotic stress-induced by expected futuristic climate conditions, which could positively affect its use for bioenergy purposes. However, the work has gone beyond the concerns over the carbon emissions and climate change brought by fossil fuels; this work has also come up with a potential alternative of lignocellulosic biomass for biorefinery solutions, while assisting in compliance with national and international recently approved bioenergy laws. Therefore, this work may contribute to better-informed decisions on energy options for the future**.**

Declaration of competing interest

The authors have no conflict of interest.

Acknowledgments

The authors thank the postgraduate students and laboratory technicians who contributed to the work and the BIOPLAN-DBQ-UEM and the FPB/B group, Wood Science Department-UBC.

Funding

The authors thank Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) (Grant 2008/57908-6, 2008/58075-8, 2014/50884, 2018/07522-6, and Process 2017/23989-9) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Grants 446357/2015-4; 465319/2014-9, 301963/2017-7).

References

(1) Intergovernmental Panel on Climate Change. Carbon Dioxide: Projected emissions and concentrations.

(2) de Sousa, A. S.; de Melo, R. R.; Miyamoto, R. Y.; Morais, M. A. B.; Andrade, L. P.; Milan, N.; de Avila, M. C.; de Souza, C. M.; Adão, R. C.; Scarpassa, J. A.; Vieira, P. S.; dos Santos, L. V.; Ramos, C. H. I.; Murakami, M. T.; Zanphorlin, L. M. A Rationally Identified Marine GH1 β-Glucosidase Has Distinguishing Functional Features for Simultaneous Saccharification and Fermentation. *Biofuels, Bioprod. Biorefining* **2020**, *14* (6), 1163–1179. https://doi.org/10.1002/bbb.2136.

(3) Magalhães, A. I.; de Carvalho, J. C.; de Melo Pereira, G. V.; Karp, S. G.; Câmara, M. C.; Medina, J. D. C.; Soccol, C. R. Lignocellulosic Biomass from Agro-Industrial Residues in South America: Current Developments and Perspectives. *Biofuels, Bioprod. Biorefining* **2019**, *13* (6), 1505–1519. https://doi.org/10.1002/bbb.2048.

(4) Ainsworth, E. A.; Ort, D. R. How Do We Improve Crop Production in a Warming World? *Plant Physiol.* **2010**, *154* (2), 526–530. https://doi.org/10.1104/pp.110.161349.

(5) Asseng, S.; Ewert, F.; Martre, P.; Rötter, R. P.; Lobell, D. B.; Cammarano, D.; Kimball, B. A.; Ottman, M. J.; Wall, G. W.; White, J. W.; Reynolds, M. P.; Alderman, P. D.; Prasad, P. V. V.; Aggarwal, P. K.; Anothai, J.; Basso, B.; Biernath, C.; Challinor, A. J.; De Sanctis, G.; Doltra, J.; Fereres, E.; Garcia-Vila, M.; Gayler, S.; Hoogenboom, G.; Hunt, L. A.; Izaurralde, R. C.; Jabloun, M.; Jones, C. D.; Kersebaum, K. C.; Koehler, A.-K.; Müller, C.; Naresh Kumar, S.; Nendel, C.; O'Leary, G.; Olesen, J. E.; Palosuo, T.; Priesack, E.; Eyshi Rezaei, E.; Ruane, A. C.; Semenov, M. A.; Shcherbak, I.; Stöckle, C.; Stratonovitch, P.; Streck, T.; Supit, I.; Tao, F.; Thorburn, P. J.; Waha, K.; Wang, E.; Wallach, D.; Wolf, J.; Zhao, Z.; Zhu, Y. Rising Temperatures Reduce Global Wheat Production. *Nat. Clim. Chang.* **2015**, *5* (2), 143–147. https://doi.org/10.1038/nclimate2470.

(6) Zhao, C.; Liu, B.; Piao, S.; Wang, X.; Lobell, D. B.; Huang, Y.; Huang, M.; Yao, Y.; Bassu, S.; Ciais, P.; Durand, J. L.; Elliott, J.; Ewert, F.; Janssens, I. A.; Li, T.; Lin, E.; Liu, Q.; Martre, P.; Müller, C.; Peng, S.; Peñuelas, J.; Ruane, A. C.; Wallach, D.; Wang, T.; Wu, D.; Liu, Z.; Zhu, Y.; Zhu, Z.; Asseng, S. Temperature Increase Reduces Global Yields of Major Crops in Four Independent Estimates. *Proc. Natl. Acad. Sci. U. S. A.* **2017**, *114* (35), 9326–9331. https://doi.org/10.1073/pnas.1701762114.

(7) Cesarino, I. Structural Features and Regulation of Lignin Deposited upon Biotic and Abiotic Stresses. *Curr. Opin. Biotechnol.* **2019**, *56*, 209–214. https://doi.org/10.1016/j.copbio.2018.12.012.

(8) Tenhaken, R. Cell Wall Remodeling under Abiotic Stress. *Front. Plant Sci.* **2015**, *5*. https://doi.org/10.3389/fpls.2014.00771.

(9) Borjas-Ventura, R.; Alves, L. R.; de Oliveira, R.; Martínez, C. A.; Gratão, P. L. Impacts of Warming and Water Deficit on Antioxidant Responses in Panicum Maximum Jacq. *Physiol. Plant.* **2019**, *165* (2), 413–426. https://doi.org/10.1111/ppl.12907.

(10) Anderson, E. K.; Voigt, T. B.; Kim, S.; Lee, D. K. Determining Effects of Sodicity and Salinity on Switchgrass and Prairie Cordgrass Germination and Plant Growth. *Ind. Crops Prod.* **2015**, *64*, 79–87. https://doi.org/10.1016/j.indcrop.2014.11.016.

(11) Sosa, L. L.; Jozami, E.; Oakley, L. J.; Montero, G. A.; Ferreras, L. A.; Venturi, G.; Feldman, S. R. Using C4 Perennial Rangeland Grasses for Bioenergy. *Biomass and Bioenergy* **2019**, *128*, 105299. https://doi.org/10.1016/j.biombioe.2019.105299.

(12) EMBRAPA. *Relatório de Avaliação Dos Impactos Das Tecnologias Geradas Pela Embrapa Gado de Corte—Capim Mombac¸a. Embrapa Gado Corte.*; 2014.

(13) Lima, M. A.; Gomez, L. D.; Steele-King, C. G.; Simister, R.; Bernardinelli, O. D.; Carvalho, M. A.; Rezende, C. A.; Labate, C. A.; Deazevedo, E. R.; McQueen-Mason, S. J.; Polikarpov, I. Evaluating the Composition and Processing Potential of Novel Sources of Brazilian Biomass for Sustainable Biorenewables Production. *Biotechnol. Biofuels* **2014**, *7* (1), 1–19. https://doi.org/10.1186/1754-6834-7-10.

(14) De Assis Prado, C. H. B.; De Camargo-Bortolin, L. H. G.; Castro, É.; Martinez, C. A. Leaf Dynamics of Panicum Maximum under Future Climatic Changes. *PLoS One* **2016**, *11* (2), 1–17. https://doi.org/10.1371/journal.pone.0149620.

(15) Habermann, E.; San Martin, J. A. B.; Contin, D. R.; Bossan, V. P.; Barboza, A.; Braga, M. R.; Groppo, M.; Martinez, C. A. Increasing Atmospheric CO 2 and Canopy Temperature Induces Anatomical and Physiological Changes in Leaves of the C 4 Forage Species Panicum Maximum. *PLoS One* **2019**, *14* (2), 1–25. https://doi.org/10.1371/journal.pone.0212506.

(16) Kimball, B. A. Theory and Performance of an Infrared Heater for Ecosystem Warming. *Glob. Chang. Biol.* **2005**, 051006062331001-??? https://doi.org/10.1111/j.1365-2486.2005.1028.x.

(17) Nakagame, S.; Chandra, R. P.; Saddler, J. N. The Effect of Isolated Lignins, Obtained from a Range of Pretreated Lignocellulosic Substrates, on Enzymatic Hydrolysis. *Biotechnol. Bioeng.* **2010**, *105* (5), 871–879. https://doi.org/10.1002/bit.22626.

(18) Sluiter, A.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D. Determination of Extractives in Biomass: Laboratory Analytical Procedure (LAP); Issue Date 7/17/2005. **2008**, No. January.

(19) Oliveira, D. M.; Mota, T. R.; Grandis, A.; de Morais, G. R.; de Lucas, R. C.; Polizeli, M. L. T. M.; Marchiosi, R.; Buckeridge, M. S.; Ferrarese-Filho, O.; dos Santos, W. D. Lignin Plays a Key Role in Determining Biomass Recalcitrance in Forage Grasses. *Renew. Energy* **2019**, *147*, 2206–2217. https://doi.org/10.1016/j.renene.2019.10.020.

(20) Ferrarese, MLL; Zottis, A; Ferrarese-Filho, O. Protein-Free Lignin Quantification in Soybean (Glycine Max) Roots. *Biologia (Bratisl).* **2002**, *57*, 541–543.

(21) Salvador, V. H.; Lima, R. B.; dos Santos, W. D.; Soares, A. R.; Böhm, P. A. F.; Marchiosi, R.; Ferrarese, M. de L. L.; Ferrarese-Filho, O. Cinnamic Acid Increases Lignin Production and Inhibits Soybean Root Growth. *PLoS One* **2013**, *8* (7), 1–10. https://doi.org/10.1371/journal.pone.0069105.

(22) Jiang, J.; Carrillo-Enríquez, N. C.; Oguzlu, H.; Han, X.; Bi, R.; Song, M.; Saddler, J. N.; Sun, R.-C.; Jiang, F. High Production Yield and More Thermally Stable Lignin-Containing Cellulose Nanocrystals Isolated Using a Ternary Acidic Deep Eutectic Solvent. *ACS Sustain. Chem. Eng.* **2020**, *8* (18), 7182–7191. https://doi.org/10.1021/acssuschemeng.0c01724.

(23) Somogyi, M. A New Reagente for the Determination of Sugars. *J Biol Chem* **1945**, *160*, 61–63.

(24) Amaral, L. I. V. do; Gaspar, M.; Costa, P. M. F.; Aidar, M. P. M.; Buckeridge, M. S. Novo Método Enzimático Rápido e Sensível de Extração e Dosagem de Amido Em Materiais Vegetais. *Hoehnea* **2007**, *34* (4), 425–431. https://doi.org/10.1590/s2236-89062007000400001.

(25) Jung, C. D.; Yu, J. H.; Eom, I. Y.; Hong, K. S. Sugar Yields from Sunflower Stalks Treated by Hydrothermolysis and Subsequent Enzymatic Hydrolysis. *Bioresour. Technol.* **2013**, *138*, 1–7. https://doi.org/10.1016/j.biortech.2013.03.033.

(26) Grandis, A.; Arenque-Musa, B. C.; Martins, M. C. M.; Maciel, T. O.; Simister, R.; Gómez, L. D.; Buckeridge, M. S. Senna Reticulata: A Viable Option for Bioenergy Production in the Amazonian Region. *Bioenergy Res.* **2020**. https://doi.org/10.1007/s12155-020-10176-x.

(27) Khatri, V.; Meddeb-Mouelhi, F.; Adjallé, K.; Barnabé, S.; Beauregard, M. Determination of Optimal Biomass Pretreatment Strategies for Biofuel Production: Investigation of Relationships between Surface-Exposed Polysaccharides and Their Enzymatic Conversion Using Carbohydrate-Binding Modules. *Biotechnol. Biofuels* **2018**, *11* (1), 1–16. https://doi.org/10.1186/s13068-018-1145-5.

(28) Khatri, V.; Meddeb-Mouelhi, F.; Beauregard, M. New Insights into the Enzymatic Hydrolysis

of Lignocellulosic Polymers by Using Fluorescent Tagged Carbohydrate-Binding Modules. *Sustain. Energy Fuels* **2018**, *2* (2), 479–491. https://doi.org/10.1039/c7se00427c.

(29) Gatt, E.; Khatri, V.; Bley, J.; Barnabé, S.; Vandenbossche, V.; Beauregard, M. Enzymatic Hydrolysis of Corn Crop Residues with High Solid Loadings: New Insights into the Impact of Bioextrusion on Biomass Deconstruction Using Carbohydrate-Binding Modules. *Bioresour. Technol.* **2019**, *282* (March), 398–406. https://doi.org/10.1016/j.biortech.2019.03.045.

(30) Hébert-Ouellet, Y.; Meddeb-Mouelhi, F.; Khatri, V.; Cui, L.; Janse, B.; Macdonald, K.; Beauregard, M. Tracking and Predicting Wood Fibers Processing with Fluorescent Carbohydrate Binding Modules. *Green Chem.* **2017**, *19* (11), 2603–2611. https://doi.org/10.1039/c6gc03581g.

(31) Bombeck, P. L.; Khatri, V.; Meddeb-Mouelhi, F.; Montplaisir, D.; Richel, A.; Beauregard, M. Predicting the Most Appropriate Wood Biomass for Selected Industrial Applications: Comparison of Wood, Pulping, and Enzymatic Treatments Using Fluorescent-Tagged Carbohydrate-Binding Modules. *Biotechnol. Biofuels* **2017**, *10* (1), 1–14. https://doi.org/10.1186/s13068-017-0980-0.

(32) Mboowa, D.; Khatri, V.; Saddler, J. N. The Use of Fluorescent Protein-Tagged Carbohydrate-Binding Modules to Evaluate the Influence of Drying on Cellulose Accessibility and Enzymatic Hydrolysis. *RSC Adv.* **2020**, *10* (45), 27152–27160. https://doi.org/10.1039/D0RA05333C.

(33) Khatri, V.; Hébert-Ouellet, Y.; Meddeb-Mouelhi, F.; Beauregard, M. Specific Tracking of Xylan Using Fluorescent-Tagged Carbohydrate-Binding Module 15 as Molecular Probe. *Biotechnol. Biofuels* **2016**, *9* (1), 1–13. https://doi.org/10.1186/s13068-016-0486-1.

(34) Valliammai, M. G.; Gopal, N. O.; Anandham, R. Elucidation of Microbial Diversity and Lignocellulolytic Enzymes for the Degradation of Lignocellulosic Biomass in the Forest Soils of Eastern and Western Ghats of Tamil Nadu, India. *Biofuels, Bioprod. Biorefining* **2021**, *15* (1), 47–60. https://doi.org/10.1002/bbb.2144.

(35) Silveira, M. H. L.; Morais, A. R. C.; Da Costa Lopes, A. M.; Olekszyszen, D. N.; Bogel-Łukasik, R.; Andreaus, J.; Pereira Ramos, L. Current Pretreatment Technologies for the Development of Cellulosic Ethanol and Biorefineries. *ChemSusChem* **2015**, *8* (20), 3366–

3390. https://doi.org/10.1002/cssc.201500282.

(36) Sosa, L. L.; Jozami, E.; Oakley, L. J.; Montero, G. A.; Ferreras, L. A.; Venturi, G.; Feldman, S. R. Using C4 Perennial Rangeland Grasses for Bioenergy. *Biomass and Bioenergy* **2019**, *128* (December 2018), 105299. https://doi.org/10.1016/j.biombioe.2019.105299.

(37) Wei Kit Chin, D.; Lim, S.; Pang, Y. L.; Lam, M. K. Fundamental Review of Organosolv Pretreatment and Its Challenges in Emerging Consolidated Bioprocessing. *Biofuels, Bioprod. Biorefining* **2020**, *14* (4), 808–829. https://doi.org/10.1002/bbb.2096.

(38) Wei, H.; Gou, J.; Yordanov, Y.; Zhang, H.; Thakur, R.; Jones, W.; Burton, A. Global Transcriptomic Profiling of Aspen Trees under Elevated [CO2] to Identify Potential Molecular Mechanisms Responsible for Enhanced Radial Growth. *J. Plant Res.* **2013**, *126* (2), 305–320. https://doi.org/10.1007/s10265-012-0524-4.

(39) Hatfield, R. D.; Rancour, D. M.; Marita, J. M. Grass Cell Walls: A Story of Cross-Linking. *Front. Plant Sci.* **2017**, *7* (January). https://doi.org/10.3389/fpls.2016.02056.

(40) Moura, J. C. M. S.; Bonine, C. A. V.; de Oliveira Fernandes Viana, J.; Dornelas, M. C.; Mazzafera, P. Abiotic and Biotic Stresses and Changes in the Lignin Content and Composition in Plants. *J. Integr. Plant Biol.* **2010**, *52* (4), 360–376. https://doi.org/10.1111/j.1744-7909.2010.00892.x.

(41) Wang, T.; McFarlane, H. E.; Persson, S. The Impact of Abiotic Factors on Cellulose Synthesis. *J. Exp. Bot.* **2016**, *67* (2), 543–552. https://doi.org/10.1093/jxb/erv488.

(42) Olivera Viciedo, D.; de Mello Prado, R.; Martínez, C. A.; Habermann, E.; de Cássia Piccolo, M. Short-Term Warming and Water Stress AffectPanicum MaximumJacq. Stoichiometric Homeostasis and Biomass Production. *Sci. Total Environ.* **2019**, *681*, 267–274. https://doi.org/10.1016/j.scitotenv.2019.05.108.

(43) Wedow, J. M.; Yendrek, C. R.; Mello, T. R.; Creste, S.; Martinez, C. A.; Ainsworth, E. A. Metabolite and Transcript Profiling of Guinea Grass (Panicum Maximum Jacq) Response to Elevated [CO 2] and Temperature. *Metabolomics* **2019**, *15* (4), 1–13. https://doi.org/10.1007/s11306-019-1511-8.

(44) Zhu, J.; Lee, B.-H.; Dellinger, M.; Cui, X.; Zhang, C.; Wu, S.; Nothnagel, E. A.; Zhu, J.-K. A

Cellulose Synthase-like Protein Is Required for Osmotic Stress Tolerance in Arabidopsis. *Plant J.* **2010**, no-no. https://doi.org/10.1111/j.1365-313X.2010.04227.x.

(45) Oliveira, D. M.; Mota, T. R.; Salatta, F. V.; Sinzker, R. C.; Končitíková, R.; Kopečný, D.; Simister, R.; Silva, M.; Goeminne, G.; Morreel, K.; Rencoret, J.; Gutiérrez, A.; Tryfona, T.; Marchiosi, R.; Dupree, P.; del Río, J. C.; Boerjan, W.; McQueen-Mason, S. J.; Gomez, L. D.; Ferrarese-Filho, O.; dos Santos, W. D. Cell Wall Remodeling under Salt Stress: Insights into Changes in Polysaccharides, Feruloylation, Lignification, and Phenolic Metabolism in Maize. *Plant. Cell Environ.* **2020**. https://doi.org/10.1111/pce.13805.

(46) Li, X.; Ximenes, E.; Kim, Y.; Slininger, M.; Meilan, R.; Ladisch, M.; Chapple, C. Lignin Monomer Composition Affects Arabidopsis Cell-Wall Degradability after Liquid Hot Water Pretreatment. *Biotechnol. Biofuels* **2010**, *3*, 1–7. https://doi.org/10.1186/1754-6834-3-27.

(47) Studer, M. H.; DeMartini, J. D.; Davis, M. F.; Sykes, R. W.; Davison, B.; Keller, M.; Tuskan, G. A.; Wyman, C. E. Lignin Content in Natural Populus Variants Affects Sugar Release. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108* (15), 6300–6305. https://doi.org/10.1073/pnas.1009252108.

(48) Anderson, E. M.; Stone, M. L.; Katahira, R.; Reed, M.; Muchero, W.; Ramirez, K. J.; Beckham, G. T.; Román-Leshkov, Y. Differences in S/G Ratio in Natural Poplar Variants Do Not Predict Catalytic Depolymerization Monomer Yields. *Nat. Commun.* **2019**, *10* (1), 1–10. https://doi.org/10.1038/s41467-019-09986-1.

(49) Tobimatsu, Y.; Schuetz, M. Lignin Polymerization: How Do Plants Manage the Chemistry so Well? *Curr. Opin. Biotechnol.* **2019**, *56*, 75–81. https://doi.org/10.1016/j.copbio.2018.10.001.

(50) Oliveira, D. M.; Mota, T. R.; Oliva, B.; Segato, F.; Marchiosi, R.; Ferrarese-Filho, O.; Faulds, C. B.; dos Santos, W. D. Feruloyl Esterases: Biocatalysts to Overcome Biomass Recalcitrance and for the Production of Bioactive Compounds. *Bioresour. Technol.* **2019**, *278* (January), 408–423. https://doi.org/10.1016/j.biortech.2019.01.064.

(51) Masarin, F.; Neves, A. S.; Milagres, A. M. F.; Ferraz, A. Evaluation of a Simple Alkaline Pretreatment for Screening of Sugarcane Hybrids According to Their in Vitro Digestibility. *Ind. Crops Prod.* **2013**, *51*, 390–395. https://doi.org/10.1016/j.indcrop.2013.09.033.

(52) Ostovareh, S.; Karimi, K.; Zamani, A. Efficient Conversion of Sweet Sorghum Stalks to

Biogas and Ethanol Using Organosolv Pretreatment. *Ind. Crops Prod.* **2015**. https://doi.org/10.1016/j.indcrop.2014.12.023.

(53) Zhao, X.; Zhang, L.; Liu, D. Biomass Recalcitrance. Part I: The Chemical Compositions and Physical Structures Affecting the Enzymatic Hydrolysis of Lignocellulose. *Biofuels, Bioprod. Biorefining* **2012**, *6* (4), 465–482. https://doi.org/10.1002/bbb.1331.

(54) Jeoh, T., Ishizawa, C. I., Davis, M. F., Himmel, M. E., Adney, W. S., & Johnson, D. K. Cellulase Digestibility of Pretreated Biomass Is Limited by Cellulose Accessibility. *Biotechnol. Bioeng.* **2007**, *98* (1), 112–122. https://doi.org/doi:10.1002/bit.21408.

(55) MILLER, G.; SUZUKI, N.; CIFTCI-YILMAZ, S.; MITTLER, R. Reactive Oxygen Species Homeostasis and Signalling during Drought and Salinity Stresses. *Plant. Cell Environ.* **2010**, *33* (4), 453–467. https://doi.org/10.1111/j.1365-3040.2009.02041.x.

(56) Gangola, M. P.; Ramadoss, B. R. Sugars Play a Critical Role in Abiotic Stress Tolerance in Plants. In *Biochemical, Physiological and Molecular Avenues for Combating Abiotic Stress Tolerance in Plants*; Elsevier, 2018; pp 17–38. https://doi.org/10.1016/B978-0-12-813066-7.00002-4.

(57) Koch, K. E. CARBOHYDRATE-MODULATED GENE EXPRESSION IN PLANTS. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1996**, *47* (1), 509–540. https://doi.org/10.1146/annurev.arplant.47.1.509.

(58) Habermann, E.; Dias de Oliveira, E. A.; Contin, D. R.; Delvecchio, G.; Viciedo, D. O.; de Moraes, M. A.; de Mello Prado, R.; de Pinho Costa, K. A.; Braga, M. R.; Martinez, C. A. Warming and Water Deficit Impact Leaf Photosynthesis and Decrease Forage Quality and Digestibility of a C4 Tropical Grass. *Physiol. Plant.* **2019**, *165* (2), 383–402. https://doi.org/10.1111/ppl.12891.

(59) Thalmann, M.; Santelia, D. Starch as a Determinant of Plant Fitness under Abiotic Stress. *New Phytol.* **2017**, *214* (3), 943–951. https://doi.org/10.1111/nph.14491.

(60) Zhang, X.; Qin, W.; Paice, M. G.; Saddler, J. N. High Consistency Enzymatic Hydrolysis of Hardwood Substrates. *Bioresour. Technol.* **2009**, *100* (23), 5890–5897. https://doi.org/10.1016/j.biortech.2009.06.082.

(61) Ferreira, R. G.; Azzoni, A. R.; Freitas, S. On the Production Cost of Lignocellulose-Degrading Enzymes. *Biofuels, Bioprod. Biorefining* **2021**, *15* (1), 85–99. https://doi.org/10.1002/bbb.2142.

(62) Oliveira, C.; Carvalho, V.; Domingues, L.; Gama, F. M. Recombinant CBM-Fusion Technology - Applications Overview. *Biotechnol. Adv.* **2015**, *33* (3–4), 358–369. https://doi.org/10.1016/j.biotechadv.2015.02.006.

(63) Cultures, P. T. Chapter 1 Plant Tissue Cultures. *Methods 715*, 1–20. https://doi.org/10.1007/978-1-61779-008-9.

(64) Knox, J. P. *In Situ Detection of Cellulose with Carbohydrate-Binding Modules*, 1st ed.; Elsevier Inc., 2012; Vol. 510. https://doi.org/10.1016/B978-0-12-415931-0.00012-4.

Table 1. Chemical composition of *Panicum maximum* in different climate conditions. Mean values ± SEM (n=3) marked with different letters in the same column are significantly different (p ≤ 0.05, Tukey's test).

	Chemical Composition (%)				
Biomass	**Total Glucan**	**Total Hemicellulose**	**Total Lignin**	**Ash**	**Extractives**
Control (C)	25.8 ± 0.5^{a}	16.5 ± 0.2^{a}	27.21 ± 1.0^{a}	11.12 ± 0.2^{a}	4.12 ± 0.1^{a}
Elevate Temperature (eT)	30.2 ± 0.03^{b}	16.8 ± 0.03^{a}	28.81 ± 0.8^{a}	11.54 ± 0.4^{a}	4.44 ± 0.05^{a}
Elevate CO_2 (eC)	27.0 ± 0.7^{a}	17.7 ± 0.05^{b}	26.76 ± 0.6^{a}	11.73 ± 0.25^{a}	4.43 ± 0.1^{a}
eTemperature+eCO_2 (eT+eC)	29.7 ± 0.4^{b}	16.6 ± 0.2^{a}	29.38 ± 0.6^{a}	11.30 ± 0.5^{a}	4.08 ± 0.1^{a}

Figures

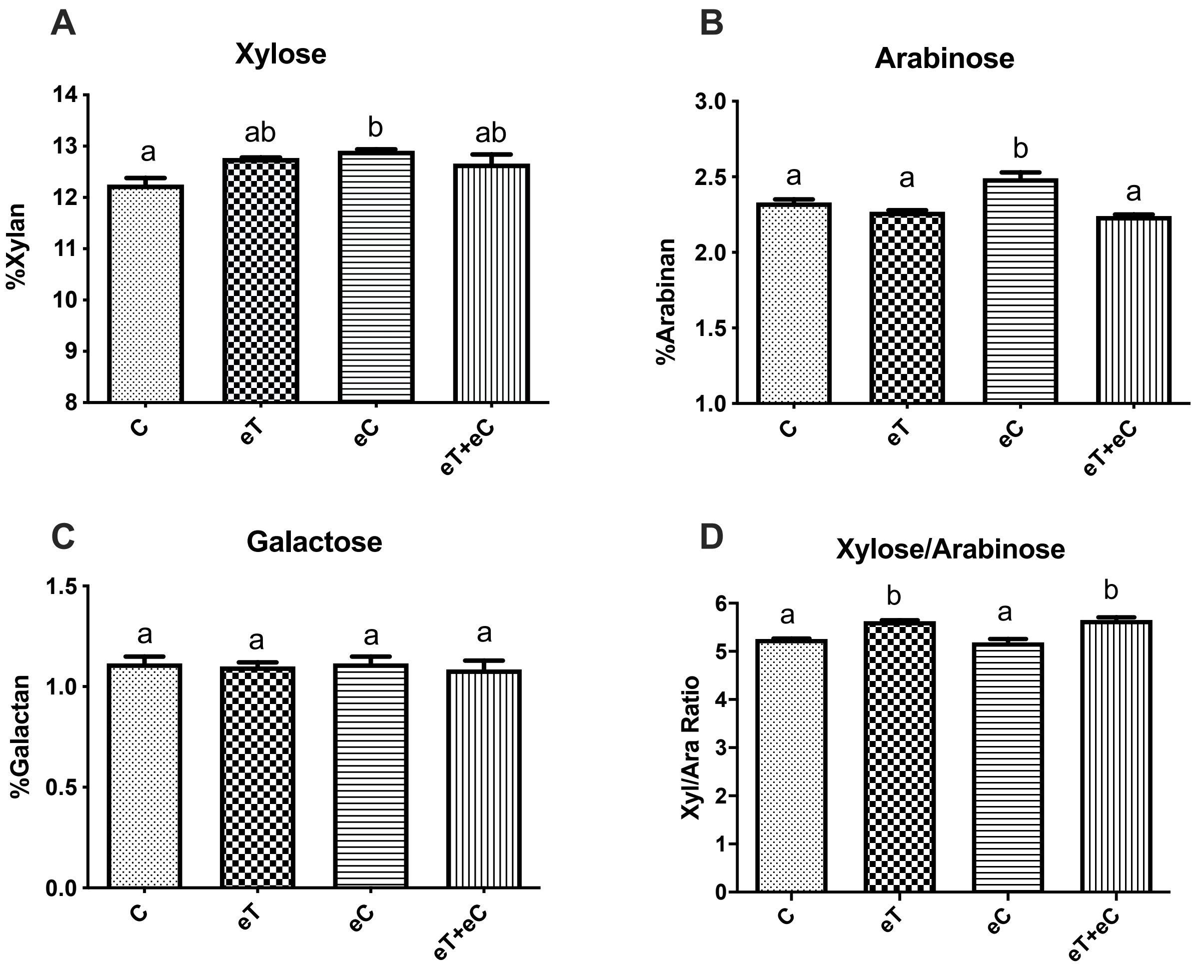


Figure 1: Monosaccharide profiles of *Panicum maximum* in different climate conditions: Control (C), Elevated Temperature (eT), Elevated CO_2 (eC) and combined treatments (eT+eC). Mean values ± SD (n=3) marked with different letters above bars symbolizes statistical differences among treatments ($p \leq 0.05$, Tukey's test).

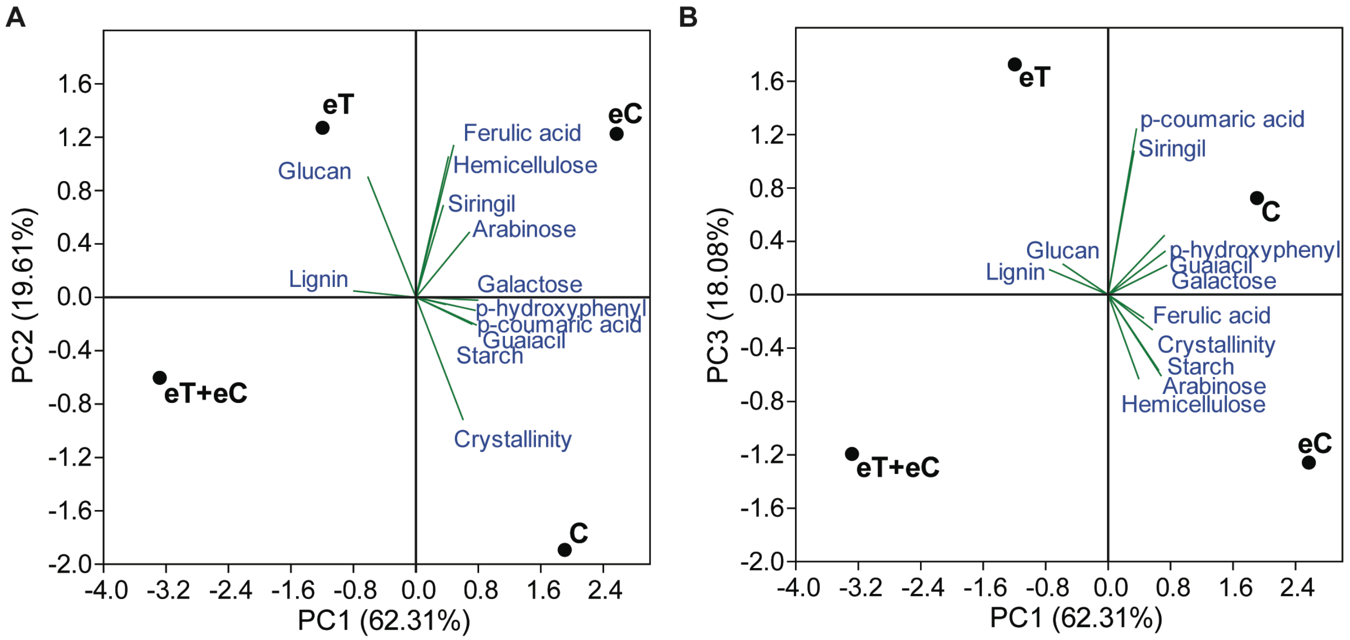


Figure 2: Principal Component Analysis of *P. maximum* cell wall composition in different climate conditions: Control (C), Elevated Temperature (eT), Elevated CO_2 (eC) and combined treatments (eT+eC).

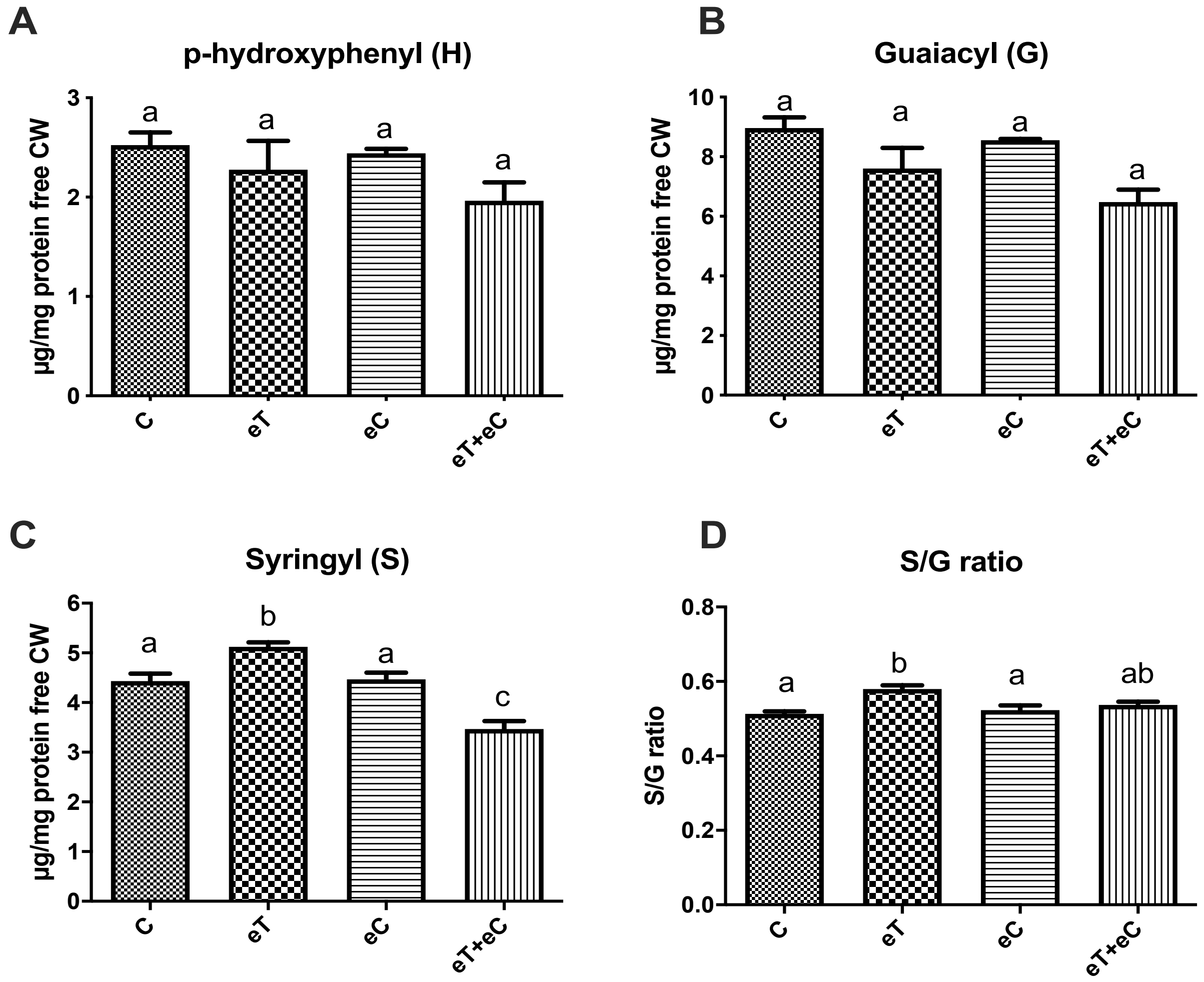


Figure 3: Lignin monomers of *P. maximum* in different climate conditions: Control (C), Elevated Temperature (eT), Elevated CO_2 (eC) and combined treatments (eT+eC). CW: Cell Wall. Mean values ± SD (n=3) marked with different letters above bars symbolize statistical differences among treatments (p≤0.05, Tukey's test).

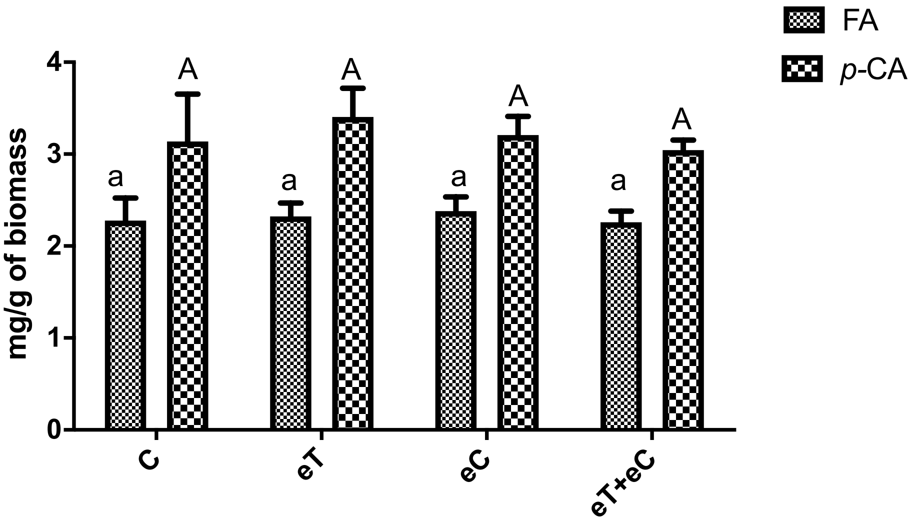


Figure 4: Profile of cell wall-bound phenolics Ferulic acid (FA) and p-coumaric acid (p-CA) of P. maximum in different climate conditions: Treatments: Control (C), Elevate Temperature (eT), Elevate CO_2 (eC) and combined treatment (eT+eC). Mean values ± SD (n=3) marked with different letters above bars symbolize statistical differences among treatments ($p \leq 0.05$, Tukey's test).

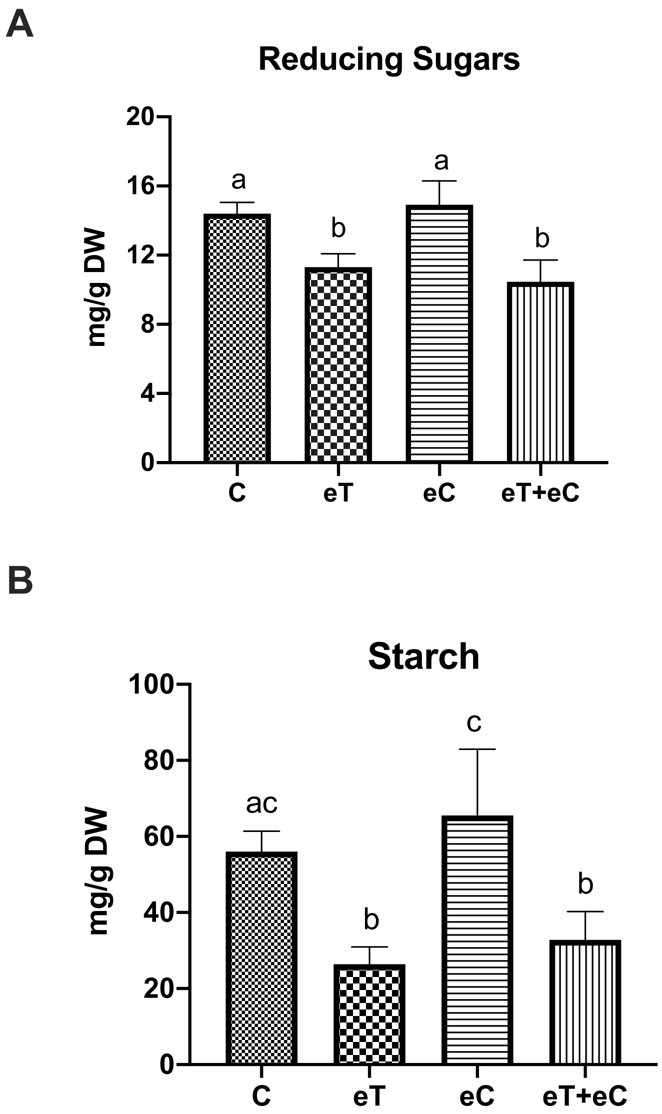


Figure 5: Carbohydrates content in leaves of *P. maximum*. Reducing sugars (A) and starch (B). Treatments: Control (C), Elevated Temperature (eT), Elevated CO_2 (eC) and combined treatments (eT+eC). Mean values ± SD (n=3) marked with different letters above bars symbolize statistical differences among treatments ($p \leq 0.05$, Tukey's test).

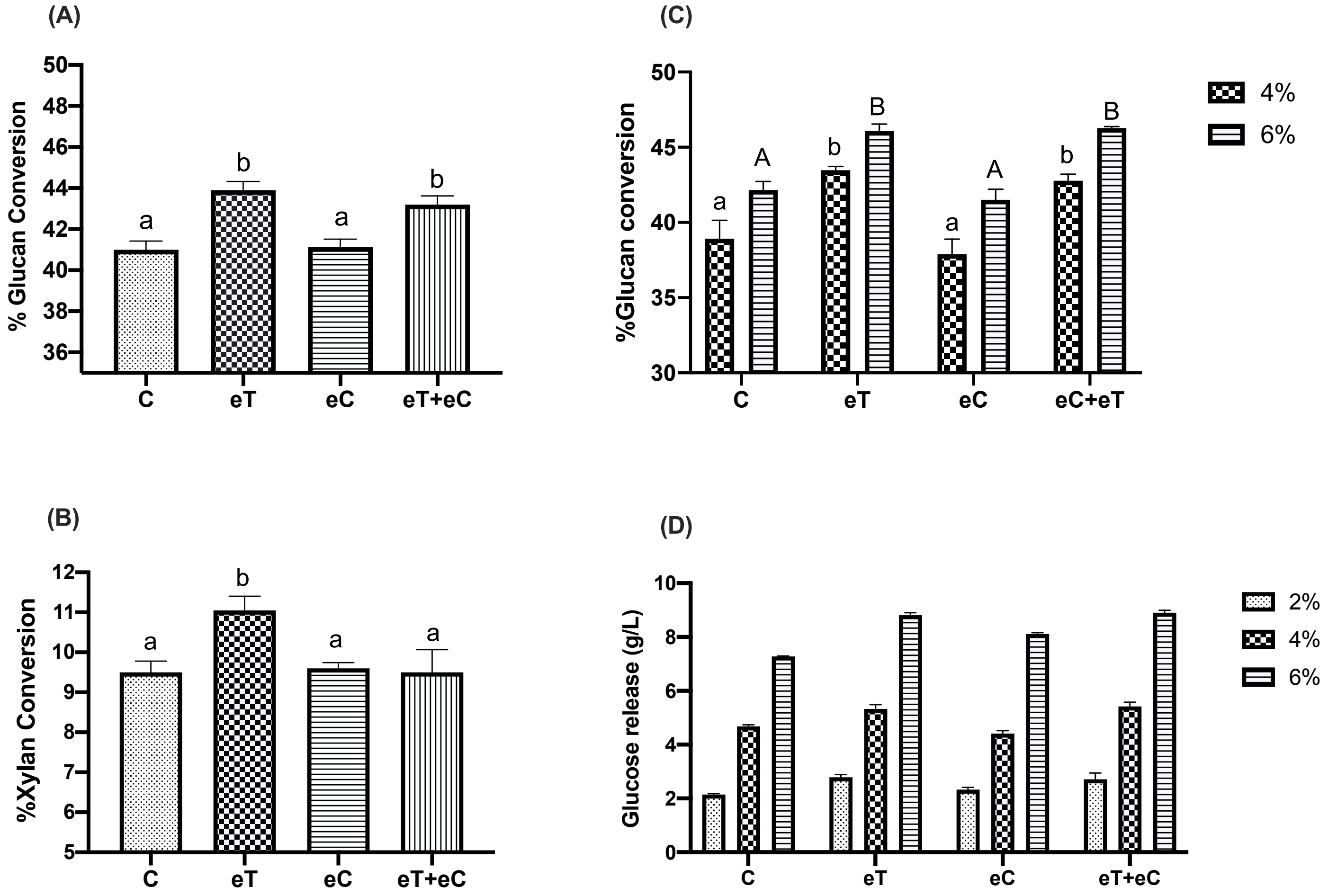


Figure 6: Glucan (A) and Xylan (B) conversion of untreated *P. maximum* after enzymatic saccharification at 72 h and 2% solid content. Glucan Conversion (%) at higher solid loadings (C), and glucose release (g/L) at 2, 4, and 6% solid loading (D). Treatments: Control (C), Elevated Temperature (eT), Elevated CO_2 (eC) and combined treatments (eT+eC). Mean values ± SD (n=3) marked with different letters above bars symbolize statistical differences among treatments.

