UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE BIOLOGIA

LÍVIA BEATRIZ BRENELLI DE PAIVA

APPLICATION OF CHEMICAL AND BIOLOGICAL APPROACHES FOR LIGNIN VALORIZATION

APLICAÇÃO DE ABORDAGENS QUÍMICAS E BIOLÓGICAS PARA VALORIZAÇÃO DA LIGNINA

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Thesis presented to the Institute of Biology of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in FUNCTIONAL AND MOLECULAR BIOLOGY, in the area of BIOCHEMISTRY under the Cotutela Agreement signed between University of Campinas and University of Copenhagen, Denmark.

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Dr. André Luis Ferraz

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RESUMO

As atividades voltadas para a produção comercial de etanol a partir de materiais lignocelulósicos criaram a oportunidade de desenvolver novas tecnologias utilizando correntes de processos com alto conteúdo de lignina. Encontrar aplicações para a lignina que possam agregar maior valor, em comparação com a queima para produção de energia, torna-se imprescindível para viabilizar estes processos no contexto de biorefinarias. A lignina é um polímero aromático com características singulares. Devido à sua estrutura molecular heterogênea, a lignina ainda é um polímero inexplorado em tecnologias de bioconversão. As estratégias emergentes para as transformações de ligninas são focadas em catálises inorgânicas, orgânicas ou na combinação de ambas. Sob esta perspectiva, este trabalho buscou desenvolver estratégias químicas e biológicas para valorizar correntes de lignina geradas no processamento do bagaço de cana-de-açúcar. Neste contexto, como estratégia química, fragmentos de lignina derivados de tratamento alcalino de bagaço de cana-de-açúcar, depois de processo de explosão a vapor, foram submetidos a um processo de acidificação. As frações solúveis produzidas em diferentes valores de pH foram caracterizadas quimicamente e a capacidade antioxidante *in vitro* contra espécies reativas de oxigênio e nitrogênio foram avaliadas. Em comparação com as outras frações obtidas neste trabalho, a fração solúvel obtida em pH 2 apresentou as melhores capacidades de eliminação contra todas as espécies de radicais testadas (10,2 ± 0,7 mmol Trolox equivalente g^{-1} para $ROO^{\bullet}$, IC_{30} = 14,9 μg mL^{-1} para H_2O_2 e IC_{50} = 2,3 μg mL^{-1} para $ONOO^-$), bem como apresentou a menor polidispersidade (1,2). Dados biofísicos mostraram que as moléculas presentes nas frações solúveis obtidas em pH 4 e 2 consistiam estruturalmente em pequenos discos com raio e espessura média de 0,31 nm e polifenóis de baixo peso molecular (~ 400 Da), enquanto que frações solúveis obtidas em pH elevados predominavam nanopartículas de lignina (discos com raio maiores que 1,1 nm e espessura em torno de 0,7 nm) e agregados com dimensão fractal em torno de 2,8 nm. Em todas as frações solúveis foram identificados compostos fenólicos e não fenólicos conhecidos como eficientes antioxidantes na literatura. Estes resultados demostraram que o tratamento de acidificação é uma estratégia simples e promissora para valorizar correntes heterogêneas de ligninas provenientes do processamento do bagaço, obtendo-se preparações com composições com reduzida polidispersidade e alta capacidade antioxidante. Neste trabalho de doutorado também foram avaliados processos enzimáticos para valorização da lignina. Foi explorado o potencial de duas lacases fúngicas juntamente com um mediador para isolar ligninas de baixo peso molecular a partir da biomassa lignocelulósica. As ligninas isoladas foram utilizadas como doadores de elétrons para a ativação de monooxigenases de polissacarídeos líticas (LPMOs). Uma correlação direta entre a lignina de baixo peso molecular obtida com o sistema mediador-lacase e o aumento na atividade de um coquetel celulolítico comercial contendo LPMOs foi encontrada quando a celulose pura foi hidrolisada. Nas condições testadas no trabalho, a co-incubação das lacases com com o coquetel contendo LPMOs resultou em uma competição pelo substrato oxigênio, inibindo as LPMOs. O tratamento com lacases podem ter causado outras modificações na presença de celulose, tornando o material mais recalcitrante para a sacarificação enzimática. Finalmente, visando a descoberta e aplicação de novos biocatalizadores envolvidos na desconstrução e valorização de material lignocelulósico, o basidiomiceto derivado do mar *Peniophora* sp. CBMAI 1063 cultivado em condições salinas teve seu genoma sequenciado, os genes anotados e o conteúdo proteico do secretoma analisado. Os estudos mostraram que o fungo possui um espectro enzimático ligninolítico completo e versátil, produzindo especialmente enzimas envolvidas na degradação da lignina. A principal lacase secretada pelo fungo mostrou potencial em tecnologias de bioconversão, promovendo modificação, despolimerização e solubilização de ligninas do bagaço.

ABSTRACT

The development of commercial production of ethanol from lignocellulosic materials has created the opportunity to increase the transformation of lignin streams into high added-value applications. Finding applications for lignin valorization instead of combustion for energy production is essential to make biorefineries an element of sustainable development. Lignin is a complex aromatic polymer with unique characteristics. However, due to its molecular structure, lignin is an untapped resource in bioconversion technologies. Emerging strategies for lignin transformations focus on inorganic or organic catalysis or a combination of both. This thesis focuses on chemical and biological strategies to add value to lignin from sugarcane bagasse. Lignin obtained from alkaline treatment of steam-exploded sugarcane bagasse was submitted to an acidification process. The soluble fractions produced at different pH were comprehensively characterized, and *in vitro* antioxidant capacity against reactive oxygen and nitrogen species was evaluated. The soluble fraction obtained at pH 2 exhibited the highest scavenging capacities against all tested species (10.2 ± 0.7 mmol Trolox equivalent g^{-1} for $ROO^{\bullet}$, IC_{30} = 14.9 μg mL^{-1} for H_2O_2 and IC_{50} = 2.3 μg mL^{-1} for $ONOO^{-}$) and the lowest polydispersity value (1.2). Biophysical data showed that the soluble fractions obtained at pH 4 and pH 2 consisted of small nanometer-sized discs with average radius and thickness of 0.31 nm made from low molecular weight polyphenolics (~400 Da). In the soluble fractions obtained at high pH larger lignin nanoparticles (average disk radius higher than 1.1 nm and disk thickness around 0.7 nm) and larger aggregates with fractal dimension of 2.8 nm were found. Phenolic and non-phenolic compounds, well-known as efficient antioxidants, were identified in all soluble fractions. The results provided, further demonstrates that acidification treatment is a promising strategy to upgrade heterogeneous lignin-enriched stream from sugarcane bagasse, such as preparations with homogeneous compositions and high antioxidant capacity. Furthermore, the potential of two fungal laccases together with a mediator to isolate low molecular weight lignin from lignocellulosic biomass was explored. These lignins were used as electron donors for activation of lytic polysaccharide monooxygenases (LPMOs). For cellulose hydrolysis a direct correlation between the low molecular weight lignin obtained with laccases and the activity of a cellulolytic cocktail containing LPMOs was found. Under the conditions tested, the co-incubation of laccases with LPMOs showed a substrate competition towards LPMOs inhibition by oxygen. Also laccase treatment may cause other modifications in presence of cellulose, rendering the material more recalcitrant for enzymatic saccharification. Finally, regarding the discovery of new enzymes for lignin deconstruction, the genome sequencing, annotation and secretome analysis of the marine-derived basidiomycete *Peniophora* sp. CBMAI 1063 grown under saline optimal conditions were performed. Omics studies showed that this fungus possesses a complete and versatile enzymatic spectrum, especially enzymes involved in lignin degradation. In addition, the major secreted laccase exhibited potential to lignocellulose bioconversion new technologies, promoting lignin modification, depolymerization and solubilization.

LIST OF ACRONYMS

^{1}H NMR	Proton nuclear magnetic resonance
AA	Ascorbic acid
AAs	Auxiliary Activities
ABTS	2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid
AT	Alkaline treatment
CAZy	Carbohydrate-Active enZYmes Database
CBM	Carbohydrate-Binding Modules
CC2	Cellic Ctec2
CDH	Cellobiose dehydrogenase
CE	Carbohydrate esterase
CG-MS	Gas chromatography-mass spectrometry
Da	Dalton
DM	Dry matter
FDR	False Discovery Rate
FPU	Filter paper cellulase units
FTIR	Fourier transform infrared
GH	Glycoside hydrolases
GPC	Gel permeation chromatography
GT	Glycosyl Transferase
HMW	High molecular weight
IL	Ionic liquids
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LMS	Laccase mediator system
LMW	Low molecular weight
LPMO	Lytic polysaccharide monooxygenases
Mb	Million barrels
Mn	Number-average molecular weight
MtL	Laccase from *Myceliophthora thermophila*
Mw	Weight- average molecular weight
ORAC	Oxygen absorbance capacity
pH	Potential of hydrogen

PL	Polysaccharide Lyases
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RP-HPLC	Reverse Phase Liquid Chromatography
SAXS	Small- angle X-ray scattering
SCB	Sugarcane Bagasse
SF	Soluble fraction
SGD	Syringaldazine
TPC	Total phenolic compounds
TvL	Laccase from *Trametes villosa*
WS	Wheat straw

CONTENTS

CHAPTER 1 – Introduction

1. A Brief Literature Review

1.1 Agro-industrial lignocellulosic biomass a key to unlock the future bio-energy

The world's economy is highly dependent on various fossil energy sources such as oil, coal and natural gas for the production of fuels, electricity and other goods (Sarkar et al., 2012; Uihlein & Schbek, 2009). The effects of this scenario added to the globalization of the growing energy demand, makes the energy shortage and climate changes common worldwide problems (Baeyens et al., 2015). The level of oil demand will increase from 85 $Mb \cdot day^{-1}$ in 2008 to 105 $Mb \cdot day^{-1}$ in 2030 and the projections show that the production of easily accessible oil and gas will not match the consumption by 2040–2050 (Bradshaw, 2010).

Bio-ethanol is considered the renewable fuel with the largest potential to replace fossil-derived fuels and significantly reduces the greenhouse gas emissions (Dias et al., 2013). Brazil and the United States (US) are leading the change in fuel usage, producing and consuming approximately 80% of the world's total amount. In the major markets of Brazil and the US, the bio-ethanol is an important part of the fuel mix and the consumption has been growing by around 10% per year (http://biofuelsassociation.com.au/).

The European Union aims to replace 10% of each European Union (EU) member state's transport fuels with renewable fuels like ethanol by 2020. The Nordic countries are at the forefront of the replacement, with Sweden for example aiming for their transport industry to be completely free of fossil fuels by 2030 (http://biofuelsassociation.com.au/).

The major traditional agricultural crops used for bio-ethanol production are maize, sugarcane and sugar beets. However, these crops are not enough to supply the global demand of ethanol, food and feed production (Gupta & Verma, 2015). Hence, lignocellulosic materials are attractive feedstocks for bio-ethanol production since it is the most abundant biomass on Earth (Hamelinck, Hooijdonk & Faaij, 2005; Pandey, Soccol & Mitchell, 2000). Rice-straw, wheat straw, sugarcane bagasse, cotton stalk, soft bamboo are examples of valuable sources of lignocellulosic materials that have been used to produce second generation bio-ethanol worldwide (Ravindranath et al., 2011).

Although the second generation bio-ethanol is considered a promising technology, the production involves primarily pretreatment steps followed by enzymatic or chemical hydrolysis, fermentation, distillation and also several challenges to make the whole process cost effective (Aditiya et al., 2016; Gupta and Verma, 2015).

To understand the composition and architecture of cell walls and understand the impacts and challenges involved in the bioconversion of lignocellulosic biomass, the structure of each main component is reported in the following section.

1.2 Composition of plant cell walls

Lignocellulose is the name given to the material present in the cell wall of higher terrestrial plants, made up of microfibriles of cellulose embebed in an amorphous matrix of hemicellulose and lignin along with smaller amounts of pectin, protein, extractives and ash (Fig. 1). The composition is in the following proportion: cellulose (30 – 50%), hemicelluloses (20 – 35%) and lignin (5 – 30%) of plant dry matter (Martínez et al., 2009).

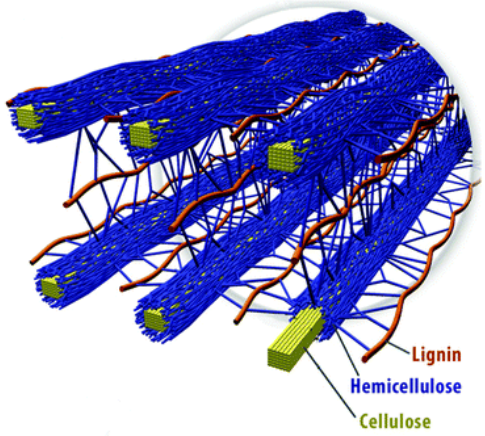

Figure 1. Schematic representation of basic plant cell wall structure showing the association between cellulose, hemicellulose and lignin. These polymers are associated with each other in a heterogeneous matrix to different degrees and varying relative composition depending on the type, species, and even source of the biomass. Reproduced from http://genomics.energy.gov.

These three types of polymers are strongly bonded to one another and their quantities vary according to the species variety, harvest season and regions of the same plant (Martins et al., 2011).

Cellulose is the most abundant organic compound on Earth and the main constituent of plant cell wall conferring structural support. The cellulose chemical structure consists of linear chains of approximately 8,000 to 12,000 residues of D-glucose linked by β-1**,** 4 bonds (Fig. 2). Considered a highly stable structure, the long-chain of cellulose are linked together by

hydrogen and van der Waals bonds, which cause the cellulose to be packed into microfibrils with high tensile strength. The interchain hydrogen bonds might introduce the crystalline region or amorphous region into the cellulose structure (Himmel et al., 2007; Vainio, 2004).

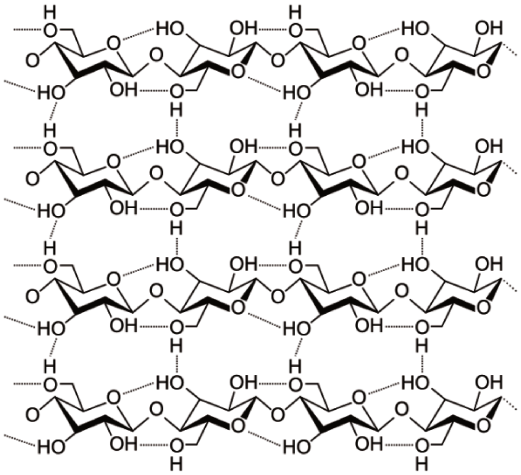

Figure 2. A strand of cellulose, showing the hydrogen bonds (dashed lines) within and between D-glucose molecules. Reproduced from http://www.wikiwand.com/en/Cellulose.

Hemicellulose is the second most abundant polymer, and differently from cellulose, is made up of non-crystalline heteropolyssacharides (Fig. 3). The common monomers constituent can be pentoses (β-D-xylose, α-L-arabinose, α-D-arabinose), hexoses (α-D-mannose, β-D-mannose) and/or uronic acids (α-D-glucuronic, α-D-4-O- methylgalacturonic, α-D- galacturonic acids) (Fig.3). Less often, other sugars such as α-L-rhamnose and α-L-fucose may also be present in small amounts (Martins et al., 2011).

Depending on the type of the constituent monomers, hemicellulose can be classified as xyloglucans, xylans, mannans and β-(1→3, 1→4)-glucans. Galactans, arabinans and arabinogalactans are included in the hemicellulose group; however, they do not share the equatorial β-1, 4 linked backbone structure (Scheller & Ulvskov, 2010). Hemicellulose from grasses and straws contain arabinan, galactan and xylan. Hardwood and softwood hemicellulose contain mannan. Although hemicelluloses cannot self-aggregate to form long, close-packed crystalline fibrils in the manner of cellulose, the chains of hemicellulosic polysaccharides can form important hydrogen bonded associations with each other in the cell wall, particularly between regions of the chains which have few side branches or axial hydroxyl groups (Anwar, Gulfraz & Irshad, 2014).

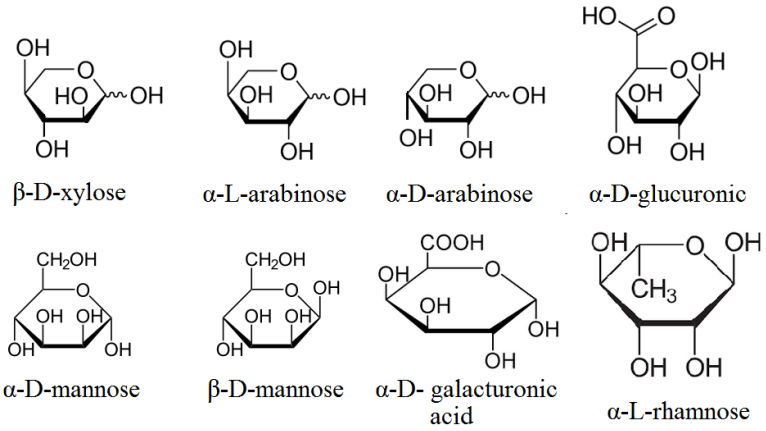

Figure 3. Examples of monomer sugars found in hemicellulose composition present in plant cell walls. Reproduced with modifications from Sjöström and Westermark (1999).

Lignin composes of ~25% of lignocellulosic biomass and is an amorphous natural polymer (Dutta et al., 2014) consisting of a 3D arrangement of methoxylated phenylpropane (Fig.4) (Boerjan, Ralph & Baucher, 2003; Abe et al., 2010).

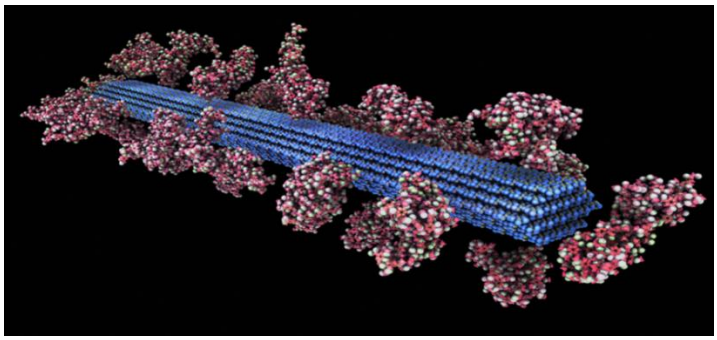

Figure 4. Interaction between cellulose fibril (blue) and lignin (pink) molecules in plant cell walls. Reproduced from https://www.olcf.ornl.gov/2014/01/02/boosting-bioenergy/.

This polymer occurs widely in the middle lamellae and secondary cell walls of higher plants and plays a key role in constructive tissues as a building material, giving the walls strength and rigidity and resistance to environmental stresses. This is due to the hydrophobic nature and insolubility in aqueous systems preventing access of degrading chemicals and organisms (Boerjan, Ralph & Baucher, 2003; Abe et al., 2010).

Lignins are built in plants by oxidative phenolic coupling reactions, starting from three basic monolignols (Fig. 5) which are: *p*-coumaryl alcohol, coniferyl alcohol and sinapyl

alcohol, with the proportions depending on the source (Ralph, Brunow & Boerjan, 2007; Boerjan, Ralph & Baucher, 2003).

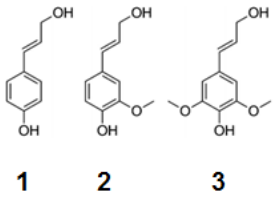

Figure 5. The structures of *p*-coumaryl alcohol (1), coniferyl alcohol (2) and sinapyl alcohol (3). Reproduced from Stanley et al. (2013).

Phenylpropane monomers, syringyl (S), guaiacyl (G), and *p*-hydroxyphenyl (H) are derived from monolignols sinapyl-, coniferyl-, and coumaryl-alcohol, respectively. Depending on the source, these units are present in different contents and interconnected through different cross-linkages and C–C interunit linkages (Constant et al., 2015) (Fig. 6).

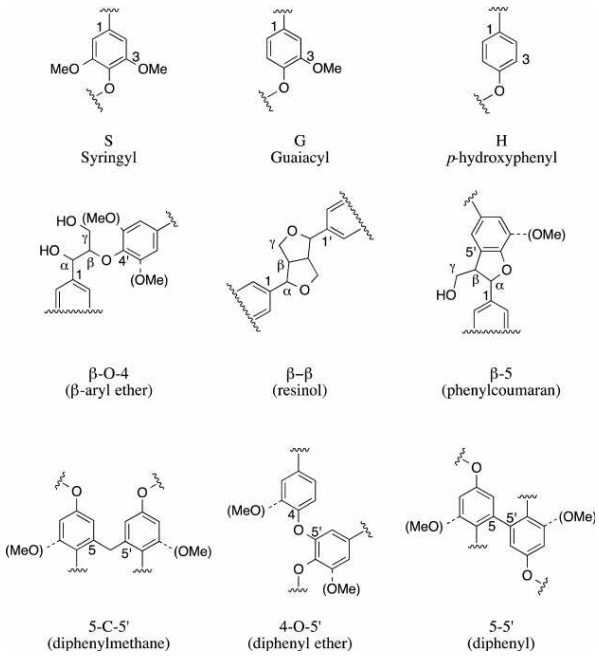

Figure 6. Main inter-units linkages and building blocks found in lignin plant cell walls. The β-O-4 linkage is dominant, consisting of more than half of all inter subunit bonds. Reproduced from Constant et al. (2015).

There are considerable differences in linkage occurrence between softwood and hardwood lignins. For example, hardwood lignin contains more β-O-4, β-1 and β-β linkages and less α-O-4, β-5, 5-5, 4-O-5 linkages compared to softwood lignin (Dutta et al., 2014). Despite many research efforts, lignin chemistry, biosynthesis and molecular biology is not fully

understood. As result, lignin structure is not completely established. Nevertheless, some representations of substructures of lignin are reported in the literature (Fig. 7).

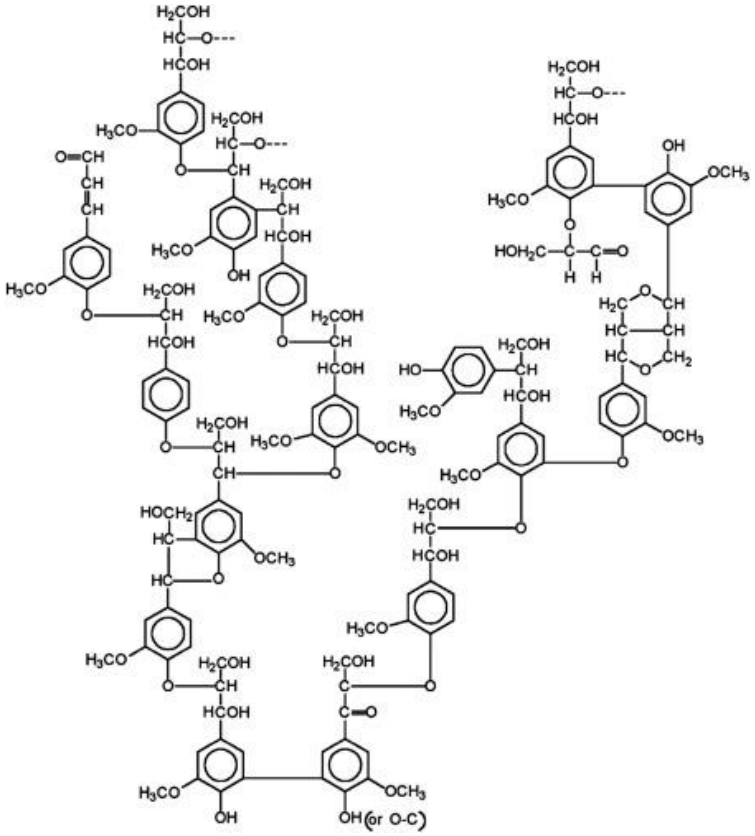

Figure 7. Schematic of lignin structure from wheat straw showing a diversity of linkages based on ether and alkane groups found in plant cell walls. Reproduced from Pouteau et al. (2003).

The complex matrix of cellulose and lignin bound by hemicelluloses chains makes the production of biofuel from the agro-residues and other sources of biomass a challenging process (Aditiya et al., 2016; Anwar, Gulfraz & Irshad, 2014; Ragauskas et al., 2014). To overcome the lignocellulose recalcitrance, scientists have worked on many strategies for biomass deconstruction and enzymatic hydrolysis to depolymerize the intact carbohydrate polymers into soluble sugars.

1.3 Bio-ethanol from agro-residues involves pretreatment steps and enzymatic hydrolysis

1.3.1 Pretreatment process technologies

Pretreatment is a key factor to improve the efficiency of the processing of agro-residues, as it is necessary to make cellulose more accessible to enzymatic hydrolysis for conversion to fuels (Agbor et al., 2011).

Since the last 10 years, a large number of pretreatment methods have been developed, including biological, mechanical, chemical and various combinations thereof, in order to change the physical and chemical structure of the lignocellulosic biomass and consequently, improve hydrolysis rates. The choice of suitable pretreatment strategies involves many factors such as nature of the lignocellulose biomass, desired products, economic assessment and environmental impact. There are other factors that need to be considered: the possibility to use high loading of biomass, no significant sugar degradation into toxic compounds, yeast and bacterial fermentation compatibility of the derived sugar syrups, lignin recovery and minimum heat and power requirements (Alvira et al., 2010; Kumar et al., 2009).

Physical pretreatments of biomass aim to increase surface area by reducing the feedstock particle size, combined with defibrilization or reduction in the crystallinity degree. The physical pretreatment technologies include mechanical, uncatalyzed steam-explosion, liquid hot water and high energy radiation. Wet milling, dry milling, vibratory ball milling and compression milling are examples of mechanical pretreatments. The disavantages of mechanical pretreatment are: (1) the high energy consumption and (2) requires additional thermal or chemical pretreatments (Gupta & Verma, 2015; Alvira et al., 2010; Silva et al., 2010). The advantages of steam explosion pretreatments are many: (1) high sugars recovery (2) industrial implementation feasibility (3) the soluble stream rich in carbohydrates (from the hemicellulose) in the form of oligomers and monomers which could be used as feedstock for the production of higher added-value products (4) lower environmental impact and (5) lower capital investment. The downside of this process is the generation of enzyme and yeast inhibitors: furfural and hydroxymethyl furfural, acetic acid, formic acid, levulinic acid and phenolic compounds derived from lignin degradation.

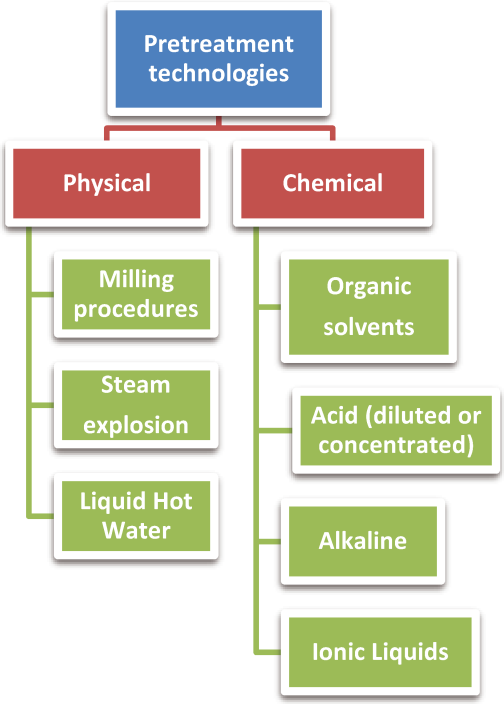

Figure 8. Example of different process of pretreatment mentioned in the text. Adapted from Gupta & Verma (2015).

Detoxification methods can be applied after the pre-treatment, representing additional costs to the overall process (Teixeira et al., 2014; Rocha et al., 2012). Liquid Hot Water pretreatments are based on the use of pressure to keep water in the liquid state at elevated temperatures (160 - 240ºC) to modify the lignin structure and to remove the hemicellulose content in the liquid fraction. The positive aspects of this pretreatment are: (1) does not use rapid decompression and chemicals (2) low-corrosion potential and consequently low cost materials and maintenance (3) higher pentosans recovery associated with the lower formation rate of inhibitors and (4) glucan content not altered at the end of the process. The negative aspect associated to the Liquid Hot Water pretreatments is the pH which should be kept at between 4.0 and 7.0 during the pretreatment to avoid the formation of inhibitors.

Chemical pretreatment technologies include acid, alkaline or ionic liquids. Acid pretreatment by using sulfuric acid is the most commonly employed chemical pretreatment for lignocellulose and can be performed under low acid concentration and high temperature or under higher acid concentration and lower temperature (Mood et al., 2013). This pretreatment hydrolyzes up to 90% of the hemicellulose fraction into monosaccharides, rendering higher accessibility of cellulose to enzyme hydrolysis (Teixeira et al., 2014; Sun & Cheng, 2002). A neutralization step that generates salt and biomass sugar degradation, formation of inhibitors,

corrosion of the equipaments along with higher maintenance costs, acid recovery and the environmental problems caused by waste streams are major drawbacks of this method (Mood et al., 2013; Alvira et al., 2010; Guo et al., 2010). The alkaline pretreatment causes less sugar degradation than the acidic pretreatments, but still requires neutralization step prior the enzymatic hydrolysis. In this pretreatment, similar to the Kraft pulping process used in the pulp and paper industries, lignin, acetyl groups and uronic acid substitution are removed from the biomass to reduce the steric hindrance of hydrolytic enzymes. The alkali pretreatment also causes partial hemicellulose removal, cellulose swelling and cellulose partial decrystallization. The process is operated at lower temperatures and does not require complex reactors; however, the reaction time frame takes long residence time (from hours to days) (Teixeira et al., 2014; Wan et al., 2011; McIntosh & Vancov, 2010). Ionic liquids (ILs)-based pretreatments are composed by organic salts (composed of cations and anions) able to dissolve carbohydrates and lignin simultaneously, altering cellulose crystallinity and structure (Tan et al., 2010). As innovative and promising biomass pretreatment technologies, ILs allows lignin recovery and can be tailored to suit the selective extraction and recovery of the biomass components (Tan et al., 2010). Although the process occurs under mild conditions and the reagents are easily recycled, ILs are still too expensive to be used for biomass pretreatment at industrial scale. The cellulase inactivation caused by ILs and high viscosity of the mixtures along the process describes some of the challenges associated to this biomass pretreatment technology (Fu & Mazza, 2011; Alvira et al., 2010; Singh et al., 2009).

In summary, from the literature cited above, only a small number of pretreatment methods have been reported as being potentially cost-effective, including uncatalyzed steam explosion, liquid hot water, concentrated and diluted acid (chemical treatments). The hydrothermal, steam explosion and acidic pretreatments remove mainly the biomass hemicellulose fraction whereas alkaline pretreatments remove lignin. On the other side, the cellulose crystallinity is not significantly reduced by pretreatments based on steam, or hydrothermal, or acidic procedures, whereas ionic liquid-based techniques can shift crystalline cellulose into amorphous cellulose. The choice of pretreatment, operational conditions and the composition of the enzyme blend used in the hydrolysis step determine the hexose and pentose sugars composition, the concentration and toxicity of the resulting biomass syrups.

1.3.2 Enzymatic hydrolysis

The second step in biochemical conversion of biomass is enzymatic hydrolysis to depolymerize the carbohydrate polymers to soluble sugars (Chundawat et al., 2011).

In nature, the biomass deconstruction through biological process involves the synergic action of cellulose, hemicellulose and lignin-degrading enzymes. Certain fungi like basidiomycetes, bacteria and insects have evolved the ability to degrade lignocellulose efficiently and for this reason, their lignocellulolytic systems have been extensively studied (Dionisi et al., 2015; Bourne & Henrissat, 2001).

In 1998, the Carbohydrate-Active enZYmes (CAZy) Database became available online to describe the families of structurally-related catalytic and carbohydrate-binding modules of enzymes able to degrade, modify, or create glycosidic bonds (Lombard et al., 2014). The enzyme classes covered by the CAZy are: Glycoside Hydrolases (GHs), Glycosyl Transferases (GTs), Polysaccharide Lyases (PLs) Carbohydrate Esterases (CEs) and Auxiliary Activities (AAs) (http://www.cazy.org) (Lombard et al., 2014). As research into carbohydrate active enzymes develops, new families are discovered.

Enzymatic release of oligosaccharides and monosaccharides from cellulose and hemicellulose is mediated by GHs, a large class of enzymes that exhibit both broad substrate specificities (Bourne & Henrissat, 2001).

According to the CAZy database, GHs are currently composed by 135 families into 14 clans (http://www.cazy.org). GHs can exhibit a diverse array of multi-modular configurations in the same genome. Polypeptides associated with plant cell wall hydrolysis commonly harbor a catalytic GH domain and a carbohydrate-binding module (CBM). CBM is defined as a contiguous amino acid sequence within a carbohydrate-active enzyme with a discreet fold having carbohydrate-binding activity (Shoseyov et al., 2006).

The classical model describing the action of free-enzyme systems involves three hydrolytic enzyme activities necessary for the complete degradation of crystalline cellulose (Fig. 9): Endoglucanase (1,4-β-D-glucan glucohydrolase), exoglucanase (1,4-β-D-glucan cellobiohydrolases) and β-glucosidase (β-D-glucoside glucohydrolase) (Bhat, 2000).

Endoglucanases randomly cleave β-1,4 glucosidic linkages within the backbone of cellulose. Exoglucanases act in a processive manner on the reducing or nonreducing ends of cellulose polysaccharide chains, liberating either glucose or cellobiose (cellobiohydrolase) as major products. Oligosaccharides released as a result of these activities are converted to glucose by the action of cellodextrinases, whereas cellobiose released mainly by the action of

cellobiohydrolases is converted to glucose by β-glucosidases (Phitsuwan & Laohakunjit, 2013; Hasunuma et al., 2013; Yeoman et al., 2010; Bhat, 2000).

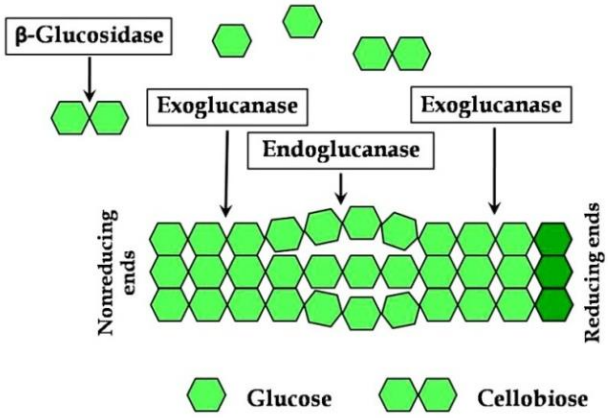

Figure 9. The classic model of enzyme degradation of cellulose. Glucose is represented by a green hexagon. Reproduced from Ratanakhanokchai et al., 2013 with modifications.

The discovery of oxidative enzymes lytic polysaccharide mono-oxygenases (LPMOs) involved in polysaccharide depolymerization revolutionized the classical knowledge of the enzymatic conversion of plant biomass (Fig. 10) (Garajova et al., 2016; Vaaje-Kolstad et al., 2010).