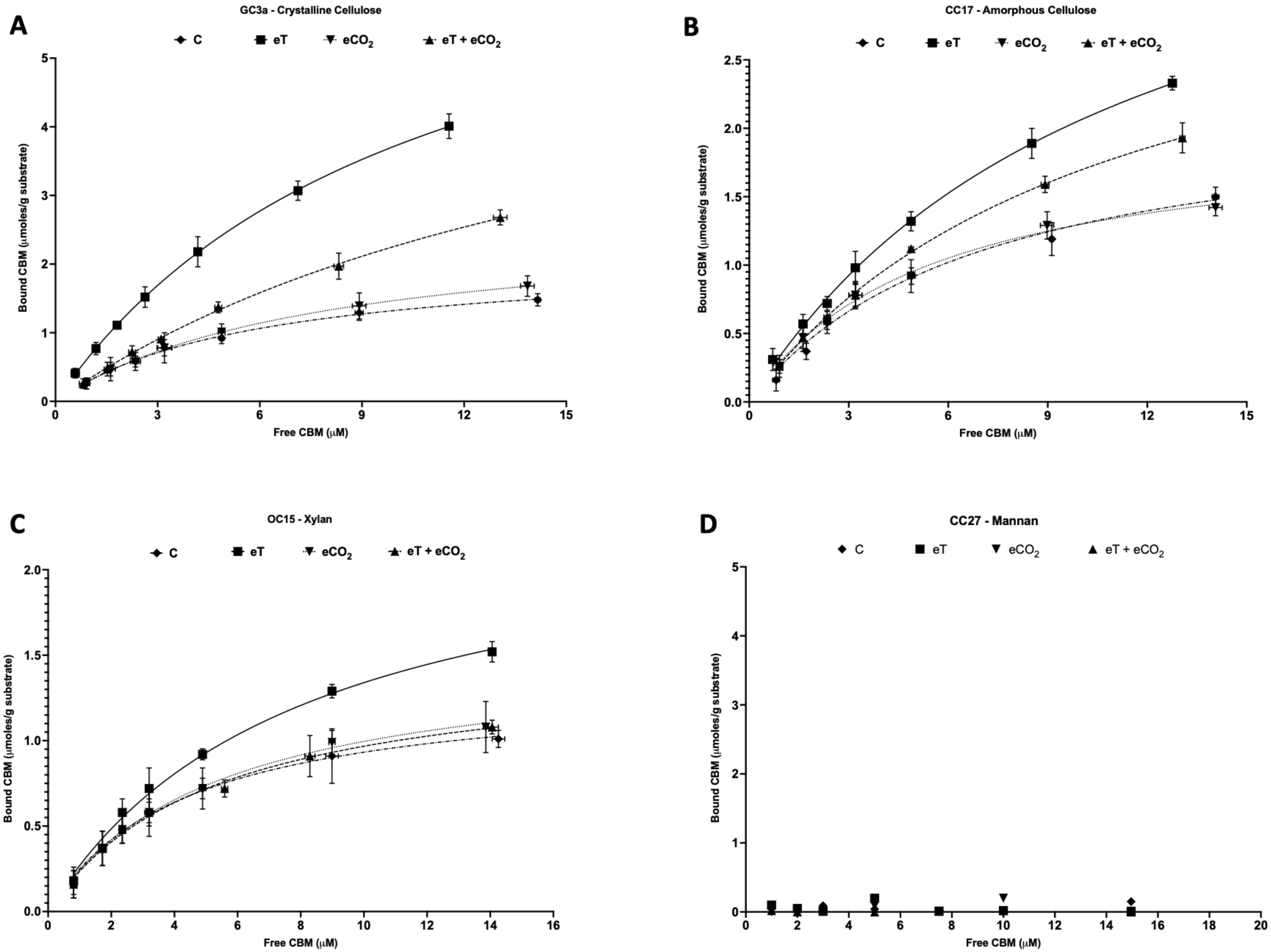


Figure 7: Binding isotherm of FTCM-depletion assay probes: GC3a (A), CC17 (B), OC15 (C) and CC27 (D) to C, eT, eC and eT+eC substrates at 25°C in a 20 mM Tris–HCl (pH 7.5) buffer containing 20 mM NaCl, 5 mM $CaCl_2$. Treatments: Elevated Temperature (eT), Elevated CO_2 (eC) and combined treatments (eT+eC).

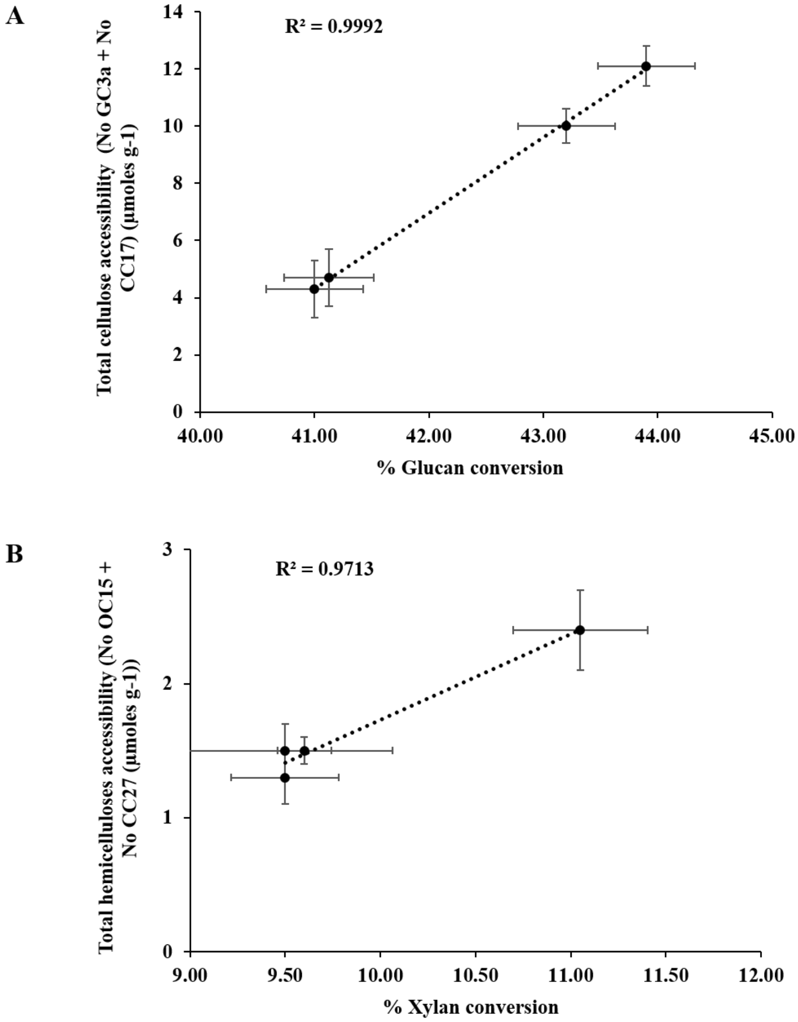


Figure 8: Correlation between accessibility (total cellulose accessibility (A) as detected by FTCM probes (GC3a + CC17) and total hemicelluloses accessibility (B) as detected by FTCM probes (OC15 + CC27)) and percent glucan/xylan hydrolysis.

SUPPLEMENTARY MATERIAL

Table S1. Binding capacity, affinity and change in Gibb's free energy (ΔG) of the GC3a, CC17, OC15 and CC27 probes as determined by FTCM-depletion assay at 25°C in a 20 mM Tris–HCl (pH 7.5) buffer containing 20 mM NaCl, 5 mM $CaCl_2$.

	C				eT				eCO_2				eT + eCO_2			
	GC 3a	CC 17	OC 15	CC 27	GC 3a	CC 17	OC 15	CC 27	GC 3a	CC 17	OC 15	CC 27	GC 3a	CC 17	OC 15	CC 27
N_o (µmoles g^{-1})	2.1 ± 0.3	2.2 ± 0.7	1.3 ± 0.2	-	7.7 ± 0.3	4.4 ± 0.4	2.4 ± 0.3	-	2.6 ± 0.4	2.1 ± 0.6	1.5 ± 0.1	-	6.4 ± 0.2	3.6 ± 0.4	1.5 ± 0.2	-
K_a (µM^{-1})	0.15 ± 0.01	0.14 ± 0.02	0.23 ± 0.04	-	0.09 ± 0.02	0.1 ± 0.02	0.13 ± 0.05	-	0.13 ± 0.03	0.18 ± 0.03	0.19 ± 0.04	-	0.08 ± 0.02	0.11 ± 0.03	0.19 ± 0.05	-
ΔG (KJ mol^{-1})	-29.5 ± 0.2	-29.4 ± 0.4	-30.6 ± 0.4	-	-28.2 ± 0.6	-28.5 ± 0.5	-29.1 ± 1.0	-	-29.1 ± 0.6	-30.0 ± 0.4	-30.1 ± 0.5	-	-27.9 ± 0.6	-28.7 ± 0.7	-30.1 ± 0.7	-
Total cellulose/hemicelluloses accessibility ($N_{o\ GC3a}$ + $N_{o\ CC17}$) ($No_{\ OC15}$ + $No_{\ CC27}$) (µmoles g^{-1})	4.3 ± 1.0		1.3 ± 0.2		12.1 ± 0.7		2.4 ± 0.3		4.7 ± 1.0		1.5 ± 0.1		10.0 ± 0.6		1.5 ± 0.2	

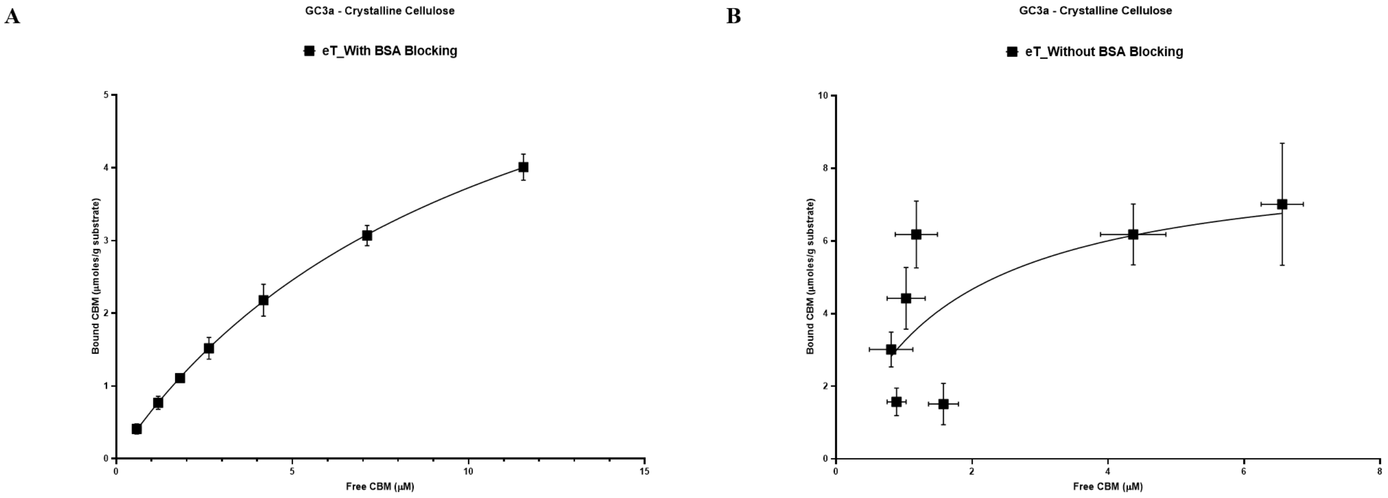


Figure S1: Binding isotherm of FTCM-depletion assay probe GC3a against eT substrate with BSA blocking (A) and without BSA blocking (B) at 25°C in a 20 mM Tris–HCl (pH 7.5) buffer containing 20 mM NaCl, 5 mM CaCl2. Treatments: Elevate Temperature (eT).

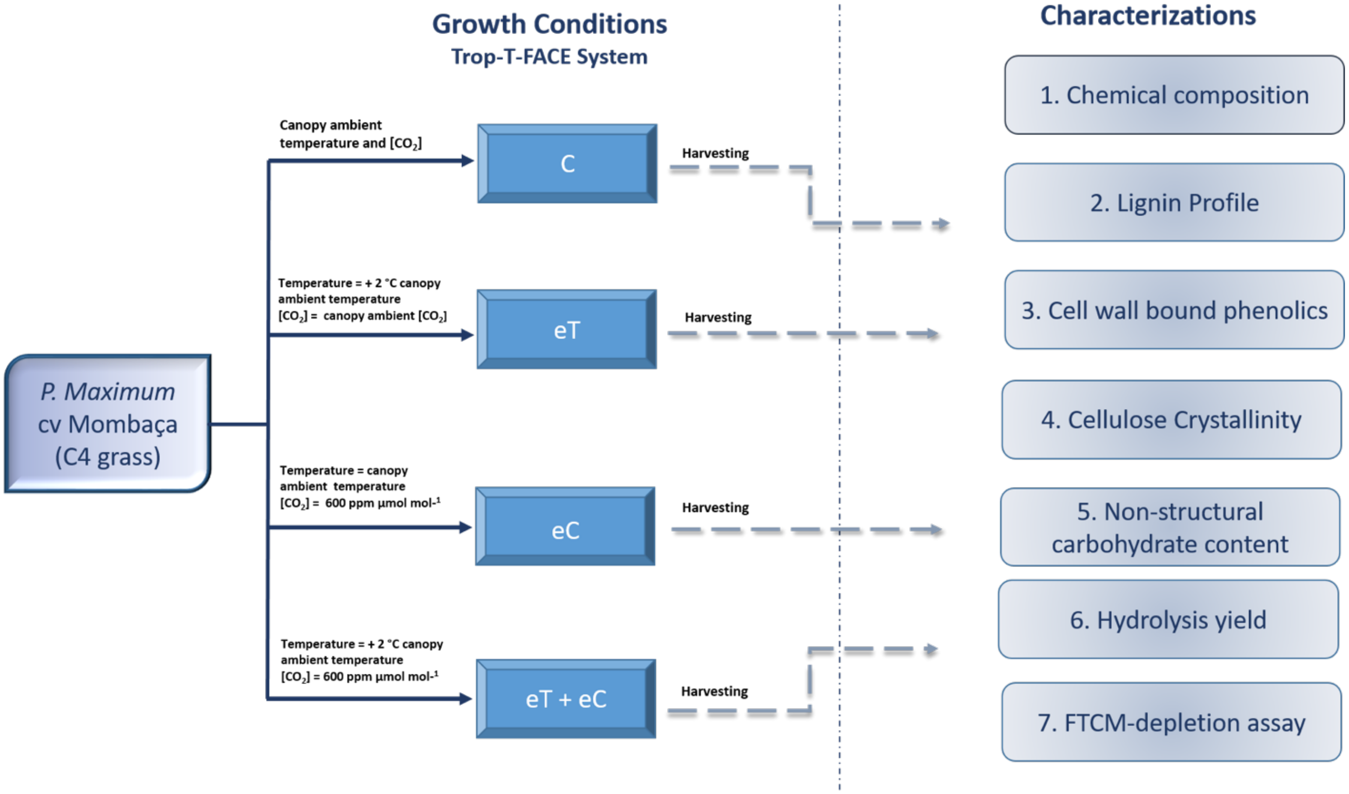


Figure S2: Schematic representation of the growth conditions and analytical parts of the study. C: control, eT: elevated temperature, eC: elevated [CO2] and eT+eC: elevated temperature and elevated [CO_2].

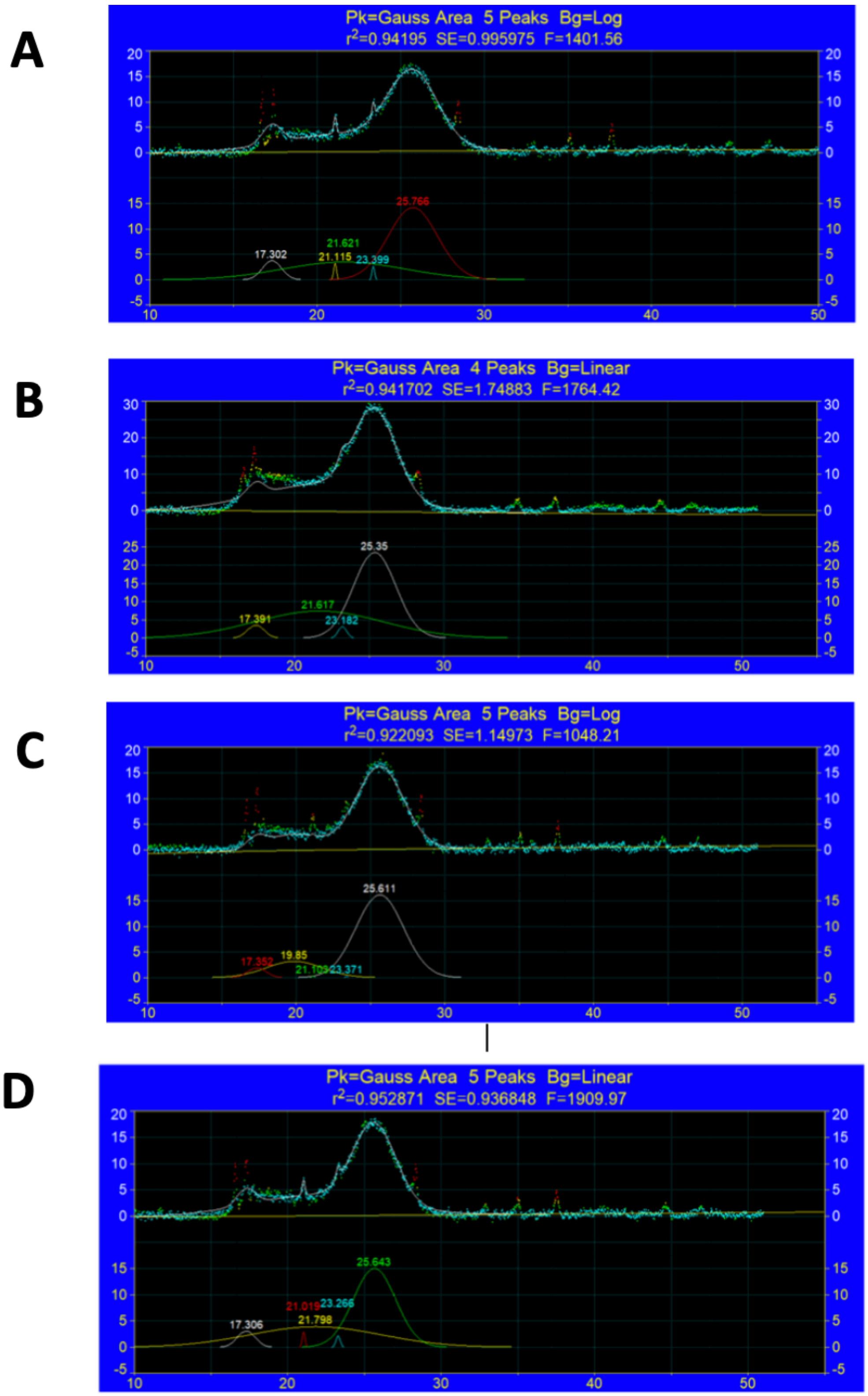


Figure S3: X-ray diffraction (XRD) patterns of Control (A), elevate temperature (B), elevate CO_2 (C) and elevate temperature + elevate CO_2 (D), analyzed by PeakFit software.

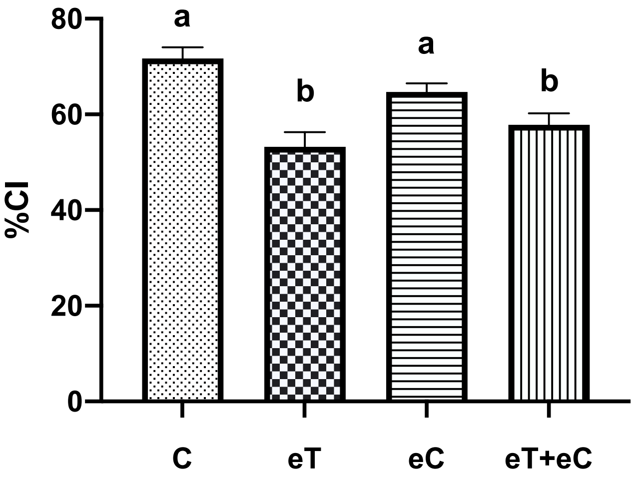


Figure S4: Crystallinity Index (CI) of Control (A), elevate temperature (B), elevate CO_2 (C) and elevate temperature + elevate CO_2 (D).

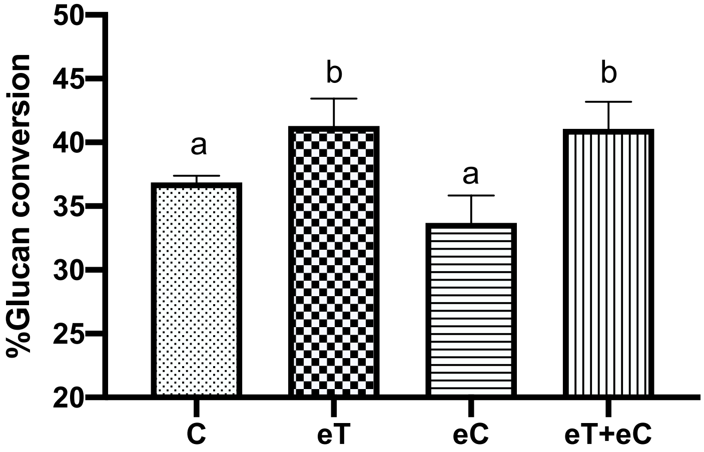


Figure S5: Glucose (A) conversion of untreated *Panicum maximum* (72 h and 2% solid content) after starch removal with dimethyl sulfoxide (DMSO). Control (A), elevate temperature (B), elevate CO_2 (C) and elevate temperature + elevate CO_2 (D).

CHAPTER II

Enzymatic pretreatment with laccases from *Lentinus sajor-caju* induces structural modification in lignin and enhances the digestibility of tropical forage grass (*Panicum maximum*) grown under future climate conditions.

This chapter was published as:

(Freitas et al. 2021) Freitas, E.N.d.; Alnoch, R.C.; Contato, A.G.; Nogueira, K.M.V.; Crevelin, E.J.; Moraes, L.A.B.d.; Silva, R.N.; Martínez, C.A.; Polizeli, M.d.L.T.M. **Enzymatic Pretreatment with Laccases from *Lentinus sajor-caju* Induces Structural Modification in Lignin and Enhances the Digestibility of Tropical Forage Grass (*Panicum maximum*) Grown under Future Climate Conditions.** *International Journal of Molecular Sciences.* 2021, 22, 9445. https://doi.org/10.3390/ijms22179445

International Journal of
Molecular Sciences

MDPI

Article

Enzymatic Pretreatment with Laccases from *Lentinus sajor-caju* Induces Structural Modification in Lignin and Enhances the Digestibility of Tropical Forage Grass (*Panicum maximum*) Grown under Future Climate Conditions

Emanuelle Neiverth de Freitas [1], Robson Carlos Alnoch [2], Alex Graça Contato [1], Karoline Maria V. Nogueira [1], Eduardo José Crevelin [3], Luiz Alberto Beraldo de Moraes [3], Roberto Nascimento Silva [1], Carlos Alberto Martínez [2] and Maria de Lourdes T. M. Polizeli [1,2,*]

[1] Departamento de Bioquímica e Imunologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto 14049-900, Brazil; emanuelleneiverthf@gmail.com (E.N.d.F.); alexgraca.contato@gmail.com (A.G.C.); karolmvnogueira@gmail.com (K.M.V.N.); rsilvausp@gmail.com (R.N.S.)

[2] Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto 14050-901, Brazil; robsonalnoch@usp.br (R.C.A.); carlosamh@ffclrp.usp.br (C.A.M.)

[3] Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto 14050-901, Brazil; ejcrevelin@ffclrp.usp.br (E.J.C.); luizmoraes@ffclrp.usp.br (L.A.B.d.M.)

* Correspondence: polizeli@ffclrp.usp.br

Citation: Freitas, E.N.d.; Alnoch, R.C.; Contato, A.G.; Nogueira, K.M.V.; Crevelin, E.J.; Moraes, L.A.B.d.; Silva, R.N.; Martínez, C.A.; Polizeli, M.d.L.T.M. Enzymatic Pretreatment with Laccases from *Lentinus sajor-caju* Induces Structural Modification in Lignin and Enhances the Digestibility of Tropical Forage Grass (*Panicum maximum*) Grown under Future Climate Conditions. *Int. J. Mol. Sci.* **2021**, *22*, 9445. https://doi.org/10.3390/ijms22179445

Academic Editor: Georg A. Sprenger

Received: 10 August 2021
Accepted: 24 August 2021
Published: 31 August 2021

Abstract: Since laccase acts specifically in lignin, the major contributor to biomass recalcitrance, this biocatalyst represents an important alternative to the pretreatment of lignocellulosic biomass. Therefore, this study investigates the laccase pretreatment and climate change effects on the hydrolytic performance of *Panicum maximum*. Through a Trop-T-FACE system, *P. maximum* grew under current (Control (C)) and future climate conditions: elevated temperature (2 °C more than the ambient canopy temperature) combined with elevated atmospheric CO_2 concentration(600 μmol mol^{-1}), name as eT+eC. Pretreatment using a laccase-rich crude extract from *Lentinus sajor caju* was optimized through statistical strategies, resulting in an increase in the sugar yield of *P. maximum* biomass (up to 57%) comparing to non-treated biomass and enabling hydrolysis at higher solid loading, achieving up to 26 g L^{-1}. These increments are related to lignin removal (up to 46%) and lignin hydrophilization catalyzed by laccase. Results from SEM, CLSM, FTIR, and GC-MS supported the laccase-catalyzed lignin removal. Moreover, laccase mitigates climate effects, and no significant differences in hydrolytic potential were found between C and eT+eC groups. This study shows that crude laccase pretreatment is a potential and sustainable method for biorefinery solutions and helped establish *P. maximum* as a promising energy crop.

Keywords: climate change; enzymatic pretreatment; laccase; dedicated energy crop; biorefinery; biofuels

1. Introduction

One of the most significant challenges humanity faces nowadays is building a sustainable future for a developing world. The increasing global consumption of fossil fuels by 50% in the next five decades, the price hikes, and the greenhouse gas emissions (i.e., CO_2, CH_4, N_2O) generated by them, accelerate climate change and has shifted the research efforts on developing new alternatives of renewable fuels and chemicals [1]. The abundant availability and high holocellulose content of lignocellulosic biomass (LCB) provide a sustainable, cheap, and carbon-neutral emission option to produce renewable fuels and chemicals within the new biorefinery and circular economy concept [2].

However, the deconstruction of LCBs into fuels and chemicals has several drawbacks to their continued growth as a feedstock for energy production that must be overcome. Both microscale (e.g., lignin-carbohydrate complexes, cellulose crystallinity) and macroscale factors (e.g., food vs. fuel conflict, harvesting, and biomass consolidation) affect the production of bioproducts by lignocellulosic sources [3].

Lignocellulosic biomasses are mainly composed of carbohydrate polymers like cellulose and hemicellulose, which are hindered by lignin that is a heteropolymer composed of three main phenylpropane units: *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) [4]. Due to the close association of lignin with cellulose microfibrils forming lignin-carbohydrate complexes (LCCs), this polymer represents a significant barrier in the enzymatic conversion of polysaccharides into sugars that can be further fermented to produce second-generation ethanol or added value green chemicals. Furthermore, lignin is responsible for the unspecific binding of hydrolytic enzymes, decreasing the enzyme concentration during the saccharification process [5]. Therefore, a pretreatment step is often applied, and most of them aim to remove lignin in order to enhance carbohydrate's accessibility and avoid enzyme losses [6].

Although there are several chemical and physical pretreatment methods for biomass delignification, all of them have limitations arising from the high energy input, saccharification and fermentation inhibitors, and the use of aggressive chemicals [7]. Thus, with the growing search for sustainable methods, the biologic pretreatment approach is a promising technology. It is an ecofriendly and cost-effective strategy due to its lower energy consumption and no release of toxic compounds to the environment. The biologic methods apply microorganisms and their biocatalysts that have an important role in the transformation of lignin due to their high substrate specificity contributing to process efficiency. In biological methods, the lignin degradation depends on lignolytic enzymes like lignin peroxidase (LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13), and laccases (EC 1.10.3.2) [8].

Laccases are multicopper oxidoreductases that oxidize a broad range of phenols substrates with the benefit of using oxygen as the electron acceptor rather than H_2O_2. Laccases have been considered as a powerful tool in many biotechnological applications such as bioremediation processes, phenolics compound degradation, and for the enzymatic oxidation of lignin, offering advantages such as low-energy demand, milder pretreatment conditions, fewer side-reactions, lower fermentable carbohydrate loss during pretreatment, and environmentally safe process [9]. To date, laccases from white-rot basidiomycetes fungi have been described as the most efficient for biotechnological applications since they have the highest redox potential (0.8 V vs. Normal Hydrogen Electrode), which enable the enzyme to subtract electrons from the nonphenolic lignin subunits. An alternative to modulate redox potential is the use of low molecular weight compounds, known as mediators, whose enzymatic oxidation produces stable high redox potential intermediates that can further oxidize (nonenzymatic) other compounds that are not oxidizable by laccases alone [10].

Several studies have shown the efficient lignin removal, and increase in the reducing sugar content after saccharification in LCB pretreated with fungal laccases [11–14]. However, there are some challenges to using laccase for LCB delignification, such as high costs for enzymatic production, stability, and low-efficiency process, which makes the area of research still at an early stage in terms of commercial application [15]. Hence, more research is required to overcome the existing limitations in using these biocatalysts on an industrial scale.

In addition to developing new sustainable and economically feasible pretreatment methods, it is equally important to explore alternative lignocellulosic feedstocks to meet the global energy demand and overcome the drawbacks related to biomass harvesting, transport, and consolidation, which currently exists. For example, fast-growing perennial

C4 grasses, such as Miscanthus spp. and switchgrass, have gained momentum as dedicated energy crops in the recent past, being widely used in Europe and the United States [16]. Likewise, *Panicum maximum* (Jacq. cv. Mombaça) is an important C4 grass used for pasture in Brazil, being rich in holocellulosic content (67%, *w/w*) and other several advantages that make it suitable for bioenergy purposes [17]. In addition, *P. maximum* has high efficiency in producing biomass through photosynthesis (30 ton/ha). It can grow in lands no longer utilized for agricultural uses, which minimize the food vs. fuel conflict and lead to cost-effective feedstock production [17,18].

Nonetheless, the climate change expected for the following years should affect growth, yield, and chemical composition since plant physiologic processes, such as photosynthesis, are sensitive to temperature and carbon dioxide (CO_2) [19]. Thus, it is also necessary to evaluate the effects of climate change in C4 grasses as an energy crop after the enzymatic pretreatment step, once both are promising alternatives to the future of renewable fuels and chemicals.

We used a Trop-T-FACE system to simulate future temperature and CO_2 atmospheric concentration under field conditions in the present work. Moreover, to achieve maximum fermentable sugar yield in the saccharification step, a response surface methodology was employed to optimize enzymatic pretreatment conditions using crude laccase from white-rot fungus. To study chemical reactions and physical rearrangements caused by pretreatment and further correlate fiber morphology, substrate composition, and lignin distribution with the enzymatic hydrolysis yield, structural (SEM/CLSM/FTIR), and compositional analysis were performed. We speculated that the crude laccase pretreatment processes could lead to chemical modification on holocellulosic composition and lignin depolymerization/modification, which might influence the climate changes effects on *P. maximum* and its potential as a dedicated energy crop. Thus, the present study comes up with approaches to address both macro and microscale factors that hamper the use of LCB for biorefinery purposes, also considering the climate change experts for the following decades, and thus, greatly helping in the establishment of lignocellulosic-based fuels and products.

2. Results

2.1. Evaluation Enzymes Produced by L. sajor caju in Orange Waste Solid-State Cultures

Lentinus sajor caju was cultivated in orange waste solid cultures for 14 days, at 28 °C. As expected, high levels of laccase activity (>20,000 U L^{-1}) were obtained in the crude extracts (Table S1). Manganese peroxidase activity was detected in small amounts (<180 U L^{-1}), whiles lignin peroxidase activity was not detected in the crude extracts. Moreover, as manganese and lignin peroxidases required hydrogen peroxide (H_2O_2) for oxidation reactions, we assumed negligible activities for these enzymes in the crude extract. Table S1 also showed the activities detected for holocellulose complex enzymes in the crude extracts, and as expected, negligible activity values were also obtained for most enzymes evaluated. Thus, these results indicated the production of the laccase-rich crude extract by *L. sajor caju* under this culture conditions.

To confirm the production of laccases by *L. sajor caju,* two chromatography steps were used to purify laccases from the crude extract. As showed in Figure 1, a band with a molecular mass of approximately 52 kDa was evidenced in SDS-PAGE (Figure 1A). Zymogram analysis confirmed the laccase activity since only one active spot over agar-ABTS was observed (Figure 1B). The difference found in protein molecular weight for Zymogram is due to the non-denaturing conditions used, impacting relative protein mobility through the polyacrylamide gel. To confirm the identity of the enzyme, purified fractions corresponding to laccase activity were analyzed by mass spectrometry (LC/MS). The mass spectra data obtained were analyzed in the TPP server platform (Uniprot database) with the XTandem peptide search engine and ProteinProphet. The mass fragments analysis gave sequence coverages of 11% for the putative laccase 4 (Uniprot

Q9HFT4) of *Lentinus (Pleurotus) sajor-caju* (Table S2). Initial sequence analysis showed a theoretical molecular weight of 49.5 kDa, as evidenced in the SDS-PAGE (Figure 1A), confirming a rich-laccase crude extract produced by *L. sajor caju.* This extract, named LacLsc, was characterized and applied in the pretreatments used in the current work.

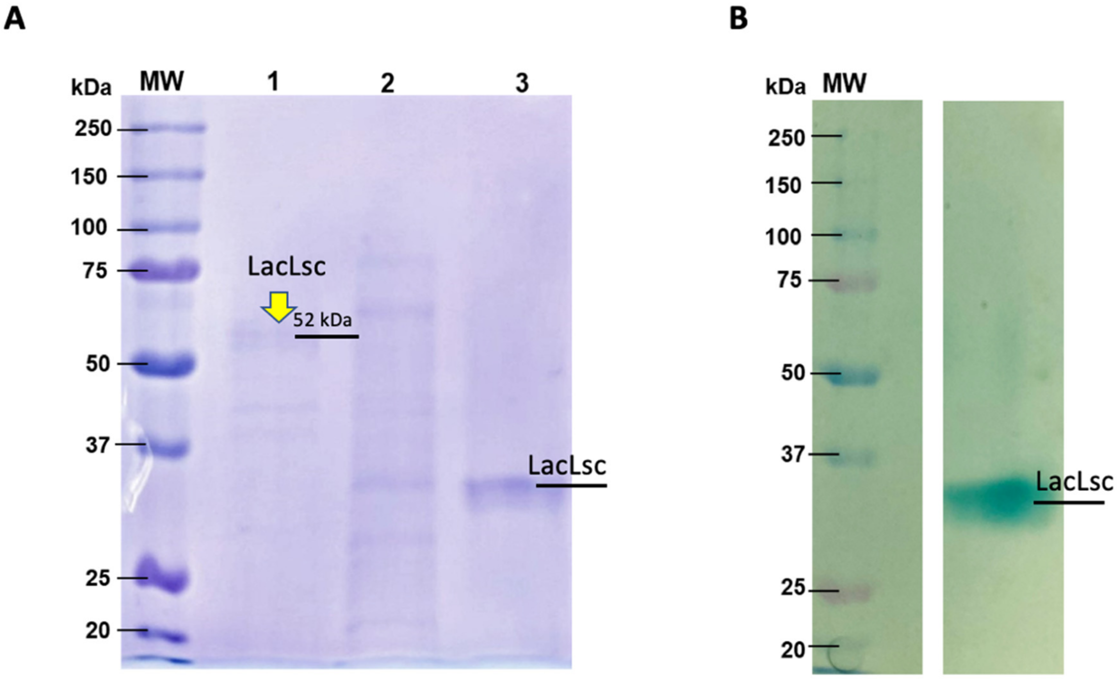


Figure 1. (**A**) SDS-PAGE of *L. sajor-caju* laccase purified through ion exchange followed by size exclusion chromatography (1), only ion exchange (2), and (3) native laccase (non-denatured) purified with ion exchange and size exclusion. (**B**) Zymogram in non-denaturing conditions visualized with ABTS-agar. The Bio-Rad standards contained 10 recombinant proteins (20–250 kDa). The SDS-PAGE gel was stained with Coomassie Blue R dye.

2.2. Laccase Activity Optimal Parameters

Both temperature and pH have great influence on enzymes activities. The pH affects active sites, which are composed of ionizable groups that must be in an acceptable ionic form to maintain the conformation of the active site and catalyze the reaction. Likewise, temperature affects the three-dimensional structure of proteins and their reaction velocity [20]. Therefore, we studied pH and thermal stability more closely to define the conditions to be applied on laccase pretreatment.

Figure 2A shows that pH 5.0 was the optimal value for *L. sajor-caju* laccase activity. Therefore the acetate buffer pH 5.0 was used for the pretreatment process. Regarding the thermal stability (Figure 2B,C), the crude laccase was stable in a temperature range of 40 to 50 °C, with a half-life time of approximately 1200 and 450 min for 40 and 50 °C, respectively. At temperatures above 50 °C, the activity drops off quickly in the first 30 min. In 24 h at 40 and 50 °C, the *L. sajor-caju* laccase presented a residual activity of 44 ± 1% and 23 ± 0.1%, respectively. Considering the above data, we kept pH 5.0 and pretreatment time at 6 h for the CCD experiment, while 41.6 to 58 °C was the temperature range tested in CCD experiments.

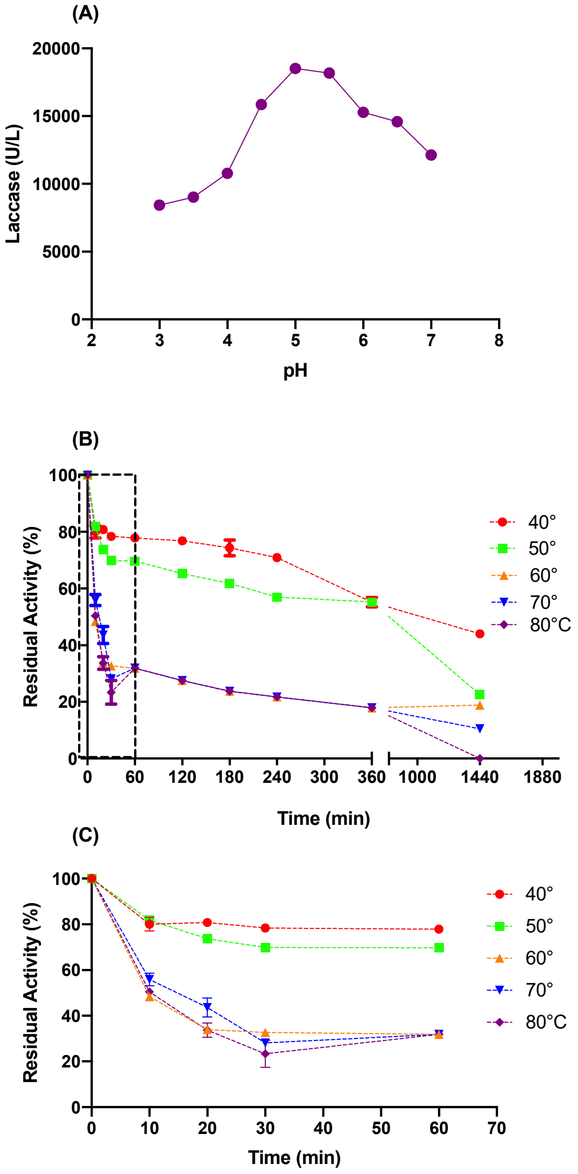


Figure 2. Optimum pH for catalytic activity (**A**) and thermal stability of *L. sajor-caju* crude laccase in longer times (**B**) and extension of shorter times (**C**). The buffer solutions for optimum pH assay were used according to their buffer range: Sodium Acetate (3–5) and Sodium Phosphate (6–8).

2.3. Optimization of Crude Laccase Pretreatment by Central Composite Design (CCD) and Response Surface Analysis

Laccase load (U g^{-1}), mediator concentration (*w*/*v*), and temperature (°C) were investigated CCD since they are the main variables that influence the pretreatment efficiency and, therefore, the hydrolysis yield of fermentable sugars. The following reduced mathematical models (considering only the significant variables, $p < 0.05$) represent the influence of the independent variables on the final response, Sugar yield (g L^{-1}). Equation (1) was obtained for *P. maximum* control group and Equation (2) for eT+eC treatment.

$$Sugar\ yield\ (g\ L^{-1}) = 10.98 + 1.29\ (X_1) - 0.97\ (X_1)^2 - 0.91\ (X_2) - 0.97(X_2)^2 + 0.73\ (X_3) - 0.85\ (X_3)^2 \quad (1)$$

$$\begin{aligned} Sugar\ yield\ (g\ L^{-1}) &\\ &= 11.67 + 1.76\ (X_1) - 0.69\ (X_1)^2 - 0.85\ (X_2) - 1.04\ (X_2)^2 + 0.85\ (X_3) - 1.47(X_3)^2 \\ &- 0.47\ (X_1)(X_3) \end{aligned} \quad (2)$$

where X_1, X_2, and X_3, correspond to the encoded values for laccase, mediator, and temperature, respectively.