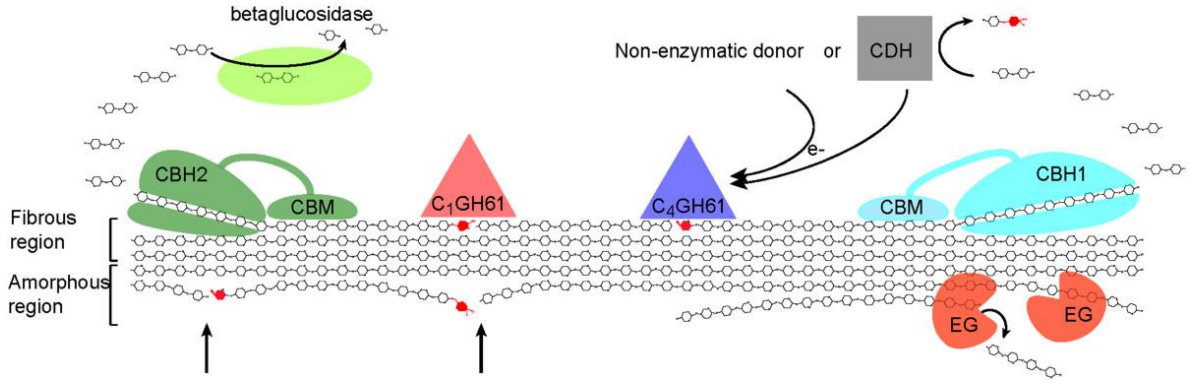

Figure 10. Current view on fungal enzymatic degradation of cellulose. Abbreviations: EG, endoglucanase; CBH, cellobiohydrolase; CDH, cellobiose-dehydrogenase; CBM, carbohydrate-binding module. The oxidation of C1 (pink) or C4 (blue) by LPMO (formerly GH61) generate non-oxidized ends for the CBH2 and the CBH1, respectively (oxidized sugars are colored red). The combined action of C1 and C4 oxidizing enzymes produce native cello-oligosaccharides from the middle of the cellulose chain. Reproduced from Horn et al. (2012).

LPMOs were classified in CAZy database in the AAs class, gathering four families (AA9, AA10, AA11 and AA13). Cellobiose dehydrogenases (CDHs), which act as electron donors to LPMOs and others oxidases, are present in the family AA3 (Levasseur et al., 2013).

LPMOs oxidize glucose-based oligosaccharides resulting in a non-reducing end and a C1-oxidized end, or a reducing end and an oxidation of the C4 at the non-reducing terminal (Fig. 11). The products of the subsequent actions of exo-cellulases and β-glucosidases are monomeric glucose molecules and their oxidized forms: gluconic acid and gemdiol 4-ketoaldose, from C1 and C4 oxidation respectively (Vaaje-Kolstad et al., 2010).

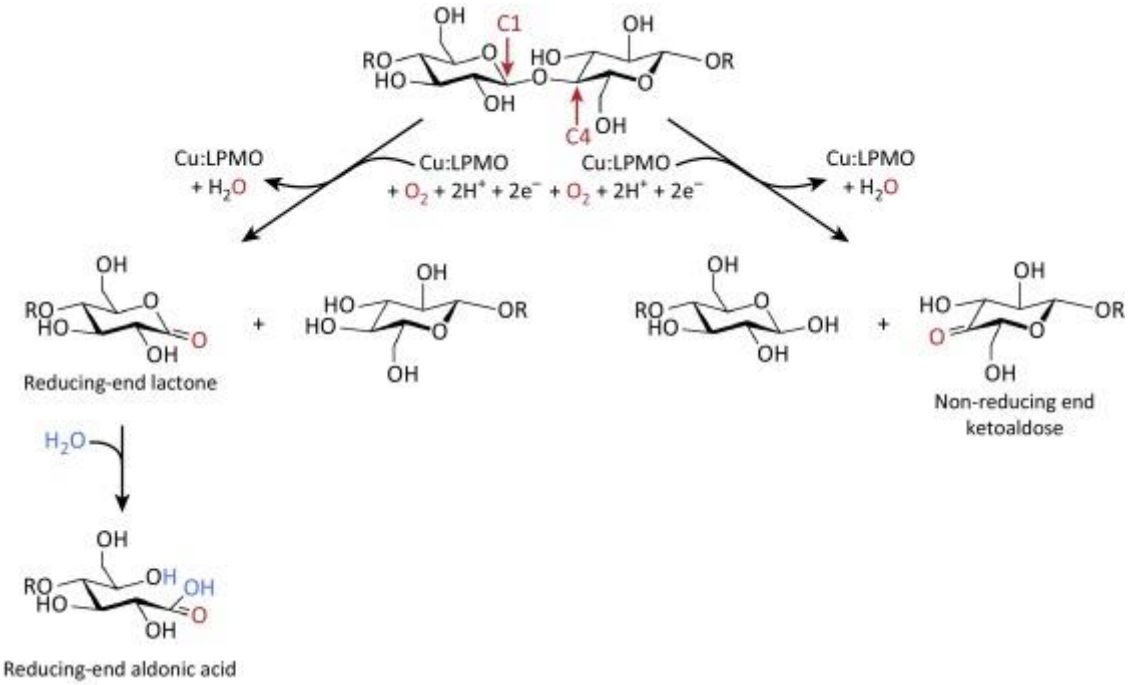

Figure 11. Oxidative cleavage of a polysaccharide chain carried out by LPMOs, showing different routes of oxidation carried out by LPMO subgroups. Reproduced from Hemsworth et al. (2015).

Because of their variable structure and organization, hemicellulose degradation requires a larger repertoire of GH and CE enzymes (Fig. 12) with diverse modes of action, which include endo-β-1,4-xylanases, xylan 1,4-β-xylosidases, α-L-arabinofuranosidases, α-glucuronidases, acetylxylan esterases, feruloyl esterases, mannan endo-1,4-β-mannanases, β-1,4-mannosidases and arabinan endo-1,5-α-L-arabinosidases (Yeoman et al., 2010; Collins et al., 2005).

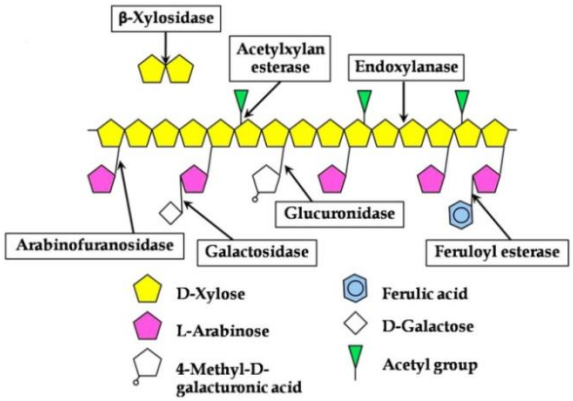

Figure 12. Enzyme systems involved in the degradation of cellulose. Reproduced from Ratanakhanokchai et al. (2013). Sugars, phenolics and acetyl groups are simplified by symbols.

For xylan deconstruction, endoxylanases cleave the backbone of xylan chains to release shorter xylooligosaccharides, which are further debranched by accessory enzymes. The release of xylose monomers from the non-reducing ends of debranched xylo-oligosaccharides is mediated by β-xylosidases (Fig. 12). α-Glucuronidases catalyze the cleavage of the α-1,2-glycosidic bond between 4-O-methyl α-glucuronic acid and the terminal non-reducing end xylopyranosyl unit of small xylo-oligosaccharides. Arabinan hydrolysis involves endo-arabinanases cleaving α-1,5 linkages between main chain arabinose residues. α-L-arabinofuranosidases release arabinose monomers by cleaving α-1,2, α-1,3, or α-1,5 linked arabinose residues from the non-reducing end (Yeoman et al., 2010).

The CAZy also included the ligninolytic enzymes among the families AA1, AA2, AA4 and AA6 since lignin is found invariably in the plant cell wall along with polysaccharides and lignin and low molecular weight lignans (LMWL) can also deliver electrons to LPMOs (Westereng-Cannella et al., 2015).

The recalcitrance of lignin to chemical and biological breakdown is the main challenge in lignocellulose conversion process. A variety of white-rot and brown-rot fungi, as well as bacteria, have been reported to degrade or modify lignin by employing different enzymes and pathways (Pollegione et al., 2015).

Lignin degradation is only partially understood and more difficult and slower due to complex structure and bonding to carbohydrate complexes. The process requires oxidative enzymes, such as lignin peroxidases (AA2), manganese peroxidases (AA2), versatile peroxidases (AA2), laccases (AA1) and several other auxiliary enzymes. The only organisms capable of substantial lignin decay are white rot fungi in the Agaricomycetes, which also

contains non–lignin-degrading brown rot and ectomycorrhizal species. These organisms employ a combination of these ligninolytic enzymes, organic acids, mediators and accessory enzymes (Floudas et al., 2012; Dashtban et al., 2010).

Lignin peroxidases are heme-containing glycoproteins. They catalyze the H_2O_2-dependent oxidative depolymerization of a variety of non-phenolic lignin compounds and a wide range of phenolic compounds with redox potentials up to 1.4 V without mediators by single electron abstraction. The substrate is oxidized by the enzyme in multi-step electron transfers and form intermediate radicals such as phenoxy radicals and veratryl alcohol radical cations. These intermediate radicals undergo non-enzymatic reactions such as radical coupling and polymerization, side-chain cleavage, demethylation and intramolecular addition and rearrangement (Brown & Chang, 2014; Wong, 2009; Piontek et al., 2001).

Manganese peroxidases are thought to play the most crucial role in lignin degradation, since it is found in all lignin-degrading white-rot fungi (Järvinen et al., 2012). This class of heme-proteins catalyzes the oxidation of Mn^{2+} −ions to highly reactive Mn^{3+}−ions. Chelated Mn^{3+} complex acts as a low molecular weight mediators able to attack phenolic structures (Fig. 13). However, the oxidation of non-phenolic substrates by Chelated Mn^{3+} complex requires chelators such as oxalate and malonate (Wong, 2009).

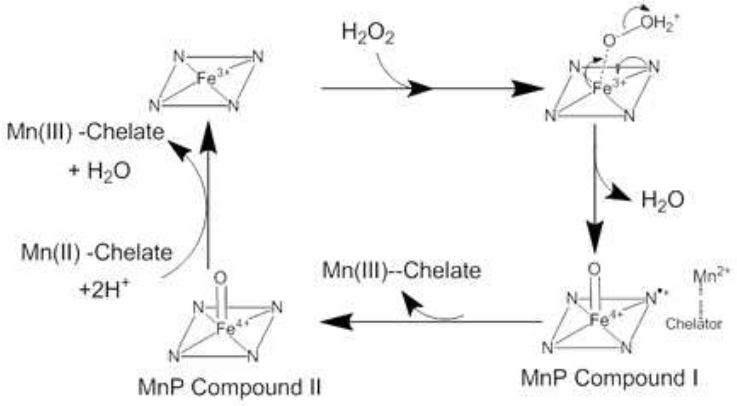

Figure 13. Manganese peroxidase mechanism with the heme cofactor represented via an iron-nitrogen complex. Reproduced from http://www.wikiwand.com/en/Manganese_peroxidase.

Versatile peroxidases combine catalytic properties of manganese peroxidase, lignin peroxidase (Mn-independent oxidation of non-phenolic aromatic compounds) and plant peroxidase (oxidation of hydro-quinones and substituted phenols). The manganese peroxidase component catalyzes the oxidation of Mn(II) to Mn(III) by H_2O_2. The highly reactive Mn(III) is stabilized via chelation in the presence of dicarboxylic acid (Pollegione et al., 2015: 2016).

The hybrid molecular structures which provide multiple binding sites for the substrates, makes these class of enzymes superior to other peroxidases, which are not able to efficiently oxidize phenolic compounds in the absence of veratryl alcohol or to oxidize phenols in the absence of Mn(ll) (Pollegione et al., 2015:2016, Dashtban et al., 2010; Camarero et al., 1999).

Comparative analyses of 31 fungal genomes suggest that lignin-degrading peroxidases expanded in the lineage leading to the ancestor of the Agaricomycetes, which is reconstructed as a white rot species, and then contracted in parallel lineages leading to brown rot and mycorrhizal species (Floudas et al., 2012).

Laccases are glycosylated blue multi-copper oxidoreductases widespread in plant and fungal sources. Fungal laccases are present in most of the wood-rotting fungi as a part of the lignin-degrading enzyme system (Heinzkill et al., 1998). In plants, laccases play a role in lignin polymerization (Ranocha et al., 1999).

Laccases catalyze monoelectronic oxidation of substrate molecules to corresponding reactive radicals with the assistance of four copper atoms that form the catalytic core of the enzyme, accompanied with the reduction of one molecule of oxygen to two molecules of water and the concomitant oxidation of four substrate molecules to produce four radicals (Fig. 14). The redox process is mediated by four copper ions in laccase catalytic center. Based on the copper's coordination and spectroscopic properties, the centers are differentiated as one type 1 (T1) or blue copper center, two type 3 (T3) and one type 2 (T2) copper ions. T1 copper confers the typical blue colour to multicopper proteins, which results from the intense electronic absorption caused by the covalent copper–cysteine bond. T2 and T3 form a trinuclear cluster coordinated by a highly conserved pattern of four histidine residues where reduction of molecular oxygen and release of water takes place (Claus et al., 2004; Kumar et al., 2003).

Because of the fact that all four copper atoms are fully oxidized (Cu^{2+}) in the enzyme native form, laccase can decarboxylate, demethylate and demethoxylate phenolic, and methoxyphenolic acids, which are important initial steps in lignin degradation (d'Acunzo, Galli, & Masci, 2002). The laccase-mediated formation of phenoxyl radicals results in cleavage of carbon–carbon and β-aryl bonds as well as aromatic rings in lignin. Due to the low redox potential of laccases (0.5–0.8 V), laccase alone can only oxidize phenolic lignin structures (Wong, 2009).

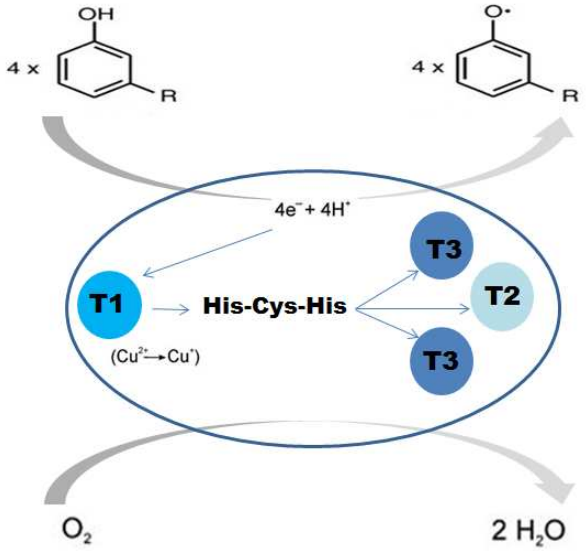

Figure 14. Catalytic cycle of laccases. The substrates are oxidized by the T1 copper and the extracted electrons are transferred, probably through a strongly conserved His-Cys-His tripeptide motif, to the T2/T3 site, where molecular oxygen is reduced to water. Adapted from Baldrian (2006).

However, in presence of low-molecular weight (LMW) chemical compounds (mediators), the substrate range of laccase can be expanded to include oxidation of non-phenolic lignin, which comprises more than 80% of the polymer composition (Wong, 2009). Therefore, the laccase-mediator-system (LMS) plays a key role in depolymerizing lignin. In addition, LMS has potential biotechnological applications such as detoxification of industrial effluents, soil bioremediation, manufacture of pharmaceuticals, food and cosmetic products (Christopher, Yao and Ji, 2014).

Nevertheless, it is difficult to predict the path of the reactions and the products that could be obtained since different reactions can occur as result of laccases or LMS action during lignin degradation. Lignin modification by laccases leads to changes in functional groups while bond cleavage may induce lignin depolymerization. Coupling events may induce grafting (changes in lignin properties) or polymerization. The nature of the lignin-derived compounds is influenced by the radical species generated along the reactions and the type of the mediator applied (Munk et al., 2015).

Employing oxidative enzymes can be considered as a promising strategy for detoxification of hydrolysate, removal of the remaining lignin from the biomass after pre-treatments and delignification steps. In particular, they are also hugely attractive to valorize lignin-enriched streams derived from biomass processing.

1.2 Biorefinery concept – lignin valorization

The major bottlenecks in cellulosic ethanol production are still associated to the processes of pretreatment and enzymatic hydrolysis. It is necessary to overcome these obstacles by cost effective pretreatment technologies and more efficient and less expensive enzymatic mixtures (Bussamra, Freitas & Carvalho da Costa, 2015; Goldbeck et al., 2014). The research and development toward commercial production of this green fuel have created the opportunity to dramatically increase the transformation of lignin to value-added products (Ayyachamy et al., 2013). The fully exploiting of lignin is indispensable to overcome the economic and sustainability challenges associated with the use of lignocellulose in the bio-product value chain (Isikgor & Becer, 2016; Brenelli et al., 2016).

Lignin is the only large-volume renewable feedstock that is composed of aromatics (Tuck et al., 2012). Nevertheless, in conventional chemical pulping processes and large-scale industrial processes that use lignocellulose, lignin is mainly used as a low-cost source of energy (Bujanovic et al., 2012).

The biorefinery concept is a new direction of research for biomass refining. The main idea is to combine process that creates separate streams of relatively clean lignocellulosic components. The carbohydrate-rich streams undergo further biochemical or chemical conversion to produce fuels/biochemicals and streams rich in lignin have high potential for the production of additional value-added products (Heiko, Decina & Crestini, 2013; Ayyachamy et al., 2013; Tuck et al., 2012).

The advantage of the biorefinery concept for lignin valorization is the possibility to choose the pretreatment technologies aiming products with tailor-made properties (Ragauskas et al., 2014). All this, combined with genetic manipulations of lignin to produce phenotypically normal plants with reduced recalcitrance, opens new prospects to valorize lignin (Fu & Mazza, 2011; Hisano et al., 2011).

The controlled breaking of C-C and C-O bonds in lignin represents a very selective depolymerization to produce different aromatic compounds and avoid the formation of a wide variety of products. However, the approach is challenging given the broad distribution of bond strengths in the various C-C and C-O linkages in the polymer and the tendency for low-molecular-weight species to undergo recondensation, often to more recalcitrant species (Fig. 6) (Ragauskas et al., 2014; Lange, Decina & Crestini, 2013).

Due to their unique properties, lignins have been recognized for many applications such as the use of lignin as a precursor for carbon fibers (Fig. 15) (Dutta et al., 2014; Khitrin et

al., 2010). Lignin by-products obtained through biomass conversion processes in the cosmetic pharmaceutical and food processing industries have been suggested. The capacity to reduce the production of radicals, stabilize reactions induced by oxygen and radical species without cytotoxic properties demonstrate their suitability for these new commercial applications (Vinardell et al., 2008; Dizhbite et al., 2004)

Lignin transformations can be performed by inorganic or organic catalysis. Inorganic catalysis includes lignin cracking, hydrolysis, reduction and oxidation. Generally, catalytic systems for lignin reduction produce bulk chemicals with reduced functionality, whereas catalytic systems for lignin oxidation produce fine chemicals with increased functionality (Zakzeski et al., 2010).

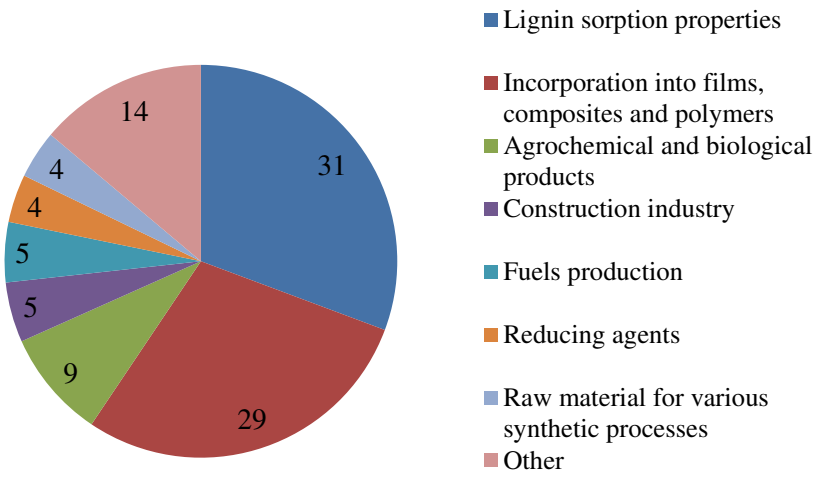

Figure 15. Distribution (%) of the applications of lignin in various industrial sectors according to Khitrin et al. (2010) for the past 10 years.

Fortunately, the emerging concept of green chemistry is designed to perform the inorganic catalysis in aqueous environments at milder reaction conditions (Gasser et al., 2012). The lack of detailed information regarding the performance of catalysts on the valorization of actual lignin streams and the influence of different pretreatments on lignin structure and undesired products present in the streams (proteins and inorganic salts) are some issues limiting the specificity of inorganic catalytic routes. In case of lignin depolymerization for example, the literature shows that single inorganic catalysts step has limited successes (Gasser et al., 2012).

Organic catalysis employing enzymes in industrial process is also challenging since the stability is lower compared to inorganic catalyst and is not easily recycled. On the other hand, biocatalysts are more selective to react with different lignin function groups and conduct

at mild reaction conditions. As described before, in nature, lignin is selectively degraded and modified by white-rot fungi through oxidative and auxiliary enzymes. In an industrial process, the paths for lignin decomposition should be suitable to the substrate and enzymes and at the same time, avoid the generation of free radical species which lead to undesired products generation (Munk et al., 2015; Lange, Decina & Crestini, 2013).

Summarizing, the lignin fractions of biomass should be transformed from a low-quality or low-price waste product into a high-quality or high-value feedstock for bulk and specialty chemicals by the development of the appropriate technology. As an essential tool to achieve the lignin valorization, biocatalysis has to be explored. Indeed, combining chemical and biological catalysis with continued advances in protein engineering, enzymology, synthetic biology and systems biology opens a new scenarium to develop and strengthen the concept of fully integrated biorefineries (Pollegione, 2016; Zhou & Tang, 2016; Dutta et al., 2014; Hisano, Nandakumar & Wang, 2011).

2. Project Justification and Contribution

The development of this PhD thesis was motivated by the commercial expansion of lignocellulosic biorefineries based on enzymatic deconstruction of plant polysaccharides, increasing the production of lignin-rich streams. Based on projected scenarios to integrate the first and second-generation ethanol production processes with the electrical cogeneration system, lignin streams are currently considered fuel in boilers to supply the required energy to run the plant. Thus, the development of strategies to fully exploit lignin potential is necessary to overcome the economic and sustainability challenges into lignocellulosic bio-product chain, as highlighted by recent publications.

The first part of this project concerns the upgrade of real lignin-enriched stream, from pilot-scale pre-treated sugarcane bagasse to value-added compounds. Several analytical methodologies were performed to evaluate lignin fractions, including small-angle X-ray scattering (SAXS), along with in vitro antioxidant capacity analysis against naturally occurring reactive oxygen and nitrogen species. This results the first comprehensive study combining biophysical properties of lignins with *in vitro* antioxidant capacity and relevant insights for research field of lignin valorization and the production of added-value products from lignocellulose.

The second part of the thesis was motivated by the lack of information in the literature on the interaction between lignin-degrading enzymes with lytic polysaccharide

monooxygenases during lignocellulose deconstruction. The discovery of AA9 class of cellulose oxidation enzymes made a revolution on the previous knowledge of plant cell wall degradation.

In the last year several articles gained the attention of the field investigating the electron delivery to LPMO enzymes. The positive synergism between lignin (working as activator) and the LPMO enzymes, on lignocellulose enzymatic hydrolysis, may be considered a key aspect for future of industrial process development. In the second study, the interplay between laccases and AA9 (LPMOs) enzymes in the plant cell wall degradation was investigated. Communities of scientists are working on lignin depolymerization, in one side, and cellulose degradation, on the other side. Laccases can assist the pretreatment of biomass and promote the subsequent enzymatic hydrolysis of cellulose by the oxidative modification of residual lignin on the biomass surface. Studies with lignin model compounds confirm the chemical possibility of a laccase-catalyzed cleavage of lignin bonds, but the strong polymerization activity of laccase counters the decomposition of lignin by repolymerizing the degradation products. Therefore, it is a key challenge to shift the catalytic performance of laccase towards lignin cleavage by optimizing the process conditions. These advances will benefit from these data showing that two oxidative systems might be connected.

The third part of the thesis arises from the necessity to explore new microorganisms as natural reservoir of new potential enzymes involved in lignin degradation and modification. Therefore, marine-derived basidiomycetes are promising sources of novel tools with biotechnological potential. In particular, enzymes isolated from extremophiles may have biological advantages over enzymes from terrestrial fungi because of their adaptations to high salinity and extremes pH. Focusing on the discovery of new enzymes with biocatalytic activities for lignocellulose deconstruction, the genome sequencing, gene annotation and secretome analysis of the marine-derived basidiomycete *Peniophora* sp. CBMAI 1063 grown under saline optimal conditions were performed. Omics analysis showed that this species has a complete versatile and unexplored ligninolytic arsenal with biotechnological potential.

3. Objective

The aim of the work described in this thesis was to study and explore chemical and biochemical routes to overcome the inherent heterogeneity of lignin-enriched streams and allow for the production of high-value and sustainable commodities.

3.1 Secondary Objectives

- Characterize by different techniques the chemical, physical and biological properties of lignin extracted from exploded sugarcane bagasse.

- Correlate the characterization data from the lignins fractions and the impact on the antioxidant properties and its biochemical conversion into value added products.

-Employ laccases and lacases-mediator system to modify and depolymerize lignin extracted from sugarcane bagasse and lignin present in lignocellulose.

-Explore a new marine-derived basidiomycete strain to identify new CAZymes, especially lignin-degrading enzymes able to modify lignins.

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CHAPTER 2 - Acidification treatment of lignin from sugarcane bagasse results in fractions of reduced polydispersity and high free-radical scavenging capacity

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Abstract

Lignin constitutes up to one-third of the material found in plant cell walls and is considered the second most abundant natural polymer in the world. Despite unique characteristics of lignin, it is mostly used for low-value commercial applications. In this study, lignin obtained after alkaline treatment of steam-exploded sugarcane bagasse was submitted to an acidification process. The soluble fractions produced at different pH values were comprehensively characterized and *in vitro* antioxidant capacity against reactive oxygen ($ROO^{\bullet}$ and H_2O_2) and nitrogen ($ONOO^-$) species was evaluated. The soluble fraction obtained at pH 2 exhibited the highest scavenging capacities against all species tested (10.2 ± 0.7 mmol Trolox equivalent g^{-1} for $ROO^{\bullet}$, IC_{30} = 14.9 μg mL^{-1} for H_2O_2 and IC_{50} = 2.3 μg mL^{-1} for $ONOO^-$) and the lowest polydispersity value (1.2) compared to others fractions. According to the SAXS data, the soluble fractions obtained at pH 4 and pH 2 consisted of small nanometer-sized discs and low molecular weight polyphenolic clusters, while soluble fractions obtained at high pH predominated wide lignin nanoparticles and larger aggregates. Mass spectroscopy analysis revealed the presence of phenolic and non-phenolic compounds, well-known as efficient antioxidants, which were identified in all soluble fractions. Collectively, our results provided further demonstration that acidification treatment is a promising strategy to upgrade heterogeneous lignin-enriched stream from sugarcane bagasse, such as preparations with homogeneous compositions and high antioxidant activity.

Abbreviations

SCB, sugarcane bagasse; AT, alkaline treatment; SF, soluble fraction; ROS, reactive oxygen species; RNS, reactive nitrogen species; TPC, total phenolic compounds; LMW, low molecular weight; ORAC, oxygen radical absorbance capacity; FTIR, Fourier transform infrared spectroscopy; GPC, gel permeation chromatography; ^{1}H NMR, proton nuclear magnetic resonance; Mn, number-average molecular weight; Mw, weight-average molecular weight; SAXS, small-angle X-ray scattering; GC–MS, gas chromatography–mass spectrometry

Keywords

Lignin; Sugarcane bagasse; Small-angle X-ray scattering; Reactive oxygen species; Reactive nitrogen species

1. Introduction

Sugarcane bagasse (SCB) is a renewable power generation source that provides energy to operate sugar and ethanol mills and bagasse-based bioelectricity, which is exported to the national grid (Bizzo et al., 2014). Furthermore, this lignocellulosic material has been considered promising resource to produce biofuel and other value-added products because of the relatively low cost, great abundance and low environmental impact (Mandelli et al., 2014 and Chundawat et al., 2011).

The biofuel production from SCB usually requires pretreatment and delignification steps, to separate lignin and hemicellulose from cellulose and reduce the crystallinity and enzymatic recalcitrance of plant biomass polysaccharides (Martínez et al., 2015, Benjamin et al., 2013 and Kuo and Lee, 2009). Most of the available SCB pretreatment technologies for ethanol production (e.g organosolv, hydrothermal, dilute acid and alkaline) often produce a large amount of lignin stream as major residue (Carvalho et al., 2015 and Dias et al., 2009). During lignocellulose pretreatment, lignin is generally extracted under conditions in which it is progressively broken down into lower molecular weight (LMW) fragments, resulting in considerable changes in physicochemical properties. Consequently, apart from the lignocellulosic biomass, lignin isolation procedure also affects the structure and purity of the final material (Doherty et al., 2011).

Lignin often constitutes up to one-third of the material found in plant cell walls and the second most abundant natural polymer in the world. This aromatic polymer is a complex polymeric and amorphous structure arising from the enzymatic dehydrogenative polymerization of coniferyl, sinapyl and *p*-coumaryl alcohol (Ayyachamy et al., 2013), although this proportion can vary depending on the source. Despite lignin's unique characteristics, it is mostly used for low-value commercial applications, such as combusted for energy production, and considered untapped biopolymers in biomass conversion technologies (Ayyachamy et al., 2013 and Doherty et al., 2011).

There are several studies demonstrating that lignin can serve as renewable resource for aromatic compounds with antioxidant capacity (García et al., 2010, Vinardell et al., 2008 and Pan et al., 2006). The recent literature has reported that the antioxidant capacity of

lignin fractions from steam-exploded bamboo stems and SCB are higher than the synthetic antioxidant dibutylhydroxytoluene (BHT) (Kaur and Uppal, 2015 and Sun et al., 2014).

Because of its high content of diverse functional groups (e.g., phenolic and aliphatic hydroxyls, carbonyls, and carboxyls) and phenylpropanoic structure, lignin can act as a neutralizer or inhibitor in oxidation processes and stabilize reactions that are induced by oxygen radicals and derivatives thereof (Randhir et al., 2004). Nevertheless, the antioxidant capacity of lignins significantly depends on the lignocellulosic material and isolation method employed (Ponomarenko et al., 2015, Li and Ge, 2012 and Dizhibite et al., 2004).

A number of studies have shown that high chemical heterogeneity of lignin, including differences in lignin macromolecule chemical structure, functionality and molecular mass distribution, can turn impractical its applicability as antioxidant in targeted systems (Ponomarenko et al., 2015 and Bikova et al., 2004). Recently, it was reported different strategies to obtain homogeneous lignin fractions with antioxidant activity, such as acid precipitation (dos Santos et al., 2014, Ma et al., 2013 and Faustino et al., 2010) and sequential solvent fractionation (Ponomarenko et al., 2015, Cui et al., 2014 and Li et al., 2012). The acid precipitation approach is based on differences in either solubility or molecular weight of lignins and it has been widely employed since 1960s for Kraft black liquor (Wada et al., 1962).

The expansion of commercial lignocellulosic biorefineries based on the enzymatic deconstruction of plant polysaccharides will increase the production of lignin-rich streams (Ragauskas et al., 2014). Based on projected scenarios for integrating first- and second-generation ethanol production processes in Brazil, with the electrical cogeneration system, lignin streams are currently considered fuel for boilers to supply the required energy power to run plants (Dias et al., 2013). Thus, the development of strategies for fully exploiting the potential of lignin is necessary to overcome the economic and sustainability challenges associated with the use of lignocellulose in the bio-product value chain.

This work focused on studying lignin derived from SCB after steam explosion and alkaline treatment (AT lignin), which is a consistent stream from the second-generation bioethanol pilot-scale study in Brazil (Rocha et al., 2012a and Rocha et al., 2012b). This AT lignin was submitted to a simple acidification method for dissolving the heterogeneous lignin mixture to generate soluble fractions with low polydispersity, similar phenolic compositions and high antioxidant capacity. In this study, the structural features of the soluble fractions were comprehensively investigated by different techniques, as well as, the *in vitro* antioxidant

capacity against reactive oxygen ($ROO^{\bullet}$ and H_2O_2) and nitrogen ($ONOO^-$) species were also evaluated.

2. Methods

2.1 Raw material

Lignin extracted from steam-exploded SCB with NaOH 1% (w/v) was provided by The Engineering School of Lorena (EEL-USP) and the process is described in detail in Rocha et al. (2012b). The material was named in this study as AT lignin (Alkaline Treatment).

2.2 Purity analysis and acid-insoluble and acid-soluble lignin determination

The impurities in the AT lignin (starting material) were defined as ash content (inorganic material), sugars (cellobiose, xylose, glucose, arabinose and galactose), acetic acid furfuraldehyde and hydroxymethylfurfural content. For ash analysis **c**rucibles were pre-dried to constant weight in a muffle furnace at 575 °C. The AT lignin samples were weighed into the crucibles and heated to 100 °C to remove moisture. The crucibles were subsequently heated at 300° C for 2 h and 800 °C for 2 h to constant weight (ASTM, 1966). The weight of the remaining ash was calculated as a percentage of the original dry weight of the sample. For insoluble and soluble lignin determination, carbohydrates, acetic acid and furfuraldehyde/hydroxymethylfurfural determination, approximately 2 g of AT lignin was treated with 10 mL of 72% sulfuric acid under vigorous mixing for 7 min in a bath at 45 °C (Gouveia et al., 2009). The reaction was interrupted thought the addition of 275 mL of distilled water and the solution was autoclaved for 15 min at 1.05 atm and 121 °C to complete the oligomer hydrolysis. The hydrolyzed material was separated from solids through filtration using a paper filter (Nalgon, 18.5 cm diameter) previously weighed. The solid in the paper filter was washed with distilled water which was added in the hydrolysate stored for further analysis. The lignin retained in paper filter was dried in oven at constant temperature of 105 °C and the content was obtained by gravimetry. Soluble lignin in the hydrolysate was determined by UV spectroscopy as previously described (Rocha et al., 2012a). The results reported as a percentage of the original dry weight of the sample. Sugars, acetic acid and furfuraldehyde / hydroxymethylfurfural content in the hydrolysate were determined through high performance liquid chromatography (HPLC) as described in Rocha et al. (2012a) and reported as a percentage of the original dry weight of the sample.

2.3 Acidification treatment

AT lignin acidification treatment was achieved by adding sulfuric acid (Fig. 1) in order to obtain the Soluble Fractions (SF) in different pH conditions. Approximately 20 g of AT lignin was dissolved in 2 L of NaOH aqueous solution (400 µL NaOH 10 mol L^{-1} in 2 L distilled water) at pH 12 (SF-pH12). Sulfuric acid 1 mol L^{-1} was slowly added to SF-pH12 under stirring until reaching a pH value of 10. The pH value was allowed to stabilize for 30 min. The solution was centrifuged at 1878 *g* for 20 min at 4 °C, and the supernatant (soluble fraction) separated from the precipitate (discarded). An aliquot (200 mL) was removed from the supernatant and named SF-pH10. The same procedure was conducted to the remainder of the solution to obtain SF-pH8, SF-pH6, SF-pH4 and SF-pH2. The pH value stabilization was allowed to stabilize for 30 min, and the pH value was adjusted if necessary. All aliquot fractions were dried at 30 °C under normal atmosphere. Dry powdered fractions were used for further analysis. The concentration of soluble lignin was determined using UV-spectroscopy as previously described (Rocha et al., 2012a).

2.4 Determination of the phenolic hydroxyl content

The phenolic hydroxyl content was determined using the differential scanning UV-spectroscopy method adapted based on the average absorptivity of the model lignin compounds (Wexler, 1964). A solution was prepared by diluting 1 mL of lignin solution (20 mg mL^{-1}) in 10.0 mL of dioxane 50% (v/v). The pH values of the solutions were adjusted to 14 with sodium hydroxide (1 mol L^{-1}). A blank of each sample was prepared in the same dilution at pH 1 using hydrochloric acid (1 mol L^{-1}). The sweep of the alkaline solution against the acid solution was performed. The results were expressed in percentage of phenolic hydroxyl. Equation 1 describes the phenolic hydroxyl content determination.

$$\%OH\text{-}Ph = (\Delta Abs_{250nm} \times 0.192) / Clig \quad (1)$$

where %OH-Ph = phenolic hydroxyl content in percentage; ΔAbs_{250nm}= absorbance of the solution at 250 nm subtracted from the relative absorbance of the baseline spectrum; Clig = Lignin concentration in g L^{-1}.

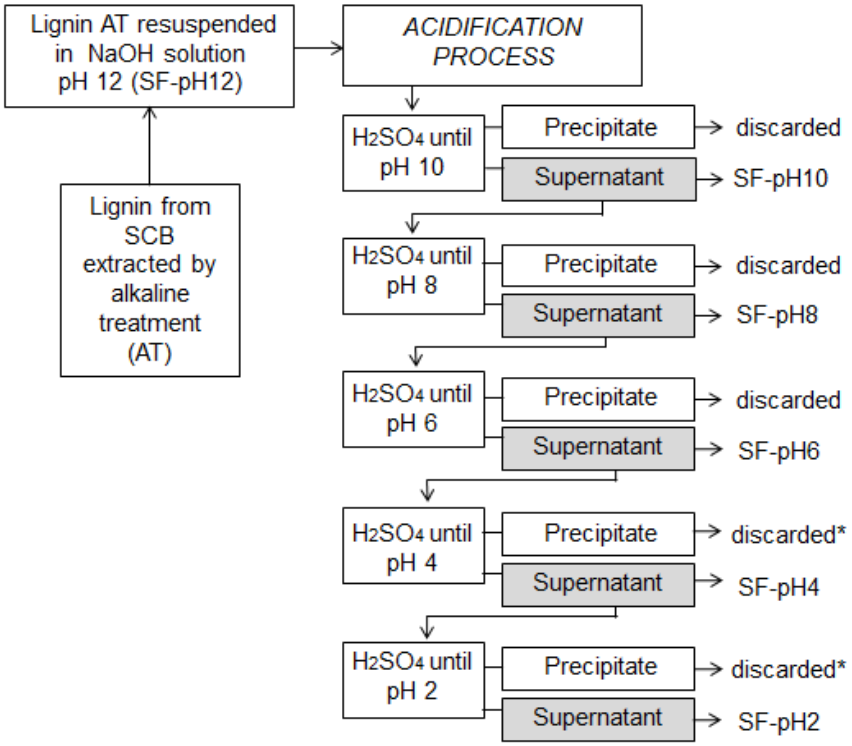

Fig.1. Scheme for the acidification process protocol of lignin. *Large amount of precipitate was observed at pH 4 and 2.

2.5 Fourier transform infrared spectroscopy (FTIR) analysis

Dry powdered fractions were dissolved in pure chloroform, filtered using a paper filter and dried at 30 °C under normal atmosphere to eliminate the sodium sulphate formed during the acidification treatment. The FTIR analyses were performed using a Perkin-Elmer FTIR Spectrum^{TM} 400 Series FTIR/FT-NIR, which was equipped with Attenuated total reflectance (ATR) and zinc selenide. The spectral resolution of the apparatus was 4.0 cm^{-1}. For each sample, 32 spectra were accumulated in the range of 500-4000 cm^{-1} and averaged. The region between 1900 cm^{-1} and 1850 cm^{-1} was chosen for normalization procedure.

2.6 Proton nuclear magnetic resonance (1H-NMR) analysis

Approximately 100 mg of lignin was acetylated in 2 mL of an acetic anhydride: pyridine solution (1:1, v/v), which was kept at room temperature for 18 h under stirring. After stripping with chloroform, pyridine was removed with hydrochloric acid solution (7%) and water. The samples were dried in vacuum and solubilized in $CDCl_3$, which contained TMS 1% (tetramethylsilane). The quantitative ^{1}H-NMR spectra of the lignin fractions were recorded on

an Agilent DD2 spectrometer operating at a Larmor frequency of 499.726 MHz at 25 °C. The parameters for data acquisition were: pulse width of 10.250°, spectral window of 8000 Hz, and 256 time increments were recorded. The spectra were calibrated based on the signal from TMS.

2.7 Gel permeation chromatography (GPC) analysis

The number-average molecular weight (Mn) and weight-average molecular weight (Mw) of acetylated lignin fractions were evaluated in a Shimadzu apparatus, which was equipped with a UV detector and an auto sampler. The samples were injected onto a series of three PLGel (500, 10^3 and 10^4 Å) columns including a guard column, eluted at 35 °C, a pressure of 71 kgf cm^{-2}, a flow rate of 1 mL min^{-1} of tetrahydrofuran (THF) and detected at 280 nm. The injection volume was 50 µL, and the concentration of the injected solutions, whether they contained standards or samples, was fixed at 40 mg of material dissolved in 1 mL of THF. Standard polystyrene (Sigma Aldrich) samples were used to construct a calibration curve (molecular weight range from 350 kDa to 165 Da). The average molar mass (M) of a polydispersed polymer results from several possible methods that average different present species according to Equation 2.

$$M = (\sum Ni\ Mi^{n+1}) / \sum (Ni\ Mi^{n}) \quad (2)$$

Due to the differences in chemical structure between the lignins and polyphenols and the calibration polystyrene standards, the data only represent the relative Mw and Mn.

2.8 Small-angle X-ray scattering (SAXS)

SAXS measurements of all studied soluble fractions were conducted at the D02A-SAXS2 beam line of the Brazilian Synchrotron Light Laboratory, Campinas, Brazil. The SF-pH4 and 2 fractions were purified to eliminate impurities using a Superdex 30 Pep Grade and NaOH 0.1 mol L^{-1} as eluent.

Soluble fractions were studied by SAXS using a monochromatic X-ray beam (wavelength λ=1.55 Å). 2D X-ray patterns were recorded by a two-dimensional CCD X-ray detector (MarResearch, USA). The sample-to-detector distance was set at 1000 mm, thus allowing for a modulus of accessible scattering vector ranging from q= 0.14 nm^{-1} up to 3.4 nm^{-1}, $q = 4\square \sin\square /\lambda$, where $\square$ is half the scattering angle. All soluble fractions were prepared in aqueous 10% (m/v) NaOH solution seven days prior to SAXS analyses (Maziero et al., 2012). The SAXS

intensity curves were determined under several dilution conditions thus yielding three different lignin solutions at 2.5, 5 and 10 g L^{-1}.

For SAXS measurements, lignin solutions and solvent were successively placed in a sample cell with two thin parallel windows (1 mm of path length) maintained at approximately 25 °C. Two successive frames of 300 s each were recorded for each sample to evaluate eventual effects of radiation damage and X-ray beam instabilities.

The experimental SAXS patterns were corrected for detector response and normalized to equivalent incident beam intensity, samples absorption and lignin concentration. The background (solvent + parasitic) scattering intensity was subtracted from the total intensity curves. The recorded isotropic 2D SAXS patterns were integrated to obtain 1D scattering intensity, $I(q)$, by using the Fit2D software (Hammersley, 1997). The fitting analysis using modelled SAXS intensities that allowed us to determine relevant structural parameters of all soluble lignin fractions was performed by applying the SASFIT package (Kohlbrecher, 2006).

2.9 Determination of the total phenolic compounds (TPC)

The Folin-Ciocalteu colorimetric method of Singleton (Singleton et al., 1999) was adapted to a microplate reader (Synergy Mx; Biotek, USA) for TPC determination. The reaction mixtures in the sample wells contained the following reagents: 150 µL of ultrapure water, 25 µL of soluble fractions in different concentrations and 25 µL of the Folin-Ciocalteu reagent. The mixture was incubated in the microplate reader, shaken for 20 s, and maintained at 25 °C for 5 min. This process was followed by adding 100 µL of a 7 % sodium carbonate solution. The absorbance signal was measured after 2 h at a wavelength of 765 nm. The results were expressed in milligrams of gallic acid equivalent per gram of lignin.

2.10 Reactive oxygen and nitrogen species scavenging (ROS and RNS) assays

A microplate reader equipped with a thermostat (Synergy HT, Biotek, Vermont, USA) for fluorescence and chemiluminescence measurements was used for all assays. Each ROS- and RNS-scavenging result corresponds to five concentrations and was performed in triplicate.

2.10.1 Peroxyl radical scavenging assay

The $ROO^{\bullet}$ scavenging capacity was measured by monitoring the effect of the soluble fractions on the fluorescence decay resulting from $ROO^{\bullet}$-induced oxidation of fluorescein, using the oxygen radical absorbance capacity (ORAC) method (Rodrigues et al., 2013). $ROO^{\bullet}$ was

generated by the thermodecomposition of α,α'-azodiisobutyramidine dihydrochloride (AAPH) at 37 °C. The reaction mixtures in the wells contained the following reagents at the indicated final concentrations (final volume of 200 µL): fluorescein (61 nmol L^{-1}), AAPH solution in phosphate buffer (19 mmol L^{-1}) and soluble fractions in different concentrations. The mixture was preincubated in the microplate reader for 10 min before the addition of AAPH. The fluorescence signal was monitored every minute for the emission wavelength of 528 ± 20 nm with excitation at 485 ± 20 nm over a period of 180 min. Trolox was used as the positive control (Net area (64 µmol L^{-1}) = 23). Briefly, the net protection provided by the extracts or Trolox (standard) was calculated using the difference between the area under the fluorescence decay curve in the presence of the sample (area under the curve $AUC_{extract}$) and in its absence (AUC_{blank}). Regression equations between the net AUC and the sample concentrations were calculated for all extracts and Trolox. The results of the peroxyl radical scavenger capacity of the extracts were expressed as Trolox equivalents in micromoles per gram of lignin.

2.10.2 Hydrogen peroxide scavenging assay

The H_2O_2 scavenging capacity of soluble fractions was measured by monitoring the H_2O_2-induced oxidation of lucigenin (Gomes et al., 2007). Reaction mixtures contained the following reagents at the final concentrations (final volume of 300 µL): 50 mmol L^{-1} Tris HCl buffer (pH 7.4), lucigenina solution in Tris HCl buffer (0.8 mmol L^{-1}), 1% (w/w) H_2O_2 and lignin in different concentrations or Trolox (five concentrations). The chemiluminescence signal was detected in the microplate reader after 5 min of incubation. Ascorbic acid was used as the positive control. The results were expressed as the inhibition in percentage (IC_{30}) of the H_2O_2-induced oxidation of lucigenin.

2.10.3 Peroxynitrite scavenging assay

The $ONOO^-$ scavenging capacity was measured by monitoring the $ONOO^-$-induced oxidation of non-fluorescent dihydrorhodamine (DHR) to fluorescent rhodamine (Gomes et al., 2007). $ONOO^-$ was synthesized as previously described (Beckman et al., 1994). The reaction mixtures contained the following reactants at the indicated final concentrations (final volume of 300 µL): DHR (5 µmol L^{-1}), $ONOO^-$ (600 nmol L^{-1}) and lignin in different concentrations or Trolox (five concentrations). The fluorescence signal was measured in the microplate reader after 5 min of incubation at emission and excitation wavelengths of 528 ± 20 nm and 485 ± 20 nm, respectively. The assays were performed in the presence of 25 mmol L^{-1} $NaHCO_3$ to simulate

the physiological CO_2 concentration once under physiological conditions; the reaction between $ONOO^-$ and bicarbonate is predominant ($k = 3–5.8 \times 10^4\ M^{-1}\ s^{-1}$), which generated nitrogen dioxide ($NO_2^{\bullet}$) and carbonate radical anion ($CO_3^{\bullet-}$). Ascorbic acid was used as the positive control ($IC_{50} = 0.31\ \mu g\ mL^{-1}$). The results were expressed as the inhibition in percentage (IC_{50}) of the $ONOO^-$-induced oxidation of non-fluorescent DHR to fluorescent rhodamine.

2.11 Gas Chromatography / Mass Spectrometry analysis (GC-MS)

The profile of soluble fractions was performed with derivatized samples by a GC-MS system (Agilent GC 6890 and MSD 5973N series, Agilent, USA), according to Suguiyama et al. (2014). The samples were derivatized with 200 µL of pyridine and 50 µL of N, O-bis[trimethylsilyl] trifluoroacetamide (BSTFA), shaken carefully and placed on a heating block at 75 °C for 1 h (tubes were shaken every 10 min in vortex). The samples were transferred to a crimp-cap GC vial and submitted for analysis. GC was performed on a 30 m DB-1701 column with 0.25 µm film thickness (Agilent Technologies, USA). The injection temperature was set at 230 °C, the interface at 250°C, and the ion source adjusted to 150 °C. Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}. The analysis was performed under the following temperature program: 5 min of isothermal heating at 70 °C, followed by a 5 °C min^{-1} oven temperature ramp to 310 °C, and a final 1 min of heating at 280° C. Mass spectra were recorded at 2 scan s^{-1} with a scanning range of 50 to 600 m/z. Both chromatograms and mass spectra were evaluated using the Chem Station program (Agilent Technologies, USA) and the software AMDIS (NIST, USA). The peaks were identified and quantified in comparison with authentic standards and the NIST Mass Spectral Library.

2.12 Correlations and statistical analysis

Triplicate analyses of the samples were performed throughout the experiments. Statistical analyses were performed using the Statistica 10.0 software at 95% confidence level. The results were expressed as the averages of triplicate experiments ± SD (standard deviation). Differences were considered significant at $p < 0.05$. A simple regression analysis with two independent variables was used for the ROS and RNS scavenging assay and TPC results. To establish the relationship among the TPC, Mw and the ROS and RNS scavenging assay results using a simple regression analysis with independent variables, we assumed an x factor [TPC/ Mw] and a y factor [ORAC/ (IC_{30} H_2O_2* IC_{50} $ONOO^-$)].

3. Results and Discussion

3.1 Alkali treatment of steam-exploded SCB was effective to produce almost pure lignin.

Future biorefineries will integrate total biomass conversion processes and provide sustainable chains for producing fuels, power, and value-added products from biomass feedstock. Thus, a large amount of lignin will be generated from this biomass conversion process, making it available for different purposes. One factor that may restrict the use of lignin fractions to produce high-value-added materials and applications is the presence of organic and inorganic impurities. Several impurities such as sugars, silicates, sulfur, ash, proteins and other compounds derived from either raw materials or the delignification process can be produced after the pretreatment step (El Mansouri and Salvadó, 2006). The lignin examined in this study was produced from pilot-scale steam explosion pretreatment of SCB followed by alkaline delignification described by Rocha et al. (2012b). According to mass balance data, approximately 91% of the lignin contained in the steam-exploded solids was solubilized by delignification, resulting in a pulp with almost 90% of cellulose content. After acidification of the black liquor, 48.3% of the lignin originally contained in the raw material was recovered (Rocha et al., 2012b).

The acid-insoluble lignin (Klason lignin), acid-soluble lignin, sugars and decomposition products content after treatment of AT lignin with 72% sulfuric acid were determined. The percentage of acid-insoluble and acid-soluble lignin in AT lignin was 91.29 ± 0.63 % and 6.02 ± 0.22 % (w/w), respectively. Xylose was the only sugar detected in the hydrolysate at 0.19 ± 0.07 % (w/w). Furan aldehydes and acetic acid was not detected. The ash content determined in solid AT lignin was 2.92 ± 0.03 % (w/w).

The low amount of impurities in the starting material indicate that the approach based on steam explosion, followed by alkali precipitation, as described in Rocha et al. (2012b), produced almost pure lignin from SCB.

3.2 Fractions with similar chemical structures were produced after the acidification steps

The potential utilization of lignin to produce value-added compounds can be affected by heterogeneous nature of lignin. In this study, AT lignin (Fig. 1) was acidified with sulfuric acid (1 mol L^{-1}) to yield soluble fractions, composed by lignins and LMW phenolic compounds, of homogeneous molecular weight and chemical functionality. This process is

based on the solubility of lignins and LMW phenolic compounds at different pH values. The initial pH of the black liquor was 12, and no precipitate formation was observed during the first solubilization step. The progressive decrease from pH 12 to 2, which was achieved by adding sulfuric acid (1 mol L^{-1}), increased formation of precipitate and lightened the liquor's color. Two zones of high lignin precipitation yield were observed at pH 4 and pH 2. The resulting soluble fractions SF-pH4 and SF-pH2 presented polyphenolics concentration of 4.44 g L^{-1} and 0.5 g L^{-1}, respectively (Table 1). In a similar experiment, García et al. (2009) used black liquor from *Miscanthus sinensis* pulping to precipitate lignin. The authors also observed different zones of meaningful precipitation, but over pH 10–11, which was attributable to the silicates in the raw material, and pH 7–4.

The soluble fraction yield (total amount of lignins and LMW phenolic compounds) in percentage of dry mass of SCB was calculated based on the mass balance of the pretreatment process (Rocha et al. 2012b). The highest yield obtained was 12% for SF-pH12 (Table 1) because in this pH condition AT lignin was totally soluble. The lowest yield was obtained at SF-pH2, about 1%.

It is known that oxidative degradation of ether linkages during alkaline treatment can increase hydroxyl groups content (Li and Ge, 2012). In this study, phenolic hydroxyl content was determined using differential scanning UV spectroscopy (Table 1). The SF-pH12 showed highest phenolic hydroxyl content (4.99%), whereas the SF-pH2 showed the lowest value (3.54%). Using a titration method, Mousavioun and Doherty (Mousavioun and Doherty, 2010) obtained a notably similar value for the phenolic hydroxyl content (5.1%) from lignin derived from SCB *in natura* after alkaline treatment.

The FTIR spectra of the soluble fractions are presented in Fig. 2. As shown in Fig.2, the polysaccharide bands are not detected in the fractions. The results showed that the linkages between lignin and polysaccharides in sugarcane bagasse were completely cleaved during lignin alkali extraction from steam-exploded SCB described in Rocha et al. (2012b). The spectral patterns between SF-pH12 and 10 fractions and between SF-pH8 and 6 were notably comparable, suggesting that these fractions possessed similar lignin chemical compositions. A strong band around 1700 cm^{-1} observed for the SF-pH2 fraction has been assigned to carbonyl stretching group in unconjugation with the aromatic ring (Faix, 1994). García et al. (2009) observed that intensity of the carbonyl group increased after application of an acidification step to lignins obtained from apple tree pruning, which suggested oxidation or degradation of some phenolics groups. Probably, a significant number of polyphenols in the SF-pH2 fraction were

likely oxidized. The bands at 1600 cm^{-1} and 1516 cm^{-1} which is attributed to aromatic skeleton vibrations and aromatic skeleton vibrations of guaiacyl and syringyl units, respectively, were observed for all fractions and with a higher intensity signals in SF-pH4 and 2 fractions (Faix, 1994). Asymmetric C-H deformations appear at 1460 cm^{-1} (Faix, 1994) in all fractions and higher intensity signals in SF-pH4 and 2 fractions. The band at 1330 cm^{-1} corresponds to the syringyl and guaiacyl condensed rings (Faix, 1994). Absorption at 1166 cm^{-1} only observed in SF-pH2 spectra indicates C=O stretching in conjugated ester groups. The bands at 1105 cm^{-1}, 1030 cm^{-1} and 830 cm^{-1} attributed to aromatic C-H in plane deformations (syringyl type) and C-H out-of-plane vibrations in position 2, 5 or 6 of guaiacyl units, respectively, were observed in all fractions spectra with stronger signals in SF-pH4 and 2 fractions (Faix, 1994).

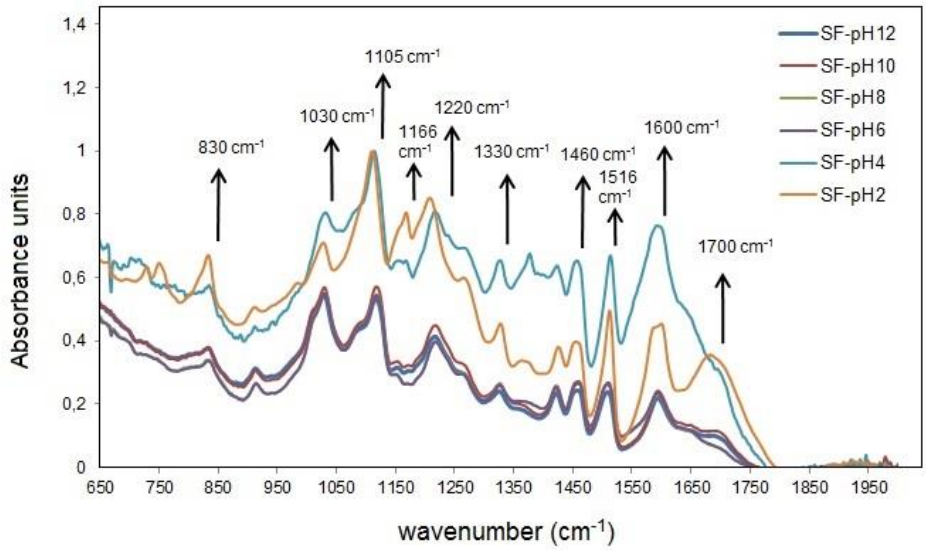

Fig. 2. FTIR spectra of soluble fractions.

The chemical structure of acetylated soluble fractions was studied using ^{1}H-NMR spectrometry; the spectra are shown in Fig. 3. The SF-pH 12-6 fractions showed similar spectra with typical regions of lignin fragments (García et al., 2009), whereas the SF-pH 4-2 fractions showed a more defined profile with the region typical of LMW phenolics (Yoshimura et al., 2015).

The integral of all signals between 6.4 and 8.0 ppm can be attributed to aromatic protons. More specifically, the integrals of the signals centered at 6.6 and 6.9 ppm observed only for SF-pH2 are assigned to aromatic protons in syringyl and condensed guaiacyl structures, respectively. The signal around 7.4 ppm in SF-pH2 spectra and discreet for SF-pH4 confirming the presence of *p*-hydroxyphenyl units and C_{α}=O groups in these fractions. The signal is

attributed to the aromatic protons in positions 2 and 6 in structures containing a C_α=O group, to aromatic protons in positions 2 and 6 of *p*-hydroxyphenyl units conjugated with a double bond and also protons in C_α=C_β structures (Sun et al., 2003). The signal around 6.0 ppm and 5.5 ppm in SF-pH2 spectra is related to Hα in β-O-4 and β-5 structures, respectively. The integral of signals between 0.85 and 1.20 ppm, more intense in SF-pH4 and 2, can be attributed to the aliphatic moiety. Methoxyl protons (OCH_3) produced an intense signal centered at 3.75 ppm in SF-pH12-6 fractions. Acetylation step was necessary to solubilize lignin and LMW phenolic compounds in $CDCl_3$; however, derivatives displaying broad proton signals were produced between 1.65 and 2.70 ppm. In addition, aromatic and aliphatic acetyl groups were not shifted differently, which led to peaks overlapping.

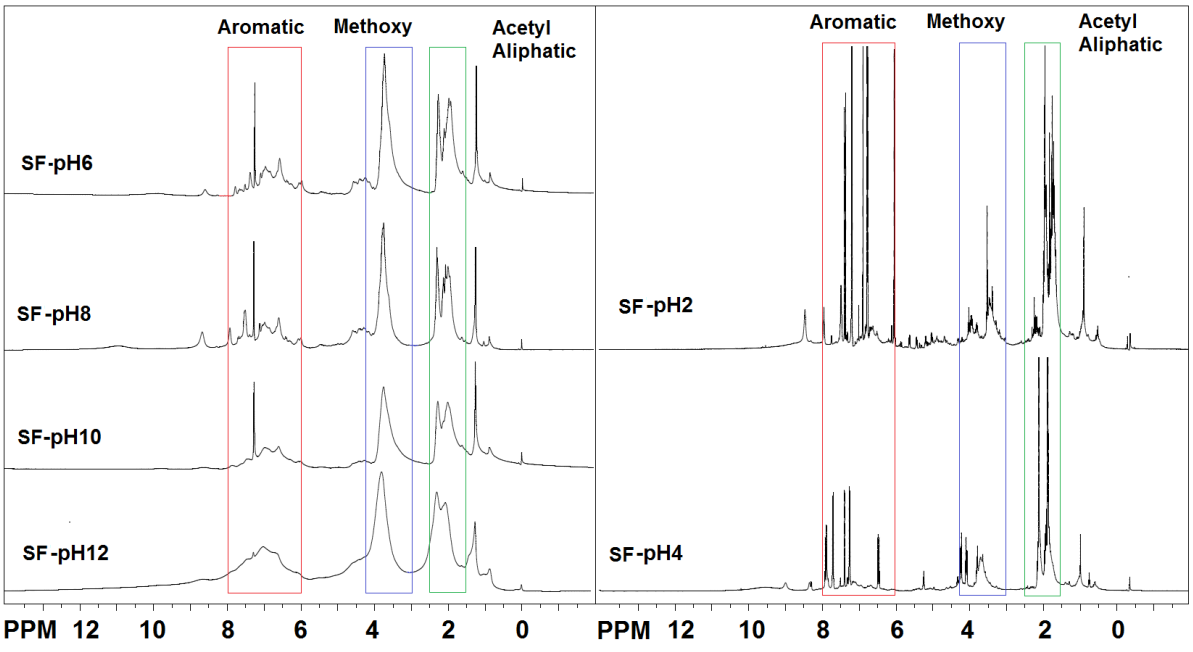

Fig. 3. ^{1}H-NMR spectra of acetylated soluble fractions.

Table 1. Chemical and physical properties and scavenging capacities of soluble fractions

	Chemical and physical properties							*Scavenging capacities* ***		
Sample	**Soluble fraction yield (%)**	**Soluble fraction concentration (g L^{-1})**	**Phenolic Hydroxyl content***	**Mw**	**Mn**	**Mw/Mn**	**TPC****	**ROO•**	**H_2O_2**	**$ONOO^-$**
SF-pH12	12	10	4.99 ± 0.10	4438	661	6.71	278.5 ± 11.3^{a}	3830.7 ± 134.2^{a}	30.0^{a}	2.4^{a}
SF-pH10	10	8.3	4.71 ± 0.07	4254	702	6.05	317.5 ± 26.5^{ade}	3765.9 ± 672.4^{ab}	30.5^{b}	2.8^{b}
SF-pH8	10	8.2	4.94 ± 0.04	9359	605	15.47	297.9 ± 20.3^{a}	2749.7 ± 223.8^{c}	34.1^{c}	3.2^{c}
SF-pH6	10	8.13	4.66 ± 0.02	9068	878	10.3	284.7 ± 18.6^{ac}	2868.9 ± 307.4^{ac}	39.9^{d}	2.3^{d}
SF-pH4	5	4.44	3.98 ± 0.03	488	342	1.43	269.1 ± 18.8^{ac}	3646.3 ± 238.1^{ab}	29.6^{e}	3.4^{e}
SF-pH2	1	0.55	3.54 ± 0.09	409	339	1.21	430.8 ± 42.8^{b}	10167.8 ± 694.7^{d}	14.9^{f}	2.3^{d}

Legend. The characteristic of the soluble preparations are presented: Soluble fraction yield (%), Soluble fraction concentration (%) in the supernatant, Phenolic hydroxyl content in the supernatant, weight average (Mw), number average molecular weight (Mn), Polydispersity (Mw/Mn) of acetylated soluble fractions, Total phenolic compounds (TPC), peroxyl radical (ORAC), hydrogen peroxide (H_2O_2)-, peroxynitrite ($ONOO^-$)- scavenging capacities of soluble fractions. Soluble fraction yield (%) expressed as percentage relative to the oven-dry mass of SCB, these results were calculated according to the mass balance data described from previous study of our group using the same raw material (Rocha et al., 2012b).

* n=3. Note: The lignin concentration in the supernatant was estimated using Equation 1 developed for the original lignin (AT). **Total phenolic compounds expressed as mg gallic acid equivalent. g $lignin^{-1}$ *** ORAC, Hydrogen Peroxide and Peroxynitrite assay were expressed as µmol TE g $lignin^{-1}$, IC_{30} IC_{50} and (µg mL^{-1}), respectively. IC_{30} and IC_{50} is inhibitory concentration, in vitro, to decrease (by 30% and 50%, respectively) the oxidative effect of reactive species in the tested media. Values represent the average of triplicates ± standard deviation; means followed by different letters in the same column differ statistically ($p < 0.05$) by the Tukey test. TE is Trolox equivalent.

3.3 Large lignins aggregates, small nanometer-sized discs and polyphenolics clusters were found in the different lignin fractions

To investigate the effect of acidification process on molecular weight of the AT lignin, soluble fractions obtained after acetylation protocol were first analyzed by GPC. Table 1 shows Mw, Mn and polydispersity (Mw/Mn) of soluble fractions.

The first two soluble fractions (SF-pH12 and SF-pH10) exhibit notably similar Mw (~4346 Da), Mn (~680 Da) and polydispersity (~6.4). According to Li and Ge (2012), similar Mw (4300 Da) and a lower polydispersity (2.86) were observed for sugarcane lignin derived from alkali process. However, Li and Ge (2012) used a different process for sugarcane bagasse pretreatment.

The SF-pH8 and SF-pH6 fractions exhibited the highest Mw (~ 9200 Da) and polydispersity (10.3 and 15.5, respectively). The highest Mw for these fractions can be attributed to acetylation step (required to perform GPC analysis) that could result in chemical modification of lignin. According to GPC data, in SF-pH4 and SF-pH2 fractions predominated LMW phenolics with low Mw (~ 448 Da), Mn (~ 340 Da) and polydispersity (1.32).

SAXS is an experimental technique widely used for structural characterization of colloidal or nanoscopic (organic or inorganic) particles in solution (Glatter and Kratky, 1982). This technique provides useful information about shape, dimension and size distribution of macromolecules in dilute solutions, as well as, the nature of molecular interactions. SAXS studies were previously applied to lignin by Harton et al. (2012) and others, to characterize lignin molecular architecture, shape, dimensions and intermolecular interactions.

We applied the SAXS technique to provide biophysical parameters from lignin and polyphenolics content of all soluble fractions describe in this remit. SAXS intensity data (function of the modulus of the scattering vector, $I(q)$) were obtained for each soluble fraction at three different concentrations of polyphenols: 2.5, 5 and 10 g L^{-1}. The SF-pH4 and 2 fractions were purified by GPC prior the analysis to eliminate the interfering inorganic components (see Supplementary Material Fig. S1).

The results were displayed as log $I(q)$ vs. log q in Fig. 4. It was observed the same q-dependence for all three concentrations, indicating dilute regime even at highest concentration of polyphenols (10 g L^{-1}).

SF-pH4 and SF-pH2 fractions yielded similar SAXS curves, but since the scattering curve corresponding from SF-pH2 fraction exhibited very high statistical errors, the SAXS data from this sample is not shown.

The qualitative analysis of SAXS curves plotted in Fig. 4 suggests that all soluble fractions exhibit typical behavior expected for nanoscopic macromolecules and their aggregates with two structural levels. At high q range ($q > 0.4$ nm^{-1}), with negative curvature, it indicates the presence of rather small macromolecules or clusters (primary structure level). The intensity in the low q range ($q < 0.4$ nm^{-1}), with positive curvature, implies occurrence of larger macromolecules or aggregates (secondary structure level).

The SASFIT package (Kohlbrecher, 2006) was used to fit simulated SAXS intensity functions to experimental curves. As approach for SAXS curves modeling, it was assumed that the system to be studied is isotropic, as well as, consists of diluted set of macromolecules or molecular clusters embedded in a homogeneous liquid solvent. It was also assumed that low-resolution shape of the primary macromolecules could be well described as a set of disks with unique thickness and distribution radius with lognormal distribution. The hypothesis of oblate ellipsoid, similar to disk-shaped lignin organization in solution, has been proposed previously (Vainio et al., 2004). The secondary structure level was considered to be composed of aggregates of lignin macromolecules with fractal structure.

The relevant parameters determined using the best fit procedure were the arithmetic average radius <R>, the standard deviation (sd_R) and the thickness (L) of the disk-shaped entities, along with the fractal dimension of molecular aggregates (D). The aggregates radius of gyration, which can be used for characterization of the average size of the aggregates (secondary structure level), were not determined because the minimum q value accessible was not low enough in our experimental setup.

Comparison of the experimental scattering curves (symbols) with modeled scattering curves (solid line) is showed in Fig. 4. The structural parameters derived from best fitting procedure are reported in Table 2. The average radius of the studied lignin and LMW phenolics is approximately the same for pH ranging from 6 to 10, <R>= 1.35-1.36 and smaller for SF-pH12, <R>= 1.13 nm. SF-pH4 and SF-pH2 exhibited smaller average radius, <R>= 0.31nm, compared to the others fractions. The disk-shaped lignin is rather thick in SF-pH12 with L= 0.81nm and becomes progressively thinner in SF-pH4 and SF-pH2 with L= 0.31nm.

These findings indicate that progressive addition of sulfuric acid (consequent decrease in final pH) caused fragmentation of lignin disk-shaped macromolecules and reduction of their thickness.

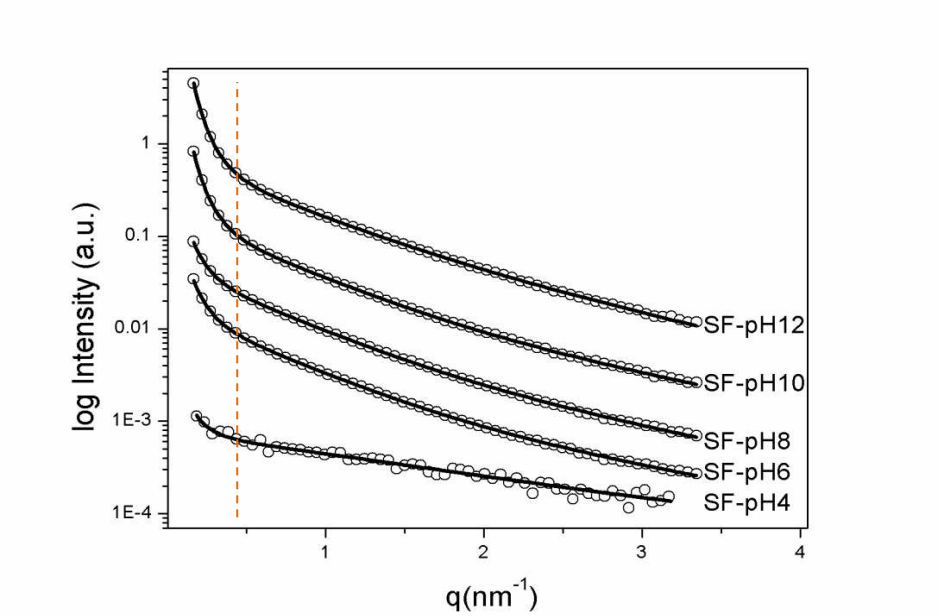

Fig. 4. Normalized SAXS in log(*I*) *q* vs. *q* scale corresponding to different soluble fractions samples. Experimental SAXS curves (symbols) and simulated SAXS functions modelled by SASFit (solid lines) exhibit excellent agreement. Separated by dotted line, the high *q* range ($q > 0.4$ nm^{-1}) indicates the presence of rather small macromolecules or clusters (primary structure level) and the low *q* range ($q < 0.4$ nm^{-1}) implies occurrence of larger macromolecules or aggregates (secondary structure level).

The fractal dimension of the lignin aggregates ranged from 2.80 to 2.71 nm in SF-pH12 and SF-pH10, respectively, and decreased during the acidification steps within D=2.15-2.19 nm range. However, the values obtained for this parameter indicate that all studied samples exhibited mass fractal structure of lignin aggregates.

Lindström (1979) explained the aggregation of alkali lignin in aqueous solutions by hydrogen bonding between neighboring lignin group carboxylic groups, ether oxygens, and hydroxylic groups. However, the same study with organosolv lignin and kraft lignin showed the intermolecular associative effects are governed by nonbonded orbital interactions (π-π interactions) among the benzene groups (Sarkanen et al., 1981). This type of interaction is a kind of π-π aggregation of the aromatic groups that induces molecular aggregation. In the case of molecular aggregation governed by hydrogen bonding proposed by Lindström (1979) is a

kind of aggregation that is not necessarily accompanied by π-π aggregation of the aromatic rings.