The determination coefficient (R^2) of the regression model was used to check its goodness of fit. The R^2 for optimization of crude laccase pretreatment was 0.835 and 0.935 for C and eT+eC models, respectively. These numbers suggest the above models have just failed to explain 16.5% and 6.5% of the total variation. Although this number is more significant for the C group, it is considered a reasonable value considering biological samples. The ANOVA results of the second-order reduced models are summarized in Tables S3 and S4. The validation of the models arises when the variance (F) of the regression is greater than its tabulated F-value, as well the F value of the residues (lack of fit) is lower than its tabulated F-value. Thus, as both conditions were fulfilled, the models are valid for both climate conditions datasets (C and eT+eC).

The interaction between independent variables and their independent influence on sugar yields of *P. maximum* biomass after laccase pretreatment was graphically denoted by response surfaces. The contour curves (Figure 3) created based on the models described above show similar trends related to sugar yields (g L^{-1}) for both climate conditions. The highest sugars yields found for C Figure 3 (A1–3) and eT+eC Figure 3 (B1–3) occur at intermediate to higher levels of laccase and temperature but in a lower concentration of Vanillin mediator.

The desirability profile (Figures S1 and S2) allows knowing the precise proportions of the independent variables that lead to the greatest levels of sugar release (response). According to this analysis for the C group, the optimum conditions for maximum sugar yield (g L^{-1}) was achieved by the model at 228 (U g^{-1}), 0.76 (% *w*/*v*), and 54.2 (°C) for laccase load, mediator concentration, and temperature (uncoded values), respectively. Likewise, to eT+eC group the optimum uncoded values were 350 (U g^{-1}), 0.76 (% *w*/*v*) and 54.2 (°C). The laccase pretreated biomasses under these established optimum conditions were then submitted to hydrolysis assays to study its influence on the hydrolytic potential of *P. maximum*, grown under different climate conditions, as an energy crop.

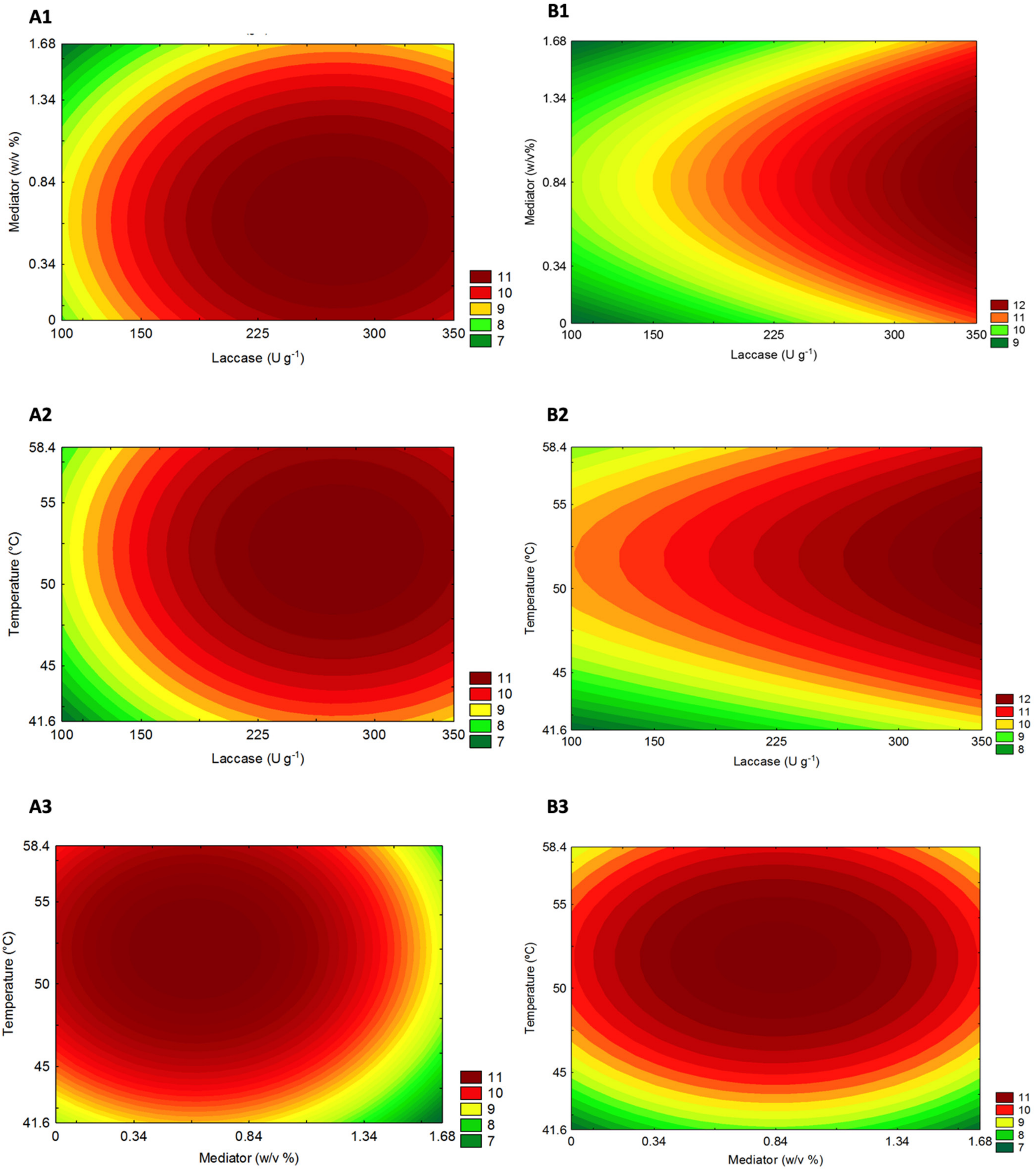


Figure 3. *Panicum maximum* response surfaces for the sugar yield obtained from hydrolysis after laccase pretreatment on (**A**) Control and (**B**) elevated temperature+elevated CO_2 (eT+eC). Correlations are shown between (**1**) Laccase and mediator, (**2**) Laccase and Temperature, and (**3**) Temperature and mediator.

2.4. Chemical Composition

Pretreatment methods are described as an important process to overcome LCB recalcitrance and increase the substrate accessibility to hydrolytic enzymes. In this study, enzymatic pretreatment was used to achieve these goals by targeting lignin removal and/or its structural modification. The chemical composition analysis of *P. maximum* grass in different climate conditions in native conditions (non-treated) and pretreated by crude laccase was carried out in terms of total anhydrousglucan, anhydrousxylan, anhydrousarabinose, anhydrousgalactose, lignin, and ash contents (wt %), as demonstrated (Table 1).

Analyzing the chemical composition (Table 1), two main effects were observed for pretreated substrates: (I) lignin removal and (II) cellulose-richer substrate, for both C, and eT+eC groups. The glucan content for laccase pretreated biomass was higher than the native material; the increase was from 26.2 to 30.4% glucan content for the C group, and 29.7 to 33.4% for the eT+eC group, on average. Moreover, laccase pretreatment was efficient for lignin removal achieving 40.8 and 46.2% delignification for C and eT+eC, respectively. Concerning the content of other components, no significant changes were found between non-treated and pretreated biomass, except for anhydrousxylose content that decreased from 17.3 to 15.7% for eT+eC climate condition. Considering the solid recovery and the chemical composition of *P. maximum* before and after pretreatment, only 7.5 to 9.2% (depending on the climate condition) of its glucan component was solubilized during crude laccase pretreatment, which is important since glucose is the most important fermentable sugar.

Table 1. Percentage (%) of the chemical composition of non-treated and pretreated *P. maximum* samples in different clime conditions. Mean values ± SD ($n = 3$) marked with other letters at the same line are significantly different ($p < 0.05$, Tukey's test).

Component (%)	**Non-Treated**		**LacPT** [c]	
	C [a]	**eT+eC** [b]	**C**	**eT+eC**
Anhydrousglucose	26.2 ± 0.5 a	29.7 ± 0.1 b	30.4 ± 0.2 b	33.4 ± 0.3 c
Anhydrousxylose	17 ± 0.3 a	17.3 ± 0.2 a	16.3 ± 0.2 a	15.7 ± 0.4 b
Anhydrousarabinose	2.4 ± 0.12 a	2.3 ± 0.15 a	2.4 ± 0.12 a	2.2 ± 0.0 a
Anhydrousgalactose	1.15 ± 0.05 a	1.12 ± 0.03 a	1.08 ± 0.05 a	1.04 ± 0.07 a
Lignin	26.3 ± 0.8 a	29.8 ± 0.9 b	21.2 ± 1.2 c	20.5 ± 1.4 c
Ash	10.8 ± 0.2 a	11.5 ± 0.4 a	9.7 ± 0.4a	9.2 ± 0.6 a
Solid yield	N/A	N/A	73.4	78.2
Glucan recovery	N/A	N/A	92.5	90.8
Lignin removal	N/A	N/A	40.8	46.2

C [a]: Control group. eT+eC [b]: elevated temperature + elevated CO_2. LacPT [c]—Crude laccase pretreatment.

2.5. Validation of Crude Laccase Pretreatment at Determined Optimal Conditions

At the optimum operating conditions for crude laccase pretreatment obtained from CCD, the maximum theoretically sugar yield predicted by the model was 11.47 and 12.39 g L^{-1} (Equations (1) and (2)) for C and eT+eC groups, respectively. To further validate the model, we carried out experiments at optimum responses conditions predicted by CCD. As a result, the optimum experimental sugar yield was 11.54 and 12.46 g L^{-1}, for C and eT+eC, respectively, with a standard error of 0.34 and 0.16 (Figure 4), which are close to the predicted response of the statistical model. Considering these values, a significant improvement of 41.2 and 36.6% in the sugar yields (g L^{-1}) was found for laccase pretreated substrate compared to non-treated substrates (Figure 4A) at the same hydrolysis conditions (5% solid load and 15 mg protein/g biomass). The glucan conversion (%) was also performed to reveal the pretreatment process efficiency and it was calculated as a percentage of the theoretical glucan presented in the substrate and expressed as glucan conversion rates. For this experiment, the Ctec® cocktail was applied in relation to the substrate glucan content (50 mg protein per gram of glucan). As a result, the glucan conversion for pretreated samples were 26.5 and 20.5% higher than non-treated substrates for C and eT+eC group, respectively (Figure 4B).

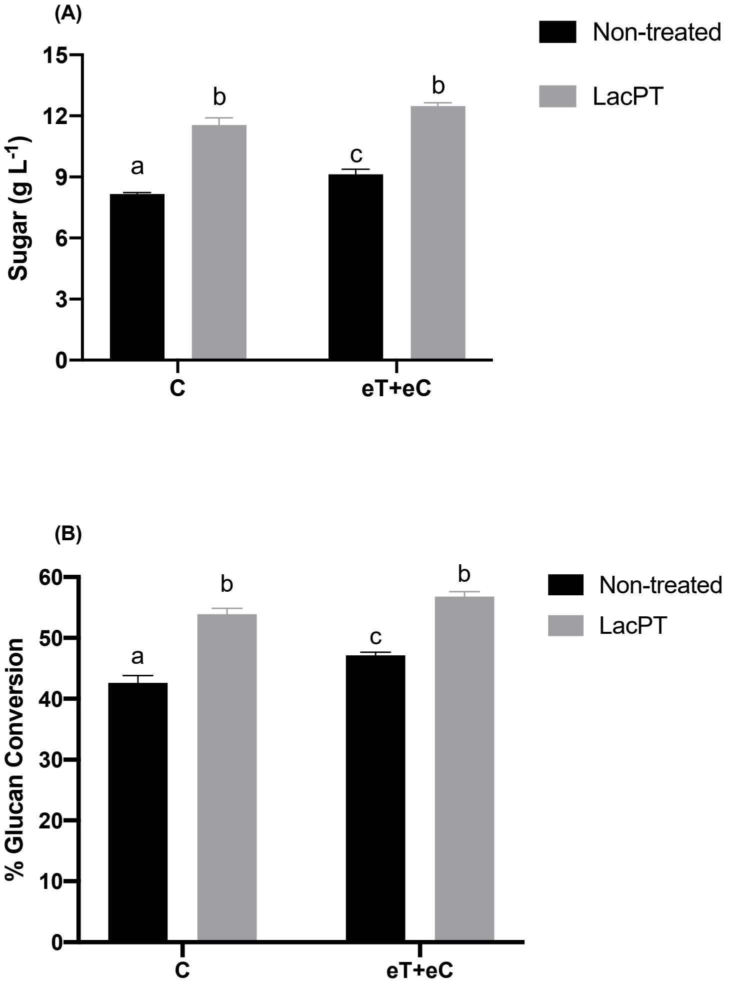


Figure 4. Sugar yield (g L^{-1}) (**A**) and Glucan conversion (%) (**B**) after enzymatic hydrolysis (5% solid load and 48 h) for non-treated and pretreated *P. maximum* at different climate conditions. Treatments: Control (C) and elevated temperature + elevated CO_2 (eT+eC). Mean values ± SD (n = 3) marked with different letters above bars symbolize statistical differences among treatments ($p \leq 0.05$, Tukey's test).

To further prove the positive effect of crude laccase pretreatment on hydrolysis sugar yields, we compared the sugar amount obtained for laccase pretreated with the ones resulted after pretreatments applying commercial laccase and inactive crude laccase (Figure 5). The experiments were performed at the same optimum parameters previously obtained in the CCD experiment and at the same hydrolysis conditions. As a result, the total sugar yields to inactivate crude laccase pretreated biomass, 7.81 and 8.48 g L^{-1} for C and eT+eC, respectively, were similar to the ones found in non-treated biomass, showing that the positive effects on sugar yields for laccase pretreated substrate are due to enzyme activity present on the crude extract of *L. sajor-caju*. Moreover, the commercial laccase pretreated samples showed significantly lower ($p < 0.05$) levels of sugar yields related to crude laccase, 9.8 and 10.26 g L^{-1} for C and eT+eC, respectively, which demonstrate that the use of crude laccase has costs and operational advantages.

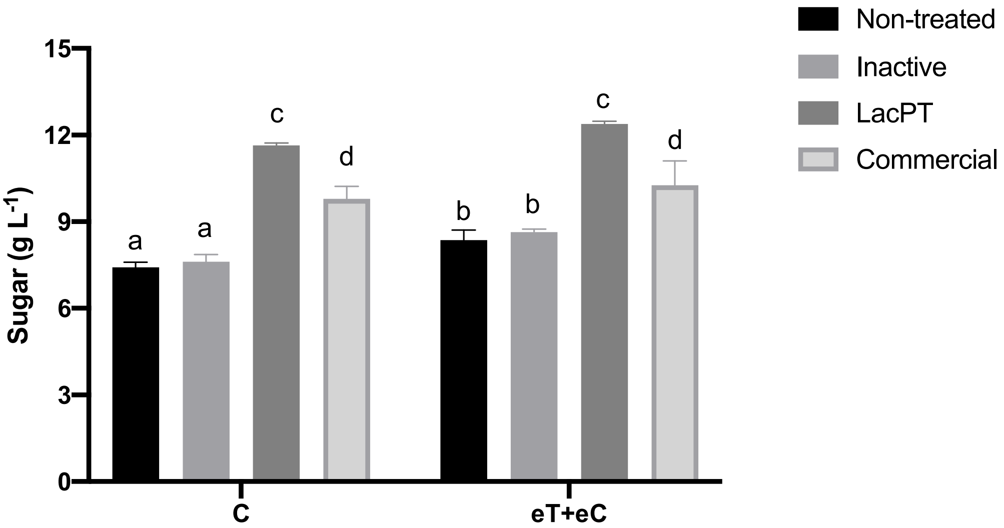


Figure 5. Sugar yield (gL^{-1}) after enzymatic hydrolysis of non-treated and pretreated by inactive crude laccase, crude, and commercial laccase. Hydrolysis conditions: 5% solid loading, 15 mg protein/g biomass for 48 h. Treatments: Control (C) and elevated temperature + elevated CO_2 (eT+eC). Mean values ± SD (n = 3) marked with different letters above bars symbolize statistical differences among treatments ($p \leq 0.05$, Tukey's test).

Regarding climate conditions, significant differences between C and eT+eC groups were found just for non-treated and inactive laccase pretreated biomass. After commercial and crude laccase no significantly differences could be observed between these groups (Figure 5).

2.6. Hydrolysis Studies of Crude Laccase Pretreated *P. maximum* Biomass

2.6.1. At Lower Protein Load

In addition to the barrier effect that lignin represents to the bioconversion of lignocellulosic feedstocks mainly due to the formation of lignin-carbohydrates complexes, this polymer has also been described to have another negative impact on enzymatic hydrolysis by promoting non-productive cellulase and xylanase adsorption through hydrophobic interactions [21]. As the latter effect is especially observed at low enzyme loadings [22], we carried out some hydrolysis experiments with reduced enzyme loading to ensure that 15 mg protein/g biomass had not "masked" any differences between the non-treated and pretreated substrates.

It was apparent that reduced protein load affects the differences found between non-treated and pretreated substrates (Figure 6). At 2 and 5 mg protein/g biomass, we found more significant relative increments on glucose and xylose releases after laccase pretreatment than non-treated biomass. At 2 mg/g biomass of protein load, the glucose and xylose releases were 41.0 and 50.1% higher for pretreated biomass of the C group and 40.5 and 57.1% for eT+eC treatment. Considering 5 mg protein/g biomass, the relative increment on glucose release for pretreated C and eT+eC were 39.1 and 35.4%, respectively, while xylose yield was 46.7 and 40.9% higher. For 10 mg protein/g biomass, the glucose and xylose yields were 29.2 and 34.4%, respectively, greater than non-treated C and 24.8 and 27.45% for eT+eC groups. Finally, at 15 mg g^{-1} of protein load, we observed similar differences to 10 mg g^{-1}. Pretreated substrates exhibited 31.1 and 20.3% more glucose in the hydrolysate for C and eT+eC group, respectively, and 14.8 and 13.35% more xylose in relation to non-treated biomass. Notably, differences in glucose and xylose yields between non-treated and pretreated substrates were higher at hydrolysis with lower cellulase load, suggesting that laccase plays a role in reducing the hydrophobic interactions that result in non-productive biding on lignin that will be further discussed.

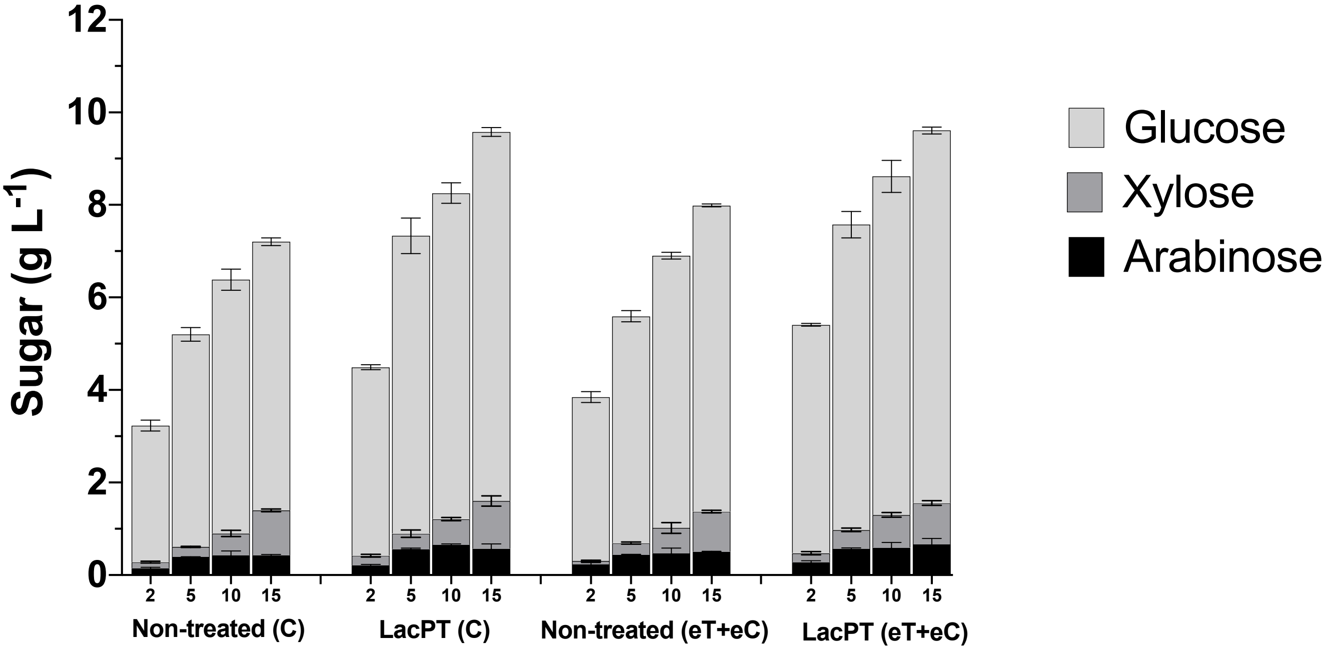


Figure 6. Glucose, xylose, and arabinose yield (g L^{-1}) after enzymatic hydrolysis of non-treated (C and eT+eC) and laccase pretreated (C and eT+eC) biomass at 2, 5, 10, and 15 mg protein/g biomass. Hydrolysis conditions: 5% solid load for 48 h. Treatments: Control (C) and elevated temperature + elevated CO_2 (eT+eC). Mean values ± SD (n = 3). The p-value was calculated using Tukey's test and is discussed in the text at the proper place (p-value, significant < 0.05).

Nonetheless, the arabinose yield (g L^{-1}) seems not to grow proportionally with the protein loading for both non-treated and pretreated substrates, and, therefore, its relative increment on laccase pretreated samples was almost constant at all conditions, being 45.3 and 27.3% (in average) higher for C and eT+eC pretreated biomass, respectively.

2.6.2. At Different Solid Load

LCB hydrolysis must be performed at a commercial scale at higher solid loading to increase sugar concentrations and, therefore, ethanol/bioproducts yields. However, work with a higher solid load is challenging since rheological problems start to occur, and achieving proper mixing during the saccharification process turn to be a difficult process [23]. Thus, to study laccase contribution to overcome this challenge, we performed hydrolysis at higher solid loading. *P. maximum* in both climate conditions was pretreated for 6 h with crude laccase at optimal conditions, washed, dried, and hydrolyzed at different solid loads. The monosaccharides in the hydrolysate (glucose, xylose, and arabinose) were quantified through HPLC, and the results are presented in Figure 7.

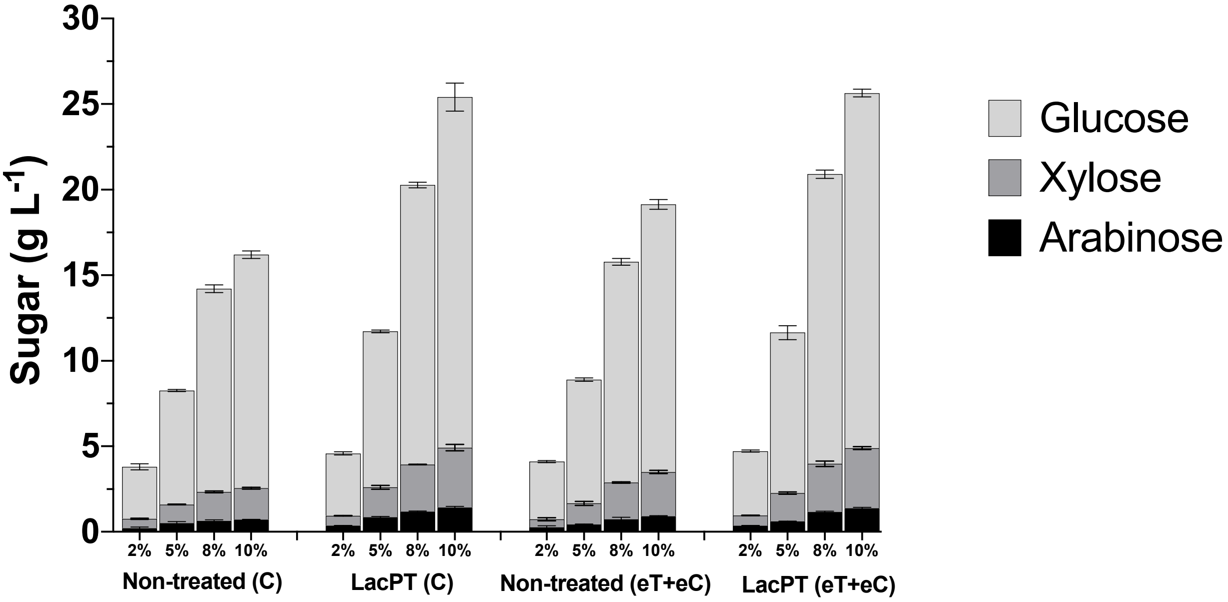


Figure 7. Glucose, xylose, and arabinose yield (g L^{-1}) after enzymatic hydrolysis of non-treated (C and eT+eC) and laccase pretreated (C and eT+eC) biomass at 2, 5, 8, and 10% solid load. Hydrolysis conditions: 15 mg protein/ g biomass for 48 h. Treatments: Control (C) and elevated temperature+elevated CO_2 (eT+eC). Mean values ± SD (n = 3). The p-value was calculated using Tukey's test and is discussed in the text at the proper place (p-value, significant < 0.05).

The pretreatment with crude laccase had a significantly positive effect ($p < 0.05$) in relation with non-treated biomass for all tested solid load (2, 5, 8, and 10%) and for all monosaccharides analyzed (glucose, xylose, and arabinose) (Figure 7). However, we observed a minor relative increment at 2% solid load, in which the pretreated biomass showed 20.2 and 11.5% higher glucose release for C and eT+eC, respectively, compared to non-treated substrates. At higher solid load, the positive effect of laccase pretreatment increases; at 5% solid load, the relative increment in glucose, xylose, and arabinose were 36.7, 59.9, and 69.3%, respectively, for the C group. Likewise, for eT+eC, the relative increment in glucose, xylose, and arabinose was found to be 29.6, 31.6, and 42.4, respectively. At 8% solid load, the relative increment is even higher 37.54, 64.02, 80.3% for the C group, and 31.2, 31.21, and 57.66% for glucose, xylose, and arabinose yields, respectively, of eT+eC treatment. The highest relative increment was found at 10%, in which the C group showed 50.15, 90.2, and 97.7%, and eT+eC 32.67, 36.35, and 69.64% higher glucose, xylose, and arabinose release, respectively. These results demonstrate that the crude laccase pretreatment approach could reduce costs and increase the efficiency of the bioconversion process, especially at higher solid load; its effects are independent of the future expected climate changes.

The above-described increments for laccase pretreated biomass result in significantly higher ($p < 0.05$) concentration of fermentable sugars in the hydrolysate yielding from 3.6 to 20.5 g L^{-1} of glucose for the C group and from 3.7 to 20.7 g L^{-1} for the eT+eC group, depending on the solid load applied. In addition, xylose was released in satisfactory concentration for both C and eT+eC groups, reaching 3.4 and 3.5 g L^{-1}, respectively, at 10% solid.

2.7. Simon's Staning

Earlier works have used Simons' staining method with the adsorption of direct orange (DO) dye to estimate the overall porosity/accessibility of pretreated lignocellulosic substrates since DO dye has molecular weight like cellulases and affinity for cellulose [22,24]. Thus, after the incubation with DO dye, it was possible to determine the amount of dye absorbed in the fiber, which is directly proportional to the enzyme accessibility. The results for Simon's Staining for both non-treated and crude laccase pretreatment are presented in Table 2. The DO adsorption values for pretreated with crude laccase significantly increased ($p < 0.05$) for both climate conditions compared to the respective non-treated biomass, indicating enhanced overall cellulose accessibility after crude laccase pretreatment, from which the main effect is to modify and remove lignin. This result agrees with the hydrolysis data in which pretreated substrates showed the highest bioconversion rates (Figures 4, 6 and 7).

Table 2. Direct orange dye adsorption of non-treated and pretreated *P. maximum* samples in different clime conditions.

Biomass	**Adsorption of Direct Orange (mg g^{-1})**	
	C^{a}	**eT+eC^{b}**
Non-treated	63.7 ± 1.4 a	76.4 ± 1.7 a
LacPT^{c}	79.9 ± 3.0 b	84.7 ± 2.9 b

Mean values ± SD (n = 3) marked with different letters at the same column are significantly different ($p < 0.05$, Tukey's test). C^{a}: Control group. eT+eC^{b}: elevated temperature + elevated CO_2. LacPT^{c}—Crude laccase pretreatment.

2.8. GC-MS Analysis of Lignin Degradation Products

After pretreatment of *P. maximum* with laccase-rich crude extract, 10 lignin degradation products were identified by GC-MS listed in Table 3 and their retention times. With the increase in pretreatment time, we observed the formation of new intermediates while some compounds found in initial reaction times were not detected later. Furthermore, as enzymatic reactions generate compounds according to enzyme-specific reaction mechanisms or substrate structural/chemical prosperities, some intermediates were found to be different between C and eT+eC climate conditions.

The intermediates found in this study have some lignin markers that include phenolic and aliphatic carboxylic acids, such as 2-ethylhexyl ester,3-phenylpropionic acid (No 2), 2-isopropyl-5-methylhexyl acetate (No 4), and 2-hydroxy-1,3-propanediyl ester-octadecanoic acid (No 10). These compounds are described to be derived from to phenylpropanoid class, which plays a role in linking lignin and carbohydrates [25]. Additionally, methylated derivatives of hydroxybenzaldehydes, as 2,4-dimethyl-benzaldehyde (No 1), could be released from the laccase catalyzed breakage of alkyl-aryl ether bonds in lignin and are associated sinapyl and coniferyl alcohol lignin precursors. In addition, the presence of the linear/branched oxygenated hydrocarbons (No 4, 6, and 10 in Table 3) suggests that lignin was further degraded by catalytic cleavage of C-O-C bonds.

Heterocyclic aromatic compounds such as 5,5,8a-trimethyl-3,5,6,7,8,8a-hexahydro-2H-chromene (No 7) and 5-methyl-indole (No 9), made of heteroatoms of oxygen and nitrogen, respectively, were observed. These intermediates could be a result of the radical polymerization process of phenylpropanoid units during laccase reactions. Another lignin degraded product in the form of phenolic ketone, 2,5-di-tert-butyl-p-quinone (No 8)was released during pretreatment, and it is usually produced by the oxidation of phenyl propane lignin monomers. The release of heterocyclic aromatic compounds and phenolic ketones positively affects on laccase pretreatment process since they are described as natural mediators that help non-phenolic lignin biodegradation [26], corroborating with the more than 40% delignification of *P. maximum* (Table 1).

The aliphatic compounds and linear alkanes found in this study (No 3, 4, 5, 6, and 10 from Table 3) resulted from ring-opening reactions through lignin depolymerization. These occurred when laccase-activated lignin subunits in the form of radicals are not stabilized by couplings reactions, but instead, they proceed to bond cleavage within lignin. This fact may lead to consecutive cleavages that facilitate ring-opening or lignin fragmentation [10]. Ring-opening products can further be involved in coupling reactions creating products such as the ones above mentioned.

An important finding from this study was that hydrolysis and fermentation inhibitors, as furfurals and hydroxymethyl furfurals produced in chemical pretreatment methods, were not observed in the GC-MS profile, illustrating that lignin has been specifically cleaved by laccase.

Table 3. Lignin degraded intermediates identified by GC-MS at different time courses of laccase pretreatment.

No	Intermediate Compounds	Control (C)			eT+eC			Retention Time (min)
		1 h	4 h	6 h	1 h	4 h	6 h	
1	2,4-imethyl-benzaldehyde	+	+	–	–	+	+	8.60
2	2-ethylhexyl ester,3-phenylpropionic acid	–	–	–	+	+	–	8.65
3	4,6-dimethyldodecane	–	–	–	–	–	+	8.81
4	2-isopropyl-5-methylhexyl acetate	–	–	–	–	–	+	9.59
5	2,3-dimethyldodecane	–	+	+	–	–	–	12.33
6	2-isopropyl-5-methyl-1-heptanol	–	+	+	–	–	–	13.39
7	5,5,8a-trimethyl-3,5,6,7,8,8a-hexahydro-2H-chromene	–	+	+	–	+	+	16.65
8	2,5-di-tert-butyl-*p*-quinone	–	–	+	–	+	+	17.84
9	5-methyl-indole	+	+	+	+	+	+	22.57
10	2-hydroxy-1,3-propanediyl ester-cctadecanoic acid	–	+	–	–	+	–	22.76

2.9. Physical Characterization of Optimized Laccase Pretreated Biomass

2.9.1. CLSM and SEM Analysis

In order to understand the morphological and structural impacts of crude laccase pretreatment under optimum conditions on *P. maximum* biomass, we carried out scanning electron microscopy (SEM) and Confocal Laser Scanning Microscopy (CLSM) analyses. Unfortunately, the climate conditions were not approached for these techniques since it was not possible to note any differences in the images between these groups.

In SEM images, non-treated biomass (Figure 8A,B) is characterized by highly ordered and tightly packed fibers that are covered by a lignin layer at the surface. However, upon the crude laccase pretreatment, the fiber structure was strongly modified to less tightly, and ordered ones with the loss of the packed assembly and with the formation of pores in the cell wall surface (Figure 8C,D) showed. These effects are likely due to the partial removal of the lignin layer that held the fibers together, thus increasing the surface area of cellulose for cellulase accessibility and, therefore, the yield of fermentable sugars.

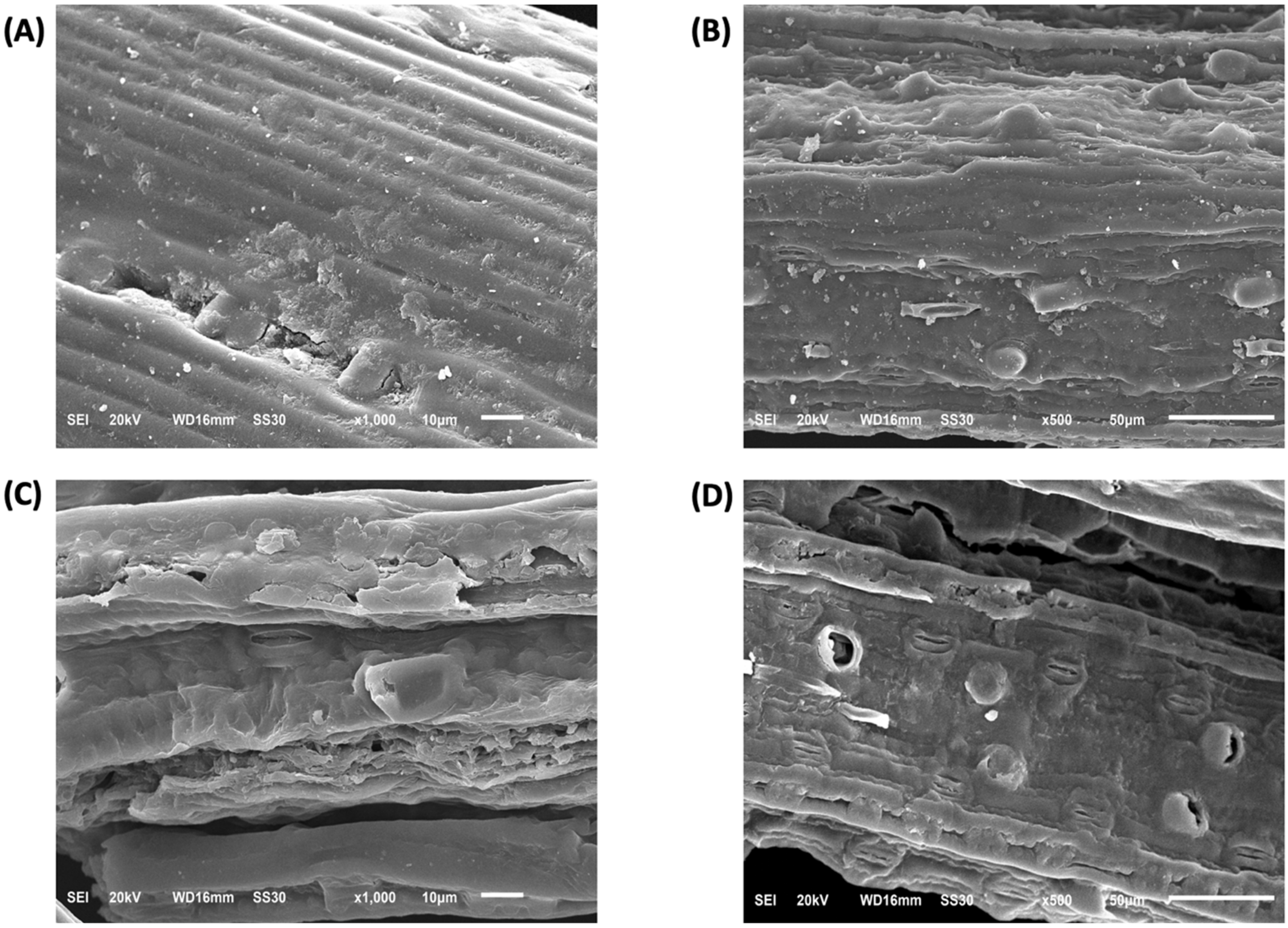


Figure 8. Scanning electron microscopy (SEM) images of (**A**,**B**) non-treated and (**C**,**D**) laccase pretreated *P. maximum* biomass.

For a more detailed study about lignin in the cell wall before and after laccase pretreatment, we performed a CLSM analysis. Since lignin is a chromophore, its distribution can be imaged and analyzed by detecting its autofluorescence. Thus, CLSM images were obtained from the surface of non-treated and pretreated biomass (Figure 9). Is it possible to observed changes in the emission spectrum of lignin where non-treated biomass (Figure 9A,B) emits fluorescence in the blue and green range (450–570 nm) of the electromagnetic spectrum with a green blush emission color, while laccase pretreated samples (Figure 9B,C) shift the fluoresce emission toward longer wavelengths, mainly in the green range (500–570 nm). In addition, it was possible to observe a reduction in fluorescence intensity for pretreated samples with some fiber sites having almost no fluorescence detected. Spectral and intensity fluoresce changes between native and pretreated samples are likely related to lignin degradation and/or structure modifications in the cell wall, also noted in SEM images.

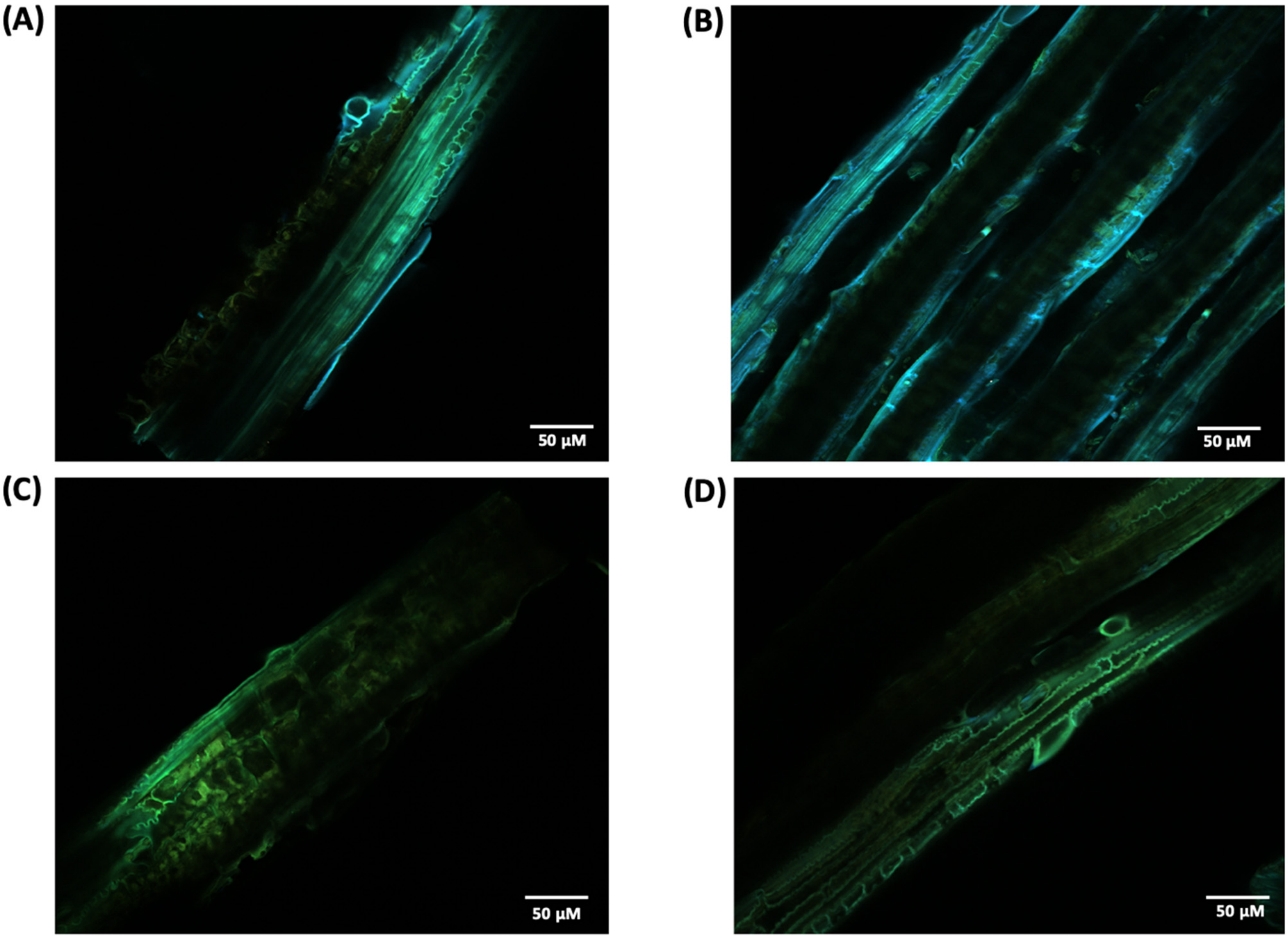


Figure 9. CLSM image of non-treated (**A**,**B**) and crude laccase pretreated (**C**,**D**) *P. maximum* biomass.

2.9.2. FTIR Analysis

The changes in the functional groups for laccase pretreated biomass in relation to the non-treated substrate was qualitatively observed by FTIR analysis aiming to identify modification in lignin structure (Figure 10A,B). For the C group (Figure 10A), after laccase pretreatment, a notable decrease in the intensity peaks was observed at 665 cm^{-1} (aromatic C–H binding of lignin), 1629 cm^{-1} (C=O stretching vibration in conjugated carbonyl of lignin), and 3396 cm^{-1} (OH stretching of lignin). However, a slight increase was detected at 1250 cm^{-1} (C-O vibration related to G lignin) and at 2919 cm^{-1} (C–H stretching of lignin). Further, no significant change was found for some functional groups belonging to holocellulose components (898 and 1727 cm^{-1}).