Table 2. Structural parameters of different soluble fractions determined by SAXS

Sample	SF-pH12	SF-pH10	SF-pH8	SF-pH6	SF-pH4/pH2
<R> (nm)	1.13	1.35	1.35	1.36	0.31
sd (nm)	0.41	0.26	0.31	0.37	0.57
L (nm)	0.81	0.66	0.71	0.58	0.31
D	2.80	2.71	2.15	2.18	2.19

Legend. Average disk radius <R>, radius standard deviation (sd), disk thickness (L) and fractal dimension (D).

Collectively, SAXS data corroborated with molecular weight distribution of soluble fractions determined by GPC analysis (Table 1). According to the structural parameters determined, the soluble fractions over the pH range of 12-6 contain rather large lignin aggregates, whereas the fractions with pH 4 and pH 2 consist of relatively small nanometer-sized LMW phenolics clusters.

3.4 TPC, ROS and RNS scavenging assays correlated to the biophysical properties of soluble fractions

Different *in vitro* assays were used to evaluate the effects of the soluble fractions on reactive oxygen and nitrogen species. *In vitro* systems have the advantage of being relatively simple and inexpensive to implement; however, it can only rank the antioxidant capacity for particular reaction system. Therefore, the correlation to *in vivo* systems must be further studied (Alves et al., 2010). Furthermore, it is considered prudent to use more than one antioxidant assay system to measure antioxidant capacities, since there may be distinct mechanisms involved that result in different outcomes depending on the test method employed (Magalhães et al., 2008). In this work, we evaluated the capacity of soluble fractions to serve as scavengers of peroxyl radical ($ROO^{\bullet}$), hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$).

The TPC and scavenging capacities of all soluble fractions are presented in Table 1. SF-pH2 showed the highest content of TPC, whereas the results were notably similar for samples SF-pH12 to 4. It is important to note TPC were determined by Folin-Ciocalteu colorimetric method. However, this method is not specific for phenolic compounds

and estimates the total reducing capacity of certain sample, i.e. the Folin reagent reacts with any reducing compound and not only with the ones possessing phenolic hydroxyl groups (Everette et al., 2010).

SF-pH2 exhibited the highest values of scavenging capacity against peroxyl radical (10167.8 ± 694.7 µmol TE. g lignin^{-1}) and hydrogen peroxide (IC_{30} = 14.9 µg mL^{-1}). In the peroxynitrite scavenging capacity assay, SF-pH6 and SF-pH2 exhibited identical capacities (IC_{50}= 2.3 µg mL^{-1}). It should be noted that the significance and relevance of an antioxidant evaluation strongly depend on the test method employed.

The correlation analysis between TPC and molecular weight was performed to understand the interrelationship between the soluble fractions and their antioxidant capacity. A good correlation was observed with the TPC and ROO$^{\bullet}$ scavenging capacity (R^2= 0.89) and TPC and hydrogen peroxide scavenging capacity (R^2= 0.71), but correlation was not found between TPC and peroxynitrite scavenging capacity (R^2= 0.19). When all assays of scavenging capacity were compared with TPC and molecular weight, the linear correlation was R^2= 0.72. These correlations suggest that TPC affects the antioxidant capacity, but more experimental data should be evaluated to confirm this hypothesis.

3.5 GC-MS analysis identified LMW compounds with antioxidant capacity

Based on the GC-MS analysis employed, it was possible to identify several phenolic and non-phenolic compounds in the soluble fractions (see Supplementary Material Table T1). Cinnamic acid was identified in all fractions, as well as, ferulic acid, excepting for SF-pH12 and SF-pH2. Caffeic acid was identified only in SF-pH4 fraction. All compounds are reported in the literature as efficient natural antioxidants (Paiva et al., 2013; Damásio et al., 2013; Sova et al., 2012 and Gülçin et al., 2006). Glycolic acid was present in all fractions. SF-pH4 and SF-pH2 fractions showed oxalic and malic acid in its composition (Table 3), which are related with antioxidant properties (Kazemi et al., 2012; Kayashima and Katayama, 2002; Morreale and Livrea 1997).

It is important to mention that the value observed for TPC in disagreement with phenolic hydroxyl content for SF-pH2 (Table 1) can be attributed to the response of non-phenolic reducing substances present in this fraction. Glycolic, oxalic and malic acid are reducing substances that react with the Folin-Ciocalteu reagent and therefore are quantified as total phenolic compounds by the colorimetric method but not by the differential scanning UV-spectroscopy method for phenolic hydroxyl content determination.

3.6 Nearly monodispersed lignin and LMW compounds recovered in soluble fractions could be useful for further applications

According to our data, the antioxidant capacity of soluble fractions may be attributed not only to the TPC and phenolic hydroxyl content but also to the ability of lignins to donate electrons (mechanism used in the inactivation of H_2O_2) and protons (mechanism used in the inactivation of $ROO^{\bullet}$ and $ONOO^-$) to radical species (Anouar et al., 2009 and Huang et al., 2005). The SAXS data obtained for the SF-pH4 and 2 fractions suggest the occurrence of a significant resonance effect in the sample, because of the difficulties of primary beam attenuation and solvent subtraction. The free phenolic hydroxyl groups are essential to antioxidant capacity of lignin and LMW phenolics, because they can scavenge radicals by forming a stable phenoxyl radical (Barclay et al., 1997). There are substituents that positively affect the stabilization of phenoxyl radicals, such as methoxyl groups at the ortho position or conjugated double bonds, whereas a conjugated carbonyl group negatively affects the antioxidant capacity (Pan et al., 2006). Li and Ge (2012) evaluated the scavenging capacity of lignin that was extracted from SCB using different chemical procedures. They demonstrated that the radical scavenging activity was more strongly affected by the hydroxyl and methoxyl content than by the molecular mass and polydispersity. Based on a series of lignins that were isolated from deciduous and coniferous wood species, Dizhbite et al. (2004) demonstrated that high molecular weight, enhancement of heterogeneity and polydispersity were the factors that decreased the radical scavenging capacity of lignins. García et al. (2010) evidenced that different extraction processes could affect the antioxidant capacity of lignin obtained from *Miscanthus sinensis*. Moreover, Hussin et al. (2014) studied ethanol organosolv lignin from oil palm fronds and concluded that the radical scavenging capacity thereof was closely related to the average molecular weight and phenolic hydroxyl content. In addition, previous studies have demonstrated that lignins prepared from SCB exhibit antioxidant capacity similar to Epicatechin (Vinardell et al., 2008) (a well-known antioxidant compound) and lack cytotoxicity (Ugartondo et al., 2008).

Finally, even with the potential health benefits and industrial applications of lignins, the great heterogeneity of lignins makes it difficult to assign their antioxidant efficiency to specific structural components compared to the activities of chemically defined molecules such as tannins and flavonoids (Sakagami et al., 2005). In this study, the phenolic and non-phenolic molecules obtained as soluble fractions can be considered high-value components, which were produced from a low cost acidification treatment of SCB lignin. Along with the nearly

monodispersed lignin with unique structural features, these preparations could be attractive for development of range of products with antioxidant and antimicrobial properties. As a preliminary study not focused on process optimization, the yield of some promising fractions were low. To achieve complete integration of lignin from SCB in the biobased economy, more focus on the development of this process in a single step for example for lignin valorization is clearly desired. The mixtures of products that will arise from lignin processing constitute also a challenge as well as new separation techniques for LWM phenolic compounds.

4. Conclusions

The acidification treatment of lignin stream, derived from steam-exploded sugarcane bagasse, resulted in low-polydispersity and low-molecular-weight preparations with high free-radical scavenging capacity. The soluble LMW compounds obtained at pH 2 exhibited highest total phenolic yield and better antioxidant properties. According to the SAXS data, the soluble fractions obtained at pH 4 and pH 2 consisted of small nanometer-sized and low molecular weight polyphenols clusters, while in soluble fractions obtained at higher pHs wide nanoparticles and larger aggregates of lignin were predominant. Analysis by GC-MS identified in all fractions phenolic and non-phenolic compounds, which are well described antioxidants, such as cinnamic acid and glycolic acid, furthermore, the SF-pH4 and SF-pH2 fractions also presented oxalic and malic acid in its composition. We expect that our findings help to accelerate the development of new routes to overcome the inherent heterogeneity of lignin-enriched streams and allow the production of higher-value and sustainable commodities.

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6. Supplementary Material

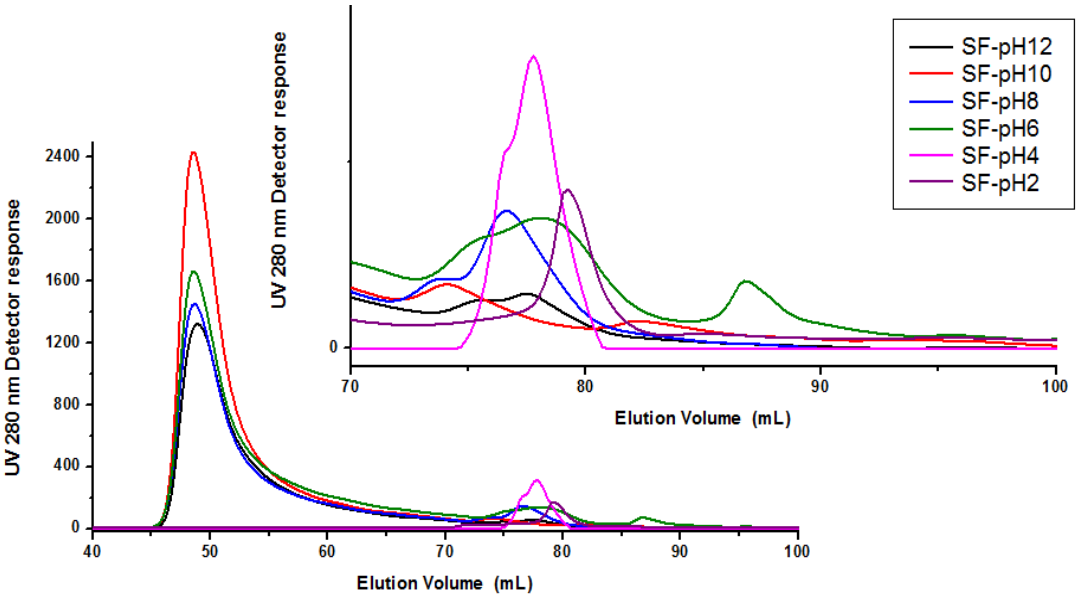


Fig. S1. The molecular distribution by gel exclusion chromatography (GPC) of soluble fractions using Superdex 30 column (65 cm x 1.6 cm) and NaOH 0.1 M as eluent in a flow of 0.1 mL min^{-1}. The SF-pH4 and 2 fractions were collected for SAXS analysis.

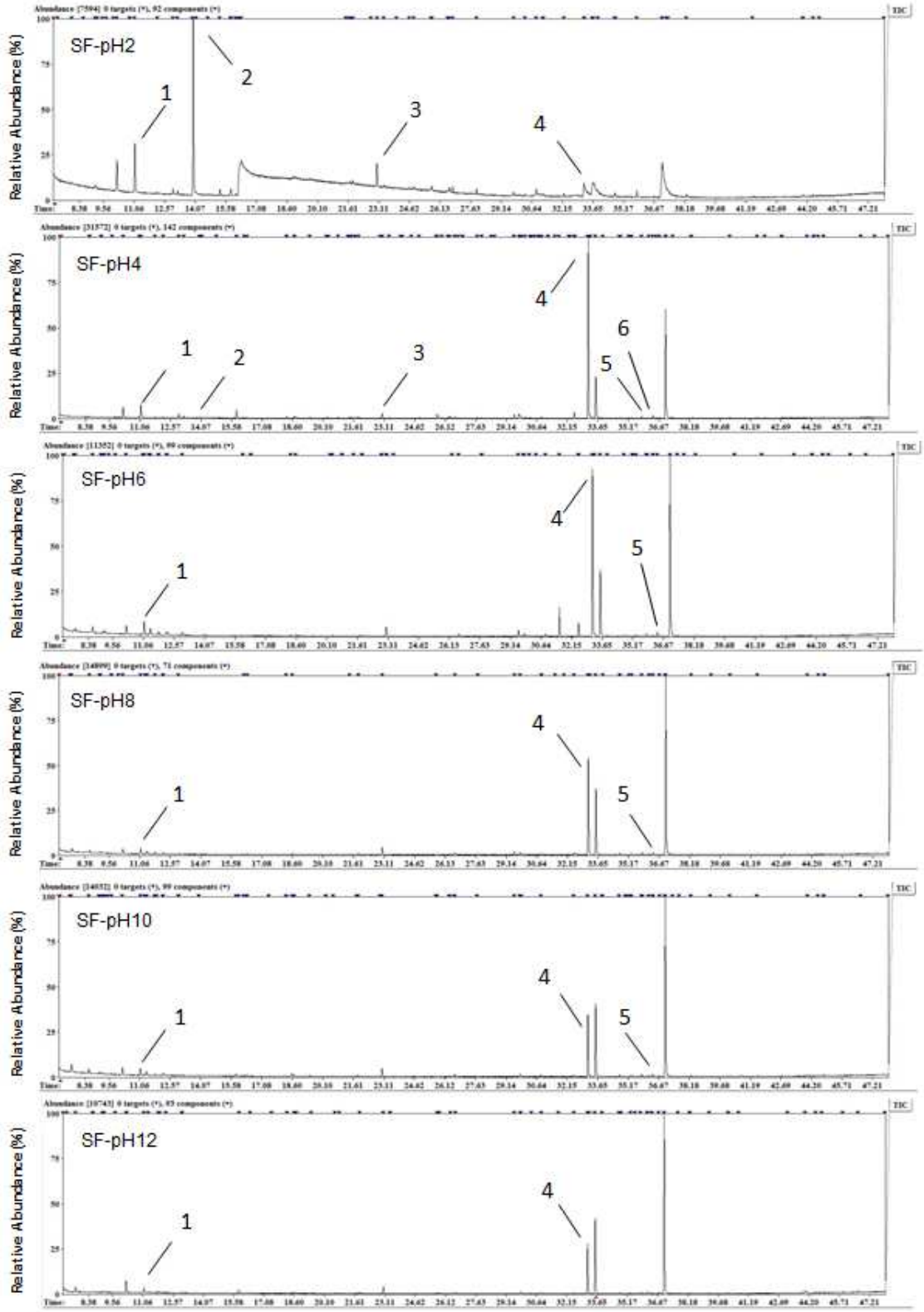
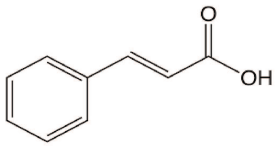
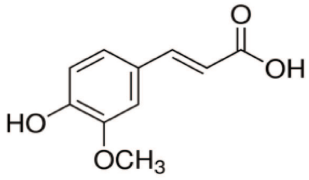
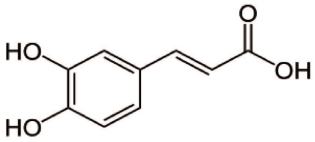
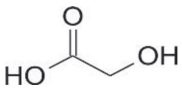
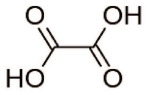
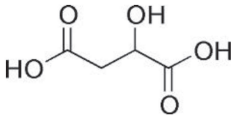


Fig. S2 GC/MS chromatograms of soluble fractions. The numerical labels of the identified compounds are: (1) glycolic acid; (2) oxalic acid; (3) malic acid; (4) cinnamic acid; (5) ferulic acid and (6) caffeic acid.

Table S1. Compounds identified by GC-MS in soluble fractions with antioxidant properties.

Sample	Fragments (m/z)	Retention time (min)	Identified molecule	Structure	Reference
SF-pH12 SF-pH10 SF-pH8 SF-pH6 SF-pH4 SF-pH2	161, 135, 131, 145, 192	33.192	cinnamic acid		Sova., 2012
SF-pH10 SF-pH8 SF-pH6 SF-pH4 SF-pH2	338, 249, 323, 293, 308	36.393	ferulic acid		Paiva et al., 2013
SF-pH4	396, 381, 219, 307, 205	36.556	caffeic acid		Gülçin et al., 2006
SF-pH12 SF-pH10 SF-pH8 SF-pH6 SF-pH4 SF-pH2	177, 205, 161, 133, 103	11.129	glycolic acid		Morreale and Livrea, 1997
SF-pH4 SF-pH2	219, 190, 175, 147, 133	13.991	oxalic acid		Kayashima and Katayama, 2002
SF-pH4 SF-pH2	233, 245, 335, 307, 217	22.931	malic acid		Kazemi et al., 2012

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CHAPTER 3 – Laccase-derived lignin compounds boost cellulose oxidative enzymes AA9

Manuscript in preparation.

Authors: Lívia Brenelli[1,2], Fabio M. Squina[3], Claus Felby[1] & David Cannella[1,2*]

(1) University of Copenhagen, Faculty of Science, Department of Geosciences and Natural Resource Management, Frederiksberg C, Denmark.
(2) Laboratório Nacional de Ciência e Tecnologia do Bioetanol (CTBE), Centro Nacional de Pesquisa em Energia e Materiais (CNPEM), Campinas, Brazil.
(3) State University of Campinas, School of Chemical Engineering, Department of Process Engineering, Campinas, Brazil.

Abstract

The discovery of lignin as activator for the redox enzyme lytic polysaccharide monooxygenases (LPMOs) in oxidation of cell wall polysaccharides opens a new scenario for investigation of the interplay between lignocellulose degrading enzymes. In many lignocellulose-degrading organisms, LPMOs are co-expressed with lignin active enzymes such as laccase. In this work, two fungal laccases together with a mediator (ABTS) were used to generate soluble lignin-derived compounds from lignocellulosic biomass. The lignin-derived compounds mixtures were used as electron donors for activation of LPMOs. A direct correlation between the low molecular weight lignin-derived compounds isolated with laccases and an increased activity of a cellulolytic cocktail containing LPMO was found when pure cellulose was hydrolyzed. Under the conditions tested, the co-incubation of laccases with LPMOs showed a substrate competition towards oxygen inhibiting the LPMO. Also laccase treatment may cause other modifications in presence of pure cellulose, rendering the material more recalcitrant for enzymatic saccharification.

Keywords

Laccase, LPMO, sugarcane bagasse, wheat straw, lignin, cellulose oxidation

1. Introduction

Over the last century our dependence on non-renewable fossil-based fuels has led to concerns over climate change effects, and increased the interest of creating a sustainable bioeconomy. Carbon-based but fossil free technologies such as the enzymatic conversion of lignocellulosic materials to fuels and value added products are therefore gaining significant attention [1,2].

Lignocellulose is a recalcitrant and complex material composed mainly of cellulose, hemicellulose and lignin. The enzymatic conversion of this material has been intensively studied, dividing it into two major classes of reactions: oxidative modification/depolymerization of lignin, and hydrolysis of polysaccharides. This paradigm was valid until the recent discovery of an oxidative enzyme active on polysaccharides, named lytic polysaccharide monooxygenase (LPMO). Today LPMOs have been found widespread in the Tree of Life: from bacteria to fungi, and often together with lignin-active enzymes e.g. ligninase, manganese- and versatile peroxidase and laccase, or in some organisms also with glucose-methanol-choline (GMC) oxidoreductases e.g. cellobiose dehydrogenase (CDH), pyranose oxidase, poly-phenol oxidases or galactose oxidase [3,4]. LPMOs oxidize glucose-based oligosaccharides resulting in a non-reducing end and a C1-oxidized end, or a reducing end and an oxidation of the C4 at the non-reducing terminal [5,6]. The products of the subsequent actions of exo-cellulases and β-glucosidases are monomeric glucose molecules and their oxidized forms: gluconic acid and gemdiol 4-ketoaldose (used as markers of LPMOs activity), from C1 and C4 oxidation respectively [7].

New generations of commercial cellulolytic cocktail contain LPMO to exploit the oxidases/hydrolases synergism [8], which has a positive impact on the industrial ethanol production, and consequently benefiting the overall CO_2 emission of the processes [9,10]. LPMOs are copper-dependent enzymes active only in the presence of molecular oxygen [11], and an electron donor, which can be delivered in different forms: from enzymes (GMC oxidoreductases) [12,13], from plant cell wall derived lignins or phenolic compounds [9,14,15], and recently from light-activated photosynthetic pigments [16,17]. Moreover, another elucidated mechanism shows that lignin and low molecular weight lignins-derived compounds (LMWL-DC) from plant cell wall can deliver electrons to LPMO [18]. In a long range electron transfer, bulk lignin works as a source of electrons, while LMWL-DC shuttle the electrons to LPMOs and being oxidized; after the oxidation these LMWL-DC are

reduced back by an electron donation from the bulk lignin, thus capable of restart a new electron delivery [13,14,18]. An interesting aspect is that these LMWL-DC i.e. caffeic acid and sinapic acid, which deliver electrons to LPMO, might be obtained from the plant cell wall upon the activity of lignin active enzymes or class II peroxidases (POD) systems [19], which are co-expressed in fungi together with LPMOs. So far these enzymes have been individually studied and few reports have focused on the interplay or synergism between different oxidases upon lignocellulose degradation, and mainly investigating the electron transfer between CDH and LPMO, and more recently the phenols mediated poly-phenol oxidases (PPO) electron transfer to LPMO [4].

In this context many aspects still need to be investigated. Laccases are multicopper oxidases catalyzing the oxidation of phenolic compounds by a one electron transfer, driven by the reduction of molecular dioxygen to water [20]. Its potential involvement in lignin depolymerization and/or modification *in vivo* has opened the field to its potential biotechnological applications [21]. In the presence of small molecules such as 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), acting as electron shuttle, laccases are able to oxidize a wider range of substrates, and it is referred to as laccase-mediator systems (LMS) [22]. Studies employing different laccases and lignocellulose materials have confirmed the potential of LMS for lignin solubilization, depolymerization and modification [23,24,25].

In this work we combine the oxidation of lignin by fungal laccases with the cellulose oxidation done by LPMO and connect the two oxidative systems by means of low molecular weight lignins-derived compounds (LMWL-DC) functioning as electron shuttles. Steam-exploded sugarcane bagasse (SCB) and hydrothermal treated wheat straw (WS) were incubated with low or high redox laccases from *Myceliophthora thermophila* and *Trametes villosa* combined with ABTS as mediator to produce soluble LMWL-DC. Gel permeation chromatography (GPC) and RP-HPLC analytical instruments were used to monitor LMS assays. The liquid-phase, rich in LMWL-DC, was used to boost the activity of LPMO in a commercial cellulase cocktail. Furthermore, co-incubation of cellulase and laccase was performed to assess potential oxygen competition. A special gas sealed reactor equipped with an oxygen sensor was used for the enzymatic hydrolysis and oxidation of lignocellulose.

2. Materials and Methods

2.2. Materials

Steam-exploded sugarcane bagasse (SCB) was kindly provided by The Engineering School of Lorena (EEL-USP) and details of the process, and the washing and pressing step to remove solubilized sugars and degradation products were described previously [26]. Wheat straw was hydrothermally pretreated (WS) in an oil bath at 194±1.5 °C with a residence time of 20 minutes at 10% dry matter content in water using a Parr reactor with 100 mL capacity. A washing and pressing step was applied after the pre-treatment to remove solubilized sugars and degradation products. The SCB and WS were dried at 30 °C and ground in a coffee grinder for 2 minutes (particle size distribution ranging from 0.5 mm to 5 mm) prior to further use. The chemical composition determined using the NREL method for lignocellulose biomasses [27] was reported to be 52% glucan, 6% xylan, 24% lignin, 0.2 % galactan, 0.5% arabinan for pretreated SCB and 54% glucan, 5% xylan, and 32% lignin for pretreated WS. The chemical composition of the raw WS and SCB was described previously in [14] and [26], respectively. Microcrystalline Avicel cellulose was used as model substrate for enzymatic hydrolysis and was purchased from Sigma-Aldrich Co. (MO, USA).

2.3. Enzymes and chemicals

The two commercial laccases used in this study were supplied from Novozymes A/S (Bagsvaerd, Denmark): *Myceliophthora thermophila* laccase (MtL) with a low redox potential (450 ±10 mV) [25] and *Trametes villosa* laccase (TvL) with a high redox potential (790 ±10 mV) [28]. The laccase activities were measured by oxidation of 1 mmol L^{-1} ABTS to its radical form by monitoring the absorbance at 420 nm (ε= 36 000 M^{-1} cm^{-1}) in 0.5 mol L^{-1} sodium acetate buffer pH 5.0 at 20 °C. One unit of activity was defined as the amount of enzyme required to oxidize 1 µmol of ABTS per minute. The cellulolytic commercial cocktail containing LPMO activity employed in this study was Cellic® Ctec2 (Novozymes A/S, Bagsvaerd, Denmark). The protein content of the enzymatic preparation was 160 mg protein g^{-1}, as determined by the bicinchoninic acid (BCA) method. The cellulase activity was 120 FPU g^{-1} of preparation measured by the filter paper assay. Cellic® CTec2 was stored at 4 °C. ABTS was purchased from Sigma-Aldrich Co. (MO, USA).

2.4. Laccase mediated system (LMS) treatments for production of LMWL-DC

Enzymatic treatments with laccases were performed always in the presence of ABTS as mediator molecule if not otherwise stated, and referred to as laccase-mediator system treatments (LMS). LMS treatments were performed on SCB, WS and Avicel at 5% (w V^{-1}) dry matter (DM) in 0.05 mol L^{-1} sodium acetate buffer pH 5.0 in 20 mL reaction volume at 40 °C, 500 rpm shaking for 6 h. MtL or TvL laccase were added at a dosage of 10 U g $cellulose^{-1}$ DM (corresponding to 500 µU ml^{-1} of enzyme) and the ABTS mediator was dosed at a final concentration of 1 mmol L^{-1}. The incubations were conducted in presence of an external air supply (flask unsealed). Incubations lacking laccase and containing only the ABTS mediator were used as controls. After the incubations, laccase activity was stopped by adding NaN_3 at a final concentration of 0.05% (w V^{-1}), and oxygen consumption measurements using an oxygen electrode chamber (Oxy-Lab Hansatech®) confirmed that laccase enzymes were inactivated. The reactions were centrifuged (1200 *g*, 10 min at 20 °C) and the supernatants rich in solubilized LMWL-DC were separated and stored at 4 °C. All pellets containing the leftover biomass were washed 3 times with pure water to remove residual laccases and mediators and stored at 4 °C prior further enzymatic hydrolysis.

2.5. Enzymatic hydrolysis of Avicel supplemented with LMWL-DC

The experimental concept is illustrated in Figure 1. The supernatants rich in solubilized LMWL-DC as result of LMS treatment of SCB and WS biomass were added to the enzymatic hydrolysis of Avicel performed at 5% (w V^{-1}) DM in 0.05 mol L^{-1} citrate buffer pH 5.5, in 1 mL reaction volume, at 50 °C, and 150 rpm using a rotary shaker. Cellic® CTec2 was added at a dosage of 5 FPU g $cellulose^{-1}$ DM, whereas 150 µl of each supernatant rich in solubilized LMWL-DC from LMS or mediator-only treatment of SCB and WS biomass were added. Enzymatic hydrolysis of Avicel without LMWL-DC rich supernatants, or supplemented only with ascorbic acid (1 mmol L^{-1} final concentration), were used as controls. After 72 hours the enzymatic hydrolysis were stopped by boiling the samples and analyzed with HPLC and HPAEC for quantification of glucose and its oxidized products. The LMS treated SCB and WS biomass separated and washed from their respective supernatants (pellets) were enzymatically hydrolyzed at 5% (w V^{-1}) DM in 0.05 mol L^{-1} citrate buffer pH 5.5, in 1 mL reaction volume, at 50 °C, and 150 rpm using a rotary shaker. Cellic® CTec2 was added to a dosage of 10 FPU g $cellulose^{-1}$ DM. After 72 hours the enzymatic hydrolysis were stopped by boiling the samples

and analyzed with analytical methods for quantification of monosaccharides and their oxidized products. All enzymatic hydrolysis was done in triplicate.

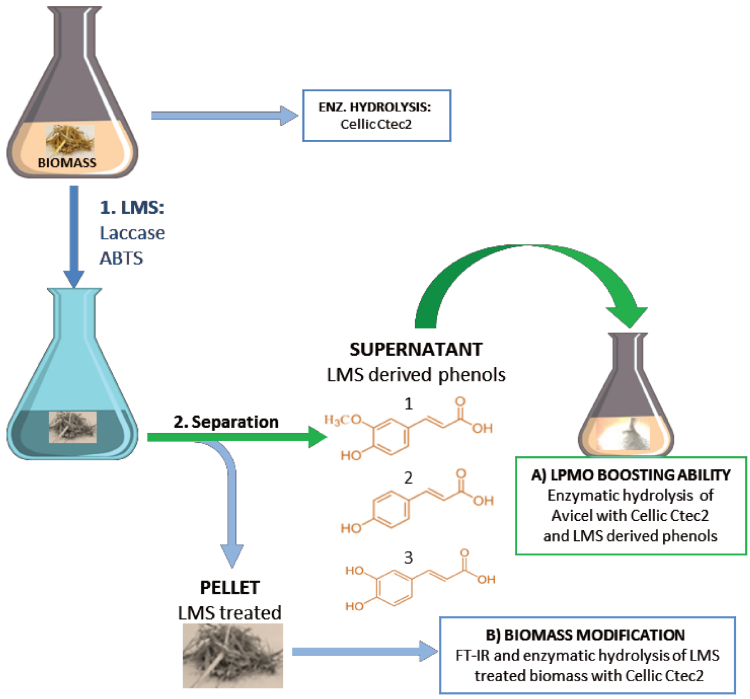

Figure 1. Flow chart of experiments investigating the ability of lignin-derived compounds generated from laccase-mediator treatments on lignocellulose to boost LPMO activity and enhance commercial cocktail hydrolysis efficiency

2.6. Oxygen consumption measurements

Oxygen consumption measurements were performed using a Chlorolab 2 System (Oxy-Lab Hansatech®, England), with an oxygen sensor mounted at the bottom of a sealed reaction chamber and the agitation provided by a magnetic stirrer. The initial oxygen concentration ($T_{0\ min}$) of each measurement was set as 100% and the following O_2 consumption calculated relative to this amount. The measurements were performed by reproducing the same conditions applied for laccases treatments (paragraph 2.3) in terms of enzymes and ABTS dosages, WS substrate was loaded at 5 % DM, 25°C (± 0.5) was chosen as temperature to maximize the oxygen availability, 100 rpm for the agitation and the reactor was kept sealed without oxygen supply. For the incubation of WS with Cellic® CTec2 the same physical parameters (agitation temperature and solids content) were kept as for laccases, and the cocktail was added to a dosage of 10 FPU g cellulose^{-1} DM in 0.05 mol L^{-1} citrate buffer pH 5.5. The control experiment was

set containing WS sodium acetate buffer and ABTS (like in the LMS treatment) without enzymes.

2.7. LMS and cellulases co-incubation experiments

2.7.1. Simultaneous LMS and enzymatic hydrolysis

SCB or WS were loaded at 5% (w V^{-1}) dry matter (DM) in 0.05 mol L^{-1} sodium acetate buffer pH 5.0 in 1 mL reaction volume using 2 ml screw cup tubes at 50 °C temperature, and 800 rpm shaking for 72 hours. MtL or TvL laccase were added at a dosage of 10 U g $cellulose^{-1}$ DM (corresponding to 500 µU ml^{-1} of enzyme) and the ABTS mediators was dosed at a final concentration of 1 mmol L^{-1}. The cellulolytic cocktail Cellic® CTec2 was added to a dosage of 10 FPU g $cellulose^{-1}$ DM.

2.7.2. Separated LMS and enzymatic hydrolysis

WS was loaded at 5% (w V^{-1}) dry matter (DM) in 0.05 mol L^{-1} sodium acetate buffer pH 5.0, in 1 mL reaction volume using 2 ml screw cup tubes at 50 °C temperature, and 800 rpm shaking together with MtL or TvL laccase added at a dosage of 10 U g $cellulose^{-1}$ DM (corresponding to 500 µU ml^{-1} of enzyme) and the ABTS mediators was dosed at a final concentration of 1 mmol L^{-1}. After 1 hour the laccase activity was stopped by adding NaN_3 at a final concentration of 0.05% (w V^{-1}), and the reaction medium re-oxygenated by fluxing oxygen gas in the empty space of the reaction tube. Then the cellulolytic cocktail Cellic® CTec2 was added to a dosage of 10 FPU g $cellulose^{-1}$ DM and the enzymatic hydrolysis was kept for 72 hours.

2.8. Analytical methods

2.8.1. Glucose quantification by HPLC

The glucose quantification was done using an Ultimate 3000 HPLC (Dionex, Germering, Germany) equipped with a refractive index detector (Shodex, Japan). The separation was performed with a Phenomenex Rezex ROA column, kept at 80 °C, with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL min^{-1}. The results were analyzed using the software Chromeleon (Dionex).

2.8.2. Oxidized monosaccharides quantification by HPAEC

The oxidized monosaccharides quantification was conducted with HPAEC chromatography using an ICS5000 system (Dionex, Sunnivale, CA, USA) equipped with a gold electrode PAD

to analyze the oxidized products. The separation was performed with a CarboPac PA1 2x250 mm analytical column (Dionex, Sunnivale, CA, USA) and a CarboPac PAC1 2x50 mm guard column, maintained at 30 °C. The gradient mixing of eluents 0.1 M NaOH and 1 M NaOAc (sodium acetate) used has been described in detail previously [8,14].

2.8.3. Molecular weight distribution of LMWL-DC by GPC

The GPC measured the molecular weight distribution of the LMWL-DC present in water solution. 200 µL of each supernatant was diluted in 200 µL in a 9:1 DMSO: water mixture containing 0.05 mol L^{-1} LiBr (the mobile phase) and transferred to a sample vial. The GPC gradient was performed isocratically with a Hitachi 7000 system set up with a PolarSil column (300 mm, 5 µm particles, 100 Å porosity from Polymer Standard Service) at 1 mL min^{-1} flow and 40 °C. Detection was obtained using a UV detector (280 nm), using tannic acid and phenol as external standards.

2.8.4. LMWL-DC analysis by RP-HPLC

The supernatants rich in solubilized LMWL-DC from LMS treatments of SCB and WS were also analyzed by Reverse Phase Liquid Chromatography (RP-HPLC). RP-HPLC was carried out with a Ascentis® Express C18 column (15 cm × 2.1 mm, 2.7 µm) (Supelco™ Analytical, Bellefonte, PA, USA) on a Shimadzu HPLC system equipped with diode array detection at a flow rate of 0.5 mL min^{-1} and 40 °C. A gradient of buffer A (2% acetonitrile and 0.2% formic acid in water w/w) and buffer B (2% water and 0.2% formic acid in acetonitrile w/w) was used as follows: 0% B over 4 min, 10% B for 15 min, 50% B for 1 min, 0% B over 20 to 50 min. The injection volume was 1 µL for samples and phenolic compounds (quinones, para-coumaric, caffeic and ferulic acid, vanillin, vanillic acid, tannic acid) from Sigma Aldrich® were used as internal standards.

2.9. FTIR and UV spectroscopy

The lignocellulosic materials recovered after LMS treatments were analyzed using a Thermo Nicolet 6700 FT-IR spectrophotometer equipped with a Golden Gate (diamond) ATR accessory and a DTGS (KBr) detector. Spectra were collected at room temperature in the 4000-800 cm^{-1} range with an average of 150 scans. A background of 150 scans was acquired, and the spectrum of each sample is reported as the average of three spectra. FTIR data were normalized by discounting the average between 1750 and 1800 cm^{-1}, where there is no signal, and dividing by

the maximum intensity value in the region from 1000 to 1250 cm^{-1}. The lignin-derived compounds and phenolics present in the supernatant from LMS or mediator-only treatment on WS and SCB were also analyzed by UV in alkaline solution (pH >12) in order to assure that the hydroxyl groups were ionized and the absorption changed towards longer wavelengths and higher intensities. The liquid fractions were diluted 36 times in NaOH 0.1 M and placed in a 1 cm quartz cuvette. The UV absorption spectrum (220 to 400 nm) was then recorded using an OceanView spectrophotometer UV-vis (Ocean Optics®, Netherlands). Reference solution was consistent with NaOH solution used for the samples.

3. Results and Discussion

3.1. Laccase mediated system (LMS) for isolation of low molecular weight lignin derived compounds (LMWL-DC)

Two laccases (MtL and TvL) together with a mediator molecule ABTS were used to generate soluble LMWL-DC from SCB and WS (as illustrated in Figure 1). The supernatant rich in lignin-derived compounds were collected and further characterized (using UV-light absorbance, gel permeation chromatography (GPC) and reverse phase chromatography RP-HPLC) before using these as booster for LPMO during enzymatic hydrolysis of Avicel. The increases in UV-light absorbance at 280 nm were detected in the supernatants of incubations of SCB or WS biomass with LMS compared to their controls lacking of laccases enzymes (results in Table 1). MtL laccases promoted an increase of more than 100% in UV absorbance from the initial values regardless the type of biomass. Also TvL laccases was active on both SCB and WS lignocelluloses, and the UV-absorbance of the reaction medium increased 20% with the respect of their control reactions lacking of laccases. This confirmed that lignin was partially degraded, leading to its solubilization into the supernatant of the reaction made of water as solvent.

The weight distributions of these LMWL-DC from the same sample were analyzed with GPC and are shown in Figure 2. The spontaneously solubilized lignin-derived compounds from both biomasses lacking of LMS treatment had a narrow molecular weight distribution, with predominance of compounds with molecular weight ranging from 1000 - 1700 Da. The incubation with LMS increased the overall amount of compounds with a broad molecular weight distribution between 10 kDa to <100 Da.

Table 1. UV-Absorbances at 280 nm of liquid fractions of LMS treated SCB or WS. The LMS treatments of biomass are based on MtL or TvL laccases incubated with ABTS for 6 hours. The control experiments lack of laccases but containing ABTS.

Sample	Abs $_{280\ nm}$
Control SCB	0.67
SCB + LMS_MtL	1.39
SCB + LMS_TvL	0.80
Control WS	0.97
WS + LMS_MtL	1.89
WS + LMS_TvL	1.18

Noteworthy is that despite the biomass MtL and TvL laccase respectively, promoted the same solubilization pattern in terms of molecular weight distribution.

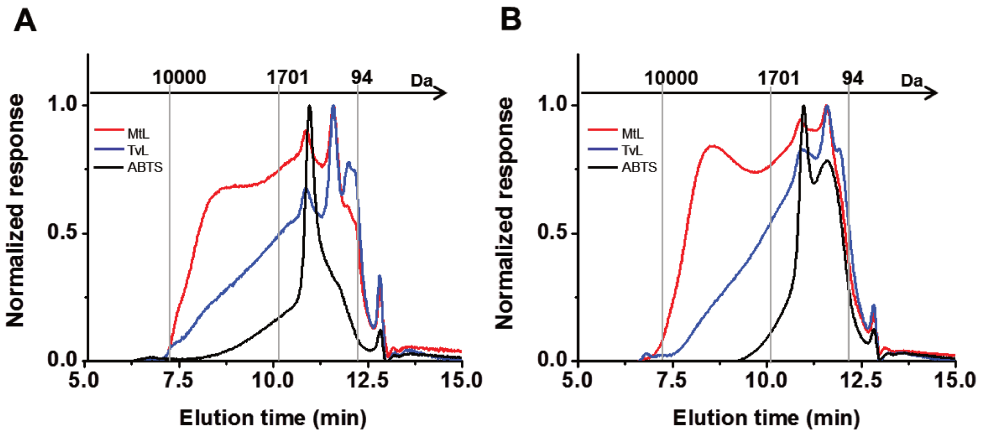

Figure 2. GPC chromatograms showing the molecular weight distribution of the soluble lignins- derived compounds in the supernatants from LMS treated SCB (A) and WS (B). Red lines are the incubations of the biomass with LMS based on MtL, and the blue lines are based on TvL laccases. Black lines refer to control experiments missing laccases. On top of the chromatograms are indicated the molecular weight distributions in Dalton (Da) which are inversely correlated with the elution time: higher molecular weight corresponds to lower elution time. The 1701 Da and 94 Da are calculated measuring the elution time of tannic acid (10.5 min) and phenol (12.3 min) respectively.

RP-HPLC was used for the characterization and identification of lignin-derived monophenolic compounds solubilized in the supernatant of the reaction containing

lignocellulose and LMS, and to confirm that all ABTS added was present at the end (Figure S1 and S2, Table S2 and S3). Mainly ferulic and coumaric acid were detected in the supernatants of WS and SCB when no LMS was present in the reaction, in agreement with previous results [14,29,30]. But after the incubation with LMS these monophenolic compounds mostly disappeared, while traces of caffeic acid (for MtL), a good electron donor for LPMO [18], and few other unknown monophenols appeared. The peak corresponding to the ABTS in the chromatograms, disappeared in the supernatants after LMS treatment (Figure S1 and S2), which means that all mediator added at the beginning of the incubation was completely consumed after 6 hours. These observations suggest that both laccases catalyzed incorporation of ABTS into the solid materials SCB and WT. These results indicate that a depolymerization/solubilization of insoluble lignin occurred, but also it cannot be ruled out that a re-polymerization phenomenon of the soluble monophenolic compounds occurred at the same time, thus generating lignin-derived compounds with molecular weight increased (in agreement with a series of previous papers [31-34]. This also explains the formation of an intermediate size of phenolic compounds around 600-1000 Da detected by the GPC but not detectable by the RP-HPLC tuned for smaller sized compounds. This pool of phenolics was suggested to be the responsible for the long range electron transfer from lignin to LPMO enzymes [18].

3.2.Lignin solubilized by laccase mediator system boost LPMO enzymes

The soluble LMWL-DC, produced after LMS treatment on pre-treated SCB and WS, were added to the enzymatic hydrolysis of Avicel cellulose (experimental scheme in Figure 1). The LPMO containing cocktail Cellic® Ctec2 was used for the enzymatic hydrolysis, and its cellulose oxidizing activity was monitored detecting gluconic acid production (C1 oxidation of glucose). After 72 hours of hydrolysis it was observed that all the reactions incubated with LMWL-DC-rich supernatants had improved cellulose conversion correlating with an increase in LPMO activity (Figure 3 and Table S1) compared to the control experiments without any electron donors added. Notably, the cellulose conversion increased 42% for the Avicel hydrolysis when Cellic® CTec2 was boosted by the LMWL-DC solubilized from pretreated SCB incubated with MtL laccase (in comparison with the control lacking of LMWL-DC (Figure 3).

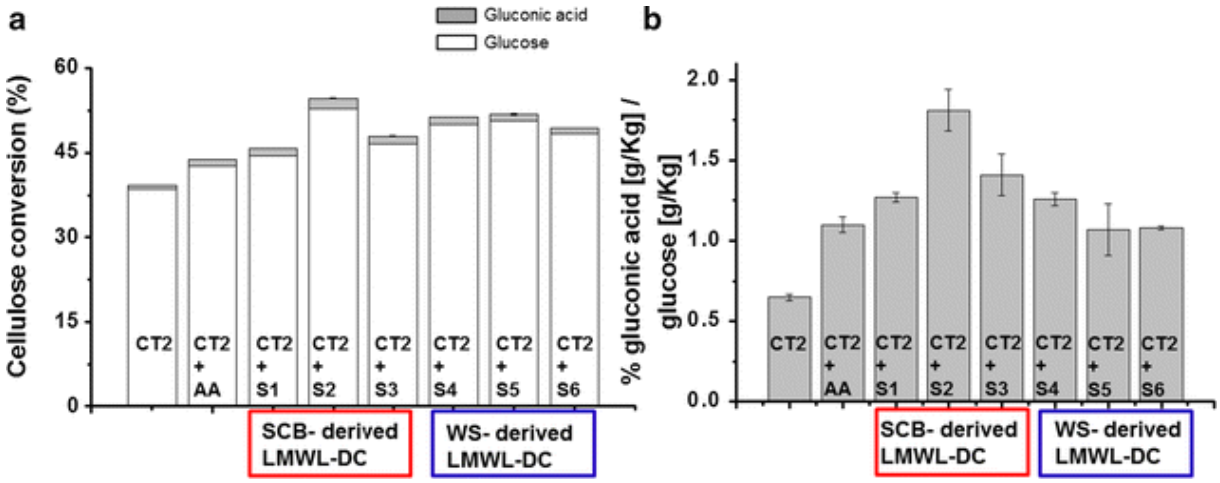

Figure 3. Enzymatic hydrolysis of Avicel incubated with supernatants rich in solubilized low molecular weight lignin-derived compounds after LMS treatments of biomass at different conditions. The enzymatic hydrolysis was run with the LPMO containing cocktail Cellic® CTec2 (CT2). A) In the y axis are reported the cellulose (white bars) and glucose oxidation (gluconic acid, grey bars) conversion yield in percentage of the maximum theoretical cellulose conversion after 72h at 50ºC. B) Relative yield of glucose oxidation. The glucose oxidation was calculated as percentage of the amount of gluconic acid over the amount of glucose hydrolyzed from cellulose. Error bars represent the standard errors based on the means of triplicate experiments. CT2: control experiment with Avicel and enzymes only; CT2+AA: contains Avicel, enzymes and ascorbic acid (AA); CT2+supernatant: contains Avicel, enzymes and supernatant rich of lignin-derived compounds from incubation of: 1) SCB and ABTS (no laccase); 2) SCB, ABTS and MtL; 3) SCB, ABTS and TvL; 4) WS and ABTS (no laccase); 5) WS, ABTS and MtL; 6) WS, ABTS and TvL.

Using TvL on the same material (SCB) to generate LMWL-DC, the yield increased 25%. Also a positive response was obtained using LMWL-DC from WS. The Avicel conversion increased 34% with the LMWL-DC solubilized with MtL laccase and also using the phenolics spontaneously solubilized, while 29% increase was obtained with LMWL-DC solubilized with TvL (Table S1). Controls containing only ABTS at 0.15 mM (the same concentration present in the reactions incubated with LMWL-DC-rich supernatants) were found effectless to the enzymatic hydrolysis and to the LPMO in agreement as previously reported [18] (data not shown).

The hydrolysis yields observed confirms that for SCB, laccase-mediator oxidation produced the pool of LMWL-DC acting as electron donors for LPMOs in the cellulolytic

cocktail Cellic® CTec2. The highest glucose oxidation was obtained for the supernatants containing the LMWL-DC isolated from SCB and MtL (1.8%), followed by SCB and TvL (1.4%) and spontaneously hydrolyzed without the LMS. Notably these data correlate with the amounts of solubilized LMWL-DC detected with UV_{280} absorbance (Table 1): at higher amounts of solubilized LMWL-DC corresponded the highest LPMO activity (detected as cellulose oxidation Table S1, supernatant 2) and consequently a higher enzymatic hydrolysis of Avicel. Also the results correlate with the presence of caffeic acid in the supernatants, produced by the activity of MtL on SCB (detected with RP-HPLC) and not present before the LMS treatment (Figure S2 and Table S3). Caffeic acid and in general plant-derived methoxylated and non-methoxylated compounds were previously found to be efficient LPMO reducing agents [18,34].

A recent paper [4] showed how polyphenol oxidases (PPO) can boost LPMO activity by hydroxylating plant derived monophenolics turning some phenolics and methoxylated phenolics into active reducing agent donating electrons to LPMO. Thus, in Figure 4 we drew a simple model to implement the PPO activity in the wider scenario of lignocellulose oxidation/hydrolysis together with laccases and LPMO.

Bulk insoluble lignin is the primary source of polyphenols which are depolymerized by the LMS activity at the expense of oxygen, the produced LMWL-DC pool contains a heterogeneous distribution of monophenolics (mono to trimmers, and also methoxylated [18]) some of which can directly activate LPMO. But also some of the LMWL-DC inefficient in donating electrons to LPMO, could also be the substrate for the PPO oxidase that after hydroxylation at the expense of oxygen, can turn these LMWL-DC in good electron donors for LPMO [4].

Since also the cellulose oxidizing activity of LPMO is heavily dependent on oxygen, the amount of dissolved oxygen and or anaerobic versus aerobic conditions should be carefully considered for any application or co-incubation of these oxidases together. Thus the next paragraph is dedicated to the competition over oxygen on co-incubations of laccases and LPMO containing cellulases cocktails.

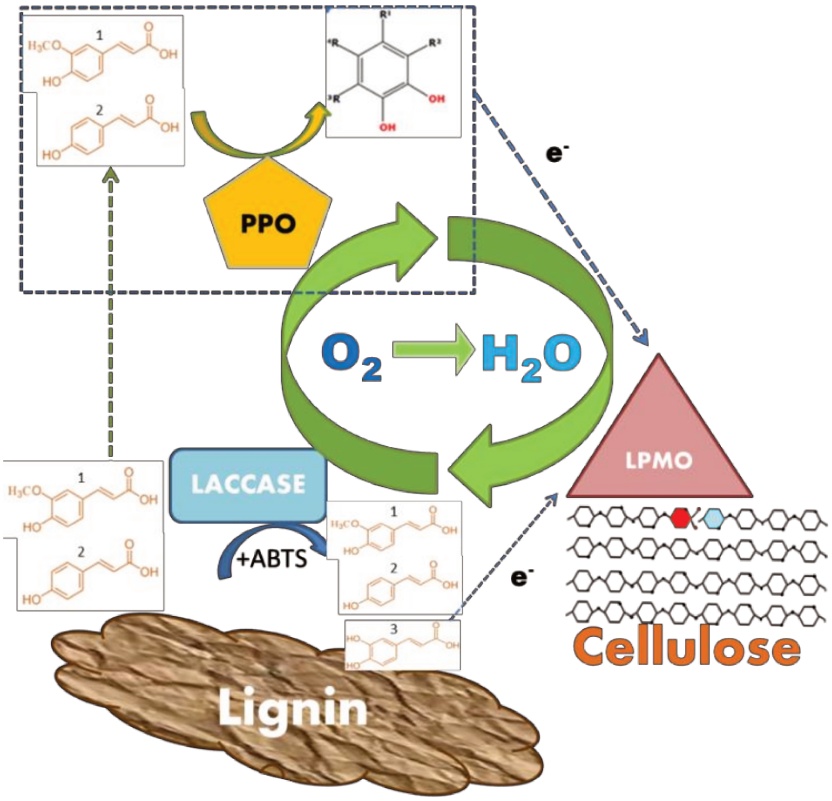

Figure 4. Simple representation of several oxidases enzymes degrading lignocellulose. The LMS system is depicted as Laccases + ABTS while depolymerizing the bulk lignin for the production of low molecular weight lignin- derived compounds (LMWL-DC) represented by the compounds identified in this study: the 2) coumaric, 3) caffeic and 1) ferulic acid, respectively the hydroxylated and methoxylated form of coumaric acid. Since caffeic acid is a good electron donor to LPMO, it can directly activate the cellulose oxidation, while coumaric and ferulic acid do not have the same favorable redox potential. Thus we hypothesize based on literature data that polyphenol oxidase PPO can be used to hydroxylate the coumaric acid and other monophenols into good electron donor for LPMO, and at the same time mitigate the repolymerization into high molecular weight lignin-derived compounds. All the enzymes depicted in the figure are all oxygen dependent (Figure adapted from [4 and 17]).

3.3.Laccase and LPMO co-incubation: oxygen competition

When co-incubating laccases and LPMOs on lignocellulosic substrates, their O_2 consumption rates should be considered to avoid any competition. Enzymatic reactions were set in a 1 ml sealed reactor-chamber equipped with an oxygen electrode disc as sensor to monitor the oxygen consumption. In this reactor it was possible to perform simultaneous hydrolysis and oxidation of lignocellulosic substrate i.e. WS or the pure cellulose Avicel. As

shown in Fig.5, 50% of the initial oxygen was consumed in 6.8 minutes during the incubation of WS with MtL laccase, and 8.7 minutes for TvL laccase both at 25 ºC of temperature. In 15 minutes, both laccases consumed all the dissolved oxygen to undetectable levels. In comparison for the LPMOs, only 10% of the oxygen available was consumed during the first 26 minutes when WS was incubated with Cellic®CTec2.

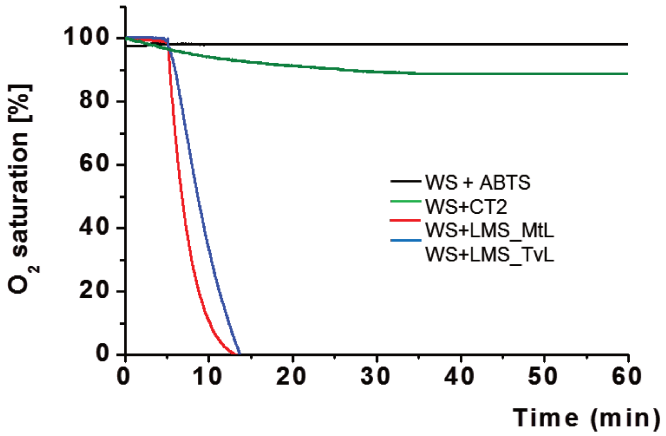

Figure 5. Oxygen consumption measurements as % decrease of O_2 saturated solution versus time. Each line is representative of average of triplicate experiments containing: WS and ABTS (black); WS and Cellic® CTec2 (green); WS, LMS_MtL laccase (red), WS, LMS_TvL laccase (blue).

Based on these results we could predict an inhibition of the LPMO enzymes contained in the cellulolytic cocktails during the hydrolysis of lignocellulosic materials if co-incubated with laccases. In fact, a decrease of enzymatic hydrolysis yield for the co-incubations of Cellic® CTec2 and laccases on hydrothermally pretreated SCB or WS substrates were observed. For SCB, 53.9% of conversion yield was obtained for control experiment incubated solely with the cellulolytic cocktail, while MtL laccases was added only 27.3% of conversion was obtained, and 34.1% when incubated with TvL (Figure 6a). For WS the inhibition of the enzymatic hydrolysis was similar: 68.1% of conversion was obtained for the control enzymatic hydrolysis, when 44.1% and 52.3% was obtained adding MtL and Tvl, respectively (Figure 6a).

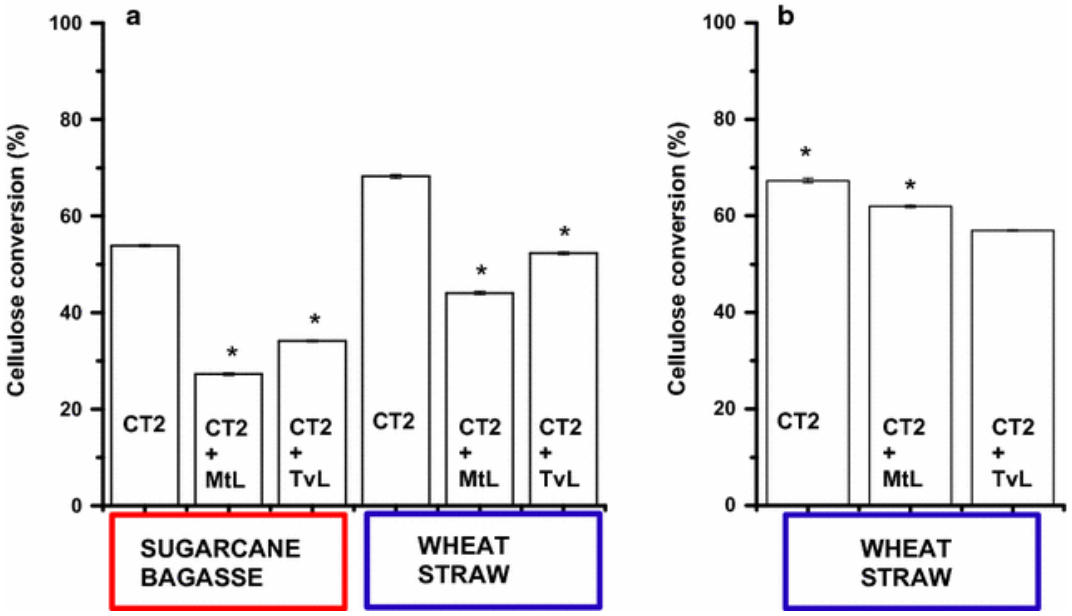

Figure 6. Enzymatic hydrolysis yields. A) Simultaneous LMS and enzymatic hydrolysis of lignocellulose: samples containing pretreated sugarcane bagasse and Cellic® CTec2 (SCB+CT2); SCB, CT2 and simultaneously treated with LMS based on either MtL or TvL laccases (SCB+CT2+LMS_MtL; SCB+CT2+LMS_TvL); pretreated wheat straw and Cellic® CTec2 (WS+CT2); WS, CT2 and simultaneously treated with LMS based on either MtL or TvL laccases (WS+CT2+LMS_MtL; WS+CT2+LMS_TvL). B) Separated LMS and enzymatic hydrolysis: samples containing pretreated wheat straw and Cellic® CTec2 (WS+CT2); WS treated with LMS based on either MtL or TvL laccases, then after inactivation of laccases hydrolyzed with Cellic® CTec2 (WS+LMS_MtL+CT2; WS+LMS_TvL+CT2). Error bars represent the standard errors of triplicate experiments. (*) The mean difference is statistically significant at the 0.05 level by the Tukey test.

The LPMOs were inhibited by the anoxic environment having the gluconic acid undetectable, despite the presence of electron donor molecules like LMWL-DC solubilized by the laccases, thus no cellulase/LPMO synergism was observed. Noteworthy is the correlation between the activity of the laccases generating LMWL-DC reported in Table 1 (despite the biomass employed) and the inhibition of the enzymatic hydrolysis caused. MtL laccases was found to be more active on SCB, which reported the lowest cellulose enzymatic conversion.

To avoid the oxygen competition between laccases and LPMO, the two enzymatic reactions were separated but performed on the same material. The hydrothermal pretreated WS was incubated with LMS, and after the inhibition of the laccase by adding sodium azide

harmless [35] to cellulolytic cocktail and re-oxygenation of the medium, the LPMO containing cellulolytic cocktail was added. Results similar to the control experiment (Cellic® CTec2 only) were obtained when using separated incubations with MtL laccase leading at 62% of cellulose hydrolysis yield, restoring 74% of the cellulose hydrolysis yield lost with the simultaneous co-incubation strategy (Figure6b). When adding on the TvL laccases for the separated strategy the final glucose yield was 57%. Despite an active LPMO i.e. presence of LMWL-DC electron donors and oxygen, the enzymatic hydrolysis yield did not increase with the separated LMS/LPMO approach. This indicates that laccase can be applied for the production of electron donors from lignin to boost LPMO, but might also cause other inhibiting effects e.g. a modification of cellulose fibers which could render the lignocellulose more recalcitrant to enzymatic hydrolysis as previously observed [29,30,31,36], or by increasing the binding of cellulases on modified lignin [37,38].

3.4. Insights on LMS detrimental effect on cellulose conversion

The negative results obtained with the co-incubation of LMS and Cellic® CTec2, and partially restored with a separate incubation, made us wonder if LMS could modify cellulose fibers contributing to the inhibition of the enzymatic hydrolysis. Pure cellulose (Avicel) was treated with LMS using both MtL and TvL laccases. The resulting cellulose was hydrolyzed with Cellic® CTec2 and the hydrolysis yield was found to be lower (12% for MtL-treated Avicel, and 20% TvL-treated Avicel respectively) compared to the untreated material (Figure 7a). Prior to the enzymatic hydrolysis the LMS treated Avicel was thoroughly washed and the surface analyzed with FT-IR spectroscopy. FTIR analysis (Figure 7b) revealed that LMS treatment of Avicel cellulose and TvL laccase decreased by 32% the hydroxyl stretching band at 3335 cm^{-1} and by 25% the band at 2850 cm^{-1} related to the symmetrical stretching vibrations of CH_2 groups (Table S4).

No difference was observed for LMS treatment of Avicel by MtL laccase. These modifications suggest that LMS treatment with TvL laccase may have decreased the free hydroxyl groups on the cellulose surface, or inter-fiber covalent bonds through hemiacetal linkages between hydroxyl groups and carbonyl groups could be formed increasing the strength of the cellulose [37,39,40,41].

RP-HPLC analysis of the liquid phase also showed that all ABTS added at the beginning of the reaction was present in the supernatant and at the end of the LMS incubation period (results not shown) and not grafted onto the material.

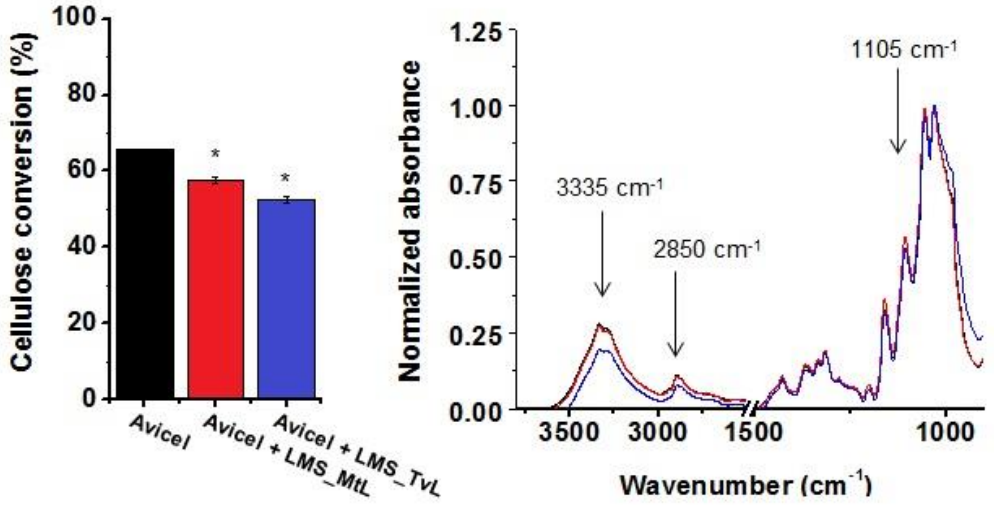

Figure 7. Enzymatic hydrolysis (A) and Fourier transforms infrared spectra (B) of Avicel treated with LMS based on MtL laccase (Avicel+LMS_MtL in A; red line in B) and LMS based on TvL laccase (Avicel+LMS_TvL in A, blue line in B), the control experiment contained Avicel and ABTS, lacking of laccases (Avicel in A; black line in B). (*) The mean difference is statistically significant at the 0.05 level by the Tukey test. The arrows indicate the main changes in the spectra after LMS treatment.

It has already been shown that oxidized groups on cellulose such as carbonyl and carboxyl groups can decrease the enzymatic hydrolysis of cellulose, inhibiting cellobiohydrolases and ß-glucosidases [31]. Here we report that the modification of OH and CH_2 groups on the surface of Avicel caused by the LMS with a high redox potential laccase can also negatively affect the enzymatic hydrolysis yield. FT-IR data of SCB and WS after LMS treatment with TvL laccase also revealed a decrease in the hydroxyl content by 17% and 50% for SCB and WS (Figure S4a and b, respectively, and Table S4) in cellulose surface. In agreement with the results observed for Avicel, the hydrolysis yield was found to be lower (15%) for TvL-treated WS compared to the untreated material (Figure S3). As observed in previous studies [37,42,43], it seems that reducing hydroxyl groups in lignocellulose surface is a hallmark of LMS treatment.

Low redox potential laccases (i.e. MtL) should be preferred over high redox potential laccase (i.e. TvL) given the reduced extends of modification caused onto cellulose fibers. These

modifications were found to be a key factor in increasing the recalcitrance of cellulose to the enzymatic hydrolysis.

4. Conclusions

The results obtained in this work show that the lignin-derived compounds and phenols released using laccase–mediator system were able to boost LPMO activity present in a commercial cellulolytic cocktail, increasing their hydrolysis efficiency of cellulose. In particular, the MtL laccase with low redox potential caused the highest release of low-molecular-weight lignin-derived compounds capable of activating LPMOs. It was also found that the co-incubation of laccase together with LPMOs containing cellulolytic cocktail led to substrate competition towards oxygen, causing an inhibition of LPMO. Thus, the LMS was applied prior to the cellulolytic enzymes on the same lignocellulosic material: the inhibition of the LPMOs was mitigated, but the overall cellulose hydrolysis did not increase. These results suggest the presence of a second inhibition caused by laccases but acting directly on the cellulose material. Laccase, especially with high redox potential TvL, could induce further chemical modification in lignocellulosic fibers increasing the recalcitrance of sugarcane bagasse and wheat straw. In conclusions for an affective exploitation of a long-range electron transfer from lignin to cellulose catalyzed by several oxidases, the enzymes should be chosen and dosed very carefully considering the substrate competition towards oxygen aiming at a co-incubation with LPMO. Moreover, the data obtained on lignin depolymerization after LMS activity suggest a potential synergy also with other oxidases (i.e., PPO) in producing low-molecular-weight lignin-derived compounds acting as a reducing agent for LPMO.

5. Acknowledgements

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6. Supplementary Material

Table S1. Enzymatic hydrolysis of Avicel supplemented with supernatants rich in solubilized low molecular weight lignin-derived compounds. Quantification of gluconic acid in g/Kg (first column), relative yield of cellulose oxidation calculated towards the amount of cellulose hydrolyzed (second column), calculated as % of maximum theoretical (third column).

Sample	Gluconic acid (g Kg^{-1})	Oxidized cellulose / total glucose released	Cellulose Conversion (%)
CT2	0.14 ± 0.01	0.65 ± 0.02	37.3 ± 0.2
CT2 + AA	0.35 ± 0.01	1.10 ± 0.05	42.7 ± 0.8
CT2 + supernatant 1 (SCB+ABTS no laccase)	0.31 ± 0.01	1.27 ± 0.03	44.5 ± 0.4
CT2 + supernatant 2 (SCB+LMS_MtL)	0.53 ± 0.04	1.81 ± 0.13	52.9 ± 0.9
CT2 + supernatant 3 (SCB+LMS_TvL)	0.36 ± 0.03	1.41 ± 0.13	46.6 ± 0.1
CT2 + supernatant 4 (WS+ABTS no laccase)	0.35 ± 0.01	1.26 ± 0.04	50.0 ± 0.8
CT2 + supernatant 5 (WS+LMS_MtL)	0.30 ± 0.05	1.07 ± 0.16	50.7 ± 0.7
CT2 + supernatant 6 (WS+LMS_TvL)	0.29 ± 0.02	1.08 ± 0.01	48.3 ± 0.3

Figure S1 and Table S2. RP-HPLC analysis of the supernatants obtained for the pre-treated sugarcane bagasse incubated with buffer and ABTS (black line), LMS_MtL (blue line) or LMS_TvL (pink line). New peaks that appeared after LMS treatment are circled by red circles. Only the retention time, wavelength of maximum absorption and peak area and peak height for each compound is reported. The height of each peak is in proportion to the amount of the component present in the sample mixture.

			Control SCB		LMS_TvL		LMS_MtL	
Name	Ret. Time	UV abs	Peak area	Peak height	Peak area	Peak height	Peak area	Peak height
p-Coumaric acid	10.976	308nm	20713	3668	1046	165	1092	194
Ferulic acid	13.022	321nm	6622	1164	-	-	-	-
Sinapic acid	13.022	322nm	6567	1160	-	-	-	-
Caffeic acid	8.038	321nm	-	-	-	-	2553	190
Vanillic acid	7.588	202nm	169820	15358	178725	15851	178713	15970
Vanillin	9.465	204nm	123846	15237	123376	14941	111727	14076

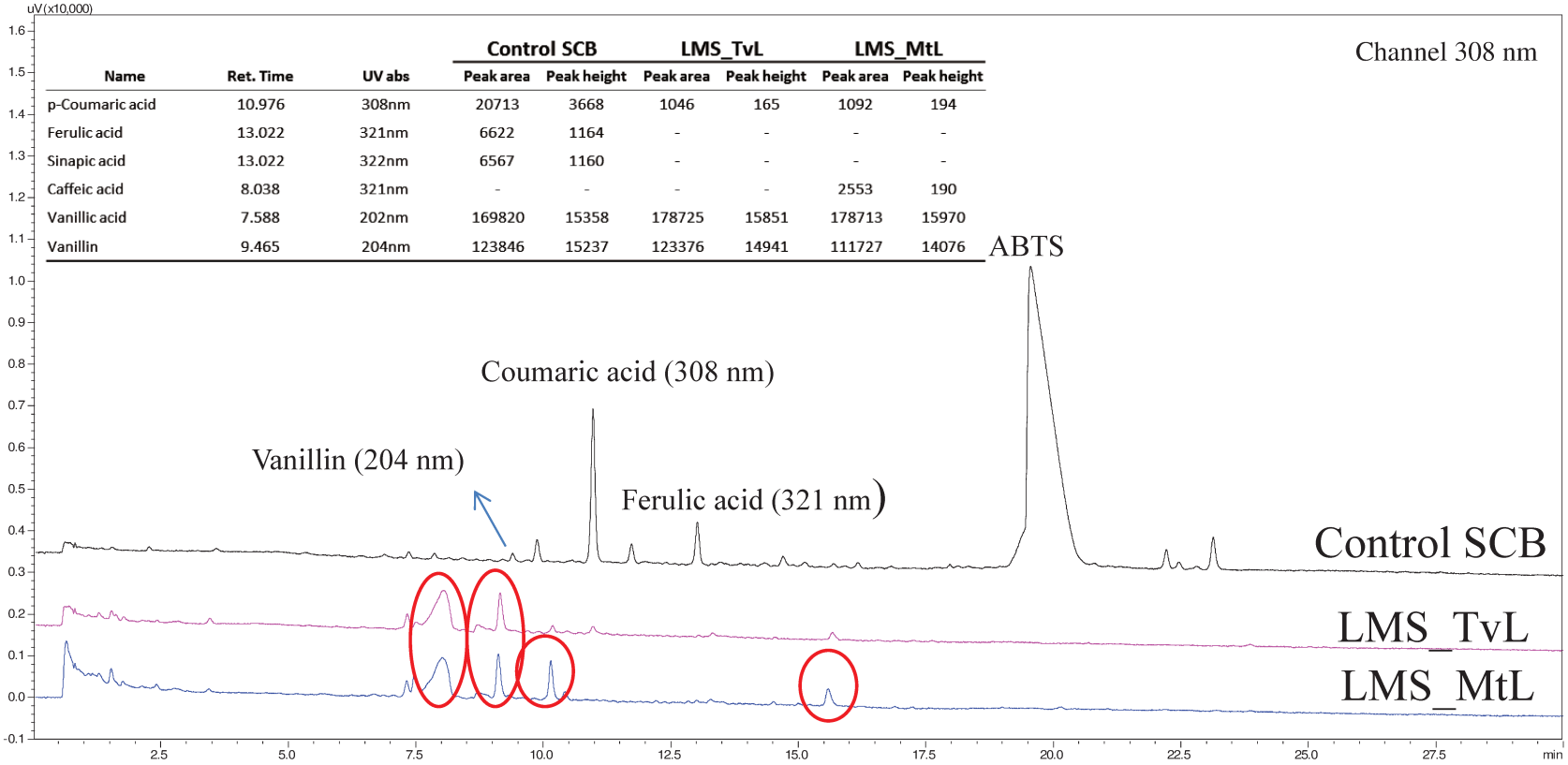

Figure S2 and Table S3. RP-HPLC analysis of the supernatants obtained for the pre-treated wheat straw incubated with buffer and ABTS (black line), LMS_MtL (blue line) or LMS_TvL (pink line). New peaks that appeared after LMS treatment are circled by red circles.