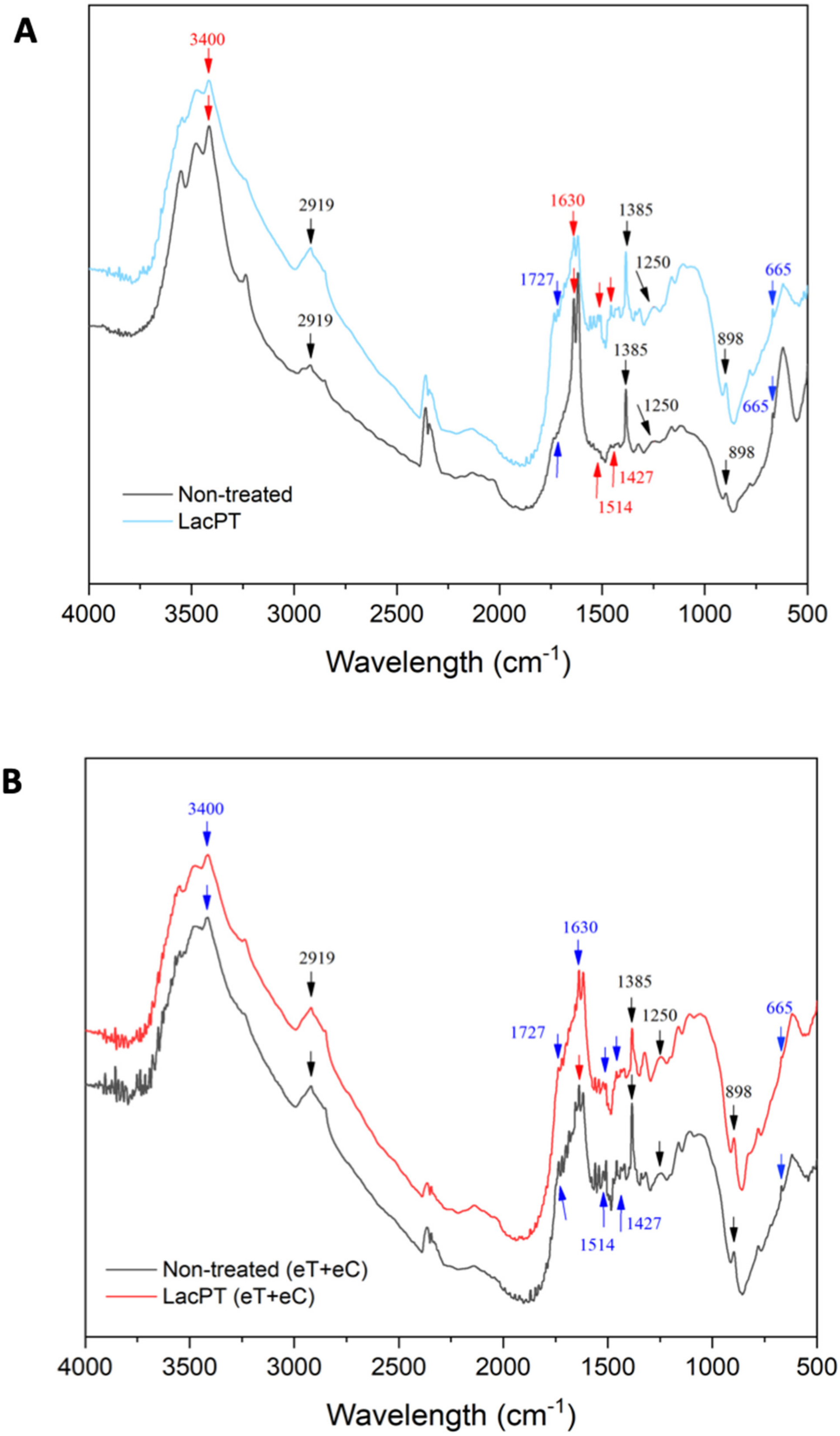


Figure 10. Fourier transform infrared spectra of raw and delignified pineapple leaf waste for Control (C) (**A**), and elevated temperature + elevated CO_2 (eT+eC) (**B**). Pretreatment conditions: Laccase load of 228 U/g for C and 350 U/g for eT+eC, 0.84% (*w*/*v*) Vanillin and 54.2 °C for both groups.

The FTIR spectrum for the eT+eC group is presented in Figure 10B. Significant decrease in the absorption peaks was detected at 665 cm^{-1} (aromatic C–H binding of lignin), 1385 cm^{-1} (phenolic hydroxyl groups), 1427 cm^{-1} (C–H in-plane deformation of lignin), 1514 cm^{-1} (C=C stretching vibrations of aromatic rings of lignin), and 1727 cm^{-1} (hemicellulose components). Further, no significant change was found for 898 cm^{-1} peaks (amorphous cellulose) and for 1250, 1630, 2929, and 3400 cm^{-1} lignin-related groups.

3. Discussion

The pretreatment step is essential to the valorization of lignocellulosic biomass. It is often applied to remove or modify lignin, increasing cellulose accessibility to biocatalysts that lead to the products of interest. Laccases are recognized for their activity against lignin using oxygen (O_2) as a final electron acceptor [9], making them a promising alternative as a biocatalyst to be applied in the pretreatment process of LCB. Furthermore, white-rot fungi laccases are described to have high redox potential (around +730 mV to +800 mV), increasing their ability to act towards lignin compounds [10]. In this work, we used a laccase-rich crude extract produced from *L. sajor-caju* using orange waste as a carbon source. The use of crude laccase was an attempt to reduce the enzyme purification steps that add significant costs to the process.

The tropical forage grass *P. maximum* is a potential source of LCB. However, the climate changes expected for the next decades can significantly impact its chemical composition and structure. Thus, the laccase-rich crude extract was applied for the pretreatment of *P. maximum,* grown under expected future climate conditions, to evaluate the potential of pretreated laccase biomass as an energy crop for the next decades.

Central composite design (CCD) and response surface analysis are considered powerful and valuable strategies to optimize biological processes; it is also timesaving related to the traditional 'one-factor-at-a-time,' helping to reduce costs in the enzymatic process [27] effectively. Therefore, we applied this statistical design to optimize laccase pretreatment, studying laccase load (U g^{-1}), mediator concentration (*w*/*v*%), and temperature (°C) as independent variables. The response (dependent variable) was the Sugar Yield (g L^{-1}) (sum of glucose, xylose, and arabinose) released after the saccharification step of the laccase pretreated substrates.

We chose to work with sugar yields rather than delignification since lignin removal is not always correlated with the greatest fermentable sugar yield [28]. Lignin rearrangements, reallocation, and structural modification are also described to lead to higher saccharification yields [29,30], but these chemicals alterations would not be quantified as delignification percentages. Nonetheless, by quantifying the total sugar yields after hydrolysis of laccase pretreated biomass, it is possible to address all laccase effects that directly or indirectly impacts enzymes accessibility and end up increasing the sugar release from LCB. All factors that could influence sugar production, except those studied by the CCD, were kept constant in pretreatment and hydrolysis steps.

Laccase pretreatment at optimized conditions led to significant changes in *P. maximum* chemical composition. Significant lignin removal (greater than 40%) was observed due to laccase action on *P. maximum* biomass (Table 1). Laccase mechanisms for lignin depolymerization and degradation include: (I) Decrease the number of aliphatic side chains describe to be involved in β-O-4′ and β-5′ interunit linkages (most common bond in lignin), suggesting Cα–Cβ cleavage; (II) Significantly removal of the three lignin monomeric units, p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S); (III) Oxidation in Cα of syringyl lignin, and (IV) Breaking down the p-coumarates and ferulates ester bonds with polysaccharides in lignin carbohydrate complexes [10,11]. Lignin degradation products found in GC-MS analysis (Table 3) have some lignin markers that corroborate with the laccase delignification mechanisms mentioned above.

The lignin removal obtained in this study is higher than those reported using commercial laccases or in laccase association with other pretreatment methods (Table 4). Al-Zuhair et al., (2015) [31] stated 9% of delignification with commercial *Trametes versicolor* laccase using HBT (hydroxybenzotriazole) as mediator. Rencoret et al., (2019) described 24% of lignin removal from *Paulownia fortune* after laccase-mediated pretreatment combined with alkaline extraction with NaOH [32], while Gutiérrez et al., (2012) reported up to 32% reduction in lignin content for Elephant grass applying the same combine pretreatment [33]. Crude laccase of white-rot fungi was also used for pretreatment resulting in 8% delignification of oil palm empty fruit bunch [34]. Rajeswari and Jacob (2020) [12] reported a 76% maximum delignification of aloe vera leaf rind. However, the amount of laccase applied was at least 3-folds higher than in this study.

Table 4. Recent reports on several laccases capable of degrading lignin.

Laccase Source	Condition for Maximal Lignin Degradation	Delignification (%)	Reference
Commercial from *Trametes versicolor*	Use of HBT (hydroxybenzotriazole) as a mediator	9	[31]
Commercial from *Myceliophthora thermophila*	Laccase-mediated pretreatment with methyl syringate (MeS) combined with alkaline extraction with NaOH	24	[32]
Commercial *Trametes villosa* laccase	Laccase-mediated pretreatment (HBT) combined with alkaline extraction	32	[33]
Crude laccase from *Pycnoporus sanguineus*	Laccase-mediated pretreatment (HBT and ABTS) combined with alkaline extraction	up to 8	[34]
Crude white-rot fungi locally isolated	Laccase load (922 U g^{-1})	76	[12]
Crude laccase from *Lentinus sajor-caju*	Laccase-mediated pretreatment (Vanillin)	up to 46.2	This work

In addition to the beneficial effects in chemical composition, the enzymatic pretreatment using *L. sajor-caju* crude laccase has several advantages. One of the main benefits of using a biocatalyst is the high carbohydrates retention in the solid fraction after pretreatment. In this work, we achieved efficient delignification with more than 73% solid and 90% cellulose recovery, likely because laccase acts specifically on lignin (Table 1), avoiding carbohydrates losses associated with physical-chemical pretreatments [35]. Therefore, enzymatic pretreatments may have overall process efficiency like those found in physical-chemical methods when considering the sugar losses in the process. Moreover, laccase pretreatment decreases the production of fermentation inhibitors, such as furfurals and organic acids, resulted from cellulose and hemicellulose degradation in physical-chemical methods [8].

The biochemical changes introduced by laccase on lignin minimize its physical and chemical barriers and maximize cellulase accessibility to cellulose fibers. As a result, we observed significantly greater total sugar yields and glucan conversation rates compared to non-treated biomass for both climate conditions, indicating greater efficiency of enzymatic hydrolysis. At 5% solid load, we obtained greater than 11 g L^{-1} sugar release and almost 60% glucan conversion for pretreated substrates (Figure 4), suggesting that crude laccase pretreatment is a potential alternative as a pretreatment strategy to enhance cellulose conversion into fermentable sugars. The saccharification performances described in this study are more outstanding than the ones reported to other laccase pretreated LCB like Elephant grass (5 g L^{-1}) [33], sugarcane bagasse (44.6% glucan conversion) [11], and wheat straw (29% glucan conversion) [36].

Although cellulases and hemicelluloses production by *L. sajor caju* were observed to be negligible (Table S1) in compassion to laccase, it appears that these enzymes together with natural mediators present in crude enzyme extract may have aided in the pretreatment process. Mediators of natural origin, like lignin degradation products, fungal secondary metabolites, and hemicellulose debranching enzymes could act synergistically with laccases to reduce biomass recalcitrance. Together with reducing purification costs, these synergistic effects represent the advantage of using crude enzymatic extracts rather than purified commercial enzymes. This is possibly why crude laccase pretreatment led to a greater increase in sugar release than the use of commercial laccase (Figure 5).

Delignification catalyzed by laccase increases sugar yields primarily by increasing the internal surface area, improving cellulase binding on cellulose. In addition, lignin is a moderate hydrophobic polymer due to its aromatic nature, resulting in the unproductive adsorption of cellulolytic enzymes through interactions with hydrophobic moieties in cellulases [21]. Therefore, laccase-catalyzed grafting is a term used to describe the attachment of low-molecular-weight compounds, mainly carboxylic acid residues, to the lignin surface through radical coupling that ends up in reducing hydrophobic lignin properties and, therefore, unproductive cellulase binding [10,37].

To evaluate the influence of laccase on lignin hydrophobicity, hydrolysis at limiting protein load was carried out. We observed that hydrolysis yields of laccase pretreated samples increased for all tested parameters in relation to non-treated biomass (Figure 6). However, the hydrophilization benefic effect was more pronounced at lower protein load (2 and 5 mg protein/g biomass), in which cellulase adsorption in lignin strongly impacts the amount of enzyme available to degrade polysaccharides. In this case, the differences in glucose and xylose yields between pretreated and non-treated samples were greater than at higher protein loading (10 and 15 mg protein/g biomass) since at the latter conditions; there were sufficiently amount of enzymes to catalyze cellulose and xylose conversion, reducing the negative effect of enzyme-lignin interaction. These results indicate that laccase-catalyzed hydrophilization effectively reduced the unproductive binding capacity of lignin of *P. maximum* substrate, which is particularly advantageous to hydrolysis at lower enzyme loadings.

The beneficial effect of laccase in sugar release due to delignification and lignin hydrophilization was also observed for high solid load hydrolysis. Cost-effective enzymatic bioconversion of LCB on a commercial scale requires high substrate concentrates. However, several factors such as limited mass transfer, end-products inhibition, and enzyme adsorption result in inefficient reaction mixing and poor enzyme distribution, reducing reaction rates. Crude laccase pretreatment seems to play an important role for overcame these challenges since up to 26 g L^{-1} total sugar yield was achieved at 10% solid load (Figure 7). Moreover, the differences between non-treated and pretreated substrates proportionally increased with increasing solid load. Therefore, we hypothesize that laccase-mediated delignification and modification in lignin chemical properties (e.g., oxidation, the addition of hydrophilic groups, and solubility) alters the rheological characteristics of *P. maximum* biomass with consequential lower viscosities, providing conditions properly to enzymatic hydrolysis while reducing unproductive enzyme adsorption.

Concerning the differences found between climate conditions, for non-treated *P. maximum* biomass Control (C) group had significantly ($p < 0.05$) lower sugar yields compared to elevated temperature + elevated CO_2 (eT+eC) for all conditions performed in this study (Figures 4–7). The possible explanations for these differences are described in detail in a previous study from our research group, in which similar hydrolysis results were found [38]. However, no differences were found between C and eT+eC groups after crude laccase pretreatment, suggesting that crude laccase pretreatment extensively modifies *P. maximum* fibers. Thus, the minor differences in chemical composition between C and eT+eC groups were not enough to result in significant differences in saccharification performance in the tested hydrolysis conditions.

Also, the relative increase in sugar yields between pretreated and non-treated biomass showed to be higher for the C group in relation to eT+eC substrates (Figures 4–7). This result indicates that laccase action was more advantageous to the C group, probably because this climate condition has a more recalcitrant structure than eT+eC for raw biomass [38]. Therefore, laccase pretreatment assisted in overcome these differences approximating the bioenergetic potential of both climate conditions.

The finds of Simon's staining, SEM, CLSM, and FTIR analysis support the greater hydrolysis yields for arabinan, xylan, and cellulose components in laccase pretreated biomass. The fibers microscopic changes (e.g., fibers disorganization and pores formation) in SEM images (Figure 8) have already been described for pretreatment methods. They are generally considered to result from lignin removal [8,26,30,39].

As lignin contains some endogenous fluorophores, particularly the monolignols, CLSM analysis can quickly identify possible alterations in fluorescence emission and intensity that suggest lignin removal and/or modification. CLSM images (Figure 9) showed changes in the emission spectrum between pretreated and untreated samples where the former had predominant emission in the green region. Previous studies reported that longer wavelength and, thus, lower energy emissions are correlated with the deconstruction of the well-structured lignin assembly in the plant cell wall [30,40].

In addition, the less lignin content observed in Table 1 is supported by the loss in fluorescence observed in laccase pretreated samples. In particular, the content of β-aryl ether linkages (β-O-4′), one of the linkages targeted by laccase, was noticed to positively correlate with fluorescence intensity [41]. Other factors, such as modification in lignin-carbohydrates complexes cross-linkages and alteration in monolignols linkages, are also described to impact lignin fluorescence. The reduction in fluorescence observed also corroborated with other studies that found similar results associated with lignin degradation after laccase pretreatment [42].

Our SEM and CLSM analysis agree with those found in FTIR (Figure 10) and CG-MS (Table 3) analysis, where the alterations in bands attributed to lignin suggest depolymerization and/or chemical modification. The major reason for the decrease absorption peaks of the FTIR spectrum has been reported to be caused by laccase cleavage of lignin side chains without many structural alterations of holocellulose components [8,12]. Moreover, we observed some differences in the FTIR spectrum between the climate conditions indicating that the laccase mode of action on lignin depends on the chemical characteristics of the substrate.

4. Materials and Methods

4.1. Reagents and Raw Materials

Orange waste used for laccase production was donated by a local restaurant and subsequently dried and milled. Cellic CTec2 enzymatic cocktail and commercial laccase (Novozymes NS-22127) were kindly donated by Novozymes® (Bagsvaerd, Denmark). The chromatography columns Hiprep Q FF and Superdex 75 10/300 GL were acquired from GE Healthcare Life Science (Chicago, IL, USA). Aminex HPX-87P column and Precision Plus Protein TM Standards were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Malt Extract Agar, Vanillin, 3,5-dinitrosalicylic acid (DNS), sodium carbonate, and the substrates for enzymatic activities, 3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 2,6-dimethoxyphenol (DMP), xylan beechwood, carboxymethylcellulose (CMC), locust bean, debranched arabinan, β-glucan, p-nitrophenyl-α-L-arabinofuranoside, p-nitrophenyl-β-D-galactopyranoside, p-nitrophenyl-β-D-glycopyranoside, p-nitrophenyl-β-D-xylanopyranoside, and p-nitrophenyl-β-D-cellobioside, were purchased from Sigma-Aldrich (St. Louis, MO, USA). All reagents used for the assays were of analytical grade.

4.2. Growth of *Panicum maximum* under Simulate Future Climate Conditions

Trop-T-FACE was used to assess the effect of two variables involved in climate change expected in the upcoming decades (i.e., elevated CO_2 atmospheric concentrations [CO_2] and global average temperature increase). In the current work, tropical grassland *Panicum maximum* cv. Mombaça was used as a model of study. Initially, plants grown for 60 days were clipped at 30 cm above the ground and cultivated for 24 days under the following treatments: (I) ambient atmospheric [CO_2] and temperature (annotated as Control: C) and (II) 600 ppm atmospheric [CO_2] and +2 °C above ambient temperature (named here as elevated temperature + elevated CO_2: eT+eC). Then, *P. maximum* leaves were milled at 20 mesh, dried at 40 °C for 24 h, and stored in a free humidity environment until analyses. Meteorological data from the whole growing season was reported in [43].

The miniFACE (free-air CO_2 enrichment) system was used to increase the atmospheric $[CO_2]$ by 600 μmol mol^{-1}; for this purpose, PVC rings of 2 m diameter punctured with micro holes fumigated the plots with CO_2. The T-FACE (temperature free-air controlled enhancement) system was used to increase the canopy temperature to +2 °C more than the ambient canopy temperature. We used a randomized four-block design with which the experiment plot being warmed by six infrared heaters mounted on reflectors in a 2-m-diameter hexagonal pattern. The control system integrates the canopy temperature of C plots and then regulates the canopy temperature of eT+eC to 2 °C over the ambient canopy temperature in warmed plots [43,44]. The trop-T-FACE facility is located at the campus of the University of São Paulo, Ribeirão Preto, SP, Brazil. The levels applied in the treatment were chosen according to Intergovernmental Panel on Climate Change models [45].

4.3. Fungal Strain, Culture Conditions, and Enzyme Extraction

The white-rot fungi *Lentinus sajor caju* (Fr.) Singer- CCB 020 was obtained from the fungi collection of the Botanical Institute, SP, Brazil. It was maintained in a malt extract agar medium at 28 °C. The production of the crude extract rich in laccase was carried out through solid-state fermentation (SSF), according to Freitas et al., (2017) [46]. In brief, cultures of *L. sajor caju* were inoculated in 250-mL Erlenmeyer flasks containing 7 g of orange waste (berry and peel) and 80% initial humidity using mineral solution [47] and incubated for 14 days at 28 °C. Crude extracts were obtained by adding 20 mL of water to each flask. The mycelia were separated by gauze filtration. Finally, the supernatant was centrifuged at 9500× *g* at 4 °C for 10 min. The supernatants, named as the laccase-rich crude extract, were stored at −20 °C until use.

4.4. Enzyme Assays

Laccase activity was determined using 3-ethylbenzothiazoline-6-sulphonic acid (ABTS) as substrate. The ABTS oxidation was monitored by increasing absorbance at 420 nm (at pH 5.0 $\varepsilon_{420\ nm} = 36\ mM^{-1}cm^{-1}$). Under assay conditions, one unit of activity (U) was defined as the liberation of 1 μmol of product equivalents per min. In addition, ligninolytic enzyme activities were measured as previously described [46]. Cellulases and hemicellulases activities were also determined using their respective substrates (synthetic and naturals) (Table S1), as describe by Contato et al., (2021) [2].

4.5. Protein Content Determination, Electrophoresis, and Zymogram Analysis

Protein content was determined using the ninhydrin assay [48], with bovine serum albumin as the standard. Electrophoresis analysis of protein samples was done with 12% SDS-PAGE carried according to Laemmli (1970) [49]. The gel was stained with Coomassie Brilliant Blue R-250. For the zymogram analysis, β-mercaptoethanol was not added to the sample buffer, and the samples were not heated before running. After electrophoresis, the gel was washed with a mixture (50:50) of isopropanol–acetate buffer (50 mmol L^{-1}, pH 5.0) for 30 min, and once with acetate buffer for 30 min, to remove the SDS. The gel was transferred into a plate with an agar-ABTS layer (1% *w/v* agar and 0.05% *w/v* ABTS) and incubated at 25 °C until green bands appearance. The apparent molecular masses of proteins were calculated by comparing their electrophoretic mobility with standard protein markers (Precision Plus Protein TM Standards Bio-Rad).

4.6. Purification and Identification of Laccase by Mass Spectrometer Analyses

Two chromatography steps were used for laccase purification. First, the crude extract was loaded on an anion exchange column (Hiprep Q FF) equilibrated with 50 mmol L^{-1}

phosphate buffer, pH 6.5, integrated into an ÄKTA Purifier 10 FPLC System UV−900 (GE Healthcare Life Science, Cranbury, NJ, USA) chromatography system. Proteins were eluted with an increasing gradient of NaCl (0.05 to 1 M), and the elution was monitored at 280 nm. Fractions of interest were selected by laccase activity and SDS-PAGE page, pooled, and concentrated in Vivaspin (GE Healthcare, Cranbury, NJ, USA) ultra-filtration devices with 10 kDa membrane. Next, the selected fraction was loaded on a size exclusion column (Superdex 75 10/300 GL) equilibrated with 50 mmol L^{-1} phosphate buffer, NaCl 150 mmol L^{-1}, pH 6.5). Protein elution was monitored at 280 nm, and the fractions with protein of interest were selected as described above.

Characterization of purified samples was carried out using a Waters Xevo TQ-S mass spectrometer system. For analysis, 100 μg of purified protein samples were diluted in a solution of Tris-HCl 100 mmol L^{-1}, CH_4N_2O 8.0 mol L^{-1}, pH 8.5. Protein cysteine residues were reduced with 100 μg dithiothreitol (DTT) at 37 °C for 60 min and then alkylated with 300 μg iodoacetamide for 60 min. The protein solution was then diluted with Tris-HCl 200 mmol L^{-1} (pH 8.0) to reduce CH_4N_2O concentration before tryptic digestion (37 °C overnight).

Solid-phase extraction was performed in Oasis HLB Cartridges (Waters). The dried sample was dissolved in 50 μL of 5% acetonitrile solution. It was chromatographed three times in the Waters Acquity UPLC I-class with a 2–50% acetonitrile gradient in 60 min using 0.1% formic acid as a modifier. The mass spectra were acquired in the Waters Xevo TQ-S mass spectrometer in survey mode with masses for parent peaks ranging from 200 to 1800 and for child peaks from 200 to 1600 with a cone voltage of 50 V and collision energy of 20 eV. The files were converted to Mzxml format using Masswolf software, and the analysis was performed on the TPP server platform with the XTandem peptide search engine. Carbamidomethyl was inserted as fixed modification, and oxidation of methionine was inserted as variable modification. Protein identification was performed considering high probability values using ProteinProphet.

4.7. Effect of the Temperature and pH on Laccase Activity and Stability

To pre-establish, the optimum performance conditions for laccase activity in the biomass pretreatment, the effect of temperature and pH on laccase activity and stability was determined. The effect of temperature (40–80 °C) and pH (3.0–7.0) were determined by measuring the laccase activity using ABTS as substrate under standard conditions. Thermal stability assays were performed at pH 5.0. The residual activity was measured as the percentage of enzyme activity after a specific time in relation to the activity in the initial time (100%) was treated identically but without incubation.

4.8. Optimization of Laccase Pretreatment

Initially, *P. maximum* samples were washed six times with 80% ethanol and then with water until the soluble sugars were entirely removed to avoid soluble sugar interference during pretreatment hydrolysis experiments. All samples were then dried in an oven at 50 °C. For the pretreatment step, we used the laccase-rich crude extract produced by *L. sajor caju* as previously described.

The optimization of crude laccase pretreatment of *P. maximum* (Control and eT+eC treatment) was carried out using 2^3 central composite design (CCD) and response surface methodology. The efficiency of laccase pretreatment was evaluated through the effect of laccase load (X_1) (100–350 U g^{-1}), mediator concentration (X_2) (0–1.68% *w*/*v*), and temperature (41.6–58.4 °C) (X_3) on the sugar yield (g L^{-1}) as the dependent variable. The mediator used was Vanillin, and all pretreatment runs were conducted for 6 h at 2% solid loading (*w*/*v*) and pH 5.

The levels of the independent variables were defined based on a complete experimental design (data not showed). A total of 18 experiments were performed. Among them, 15 experiments were structured in a factorial design (including 8 factorial points, 6 axial points, and 1 central point) and 3 experiments represent the replicates of central points. Table 5 represents the uncoded and coded levels for each run in the CCD.

Table 5. Experimental design (conditions and responses) for crude laccase pretreatment of *Panicum maximum* biomass.

	Independent Variables			**Sugar Yield for C Group (g L^{-1}) [d]**		**Sugar Yields for eT+eC Group (g L^{-1}) [e]**	
Run Order	**Laccase (X_1) [a]**	**Mediator (X_2) [b]**	**Temperature (X_3) [c]**	**Experimental**	**Predicted**	**Experimental**	**Predicted**
1	150(−1)	0.34(−1)	45(−1)	8.46	9.03	8.34	8.95
2	300(+1)	0.34(−1)	45(−1)	9.94	10.33	11.2	11.18
3	150(−1)	1.34(+1)	45(−1)	8.3	8.11	8.21	8.56
4	300(+1)	1.34(+1)	45(−1)	9.13	9.41	9.88	9.85
5	150(−1)	0.34(−1)	55(+1)	9.91	9.76	9.54	9.8
6	300(+1)	0.34(−1)	55(+1)	10.77	11.06	11.56	12.03
7	150(−1)	1.34(+1)	55(+1)	8.33	8.84	9.42	9.41
8	300(+1)	1.34(+1)	55(+1)	9.48	10.14	10.77	10.71
9	100(−1.68)	0.84(0)	50 (0)	8.69	8.53	9.75	9.22
10	350(+1.68)	0.84(0)	50(0)	11.39	10.71	12.20	12.17
11	225(0)	0(−1.68)	50(0)	10.74	10.38	11.51	10.91
12	225(0)	1.68(+1.68)	50(0)	9.32	8.84	9.43	9.46
13	225(0)	0.84(0)	41.6(−1.68)	9.51	9.17	9.23	8.87
14	225(0)	0.84(0)	58.4(+1.68)	10.89	10.39	10.51	10.31
15	225(0)	0.84(0)	50(0)	11.03	10.98	11.84	11.67
16	225(0)	0.84(0)	50(0)	10.98	10.98	11.59	11.67
17	225(0)	0.84(0)	50(0)	10.52	10.98	11.40	11.67
18	225(0)	0.84(0)	50(0)	11.25	10.98	11.75	11.67

[a] (U g^{-1}), [b] (% *w*/*v*) and [c] (°C). C [d]: Control group. eT+eC [e]: elevated temperature + elevated CO_2 treatment.

Response surface methodology was used to analyze the experimental data can be approximated by the quadratic polynomial as expressed below (Equation (3)).

$$Y = \beta_0 + \sum_j \beta_j x_j + \sum_{i<j} \beta_{\{ij\}}\, x_i x_j + \sum_j \beta_{jj} x_j^2 \tag{3}$$

where: Y denotes the dependent variable (Sugar Yield), β_0, β_j, β_{jj}, and β_{ij} represent the constant co-efficient and X_i and X_j stand for the coded independent variables that have influenced the response of variable Y.

Following the pretreatments performed at the conditions established in Table 5 for X_1, X_2, and X_3, *P. maximum* pretreated biomasses were vacuum filtrated, washed with 0.1 L of water, and dried at 50 °C. Samples were then subjected to enzymatic hydrolysis using Cellic CTec2 enzymatic cocktail (Novozymes®, Bagsvaerd, Denmark) at 15 mg protein per gram of biomass. The hydrolysis was carried with an initial solid loading of 5% (*w*/*v*) of the laccase pretreated biomass in 50 mmol L^{-1} sodium acetate buffer (pH 4.8) at 50 °C for 48 h. The dependent variable (Y) was the sum of glucose, xylose, and arabinose obtained after the Ctec2 hydrolysis called here as Sugar Yield (g L^{-1}). It is important to note that all paraments from pretreatment and hydrolysis were kept constants for all 18 runs except for the studied independent variables (laccase load, mediator concentration, and temperature).

4.9. Chemical Characterization

The chemical composition of *P. maximum* was determined for untreated, and laccase pretreated biomass in three independent replicates. The average composition analysis was established using the Klason lignin method derived from the TAPPI standard method

T222 om-88 [50]. Ash content was measured according to the NREL/TP-510-42622 method [51].

Glucan and lignin recoveries in the laccase pretreated materials were obtained according to the following equation:

$$Recovery\ (\%) = \frac{DM_{PT}\ x\ C_{PT}}{DM_I\ x\ C_I} \times 100 \quad (4)$$

where DM_{PT} is the dry mass after pretreatment, DM_I is the initial dry mass, C_{PT} is the glucan or lignin content after pretreatment, and C_I is the initial glucan or lignin content. Lignin removal was calculated by subtracting the lignin recovery from 100% (maximum yield).

4.10. Enzymatic Hydrolysis Studies of Crude Laccase Pretreated Biomass

To better understand the effects of crude laccase pretreatment on the potential of *P. maximum* as LCB, we performed enzymatic hydrolysis in different protein and solid loading. The experiments sets were carried out using raw (ethanol and water pre-washed) and crude laccase pretreated biomass at optimum conditions. For enzymatic hydrolysis studies, Cellic CTec2 was used (Novozymes®, Bagsvaerd, Denmark) in 50 mmol L^{-1} sodium acetate buffer (pH 4.8) for 48 h at 50 °C and 150 rpm. After hydrolysis, enzymes were centrifugated (10,000× *g* 5 min) and heated for 30 min at 100 °C for inactivation. The hydrolysate was stored at −20 °C for further analysis. We also performed hydrolysis with biomass pretreated by inactivated crude laccase (98 °C for 24 h) and commercial laccase (Novozymes NS-22127), all of them under optimized conditions.

4.11. Determination of Monosaccharides

The quantitative analysis of monosaccharides (xylose, arabinose, galactose, and mannose) in the Klason and saccharification hydrolysate was determined by a high-performance liquid chromatography system (HPLC- YL9100 model) equipped with an Aminex HPX-87P column at 85 °C, which was preceded with the appropriate pre-column and eluted with HPLC grade water at a flow rate of 0.6 mL min^{-1}. The components were detected by a refractive index detector and quantified by external calibration.

4.12. Simon’s Stain

To measure the cellulose accessibility before and after laccase pretreatment, we applied Simon’s stain procedure using Direct Orange 15 (DO), according to previous studies by Chandra et al. [52]. For this experiment, 10 mg of raw and pretreated samples were mixed with PBS buffer and DO dye in increasing concentrations. The mixture was incubated at 70 °C and 180 rpm overnight. The tubes were then centrifuged, and the absorbance of supernatant at 450 nm was measured.

4.13. GC-MS Analysis of Lignin Degradatory Products

The pretreatment liquors were collected at different times and analyzed by GC-MS to analyze lignin degradation products generated by laccase activity. First, *P. maximum* biomass (C and eT+eC groups) were subjected to laccase pretreatment in the optimized conditions, and five hundred microliters of the supernatant were collected at 0, 1, 2, 4, and 6 h of pretreatment. The supernatants were then acidified to pH 1.0–2.0 by adding a few drops of 1 mol L^{-1} of HCl and 0.2 g of NaCl were then added. Next, the extraction was repeated three times using two volumes of ethyl acetate with vigorous shaking, the organic phase was separated, and 0.6 g of Na_2SO_4 was added. Finally, the volume of ethyl acetate was totally evaporated at ambient temperature, and the samples were resuspended in 100 μL of the same solvent. GC–MS analysis was carried out using a Shimadzu QP2010Plus mass spectrophotometer (Shimadzu Corporation, Kyoto, Japan) as described in detail in [53]. Lignin-degrading compounds were performed by comparing mass spectra to the NIST library (version 2.0) of the GC–MS.

4.14. Physical Characterization of Optimized Laccase Pretreated Biomass

4.14.1. SEM and FTIR Analysis

Scanning electron microscopy (SEM) was conducted to analyze the surface morphology of *P. maximum* biomass before and after laccase pretreatment using a JSM-6610LV microscope. Dried samples were placed on an SEM stub and gold-coated before the observation. FTIR (Fourier transformed infrared spectroscopy) analysis in dried samples of raw and laccase pretreated biomass using FTIR spectrometer (Cary 600 Series, Agilent Technologies, Santa Clara, CA, USA). The samples were molded into KBr discs, and the FTIR spectrum was obtained by scanning within a range of 500–4000 cm^{-1}. Lignin and carbohydrate peaks were chosen based on previous studies [8,26,39] and analyzed utilizing OriginLab version 8.0 software.

4.14.2. Confocal Microscopy

Confocal laser scanning microscopy (CLSM) was applied to analyze lignin distribution with the cell wall before and after enzymatic pretreatment. CLSM allows mapping lignin by measuring its fluorescence emission spectra. The images from CLSM were collected from biomass surface using a Zeiss LSM 780 confocal microscope with a Coherent Chameleon laser (Tisapphire) for a two-photons (2 P) excitation and a Plan-Apochromat objective lens (20×) as described previously [5,30]. In addition, fluorescence measurements were performed for all non-treated and pretreated samples with the same acquisition parameters.

4.15. Statistics and Numerical Analysis

Data were expressed as the mean of three replicates ± standard deviations of the means (SD). The significance of the observed differences was tested using analysis of variance (ANOVA) from the Graph-Pad Prism 8.0 software (San Diego, CA, USA). The Tukey test availed differences among the studied parameters, and p values <0.05 were considered statistically significant.

Statistica 8.0 was the software used for response surface methodology analysis. The response surfaces were generated once the statistical significance was determined by the analysis of variance (ANOVA) and using the F test (Fisher's test).

To better understand the methods used, a flow chart of the experimental design is presented in Figure S3.

5. Conclusions

The steric hindrance imposed by lignin and its hydrophobic properties plays an important role in restricting cellulase access to cellulose and the unproductive binding of enzymes on lignin. Laccase pretreatment is a sustainable alternative to remove the lignin barrier to cellulose and has several advantages in relation to the conventional pretreatment process, such as specific activity towards lignin that reduces carbohydrate losses, besides the less production of fermentation inhibitors. Here, we applied a crude laccase of *L. sajor-caju* for the pretreatment of *P. maximum* biomass at the optimal conditions that led to significant lignin removal (up to 46%). The delignification and laccase-catalyzed lignin hydrophilization enabled *P. maximum* hydrolysis at up to 10% solid loading yielding up to 26 g L^{-1} of total fermentable sugars. In addition, for all tested conditions, enzymatic hydrolysis of arabinan, xylan, and glucan components greatly increased compared to non-treated biomass, especially at low cellulase loading and high solid load. Physical characterization of optimized laccase pretreated biomass through FTIR, SEM, CLSM, and CG-MS analysis agrees with hydrolysis results, showing clear evidence of lignin removal and chemical modification, showing fibers more accessible for enzymatic hydrolysis. Laccase pretreatment also contributes to mitigating the climate changes effects on the potential of *P. maximum* since no significant differences were found between C and eT+eC climate conditions after laccase pretreatment, showing that laccase could overcome higher recalcitrance properties found for non-treated C group due to modification induced on lignin and LCCs.

Therefore, our work contributed to the development of a technology that significantly reduces the operating costs of the pretreatment step since the use of crude laccase reduces protein purification costs, the method is operated under mild temperatures, and no chemicals are applied in the process. Furthermore, this study suggests that *P. maximum* is forage grass with a high potential for cellulosic ethanol production, showing similar or more excellent hydrolysis performance than many energy crops widely used worldwide.

Supplementary Materials: The following are available online at www.mdpi.com/article/10.3390/ijms22179445/s1.

Author Contributions: Data Curation: E.N.d.F., R.C.A., A.G.C., and K.M.V.N.; Methodology: E.N.d.F., R.C.A., A.G.C., K.M.V.N., E.J.C., and L.A.B.d.M.; Supervision and responsible for the financial support: M.d.L.T.M.P., C.A.M., and R.N.S.; Writing—Original Draft: E.N.d.F. and R.C.A. Writing—Review and Editing: M.d.L.T.M.P. All authors have read and agreed to the published version of the manuscript.

Funding: The authors thank Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) (Grant 2008/57908-6, 2008/58075-8, 2014/50884, 2018/07522-6, 2017/23989-9, 2019/07141-5, and 2020/00081-4), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Grants 446357/2015-4; 465319/2014-9, and 301963/2017-7).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors thank the postgraduate students, laboratory technicians who contributed to the work, and Regina Teresa Rosim Monteiro (CENA/USP) kindly provide *Lentinus sajor-caju* used in this study.

Conflicts of Interest: The authors have no conflict of interest.

References

1. Patel, S.K.S.; Das, D.; Kim, S.C.; Cho, B.K.; Kalia, V.C.; Lee, J.K. Integrating strategies for sustainable conversion of waste biomass into dark-fermentative hydrogen and value-added products, Renew. *Sustain. Energy Rev.* **2021**, *150,* 111491, doi:10.1016/j.rser.2021.111491.
2. Contato, A.; de Oliveira, T.; Aranha, G.; de Freitas, E.; Vici, A.; Nogueira, K.; de Lucas, R.; Scarcella, A.; Buckeridge, M.; Silva, R.; et al. Prospection of fungal lignocellulolytic enzymes produced from jatoba (*Hymenaea courbaril*) and tamarind (*Tamarindus indica*) seeds: scaling for bioreactor and saccharification profile of sugarcane bagasse. *Microorganisms* **2021**, *9*, 533, doi:10.3390/microorganisms9030533.
3. Chundawat, S.P.; Beckham, G.T.; Himmel, M.E.; Dale, B.E. Deconstruction of lignocellulosic biomass to fuels and chemicals. *Annu. Rev. Chem. Biomol. Eng.* **2011**, *2*, 121–145, doi:10.1146/annurev-chembioeng-061010-114205.
4. Kumar, V.; Patel, S.K.S.; Gupta, R.K.; Otari, S.; Gao, H.; Lee, J.; Zhang, L. Enhanced saccharification and fermentation of rice straw by reducing the concentration of phenolic compounds using an immobilized enzyme cocktail. *Biotechnol. J.* **2019**, *14*, e1800468, doi:10.1002/biot.201800468.
5. Santo, M.C.E.; Fockink, D.H.; Pellegrini, V.O.; Guimaraes, F.E.; Deazevedo, E.R.; Ramos, L.P.; Polikarpov, I. Physical techniques shed light on the differences in sugarcane bagasse structure subjected to steam explosion pretreatments at equivalent combined severity factors. *Ind. Crop. Prod.* **2020**, *158*, 113003, doi:10.1016/j.indcrop.2020.113003.
6. Fillat, U.; Ibarra, D.; Eugenio, M.E.; Moreno, A.D.; Tomás-Pejó, E.; Martín-Sampedro, R. Laccases as a potential tool for the efficient conversion of lignocellulosic biomass: a review. *Fermentation* **2017**, *3*, 17, doi:10.3390/fermentation3020017.
7. Sindhu, R.; Binod, P.; Pandey, A. Biological pretreatment of lignocellulosic biomass—An overview. *Bioresour. Technol.* **2016**, *199*, 76–82, doi:10.1016/j.biortech.2015.08.030.
8. Banerjee, R.; Chintagunta, A.D.; Ray, S. Laccase mediated delignification of pineapple leaf waste: an ecofriendly sustainable attempt towards valorization. *BMC Chem.* **2019**, *13*, 58, doi:10.1186/s13065-019-0576-9.
9. Wang, F.; Xu, L.; Zhao, L.; Ding, Z.; Ma, H.; Terry, N. Fungal Laccase production from lignocellulosic agricultural wastes by solid-state fermentation: a review. *Microorganisms* **2019**, *7*, 665, doi:10.3390/microorganisms7120665.
10. Munk, L.; Sitarz, A.K.; Kalyani, D.; Mikkelsen, J.D.; Meyer, A.S. Can laccases catalyze bond cleavage in lignin? *Biotechnol. Adv.* **2015**, *33*, 13–24, doi:10.1016/j.biotechadv.2014.12.008.

11. Rencoret, J.; Pereira, A.; del Río, J.C.; Martínez, T.; Gutiérrez, A. Delignification and saccharification enhancement of sugarcane byproducts by a laccase-based pretreatment. *ACS Sustain. Chem. Eng.* **2017**, *5*, 7145–7154, doi:10.1021/acssuschemeng.7b01332.
12. Rajeswari, G.; Jacob, S. Deciphering the aloe vera leaf rind as potent feedstock for bioethanol through enzymatic delignification and its enhanced saccharification. *Ind. Crop. Prod.* **2019**, *143*, 111876, doi:10.1016/j.indcrop.2019.111876.
13. Zhang, R.; Lv, C.; Lu, J. Studies on laccase mediated conversion of lignin from ginseng residues for the production of sugars. *Bioresour. Technol.* **2020**, *317*, 123945, doi:10.1016/j.biortech.2020.123945.
14. Giacobbe, S.; Pezzella, C.; Lettera, V.; Sannia, G.; Piscitelli, A. Laccase pretreatment for agrofood wastes valorization. *Bioresour. Technol.* **2018**, *265*, 59–65, doi:10.1016/j.biortech.2018.05.108.
15. Malhotra, M.; Suman, S.K. Laccase-mediated delignification and detoxification of lignocellulosic biomass: removing obstacles in energy generation. *Environ. Sci. Pollut. Res.* **2021**, 1–16, doi:10.1007/s11356-021-13283-0.
16. Sosa, L.L.; Jozami, E.; Oakley, L.J.; Montero, G.; Ferreras, L.A.; Venturi, G.; Feldman, S.R. Using C4 perennial rangeland grasses for bioenergy. *Biomass Bioenergy* **2019**, *128*, 105299, doi:10.1016/j.biombioe.2019.105299.
17. Lima, M.A.; Gomez, L.D.; Steele-King, C.G.; Simister, R.; Bernardinelli, O.D.; Carvalho, M.A.; Rezende, C.A.; Labate, C.A.; De Azevêdo, E.R.; McQueen-Mason, S.J.; et al. Evaluating the composition and processing potential of novel sources of Brazilian biomass for sustainable biorenewables production. *Biotechnol. Biofuels* **2014**, *7*, 10, doi:10.1186/1754-6834-7-10.
18. Yang, Y.; Reilly, E.C.; Jungers, J.M.; Chen, J.; Smith, T.M. Climate benefits of increasing plant diversity in perennial bioenergy crops. *One Earth* **2019**, *1*, 434–445, doi:10.1016/j.oneear.2019.11.011.
19. Sage, R.F.; Way, D.A.; Kubien, D.S. Rubisco, Rubisco activase, and global climate change. *J. Exp. Bot.* **2008**, *59*, 1581–1595, doi:10.1093/jxb/ern053.
20. Daniel, R.M.; Danson, M.J.; Eisenthal, R.; Lee, C.K.; Peterson, M.E. The effect of temperature on enzyme activity: New insights and their implications. *Extremophiles* **2007**, *12*, 51–59, doi:10.1007/s00792-007-0089-7.
21. Guo, F.; Shi, W.; Sun, W.; Li, X.; Wang, F.; Zhao, J.; Qu, Y. Differences in the adsorption of enzymes onto lignins from diverse types of lignocellulosic biomass and the underlying mechanism. *Biotechnol. Biofuels* **2014**, *7*, 38, doi:10.1186/1754-6834-7-38.
22. Wu, J.; Chandra, R.; Saddler, J. Alkali–oxygen treatment prior to the mechanical pulping of hardwood enhances enzymatic hydrolysis and carbohydrate recovery through selective lignin modification. *Sustain. Energy Fuels* **2018**, *3*, 227–236, doi:10.1039/c8se00452h.
23. Du, J.; Cao, Y.; Liu, G.; Zhao, J.; Li, X.; Qu, Y. Identifying and overcoming the effect of mass transfer limitation on decreased yield in enzymatic hydrolysis of lignocellulose at high solid concentrations. *Bioresour. Technol.* **2017**, *229*, 88–95, doi:10.1016/j.biortech.2017.01.011.
24. Tian, D.; Chandra, R.P.; Lee, J.-S.; Lu, C.; Saddler, J.N. A comparison of various lignin-extraction methods to enhance the accessibility and ease of enzymatic hydrolysis of the cellulosic component of steam-pretreated poplar. *Biotechnol. Biofuels* **2017**, *10*, 157, doi:10.1186/s13068-017-0846-5.
25. Avanthi, A.; Banerjee, R. A strategic laccase mediated lignin degradation of lignocellulosic feedstocks for ethanol production. *Ind. Crop. Prod.* **2016**, *92*, 174–185, doi:10.1016/j.indcrop.2016.08.009.
26. Sherpa, K.; Ghangrekar, M.M.; Banerjee, R. A green and sustainable approach on statistical optimization of laccase mediated delignification of sugarcane tops for enhanced saccharification. *J. Environ. Manag.* **2018**, *217*, 700–709, doi:10.1016/j.jenvman.2018.04.008.
27. Monteiro, L.M.O.; Vici, A.C.; Messias, J.M.; Heinen, P.R.; Pinheiro, V.E.; Rechia, C.G.V.; Buckeridge, M.S.; Polizeli, M.LT.M. Increased *Malbranchea pulchella* β-glucosidase production and its application in agroindustrial residue hydrolysis: A research based on experimental designs. *Biotechnol. Rep.* **2021**, *30*, e00618, doi:10.1016/j.btre.2021.e00618.
28. Schmatz, A.A.; Tyhoda, L.; Brienzo, M. Sugarcane biomass conversion influenced by lignin. *Biofuels Bioprod. Biorefining* **2019**, *14*, 469–480, doi:10.1002/bbb.2070.
29. Takada, M.; Chandra, R.P.; Saddler, J.N. The influence of lignin migration and relocation during steam pretreatment on the enzymatic hydrolysis of softwood and corn stover biomass substrates. *Biotechnol. Bioeng.* **2019**, *116*, 2864–2873, doi:10.1002/bit.27137.
30. Brar, K.K.; Santo, M.E.; Pellegrini, V.O.; Deazevedo, E.R.; Guimaraes, F.E.; Polikarpov, I.; Chadha, B.S. Enhanced hydrolysis of hydrothermally and autohydrolytically treated sugarcane bagasse and understanding the structural changes leading to improved saccharification. *Biomass Bioenergy* **2020**, *139*, 105639, doi:10.1016/j.biombioe.2020.105639.
31. Al-Zuhair, S.; Abualreesh, M.; Ahmed, K.; Razak, A.A. Enzymatic delignification of biomass for enhanced fermentable sugars production. *Energy Technol.* **2015**, *3*, 121–127, doi:10.1002/ente.201402138.
32. Rencoret, J.; Pereira, A.; Marques, G.; del Río, J.C.; Martínez, T.; Gutiérrez, A. A commercial laccase-mediator system to delignify and improve saccharification of the fast-growing *Paulownia fortunei* (Seem.) Hemsl. *Holzforschung* **2018**, *73*, 45–54, doi:10.1515/hf-2018-0095.
33. Gutiérrez, A.; Rencoret, J.; Cadena, E.M.; Rico, A.; Barth, D.; del Río, J.C.; Martínez, A.T. Demonstration of laccase-based removal of lignin from wood and non-wood plant feedstocks. *Bioresour. Technol.* **2012**, *119*, 114–122, doi:10.1016/j.biortech.2012.05.112.
34. Zanirun, Z.; Bahrin, E.K.; Lai-Yee, P.; Hassan, M.A.; Abd-Aziz, S. Enhancement of fermentable sugars production from oil palm empty fruit bunch by ligninolytic enzymes mediator system. *Int. Biodeterior. Biodegrad.* **2015**, *105*, 13–20, doi:10.1016/j.ibiod.2015.08.010.

35. Meenakshisundaram, S.; Fayeulle, A.; Leonard, E.; Ceballos, C.; Pauss, A. Fiber degradation and carbohydrate production by combined biological and chemical/physicochemical pretreatment methods of lignocellulosic biomass—A review. *Bioresour. Technol.* **2021**, *331*, 125053, doi:10.1016/j.biortech.2021.125053.
36. Rencoret, J.; Pereira, A.; del Río, J.C.; Martínez, A.T.; Gutiérrez, A. Laccase-Mediator Pretreatment of wheat straw degrades lignin and improves saccharification. *BioEnergy Res.* **2016**, *9*, 917–930, doi:10.1007/s12155-016-9745-z.
37. van der Zwan, T.; Chandra, R.P.; Saddler, J.N. Laccase-mediated hydrophilization of lignin decreases unproductive enzyme binding but limits subsequent enzymatic hydrolysis at high substrate concentrations. *Bioresour. Technol.* **2019**, *292*, 121999, doi:10.1016/j.biortech.2019.121999.
38. Freitas, E.N.; Khatri, V.; Contin, D.; Oliveira, T.; Contato, A.; dos Santos, W.R.P.; Martínez, C.; Saddler, J.; Polizeli, M.L.T. Climate change affects cell wall structure and hydrolytic performance of a tropical forage grass as an energy crop. *Prepr. Author.* **2020**, doi:10.22541/au.160629306.65292242/v1.
39. Castoldi, R.; Bracht, A.; de Morais, G.R.; Baesso, M.L.; Corrêa, R.C.G.; Peralta, R.; de Fátima Peralta MunizMoreira, R.; Polizeli, M.L.T.M.; de Souza, C.G.M.; Peralta, R.M. Biological pretreatment of *Eucalyptus grandis* sawdust with white-rot fungi: study of degradation patterns and saccharification kinetics. *Chem. Eng. J.* **2014**, *258*, 240–246, doi:10.1016/j.cej.2014.07.090.
40. Santo, M.E.; Rezende, C.A.; Bernardinelli, O.D.; Pereira, N.; Curvelo, A.A.; Deazevedo, E.R.; Guimarães, F.E.; Polikarpov, I. Structural and compositional changes in sugarcane bagasse subjected to hydrothermal and organosolv pretreatments and their impacts on enzymatic hydrolysis. *Ind. Crop. Prod.* **2018**, *113*, 64–74, doi:10.1016/j.indcrop.2018.01.014.
41. Auxenfans, T.; Terryn, C.; Paës, G. Seeing biomass recalcitrance through fluorescence. *Sci. Rep.* **2017**, *7*, 8838, doi:10.1038/s41598-017-08740-1.
42. Rajak, R.C.; Banerjee, R. Enzyme mediated biomass pretreatment and hydrolysis: A biotechnological venture towards bioethanol production. *RSC Adv.* **2016**, *6*, 61301–61311, doi:10.1039/c6ra09541k.
43. Habermann, E.; Martin, J.A.B.S.; Contin, D.R.; Bossan, V.P.; Barboza, A.; Braga, M.R.; Groppo, M.; Martinez, C.A. Increasing atmospheric CO_2 and canopy temperature induces anatomical and physiological changes in leaves of the C4 forage species *Panicum maximum*. *PLoS ONE* **2019**, *14*, e0212506, doi:10.1371/journal.pone.0212506.
44. de Assis Prado, C.H.B.; De Camargo-Bortolin, L.H.G.; Castro, E.; Martínez, C.A. Leaf dynamics of *Panicum maximum* under future climatic changes. *PLoS ONE* **2016**, *11*, e0149620, doi:10.1371/journal.pone.0149620.
45. IPCC. *Climate Change 2014 Synthesis Report Summary Chapter for Policymakers*; IPCC: Geneva, Switzerland, 2014.
46. Freitas, E.N.; Bubna, G.A.; Brugnari, T.; Kato, C.G.; Nolli, M.; Rauen, T.G.; de FátimaPeralta Muniz Moreira, R.; Peralta, R.; Bracht, A.; de Souza, C.G.; et al. Removal of bisphenol A by laccases from *Pleurotus ostreatus* and *Pleurotus pulmonarius* and evaluation of ecotoxicity of degradation products. *Chem. Eng. J.* **2017**, *330*, 1361–1369, doi:10.1016/j.cej.2017.08.051.
47. Vogel, H. A convenient growth medium for Neurospora (Medium N). *Microb. Genet. Bull.* **1956**, *14*, 42–43.
48. Starcher, B. A ninhydrin-based assay to quantitate the total protein content of tissue samples. A ninhydrin-based assay to quantitate the total protein content of tissue samples. *Anal. Biochem.* **2001**, *292*, 125–129, doi:10.1006/abio.2001.5050.
49. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685, doi:10.1038/227680a0.
50. Nakagame, S.; Chandra, R.P.; Saddler, J.N. The effect of isolated lignins, obtained from a range of pretreated lignocellulosic substrates, on enzymatic hydrolysis. *Biotechnol. Bioeng.* **2010**, *105*, 871–879, doi:10.1002/bit.22626.
51. Sluiter, D.; Hames, A.; Ruiz, B.; Scarlata, R.; Sluiter, C.; Templeton, J. *Determination of Ash in Biomass*; National Renewable Energy Laboratory (NREL): Golden, CO, USA, 2008.
52. Chandra, R.P.; Saddler, J.N. Use of the Simons' staining technique to assess cellulose accessibility in pretreated substrates. *Ind. Biotechnol.* **2012**, *8*, 230–237, doi:10.1089/ind.2012.0016.
53. Tahir, A.A.; Barnoh, N.F.M.; Yusof, N.; Said, N.N.M.; Utsumi, M.; Yen, A.M.; Hashim, H.; Noor, M.J.M.M.; Akhir, F.N.M.; Mohamad, S.E.; et al. Microbial diversity in decaying oil palm empty fruit bunches (opefb) and isolation of lignin-degrading bacteria from a tropical environment. *Microbes Environ.* **2019**, *34*, 161–168, doi:10.1264/jsme2.ME18117.

CHAPTER III

Structural and compositional changes induced by hydrothermal and organosolv pretreatments impacts enzymatic hydrolysis of a tropical forage grass grown under future climate conditions

This chapter was published as:

Freitas, E.N. de; Khatri, V; Wu, J; Takada, M; Scarcella, A.S.A.; Martinez, C.A.; Saddler, J.N.; Polizeli, M.d.L.T.M, **Structural and compositional changes induced by hydrothermal and organosolv pretreatments impacts enzymatic hydrolysis of a tropical forage grass grown under future climate conditions.** *Industrial Crops and Products*. 2021, 171, p. 113937. https://doi.org/10.1016/j.indcrop.2021.113937

Structural and compositional changes induced by hydrothermal and organosolv pretreatments impacts enzymatic hydrolysis of a tropical forage grass grown under future climate conditions

Emanuelle N. de Freitas[a,b], Vinay Khatri[b*], Jie Wu[b], Masatsugu Takada[b], Ana Silvia de A. Scarcella[a], Carlos Alberto Martinez[a], Jack N. Saddler[b] and Maria de Lourdes T. M. Polizeli[a]

[a] *Department of Biology, FFCLRP, University of São Paulo, Ribeirão Preto, São Paulo, Brazil*
[b] *Department of Wood Science, Faculty of Forestry, University of British Columbia, Vancouver, British Columbia, Canada*
* Present Address: Department of Chemistry, Faculty of Sciences, University of Quebec, Montreal, SB-2540, President-Kennedy Pavilion, Montreal, Quebec, Canada

Corresponding author: polizeli@ffclrp.usp.br

Abstract

Panicum maximum represents an alternative as a dedicated energy crop for biorefinery. However, both pretreatment step and climate conditions impact its chemical composition and possibly its hydrolytic performance. Therefore, this study looks into integrating the future effect of the climate conditions and the pretreatment methods on the hydrolytic performance of *P. maximum*. Through a Trop-T-FACE system, *P. maximum* grown under three hypothetical future climate conditions: elevated temperature ((eT), which is 2°C more than the ambient canopy temperature), elevated atmospheric CO_2 concentration ((eC) up to 600 μmol mol-1) and the combined effects (eT+eC). Subsequently, the groups were subjected to the liquid hot water (LHW) and organosolv (ORG) pretreatments, which enhanced biomass digestibility by mostly hemicellulose and lignin removal, respectively. Furthermore, optimized hydrolysis and fluorescent protein-tagged carbohydrate-binding modules binding suggested that *P. maximum* pretreated by LHW has greater saccharification yields and higher cellulose surface accessibility/exposure. This shows that hemicellulose directly impacts *P. maximum* recalcitrance, and its removal was the optimum pretreatment strategy. Concerning the studied expected future climate conditions, LHW samples did not show significant differences in hydrolysis yields at low solids loading (2%). However, at 10% solids, eC had higher conversion yields (likely due to minimum end-product inhibition). Similarly, for organosolv pretreated samples, eC (at 2 and 10% solid loading) exhibited greater cellulose-glucan conversion yields among the climate conditions groups and the highest cellulose accessibility. Thus, this work contributes to understand better the influence of both studied pretreatment methods and future climate conditions on the conversion efficiency of a critical energy crop and might assist in selecting the best pretreatment method to mitigate the climate effects and achieve greater hydrolysis yields.

Keywords: climate change, enzymatic hydrolysis, carbohydrate-binding modules, pretreatment, dedicated energy crop, biorefinery, biofuels.

1. Introduction

The global rise in energy demands and consumption, the depletion of fossil fuel with low cost of extraction, and the climate changes have converged the search for renewable energy sources as a priority, aiming to enhance the efficient production of energy and mitigate carbon dioxide emissions (Alper et al., 2020). In the context of climate change, lignocellulosic materials, such as forestry and agroindustry residues, have been investigated in the development of renewable and neutral carbon cycle products (Scopel and Rezende, 2021). Lignocellulosic biomass (LCB) is widely available at a relatively low cost and represents the basis of bioeconomy development. The main components of LCB cell walls (mostly cellulose, hemicellulose, and lignin) make them suitable biorefineries materials (Paloma Manzanares, 2020) since their polysaccharides can be converted into fermentable sugars, chemicals, and biomaterials. The residual lignin could also be a source of value-added products, such as organic acids, vanillin, and epoxy resins (Nguyen et al., 2021).

Non-wood LCBs, such as fast-growing perennial C4 grasses, represent an effective alternative of feedstock that can be processed in biorefineries. For example, *Miscanthus* spp. and switchgrass are widely used in Europe and the United States as dedicated bioenergy crops (Sosa et al., 2019; van der Weijde et al., 2013). In this context*,* *Panicum maximum* (Jacq. cv. Mombaça) is an important C4 grass species used as pasture in South America, particularly in Brazil, and has several advantages on the use for bioenergy purposes as high efficiency to convert solar energy in biomass through photosynthesis (30 ton/ha); similar lignocellulosic composition and bioethanol yields to sugarcane bagasse (Lima et al., 2014); low mineral-nutrient inputs that make them able to grow in marginal lands abandoned from agricultural uses which helps to minimize the food *vs*. fuel conflict, and the cost-effective production, making the final product competitive (Yang et al., 2019). Moreover, ethanol produced from grasses is reported to lead to a potential reduction in greenhouse gas emissions from 97% to 77% during their life cycle compared to petroleum-based fuels (Wang et al., 2015).

In general, LCB cell wall components are closely associated with each other resulting in a recalcitrant structure with low accessibility to hydrolases (Bhatia et al., 2020). Thus, a pretreatment method is necessary before enzymatic conversion of biomass to minimize the physical and chemical barriers and maximize cellulase accessibility to cellulose fibers (Wu et al., 2019). Pretreatment can be classified in biological, physical, chemical processes, or combinations of these approaches (Agbor et al., 2011).

Organosolv pretreatment (ORG) is a chemical method that significantly impacts lignin removal and hemicellulose solubilization in pretreatment liquor. It utilizes organic solvents such as ethanol, methanol, and acetone either in the absence or presence of water, promoting the break-in internal bonds of hemicelluloses and lignin. Among these solvent options, ethanol is harmless due to its low toxicity and was chosen as the solvent in this work (Wei Kit Chin et al., 2020). On the other hand, liquid hot water (LHW) pretreatment efficiently promotes hemicellulose removal by converting it into mono and oligosaccharides. These can be further used in high-value chemicals/bio-products production while promoting structural and chemical changes in the lignin fraction (Silveira et al., 2015). Furthermore, LHW is considered an environmentally friendly process because it does not use chemical reagents and has effectively increased enzymatic hydrolysis of several types of raw materials, such as corn stover sugarcane bagasse (Buruiana et al., 2014; Hongdan et al., 2013).

However, not only the pretreatment process but also climate change can influence LCB bioenergetic potential. Plant physiologic processes, such as photosynthesis, are influenced by temperature and [CO_2]. Therefore, the climate change expected in the following years could affect the growth, yield, and chemical composition of lignocellulosic materials (Sage et al., 2008). In addition, abiotic stress induced by climate changes, especially heat stress (i.e., eT), can influence LCB chemical composition and structure, as cell wall remodeling represents an important stress tolerance mechanism applied by plants (Moura et al., 2010; Tenhaken, 2015; Wang et al., 2016). Transcriptome studies found that genes of several enzymes involved in cellulose, hemicellulose, and pectin biosyntheses had transcript levels affected by elevated CO_2 and temperature (Wei et al., 2013; Zhu et al., 2010).

Nonetheless, it is unclear how dedicated energy crop composition and hydrolytic performance will respond to both circumstances (climate change and pretreatment process). Hence, since *P.* maximum cv. Mombaça represents a vital alternative of a reliable energy crop; the impact of climate changes and pretreatment on its use for bioenergy purposes must be investigated.

This study intends to understand how the effects of climate changes and pretreatment processes affect synergistic associations between cellulolytic and accessory enzymes during saccharification and, therefore, the sugar yields of *P. maximum* forage grass. *P. maximum* perennial C4 grass, using the Trop-T-FACE system, grew under expected future climate conditions (i.e., elevated CO_2 and temperature). All treatments were subjected to two individual pretreatment strategies with low impact on climate change, ORG and LHW. In addition, the pretreated biomasses had their chemical composition determined and further subjected to optimized enzymatic hydrolysis and the characterization of its lignocellulosic components accessibility to enzymes using carbohydrate-binding modules (CBMs) approach. We speculated that studied pretreatment processes could influence the effect of the climate changes on *P. maximum* as a source of energy crop. As far as we know, the present study is the first to provide experimental evidence of the use of forage grasses for bioenergy, considering the effect of pretreatment choice and climate change on sugar production.

2. Materials and methods

2.1 Plant Material and TROP-T-FACE system

We evaluated the effect of two climate change elements projected for the upcoming decades (i.e., elevate CO_2 atmospheric concentrations [CO_2] and temperature increase (+2 °C)) in the tropical grassland *Panicum maximum* cv. Mombaça. The experiments were conducted at the University of São Paulo, Ribeirão Preto, SP, Brazil, using a Trop-T-FACE facility as described elsewhere (de Assis Prado et al., 2016; Habermann et al., 2019). A total of 16 plots were randomly distributed in the area as **Fig. S1**. The following treatments were applied: (i) ambient [CO_2] and temperature (Control: C), (ii) ambient [CO_2] and 2 °C above ambient temperature (Elevate temperature: eT), (iii) 600 ppm atmospheric [CO_2] and ambient temperature (Elevate CO_2: eC) and (iv) 600 ppm atmospheric [CO_2] and +2 °C above ambient temperature (eT+eC). Plants were under satisfactory conditions of water availability during all experiments.

The plants grew for 2 months and were clipped at 30 cm above the ground. After that, the treatments described above were initiated. Later with 24 days of the experiment, *P. maximum* leaves were collected at 12 p.m, milled at 20 mesh, dried, and stored in a free humidity environment until analyses. Meteorological data from the whole growing season is discussed in Habermann et al. (2019).

2.2 Liquid Hot Water (LHW) and Organosolv (ORG) pretreatments

P. maximum under different climate treatments was subjected to LHW and ORG pretreatments. The pretreatment processes were applied separately under optimal conditions that were empirically determined to boost total sugar recovery and allow cellulose hydrolysis with relatively low enzyme loadings in shorter times.

LHW was conducted in a 5.5 L stainless steel reactor (PARR Instrument Company, Moline IL, USA, model 4584) at 195°C for 10 minutes, 150 rpm. The pressure was kept at 7 bar, and a 1:10 solid to liquid ratio (grams of biomass/mL of water) was used (dos Santos Rocha et al., 2017; Pratto et al., 2016). ORG treatment was carried out in a 200 mL stainless steel cylinder reactor (working volume of 50 mL) with ethanol/water solutions (50% v/v) and a 1:10 solid to liquid ratio. The reactor was submerged in a glycerin bath with an open heating circulator (Julabo Labortechnik GmbH, Seelbath, Germany) and heated at 190°C for 1 hour (Espirito Santo et al., 2018).

At the end of each pretreatment, the solids fractions were washed with ethanol (ORG) and water (ORG and LHW) to remove inhibitory compounds solubilized during the pretreatment step until it reaches neutral pH. The solids were dried in an oven at 50 °C for 24 h and stored for chemical composition analysis and enzymatic hydrolysis. Glucan, hemicellulose, and lignin recoveries in the pretreated materials were obtained according to the following equation.

$$Recovery\ (\%) = \frac{DM_{PT}\ x\ C_{PT}}{DM_I\ x\ C_I}\ \times\ 100$$

(Eq. 1)

Where: DM_{PT} is the dry mass after pretreatment, DM_I is the initial dry mass, C_{PT} is the glucan, hemicellulose, and lignin content after pretreatment, and C_I is the initial glucan, hemicellulose, and lignin content. Lignin and hemicellulose removal was calculated by subtracting their recovery from 100% (maximum yield).

2.3 Characterization of chemical composition

The average composition analysis was always obtained from three independent replicates. In addition, the chemical composition of the water-insoluble fraction of *P. maximum* after LHW and ORG was carried out using the modified Klason lignin method derived from the TAPPI standard method T222 om-88 ((Nakagame et al., 2010). Ash content was determined according to the NREL/TP-510-42622 method (Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., & Templeton, 2008).

The biomass composition of non-treated *P. maximum* in the different climate conditions has already been reported in our previous study by the same method described in this study (Freitas, Emanuelle N., Khatri, Vinay, Contin, Daniele, Oliveira, Tassio, Contato, Alex, Peralta, Rosane, dos Santos, Wanderley, Martínez, Carlos, Saddler, Jack, Polizeli, n.d.).

2.4 Enzymes used for hydrolysis experiments

Cellulase enzyme mixtures Celluclast® 1.5 L and CTec3® were kindly provided by Novozymes, Franklinton, NC, USA. Celluclast (cellulase from *Trichoderma reesei*) was applied in a variety of protein loadings (per gram of glucan) with β-glucosidase supplementation (Novozymes 188) (2 CBU (cellobiase unit) of β-glucosidase per 1 FPU (filter paper unit)) to avoid inhibition from cellobiose accumulation.

The addition of accessory enzymes was tested to boost the effect of the cellulase mixture that was previously optimized. These enzymes were added employing a replacement strategy, which comprises replacing amounts of the enzyme from an optimized combination with the accessory enzymes as hemicellulolytic and AA9 but maintaining the total protein loading at the same level. Thus, HTec was the hemicellulolytic mixture, and *Thermoascus aurantiacus* Auxiliary Activity Family (AA9) was the Lytic Polysaccharide Monooxygenase (LPMOs) (Novozymes®).

The protein concentration in enzyme mixtures was quantified using the ninhydrin assay and bovine serum albumin (BSA) as the protein standard (Starcher, 2001).

2.5 Enzymatic hydrolysis studies of pretreated biomass

Initially, to optimize enzymatic hydrolysis, the experiments were carried with initial solid loading of 2% (w/v) in 50 mM sodium acetate buffer (pH 4.8) at 50°C for up to 72 hours. Then, different cellulase loading was tested to obtain the best glucan bioconversion rates for each pretreatment. After the optimal cellulase loading was selected, the supplementation of enzyme mixture with accessory enzymes was evaluated using above mentioned replacement strategy. First, the addition of Htec was evaluated, followed by AA9, as an attempt to reduce the amount of protein needed for effective glucan conversion.

The optimal enzyme mixture was select based on efficient bioconversion yields along with less amount of protein needed. The optimal holocellulolytic combinations for each climate condition and pretreatment were subsequently applied in comparison studies with untreated *P. maximum* biomass and hydrolysis at 10% solid loading, which was also performed in 50 mM sodium acetate buffer (pH 4.8) at 50°C for 72 hours.

The extent of glucan hydrolysis of pretreated biomass was calculated as a percentage of the theoretical glucan presented in the substrate and expressed as glucan conversion rates. The proportion of each enzyme used in the enzyme cocktail was adjusted based on the chemical composition of *P. maximum,* and for each climate, condition studied.

2.6 Determination of monosaccharides

The quantitative analysis of monosaccharides of pretreated *P. maximum* biomass after the Klason procedure was carried out by a high-performance anion-exchange chromatography system (Dionex DX-3000, Sunnyvale, CA) (Boussaid et al., 1999). In addition, the quantitative analysis of glucose concentration in the hydrolysate was performed through YSI 2700D Select Biochemistry Analyzer.

2.7 Scanning electron microscopy images

SEM observations were conducted using a Hitachi S-4700 Field Emission SEM (Hitachi) to assess possible changes in the surface structure of pretreated substrates. Samples were freeze-dried before being placed on an SEM stub then gold-coated using a Cressington 208HR High-Resolution Sputter Coater. Observations were performed at a working distance of 15 mm and accelerating voltages between 5 kV.

2.8 Holocellulose (cellulose + hemicellulose) accessibility using FTCM-depletion assay

The "fluorescent protein-tagged carbohydrate-binding modules method" (FTCM) and its adaptation FTCM-depletion assay were the method applied to evaluate the hemicellulose and cellulose surface accessibility in non-treated and pretreated *P. maximum* fibers in four treatments (C, eT, eC, and eT+eC) before enzymatic hydrolysis. The assay proceeded using four different FTCM probes: GC3a (fluorescent protein eGFP genetically linked with CBM3a), CC17 (construct of the fluorescent protein mCherry and CBM17), OC15 (a chimera made of mOrange2 fluorescent protein and CBM15), and CC27 (composed of eCFP and CBM27), targeting crystalline cellulose, amorphous cellulose (non-crystalline), xylan and mannan hemicellulose, respectively (Khatri et al., 2018a). The purification, production, characterization, and fluorescence measurements of CBM probes are described in earlier reports (Bombeck et al., 2017; Hébert-Ouellet et al., 2017; Khatri et al., 2018a, 2018b). In addition, to order to prevent the non-specific binding of CBM probes to lignin, the latter was blocked by incubating all substrates with bovine serum albumin (BSA) previously to FTCM-depletion assay (Khatri et al., 2018a).

2.9 Statistics and numerical analysis

Data were expressed as the mean of three replicates ± standard deviations of the means (SD). The significance of the observed differences was tested using analysis of variance (ANOVA) from the Graph-Pad Prism 8.0 software (San Diego, EUA). Differences among the studied parameters were evaluated by the Tukey test, and *p* values ≤ 0.05 were considered statistically significant.

3. Results and discussion

3.1 Compositional analysis of *Panicum maximum* biomass

Pretreatment methods were applied in this study to increase substrates' accessible surface area and achieve high sugar yields in all future climate conditions. Since several studies have reported that lignin removal from lignocellulosic biomass has a positive relationship with holocellulose hydrolysability, the ORG method was chosen to solubilize hemicellulose. Still, most remove lignin leaving cellulose-rich substrate after pretreatment (Zhao et al., 2009). LHW is a physicochemical process known for its effects on hemicellulose removal, lignin solubilization, and redistribution in biomass (Malgas et al., 2017). Therefore, it was chosen as the second pretreatment strategy used in this study. These two different pretreatment strategies (delignification and hemicellulose solubilization *vs.* hemicellulose solubilization and lignin redistribution) were selected to understand better the effects of these procedures on enzymatic hydrolysability by glycoside hydrolases (GH).

The chemical composition analysis of the non-treated and pretreated (by ORG and LHW) *P. maximum* grass in different climate conditions was carried out in terms of total glucan, hemicellulose, lignin, and ash contents, as demonstrated in Table 1.

The main effect of LHW pretreatment was hemicellulose removal with minor lignin solubilization. More than 70.5% of the original hemicellulose component was removed by LHW samples with a decrement from 16.9 to 6.75 wt% (on average) compared to the non-treated grass. Hemicellulose dissolution in LHW uses hydronium ions as catalysts. These ions are responsible for the cleavage of hemicellulose substitutions, resulting in the production of organic acids, which catalyzes the hydrolysis of linkages between hemicellulose and lignin, subsequently converting hemicelluloses into soluble mono and oligosaccharides (Ewanick and Bura, 2010). Also, in hydrothermal pretreatments, the lignin turns into a fluid-like state (depending on its glass transition temperature (Tg)), which may lead to lignin reallocation on the cell wall during pretreatment heating and cooling, with simultaneously a small amount of lignin being dissolved in hot water due to acid-catalyzed cleavage of β-O-4 linkages in lignin (Ruiz et al., 2020).

A relatively comparable higher lignin and cellulose content could be detected in the LHW pretreated biomass because of the extensive removal of hemicelluloses (more than 70%) along with excellent cellulose recovery (up to 89.1%) **(Table 1).** The content of cellulose in LHW substrates increased to 36.8 wt% (on average) as well as the lignin content (increased to 36.3 wt% on average) when compared to non-treated grass, indicating that only a limited proportion of lignin could be solubilized.

Concerning to climate conditions, the hemicellulose content for LHW pretreated samples was quite similar among the groups (Control (C), elevated temperature (eT), elevated [CO_2] (eC) and elevated temperature + elevated [CO_2] (eT+eC)) with a significant difference found between the C and eT+eC groups ($p < 0.05$). In addition, the C group exhibited higher lignin content (38.8 ± 0.1 wt%). Regarding glucan composition, eT (38.5 ± 0.6 wt%) and eT+eC (37.2 ± 0.2 wt%) samples showed the highest percentage of this sugar compared to C and eC groups (35.3 ± 1.5 and 36.3 ± 0.2 wt% respectively), which is likely due to non-treated eT and eT+eC samples also have higher cellulose content, which was preserved in pretreated samples probably due to the similar hemicellulose removal for all of them (Table 1).

On the contrary, the main effect of ORG was lignin removal (up to 51.7%), but also a considerable amount of hemicellulose solubilization was observed (42% on average) along with excellent cellulose recovery (up to 94.1%). Thus, for ORG pretreated substrates, the overall cellulose contents were higher (42.7 wt% on average) compared to that of non-treated and LHW samples, regardless of the climate conditions ($p < 0.05$) (**Table 1**). This glucan content increase was described as being due to delignification and solubilization of hemicellulose (Park et al., 2017). Nonetheless, the hemicellulose and lignin content of ORG pretreated grasses were similar to the non-treated ones, which could be explained by the fact that the ORG process removed both lignin and hemicellulose from the native grasses. Lignin removal during ORG pretreatment occurs primarily by breaking down α and β-aryl ether linkages in lignin and the ether 4-*O*-methyl glucuronic acid ester bonds between lignin and hemicellulose, which promotes the dissolution of lignin and hemicellulose fragments to the aqueous phase (Agbor et al., 2011).

Concerning the differences in the chemical composition of C, eT, eC, and eT+eC groups pretreated by the ORG process, we found that eC was the group with the lowest hemicellulose (15.1 ± 0.0 wt%) and glucan content (40.6.± 0.3 wt%) ($p < 0.05$). The explanation for this effect should be further investigated. In the meantime, no difference in glucan and hemicellulose ($p > 0.05$) as well as lignin ($p < 0.05$) contents was found among C, eT, and eT+eC.

3.2 Effect of pretreatments on monosaccharide profile

Pretreated and non-treated grasses exhibited similar monosaccharide profiles, which are mainly composed of xylose, arabinose, glucose, and galactose (**Fig. 1**). Mannose and rhamnose were not found in detectable quantities by the method employed. Because the grass hemicelluloses are mainly composed of arabinoxylan residues (AXs) (Vogel, 2008), and the glucose contribution for hemicellulose monosaccharide profile is meager, we represented it like total glucan content.

Regarding ORG pretreated grasses, the amount of xylose (**Fig. 1A**) for ORG samples was higher than non-treated samples for C, eT, and eT+eC groups ($p < 0.05$), with just the eC group exhibiting the same levels of xylose as non-treated grasses ($p > 0.05$). These results indicate that

ORG affects the xylose content to a lesser extent, where up to 79.4% of initial content is retained in ORG pretreated samples (**Table S1**).

On the contrary, arabinose and galactose content (**Figs. 1B and C**) were reduced in ORG pretreated samples compared to non-treated groups. Arabinose and galactose were significantly removed during ORG pretreatments. Only 39.7 and 29.4% (the average among climate conditions), respectively, of the initial content, was retained in the solid pretreated fraction (**Table S1**). Meanwhile, no difference was found in arabinose and galactose content among the climate conditions groups. Arabinose and galactose saccharides are easily removed because glycosidic linkages with ramification groups are usually more sensitive toward acidic hydrolysis than β-1,4 linkages in the xylose backbone. In addition, arabinose side-chain units are extremely labile toward acid due to their furanosidic structure and are thus cleaved already at the early stages of the pretreatment process (Marchessault, 1994).

As a consequence of the great arabinose removal (more than 60%), ORG pretreatment increases the xylose/arabinose (Xyl/Ara) ratio (**Fig 1D**) that has a strong relation with better hydrolysis yields since ferulic acid (FA) can esterify with C5 of arabinofuranose side-chains of arabinoxylans (AXs), leading to an intermolecular cross-linking between AXs and lignin (Karlen et al., 2016). The cross-linking of AXs with lignin contributes to grass biomass recalcitrance and thus leads to reduced enzymatic saccharification (Oliveira et al., 2019).

Regarding LHW, as expected, the pretreatment led to a significantly reduced in xylose, arabinose, and galactose content, achieving in average 68.5, 92, and 74.9% of removal, respectively, during the pretreatment process (**Figure 1; Table S1**). While no significant difference ($p > 0.05$) was found in their range among the studied climate conditions concerning LHW pretreatment (**Fig. 1A-C**). Besides, Xyl/Ara ratio (**Fig. 1D**) had an enormous increase compared with non-treated biomass (3.87 fold higher in average), which was much higher ($p < 0.05$) than the one promoted by ORG pretreatment (1.93 fold higher in average). As discussed before Xyl/Ara ratio is an essential factor influencing enzymatic hydrolysis and can significantly impact the saccharification process.

3.3 Optimization of enzymatic hydrolysis of *Panicum maximum* grass

A series of experiments were performed to find out the optimized use of enzyme cocktails. The objective was to achieve higher hydrolysis yield for each pretreatment and climate condition to better understanding how chemical modifications in *P. maximum* biomass (by applying different pretreatment technologies) affect the saccharification levels by cellulolytic and hemicellulolytic enzymes.

3.3.1 Effect of enzyme loading on the hydrolysis yields

First, we evaluated the glucan conversion using Celluclast® and β-glucosidase as a cellulolytic core set for both pretreatments ORG and LHW at 2% solid loading and 72 hours hydrolysis. Celluclast® is a preparation used for breaking down cellulose into glucose, cellobiose, and longer glucose polymers and contains minimum xylanase activity. It was used over a range of protein loadings (10, 20, 30, 40 mg, and 50 mg protein per gram of glucan) with enough β-glucosidase supplementation to verify the lowest protein loading necessary to obtain 70% or more of glucan conversion (**Fig. 2**).

For ORG pretreatment, by increasing the enzyme load, sugar yields tend to plateau at 40 mg protein/g glucan except for eC climate condition, in which glucan conversion yields continue to rise even at 50 mg protein/g glucan loading with a maximum yield of 76.61 ± 0.86 ($p < 0.05$). The saccharification performance for C, eT, and eT+eC groups was similar, and no significant difference ($p > 0.05$) was found among the groups in any protein loading tested in this study. Nonetheless, eC exhibited the highest yields in all tested conditions ($p < 0.05$) (Fig. 2A).

A target of 70% of cellulose hydrolysis after 72 hours has been suggested to be a good result since complete hydrolysis with low enzyme loading within and short time might be challenging to be successful (Hu et al., 2015a). Interestingly, 70% saccharification for ORG pretreated substrates was only achieved for the eC group at 40 mg protein/g glucan. C, eT, and eT+eC did not reach 70% hydrolysis even at 50 mg protein/ g glucan (**Fig 2A**). It was apparent that higher protein loadings were required for efficient degradation of C, eT, and eT+eC compared to those required for eC. This fact can not be explained just based on the chemical composition of ORG samples. Thus, further studies of cell wall structure that could restrict enzyme accessibility should be performed. In addition, eC group showed the significant increase ($p < 0.05$) in the bioconversion yield at 40 (8.43 ± 2.13%) and 50 mg protein/g cellulose (13.5 ± 0.86%) compared to C group. No positive effect concerning the C group was found for eT and eT+eC at any protein loading.

Regarding LHW pretreatment, cellulose hydrolysis yields tend to plateau at 30 mg protein/g glucan for all climate conditions **(Fig. 2B)**. Moreover, LHW pretreated samples required less protein amount to achieve 70% glucan conversion. At 20 mg protein/g glucan, more than 70% glucan conversion was reached, and at 30 mg/g, the observed glucan bioconversion was 90 - 95% among all the climate conditions. For LHW samples, at 20 mg protein/g cellulose, the eC group exhibited better hydrolysis yield ($p < 0.05$) than other groups. These results indicate that LHW pretreatment extensively modifies *P. maximum* fibers. The minor differences in chemical composition among C, eT, eC, and eT+eC were not enough to result in significant differences in saccharification performance in the tested hydrolysis conditions.

3.3.2 Enhancement of saccharification efficiency by the addition of hemicellulolytic enzymes

Hemicellulose is described to restrict the access of cellulases to their substrate in lignocellulosic biomass (Houfani et al., 2020). Therefore, Htec® was incorporated into the enzyme mixture as the hemicellulolytic cocktail aiming to achieve further hydrolysis yields through the synergism between the cellulolytic and xylanolytic enzymes core set and reduce the amount of protein needed to accomplish 70% hydrolysis of ORG and LHW pretreated samples. A replacement strategy was used with no increment in the total protein/enzyme loading in which cellulase mixture was replaced with an equivalent amount (percentage basis) of Htec®.

The addition of Htec® led to increased cellulose hydrolysis (2% solid loading) for both pretreatments (**Fig. 3**). Concerning ORG samples, protein loading of 30 mg of protein/g glucan was chosen to further experiments since it led to ~70% glucan conversion using just Celluclast® (**Fig.2A**). The replacement of cellulase mixture (Celluclast®) by hemicellulolytic mixture (Htec®) significantly improved ($p < 0.05$) the glucose release in all climate conditions (**Fig. 3A**). An increase from 27.3% (C) to 42.8% (eC) was found with 5 and 10% replacement compared to hydrolysis without Htec®. However, with the increased amounts of hemicellulase supplementation, no beneficial effect was observed, showing that in this case, there was not enough cellulase for cellulose hydrolysis.

The ORG eC group was still the group with significantly higher ($p < 0.05$) glucan conversion rates (95.89 ± 0.15% for Htec at 5%) in all replacements levels while no significant difference was found for C, eT, and eT+eC. Furthermore, only eC group demonstrated an increase on conversion rates in relation to C group both at 5% Htec (15.25 ± 0.77%) and 10% Htec (16.17 ± 0.47%).

Regarding LHW pretreatment, the replacement strategy was applied under 20 mg protein/g of glucan. Under lower protein loading (i.e., 10 mg protein/g of glucan), cellulase replacement with xylanase resulted in significantly lower conversion rates (**Fig. S2**). Minimal replacement of cellulase with hemicellulases improved sugar release. The replacement of 5% of cellulase by an equivalent amount of Htec® increased the conversion of LHW samples to 100% of the theoretical glucan conversion yields for all climate conditions **(Fig. 3B).** No significant difference was observed at 5, 10, and 20% Htec® replacement among all the climate conditions. It was apparent that hemicellulases supplementation to the cellulase mixture had a more significant positive effect on the pretreated LHW substrate, even if the hemicellulose content of these samples was low, showing the vital role that hemicellulose play by disrupting the restrictive access of hydrolytic enzymes to cellulose microfibrils.

3.3.3 Enhancement of saccharification efficiency by the addition of LPMOs accessory enzymes

Previous work has described that Lytic Polysaccharide Monooxygenase (LPMOs), such as AA9, can cleave the ordered crystalline cellulose regions by oxidative mechanisms, improving cellulose accessibility to hydrolytic enzymes in a complementary manner with them (Hu et al., 2015a). Therefore, we next investigated whether replacing more of the cellulase mixture with various amounts of AA9 might further enhance pretreated *P. maximum* hydrolysis by setting the synergistic interaction with cellulases.

The optimized Celluclast-Htec® mixture for the LHW and ORG pretreated substrates (**Fig. 3**) were assessed for the effect of partial replacement of cellulase core set by LPMOs. The inclusion of LPMOs into the enzyme mixture attempted to reduce the amount of protein required to efficiently hydrolyze *P.maximum* substrates since cellulase and xylanase mixture already led to 90-100% conversion rates. Therefore, we chose to work with the following protein loading on LPMO replacement trials: ORG: 20mg/g and LHW: 10 mg/g, both of them with 5% Htec replacement (**Fig. 4**).

Concerning ORG pretreated samples, a relative significative increase ($p < 0.05$) on hydrolysis yields for C, eT, and eT+eC groups has been observed when 10% cellulase was replaced by AA9. In these cases, the glucan conversion rates increase approximately 9% after AA9 addition to the enzyme mixture (**Fig. 4A**). Moreover, the eC group exhibited the highest glucan yields, showing a relative significative increase ($p < 0.05$) of 9.2% and 12.9% at 5 and 10% AA9 replacement to 20 mg/g (5% Htec) without AA9. However, the glucan conversion rates found in AA9 trials ORG samples were above the ones achieved at 30 mg protein/g glucan and 5% Htec mixture (**Fig. 3B**).