			Control WS		LMS_TvL		LMS_MtL	
Name	Ret. Time	UV abs	Peak area	Peak height	Peak area	Peak height	Peak area	Peak height
p-Coumaric acid	10.976	308nm	4136	747	1271	213	1126	200
Ferulic acid	13.022	321nm	1502	239	-	-	1509	211
Sinapic acid	13.022	322nm	2625	460	-	-	1095	154
Caffeic acid	8.038	321nm	-	-	-	-	4124	528
Vanillic acid	7.588	202nm	814027	50846	755795	44950	744693	44453
Vanillin	9.465	204nm	488443	40457	113915	13957	398343	35625

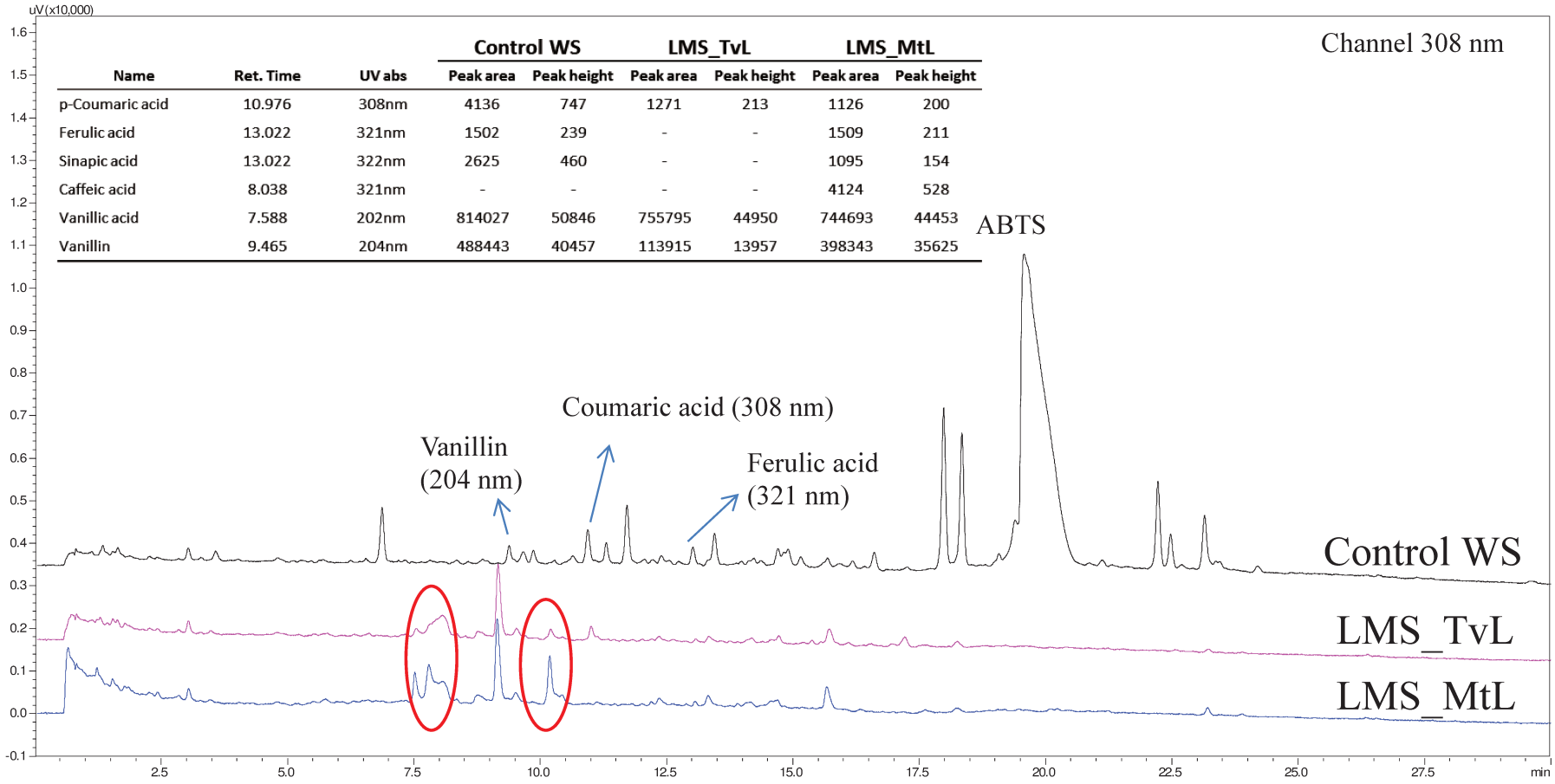

Table S4. Relative absorbance of bands in the infrared spectrum of different groups in the untreated and LMS -pretreated Avicel samples.

Wavelenght	3335 cm^{-1}	2850 cm^{-1}	1105 cm^{-1}
Assignment	OH stretching	CH_2 symmetrical stretching	C-O-C glycosidic
Relative Absorbance*			
Avicel + ABTS	0.28	0.09	0.52
Avicel + LMS_MtL	0.26	0.08	0.56
Avicel + LMS_TvL	0.19	0.06	0.53

Legend: LMS_MtL – MtL laccase mediator system treatment, LMS_TvL – TvL laccase mediator system treatment.

*From the normalized spectra.

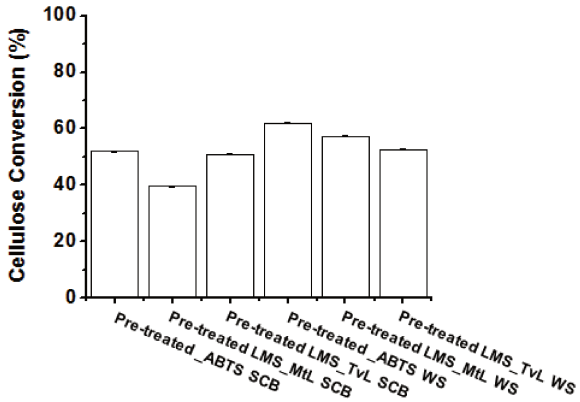

Figure S3. Cellulose conversion % of hydrothermal sugarcane bagasse and wheat straw treated with laccase mediator system, followed by laccase inactivation, washing procedure and hydrolyzed using Cellic® CTec2 for 72h. Error bars represent the standard errors of the means of triplicate experiments. Legend: CT2 - Cellic® CTec2, SCB – pre-treated sugarcane bagasse, WS – pre-treated wheat straw, LMS_MtL – MtL laccase mediator system treatment, LMS_TvL – TvL laccase mediator system treatment, ABTS – only mediator treatment.

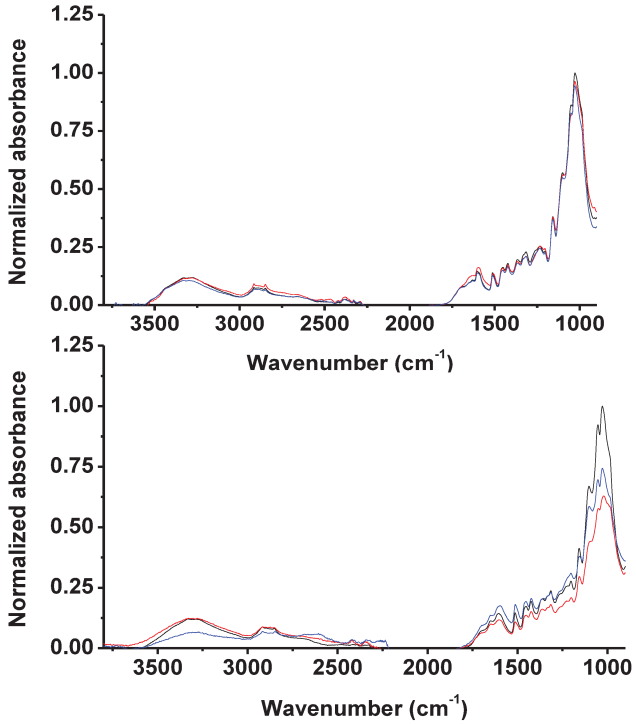

Figure S4. Fourier transforms infrared (FTIR) spectra of pre-treated sugarcane bagasse (A) and wheat straw (B) after ABTS only (black), LMS_MtL (red) and LMS_TvL (blue) treatment.

Table S5. Relative absorbance of bands in the infrared spectrum of different groups in the untreated and LMS -pretreated sugarcane bagasse and wheat straw samples.

Wavelenght	3500 cm^{-1}	2850 cm^{-1}	1604 cm^{-1}	1150 cm^{-1}	1030 cm^{-1}
Assignment	OH stretching	CH_2 symmetrical stretching	C-phenolic vibration (aromatic ring)	O-C=O esters linkages	C-O-C ether linkages
Relative Absorbance*					
SCB + ABTS	0.12	0.07	0.14	0.36	0.99
SCB + LMS_MtL	0.11	0.09	0.16	0.37	0.96
SCB + LMS_TvL	0.10	0.07	0.14	0.35	0.94
WS + ABTS	0.12	0.08	0.14	0.39	1.0
WS + LMS_MtL	0.12	0.09	0.12	0.29	0.62
WS + LMS_TvL	0.06	0.07	0.17	0.38	0.74

Legend: SCB: sugarcane bagasse, WS: wheat straw, LMS_MtL – MtL laccase mediator system treatment, LMS_TvL – TvL laccase mediator system treatment.*From the normalized spectra.

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CHAPTER 4 – Genomic, transcriptomic and proteomic analysis and biotechnological application of the marine-derived basidiomycete producer of ligninocellulytic enzymes *Peniophora* sp. CBMAI 1063

Manuscript in preparation.

Authors: Lívia B. Brenelli[1,2,6], Gabriela F. Persinoti[2], Igor V. R. Otero[3], Pedro H. Mainardi[3], João Paulo L. F. Cairo[1,2], Thiago Gonçalves[1,2], Marcelo V. Liberato[2], Adriana F. Paes Leme[4], Claus Felby[5], Lara D. Sette[3]& Fabio M. Squina[6*]

[1]Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brazil.
[2]Laboratório Nacional de Ciência e Tecnologia do Bioetanol (CTBE), Centro Nacional de Pesquisa em Energia e Materiais (CNPEM), Campinas, SP, Brazil.
[3]Universidade Estadual Paulista (UNESP/RC/IB), Rio Claro, SP, Brazil.
[4]Laboratório Nacional de Biociências (LNBio), Centro Nacional de Pesquisa em Energia e Materiais (CNPEM), Campinas, SP, Brazil.
[5]University of Copenhagen, Faculty of Science, Department of Geosciences and Natural Resource Management, Frederiksberg C, Denmark.
[6]Programa de Processos Tecnológicos e Ambientais, Universidade de Sorocaba, Sorocaba, SP, Brazil.

Abstract

The environment has enormous microbial diversity that remains unexplored. In particular, marine-derived fungal species are potential sources for novel enzymes with biotechnological applications, due to their unique properties such as high salt tolerance, thermostability, barophilicity and cold activity. In this work, we report the genome sequencing and gene annotation of the marine-derived basidiomycete *Peniophora* sp. CBMAI 1063 as well as the transcriptome and the secretome analyses of the fungus grown under its optimal cultivation condition. The resulting draft genome assembly of *Peniophora* sp. CBMAI 1063 is 46,982,497 bp in length with an N50 of 155,805 bp, and GC content of 55.21%. The genome encodes 17,714 protein-coding genes with 11,802 clusters of orthologous. The comparison of the predicted genes against the Carbohydrate-Active Enzymes (CAZy) database identified a total of 310 proteins related to lignocellulose degradation and cell wall modifications. Transcriptomic analysis showed that 98% of these CAZymes were expressed under optimal cultivation conditions, with the most CAZy classes represented in the transcriptome. Secretome

analyses revealed that 48% of the identified proteins are related to lignocellulose degradation, especially lignin degradation and modification. Aiming to disclose new oxidative enzymes with biocatalytic activities for lignocellulose deconstruction, the major laccase secreted by the fungus (Pnh_Lac1) grown under optimal cultivation condition was purified and its effects on lignin degradation were investigated. In addition, the three-dimensional structure of Pnh_Lac1 was generated from homology modeling. The "omics" analysis collectively show that *Peniophora* sp. CBMAI 1063 possesses a complete and versatile ligninolytic enzymatic spectrum with biotechnological potential to be explored, and the major secreted laccase can be applied for deconstruction of lignocellulose into fuels and chemicals.

Keywords

Marine fungi, genome annotation, oxidoreductases, laccases, CAZymes, lignocellulose, Biotechnology

1. Introduction

Lignocellulose is the major component of biomass, representing the most abundant renewable organic resource on Earth (Sánchez, 2009). In general, it is composed by four polymeric constituents: cellulose, hemicellulose, pectin, and lignin (Chundawat et al. 2011). Among all these polymers, the lignin is highly resistant towards biological degradation due to its complex aromatic structure network (Doherty et al. 2011), and to date, basidiomycetes are the most efficient naturally-found lignin degraders. According to their lifestyle and ability to degrade polymeric constituents, these microorganisms have been classified into two major groups, white-rot and brown-rot fungi (Henrissat et al. 2012; Ohm et al. 2014). Brown-rot fungi initiate degradation of cellulose through non-enzymatic Fenton mechanism without degrading the lignin (Lundell et al. 2014; Martínez et al. 2005), while many white-rot fungi simultaneously attack lignin, hemicellulose and cellulose or preferentially degrade lignin (Dashtban et al. 2010).

Currently it is known that the cellulose and hemicellulose degradation by white-rot fungi involves enzymes from the Glycoside Hydrolase (GH) and Auxiliary Activities (AA) families via hydrolytic and oxidative mechanisms, respectively. The discovery that oxidative enzymes named lytic polysaccharide mono-oxygenases (LPMOs) are active on polysaccharides (Vaaje-Kolstad et al. 2010) demanded a reclassification of these proteins by the Carbohydrate-Active Enzymes database (CAZy) (Lombard et al. 2014), once they were previously classified

as GHs or carbohydrate-binding module (CBM). Then, the new class AA was created and organized by families, gathering four families of LPMOs (AA9, AA10, AA11 and AA13), cellobiose dehydrogenases (CDHs) and glucose oxidases (GOx) in the family AA3, which act as electron donors to LPMOs and others oxidases (Levasseur et al. 2013). The CAZy also included the ligninolytic enzymes among the families AA1, AA2, AA4, AA6, AA8 and AA12, since lignin is found invariably in the plant cell wall along with polysaccharides and lignin and low molecular weight lignans (LMWL) can deliver electrons to LPMOs (Westereng-Cannella et al. 2015).

White-rot fungi generally have more encoding genes for hydrolytic and oxidative enzymes and cellulose binding domains (CBM) comparing to brown-rot fungi. Cellobiohydrolases from families GH6 and GH7, as well CDHs from subfamily AA3_1, are present in white-rot and reduced or absent in brown-rot fungi. (Riley et al. 2014; Levasseur et al. 2013).

Recently, extensive samplings of basidiomycete genomes have demonstrated that the classification "white-rot" and" brown-rot" for wood decay fungi should be reviewed. Several white-rot species lack lignin-degrading peroxidases (family AA2), and thus resemble brown-rot fungi. However, these fungi appear to degrade lignin without AA2 and the others cell wall components by using a cellulose-degrading apparatus typical of white-rot fungi (Riley et al. 2014; Floudas et al. 2012).

Although the family AA2 joins important enzymes for lignin degradation by basidiomycetes, there are other classes of enzymes able to degrade or to modify lignins. The multicopper oxidases of family AA1, specifically laccases (subfamily AA1_1), is an example, once they are more abundant in white-rot fungi rather than brown-rot fungi. Laccases are able to oxidize a wide range of aromatic compounds and lignin using oxygen as the electron acceptor (Munk et al. 2015; Christopher et al. 2014; Li et al. 1999).

Besides being important in lignin degradation, fungal laccases exhibit potential in several applications such as chemical, fuel, food, agricultural, paper, textile and cosmetic sectors (Gutiérrez et al. 2012; Sette et al. 2008; Couto & Herrera, 006). A multiple sequence alignment of more than 100 laccases revealed a set of four ungapped sequence regions (L1–L4) that can be used to identify laccases (Kumar et al. 2003). The 12 amino acid residues responsible for copper linkage are hosted in these four conserved regions where L2 and L4 is reported as being the signature sequences of multi-copper oxidases and L1 and L3 as distinctive to laccases (Kumar et al. 2003).

Analyses of laccase gene diversity have mostly been performed on basidiomycetes and xylariaceous ascomycetes from soil (Kellner, Luis & Buscot, 2007; Pointing et al. 2005; Luis et al. 2004). Therefore, the analysis for marine-derived basidiomycete species are still required (Bonugli-Santos et al. 2015; D'Souza-Ticlo, Sharma & Raghukumar, 2009).

Marine-derived fungal species have been considered attractive producers of ligninolytic and hemicellulolytic enzymes and others industrial enzymes, offering different properties from the terrestrial origin such as high salt tolerance, thermoestability, barophilicity and cold activity (Bonugli-Santos et al. 2015; 2012; Trincone, 2010; Raghukumar, D'Souza-Ticlo & Verma, 2008).

The basidiomycete *Peniophora* sp. was isolated from the marine sponge *Amphimedon viridis* and taxonomically identified as previously reported by Bonugli-Santos et al. (2010a). Thereafter, it was described the production of ligninolytic enzymes under saline and non-saline conditions by *Peniophora* sp. CBMAI 1063 as others basidiomycetes recovered from marine sponges and the potential to be applied in textile dye decolorization (Bonugli-Santos et al. 2016). Despite the recent reports on *Peniophora* sp. CBMAI lignolytic capacity, the molecular mechanisms underlying this characteristic are scarce.

In this report, we unveiled the enzymatic repertoire in the genome, transcriptome and secretome of the marine-derived basidiomycete *Peniophora* sp. CBMAI grown under optimal cultivation conditions. We focused on the oxidative enzymes aiming biotechnological applications for the *Peniophora* sp. CBMAI for value-added products. The major laccase secreted by the fungus (Pnh_Lac1) has had its three-dimensional structure generated from homology modeling and tested for lignin degradation.

2. Material and Methods

2.1 Microorganism

The marine-derived fungus *Peniophora* sp. CBMAI 1063 was isolated from the Brazilian sponge *Amphimedon viridis* collected in the town of São Sebastião, São Paulo State, Brazil (Menezes et al. 2010) The strain was deposited at the Brazilian Collection of Microorganisms from Environment and Industry—CBMAI under the accession number CBMAI 1063.

2.2 Fungal growth

The solid cultivations of *Peniophora* sp. CBMAI 1063 were carried out in Petri dish plates containing malt extract (20 g L^{-1}) and agar-agar (15 g L^{-1}) at 28 °C. After 7 days of growth, six

agar-plugs with approximately 0.9 cm of diameter were inoculated in non-baffled 125 mL Erlenmeyer flasks containing 50 mL of an optimized medium contained yeast extract (2.0 g L^{-1}), peptone extract from casein (2.7 g L^{-1}), malt extract powder (1.4 g L^{-1}) and glucose (2.7 g L^{-1}). The reagents were solubilized in distilled water containing 65% (v/v) of adapted Kester's et al. (1967) artificial seawater (ASW), containing: $MgCl_2$ (10.83 g L^{-1}), $CaCl_2$ (1.51 g L^{-1}), $SrCl_2$ (0.02 g L^{-1}), NaCl (23.9 g L^{-1}), Na_2SO_4 (4,0 g L^{-1}), KCl (0.68 g L^{-1}), $NaHCO_3$ (0.2 g L^{-1}), KBr (0.1 g L^{-1}) and H_3BO_3 (0.03 g L^{-1}). The culture medium was supplemented with aqueous $CuSO_4$ to a final copper concentration of 2 mM. The flasks were kept at 28 °C under constant agitation (140 rpm) for 5 days. The inoculated flasks were used as inoculum in a bioreactor Fermac320 (Electrolab Limited, UK) with 6.4 L of total volume at an equivalent proportion of 8.5 % (v/v) for 5 days. The bioreactor was placed in a temperature-controlled-room at 20°C and the incoming air was filtered through two 0.2 µm filters (Sartorius-GER) and injected to the system through an "L" type sparger with 5 bottom roles.

2.3 DNA preparation and sequencing

Genomic fungal DNA for sequencing was extracted from the culture using the cetyltrimethyl ammonium bromide (CTAB) method adapted from Moller et al. (1992) and Gerardo et al. (2004) and purified using a Power Clean® DNA Clean-Up Kits (Mo Bio Laboratories). High-quality genomic DNA was then submitted to construction of a sequencing library using Nextera DNA library preparation Kit (Illumina). Next, the library was submitted to sequencing on Hiseq 2500 instrument, with the HiSeq Rapid kit v2 chemistry in paired-end mode (2X100 pb), available at the Brazilian Bioethanol Science and Technology Laboratory (CTBE/CNPEM).

2.4 Genome assembly

The genome of *Peniophora* sp. CBMAI was sequenced on the Illumina HiSeq 2500 system at NGS sequencing facility at Brazilian Bioethanol Science and Technology Laboratory (CTBE), generating 55 million paired end reads (2 x 100 bp). Reads were processed with Trimmomatic 0.32 to remove low quality reads and adapter sequences, resulting in 39 million quality filtered reads. The genome size was estimated to be 47.74 Mbp based on kmer counts statistics (kmergenie), with an estimated coverage of 165 X. Before the assembly, reads were normalized based on kmer abundance with Khmer to a coverage of 20X and subjected to assembly using Velvet version 1.2.10 with k=55. The complete subset of quality filtered reads was used to improve the assembly and for scaffolding using Pilon version 1.16 and SSPACE version 3.0.

2.5 Gene prediction and Annotation

RNA-seq data (kindly provided by Professor Lara Sette) was used as evidence for gene prediction. QC reads were aligned to the scaffolds produced by SSPACE using Hisat2 and subjected to BRAKER1 genome annotation pipeline that the spliced alignments to the genome, GeneMark-ET and Augustus for gene prediction (Hoff et al. 2015). Non coding rRNA genes were identified using both ITSx version 1.0.11 and RNAmmer version 1.2, and tRNA genes were annotated using tRNAscan version 1.3.1. The completeness of the genome gene set was estimated using BUSCO version 1.1, which revealed that the current assembly is 93% complete based on 1438 single-copy orthologous genes markers from Fungi. Functional annotation was performed by comparison of the predicted protein sequences with SwissProt database (The UniProt. 2017), UniRef90 database (Suzek et al. 2015), PFAM (Finn et al. 2016), dbCAN (Yin et al. 2012) and EggNOG databases (Huerta-Cepas et al. 2016).

2.6 Phylogenetic analysis

The phylogenetic analysis was conducted using 38 organisms from *Basidiomycota phylum* with available genomes sequences, being 2 genomes sequenced at CTBE and 36 sequenced by JGI. Among the genomes analyzed, 34 are from Agaricomycetes class and 2 from Ustilaginomycotina as out group. A set of 92 single copy genes markers was used to perform the phylogenetic analysis. The protein sequences of each marker gene were aligned using MAFFT version v7.299b, and concatenated into a super matrix using FASconCAT-G version 1.2. The phylogenetic inference was performed using RAxML version 8.2.0 with PROTGAMMAWAG model.

2.7 Secretome identification - proteomic analysis

Firstly, the crude fungal extract was filtered using Whatman paper filter n.1 and 1 L of supernatant was concentrated 20 times through an Amicon® membrane (30 kDa cut-off – Millipore) and submitted to electrophoresis analysis in polyacrylamide gel containing 0.1 % (*w/v*) sodium dodecyl sulfate (SDS-PAGE). The gel-bands were excised from the SDS-PAGE and the protein digestion for the mass spectrometry-based analyses were performed in two steps as described in details by Gonçalves et al. (2012). The samples were stored at −20 °C until further analysis by LC–MS/MS. Each sample was mixed with 12 μL of 0.1 % (v/v) formic acid, and 4.5 μL of the peptide mixture were injected into the LC–MS/MS chromatograph (RP-nanoUPLC, nanoAcquity, Waters, Milford, MA). Peptide separations were performed on a C18

column (100 nm × 100 mm) previously equilibrated with a 0.1 % (v/v) formic acid buffer. The elution gradient ranged from 2 to 90 % (v/v) acetonitrile in 0.1 % (v/v) formic acid at 0.6 μL/min. Eluted peptides were analyzed in a quadrupole time of flight (Q-TOF) spectrometer (Ultima Mass Spectrometer, Waters Milford, MA) operating in the "top three-MS and MS/MS mode" (Ultima Mass Spectrometer, Waters software). The spectra were acquired using the Mass Lynx v.4.1 software (Waters, Milford, MA, USA), and the raw data were converted to "peak list format (mgf)" using the Mascot Distiller software v.2.3.02, 2009 (Matrix Science Ltd., London, UK). The results were processed by the Mascot v.2.3.02 engine software (Matrix Science Ltd.) against *Peniophora* sp CBMAI 1063 genome sequence database generated in this work. The following parameters used in this process: carbamidomethylation as a fixed modification, oxidation of methionine as a variable modification, one trypsin cleavage error and a maximum allowable peptide mass error 0.1 Da. The resulting Mascot data were analyzed for protein identification using Scaffold 3.5.1 (Proteome Software, Portland, OR). The defined parameters were as follows: a minimum protein probability of 80 %, a minimum peptide probability of 90 % and a uniquely different minimum peptide of 1. Proteins with scores up to 10 % FDR (false discovery rate) for a protein and 5 % FDR for a peptide were accepted.
The presence of a signal peptide of secreted proteins was predicted by SignalP v.4.0 (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al. 2011) and the subcellular localization of proteins was predicted by YLoc (Interpretable Subcellular Localization Prediction) (abi.inf.unituebingen.de/Services/YLoc/webloc.cgi) (Briesemeister, Rahnenführer, & Kohlbacher, 2010).

2.8 Laccase purification for the enzymatic assays

Laccase activity and putative laccase genes in *Peniophora* sp. CBMAI has already been reported and characterized previously (Bonugli-Santos et al. 2012: 2010). The crude fungal extract was filtered using Whatman paper filter n.1 and the supernatant was concentrated through an Amicon® membrane (30 kDa cut-off – Millipore) and buffer-exchanged into 50 mM Tris HCl buffer pH 7. The enzyme was submitted an ion exchange chromatography (DEAE - Sepharose Fast Flow GE Healthcare 20 mL) in 1.0 M NaCl gradient (0 to 100%) followed by size exclusion chromatography (Superdex200 HiLoad GE Healthcare 124 mL) using Tris HCl 50 mM / 0.15 M NaCl pH 7 buffer as eluent. Both purification steps were carried out in an automated AKTA™ Purifier system (GE Healthcare) equipped with UV detector (280nm) at flow rate of 1 mL min^{-1}. To determine the purity of the protein preparation and its molecular

weight, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 12% polyacrylamide gel containing 0.1% SDS. Laccase was treated before it was loaded onto the gel with β-mercaptoethanol and boiled at 100°C for 10 min. The protein was visualized by staining the gel with Coomassie blue G-250 (Bio-Rad) and compared with molecular weight markers (PageRuler Unstained Protein Ladder Thermo Scientific). Protein concentration was determined by Bradford method (Bradford, 1976).

2.8.1 Laccase assay

The laccase activity was determined using syringaldazine (SGD) as substrate (Felby et al. 1997). Oxidation of SGD was monitored at 525 nm for 5 minutes (readings every 20 seconds) at room temperature in a spectrophotometer Tecan® Infinite. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 µmol of SGD per minute.

2.8.2 Laccase-Mediator System (LMS) assays and lignin modification

The alkali-lignin used in this study (96% purity) was extracted from stream-exploded sugarcane bagasse according Brenelli et al. 2016. Lignin stock solution at 10 mg mL^{-1} was prepared in NaOH 0.1 mol L^{-1}. In the reaction system, 100 µL of the lignin stock solution was incubated with sodium acetate buffer 0.05 M pH 5.0, purified laccase at 0.6 U g $lignin^{-1}$ and 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Sigma-Aldrich®) as mediator at 1 mmol L^{-1} final concentration. The 1.0 mL reaction volume was incubated at 30 °C, 1000 rpm shaking in a Thermomixer (Eppendorf®) for 72 h. Incubations containing inactivated laccase and ABTS mediator were used as controls. After the incubations, the laccase activity was stopped by adding NaN_3 at a final concentration of 0.05% (w V^{-1}) (Johannes et al. 2000). The reaction was centrifuged (12, 000 x *g* for 10 min at 4 °C) and the supernatant collected for subsequent analysis. Changes in the molecular weight distribution of the soluble lignins and lignins-derived compounds was detected by gel exclusion chromatography (GPC) using Superdex 30 column (65 cm x 1.6 cm) in an automated AKTA™ Purifier system (GE Healthcare) equipped with UV detector (280nm) and NaOH 0.1 M as eluent. The flow was of 0.1 mL min^{-1} at room temperature and 500 µL of sample injection volume. Tannic acid, ferulic acid, coumaric acid, cinnamic acid, hydroquinone and vanillin from Sigma Aldrich® and lignins with known molecular weight were used as internal standards. UV-vis spectroscopy analysis was performed in a spectrophotometer Tecan® Infinite. The samples were diluted in 20 times

NaOH 0.1 mol L^{-1} and the UV-vis absorption spectrum (220 to 500 nm) was recorded using quartz cuvette 1 cm.

2.8.3 LMS as pre-treatment step for enzymatic hydrolysis of steam-exploded sugarcane bagasse

Steam-exploded sugarcane bagasse (SCB) was kindly provided by The Engineering School of Lorena (EEL-USP) and details of the process and the washing and pressing step to remove solubilized sugars and degradation products were described previously (Rocha et al. 2012). The SCB chemical composition was determined using the NREL method (Sluiter et al. 2011) for lignocellulose biomasses, and reported to be 52% glucan, 6% xylan, 24% lignin, 0.2 % galactan, 0.5% arabinan. The cellulolytic commercial cocktails employed in this study were Accellerase® (DuPont) and Cellic® Ctec2 (Novozymes A/S, Bagsvaerd, Denmark). The cellulase activities of the enzymatic preparations were 168 and 250 FPU mg protein mL^{-1}, respectively, measured by the filter paper assay. Both preparations were stored at 4 °C until the enzymatic hydrolysis assay. First, laccase-mediator treatment was performed in 2.0 mL tubes at 2.5 % (w V^{-1}) dry matter (DM) in sodium acetate buffer pH 5.0 in 1 mL reaction volume at 30 °C, 1000 rpm shaking in a Thermomixer® (Eppendorf). Purified laccase were added to a dosage of 0.02 U g $biomass^{-1}$ and ABTS mediator at 1 mmol L^{-1} final concentration. Incubations containing inactivated laccase and ABTS mediator were used as controls. After 72h, laccase activity was stopped by adding NaN_3 (sodium azide) at a final concentration of 0.05% (w V^{-1}) and the commercial cocktails Accellerase® or Cellic® CTec2 were added in a low dosage of 2.5 FPU g $cellulose^{-1}$ DM and incubated for 72h (50 °C, 1000 rpm) in a ThermoMixer® (Eppendorf). The supernatant was separated by centrifugation and filtered (0.45µM, Millipore®). The reducing sugars was measured reacting 100 µL of the supernatant with 100 µL of 3,5-dinitrosalicylic acid for 5 min at 99 °C (Miller, 1959). The cooled solution was analyzed at 540 nm in an Infinite M200® spectrophotometer (Tecan-Switzerland) and compared with an internal calibration curve using glucose. All enzymatic hydrolyses were carried out in triplicate and the average and standard deviation values were presented. The supernatant from LMS-treated SCB not hydrolyzed was analyzed by GPC as described in 2.8.2.

2.8.4 Statistical analysis

Statistical analysis was performed by variance analysis (One-way ANOVA) with a probability level (P) less than 5% (P< 0.05) using the program STATISTICA 5.5 from StatSoft Inc. (Tulsa, OK, USA).

3. Results and Discussion

3.1 *Peniophora sp.* CBMAI 1063 draft genome feature

The genome of the marine *Peniophora* sp. CBMAI 1063was sequenced using Illumina sequencing, with 165X coverage and reaching 93% of genome completeness. The resulting draft genome assembly of *Peniophora* sp. CBMAI 1063 is 47,9 Mb in length with an N50 of 155,8 Kb, and average G + C content of 55% (Table S1). The genome size of the fungus is similar to the other two *Peniophora* genomes available at JGI Mycocosm portal: 48.4 Mb for *Peniophora* sp. CONTA (Lopni1) and 46.0 Mb for *Peniophora* aff. *cinerea* (Ricme1), both plant pathogens (Figure 1).

The number of specific genes for each strain was very similar, contrasting with the number of orthologous genes shared only with one of the strains, which revealed that the two *Peniophora* sp. plant-pathogen strains shared a high number of orthologous genes (Figure 1). Moreover, phylogenetic analysis using a set of 92 single-copy genes markers and 38 genomes from Basidiomycetes phylum (Figure 2) indicated that the *Peniophora* sp. CBMAI 1063 clustered together with the other two *Peniophora* species previously sequenced by JGI, forming a monophyletic clade in the Russulales order

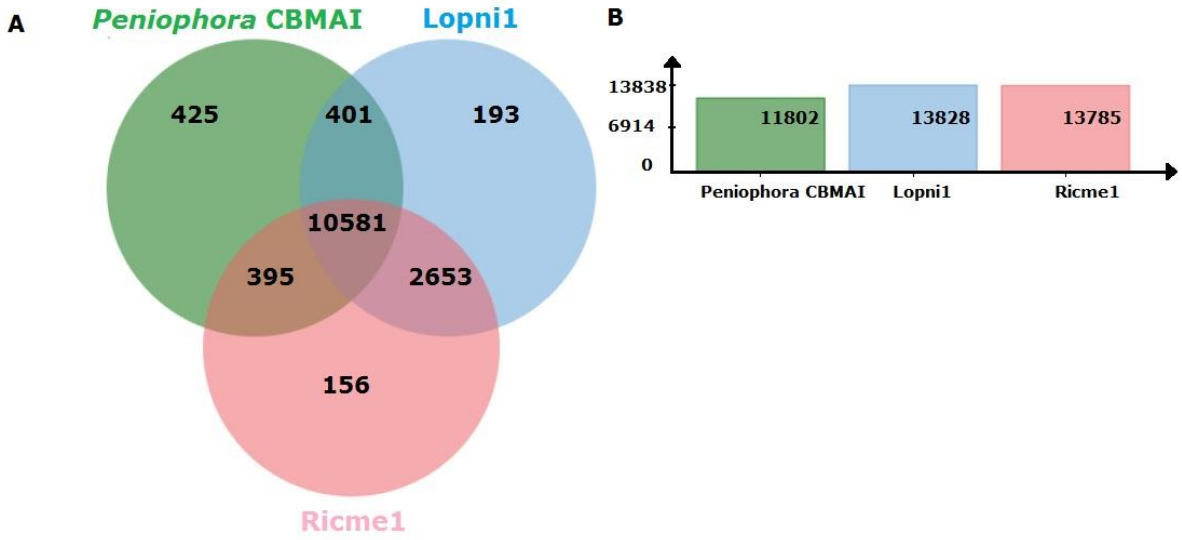

Figure 1. Comparison of orthologous gene clusters of *Peniophora* sp. CBMAI 1063, *Peniophora* Lopini1, and *Peniophora* Ricme1 (a) and the total number of orthologous gene clusters of these three organisms (b). These genomes share 10581 orthologous clusters.

3.2 Prediction of genes related to degradation of lignocellulose

The cell wall components (cellulose, hemicellulose, pectin and lignin) have limited enzyme accessibility due to the covalent cross-linkages among its constituents, which create an intricate network and a physical barrier resistant to microbial degradation (Martínez et al. 2009; Ruiz-Dueñas & Martínez, 2009). The genome of *Peniophora* sp. CBMAI 1063 encodes 17,714 genes, being 11,802 clusters of orthologous. The comparison of the predicted genes against the CAZy database profiles available at dbCAN, identified a total of 310 predicted coding genes related to lignocellulose degradation (Figure 3).

Among the predicted lignin-degrading genes, it was found, in *Peniophora* sp. CBMAI 1063 genome, domains for the families AA1, 2, 3, 4, 5 and 6 (Figure 3). It was found 18 genes encoding enzymes for the family AA1, 17 genes for AA2 and 3 genes for AA4 (vanillyl-alcohol oxidase) (Figure 3). According to the typical classification, representatives of *Peniophora* genus are white-rot fungus *with a versatile arsenal for CAZymes,* drawing attention for the biotechnological potential, not only for biodegradation of recalcitrant pollutants and treatment of textile effluents, but also for biomass degradation for second generation bioethanol.