Concerning LHW pretreatment, no beneficial effect was observed for LHW pretreated substrates from the addition of AA9 to the cellulase and xylanase mixture. Indeed, for eC and eT+eC group a significant reduction was demonstrated ($p < 0.05$) after AA9 replacement (**Fig 4B**). Further replacement of AA9 (20 and 30%) was also used for LHW and ORG samples and is illustrated in **Fig. S3,** but no positive effect was found in these cases.

Although AA9 and cellulase enzymes act synergistically to deconstruct the cellulosic fraction of LCB, which was evidenced in this study by the increase in glucose yields for ORG samples, the extent of enhancement is highly substrate-dependent. Recently, the AA9 "boosting effect" during hydrolysis has been described to depends on several factors such as the amount of exogenous reducing cofactor, fiber characteristics, cellulose accessibility, degree of polymerization, and crystallinity (Hu et al., 2014). Therefore differences in those substrates' properties are probably responsible for the differences found in the AA9 effect for hydrolysis yield of ORG and LHW substrates. However, further studies should be performed to determine the main factor responsible for the lack of improvement in glucan conversion rates for LHW samples after AA9 addition.

3.3.4 Comparison of hydrolytic efficiency of the optimized mixture of pretreated substrates to untreated biomass

Hydrolysis of non-treated *P. maximum* biomass in all climate conditions was assessed against the hydrolysis of LHW and ORG pretreated samples (**Fig. 5**). The saccharification experiments of pretreated biomass were performed in optimal conditions that were previously determined (ORG: 30 mg/g with 5% Htec, LHW: 20 mg/g with 5% Htec), and hydrolysis of non-treated *P. maximum* was evaluated using the same protein loading under identical conditions of each pretreated sample. The final enzyme mixture only contains cellulase and xylanases since accessory enzymes generally add a high cost to the enzyme mixture and, here, the increment in bioconversion yields was not satisfactory when adding AA9.

Glucose release under the action of the optimal enzyme mixture at 72 hours of hydrolysis for non-treated biomass ranged from 36.1 ± 0.2%(C) to 39.8 ± 0.3% (eT) at 30 mg/g protein loading and 5% Htec replacement (optimized mixture for ORG) (**Fig. 5A**). Although ORG pretreated samples showed higher enzymatic saccharification in comparison with non-treated biomass, the best relative improvement seen in glucan conversion rates was for the eC group (159.8 ± 0.7%), followed by C (130.4 ± 0.2%), eT (120.8 ± 0.2%), and eT+eC (116.7 ± 2.5%) with no significant difference ($p > 0.05$) between the last two climate conditions groups.

Liquid hot water pretreatment strongly enhanced lignocellulose saccharification when the optimal enzyme mixture was applied, leading to 100% of theoretical cellulose hydrolysis for all climate conditions (**Fig. 5B**), showing a relative improvement of hydrolysis that ranged from 212.5 ± 1.37 (eT+eC) to 226,05 ± 1.35 (eC) compared to non-treated samples, with no significant difference ($p > 0.05$) between climate conditions.

The saccharification performances described in this study are more outstanding than the ones reported to other LHW pretreated forage grasses like switchgrass (75%) and *Pennisetum purpureum* (60%) at similar hydrolysis conditions and protein loading (Yu et al., 2016). It was apparent that other energy crops showed lower or similar hydrolysis yields as the one reported in this study at similar hydrolysis conditions. For example, corn stover pretreated by ORG and steam explosion (a hydrothermal method such as LHW) displayed hydrolysis yields of 60 and 80%, respectively (Hu et al., 2015b). Similarly, steam pretreated sugarcane bagasse showed 96% glucan conversion rates at 20 mg/g glucan of protein loading after 72 hours of hydrolysis (Espírito Santo et al., 2019). Therefore, the results from our study indicate that *P. maximum* is a forage grass with high potential for cellulosic ethanol production, showing similar or more outstanding hydrolysis performance than many energy crops widely used around the world, such as corn stover and sugar cane bagasse, in similar conditions of enzymatic saccharification.

The high hydrolysis sugar yields of pretreated *P. maximum* biomass in different climate conditions indicated that its hollocelulose fraction was more accessible to enzymes after ORG and LHW pretreatment. Nonetheless, LHW that increases enzyme accessibility by targeting hemicellulose removal and lignin redistribution had significantly higher glucan conversion rates (14.4% on average) than the ORG process that enhances biomass digestibility by delignification and hemicellulose solubilization (**Fig. 5**).

In order to better understand the morphological changes caused by the pretreatments, we analyzed *P. maximum* samples before and after pretreatment steps using Scanning Electron Microscopy (SEM) (**Fig. 6**). In non-treated samples, a dense and highly ordered surface layer covers the fiber bundles (**Fig. 6A**). Nonetheless, the samples that underwent LHW pretreatment showed fiber surface beginning to unattached, indicating considerable disorder in lignin structure **(Fig. 6B).** However, the removal of the surface layer is inhomogeneous, with some regions (indicated by the yellow arrows) presenting considerably greater removal than others. This may mean lignin redistribution and/or modification that lead to fiber loosening and, to a lesser extent, lignin solubilization, as presented in Table 1. In contrast, ORG pretreated samples **(Fig. 6C)** showed the surface layer being gradually removed over the whole fiber, which appeared to be to less tightly with loss of the packed assembly. These effects are likely due to the removal of the lignin layer that held the fibers together.

Lignin is identified as a significant deterrent to the conversion of polysaccharides into fermentable sugars due to its close association with cellulose microfibrils acting as a physical barrier (Ye et al., 2021). Also, lignin leads to the non-productive binding of cellulolytic enzymes (Lai et al., 2019). The effects of ORG and LHW pretreatments, lignin removal, redistribution, and chemical modification, as observed in Figure 6, increase the surface area for cellulase accessibility, and the pretreated biomass becomes more digestible than non-pretreated ones even though it may have approximately the same or more lignin content as raw biomass. Therefore, the yield of fermentable sugars increases significantly for pretreated substrates (Figure 5).

Hemicellulose also exerts a significant influence on restricting the enzyme accessibility to the cellulose microfibrils since this component is closely associated with cellulose in the cell walls, hiding part of the microfibrils (Agbor et al., 2011). Hemicellulose is among the critical components of lignocellulose which is also considered as the most thermo-chemically sensitive constituent. Therefore, it is possible to remove it using different temperature and pressure conditions (Hendriks and Zeeman, 2009). Nevertheless, severity parameters must be controlled to avoid the formation of hemicellulose degradation products such as furfurals that have been reported to inhibit the fermentation process (Fujimoto et al., 2018). In addition, some studies have been reported that hemicellulose removal overmatches delignification in many lignocellulosic substrates with effects that facilitate cellulose hydrolysis (Guo et al., 2017; Sindhu et al., 2016) by increasing the porosity, removal of acetyl groups which are reported as an effective binding inhibitor, and lowering glucuronic acid side chain that confers charge to the hemicellulose surface and might link to lignin (Oinonen et al., 2015; Pan et al., 2006).

In this study, LHW was the best pretreatment strategy leading to higher glucan conversion rates for *P. maximum* in all climate conditions (**Fig. 5**). The major impact of LHW is the removal of hemicellulose, and it also leads to the redistribution and solubilization of lignin, even the latter being to a lesser extent. Additionally, even with higher lignin content than non-treated biomass, the LHW pretreated substrates converted 100% of the theoretical glucan content into glucose (**Figs. 3 and 5**). These results suggest that for *P. maximum* grass, extensively hemicellulose removal (up to 76.2%) along with structural modification of lignin overmatches lignin removal, which is the manly effect of ORG pretreatment (delignification strategy) since ORG samples showed significantly ($p < 0.05$) lower yields of glucan conversion rates than LHW. Moreover, a specific lignin fraction, specially the solvent extractable lignin, in residual lignin after pretreatment have been described to result in a stimulatory effect to enzymatic hydrolysis, by relieving the non-productive binding of cellulases (Lai et al., 2015). Such positive effect was previously found for hydrothermal pretreatment (Ewanick et al., 2007) and could also played a role for LHW greater hydrolysis yields.

Furthermore, most biopolymers, including cellulose, hemicellulose, and lignin, are intimately interconnected in lignin–carbohydrate complexes (LCC). The interactions among them are a critical factor in plant biomass recalcitrance (Houfani et al., 2020). LHW pretreatment resulted in a greater Xyl/Ara ratio which means fewer arabinosyl residues in side chains of the xylan backbone (**Fig. 1D**). The less content of arabinosyl residues in the fiber likely led to a more disconnected lignocellulose structure since arabinose is described to be involved in xylan-lignin cross-linking through ferulic acid (as discussed in Section 2.2), which turn the remaining fraction of the lignocellulose more accessible to the hydrolytic enzymes and as a consequence we observed higher conversion yields for LHW samples in comparison with the ORG samples which has smaller Xyl/Ara ratio (**Fig 1**).

The work support results from the critical role that hemicellulose plays in grasses already discussed in other studies (Guo et al., 2017; Yu et al., 2016). Although AXs or lignin removal enhances the saccharification rate, the AXs removal directly impacts glucan chain accessibility in grasses. Hence, removing xylan is more advantageous than lignin removal aside from reduced accessory enzymes (**Fig. 4**) (Sindhu et al., 2016).

3.3.5 Panicum maximum hydrolysis at a higher solid loading

In order to achieve commercial ethanol yields, a high initial sugar concentration is required. Therefore, increasing sugar concentration during hydrolysis and improving the final ethanol content after fermentation makes it possible to achieve economic savings in the bioconversion process, such as reducing hydrolysis and fermentation processes and minimizing energy consumption in downstream processes (Hu et al., 2015a). However, hydrolysis of lignocellulosic materials at high loadings is challenging to achieve due to problems such as rheological ones that could lead to inefficient mass transfer, the inherent substrate recalcitrance, and the large quantity of levels enzymes inhibition products like sugars and degradation products (Zhang et al., 2009). Therefore, higher substrate solid consistency of 10% was selected as an attempt to accomplish high glucose concentrations without greatly impacting cellulose hydrolysis yields for both pretreatments (LHW and ORG) (**Fig. 7**).

Once substrate concentration was raised to 10% substrate consistency, a lower cellulose-to-glucose conversion was achieved. Therefore, the target of 70% hydrolysis could not be reached contrary to 2% solid loading, in which case it was possible to achieve up to 100% conversion yield (**Fig. 3**). Approximately 50% and 60% hydrolysis yields of cellulose were observed after 72 hours, at 10% solid load from ORG and LHW pretreated substrates, respectively (**Fig. 7A-B**). This is probably due to the fiber–fiber interaction that occurs when the substrate consistency increases to above 8% and end-product inhibition derived from the glucose and cellobiose produced during the hydrolysis (Xiao et al., 2004; Zhang et al., 2009).

Interestingly, it was apparent that in ORG pretreated substrates, there was a significant difference in bioconversion yields between 24 and 72 hours of hydrolysis (**Fig. 7A**). However, LHW substrates did not show a substantial difference during hydrolysis time (**Fig. 7B**). This might be explained due to the inhibitor effects from the sugars released during hydrolysis as cellobiose and glucose. More than 50% of the cellulose component from LHW-pretreated substrate was hydrolyzed in the first 24 hours, generating high cellobiose and glucose concentrations. A synergistic phenomenon is widely observed in cellulose hydrolysis between endo-exoglucanases, cellobiase, and β-glucosidase. The increased glucose content in the hydrolysate could affect this synergism through end-product inhibition effects and impact hydrolysis efficiency (Xiao et al., 2004).

The same reason could be applied to explain the differences in cellulose hydrolysis between the climate conditions at higher solid loading (10%). The LHW samples had no significant difference in bioconversion rates for all climate conditions studied at 2% solid. However, at higher solid consistency, the C and eC groups have significantly better yields ($p < 0.05$) than eT and eT+eC at 24 and 72 hours **(Fig. 7B).** As previously discussed (**Table 1**), the eT and eT+eC group showed significant higher glucan content ($p < 0.05$). This difference did not impact the conversation rates at 2% (w/w). However, at 10%, the effect of greater glucan content in these groups is more prominent, resulting in more end-product inhibition since higher glucan content might lead to higher glucose released in the reaction media.

3.4 Evaluating the accessibility of the lignocellulosic components using FTCM-depletion assay

Enzymatic hydrolysis of any given biomass depends on the enzyme's accessibility to the lignocellulosic constituents (Khatri et al., 2018a). Carbohydrate-binding modules (CBMs), which consist is an integral part of the catalytic domain (CD) of glycoside hydrolases (GHs) but without having catalytic activity, have high specificity and affinity toward lignocellulosic polymers (Boraston et al., 2004). Hence, the use of CBMs represents a critical tool to investigate the chemistry and structure effects caused by the pretreatment(s) on LCB hydrolytic performance and its accessibility to cellulase enzymes (Knox, 2012; Oliveira et al., 2015).

Therefore, to investigate and elaborate the cause of the changes in the enzymatic hydrolysis data (above discussed), we used the fluorescent protein-tagged carbohydrate-binding modules (FP-CBM) approach to assess the accessibility profile of lignocellulosic components. Specifically, the *P. maximum* fibers were characterized via the FTCM-depletion assay using four highly specific FP-CBM molecular probes (i.e., GC3a, CC17, OC15, and CC27). The FTCM-depletion assay of *P. maximum* fibers fits a single-site binding model (**Figs. S4 and S5**) like described elsewhere (Mboowa et al., 2020). The parameters resulted from these fits (N_o (µmoles g^{-1}), K_a (μM^{-1}), ΔG (KJ mol^{-1}) are summarized in **Tables 2 and 3**.

In case of ORG pretreatment, the binding capacity or the total concentration of the available binding sites (No) of GC3a (crystalline cellulose accessibility recognition probe) were 11.2 ± 0.4, 12.7 ± 0.4, 14.4 ± 0.5, and 10.2 ± 0.4 (μmoles g^{-1}) for C, eT, eC and eT + eC, respectively (**Table 2**). It was apparent that eC biomass exhibited the highest crystalline cellulose accessibility, followed by eT. Similar trends were observed with the binding of the

CC17 probe (amorphous cellulose accessibility recognition probe), where eC showed the highest amorphous cellulose accessibility. However, the amorphous cellulose accessibility was at least 63% lower than crystalline cellulose accessibility. The binding of the xylan accessibility recognition probe (OC15) demonstrated that eC biomass had significantly higher xylan accessibility when compared to other climate conditions where no significant difference ($p >$ 0.05) in the surface exposure/accessibility of xylan components were found. Differently, we found no binding of CC27 (mannan recognition probe) probes (**Table 2**), in agreement with the chemical composition analysis **(Table 1),** which exhibited the absence of mannan in *P. maximum* fibers. Moreover, the total cellulose accessibility was obtained by adding crystalline cellulose (i.e., No of GC3a) and amorphous cellulose accessibility (i.e., No of CC17), while total hemicellulose accessibility was derived from the sum of xylan (i.e., No of OC15) and mannan accessibility (i.e., No of CC27) (**Table 2**). Since the total cellulose accessibility was greatly higher than the total hemicellulose accessibility (at least ~76%), it is possible to propose that that cellulose exposure was abundantly higher than hemicellulose on ORG pretreated fibers surface. Also, eC biomass exhibited the highest total cellulose accessibility, followed by eT climate conditions. This explains the highest percent glucan conversion in eC biomass as described in sections 3.3.1, 3.3.2 & 3.3.3 and Figure 2A, 3A & 4A. There was no significant difference in the total cellulose accessibility of C and eT + eC climate conditions ($p < 0.05$), which also strongly correlates with the hydrolysis yields observed above. Likewise, significantly higher xylan accessibility in eC biomass could explain the improved hydrolysis of eC biomass in the presence of hemicellulolytic (Htec®) accessory enzyme as described in section 3.3.2 and Figure 3A.

In the case of LHW pretreatment, crystalline cellulose accessibility was significantly higher ($p < 0.05$) in the eC and eT climate conditions when compared to C and eT + eC conditions (**Table 3**). On the contrary, no significant differences were observed in the amorphous, xylan and total cellulose accessibility of all the studied climate conditions ($p > 0.05$). This suggests that, unlike ORG, the LHW pretreatment did not change the total cellulose and/or total hemicellulose accessibility among all the studied climate conditions. However, the crystalline cellulose accessibility in LHW pretreated biomass was almost similar to the ORG pretreatment. Contrarily, the amorphous cellulose accessibility was 38-335% higher for LHW pretreated biomass when compared to ORG biomass. Similarly, the total cellulose accessibility was also found to be higher (8-58%) in the LHW pretreated biomass, which complements the enhanced enzymatic hydrolysis of LHW compared to ORG substrates as discussed in section 3.3.1, 3.3.2, and 3.3.3 (**Figs. 2, 3, 4 and 5**). On the other hand, the xylan accessibility was observed to be 35-64% lower when compared to ORG pretreatment (**Tables 2 and 3**). This suggests that LHW pretreatment mainly removed the hemicellulose (i.e., xylan) exposure which also complements the significant reduction in the bulk hemicellulose (wt%) composition of LHW pretreated biomass (**Table 1**).

The results obtained from the FTCM-depletion assay strongly correlate with those of the hydrolysis experiments. For ORG samples, eC was the condition with higher total cellulose and hemicellulose exposure and also the one with the higher hydrolysis yields in all tested enzyme mixtures (**Figs 2-4**). Besides, eT had the second-best cellulose accessibility, which seems to make a difference at higher solid loading since eT showed greater cellulose conversion yields after eC (**Fig. 7A**). Furthermore, CBM results confirm that LHW led to a higher increase of biomass accessibility (**Table 3**) due to changes in the compositions and structure of *P. maximum* fibers, showing that lignin redistribution and hemicellulose removal had a more significant positive effect in increasing fiber accessibility to hydrolytic enzymes. Also, FTCM-depletion assay data shows that both ORG and LHW pretreatments considerably improved total cellulose accessibility compared to non-treated biomass (Freitas et al., 2020), with LHW exhibiting higher increment than ORG.

4. Conclusion

Pretreatment processes greatly enhanced biomass digestibility by mainly exposing cellulose or modifying the material porosity. This fact facilitated the access of the hydrolytic enzyme to its substrate. Here, we applied two pretreatment methods that increase cellulose accessibly by promoting hemicellulose removal and lignin redistribution (LHW) or by manly removing lignin if some hemicellulose solubilization (ORG). Hemicellulose removal and Xyl/Ara increment seem to overmatches delignification in *P.maximum* grasses, as the higher glucan conversion rates and cellulose surface exposure/accessibility (evaluated via FTCM-depletion assay) was observed in liquid hot water pretreatment samples, demonstrating that hemicellulose directly affects *P.maximum* digestibility by restricting the access of enzymes and by increase hemicellulose-lignin cross-links. This hypothesis is supported by the fact that the addition of xylanases to the enzyme mixture led to a significant rise in saccharification performance in both pretreated samples.

Furthermore, for ORG pretreated samples, the elevate [CO_2] (eC) exhibited higher total cellulose and hemicellulose exposure and greater cellulose hydrolysis yield. Also, our data suggest that elevated temperature has a dominant effect over elevated CO_2 even in pretreated substrates since the eT+eC treatment presented similar profiles as the eT group. Therefore, this study provides clear evidence that differences in the chemical composition and component's surface accessibility of untreated biomass, caused by future climatic conditions, can influence the pretreatment processes depending on the method applied. Lately, this work has gone beyond the concerns over the better pretreatment choice for *P. maximum* grass and helped to analyze the influence of hypothetical future climate conditions on fermentable sugar yields of pretreated substrates, contributing to well-informed decisions on pretreatment and energy crops alternatives for the future.

Declaration of competing interest

The authors have no conflict of interest.

Acknowledgments

The authors thank the postgraduate students and laboratory technicians who contributed to the work and the FPB/B group, Wood Science Department-UBC. The authors would also like to thank Novozymes (Davis, CA) for kindly provide the enzymes for this study.

Funding

The authors thank Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) (Grant 2008/57908-6, 2008/58075-8, 2014/50884, 2018/07522-6, Process 2017/23989-9 and 2019/07141-5), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Grants 446357/2015-4; 465319/2014-9, 301963/2017-7).

References

Agbor, V.B., Cicek, N., Sparling, R., Berlin, A., Levin, D.B., 2011. Biomass pretreatment: Fundamentals toward application. Biotechnol. Adv. 29, 675–685. https://doi.org/10.1016/j.biotechadv.2011.05.005

Alper, K., Tekin, K., Karagöz, S., Ragauskas, A.J., 2020. Sustainable energy and fuels from biomass: A review focusing on hydrothermal biomass processing. Sustain. Energy Fuels 4, 4390–4414. https://doi.org/10.1039/d0se00784f

Bhatia, S.K., Jagtap, S.S., Bedekar, A.A., Bhatia, R.K., Patel, A.K., Pant, D., Rajesh Banu, J., Rao, C. V., Kim, Y.G., Yang, Y.H., 2020. Recent developments in pretreatment technologies on lignocellulosic biomass: Effect of key parameters, technological improvements, and challenges. Bioresour. Technol. 300, 122724. https://doi.org/10.1016/j.biortech.2019.122724

Bombeck, P.L., Khatri, V., Meddeb-Mouelhi, F., Montplaisir, D., Richel, A., Beauregard, M., 2017. Predicting the most appropriate wood biomass for selected industrial applications: Comparison of wood, pulping, and enzymatic treatments using fluorescent-tagged carbohydrate-binding modules. Biotechnol. Biofuels 10, 1–14. https://doi.org/10.1186/s13068-017-0980-0

Boraston, A.B., Bolam, D.N., Gilbert, H.J., Davies, G.J., 2004. Carbohydrate-binding modules: Fine-tuning polysaccharide recognition. Biochem. J. 382, 769–781.

https://doi.org/10.1042/BJ20040892

Boussaid A, Robinson J, Cai Yj, Gregg DJ, S.J., 1999. Fermentability of the hemicellulose-derived sugars from steam-exploded softwood (douglas fir). Biotechnol Bioeng. 64, 284-289.

Buruiana, C.T., Vizireanu, C., Garrote, G., Parajó, J.C., 2014. Optimization of corn stover biorefinery for coproduction of oligomers and second generation bioethanol using non-isothermal autohydrolysis. Ind. Crops Prod. 54, 32–39. https://doi.org/10.1016/j.indcrop.2014.01.003

Cultures, P.T., n.d. Chapter 1 Plant Tissue Cultures. Methods 715, 1–20. https://doi.org/10.1007/978-1-61779-008-9

De Assis Prado, C.H.B., De Camargo-Bortolin, L.H.G., Castro, É., Martinez, C.A., 2016. Leaf Dynamics of Panicum maximum under Future Climatic Changes. PLoS One 11, 1–17. https://doi.org/10.1371/journal.pone.0149620

dos Santos Rocha, M.S.R., Pratto, B., de Sousa, R., Almeida, R.M.R.G., Cruz, A.J.G. da, 2017. A kinetic model for hydrothermal pretreatment of sugarcane straw. Bioresour. Technol. 228, 176–185. https://doi.org/10.1016/j.biortech.2016.12.087

Espírito Santo, M.C. do, Cardoso, E.B., Guimaraes, F.E.G., deAzevedo, E.R., Cunha, G.P. da, Novotny, E.H., Pellegrini, V. de O.A., Chandel, A.K., Silveira, M.H.L., Polikarpov, I., 2019. Multifaceted characterization of sugarcane bagasse under different steam explosion severity conditions leading to distinct enzymatic hydrolysis yields. Ind. Crops Prod. 139, 111542. https://doi.org/10.1016/j.indcrop.2019.111542

Espirito Santo, M., Rezende, C.A., Bernardinelli, O.D., Pereira, N., Curvelo, A.A.S., deAzevedo, E.R., Guimarães, F.E.G., Polikarpov, I., 2018. Structural and compositional changes in sugarcane bagasse subjected to hydrothermal and organosolv pretreatments and their impacts on enzymatic hydrolysis. Ind. Crops Prod. 113, 64–74. https://doi.org/10.1016/j.indcrop.2018.01.014

Ewanick, S., Bura, R., 2010. Hydrothermal pretreatment of lignocellulosic biomass. Bioalcohol Prod. Biochem. Convers. Lignocellul. Biomass 3–23. https://doi.org/10.1533/9781845699611.1.3

Ewanick, S.M., Bura, R., Saddler, J.N., 2007. Acid-catalyzed steam pretreatment of lodgepole pine and subsequent enzymatic hydrolysis and fermentation to ethanol. Biotechnol. Bioeng. 98, 737–746. https://doi.org/10.1002/bit.21436

Freitas, Emanuelle N., Khatri, Vinay, Contin, Daniele, Oliveira, Tassio, Contato, Alex, Peralta, Rosane, dos Santos, Wanderley, Martínez, Carlos, Saddler, Jack, Polizeli, M. de L.T.M., n.d. Climate Change Affects Cell Wall Structure and Hydrolytic Performance of a Tropical Forage Grass as an Energy Crop. Prepr. Authorea. https://doi.org/https://doi.org/10.22541/au.160629306.65292242/v1

Fujimoto, S., Inoue, S., Yoshida, M., 2018. High solid concentrations during the hydrothermal pretreatment of eucalyptus accelerate hemicellulose decomposition and subsequent enzymatic glucose production. Bioresour. Technol. Reports 4, 16–20. https://doi.org/10.1016/j.biteb.2018.09.006

Guo, H., Wu, Y., Hong, C., Chen, H., Chen, X., Zheng, B., Jiang, D., Qin, W., 2017. Enhancing digestibility of Miscanthus using lignocellulolytic enzyme produced by Bacillus. Bioresour. Technol. 245, 1008–1015. https://doi.org/10.1016/j.biortech.2017.09.034

Habermann, E., San Martin, J.A.B., Contin, D.R., Bossan, V.P., Barboza, A., Braga, M.R., Groppo, M., Martinez, C.A., 2019. Increasing atmospheric CO 2 and canopy temperature induces anatomical and physiological changes in leaves of the C 4 forage species Panicum maximum. PLoS One 14, 1–25. https://doi.org/10.1371/journal.pone.0212506

Hébert-Ouellet, Y., Meddeb-Mouelhi, F., Khatri, V., Cui, L., Janse, B., Macdonald, K., Beauregard, M., 2017. Tracking and predicting wood fibers processing with fluorescent carbohydrate binding modules. Green Chem. 19, 2603–2611. https://doi.org/10.1039/c6gc03581g

Hendriks, A.T.W.M., Zeeman, G., 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour. Technol. 100, 10–18. https://doi.org/10.1016/j.biortech.2008.05.027

Hongdan, Z., Shaohua, X., Shubin, W., 2013. Enhancement of enzymatic saccharification of sugarcane bagasse by liquid hot water pretreatment. Bioresour. Technol. 143, 391–396. https://doi.org/10.1016/j.biortech.2013.05.103

Houfani, A.A., Anders, N., Spiess, A.C., Baldrian, P., Benallaoua, S., 2020. Insights from enzymatic degradation of cellulose and hemicellulose to fermentable sugars– a review. Biomass and Bioenergy 134. https://doi.org/10.1016/j.biombioe.2020.105481

Hu, J., Arantes, V., Pribowo, A., Gourlay, K., Saddler, J.N., 2014. Substrate factors that influence the synergistic interaction of AA9 and cellulases during the enzymatic hydrolysis of biomass. Energy Environ. Sci. 7, 2308–2315. https://doi.org/10.1039/c4ee00891j

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

Hu, J., Gourlay, K., Arantes, V., Van Dyk, J.S., Pribowo, A., Saddler, J.N., 2015b. The accessible cellulose surface influences cellulase synergism during the hydrolysis of lignocellulosic substrates. ChemSusChem 8, 901–907. https://doi.org/10.1002/cssc.201403335

Karlen, S.D., Zhang, C., Peck, M.L., Smith, R.A., Padmakshan, D., Helmich, K.E., Free, H.C.A., Lee, S., Smith, B.G., Lu, F., Sedbrook, J.C., Sibout, R., Grabber, J.H., Runge, T.M., Mysore, K.S., Harris, P.J., Bartley, L.E., Ralph, J., 2016. Monolignol ferulate conjugates are naturally incorporated into plant lignins. Sci. Adv. 2, 1–10. https://doi.org/10.1126/sciadv.1600393

Khatri, V., Meddeb-Mouelhi, F., Adjallé, K., Barnabé, S., Beauregard, M., 2018a. Determination of optimal biomass pretreatment strategies for biofuel production: Investigation of relationships between surface-exposed polysaccharides and their enzymatic conversion using carbohydrate-binding modules. Biotechnol. Biofuels 11, 1–16. https://doi.org/10.1186/s13068-018-1145-5

Khatri, V., Meddeb-Mouelhi, F., Beauregard, M., 2018b. New insights into the enzymatic hydrolysis of lignocellulosic polymers by using fluorescent tagged carbohydrate-binding modules. Sustain. Energy Fuels 2, 479–491. https://doi.org/10.1039/c7se00427c

Knox, J.P., 2012. In situ detection of cellulose with carbohydrate-binding modules, 1st ed, Methods in Enzymology. Elsevier Inc. https://doi.org/10.1016/B978-0-12-415931-0.00012-4

Lai, C., Tu, M., Yong, Q., Yu, S., 2015. Disparate roles of solvent extractable lignin and residual bulk lignin in enzymatic hydrolysis of pretreated sweetgum. RSC Adv. 5, 97966–97974. https://doi.org/10.1039/c5ra22308c

Lai, C., Yang, B., Lin, Z., Jia, Y., Huang, C., Li, X., Song, X., Yong, Q., 2019. New strategy to elucidate the positive effects of extractable lignin on enzymatic hydrolysis by quartz crystal microbalance with dissipation. Biotechnol. Biofuels 12, 1–12. https://doi.org/10.1186/s13068-019-1402-2

Lima, M.A., Gomez, L.D., Steele-King, C.G., Simister, R., Bernardinelli, O.D., Carvalho, M.A., Rezende, C.A., Labate, C.A., Deazevedo, E.R., McQueen-Mason, S.J., Polikarpov, I., 2014. Evaluating the composition and processing potential of novel sources of Brazilian biomass for sustainable biorenewables production. Biotechnol. Biofuels 7, 1–19. https://doi.org/10.1186/1754-6834-7-10

Malgas, S., Chandra, R., Van Dyk, J.S., Saddler, J.N., Pletschke, B.I., 2017. Formulation of an optimized synergistic enzyme cocktail, HoloMix, for effective degradation of various pretreated hardwoods. Bioresour. Technol. 245, 52–65. https://doi.org/10.1016/j.biortech.2017.08.186

Manzanares, Paloma., 2020. The role of biorefinering research in the development of a modern bioeconomy. Acta Innov. 37, 47–56. https://doi.org/doi.org/10.32933/actainnovations.37.4

Marchessault, R.H., 1994. Wood chemistry, fundamentals and applications, Carbohydrate Research. https://doi.org/10.1016/0008-6215(94)90030-2

Mboowa, D., Khatri, V., Saddler, J.N., 2020. The use of fluorescent protein-tagged carbohydrate-binding modules to evaluate the influence of drying on cellulose accessibility and enzymatic hydrolysis. RSC Adv. 10, 27152–27160. https://doi.org/10.1039/D0RA05333C

Moura, J.C.M.S., Bonine, C.A.V., de Oliveira Fernandes Viana, J., Dornelas, M.C., Mazzafera, P., 2010. Abiotic and Biotic Stresses and Changes in the Lignin Content and Composition in Plants. J. Integr. Plant Biol. 52, 360–376. https://doi.org/10.1111/j.1744-7909.2010.00892.x

Nakagame, S., Chandra, R.P., Saddler, J.N., 2010. The effect of isolated lignins, obtained from a range of pretreated lignocellulosic substrates, on enzymatic hydrolysis. Biotechnol. Bioeng. 105, 871–879. https://doi.org/10.1002/bit.22626

Nguyen, L.T., Phan, D.P., Sarwar, A., Tran, M.H., Lee, O.K., Lee, E.Y., 2021. Valorization of industrial lignin to value-added chemicals by chemical depolymerization and biological conversion. Ind. Crops Prod. 161, 113219. https://doi.org/10.1016/j.indcrop.2020.113219

Oinonen, P., Zhang, L., Lawoko, M., Henriksson, G., 2015. On the formation of lignin polysaccharide networks in Norway spruce. Phytochemistry 111, 177–184. https://doi.org/10.1016/j.phytochem.2014.10.027

Oliveira, C., Carvalho, V., Domingues, L., Gama, F.M., 2015. Recombinant CBM-fusion technology - Applications overview. Biotechnol. Adv. 33, 358–369. https://doi.org/10.1016/j.biotechadv.2015.02.006

Oliveira, D.M., Mota, T.R., Oliva, B., Segato, F., Marchiosi, R., Ferrarese-Filho, O., Faulds, C.B., dos Santos, W.D., 2019. Feruloyl esterases: Biocatalysts to overcome biomass recalcitrance and for the production of bioactive compounds. Bioresour. Technol. 278, 408–423. https://doi.org/10.1016/j.biortech.2019.01.064

Pan, X., Gilkes, N., Saddler, J.N., 2006. Effect of acetyl groups on enzymatic hydrolysis of cellulosic substrates. Holzforschung 60, 398–401. https://doi.org/10.1515/HF.2006.062

Park, Y.C., Kim, T.H., Kim, J.S., 2017. Effect of organosolv pretreatment on mechanically pretreated biomass by use of concentrated ethanol as the solvent. Biotechnol. Bioprocess

Eng. 22, 431–439. https://doi.org/10.1007/s12257-017-0088-1

Pratto, B., de Souza, R.B.A., Sousa, R., da Cruz, A.J.G., 2016. Enzymatic Hydrolysis of Pretreated Sugarcane Straw: Kinetic Study and Semi-Mechanistic Modeling. Appl. Biochem. Biotechnol. 178, 1430–1444. https://doi.org/10.1007/s12010-015-1957-8

Ruiz, H.A., Conrad, M., Sun, S.N., Sanchez, A., Rocha, G.J.M., Romaní, A., Castro, E., Torres, A., Rodríguez-Jasso, R.M., Andrade, L.P., Smirnova, I., Sun, R.C., Meyer, A.S., 2020. Engineering aspects of hydrothermal pretreatment: From batch to continuous operation, scale-up and pilot reactor under biorefinery concept. Bioresour. Technol. 299, 122685. https://doi.org/10.1016/j.biortech.2019.122685

Sage, R. F., Way, D. A., & Kubien, D.S., 2008. Rubisco, Rubisco activase, and global climate change. J. Exp. Bot. 59, 1581–1595.

Scopel, E., Rezende, C.A., 2021. Biorefinery on-demand: Modulating pretreatments to recover lignin, hemicellulose, and extractives as co-products during ethanol production. Ind. Crops Prod. 163. https://doi.org/10.1016/j.indcrop.2021.113336

Silveira, M.H.L., Morais, A.R.C., Da Costa Lopes, A.M., Olekszyszen, D.N., Bogel-Łukasik, R., Andreaus, J., Pereira Ramos, L., 2015. Current Pretreatment Technologies for the Development of Cellulosic Ethanol and Biorefineries. ChemSusChem 8, 3366–3390. https://doi.org/10.1002/cssc.201500282

Sindhu, R., Binod, P., Pandey, A., 2016. Biological pretreatment of lignocellulosic biomass - An overview. Bioresour. Technol. 199, 76–82. https://doi.org/10.1016/j.biortech.2015.08.030

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., & Templeton, D., 2008. Determination of ash in biomass. NREL TP-510-42622. Golden.

Sosa, L.L., Jozami, E., Oakley, L.J., Montero, G.A., Ferreras, L.A., Venturi, G., Feldman, S.R., 2019. Using C4 perennial rangeland grasses for bioenergy. Biomass and Bioenergy 128, 105299. https://doi.org/10.1016/j.biombioe.2019.105299

Starcher, B., 2001. 1. Starcher B. A ninhydrin-based assay to quantitate the total protein content of tissue samples. Anal Biochem. 2001;292:125–129. A ninhydrin-based assay to quantitate the total protein content of tissue samples. Anal. Biochem. 292, 125–129.

Tenhaken, R., 2015. Cell wall remodeling under abiotic stress. Front. Plant Sci. 5. https://doi.org/10.3389/fpls.2014.00771

van der Weijde, T., Alvim Kamei, C.L., Torres, A.F., Vermerris, W., Dolstra, O., Visser, R.G.F., Trindade, L.M., 2013. The potential of C4 grasses for cellulosic biofuel production. Front. Plant Sci. 4, 1–18. https://doi.org/10.3389/fpls.2013.00107

Vogel, J., 2008. Unique aspects of the grass cell wall. Curr. Opin. Plant Biol. 11, 301–307.

https://doi.org/10.1016/j.pbi.2008.03.002

Wang, M., Han, J., Dunn, J.B., Cai, H., Elgowainy, A., 2015. Well-to-wheels energy use and greenhouse gas emissions of ethanol from corn, sugarcane and cellulosic biomass for US use. Effic. Sustain. Biofuel Prod. Environ. Land-Use Res. 249–280. https://doi.org/10.1088/1748-9326/7/4/045905

Wang, T., McFarlane, H.E., Persson, S., 2016. The impact of abiotic factors on cellulose synthesis. J. Exp. Bot. 67, 543–552. https://doi.org/10.1093/jxb/erv488

Wei, H., Gou, J., Yordanov, Y., Zhang, H., Thakur, R., Jones, W., Burton, A., 2013. Global transcriptomic profiling of aspen trees under elevated [CO2] to identify potential molecular mechanisms responsible for enhanced radial growth. J. Plant Res. 126, 305–320. https://doi.org/10.1007/s10265-012-0524-4

Wei Kit Chin, D., Lim, S., Pang, Y.L., Lam, M.K., 2020. Fundamental review of organosolv pretreatment and its challenges in emerging consolidated bioprocessing. Biofuels, Bioprod. Biorefining 14, 808–829. https://doi.org/10.1002/bbb.2096

Wu, J., Chandra, R., Saddler, J., 2019. Alkali-oxygen treatment prior to the mechanical pulping of hardwood enhances enzymatic hydrolysis and carbohydrate recovery through selective lignin modification. Sustain. Energy Fuels 3, 227–236. https://doi.org/10.1039/c8se00452h

Xiao, Z., Zhang, X., Gregg, D.J., Saddler, J.N., 2004. Effects of Sugar Inhibition on Cellulases and β-Glucosidase During Enzymatic Hydrolysis of Softwood Substrates. Appl. Biochem. Biotechnol. 115, 1115–1126. https://doi.org/10.1385/ABAB:115:1-3:1115

Yang, Y., Reilly, E.C., Jungers, J.M., Chen, J., Smith, T.M., 2019. Climate Benefits of Increasing Plant Diversity in Perennial Bioenergy Crops. One Earth 1, 434–445. https://doi.org/10.1016/j.oneear.2019.11.011

Ye, K., Tang, Y., Fu, D., Chen, T., Li, M., 2021. Effect of magnesium oxide pretreatment on the delignification and enzymatic hydrolysis of corncob. Ind. Crops Prod. 161, 113170. https://doi.org/10.1016/j.indcrop.2020.113170

Yu, Q., Liu, J., Zhuang, X., Yuan, Z., Wang, W., Qi, W., Wang, Q., Tan, X., Kong, X., 2016. Liquid hot water pretreatment of energy grasses and its influence of physico-chemical changes on enzymatic digestibility. Bioresour. Technol. 199, 265–270. https://doi.org/10.1016/j.biortech.2015.07.086

Zhang, X., Qin, W., Paice, M.G., Saddler, J.N., 2009. High consistency enzymatic hydrolysis of hardwood substrates. Bioresour. Technol. 100, 5890–5897. https://doi.org/10.1016/j.biortech.2009.06.082

Zhao, X., Cheng, K., Liu, D., 2009. Organosolv pretreatment of lignocellulosic biomass for enzymatic hydrolysis. Appl. Microbiol. Biotechnol. 82, 815–827. https://doi.org/10.1007/s00253-009-1883-1

Zhu, J., Lee, B.-H., Dellinger, M., Cui, X., Zhang, C., Wu, S., Nothnagel, E.A., Zhu, J.-K., 2010. A cellulose synthase-like protein is required for osmotic stress tolerance in Arabidopsis. Plant J. no-no. https://doi.org/10.1111/j.1365-313X.2010.04227.x

Figures

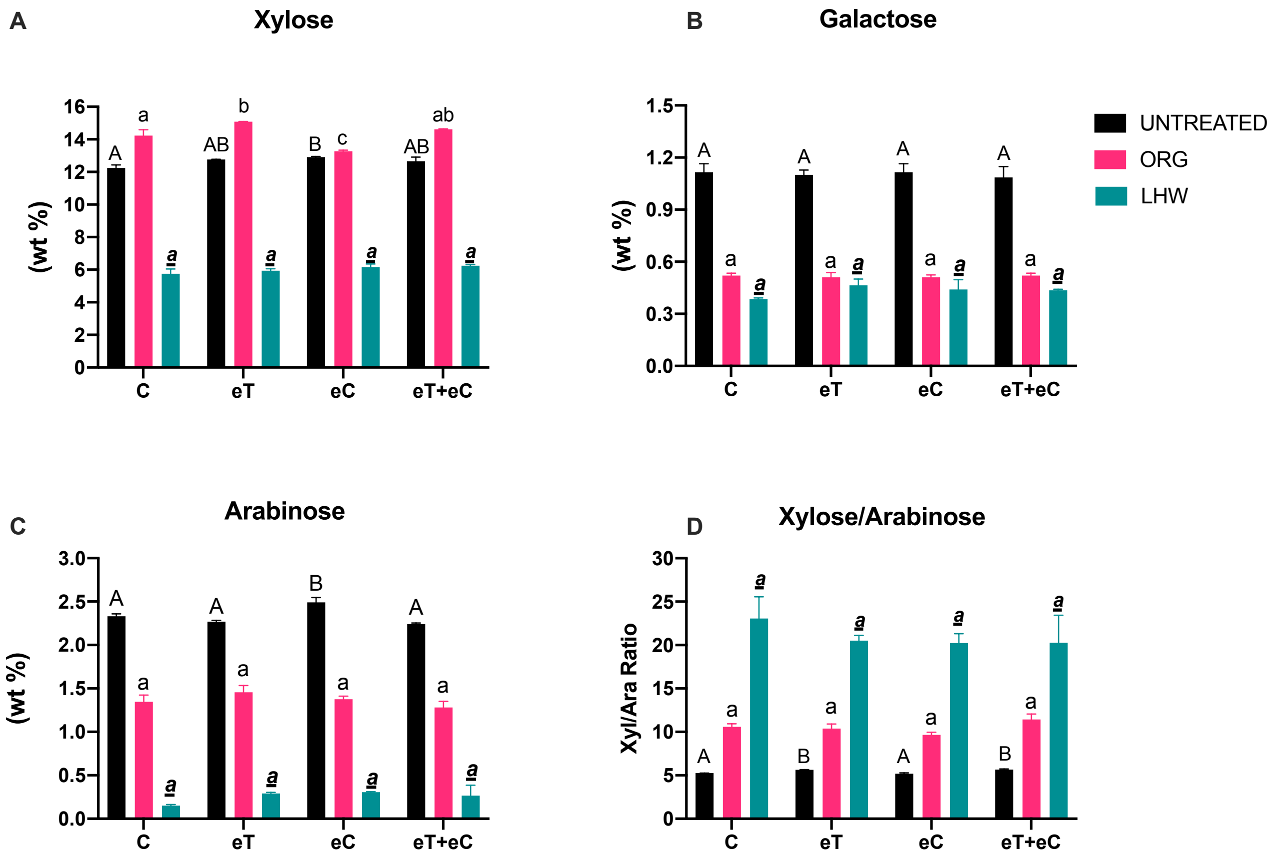
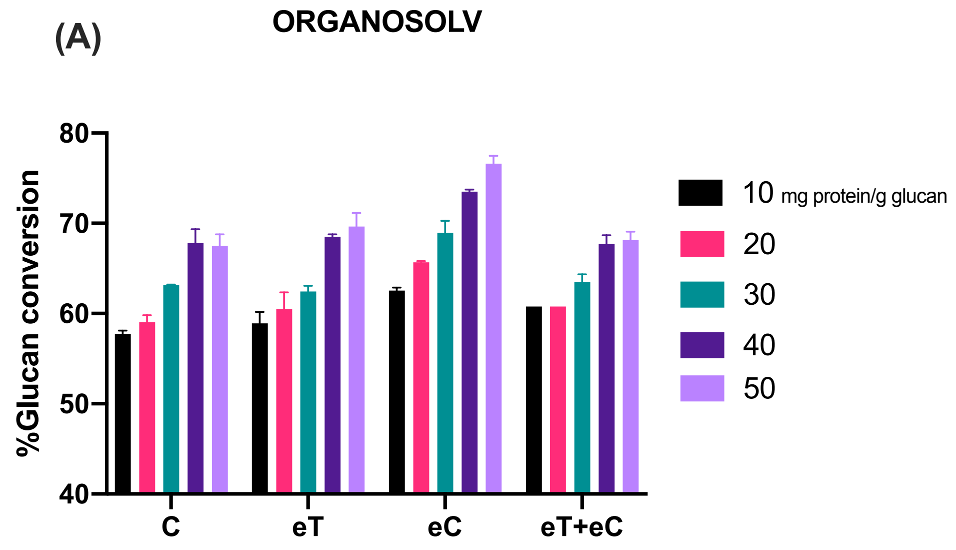


Figure 1. Monosaccharide profiles of non-treated and pretreated *P. maximum* in different climate conditions: Control (C), Elevate Temperature (eT), Elevate CO_2 (eC) and combined treatments (eT+eC). Pretreatments: liquid hot water (LHW) and organosolv (ORG). Mean values ± SD (n=3). Uppercase letters indicate significant differences between untreated samples; lowercase letters indicate significant differences between ORG pretreated and bold and underline lowercase letters indicate significant differences between LHW pretreated samples ($p < 0.05$, Tukey's test). Significant differences between pretreatments are discussed in the text in the proper place.



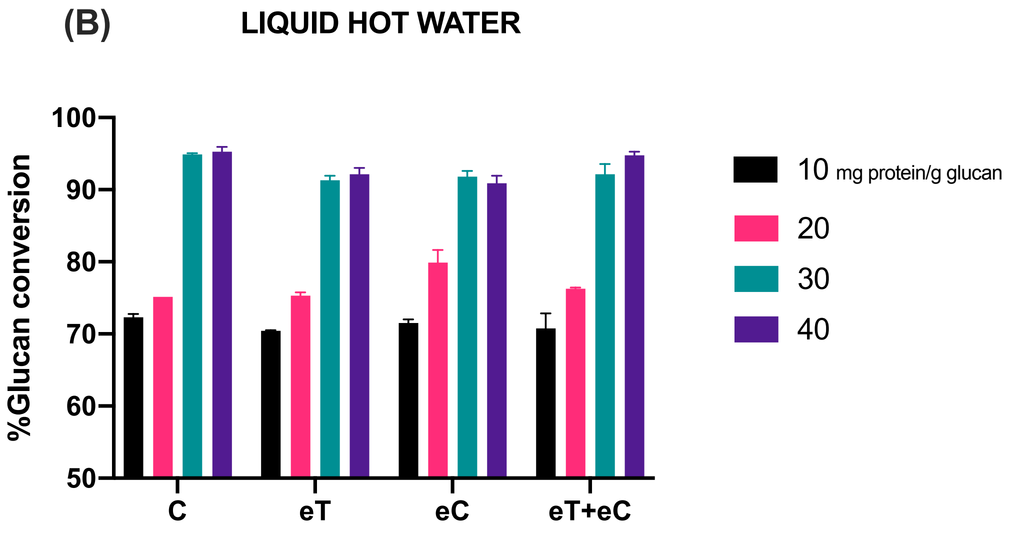


Figure 2. Glucan bioconversion using Celluclast® of *P. maximum* pretreated by (A) organosolv (ORG) and (B) liquid hot water (LHW) in different enzyme loadings (10,20,30,40 and 50 mg protein/g glucan). Climate Conditions: Control (C), elevate temperature (eT), elevate CO_2 (eC) and combined treatments (eT+eC). Hydrolysis conditions: 2% solid (w/v) and 72 hours. Mean values ± SD (n=3). The p-value was calculated using Tukey's test and is discussed in the text at the proper place (p-value, significant < 0.05).

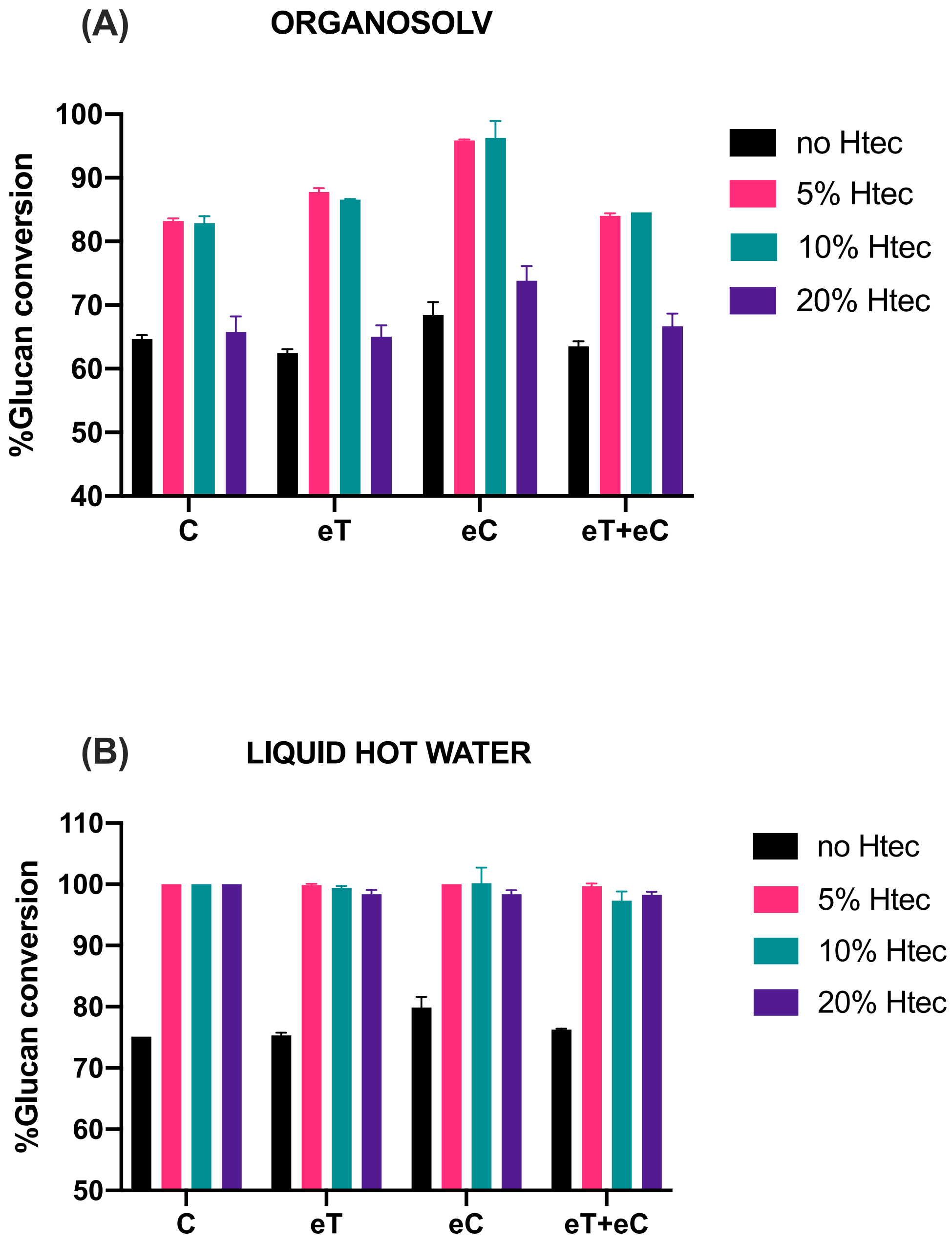
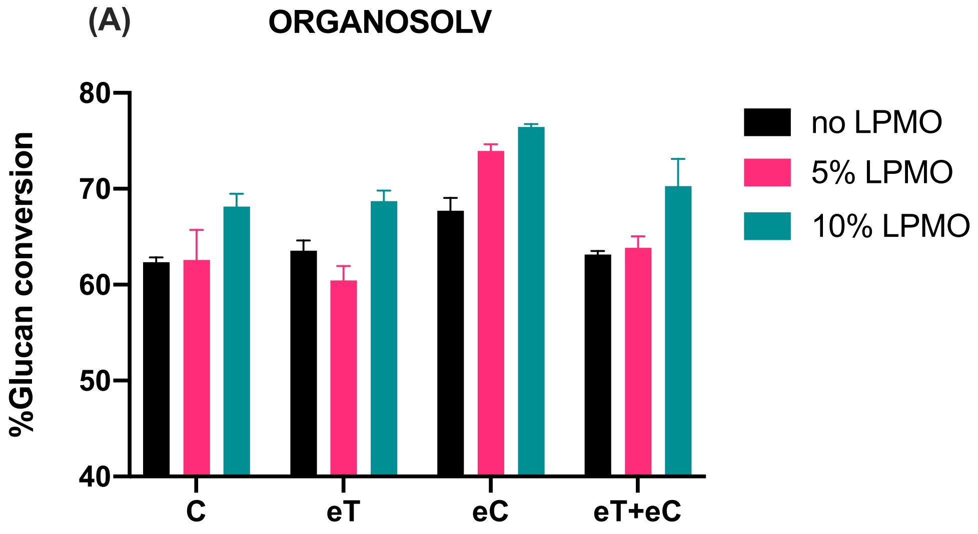


Figure 3. Glucan bioconversion using cellulase replacement by an equivalent amount of xylanase (Htec®) at 5, 10, and 20%. (A) *P. maximum* pretreated by organosolv (ORG) and (B) liquid hot water (LHW). Climate Conditions: Control (C), elevate temperature (eT), elevate CO_2 (eC) and combined treatments (eT+eC). Hydrolysis conditions: 2% solid (w/v) and 72hours. Mean values ± SD (n=3). The p-value was calculated using Tukey's test and is discussed in the text at the proper place (p-value, significant <0.05).



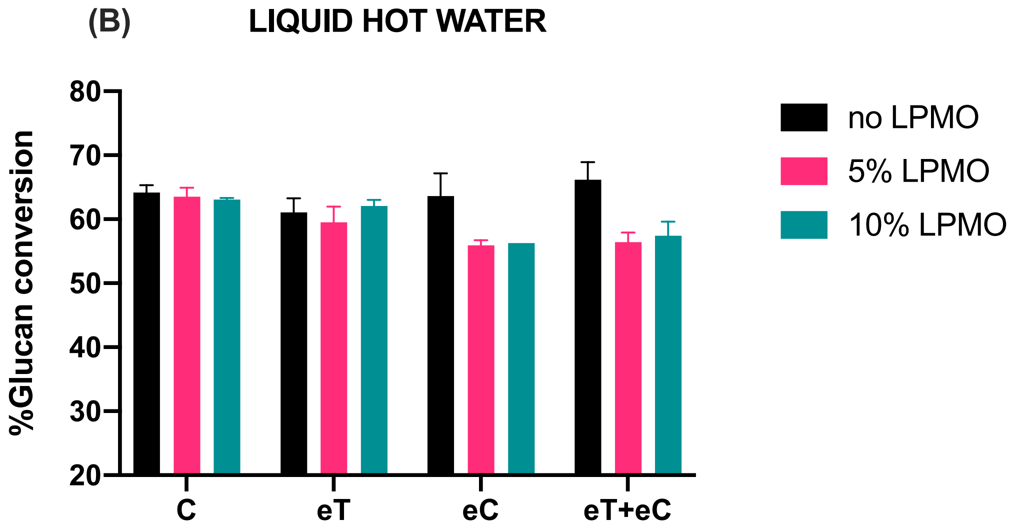


Figure 4. Glucan bioconversion of *P. maximum* pretreated by (A) organosolv and (B) liquid hot water (LHW) using cellulase replacement by an equivalent amount of the Lytic Polysaccharide Monooxygenase (LPMO) (5 and 10%). Climate Conditions: Control (C), Elevate Temperature (eT), Elevate CO_2 (eC) and combined treatments (eT+eC). Hydrolysis conditions: 20mg/g cellulose 5% Htec (Organosolv) and 10 mg/g cellulose 5% Htec (LHW), for both 2% solid (w/v) and 72hours. Mean values ± SD (n=3). The p-value was calculated using Tukey's test and is discussed in the text at the proper place (p-value, significant <0.05).

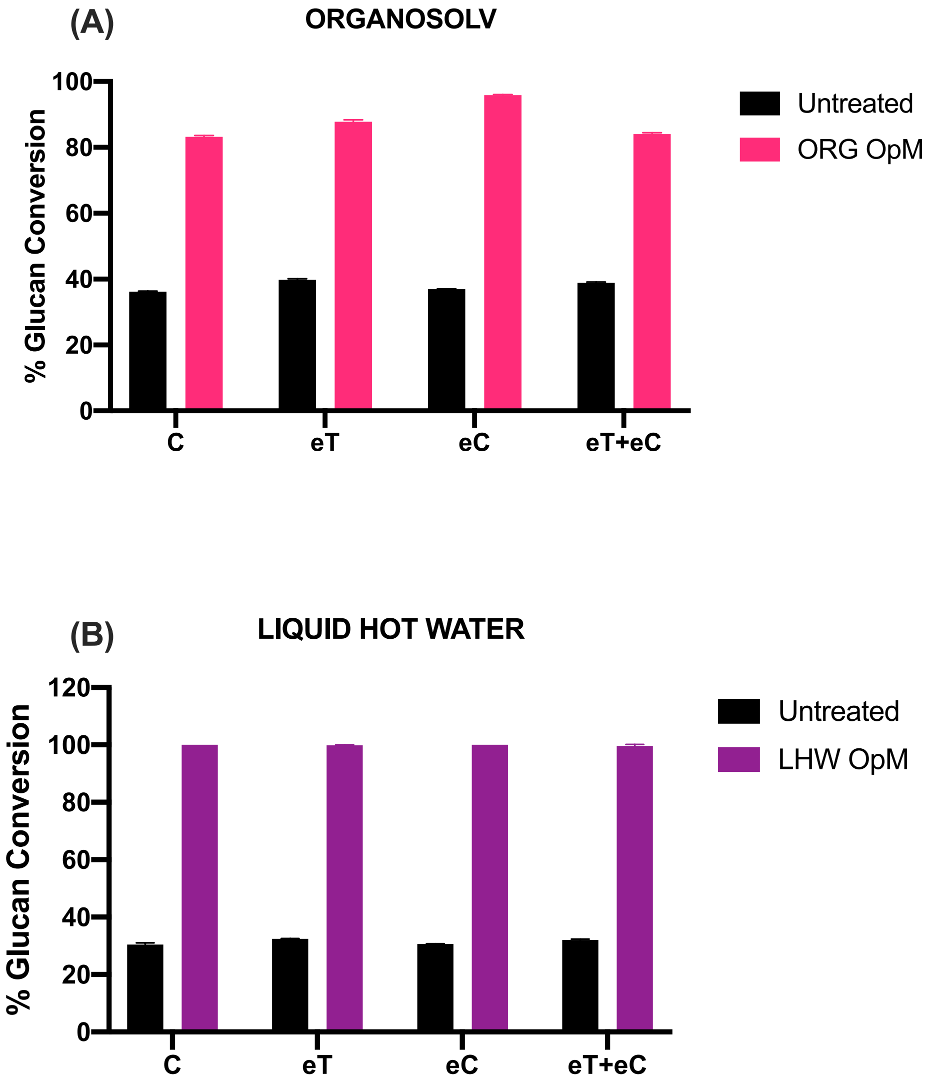


Figure 5. Glucan bioconversion using optimized enzyme mixture (OpM) for non-treated and pretreated *P. maximum* organosolv (A) and liquid hot water (B). Climate Conditions: Control (C), Elevate Temperature (eT), Elevate CO_2 (eC) and combined treatments (eT+eC). Hydrolysis conditions at 2% solid (w/v) and 72hours. Mean values ± SD (n=3). The p-value was calculated using Tukey's test and is discussed in the text at the proper place (p-value, significant <0.05).

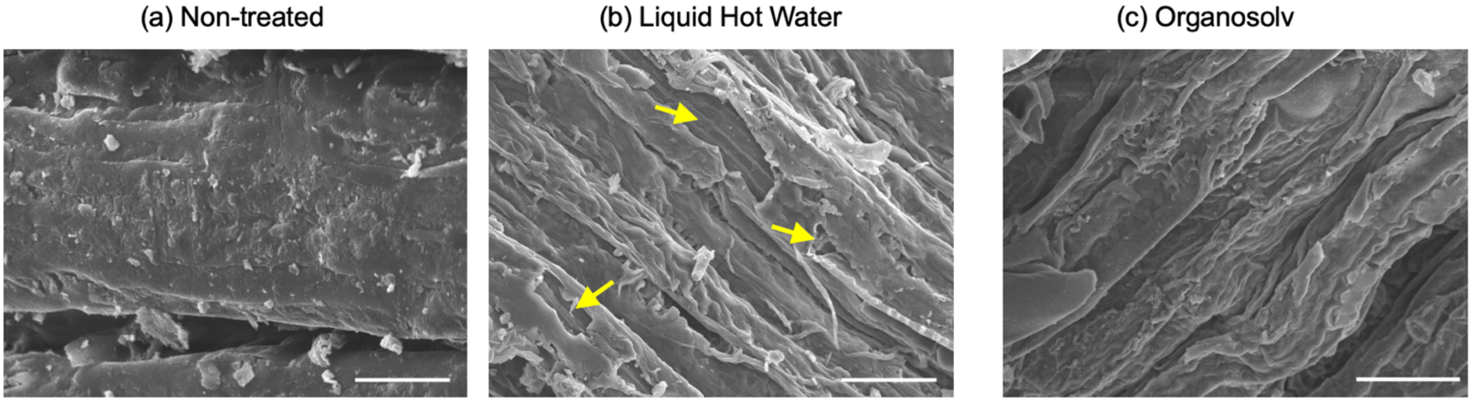


Figure 6. Field Emission Scanning Electron Microscopy (FESEM) images. (a) A general view of the non-treated, (b) organosolv pretreated, (c) liquid hot water pretreated *P. maximum*. The micrographs are showing a more advanced stage of lignin redeposition (yellow arrows). Scale bars indicate 20 μm.

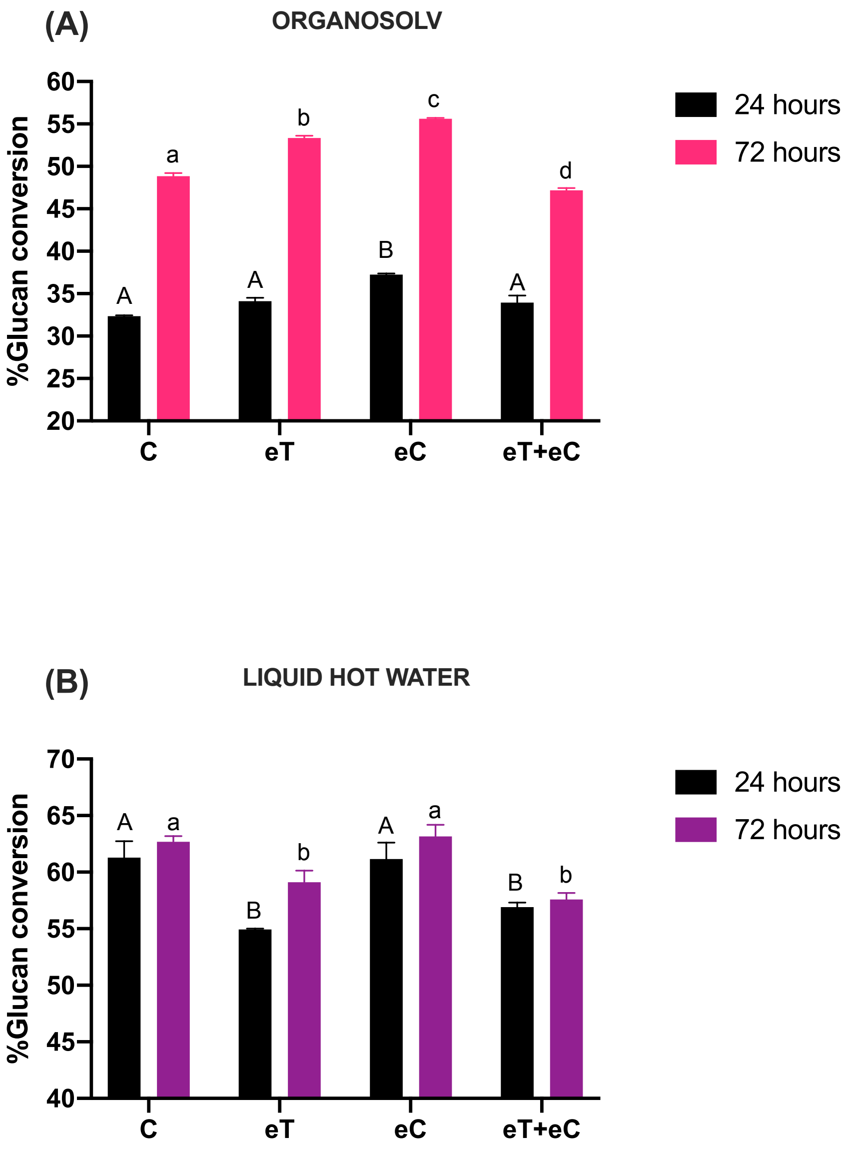


Figure 7. Glucan bioconversion using optimized enzyme mixture of *P. maximum* pretreated by (A) organosolv and (B) Liquid Hot Water in higher solid loading (10%). Climate Conditions: Control (C), Elevate Temperature (eT), Elevate CO_2 (eC) and combined treatments (eT+eC). Mean values ± SD (n=3). Uppercase letters indicate significant differences between 24 hours hydrolysis time; lowercase letters indicate significant differences between 72 hours hydrolysis time ($p < 0.05$, Tukey's test).

Table 1. Percentage (wt%) of chemical composition of non-treated and pretreated *P. maximum* samples in different clime conditions. Mean values ± SD (n=3) marked with different letters in the same pretreatment column are significantly different ($p < 0.05$, Tukey's test).

Pretreatment	Substrates	Sugar and lignin composition of water insoluble component of pretreated substrates				Glucan recovery (%)	Hemicellulose removal (%)	Lignin removal (%)
		Glucan (wt%)	Hemicellulose (wt%)	Lignin (wt%)	Ash (wt%)			
Liquid Hot Water	**C**	35.3 ± 1.5a	6.3 ± 0.5a	38.8 ± 0.1a	8.4 ± 0.3a	85.2	76.2	11.2
	eT	38.5 ± 0.6b	6.7 ± 0.2ab	36.2 ± 0.3b	8.5 ± 0.5a	85.5	73.3	15.8
	eC	36.3 ± 0.2a	6.9 ± 0.2ab	35.2 ± 0.2bc	8.6 ± 0.7a	89.1	74.2	12.8
	eT+eC	37.2 ± 0.2c	7.1 ± 0.1b	34.8 ± 0.4c	8.9 ± 0.5a	86.5	70.5	18.2
Organosolv	**C**	43.8 ± 1.7a	16.2 ± 0.4a	23.7 ± 0.1a	7.3 ± 0.4a	94.1	45.6	51.7
	eT	43.7 ± 0.5a	17.0 ± 0.1a	23.1 ± 0.1a	7.7 ± 0.7a	90.2	36.9	50.0
	eC	40.6 ± 0.3b	15.1 ± 0.0b	24.2 ± 0.3a	7.9 ± 0.3a	92.4	47.6	44.5
	eT+eC	42.7 ± 0.1a	16.43 ± 0.1a	23.8 ± 0.5a	7.7 ± 0.6a	89.0	38.8	49.8
Non-treated*	**C**	25.8 ± 0.5a	16.5 ± 0.2a	27.21 ± 1.0a	11.2 ± 0.2a	N/A	N/A	N/A
	eT	30 ± 0.03b	16.8 ± 0.03a	28.81 ± 0.8a	11.5 ± 0.4a	N/A	N/A	N/A
	eC	27.0 ± 0.7a	17.7 ± 0.05b	26.76 ± 0.6a	11.7 ± 0.3a	N/A	N/A	N/A
	eT+eC	29.7 ± 0.4b	16.6 ± 0.2a	29.38 ± 0.6a	11.3 ± 0.5a	N/A	N/A	N/A

Total Hemicellulose content: Arabinose+Galactose+Xylose and Total Lignin content :Acid Soluble lignin+Acid Insoluble Lignin

C, Control; eT, elevate Temperature; eC, elevate CO_2; eT+eC, elevate Temperature+ elevate CO_2

N/A: Not Applied

*Chemical composition of non-treated biomass was previously reported by (Freitas et al. 2020)[28]

Table 2. Binding capacity, affinity and change in Gibb's free energy (ΔG) of the GC3a, CC17, OC15 and CC27 probes as determined by FTCM-depletion assay against organosolv (ORG) pretreated substrates at 25°C in a 20 mM Tris–HCl (pH 7.5) buffer containing 20 mM NaCl, 5 mM $CaCl_2$.

	C				**eT**				**eC**				**eT + eC**			
	GC3a	**CC17**	**OC15**	**CC27**	**GC3a**	**CC17**	**OC15**	**CC27**	**GC3a**	**CC17**	**OC15**	**CC27**	**GC3a**	**CC17**	**OC15**	**CC27**
N_o (*μ*moles g^{-1})	11.2 ± 0.4	2.0 ± 0.2	2.3 ± 0.3	-	12.7 ± 0.4	4.1 ± 0.3	2.7 ± 0.2	-	14.4 ± 0.5	5.3 ± 0.4	4.7 ± 0.6	-	10.2 ± 0.4	2.8 ± 0.2	2.6 ± 0.3	-
K_a (*μ*M^{-1})	0.13 ± 0.02	0.14 ± 0.01	0.11 ± 0.03	-	0.18 ± 0.03	0.16 ± 0.01	0.11 ± 0.01	-	0.16 ± 0.04	0.07 ± 0.02	0.10 ± 0.01	-	0.13 ± 0.02	0.11 ± 0.03	0.09 ± 0.01	-
ΔG (KJ mol^{-1})	-29.2 ± 0.4	-29.4 ± 0.2	-28.7 ± 0.7	-	-30.0 ± 0.4	-29.7 ± 0.2	-28.8 ± 0.2	-	-29.7 ± 0.6	-27.6 ± 0.7	-28.5 ± 0.2	-	-29.2 ± 0.4	-28.7 ± 0.7	-28.3 ± 0.3	-
Total cellulose and xylan accessibility* (*μ*moles g^{-1})	13.2 ± 0.6		2.3 ± 0.3		16.8 ± 0.7		2.7 ± 0.2		19.7 ± 0.9		4.7 ± 0.6		13.0 ± 0.6		2.6 ± 0.3	

*Total cellulose accessibility: N_O GC3a+ N_OCC17 and Total xylan accessibility N_OOC15+ N_OCC27

Table 3. Binding capacity, affinity and change in Gibb's free energy (ΔG) of the GC3a, CC17, OC15 and CC27 probes as determined by FTCM-depletion assay against liquid hot water (LHW) pretreated substrates at 25°C in a 20 mM Tris–HCl (pH 7.5) buffer containing 20 mM NaCl, 5 mM $CaCl_2$.

	C				eT				eC				eT + eC			
	GC3a	**CC17**	**OC15**	**CC27**	**GC3a**	**CC17**	**OC15**	**CC27**	**GC3a**	**CC17**	**OC15**	**CC27**	**GC3a**	**CC17**	**OC15**	**CC27**
N_o (μmoles g^{-1})	12.2 ± 0.2	8.7 ± 0.8	1.5 ± 0.1	-	13.8 ± 0.4	7.7 ± 0.6	1.4 ± 0.3	-	14.0 ± 0.2	7.3 ± 0.9	1.7 ± 0.4	-	12.4 ± 0.4	7.8 ± 0.5	1.5 ± 0.2	-
K_a (μM^{-1})	0.16 ± 0.03	0.07 ± 0.01	0.12 ± 0.01	-	0.13 ± 0.02	0.07 ± 0.01	0.10 ± 0.01	-	0.13 ± 0.03	0.06 ± 0.01	0.05 ± 0.01	-	0.19 ± 0.04	0.05 ± 0.01	0.14 ± 0.01	-
ΔG (KJ mol^{-1})	-29.7 ± 0.5	-27.6 ± 0.4	-28.8 ± 0.2	-	-29.2 ± 0.4	-27.6 ± 0.4	-28.5 ± 0.2	-	-29.1 ± 0.6	-27.3 ± 0.4	-26.8 ± 0.5	-	-30.1 ± 0.5	-26.8 ± 0.5	-29.4 ± 0.2	-
Total cellulose and xylan accessibility* (μmoles g^{-1})	20.9 ± 1.0		1.5 ± 0.1		21.5 ± 1.0		1.4 ± 0.3		21.3 ± 1.1		1.7 ± 0.4		20.2 ± 0.9		1.5 ± 0.2	

*Total cellulose accessibility: N_O GC3a+ N_OCC17 and Total xylan accessibility N_OOC15+ N_O CC27

SUPPLEMENTARY MATERIAL

Table S1. Effects of Liquid Hot Water and Organosolv on monosaccharides in relation to the non-treated *P. maximum* biomass.

Pretreatments	Substrates	Monosaccharides retained after pretreatment (%)		
		Xylose	**Arabinose**	**Galactose**
Liquid Hot Water	**C**	29.1	5.4	22.7
	eT	31.1	9.1	30.5
	eC	31.6	8.0	22.1
	eT+eC	34.3	9.4	25.1
Organosolv	**C**	71.9	35.2	28.3
	eT	79.1	45.7	30.5
	eC	67.8	37.1	27.6
	eT+eC	79.4	40.8	31.4

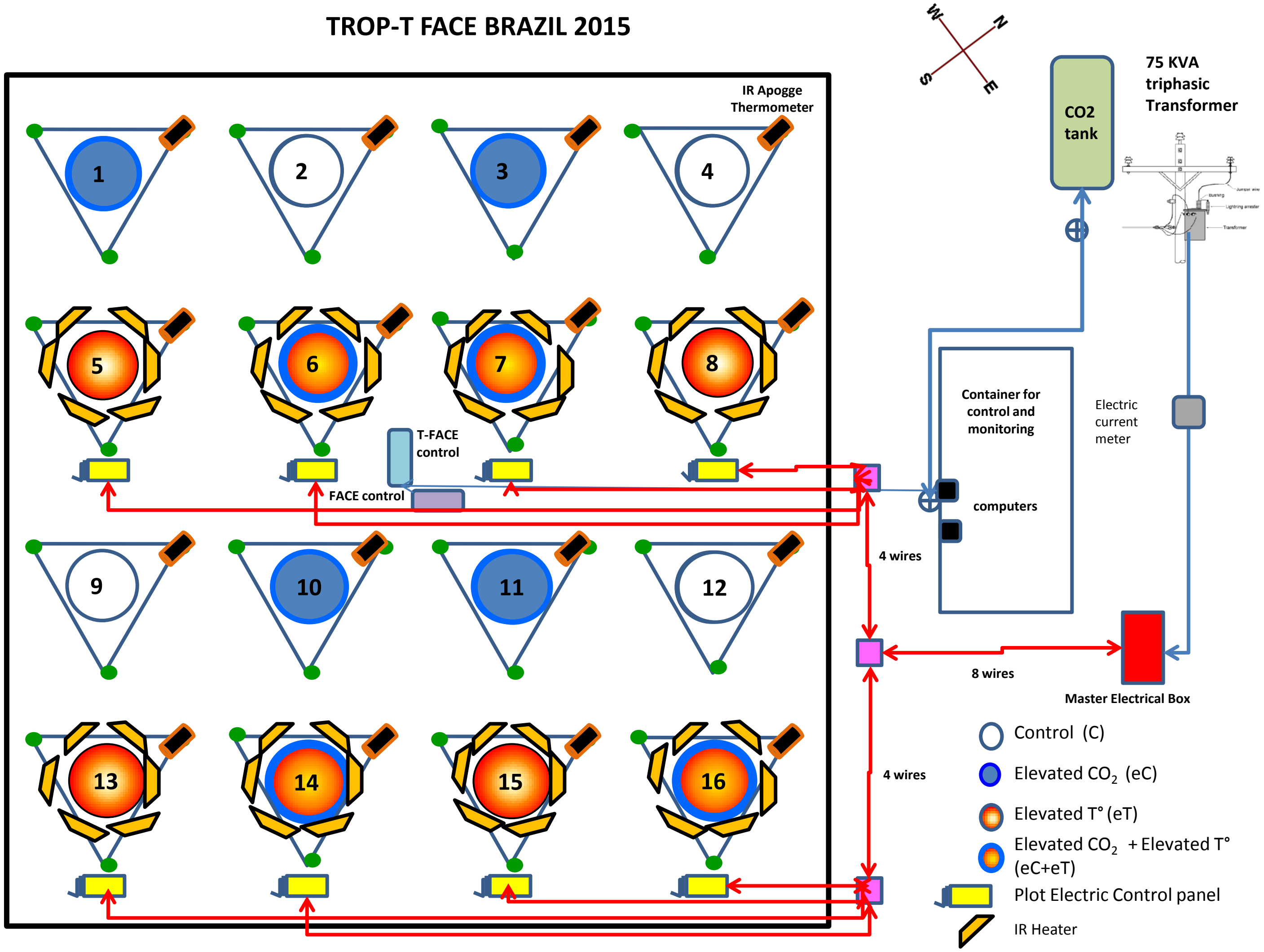
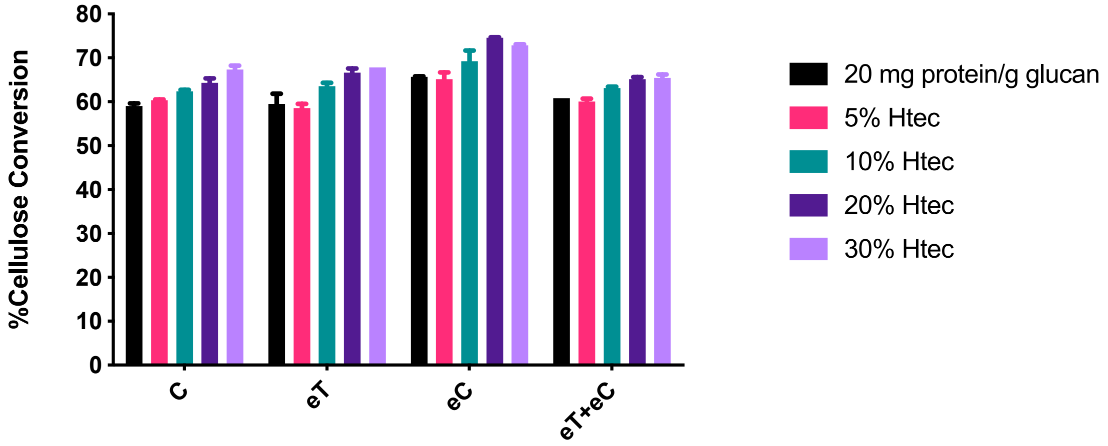


Figure S1: Schematic representation of TROP-T-FACE experiment.

(A)

ORGANOSOLV



(B) LIQUID HOT WATER

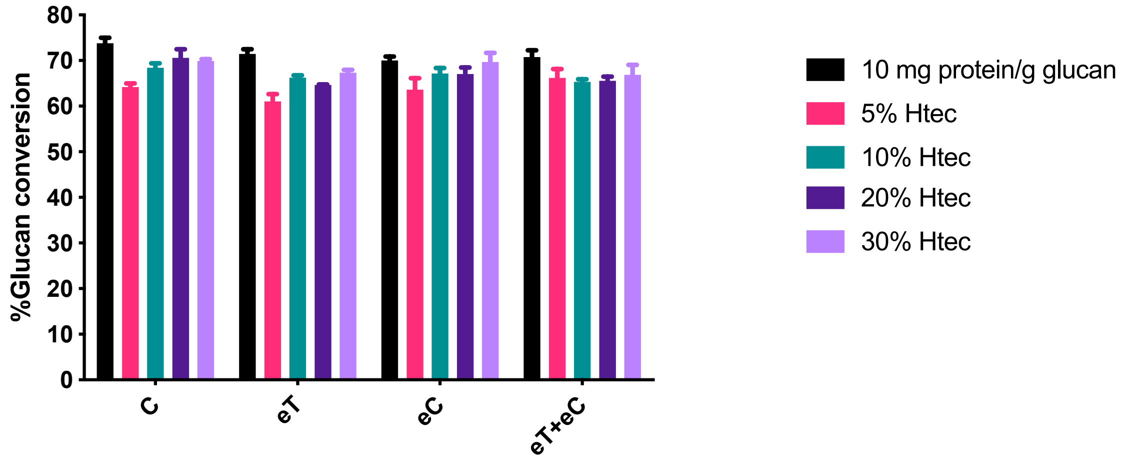


Figure S2: Htec® replacement (5, 10, 20 and 30%) for (A) liquid hot water (LHW) at 10 mg protein/ g glucan and (B) organosolv (ORG) at 20 protein/ g glucan of total protein loading.

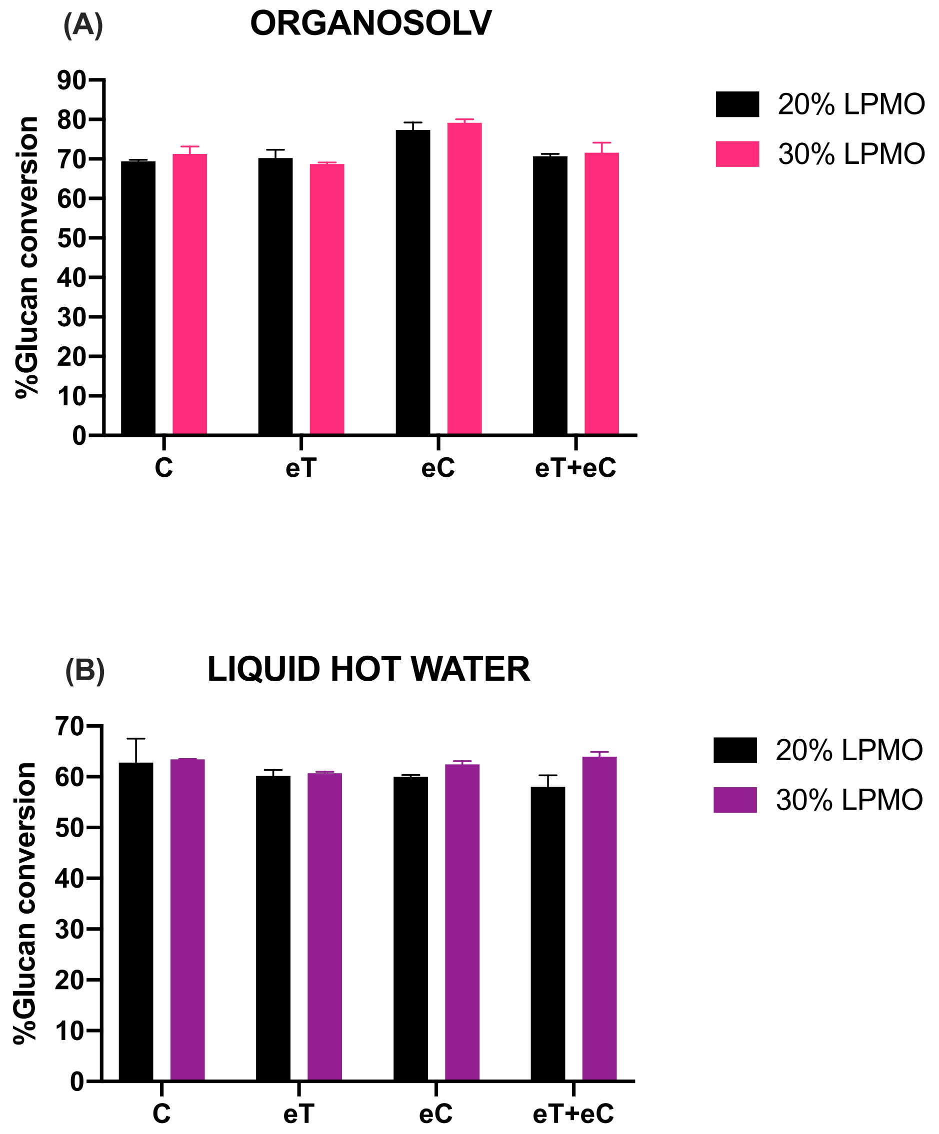


Figure S3: Lytic polysaccharide monooxygenase (LPMO) replacement (20 and 30%) for (A) liquid hot water (LHW) at 20 mg protein/ g glucan and (B) organosolv (ORG) at 30 protein/ g glucan of total protein loading and 5% Htec® replacement for both pretreatments.

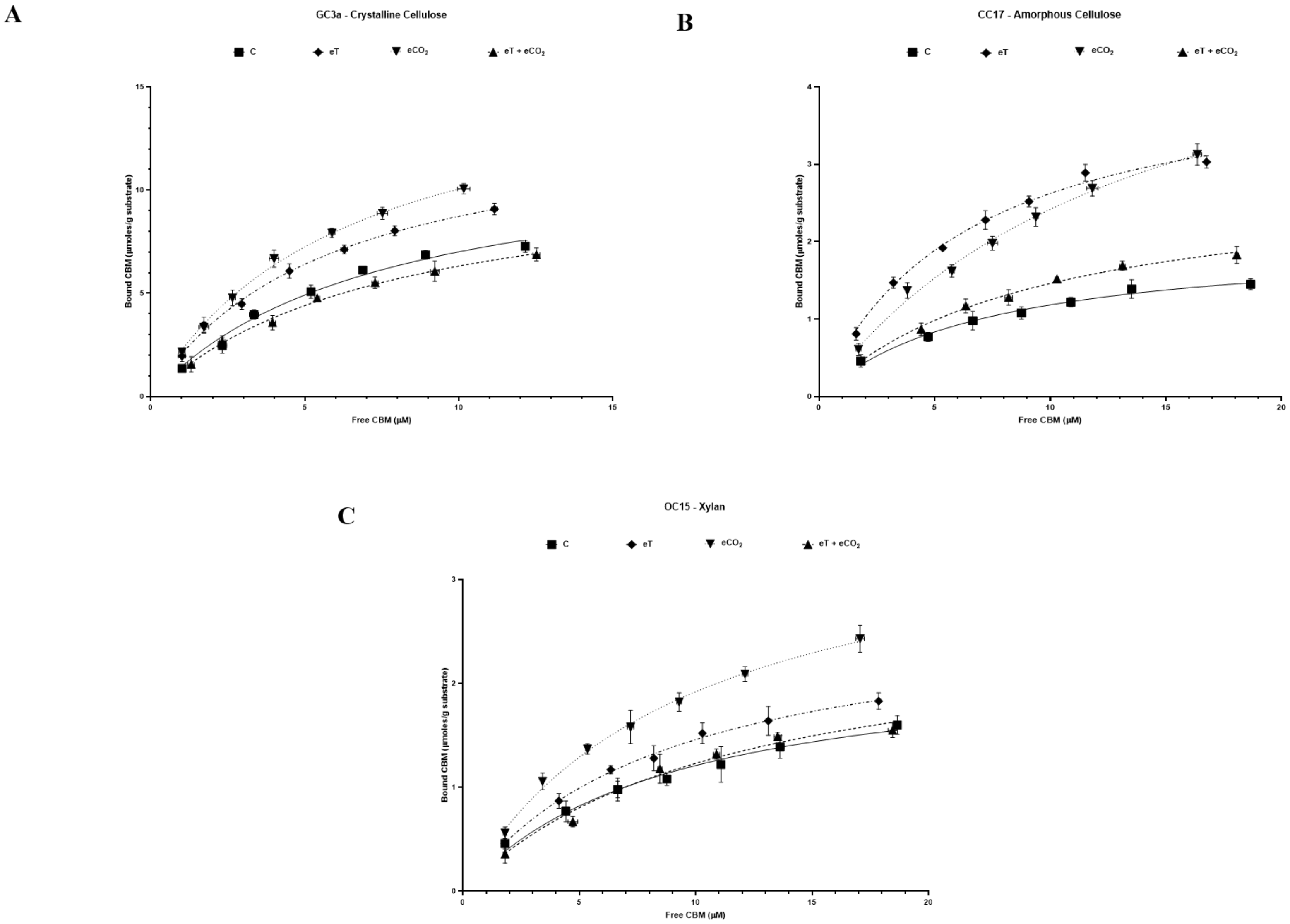


Figure S4: Binding isotherm of FTCM-depletion assay probes: GC3a (A), CC17 (B) and OC15 (C) to ORG pretreated C, eT, eC and eT+ eC climate conditions at 25°C in a 20 mM Tris–HCl (pH 7.5) buffer containing 20 mM NaCl, 5 mM CaCl2. Climate Conditions: Control (C), Elevate Temperature (eT), Elevate CO2 (eC) and combined treatments (eT+eC).