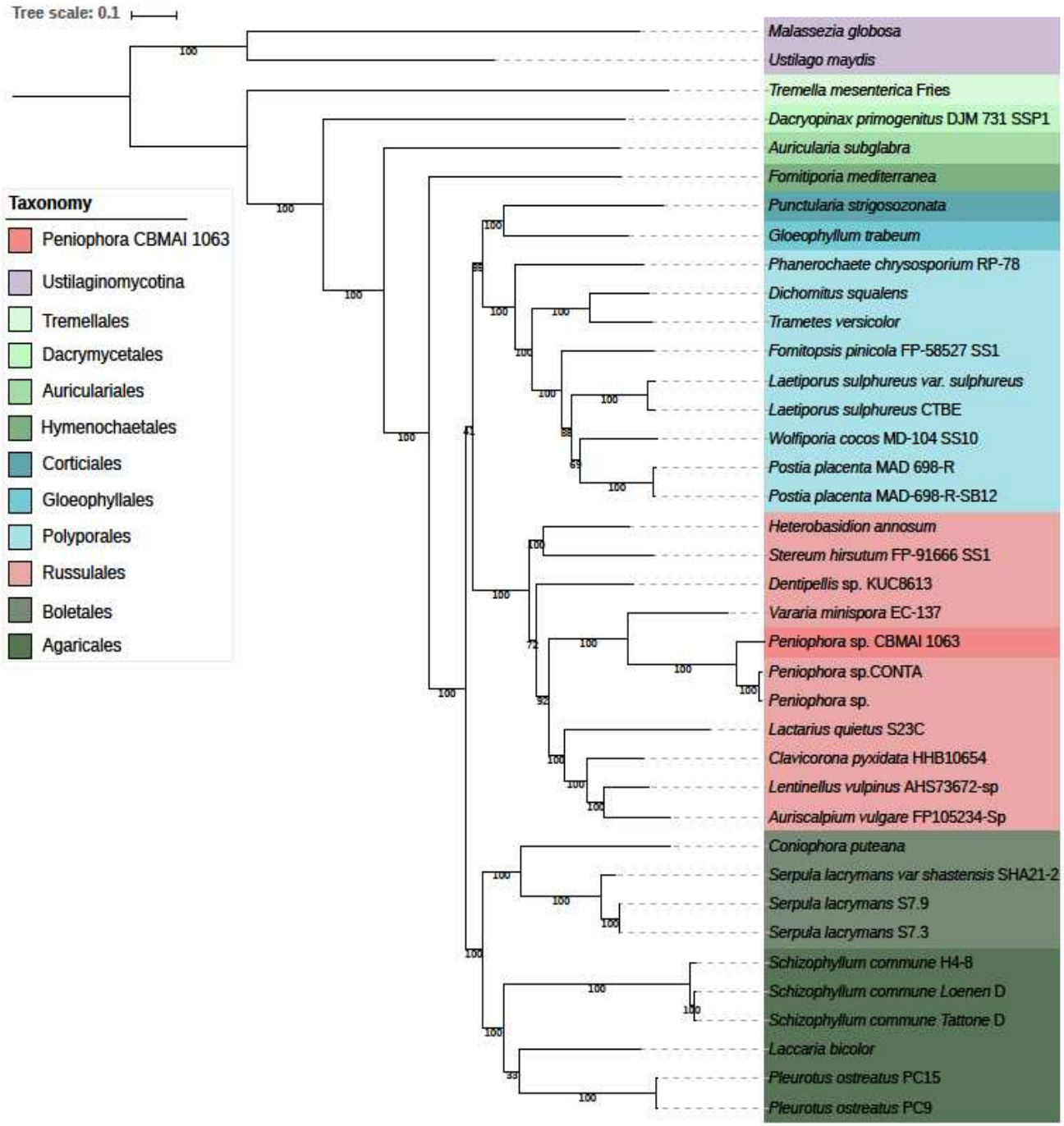

Figure 2. Phylogenetic tree of Agaricomycetes class based on 38 fungi genomes distributed among the *Basidiomycota phylum*. A set of 92 single copy genes markers was used to perform the phylogenetic analysis. Bootstrap values for 1000 replicates are show in the branches.

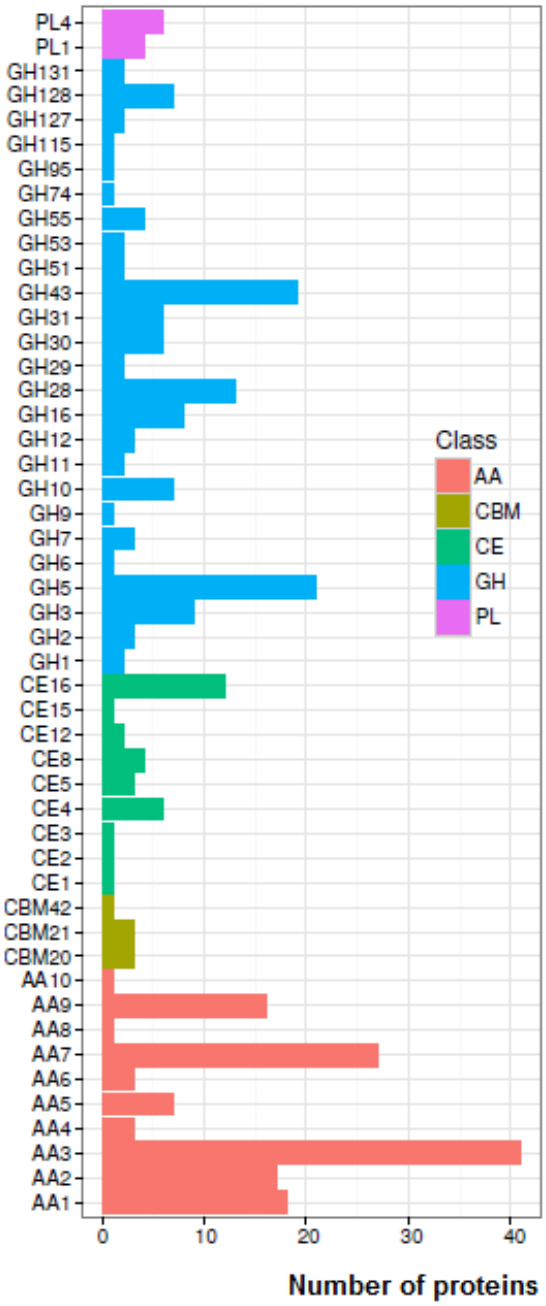

Figure 3. Number of predicted genes encoding CAZymes in *Peniophora* sp. CBMAI 1063 genome among the classes covered by the CAZy database (http://www.cazy.org/): GH- Glycoside Hydrolases, PL- Polysaccharide Lyases, CE- Carbohydrate Esterases, AA- Auxiliary Activities and CBM- Carbohydrate-binding module.

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We found, among the CAZy families, potential modules or domains for glycoside hydrolases, such as GH5, GH6, GH7, GH10, GH11, GH28 and GH43, carbohydrate esterases (CE) from families 4, 8 and 16, CBM families 20 and 21, and auxiliary activities families AA3, AA8, AA9 and putative AA10 (Figure 3). LPMOs have been found widespread in Tree of Life from bacteria to fungi (Cragg et al. 2015) and often co-expressed with sugar oxidases, such as from families AA3, AA7 and AA8 (Bey et al. 2013). Remarkably, 41 coding genes for protein families AA3 along with 16 genes for AA9, 27 genes for AA7 and 1 gene for AA8 were present in the genome of *Peniophora* sp. CBMAI 1063 (Figure 3).

3.3 Transcriptomic analysis of CAZymes

The transcriptomic analysis of *Peniophora* sp. CBMAI 1063 growth in optimized medium was performed previously (Otero et. al., 2017). This previous RNA-Seq data was used to genome annotation and to get insights about the expression profile of the CAZy genes. From the 310 CAZy genes related to lignocellulose degradation, predicted in the genome, 303 were expressed under optimal cultivation conditions (Figure 4 and Figure S1).

Among the GH families involved in cellulose, hemicellulose and pectin degradation, the expression of members from families GH1, GH3, GH5, GH7, GH10, GH11, GH43 and GH51 were detected. The gene expression of carbohydrate esterases from several families was also reported, along with the expression of genes involved in lignocellulose oxidation (Figure 4 and Figure S1). All the families from AA class predicted in the *Peniophora* sp. CBMAI 1063 genome were assigned in the transcriptomic analysis (Figure 4). The LPMOs from families AA9 and AA10 had their expression reported together with the genes coding for AA3, AA7, and AA8, their putative protein partners for the electron donors.

The gene with the highest number of TPM (Transcripts Per Million) among all the CAZy genes (around 4,500 TPM) (Figure S1) encodes a cytoplasmatic AA3 enzyme g15979t1, containing a GMC_oxiduretucase Pfam domain, suggesting its role in the lignin metabolism.

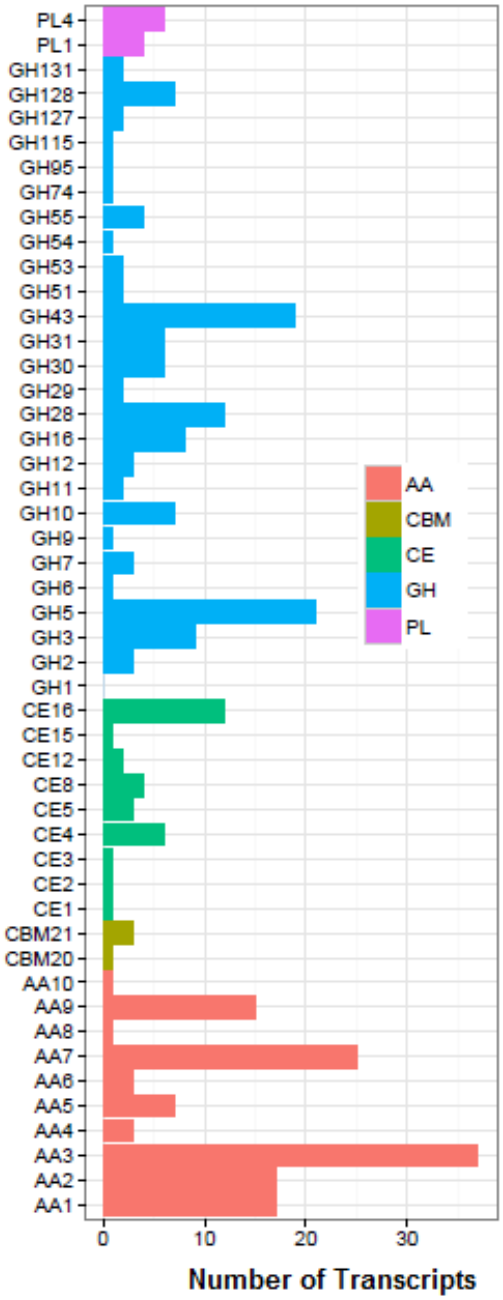

Figure 4. Number of transcripts for CAZymes in *Peniophora* sp. CBMAI 1063 genome among the classes covered by the CAZy database (http://www.cazy.org/): GH- Glycoside Hydrolases, PL- Polysaccharide Lyases, CE- Carbohydrate Esterases, AA- Auxiliary Activities and CBM- Carbohydrate-binding module.

The genes encoding ligninolytic enzymes from families AA1, AA2, AA4, and AA6 were also expressed (Figure 4). *Peniophora* sp. CBMAI 1063 is known for exhibit high laccase activity (Bonugli-Santos et al. 2012: 2010). Although Otero et al. (2017) reported eight laccase

genes on the transcriptome of *Peniophora* sp. CBMAI 1063 in the present study 18 predicted laccase genes from family AA1 were found and all of them were expressed under optimized cultivation (Figure 4). This discrepancy may be due to the different annotation methods applied. The laccase genes 15721.t1, g1591.t1, and g17194.t1 were the most abundant (number of TPMs) among the 18 genes predicted as AA1 (Figure S1). Concerning the AA2 coding genes, the peroxidases g10529.t1, g14863.t1, and g8820.t1 were the most abundant, however with less TPMs counts than the laccases from AA1 family (Figure 4 and Figure S1).

3.4 Secretomic analyses

A wide variety of CAZymes and oxidoreductases are produced by Basidiomycota species to degrade the plant polysaccharides and lignin. However, depending on species and lifestyles, the repertoire of enzymes and their gene numbers differ significantly (Lundell et al. 2010). In this work, proteomic analysis was performed with a secretome from *Peniophora* sp. CBMAI 1063 obtained under optimized growth medium conditions for oxidases expression i.e. laccases. A databank generated using RNAseq (not shown) and genomic data was used to identify the proteins in *Peniophora* sp. CBMAI 1063 secretome. It was identified 126 proteins, in which 57 are CAZymes (Table S2), 57 are non-CAZymes and 12 are hypothetical proteins (Figure 5).

The False Discovery Rate (FDR) of peptide identification based on the use of randomized decoy databases was 0.03 %, indicating that the database employed was adequate. Among the CAZymes, the secretome contained 38 proteins classified in different families of GHs, which are involved in cellulose and hemicellulose breakdown (Tables 1, 2 and 4). Predicted by SignalP v.4.0 (Petersen et al. 2011) and Yloc (Briesemeister, Rahnenführer, & Kohlbacher, 2010), most of the enzymes exhibit signal peptide of secreted proteins. Five GHs exhibited CBM: GH18 with CBM5, GH72 with CBM43, GH43 with CBM35, GH72 with CBM43 and GH15 with CBM20. Carbohydrate esterases (CE) from family 4 and 8, involved in deacetylating hemicelluloses (excluding pectin), and a **polysaccharide lyase (PL) family 22** (oligogalacturonate lyase) were also identified (Tables 2, 4 and 5).

Among the lignin-active enzymes, it was identified 2 enzymes AA1, 4 enzymes AA5 and 3 enzymes from AA7 family (Table 3). According to the analysis using SignalP v.4.0 and Yloc these enzymes have signal peptide of secreted proteins.

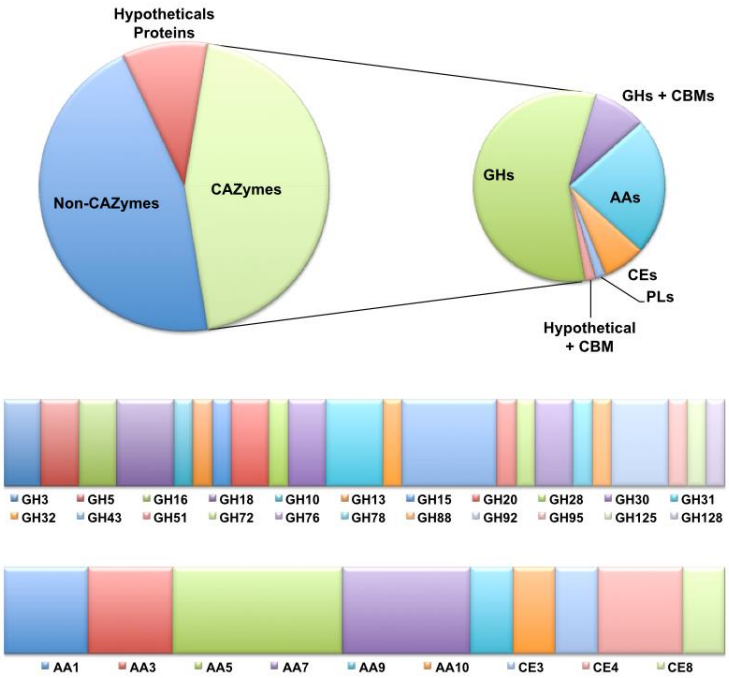

Figure 5. Distribution of proteins identified in *Peniophora* sp. CBMAI 1063 secretome by LC-MS/MS. Non-hypothetical proteins were classified into non-CAZymes and CAZymes groups according to the CAZy database (http://www.cazy.org/). GH: Glycoside Hydrolases; PL: Polysaccharide Lyases; CE: Carbohydrate Esterases; AA: Auxiliary Activities; CBM: Carbohydrate-binding module.

The laccase g1591.t1 exhibited the highest spectrum counts (Table 3) among all the CAZymes identified, containing 12 unique peptides, covering 41% of the protein sequence (Figure 6). Proteins and peptides from peroxidases AA2 were not found in the secretome, despite its presence in the genome and transcriptome analyses.

As a result, our finds confirmed that *Peniophora* sp. CBMAI 1063 has the ability to secrete a powerful set of oxidative enzymes, suggesting its use as additive in enzymatic cocktails for biomass conversion into sugar and others value-added products.

OMICs approaches can provide hypotheses concerning function for the large number of genes predicted from genome sequences (Ge et al. 2001). The comparison between genomic, transcriptomic and proteomic data was made cautiously since it was obtained from the *Peniophora* sp. CBMAI 1063 grown in optimized medium conditions to produce oxidases (specifically for laccases). It was found 18 genes encoding enzymes for the AA1 family in the

genome, 5 transcripts expressed and only 2 proteins identified in the proteomic analysis (Figure 7 and S1). For the AA5 family, 4 proteins were secreted from 7 encoding genes.

MSSSWSLLTL FSLVATGAFA AIGPTAQLTI TNANIAPDGL SRSSVLANGV
FPAPLITGNK GDAFSLTVAD SLTDTTMDLV TSIHWHGLFQ KSTNYADGVS
GVTQCPIVPD NSFEYAFSVP GQAGTFWYHS HYHAQYCDGL RGALVIYDPA
DPAASLYDVD DDSTVITLAD WYHYTSKNAP AIPAPSATLI NGLGRYSGGP
ASDLAVINVT KGTRYRFRLV SISCDTNFIF SIDNHKFSVI EVDGVNHKPQ
PIDNVQIFAG QRYSLVMTAD QDVGNYWVRA QPNNIAATFD GGLNSAILRY
KDATVADPTT TSSLSLALNE QDLHPLESVD YLSDKTPGGA DVNLELDVTF
TGGLFAVNGK SFEAPDVPVL LQILSGTPPA SLLPNGSVQL LPPNAVVEIA
IPGGVAAGPH PIHLHGHTFS VVRSAGNATY NYENPPIRDV VSIGTAATDR
TTIRFRTDNA GPWFMHCHID WHLTAGFAVV MAEDSEDVPN DVHPTDNWNA
LCPAWNTYSS TTGITQGGLK PIKAT

Figure 6. The aminoacid sequence of the laccase with the highest spectrum counts (g1591.t1). The peptides identified by LC-MS/MS analysis are shown in bold red. The coverage for this protein was 41%.

The family AA4 was not identified in the proteomic, although presenting 3 genes in the genome and one transcript expressed. The same was observed for AA2, AA6 and AA8, whose transcripts were expressed, but the proteins were not identified (Figure 7 and S1). In the case of CBMs, for example, it was found only predicted genes for CBM 20, 21 and 42. Secretome analysis identified CBM 5, 20 and 35, while transcriptomic data found only 4 transcripts for CBM 21 and one for CBM 20 (Figure 7 and S1).

The oceans are the single largest source of biogenic organohalogens containing chlorine or bromine, which are biosynthesized by myriad seaweeds, sponges, corals, tunicates, bacteria, and other marine life (Gribble, 2003). Sponges are frequently colonized by bacteria or fungi, that may be pathogenic to them. Organohalogens function is presumably to resist feeding by fish and fouling by barnacles, bacteria, and fungi (Caballero-George et al. 2010; Faulkner et al. 1993). Due to their enormous reactivity towards electrophilic halogenation reactions, pyrroles, indoles, phenols, and tyrosines are commonly found to be halogenated in sponges. It is evident that bacteria, microalgae or fungi associated with the host sponge are able to biosynthesize specific metabolites (Kelman et al. 2001; Faulkner et al. 2000; 2001; 1993). A useful group of oxidoreductive enzymes may be important in enabling *Peniophora* sp. CBMAI 1063 to live in close association with its sponge host (*Amphimedon viridis*) in a marine environment.

Table 1. Identified Cellulose-Active enzymes and spectrum counts of *Peniophora* sp. CBMAI cultivated in bioreactor under saline conditions.

Accession Number	Molecular Weight[a]	Amino acid length[a]	dbCAN[b]	PFAM[c]	PFAM description	Signal Peptide[d]	Location[e]	Unique peptides	Spectrum counts
Cellulose-Active Enzymes									
g13682.t1	71 kDa	640	AA3	PF05199 PF00732	glucose-methanol-choline oxidoreductase	NO	C	18	19
g16244.t1	62 kDa	585	AA3	PF00732 PF05199	glucose-methanol-choline oxidoreductase	NO	SP	6	7
g6504.t1	26 kDa	242	AA9	PF03443	Glycoside hydrolase family 61	YES	SP	3	71
g11705.t1	81 kDa	760	GH3	PF01915 PF00933 PF14310	Glycoside hydrolase family 3	YES	SP	9	18
g15376.t1	93 kDa	879	GH3	PF01915 PF00933 PF14310	Glycoside hydrolase family 3	YES	SP	2	1
g1658.t1	37 kDa	353	GH5	PF00150	Glycoside hydrolase family 5	YES	SP	2	2
g5589.t1	49 kDa	452	GH5	PF00150	Glycoside hydrolase family 5	YES	SP	4	7
g6425.t1	35 kDa	348	AA10	PF03067	Lytic polysaccharide monooxygenase	YES	SP	2	1

Legend. [a]Molecular Weight and [a]Amino acid length determined by LC-MS/MS. The results were processed by Mascot v.2.3.01 engine (Matrix Science Ltd.) software against the genome sequencing database of *Peniophora* sp. CBMAI and Scaffold – Proteome Software (version Scaffold_4.3.2 20140225). [b]Web server and database for automated carbohydrate-active enzyme annotation generated based on the family classification from CAZy database: GH- Glycoside Hydrolases; AA- Auxiliary Activities. [c]Protein Family Domain analysis. [d]The presence of a signal peptide of secreted proteins predicted by SignalP v.4.0. [e]The subcellular localization of proteins predicted by YLoc (Interpretable Subcellular Localization Prediction): SP – secreted pathway; C – cytoplasm; M – mitochondrial location.

Table 2. Identified Hemicellulose-Active enzymes and spectrum counts of *Peniophora* sp. CBMAI cultivated in bioreactor under saline conditions.

Accession Number	Molecular Weight[a]	Amino acid length[a]	dbCAN[b]	PFAM[c]	PFAM description	Signal Peptide[d]	Location[e]	Unique peptides	Spectrum counts
Hemicellulose-Active Enzymes									
g14451.t1	46 kDa	429	CE3	PF13472 PF00657	GDSL-like Lipase/ Acyl hydrolase	YES	SP	7	44
g10047.t1	55 kDa	513	CE4		Carbohydrate esterase	YES	SP	3	5
g4642.t1	38 kDa	354	CE4	PF01522	Polysaccharide deacetylase	YES	SP	6	14
g11401.t1	37 kDa	345	GH10	PF00331	Glycoside hydrolase family 10	YES	SP	4	8
g11177.t1	59 kDa	540	GH125	PF06824	Protein of unknown function	NO	SP	5	4
g14331.t1	30 kDa	277	GH128	PF11790	Glycoside hydrolase catalytic core	YES	SP	2	6
g7518.t1	34 kDa	318	GH16		Glycoside hydrolases family 16	YES	SP	4	18
g7519.t1	33 kDa	313	GH16		Glycoside hydrolases family 16	YES	SP	6	66
g16729.t1	57 kDa	524	GH30	PF02055	Glycoside hydrolase family 30	YES	SP	3	3
g3624.t1	53 kDa	499	GH30	PF14587	O-Glycoside hydrolase family 30	YES	SP	10	57
g14525.t1	36 kDa	341	GH43	PF04616	Glycoside hydrolases family 43	YES	SP	2	2
g4370.t1	33 kDa	321	GH43	PF04616	Glycoside hydrolases family 43	YES	SP	4	54
g5765.t1	33 kDa	310	GH43	PF04616	Glycoside hydrolases family 43	YES	SP	5	55
g9420.t1	35 kDa	329	GH43	PF04616	Glycoside hydrolases family 43	YES	SP	2	13
g15433.t1	47 kDa	444	GH43 CBM35	PF04616	Glycoside hydrolases family 43	YES	SP	2	2
g3571.t1	69 kDa	638	GH51	PF06964	α-L-arabinofuranosidase	YES	SP	10	33
g14175.t1	58 kDa	564	GH72	PF03198	Glucanosyltransferase	YES	SP	4	3

			CBM43	PF07983	X8 domain				
g11973.t1	40 kDa	375	GH76	PF03663	Glycoside hydrolase family 76	YES	SP	5	8
g4124.t1	40 kDa	370	GH76	PF03663	Glycoside hydrolase family 76	YES	SP	4	5
g11336.t1	91 kDa	827	GH92	PF07971	Glycoside hydrolase family 92	YES	SP	13	14
g1967.t1	67 kDa	606	GH92	PF07971	Glycoside hydrolase family 92	NO	SP	10	10
g5807.t1	88 kDa	812	GH92	PF07971	Glycoside hydrolase family 92	YES	SP	13	15
g5441.t1	186 kDa	1691	GH95	PF14498	Glycoside hydrolase family 95	NO	SP	2	1

Legend. [a]Molecular Weight and [a]Amino acid length determined by LC-MS/MS. The results were processed by Mascot v.2.3.01 engine (Matrix Science Ltd.) software against the genome sequencing database of *Peniophora* sp. CBMAI and Scaffold – Proteome Software (version Scaffold_4.3.2 20140225). [b]Web server and database for automated carbohydrate-active enzyme annotation generated based on the family classification from CAZy database: GH - Glycoside Hydrolases; CE - Carbohydrate Esterases; CBM: Carbohydrate-binding module. [c]Protein Family Domain analysis. [d]The presence of a signal peptide of secreted proteins predicted by SignalP v.4.0. [e]The subcellular localization of proteins predicted by YLoc (Interpretable Subcellular Localization Prediction): SP – secreted pathway; C – cytoplasm; M – mitochondrial location.

Table 3. Identified Lignin-Active enzymes and spectrum counts of *Peniophora* sp. CBMAI cultivated in bioreactor under saline conditions.

Accession Number	Molecular Weight[a]	Amino acid length[a]	dbCAN[b]	PFAM[c]	PFAM description	Signal Peptide[d]	Location[e]	Unique peptides	Spectrum counts
Lignin-Active Enzymes									
g1591.t1	58 kDa	546	AA1	PF00394 PF07731 PF07732	Multicopper oxidase	YES	SP	12	255
g17194.t1	58 kDa	540	AA1	PF00394 PF07732 PF07731	Multicopper oxidase	YES	SP	3	2
g5706.t1	71 kDa	671	AA5	PF07250 PF09118	Glyoxal oxidase N- terminus	YES	SP	2	1
g5707.t1	81 kDa	769	AA5	PF07250 PF09118	Glyoxal oxidase N terminus	YES	SP	3	1
g5709.t1	84 kDa	799	AA5	PF07250 PF09118	Glyoxal oxidase N-terminus	YES	SP	2	1
g9556.t1	59 kDa	554	AA5	PF07250 PF09118	Glyoxal oxidase N-terminus	YES	SP	4	5
g13672.t1	60 kDa	567	AA7	PF01565 PF08031	FAD binding domain Berberine	YES	SP	5	6
g17067.t1	51 kDa	485	AA7	PF01565	FAD binding domain	YES	SP	3	5
g7475.t1	59 kDa	548	AA7	PF01565 PF08031	FAD binding domain Berberine	YES	SP	3	1

Legend. [a]Molecular Weight and [a]Amino acid length determined by LC-MS/MS. The results were processed by Mascot v.2.3.01 engine (Matrix Science Ltd.) software against the genome sequencing database of *Peniophora* sp. CBMAI and Scaffold – Proteome Software (version Scaffold_4.3.2 20140225). [b]Web server and database for automated carbohydrate-active enzyme annotation generated based on the family classification from CAZy database: AA –Auxiliary Activity. [c]Protein Family Domain analysis. [d]The presence of a signal peptide of secreted proteins predicted by SignalP v.4.0. [e]The subcellular localization of proteins predicted by YLoc (Interpretable Subcellular Localization Prediction): SP – secreted pathway; C – cytoplasm; M – mitochondrial location.

Table 4. Identified Chitin, Starch & Others Carbohydrate-Active enzymes and spectrum counts of *Peniophora* sp. CBMAI cultivated in bioreactor under saline conditions.

Accession Number	Molecular Weight[a]	Amino acid length[a]	dbCAN[b]	PFAM[c]	PFAM description	Signal Peptide[d]	Location[e]	Unique peptides	Spectrum counts
Chitin, Starch & Others Carbohydrate-Active Enzymes									
g13668.t1	61 kDa	580	CBM20 GH15	PF00723 PF00686 PF17168	Glycoside hydrolases family 15	YES	SP	20	197
g7315.t1	82 kDa	769	CBM5	PF16335 PF08760	Carbohydrate binding domain	YES	SP	10	15
g7861.t1	50 kDa	474	GH13	PF02839 PF00128 PF02806	Alpha amylase	YES	SP	5	18
g11641.t1	97 kDa	890	GH31	PF01055 PF16863 PF13802	Glycoside hydrolases family 31	YES	SP	3	4
g15819.t1	109 kDa	997	GH31	PF01055 PF16863	Glycoside hydrolases family 31	NO	SP	12	9
g15820.t1	104 kDa	944	GH31	PF01055 PF16863	Glycoside hydrolases family 31	YES	SP	6	6
g11828.t1	60 kDa	547	GH32	PF00251	Glycoside hydrolases family 32	NO	SP	8	35
g11153.t1	49 kDa	460	GH88	PF07470	Glycoside Hydrolase Family 88	YES	SP	3	3
g11942.t1	45 kDa	418	GH18	PF00704	Glycoside hydrolases family 18	YES	SP	2	12
g11425.t1	49 kDa	472	GH18; CBM5	PF02839	Carbohydrate binding domain	YES	SP	2	5

g6083.t1	136 kDa	1254	GH18; CBM5	PF00009 PF03764 PF14492 PF00679 PF00704 PF02839 PF03144	Elongation factor Tu GTP binding domain18	YES	M	7	23
g10368.t1	60 kDa	560	GH20	PF00728 PF14845	Glycoside hydrolase family 20	YES	SP	5	2
g15989.t1	134 kDa	1230	GH20	PF00728 PF14845 PF02838	Glycoside hydrolase family 20	YES	SP	7	9

Legend. [a]Molecular Weight and [a]Amino acid length determined by LC-MS/MS. The results were processed by Mascot v.2.3.01 engine (Matrix Science Ltd.) software against the genome sequencing database of *Peniophora* sp. CBMAI and Scaffold – Proteome Software (version Scaffold_4.3.2 20140225). [b]Web server and database for automated carbohydrate-active enzyme annotation generated based on the family classification from CAZy database: GH - Glycoside Hydrolases; CBM- Carbohydrate-binding module. [c]Protein Family Domain analysis. [d]The presence of a signal peptide of secreted proteins predicted by SignalP v.4.0. [e]The subcellular localization of proteins predicted by YLoc (Interpretable Subcellular Localization Prediction): SP – secreted pathway; C – cytoplasm; M – mitochondrial location.

Table 5. Identified Pectin-Active enzymes and spectrum counts of *Peniophora* sp. CBMAI cultivated in bioreactor under saline conditions.

Accession Number	Molecular Weight[a]	Amino acid length[a]	dbCAN[b]	PFAM[c]	PFAM description	Signal Peptide[d]	Location[e]	Unique peptides	Spectrum counts
Pectin-Active Enzymes									
g8265.t1	35 kDa	330	CE8	PF01095	Pectin esterase	YES	SP	2	2
g8087.t1	70 kDa	665	GH78	PF05592	Bacterial alpha-L- rhamnosidase	YES	SP	7	30
g10469.t1	74 kDa	673	PL22	PF07676	WD40-like Beta Propeller Repeat	YES	SP	2	1
g1538.t1	45kDa	413	GH28	PF00295	Glycoside hydrolase family 28	YES	SP	2	1

Legend. [a]Molecular Weight and [a]Amino acid length determined by LC-MS/MS. The results were processed by Mascot v.2.3.01 engine (Matrix Science Ltd.) software against the genome sequencing database of *Peniophora* sp. CBMAI and Scaffold – Proteome Software (version Scaffold_4.3.2 20140225). [b]Web server and database for automated carbohydrate-active enzyme annotation generated based on the family classification from CAZy database: GH- Glycoside Hydrolases; PL- Polysaccharide Lyases; CE- Carbohydrate Esterases. [c]Protein Family Domain analysis. [d]The presence of a signal peptide of secreted proteins predicted by SignalP v.4.0. [e]The subcellular localization of proteins predicted by YLoc (Interpretable Subcellular Localization Prediction): SP – secreted pathway; C – cytoplasm; M – mitochondrial location.

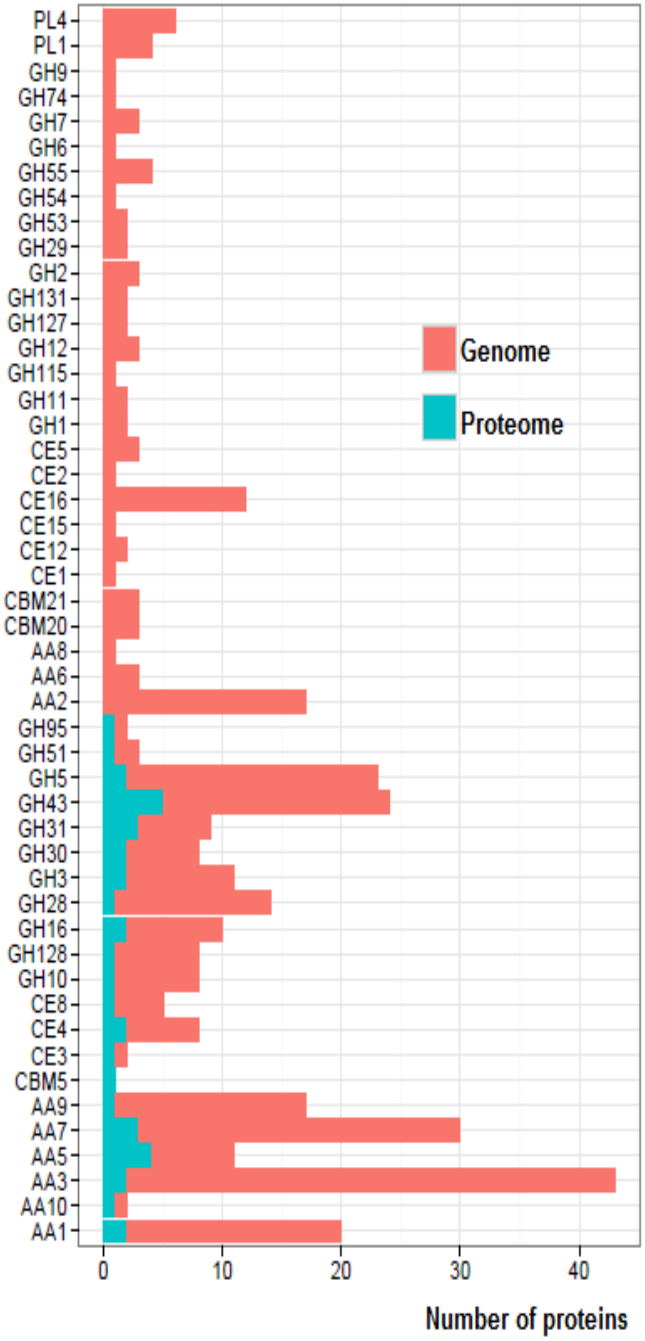

Figure 7. Number of predicted genes (orange) encoding CAZymes in *Peniophora* sp. CBMAI 1063 genome and number of identified CAZymes (blue) in *Peniophora* sp. CBMAI 1063 secretome. The enzymes were classified according to the CAZy database (http://www.cazy.org/): GH- Glycoside Hydrolases, PL- Polysaccharide Lyases, CE- Carbohydrate Esterases, AA- Auxiliary Activities and CBM- Carbohydrate-binding module.

3.5 The major laccase (Pnh_Lac1) secreted by *Peniophora* sp. CBMAI: Structural identity and effects on lignin depolymerization and enzymatic digestibility of steam-exploded sugarcane bagasse.