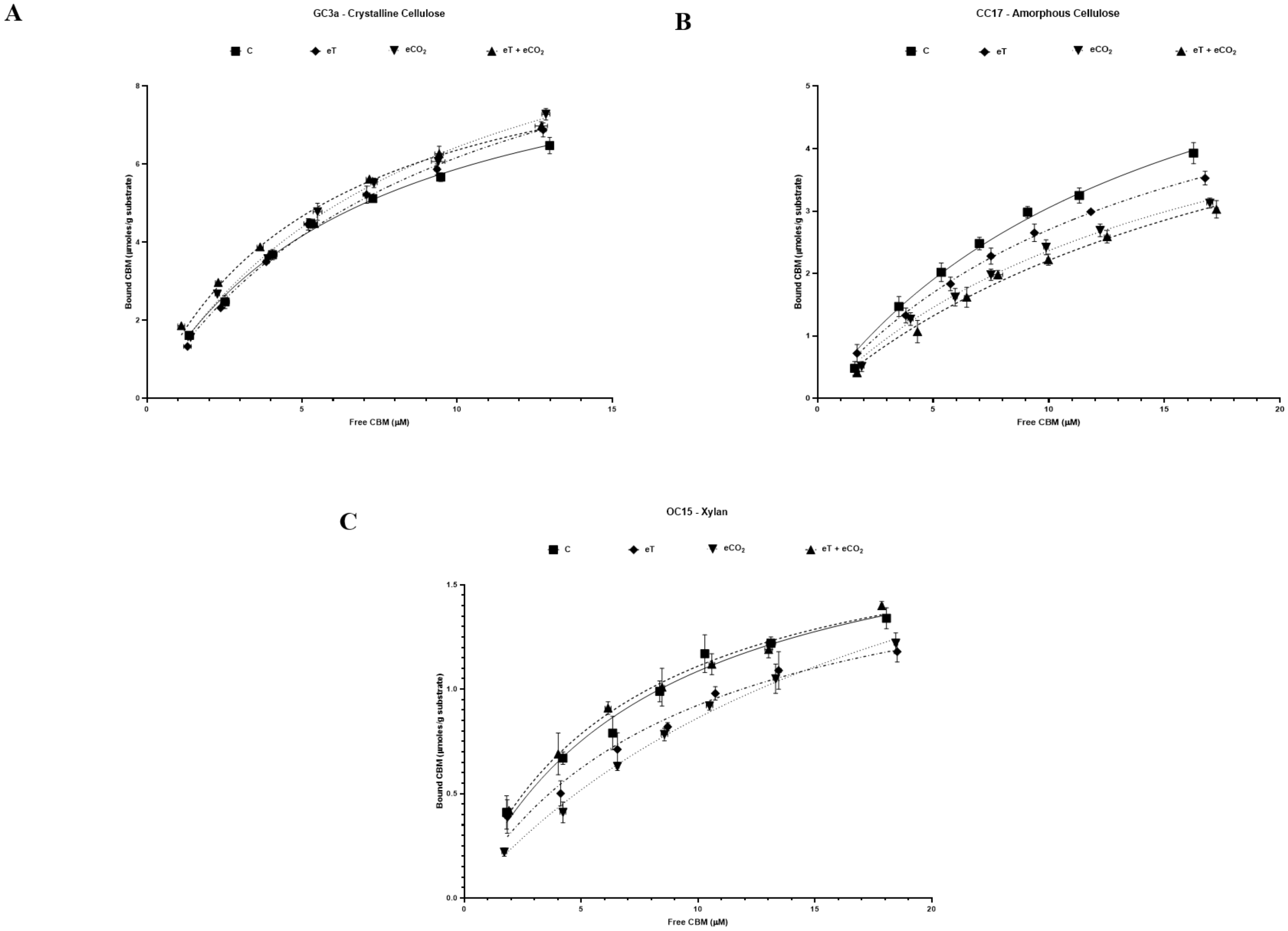


Figure S5: Binding isotherm of FTCM-depletion assay probes: GC3a (A), CC17 (B) and OC15 (C) to LHW pretreated C, eT, eC and eT+ eC climate conditions at 25°C in a 20 mM Tris–HCl (pH 7.5) buffer containing 20 mM NaCl, 5 mM CaCl2. Climate Conditions: Control (C), Elevate Temperature (eT), Elevate CO2 (eC) and combined treatments (eT+eC).

IV. GENERAL CONCLUSIONS

- Chapter I demonstrates that abiotic stress affects the plant cell wall architecture of *P. maximum* in several levels, including cellulose and hemicellulose content, lignin profile, cellulose crystallinity, and surface exposure/accessibility. The alterations in these factors impacted glucan conversion and fermentable sugar yield among the climate conditions studied, in which eT and eT+eC treatments exhibited higher saccharification yields than control and eC treatment. Furthermore, the elevated temperature seems to have a dominant role over elevated CO_2 since the eT+eC group showed similar trends compared to the eT group. Thus, our data provide significant evidence that cell wall remodeling is an essential adjustment mechanism under abiotic stress induced by expected futuristic climate conditions, which might affect the hydrolytic performance of C4 fast-growing grasses.
- In chapter II, we optimized a crude laccase pretreatment using response surface methodology to develop a sustainable and economically feasible method for the pretreatment of forage grasses. Laccase pretreatment has several positive effects on *P. maximum* lignocellulosic biomass, such as delignification, minimizing the unproductive binding of celluloses on lignin, enabling the increment of solid loading in the hydrolysis step, and reduced carbohydrate losses during the pretreatment process. Consequently, enzymatic hydrolysis yield of arabinose, xylose, and glucose significantly increased compared to non-treated biomass for all tested conditions. Moreover, crude laccase pretreatment contributed to mitigating the climate change effects on *P. maximum* hydrolytic performance since no significant differences were found between C and eT+eC climate conditions after laccase pretreatment.
- Chapter III provided evidence that the modifications induced in the chemical composition and component's surface accessibility of untreated biomass, caused by future climatic conditions, can influence synergistic associations between cellulolytic and accessory enzymes during saccharification even after the pretreatment process. However, the extension

of the effect will depend on the pretreatment method. In addition, we demonstrated that hemicellulose removal and lignin redistribution overmatches delignification in *P. maximum* grasses since higher glucan conversion rates and cellulose surface exposure/accessibility were obtained for liquid hot water compared to organosolv pretreatment. These results suggest that hemicellulose plays an essential role in restricting enzymatic digestibility, mainly due to hemicellulose-lignin cross-links.

V. ADDITIONAL INFORMATION

1. Articles published in journals (related to this thesis)

Freitas, E.N.d.; Alnoch, R.C.; Contato, A.G.; Nogueira, K.M.V.; Crevelin, E.J.; Moraes, L.A.B.d.; Silva, R.N.; Martínez, C.A.; Polizeli, M.d.L.T.M. **Enzymatic Pretreatment with Laccases from *Lentinus sajor-caju* Induces Structural Modification in Lignin and Enhances the Digestibility of Tropical Forage Grass (*Panicum maximum*) Grown under Future Climate Conditions**. *International Journal of Molecular Sciences.* 2021, 22, 9445. https://doi.org/10.3390/ijms22179445

Freitas, E.N. de; Khatri, V; Wu, J; Takada, M; Scarcella, A.S.A.; Martinez, C.A.; Saddler, J.N.; Polizeli, M.d.L.T.M, **Structural and compositional changes induced by hydrothermal and organosolv pretreatments impacts enzymatic hydrolysis of a tropical forage grass grown under future climate conditions**. *Industrial Crops and Products*. 2021, 171, p. 113937. https://doi.org/10.1016/j.indcrop.2021.113937

Freitas, E.N.d.; Khatri, V.; Contin, D.R.; Oliveira, T.B. de; Contato, A.G.; Peralta, R.M.; Santos, W.D. dos; Martinez, C.A.; Saddler, J.N.; Polizeli, M.L.T.M**. Climate change affects cell-wall structure and hydrolytic performance of a perennial grass as an energy crop**. Biofuels, Bioproducts & Biorefining, 2021. https://doi.org/10.1002/bbb.2312.

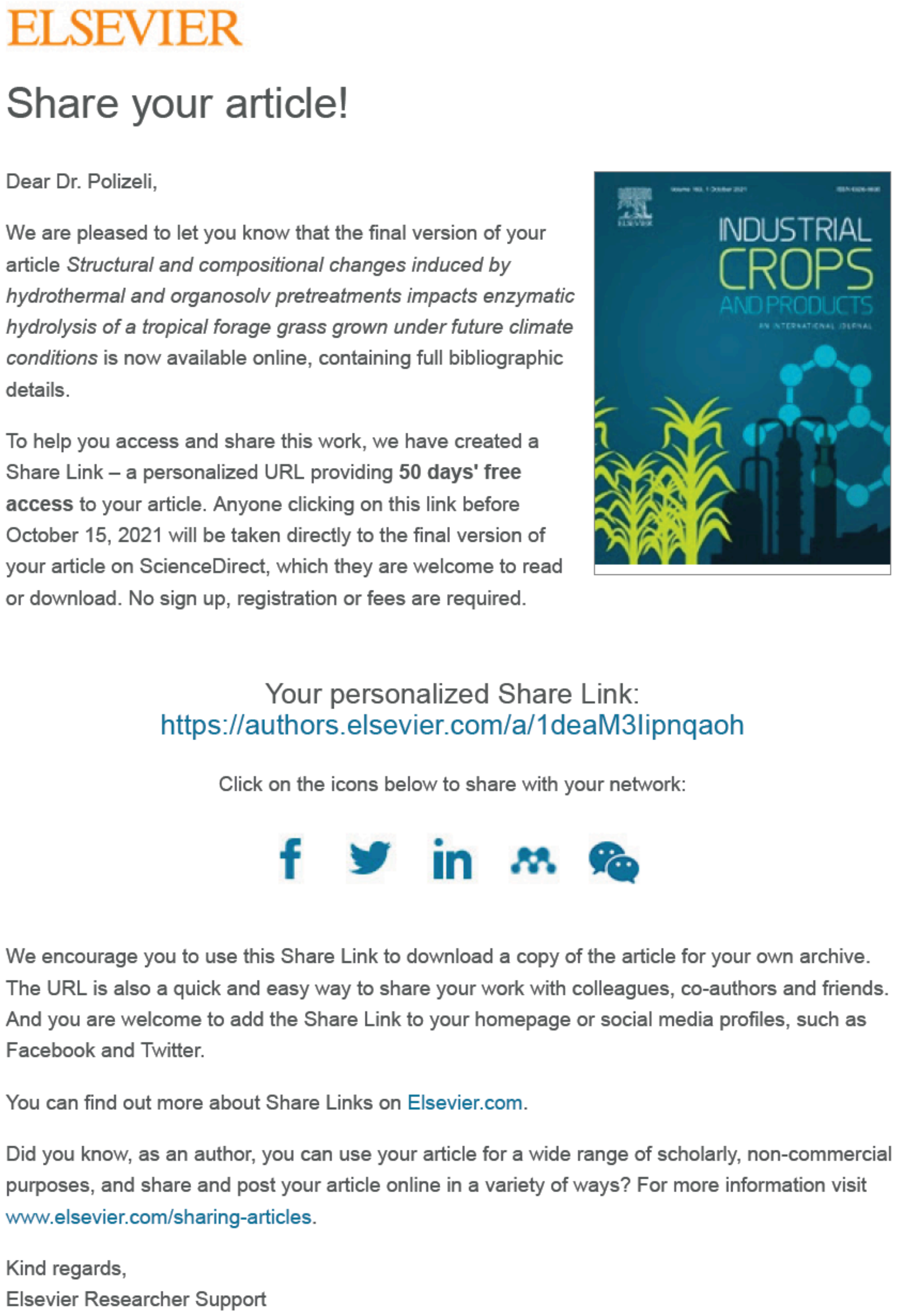
Freitas, E.N.d.; Salgado, J.C.S.; Alnoch, R.C.; Contato, A.G.; Habermann, E.; Michelin, M.; Martínez, C.A.; Polizeli, M.d.L.T.M. **Challenges of Biomass Utilization for Bioenergy in a Climate Change Scenario.** Biology. 2021, 10, 1277. https://doi.org/10.3390/biology10121277.

2. Articles published in journals (Not related to this thesis)

Contato, A. G.; de Oliveira, T. B.; Aranha, G. M.; de Freitas, E. N.; Vici, A. C.; Nogueira, K. M. V.; de Lucas, R. C.; Scarcella, A. S. de A.; Buckeridge, M. S.; Silva, R. N.; Polizeli, M. de L. T. de M. **Prospection of Fungal Lignocellulolytic Enzymes Produced from Jatoba (*Hymenaea Courbaril*) and Tamarind (*Tamarindus Indica*) Seeds: Scaling for Bioreactor and Saccharification Profile of Sugarcane Bagasse.** *Microorganisms* 2021, 9 (3), 1–16. https://doi.org/10.3390/microorganisms9030533.

Scarcella, A. S. de A.; Pasin, T. M.; de Oliveira, T. B.; de Lucas, R. C.; Ferreira-Nozawa, M. S.; Freitas, E. N. de; Vici, A. C.; Buckeridge, M. S.; Michelin, M.; Polizeli, M. de L. T. de M. **Saccharification of Different Sugarcane Bagasse Varieties by Enzymatic Cocktails Produced by *Mycothermus Thermophilus* and *Trichoderma Reesei* RP698 Cultures in Agro-Industrial Residues.** *Energy* 2021, 226. https://doi.org/10.1016/j.energy.2021.120360.

VI. ATTACHMENTS



Share your article!

Dear Dr. Polizeli,

We are pleased to let you know that the final version of your article *Structural and compositional changes induced by hydrothermal and organosolv pretreatments impacts enzymatic hydrolysis of a tropical forage grass grown under future climate conditions* is now available online, containing full bibliographic details.

To help you access and share this work, we have created a Share Link – a personalized URL providing **50 days' free access** to your article. Anyone clicking on this link before October 15, 2021 will be taken directly to the final version of your article on ScienceDirect, which they are welcome to read or download. No sign up, registration or fees are required.

Your personalized Share Link:
https://authors.elsevier.com/a/1deaM3Iipnqaoh

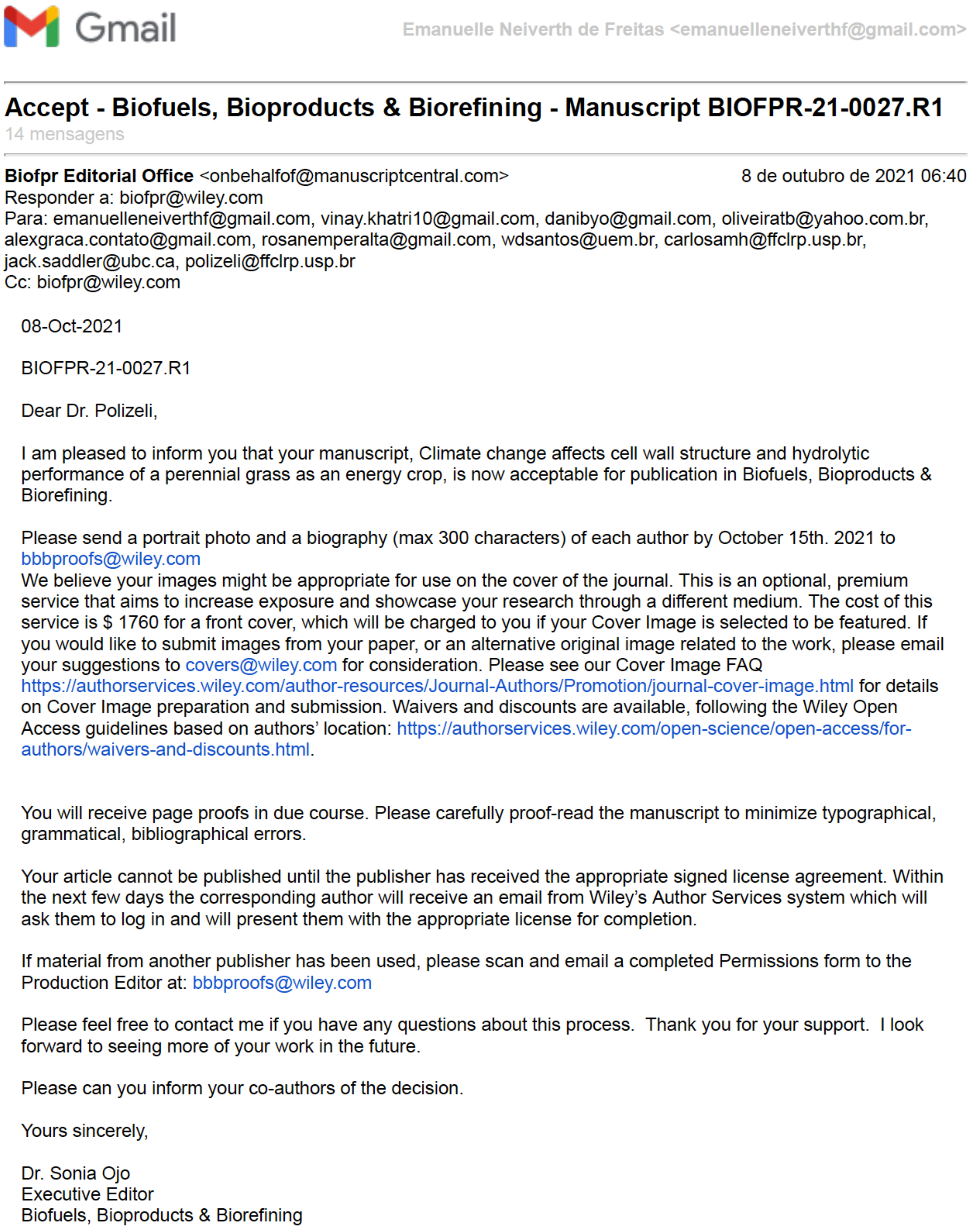
Click on the icons below to share with your network:

We encourage you to use this Share Link to download a copy of the article for your own archive. The URL is also a quick and easy way to share your work with colleagues, co-authors and friends. And you are welcome to add the Share Link to your homepage or social media profiles, such as Facebook and Twitter.

You can find out more about Share Links on Elsevier.com.

Did you know, as an author, you can use your article for a wide range of scholarly, non-commercial purposes, and share and post your article online in a variety of ways? For more information visit www.elsevier.com/sharing-articles.

Kind regards,
Elsevier Researcher Support



Gmail

Emanuelle Neiverth de Freitas <emanuelleneiverthf@gmail.com>

Accept - Biofuels, Bioproducts & Biorefining - Manuscript BIOFPR-21-0027.R1

14 mensagens

Biofpr Editorial Office <onbehalfof@manuscriptcentral.com> 8 de outubro de 2021 06:40

Responder a: biofpr@wiley.com

Para: emanuelleneiverthf@gmail.com, vinay.khatri10@gmail.com, danibyo@gmail.com, oliveiratb@yahoo.com.br, alexgraca.contato@gmail.com, rosanemperalta@gmail.com, wdsantos@uem.br, carlosamh@ffclrp.usp.br, jack.saddler@ubc.ca, polizeli@ffclrp.usp.br

Cc: biofpr@wiley.com

08-Oct-2021

BIOFPR-21-0027.R1

Dear Dr. Polizeli,

I am pleased to inform you that your manuscript, Climate change affects cell wall structure and hydrolytic performance of a perennial grass as an energy crop, is now acceptable for publication in Biofuels, Bioproducts & Biorefining.

Please send a portrait photo and a biography (max 300 characters) of each author by October 15th. 2021 to bbbproofs@wiley.com
We believe your images might be appropriate for use on the cover of the journal. This is an optional, premium service that aims to increase exposure and showcase your research through a different medium. The cost of this service is $ 1760 for a front cover, which will be charged to you if your Cover Image is selected to be featured. If you would like to submit images from your paper, or an alternative original image related to the work, please email your suggestions to covers@wiley.com for consideration. Please see our Cover Image FAQ https://authorservices.wiley.com/author-resources/Journal-Authors/Promotion/journal-cover-image.html for details on Cover Image preparation and submission. Waivers and discounts are available, following the Wiley Open Access guidelines based on authors' location: https://authorservices.wiley.com/open-science/open-access/for-authors/waivers-and-discounts.html.

You will receive page proofs in due course. Please carefully proof-read the manuscript to minimize typographical, grammatical, bibliographical errors.

Your article cannot be published until the publisher has received the appropriate signed license agreement. Within the next few days the corresponding author will receive an email from Wiley's Author Services system which will ask them to log in and will present them with the appropriate license for completion.

If material from another publisher has been used, please scan and email a completed Permissions form to the Production Editor at: bbbproofs@wiley.com

Please feel free to contact me if you have any questions about this process. Thank you for your support. I look forward to seeing more of your work in the future.

Please can you inform your co-authors of the decision.

Yours sincerely,

Dr. Sonia Ojo
Executive Editor
Biofuels, Bioproducts & Biorefining

VII. REFERENCES

[1] S. S. Hassan, G. A. Williams, and A. K. Jaiswal, "Emerging technologies for the pretreatment of lignocellulosic biomass," *Bioresour. Technol.*, vol. 262, no. March, pp. 310–318, 2018, doi: 10.1016/j.biortech.2018.04.099.

[2] "International Energy Outlook 2016. Report Number: DOE/EIA-0484."

[3] G. De Bhowmick, A. K. Sarmah, and R. Sen, "Lignocellulosic biorefinery as a model for sustainable development of biofuels and value added products," *Bioresour. Technol.*, vol. 247, no. June 2017, pp. 1144–1154, 2018, doi: 10.1016/j.biortech.2017.09.163.

[4] R. Schnepf and B. D. Yacobucci, "CRS Report for Congress Renewable Fuel Standard (RFS): Overview and Issues," 2013.

[5] R. Baños, F. Manzano-Agugliaro, F. G. Montoya, C. Gil, A. Alcayde, and J. Gómez, "Optimization methods applied to renewable and sustainable energy: A review," *Renew. Sustain. Energy Rev.*, vol. 15, no. 4, pp. 1753–1766, May 2011, doi: 10.1016/j.rser.2010.12.008.

[6] E. Habermann *et al.*, "Increasing atmospheric CO_2 and canopy temperature induces anatomical and physiological changes in leaves of the C4 forage species *Panicum maximum*", *PLoS One*, vol. 14, no. 2, pp. 1–25, 2019, doi: 10.1371/journal.pone.0212506.

[7] J. Amoah, P. Kahar, C. Ogino, and A. Kondo, "Bioenergy and Biorefinery: Feedstock, Biotechnological Conversion, and Products," *Biotechnol. J.*, vol. 14, no. 6, 2019, doi: 10.1002/biot.201800494.

[8] M. Galbe and O. Wallberg, "Pretreatment for biorefineries: A review of common methods for efficient utilisation of lignocellulosic materials," *Biotechnol. Biofuels*, vol. 12, no. 1, pp. 1–26, 2019, doi: 10.1186/s13068-019-1634-1.

[9] "Bio-based chemicals. Value added products from biorefineries." Available in: https://www.ieabioenergy.com/wp-content/uploads/2013/10/Task-42-Biobased-Chemicals-value-added-products-from-biorefineries.pdf

[10] M. FitzPatrick, P. Champagne, M. F. Cunningham, and R. A. Whitney, "A biorefinery processing perspective: Treatment of lignocellulosic materials for the production of value-added products," *Bioresour. Technol.*, vol. 101, no. 23, pp. 8915–8922, Dec. 2010, doi: 10.1016/j.biortech.2010.06.125.

[11] Wagemann, Kurt ; Tippkötter, N. Biorefineries: A Short Introduction. In: Biorefineries. Editors: Wagemann, Kurt and Tippkötter, N. Springer International Publishing, Frankfurt, 2019, p. 547.

[12] Biofuel International, "Next generation enzymes," 2014. Available in: https://www.dyadic.com/wp-content/uploads/2014/11/Next-Generation-Enzymes.pdf.

[13] A. Brandt, J. Gräsvik, J. P. Hallett, and T. Welton, "Deconstruction of lignocellulosic biomass

with ionic liquids," *Green Chem.*, vol. 15, no. 3, p. 550, 2013, doi: 10.1039/c2gc36364j.

[14] V. Menon and M. Rao, "Trends in bioconversion of lignocellulose: Biofuels, platform chemicals & biorefinery concept", *Prog. Energy Combust. Sci.*, vol. 38, no. 4, pp. 522–550, Aug. 2012, doi: 10.1016/j.pecs.2012.02.002.

[15] K. Karimi and M. J. Taherzadeh, "A critical review of analytical methods in pretreatment of lignocelluloses: Composition, imaging, and crystallinity," *Bioresour. Technol.*, vol. 200, pp. 1008–1018, 2016, doi: 10.1016/j.biortech.2015.11.022.

[16] S. P. S. Chundawat, G. T. Beckham, M. E. Himmel, and B. E. Dale, "Deconstruction of lignocellulosic biomass to fuels and chemicals," *Annu. Rev. Chem. Biomol. Eng.*, vol. 2, pp. 121–145, 2011, doi: 10.1146/annurev-chembioeng-061010-114205.

[17] Q. Liu, L. Luo, and L. Zheng, "Lignins: Biosynthesis and Biological Functions in Plants," *Int. J. Mol. Sci.*, vol. 19, no. 2, p. 335, Jan. 2018, doi: 10.3390/ijms19020335.

[18] Y.-H. P. Zhang, "Reviving the carbohydrate economy via multi-product lignocellulose biorefineries," *J. Ind. Microbiol. Biotechnol.*, vol. 35, no. 5, pp. 367–375, May 2008, doi: 10.1007/s10295-007-0293-6.

[19] D. M. de Oliveira *et al.*, "Ferulic acid: a key component in grass lignocellulose recalcitrance to hydrolysis," *Plant Biotechnol. J.*, vol. 13, no. 9, pp. 1224–1232, Dec. 2015, doi: 10.1111/pbi.12292.

[20] J. Hong, X. Ye, and Y.-H. P. Zhang, "Quantitative Determination of Cellulose Accessibility to Cellulase Based on Adsorption of a Nonhydrolytic Fusion Protein Containing CBM and GFP with Its Applications," *Langmuir*, vol. 23, no. 25, pp. 12535–12540, Dec. 2007, doi: 10.1021/la7025686.

[21] B. Yang, Z. Dai, S. Y. Ding, and C. E. Wyman, "Enzymatic hydrolysis of cellulosic biomass," *Biofuels*, vol. 2, no. 4, pp. 421–449, 2011, doi: 10.4155/bfs.11.116.

[22] S. P. Long *et al.*, "Chapter 10: Feedstocks for Biofuels and Bioenergy," *Bioenergy Sustain. Bridg. gaps*, pp. 302–346, 2015. Available in: http://bioenfapesp.org/scopebioenergy/images/chapters/bioen-scope_chapter10.pdf

[23] T. van der Weijde *et al.*, "The potential of C4 grasses for cellulosic biofuel production," *Front. Plant Sci.*, vol. 4, pp. 1–18, 2013, doi: 10.3389/fpls.2013.00107.

[24] L. L. Sosa *et al.*, "Using C4 perennial rangeland grasses for bioenergy," *Biomass and Bioenergy*, vol. 128, p. 105299, Sep. 2019, doi: 10.1016/j.biombioe.2019.105299.

[25] EMBRAPA, "Relatório de Avaliação dos impactos das tecnologias geradas pela Embrapa Gado de Corte—Capim Mombaça. Embrapa Gado Corte.," 2014.

[26] M. A. Lima *et al.*, "Evaluating the composition and processing potential of novel sources of Brazilian biomass for sustainable biorenewables production," *Biotechnol. Biofuels*, vol. 7,

no. 1, pp. 1–19, 2014, doi: 10.1186/1754-6834-7-10.

[27] R. D. Hatfield, D. M. Rancour, and J. M. Marita, “Grass cell walls: A story of cross-linking,” *Front. Plant Sci.*, vol. 7, no. January, 2017, doi: 10.3389/fpls.2016.02056.

[28] R. Sindhu, P. Binod, and A. Pandey, “Biological pretreatment of lignocellulosic biomass – An overview,” *Bioresour. Technol.*, vol. 199, pp. 76–82, Jan. 2016, doi: 10.1016/j.biortech.2015.08.030.

[29] M. Adsul, S. K. Sandhu, R. R. Singhania, R. Gupta, S. K. Puri, and A. Mathur, “Designing a cellulolytic enzyme cocktail for the efficient and economical conversion of lignocellulosic biomass to biofuels,” *Enzyme Microb. Technol.*, vol. 133, no. August 2019, p. 109442, 2020, doi: 10.1016/j.enzmictec.2019.109442.

[30] P. Vyas, A. Kumar, and S. Singh, “Biomass breakdown: A review on pretreatment, instrumentations and methods,” *Front. Biosci. - Elit.*, vol. 10, no. 1, pp. 155–174, 2018, doi: 10.2741/e815.

[31] A. Várnai, M. Siika-aho, and L. Viikari, “Restriction of the enzymatic hydrolysis of steam-pretreated spruce by lignin and hemicellulose,” *Enzyme Microb. Technol.*, vol. 46, no. 3–4, pp. 185–193, Mar. 2010, doi: 10.1016/j.enzmictec.2009.12.013.

[32] L. J. Jönsson, B. Alriksson, and N.-O. Nilvebrant, “Bioconversion of lignocellulose: inhibitors and detoxification,” *Biotechnol. Biofuels*, vol. 6, no. 1, p. 16, Dec. 2013, doi: 10.1186/1754-6834-6-16.

[33] S. Raghavi, R. Sindhu, P. Binod, E. Gnansounou, and A. Pandey, “Development of a novel sequential pretreatment strategy for the production of bioethanol from sugarcane trash,” *Bioresour. Technol.*, vol. 199, pp. 202–210, Jan. 2016, doi: 10.1016/j.biortech.2015.08.062.

[34] P. Alvira, E. Tomás-Pejó, M. Ballesteros, and M. J. Negro, “Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review,” *Bioresour. Technol.*, vol. 101, no. 13, pp. 4851–4861, Jul. 2010, doi: 10.1016/j.biortech.2009.11.093.

[35] W. Kong *et al.*, “A novel and efficient fungal delignification strategy based on versatile peroxidase for lignocellulose bioconversion,” *Biotechnol. Biofuels*, vol. 10, no. 1, p. 218, Dec. 2017, doi: 10.1186/s13068-017-0906-x.

[36] H. Guo, Y. Chang, and D. J. Lee, “Enzymatic saccharification of lignocellulosic biorefinery: Research focuses,” *Bioresour. Technol.*, vol. 252, no. November 2017, pp. 198–215, 2018, doi: 10.1016/j.biortech.2017.12.062.

[37] M.-F. Li, S. Yang, and R.-C. Sun, “Recent advances in alcohol and organic acid fractionation of lignocellulosic biomass,” *Bioresour. Technol.*, vol. 200, pp. 971–980. 2016a, doi: 10.1016/j.biortech.2015.10.004.

[38] Y. Li, C. Liu, F. Bai, and X. Zhao, “Overproduction of cellulase by *Trichoderma reesei* RUT C30 through batch-feeding of synthesized low-cost sugar mixture,” *Bioresour. Technol.*, vol. 216, pp. 503–510, 2016b, doi: 10.1016/j.biortech.2016.05.108.

[39] S. Soleimani and S.-O. Ranaei-Siadat, “Preparation and optimization of cellulase cocktail to improve the bioethanol process,” *Biofuels*, vol. 8, no. 2, pp. 291–296, Mar. 2017, doi: 10.1080/17597269.2016.1224293.

[40] A. K. Chandel, J. Q. Albarelli, D. T. Santos, S. P. Chundawat, M. Puri, and M. A. A. Meireles, “Comparative analysis of key technologies for cellulosic ethanol production from Brazilian sugarcane bagasse at a commercial scale,” *Biofuels, Bioprod. Biorefining*, vol. 13, no. 4, pp. 994–1014, Jul. 2019, doi: 10.1002/bbb.1990.

[41] A. K. Chandel, G. Chandrasekhar, M. B. Silva, and S. Silvério da Silva, “The realm of cellulases in biorefinery development,” *Crit. Rev. Biotechnol.*, vol. 32, no. 3, pp. 187–202, Sep. 2012, doi: 10.3109/07388551.2011.595385.

[42] M. L. T. M. Polizeli, A. C. Vici, A. S. A. Scarcella, M. Cereia, and M. G. Pereira. Enzyme System from *Aspergillus* in Current Industrial Uses and Future Applications in the Production of Second-Generation Ethanol. In: New and Future Developments in Microbial Biotechnology and Bioengineering: *Aspergillus* System Properties and Applications. Ed.: V.K. Gupta. Elsevier. 2016.

[43] M. E. Himmel *et al.*, “Biomass Recalcitrance: Engineering Plants and Enzymes for Biofuels Production,” *Science*, vol. 315, no. 5813, pp. 804–807, Feb. 2007, doi: 10.1126/science.1137016.

[44] P. R. Heinen *et al.*, “GH11 xylanase from *Aspergillus tamarii* Kita: Purification by one-step chromatography and xylooligosaccharides hydrolysis monitored in real-time by mass spectrometry,” *Int. J. Biol. Macromol.*, vol. 108, pp. 291–299, Mar. 2018, doi: 10.1016/j.ijbiomac.2017.11.150.

[45] R. Carapito *et al.*, “Molecular Basis of Arabinobio-hydrolase Activity in Phytopathogenic Fungi,” *J. Biol. Chem.*, vol. 284, no. 18, pp. 12285–12296, May 2009, doi: 10.1074/jbc.M900439200.

[46] B. C. Saha, “α-l-Arabinofuranosidases,” *Biotechnol. Adv.*, vol. 18, no. 5, pp. 403–423, Aug. 2000, doi: 10.1016/S0734-9750(00)00044-6.

[47] M. L. T. M. Polizeli, “Biorefinery of alternative resources: Targeting green fuels and platform chemicals”. In: Biorefinery of Alternative Resources: Targeting Green Fuels and Platform Chemicals. Eds.: S. Nanda, N. Sonil, D. Vo, P.K. Sarangi. Springer Singapore. 2020, pp.1–494.

[48] G. Müller, P. Chylenski, B. Bissaro, V. G. H. Eijsink, and S. J. Horn, “The impact of

hydrogen peroxide supply on LPMO activity and overall saccharification efficiency of a commercial cellulase cocktail," *Biotechnol. Biofuels*, vol. 11, no. 1, p. 209, Dec. 2018, doi: 10.1186/s13068-018-1199-4.

[49] J. Hu, V. Arantes, A. Pribowo, K. Gourlay, and J. N. Saddler, "Substrate factors that influence the synergistic interaction of AA9 and cellulases during the enzymatic hydrolysis of biomass," *Energy Environ. Sci.*, vol. 7, no. 7, pp. 2308–2315, 2014, doi: 10.1039/C4EE00891J.

[50] P. Biely, M. Mastihubová, M. Tenkanen, J. Eyzaguirre, X.-L. Li, and M. Vršanská, "Action of xylan deacetylating enzymes on monoacetyl derivatives of 4-nitrophenyl glycosides of β-d-xylopyranose and α-l-arabinofuranose," *J. Biotechnol.*, vol. 151, no. 1, pp. 137–142, Jan. 2011, doi: 10.1016/j.jbiotec.2010.10.074.

[51] Y.-M. Qin, H. Tao, Y.-Y. Liu, Y.-D. Wang, J.-R. Zhang, and A.-X. Tang, "A novel non-hydrolytic protein from *Pseudomonas oryzihabitans* enhances the enzymatic hydrolysis of cellulose," *J. Biotechnol.*, vol. 168, no. 1, pp. 24–31, Oct. 2013, doi: 10.1016/j.jbiotec.2013.07.028.

[52] J. M. Morrison, M. S. Elshahed, and N. H. Youssef, "Defined enzyme cocktail from the anaerobic fungus *Orpinomyces* sp. Strain C1A effectively releases sugars from pretreated corn stover and switchgrass," *Sci. Rep.*, vol. 6, no. June, pp. 1–12, 2016, doi: 10.1038/srep29217.

[53] Y. Su, X. Yu, Y. Sun, G. Wang, H. Chen, and G. Chen, "Evaluation of Screened Lignin-degrading Fungi for the Biological Pretreatment of Corn Stover," *Sci. Rep.*, vol. 8, no. 1, p. 5385, Dec. 2018, doi: 10.1038/s41598-018-23626-6.

[54] A. Oliva-Taravilla, E. Tomás-Pejó, M. Demuez, C. González-Fernández, and M. Ballesteros, "Inhibition of cellulose enzymatic hydrolysis by laccase-derived compounds from phenols," *Biotechnol. Prog.*, vol. 31, no. 3, pp. 700–706, May 2015, doi: 10.1002/btpr.2068.

[55] IPCC, "Special report on the impacts of global warming of 1.5°C above pre-industrial levels and related global greenhouse gas emission pathways, in the context of strengthening the global response to the threat of climate change, sustainable development, and eff," 2018.

[56] F. M. DaMatta, A. Grandis, B. C. Arenque, and M. S. Buckeridge, "Impacts of climate changes on crop physiology and food quality," *Food Res. Int.*, vol. 43, no. 7, pp. 1814–1823, 2010, doi: 10.1016/j.foodres.2009.11.001.

[57] R. F. Sage and D. S. Kubien, "The temperature response of C3 and C4 photosynthesis," *Plant, Cell Environ.*, vol. 30, no. 9, pp. 1086–1106, 2007, doi: 10.1111/j.1365-3040.2007.01682.x.

[58] E. A. Ainsworth and A. Rogers, "The response of photosynthesis and stomatal conductance to rising [CO_2]: Mechanisms and environmental interactions," *Plant, Cell Environ.*, vol. 30,

no. 3, pp. 258–270, 2007, doi: 10.1111/j.1365-3040.2007.01641.x.

[59] N. A. Streck, “Climate change and agroecosystems: the effect of elevated atmospheric CO_2 and temperature on crop growth, development, and yield,” *Ciência Rural*, vol. 35, no. 3, pp. 730–740, 2005, doi: 10.1590/s0103-84782005000300041.

[60] D. S. Sage, R. F., Way, D. A., & Kubien, “Rubisco, Rubisco activase, and global climate change,” *J. Exp. Bot.*, vol. 59, pp. 1581–1595, 2008.

[61] R. Borjas-Ventura, L. R. Alves, R. de Oliveira, C. A. Martínez, and P. L. Gratão, “Impacts of warming and water deficit on antioxidant responses in *Panicum maximum* Jacq,” *Physiol. Plant.*, vol. 165, no. 2, pp. 413–426, 2019, doi: 10.1111/ppl.12907.

[62] S. M. Tubiello, F. N., Soussana, J.-F., & Howden, “Crop and pasture response to climate change,” *Proc. Natl. Acad. Sci.*, vol. 104, pp. 19686–19690, 2007.

[63] H. Polley, “Implications of atmospheric and climate change for crop yield,” *Crop Sci.*, vol. 42, pp. 131–140, 2002.

[64] S. Ainsworth, EA, Leakey, ADB, Ort, DR, Long, “FACE-ing the facts: Inconsistencies and interdependence among field, chamber and modeling studies of elevated [CO_2] impacts on crop yield and food supply,” *New Phytol.*, no. 179, pp. 5–9, 2008.

[65] E.N. Freitas, J.C.S. Salgado, R.C. Alnoch, A.G. Contato, E. Habermann, M. Michelin, C.A. Martínez, M.L.T.M. Polizeli, "Challenges of Biomass Utilization for Bioenergy in a Climate Change Scenario. *Biology*, vol. 10, no. 12:127, 2021.

[66] D. M. Oliveira *et al.*, “Cell wall remodeling under salt stress: Insights into changes in polysaccharides, feruloylation, lignification, and phenolic metabolism in maize,” *Plant. Cell Environ.*, vol. 43, no. 9, pp. 2172–2191, Sep. 2020, doi: 10.1111/pce.13805.

[67] R. Tenhaken, “Cell wall remodeling under abiotic stress,” *Front. Plant Sci.*, vol. 5, Jan. 2015, doi: 10.3389/fpls.2014.00771.

[68] J. Zhu *et al.*, “A cellulose synthase-like protein is required for osmotic stress tolerance in *Arabidopsis*”, *Plant J.*, p. no-no, Apr. 2010, doi: 10.1111/j.1365-313X.2010.04227.x.

[69] H. Le Gall, F. Philippe, J. M. Domon, F. Gillet, J. Pelloux, and C. Rayon, “Cell wall metabolism in response to abiotic stress,” *Plants*, vol. 4, no. 1, pp. 112–166, 2015, doi: 10.3390/plants4010112.

[70] H. Wei *et al.*, “Global transcriptomic profiling of aspen trees under elevated [CO_2] to identify potential molecular mechanisms responsible for enhanced radial growth,” *J. Plant Res.*, vol. 126, no. 2, pp. 305–320, 2013, doi: 10.1007/s10265-012-0524-4.

[71] R. Suwa *et al.*, “High temperature effects on photosynthate partitioning and sugar metabolism during ear expansion in maize (*Zea mays* L.) genotypes,” *Plant Physiol. Biochem.*, 2010, doi: 10.1016/j.plaphy.2009.12.010.

[72] D. M. Oliveira *et al.*, “Cell wall remodeling under salt stress: Insights into changes in polysaccharides, feruloylation, lignification, and phenolic metabolism in maize,” *Plant. Cell Environ.*, 2020, doi: 10.1111/pce.13805.

[73] W. J. R. Ford C.W., Morrison I.M., “Temperature effects on lignin, hemicellulose and cellulose in tropical and temperate grasses.,” *Aust J Agr Res.*, vol. 30, no. 4, pp. 621–634, 1979.