Lignin constitutes up to one-third of the material found in plant cell walls and is considered the second most abundant natural polymer in the world (Christopher et al. 2014). Despite its unique characteristics, the large amount of lignin stream generated from pretreatment and delignification technologies for ethanol production is mostly used for low-value commercial applications, such as combusted for energy production (Christopher et al. 2014; Ayyachamy et al. 2013). The limited utilization of technical lignins is hampered by its chemical heterogeneous nature, low solubility and low reactivity (Doherty et al. 2011). In this way, lignins must be modified before application. Biological modifications using oxidative enzymes are gaining industrial and scientific interest to improve lignin properties (Ortner et al. 2015). Principally Laccases (EC 1.10.3.2), which are copper-containing oxidase enzymes able to catalyze the oxidation of phenolic compounds. However, the major disadvantage of these enzymes is their low redox potential compared to other oxidative enzymes, which can be overcome in the presence of small redox molecules (mediators). Mediators act as shuttle electrons between laccase and substrate and modulate the redox potential of the reaction system. Laccase Mediator System (LMS) enable a faster reaction time and are able to oxidise non-phenolic structures in lignin (Couto & Herrara, 2006; Li et al. 1999).

As previously mentioned, the amino acid sequence of one laccase (accession number g1591.t1), named as Pnh_Lac1, exhibited the highest spectrum counts (Table 3, Figure 6) among all the CAZymes identified. Consequently, we decided to investigate the structural identity of this enzyme and its biological role on lignin degradation.

The three-dimensional structure of Pnh_Lac1 was generated from homology modelling, performed by the I-TASSER server (Yang 2015). The final model was based on the crystallographic structure of five laccases from AA1.1 family (PDBid: 1A65, 1V10, 2QT6, 5DAO and 5E9N) and one unclassified laccase (PDBid: 2HRG) due to sequence similarity. The Pnh_Lac1 structure was considered as reliable, according to the C-score value of 0.7 (range from -5 to 2) (Yang 2015). Finally, Pnh_Lac1 presented the typical folding of laccases, consisting of three cupredoxin domains with a mononuclear copper center located in domain 3 and a trinuclear center in between domains 1 and 3 (Bento 2010) (Figure 8a). In addition, the signature composed of four ungapped sequence segments L1–L4 is present. The segments L1-L4 contain the amino acids that bind to the copper centers: H64 and H66 from L1; H109 and

H111 from L2; H390, H393 and H395 from L3; and H448 and H452 from L4 (Figure 8b). The sequential alignment of the conserved regions (figure S2), as well as the structural alignment of Pnh_Lac1 with other members of the AA1.1 family (Figure S3) confirmed the identity and classification, previously predicted by DBCAN (Table 3). Although the sequence identity varies between 50.6 and 58.1%, the highest Cα root-mean-square deviation observed (0.65 Å) proves that the general structure, regardless of region L1-L4, is also extremely conserved.

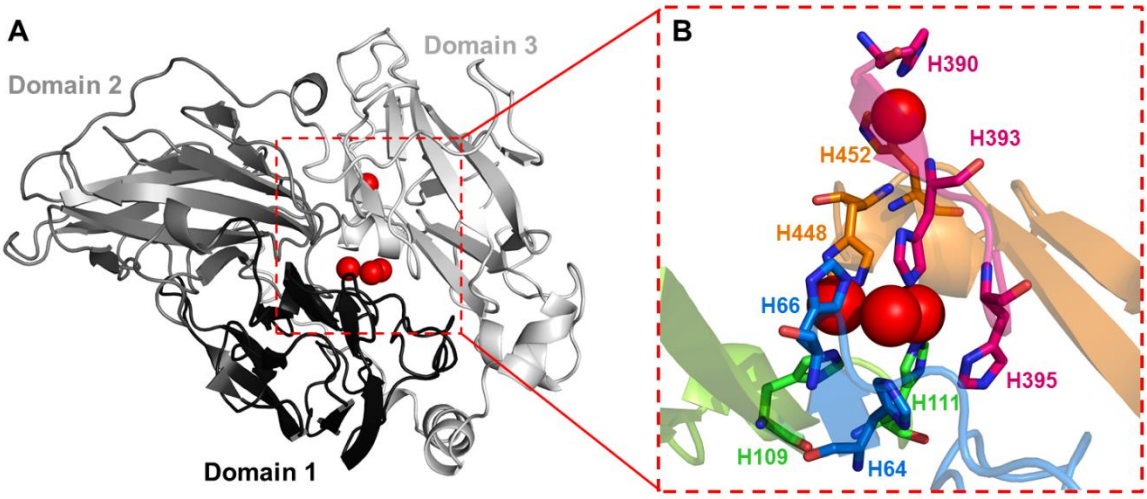

Figure 8. Three dimensional structure of Pnh_Lac1 generated by homology modelling. (A) General structure of laccases, consisting of three cupredoxin domains. (B) Zoom into the copper ions centers surrounded by the fungal laccase signature segments L1 (blue), L2 (green), L3 (magenta) and L4 (orange). The ions coordination is performed by the histidines presented in the L1-L4 regions.

Prospective assays with purified Pnh_Lac1 in combination with a synthetic mediator (ABTS) were performed on lignin extracted from sugarcane bagasse (SCB) (Brenelli et al. 2016) in order to explore some biotechnological applications. The supernatant from the LMS-treatment was analyzed by UV-light absorbance and GPC to confirm if LMS was able to promote lignin depolymerization. An increase of 50% in UV-light absorbance at 280 nm was detected after incubation of lignin from SCB with LMS, compared to the control lacking laccase (Figure 9). Intense spectra changes were detected between 260 - 270 nm in LMS-treated samples, suggesting that laccase introduced new functional groups into the side-chain of the phenylpropane unit and the aromatic ring (Figure 9).

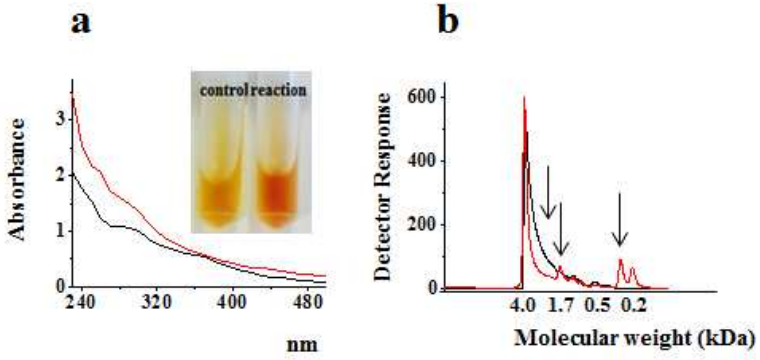

Figure 9. UV-light absorbance (a) and GPC chromatograms (b) showing the spectroscopic profile and molecular weight distribution of the lignins from SCB after LMS treatment. Red lines refer to the incubation of the lignin with LMS and black line to the control experiment without laccase.

This region is attributed to the introduction of auxochrome groups such as carbonyl in the Cα position of the side chain, new nonconjugated hydroxyl groups in the side-chain phenylpropane unit or new Cα–Cβ double bonds (Arzola et al. 2006). Spectral changes in the visible region were also detected, especially at 480 nm, which means that new quinones structures were introduced by laccase (Polcin and Rapson 1969). The chromogene groups, which contribute to red color, can be observed in Figure 9. GPC analysis shows that soluble low molecular weight-lignin derived compounds (< 500 Da) were generated after LMS treatment (Figure 9), indicated by arrows. The intense peak corresponding to HMW lignins (~ 4 kDa) appeared thinner compared to the untreated sample, indicating depolymerization. In agreement, both analyses showed evidences that the major laccase secreted by *Peniophora* sp. CBMAI, combined with a mediator, promoted lignin depolymerization under the tested conditions.

Recently, studies have shown that LMS treatments can improve the enzymatic hydrolysis of some lignocellulose materials by mechanisms involving lignin solubilization, depolymerization and modification (Moilanen et al. 2014; Chen et al. 2012). In this work, the effects of LMS treatment on SCB digestibility were also evaluated. SCB was firstly treated with laccase and ABTS, and hydrolyzed with two different commercial cocktails.

After 72 h of hydrolysis, it was observed for LMS-treated SCB an increase of 1.9% and 11.0% in hydrolysis yield using Accellerase® 1500 and Cellic Ctec2, respectively, compared to untreated samples (Figure 10). GPC analysis of the supernatant after LMS

treatment confirmed that lignin was partially degraded, leading to its solubilization into the supernatant of the reaction performed in aqueous medium (Figure 10).

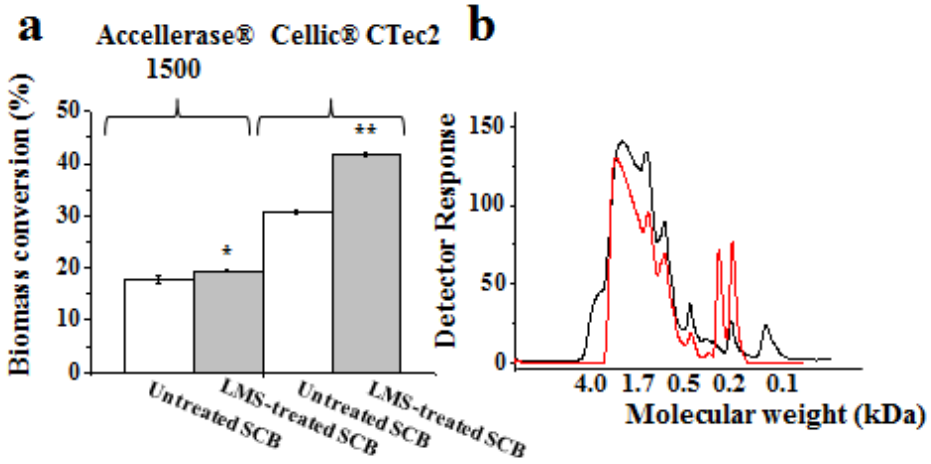

Figure 10. (a) Enzymatic hydrolysis of steam-exploded sugarcane bagasse treated with LMS with commercial cocktails at low dosage. In the y axe is reported the cellulose conversion yield in percentage of the maximum theoretical cellulose conversion after 72h at 50 ºC without (white bars) and after LMS (grey bars) treatment. Error bars represent the standard errors of the means of triplicate experiments (b) GPC chromatograms showing the molecular weight distribution of the water soluble lignins in the supernatants from LMS treated SCB. Red line is the incubation of the biomass with LMS and black line refer to control experiments missing laccase.

New peaks corresponding to soluble LMW phenolic compounds, between 400 - 200 Da (indicated by arrows), were detected in the LMS-treated sample, while HMW lignins fragments around 4 - 1.7 kDa appeared less intense in the chromatogram. Interesting, the peak corresponding to phenolic compounds with the lowest molecular weight (around 100 Da) in the untreated sample is absent in LMS-treated supernatant. Pnh_Lac1 showed potential to be applied in bioconversion technologies, promoting lignin depolymerization and solubilization in the lignocellulosic material tested, facilitating the action of cellulolytic enzymes. However, more studies are necessary combining the laccase with different mediators and high enzyme loadings to evaluate the real effects of this enzymatic pre-treatment.

4. Conclusions

Genomic, transcriptomic and secretomic studies of *Peniophora* sp. CBMAI 1063, grown under optimal cultivation condition for laccase production, revealed for the first time, its robust and versatile white-rot apparatus arsenal of CAZymes, especially the enzymes involved in lignin degradation. Interestingly, this marine fungus presented a higher number of unique orthologous gene clusters comparing to other two *Peniophora* species, demonstrating its singularity for biotechnological applications. In addition, the major laccase secreted by *Peniophora* sp. CBMAI 1063 exhibited potential to lignocellulose bioconversion technologies by promoting lignin modification, depolymerization and solubilization.

5. Acknowledgements

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6. Supplementary Material

Table S1. Statistics of the marine *Peniophora* sp. CBMAI 1063 draft genome assembly.

Assembly statistics	*Peniophora* sp. CBMAI 1063
# Number of QC reads	39442518
Estimated coverage	165X
# contigs (>= 0 bp)	939
# scaffolds	833
# contigs (>= 5000 bp)	618
# contigs (>= 10000 bp)	509
# contigs (>= 25000 bp)	376
# contigs (>= 50000 bp)	249
Total length (bp)	46982497
Largest contig (bp)	1115204
GC (%)	55,21
N50	155805
N75	73455
L50	84
L75	191
Number of predicted genes	17219
Number of predicted transcripts	17714
Average gene length (bp)	1416
rRNA	1
tRNA	74
Genome completeness (%)	93

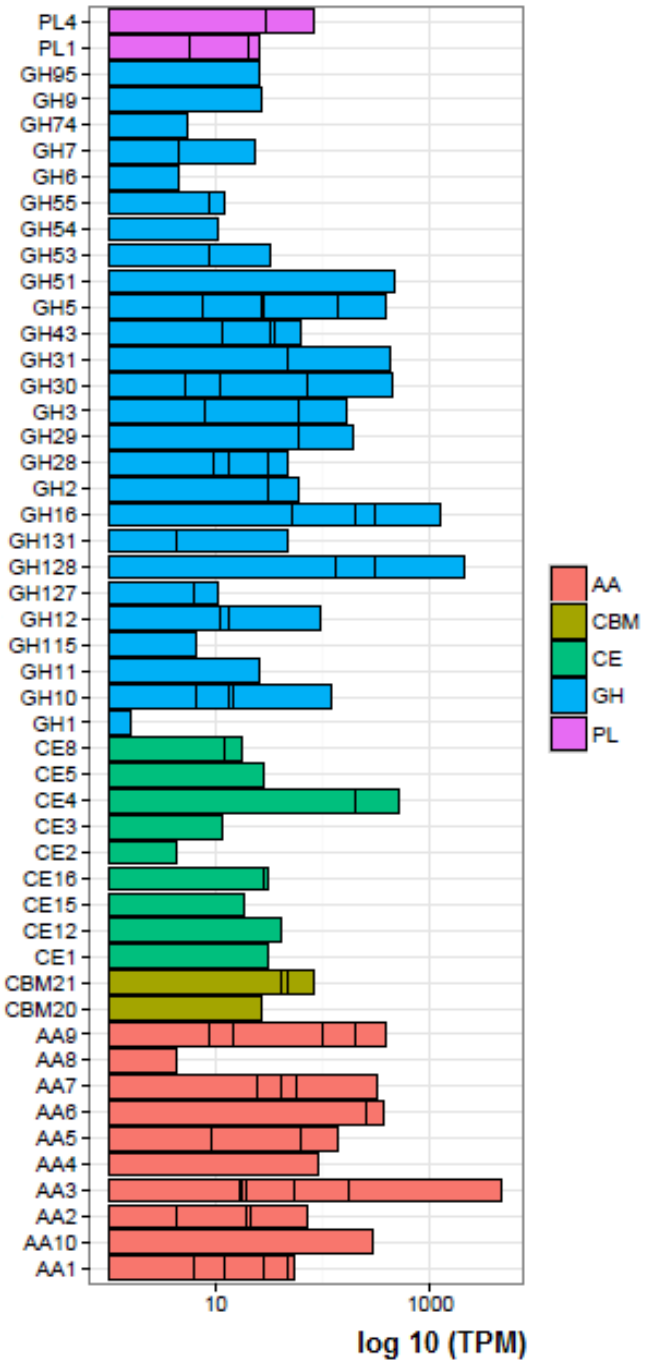

Figure S1. Number of transcripts encoding CAZymes in *Peniophora* sp. CBMAI 1063 genome in $\log_{10}$ of transcripts per million. The classification was made according to the CAZy database (http://www.cazy.org/): GH- Glycoside Hydrolases, PL- Polysaccharide Lyases, CE- Carbohydrate Esterases, AA- Auxiliary Activities and CBM- Carbohydrate-binding module.

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Figure S2. Amino acid sequence alignment of Pnh_Lac1 with eight laccases from family AA1.1. Although the identity between Pnh_Lac1 with each one is about 55 %, the fungal laccase signature segments, highlighted in blue (L1), green (L2), magenta (L3) and orange (4), showed high identity. Each laccase, excepting Pnh_Lac1, is described by its genbank accession code.

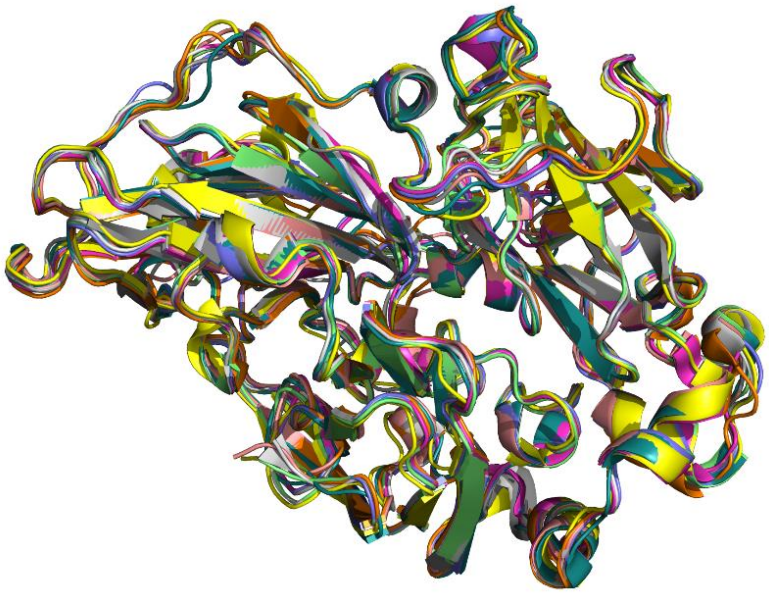

Figure S3. Structural alignment of Pnh_Lac1 model with the structures of the eight laccases from family AA1.1, used in the sequence alignment described in figure S2. The Pnh_Lac1 structure, generated by homology modelling, resulted in a high structural identity (RMSD about 0.5 Å) with the other members, evidencing the conserved folding of the family and the reliability of the model.

Table S2. Identified non-CAZymes and spectrum counts of *Peniophora* sp. CBMAI cultivated in bioreactor under saline conditions

Accession Number	Molecular Weight[a]	Amino acid length[b]	PFAM[c]	PFAM description	Signal Peptide[d]	Location[e]	Unique peptides	Spectrum counts
g1019.t1 (+4)	34 kDa	305	PF00240 PF11976 F14560 PF13881	Ubiquitin family	NO	C	2	43
g10257.t1	42 kDa	394	PF01161	Phosphatidylethanolamine-binding protein	NO	SP	2	1
g10717.t1	45 kDa	424	PF00026 PF14543	Eukaryotic aspartyl protease Xylanase inhibitor N-terminal	YES	SP	3	3
g11007.t1	36 kDa	321	PF00248	Aldo/keto reductase family	NO	C	3	9
g11209.t1	44 kDa	393	PF04389 PF01546	Peptidase family M28/ M20/M25/M40	YES	SP	5	11
g11268.t1	120 kDa	1084	PF00282	Pyridoxal-dependent decarboxylase conserved domain	NO	C	15	20
g1175.t1	29 kDa	258	PF01182	Glucosamine-6-phosphate isomerases 6-phosphogluconolactonase	NO	C	2	9
g12242.t1	53 kDa	505	PF07992 PF02852 PF00070 PF12831 PF01134 PF00890	Pyridine nucleotide-disulphide oxidoreductase; FAD dependent oxidoreductase:	NO	M	8	10
g1234.t1	31 kDa	279	PF01738	Dienelactone hydrolase Family	NO	M	4	13
g12438.t1	14 kDa	135	PF07249	Cerato-platanin	YES	SP	3	29
g12440.t1	14 kDa	135	PF07249	Cerato-platanin	YES	SP	2	26
g1251.t1	39 kDa	397			YES	SP	3	8
g12536.t1	166 kDa	1524	PF00168	C2 domain	NO	X	4	9
g13042.t1	43 kDa	395	PF06516	Purine nucleoside permease (NUP)	NO	SP	4	13

g1333.t1	61 kDa	561	PF02878 PF02880 PF02879	Phosphoglucomutase/phosphomannomutase, alpha/beta/alpha domain I	NO	C	3	4
g13681.t1	87 kDa	793			NO	C	4	2
g13797.t1	39 kDa	379			YES	SP	3	58
g13878.t1	52 kDa	479	PF04389	Peptidase family M28	YES	SP	4	7
g14070.t1	26 kDa	231	PF13417 PF13409	Glutathione S-transferase	NO	M	2	6
g14182.t1	21 kDa	200	PF02798		YES	SP	2	7
g14619.t1	61 kDa	548	PF00262	Calreticulin Family	YES	SP	2	3
g14686.t1	38 kDa	358	PF10282	Lactonase, 7-bladed beta-propeller	NO	SP	4	3
g14843.t1	22 kDa	198			NO	M	3	43
g14865.t1	34 kDa	317	PF01014 PF01014	Uricase;Uricase	NO	M	3	5
g15561.t1	38 kDa	374			YES	SP	3	5
g16265.t1	47 kDa	438	PF01546 PF07687	Peptidase family M20/M25/M40	NO	C	2	1
g16319.t1	26 kDa	253	PF00314	Thaumatin Family	YES	SP	3	9
g16410.t1	17 kDa	152	PF00334	Nucleoside diphosphate kinase	NO	M	2	7
g16808.t1	26 kDa	236	PF13419 PF00702 PF13242	Haloacid dehalogenase-like hydrolase	NO	C	2	6
g16849.t1	67 kDa	617	PF09423 PF16655	PhoD-like phosphatase	YES	SP	7	10
g1891.t1	27 kDa	256	PF00314	Thaumatin Family	YES	SP	3	18
g312.t1	62 kDa	581	PF12222	Peptide N-acetyl-beta-D-glucosaminyl asparaginase amidase A	YES	SP	2	4
g329.t1	76 kDa	696	PF03571	Peptidase family M49	NO	C	16	13
g3731.t1	54 kDa	497	PF01266	FAD dependent oxidoreductase	YES	SP	2	1
g4046.t1	33 kDa	305			YES	SP	3	2
g414.t1	35 kDa	348	PF03330	Lytic transglycolase	YES	SP	2	1

g4288.t1	35 kDa	322	PF03372	Endonuclease/Exonuclease/phosphatase family	NO	C	11	68
g514.t1	41 kDa	396			YES	SP	6	25
g5214.t1	61 kDa	568	PF00026	Eukaryotic aspartyl protease	YES	SP	2	1
g5591.t1	55 kDa	515	PF00883 PF02789	Cytosol aminopeptidase family, catalytic domain	NO	M	2	1
g5969.t1	11 kDa	99	PF11976 PF00240	Ubiquitin-2 like Rad60 SUMO-like	NO	N	2	7
g6000.t1	19 kDa	168	PF14566	Inositol hexakisphosphate	NO	C	3	14
g620.t1	44 kDa	405	PF08450 PF03088	SMP-30/Gluconolaconase/LRE-like region;Strictosidine synthase	YES	M	4	4
g6204.t1	36 kDa	343			YES	SP	2	2
g621.t1	44 kDa	409	PF08450 PF03088	SMP-30/Gluconolaconase/LRE-like region;Strictosidine synthase	YES	SP	3	2
g690.t1	97 kDa	905	PF13449 F01266	Esterase-like activity of phytase;FAD dependent oxidoreductase	YES	SP	18	42
g7075.t1	24 kDa	227			YES	SP	4	13
g7191.t1	58 kDa	529	PF05577	Serine carboxypeptidase S28	YES	SP	5	7
g7314.t1	74 kDa	691	PF17168 PF16335 PF08760	Domains of unknown function	YES	SP	6	13
g7338.t1	74 kDa	687	PF03372	Endonuclease/Exonuclease/phosphatase family	NO	C	2	2
g7407.t1	106 kDa	960	PF04253 PF04389 PF02225	Transferrin receptor-like dimerisation domain; Peptidase family M28;PA domain	NO	N	5	8
g7610.t1	68 kDa	615	PF00149	Calcineurin-like phosphoesterase	YES	SP	3	2
g7664.t1	39 kDa	403			YES	SP	4	5
g780.t1	37 kDa	338	PF07991 PF01450 PF03807 PF03446	Acetohydroxy acid isomeroreductase, NADPH-binding domain	NO	C	4	6

g7893.t1	63 kDa	590	PF09286 PF00082 PF07992		YES	SP	8	13
g8153.t1	51 kDa	470	PF02852 PF00070 PF13738 PF03486 PF13434	Pyridine nucleotide-disulphide oxidoreductase	NO	C	10	16
g8392.t1	102 kDa	934			NO	SP	7	6
g8584.t1	87 kDa	774	PF01432	Peptidase family M3	NO	SP	4	3
g8812.t1	100 kDa	903	PF01433 PF11838	Peptidase family M1;ERAP1-like C-terminal domain	NO	C	6	3
g8977.t1	64 kDa	580	PF05577	Serine carboxypeptidase S28	NO	C	3	2
g9286.t1	33 kDa	308			YES	SP	2	2
g9390.t1	44 kDa	427	PF00445	Ribonuclease T2 family	YES	SP	6	28
g9486.t1	42 kDa	389	PF01263	Aldose 1-epimerase	YES	SP	3	3
g9536.t1	50 kDa	466	PF00557	Metallopeptidase family M24	NO	N	5	9
g9683.t1	14 kDa	125	PF00173	Cytochrome b5-like Heme/Steroid binding domain	NO	M	2	7
g1396.t1	58 kDa	541	PF00135 PF07859	Carboxylesterase family 10	YES	SP	2	3
g1415.t1	58 kDa	541	PF00135 PF07859	Carboxylesterase family 10	YES	SP	4	5
g1747.t1	59 kDa	549	PF00135 PF07859	Carboxylesterase family 10	YES	SP	3	4
g5988.t1	63 kDa	584	PF00135	Carboxylesterase family 10	NO	SP	8	8

Legend. [a]Molecular Weight and [b]Amino acid length determined by LC-MS/MS. The results were processed by Mascot v.2.3.01 engine (Matrix Science Ltd.) software against the genome sequencing database of *Peniophora* sp. CBMAI and Scaffold – Proteome Software (version Scaffold_4.3.2 20140225). [b]Web server and database for automated carbohydrate-active enzyme annotation generated based on the family classification from CAZy database. [c]Protein Family Domain analysis. [d]The presence of a signal peptide of secreted proteins predicted by SignalP v.4.0. [e]The subcellular localization of proteins predicted by YLoc (Interpretable Subcellular Localization Prediction): SP – secreted pathway; C – cytoplasm; M – mitochondrial location; N – Nucleus

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CHAPTER 5

1. Concluding Remarks

The results presented in this thesis contribute to reveal lignin transformation opportunities for modern biorefinery systems. The second chapter described the composition of lignin-enriched stream produced during pilot-scale pre-treatment of sugarcane bagasse. Meaningful studies on structural characterization, biophysical properties and *in vitro* free-radical scavenging capacity of lignins of pretreated sugarcane bagasse was obtained. It was demonstrated that separation by acid gradient precipitation has a potential to upgrade heterogeneous lignin-enriched stream from sugarcane bagasse in fractions of reduced polydispersity and high free-radical scavenging capacity. The tendency to form in solution large aggregates assuming spherical shape with unexposed hydroxyl groups could be closely affected lignin free-radical scavenging capacity (Brenelli et al., 2016).

The heterogeneous nature and low solubility of the starting material used in this study demanded the combination of different analytical techniques to give information on the chemical composition and biophysical parameters. Small-angle X-ray scattering (SAXS) demonstrated to be a valuable tool to determine relevant structural parameters of isolated lignins and should be more explored for this purpose. SAXS does not require acetylation steps or chemical modifications like GPC or NMR does (Maziero et al., 2012). Sample derivatization through acetylation to achieve adequate solubility has influence on the apparent molecular weight of the lignins. For example, it was observed high polydispersity and Mw for the acetylated-fractions obtained at pH 6 and 8. The possibility of re-organization of the lignin structure could partly be induced by increasing opportunities for $n \rightarrow \sigma^*$ and $n \rightarrow \pi^*$ interactions, where n is the lone pair bonding orbital of the hydrogen bond acceptor (such as oxygen), σ^* is the anti-boding orbital of the hydrogen bond donor (hydrogen or phenolic hydroxyl, for example) and π^* is the anti-bonding orbital of the carbonyl carbon (such as a carbon from a carboxyl group). In summary, non-covalent interactions are very important on lignin aggregation behaviour in solutions (Bylin et al., 2014).

There are many possibilities to use both soluble and insoluble fractions obtained from the acidification treatment by different industries. The soluble fractions obtained at acid pH contain attractive lignin-derived molecules for pharmaceutical, cosmetic and food industries due to its antioxidant properties (Faustino et al., 2010). However, methods for separation and recovery of the molecules are essential for these purposes (Toledano et al., 2010). The insoluble fractions are composed by high molecular weight lignins and can be used as a binding and

dispersing agent in different industries without purification steps (Calvo-Flores & Dobado, 2010).

The second study described in this thesis (third chapter) raise issues about the interplay between laccases and AA9 (LPMOs) enzymes during the plant cell wall degradation. LPMOs are considered the central component present in the Cellic CTec® enzyme products which are used in several large-scale plants for the industrial production of lignocellulosic ethanol (Rodríguez-Zúñiga et al., 2015). On the other hand, laccases are considered an important group of oxidoreductive enzymes involved during lignin degradation and depolymerization (Yao & Ji, 2014). Both copper-dependent enzymes use oxygen in the catalytic reaction cycle as the final electron acceptor (Frandsen et al., 2016; Baldrian, 2006). In particular, hydrogen peroxide also acts as a co-substrate for LPMOs instead of molecular oxygen (Bissarro et al., 2016).

The recent literature describes positive or negative effects of laccase on lignin depolymerization and enzymatic hydrolysis depending on the biomass. While the positive effects are associated to lignin degradation or modification, the downside are associated to the competition of cellulose binding sites between laccases and cellulases and/or grafting process of phenoxy radicals onto the lignin fiber decreasing the accessibility of cellulases to cellulose present in the material (Oliva-Taravilla et al., 2016; Moilanen et al., 2014). Combining pretreated sugarcane bagasse or wheat straw as substrate, two fungal laccases with high or low redox potential and mediator (LMS), low molecular weight lignins was produced. These new lignin fragments were able to boost LPMO activity present in a commercial cellulolytic cocktail, increasing their hydrolysis efficiency of pure cellulose. However, the co-incubation of laccase together with LPMOs containing cellulolytic cocktail led to substrate competition towards oxygen, causing an inhibition of LPMO. Even applying the LMS prior to the cellulolytic enzymes on the same lignocellulosic material the overall cellulose hydrolysis did not increase. These results suggest that both fungi laccases chose in this work could induce further chemical modification in lignocellulosic fibers increasing the recalcitrance of sugarcane bagasse and wheat straw. As result, the negative effect was not overcome by the removal of lignin.

The important role of LPMOs in cellulose degradation in nature is highlighted by the wide distribution and often numerous occurrences in the genomes of almost all lignocellulose-degrading fungi: the literature reports that approximately ninety percent contain genes encoding LPMOs (Kracher et al., 2016). The choose among the three functional electron

transfer systems available for LPMOs (cellobiose dehydrogenase, glucose-methanol-choline oxidoreductases and phenolics) depend on fungal lifestyle as well as the availability of extracellular electron donors (Cragg et al., 2015).

Lignin and the primary lignin building blocks (monophenolics) are an intrinsic part of the plant cell wall and as the same time, act as natural electron donors for LPMOs (Cragg et al., 2015). However, some monophenols exhibit high redox potential and are not optimal electron donors for LPMO activity. Contrarily, compounds with a 1,2-benzenediol or a 1,2,3-benzenetriol moiety have a lower redox potential compared to monophenols and are able to reduce the copper ion in the LPMO-active sites. It has been previously reported that enzymes highly active towards methoxylated monophenols such as tyrosinases can boost LPMOs activity since the resulting hydroxylated monophenols are excellent electron donors (Frommel et al., 2017).

In addition, it has been speculated that H_2O_2 can also act as co-substrate instead of O_2 for LPMOs in presence of reducing agents, changing the perspectives regarding the mode of action of these enzymes (Bissarro et al., 2016). The comprehension of electron donation systems involving LPMOs is highly relevant in order to optimize the activity of these enzymes.

In the fourth chapter was presented the genome, transcriptome and proteome of *Peniophora* sp. CBMAI 1063, a new marine-derived basidiomycete, along with the potential of the fungi to produce lignin-degrading enzymes. The optimized saline grown conditions induced the fungus to produce laccases and others CAZymes, as confirmed by mass spectrometry analyses. Fungi from marine environments are adapted to high pressure and salinity conditions (Bonugli-Santos et al., 2012). Thus, the ligninolytic enzymes produced by these microorganisms may be applied in the bioremediation of saline and alkaline effluents, molasses-based distilleries, and textile mills (Bonugli-Santos et al., 2016). *Peniophora* sp. CBMAI 1063 is also able to secrete key enzymes involved in cellulose and hemicellulose degradation such as AA9, AA3 and AA10 among GH from several families. This fungus has a versatile enzymes spectrum which deserves attention to prospect new enzymes with new activities.

Although the heterogeneous composition and the molecular structure of lignin are considered challenges in biomass conversion technologies, lignins offer unique opportunities to produce fine aromatics, antioxidant and a wide range of value-added compounds.

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Annex 1

Scientific documents published during the development of the PhD thesis

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Review

Ferulic acid and derivatives: molecules with potential application in the pharmaceutical field

Lívia Brenelli de Paiva[1], Rosana Goldbeck[1], Wanderley Dantas dos Santos[2], Fabio Marcio Squina[1,*]

[1]Brazilian Bioethanol Science and Technology Laboratory, CTBE, Brazilian Centre of Research in Energy and Materials, Campinas, SP, Brazil, [2]Plant Biochemistry and Bioenergy Laboratory, Department of Agronomical Sciences, State University of Maringá, Maringá, PR, Brazil

Ferulic acid is a phenolic acid widely distributed in the plant kingdom. It presents a wide range of potential therapeutic effects useful in the treatment of cancer, diabetes, lung and cardiovascular diseases, as well as hepatic, neuro and photoprotective effects and antimicrobial and anti-inflammatory activities. Overall, the pharmaceutical potential of ferulic acid can be attributed to its ability to scavenge free radicals. However, recent studies have revealed that ferulic acid presents pharmacological properties beyond those related to its antioxidant activity, such as the ability to competitively inhibit HMG-CoA reductase and activate glucokinase, contributing to reduce hypercholesterolemia and hyperglycemia, respectively. The present review addresses ferulic acid dietary sources, the pharmacokinetic profile, antioxidant action mechanisms and therapeutic effects in the treatment and prevention of various diseases, in order to provide a basis for understanding its mechanisms of action as well as its pharmaceutical potential.

Uniterms: Ferulic acid/properties. Ferulic acid/antioxidant activity. Ferulic acid/dietary sources. Ferulic acid/therapeutic effects. Natural products. Pharmacognosy.

O ácido ferúlico é um ácido fenólico amplamente distribuído no reino vegetal. Ele apresenta uma ampla gama de potenciais efeitos terapêuticos úteis no tratamento do câncer, diabetes, doenças pulmonares e cardiovasculares, bem como efeitos hepáticos, neuro e fotoprotetores, atividades antimicrobianas e anti-inflamatórias. O potencial farmacêutico do ácido ferúlico pode ser atribuído a sua capacidade em sequestrar radicais livres. No entanto, estudos recentes revelaram que o ácido ferúlico apresenta propriedades farmacológicas, além da sua atividade antioxidante, como a capacidade de inibir competitivamente a HMG-CoA redutase e ativar a glucoquinase, contribuindo para reduzir a hipercolesterolemia e hiperglicemia, respectivamente. A presente revisão aborda as fontes dietéticas de ácido ferúlico, o perfil farmacocinético, os mecanismos de ação como antioxidante e efeitos terapêuticos no tratamento e prevenção de várias doenças, de modo a proporcionar uma base para a compreensão dos seus mecanismos de ação, bem como os seus potenciais farmacêuticos.

Unitermos: Ácido ferúlico/propriedades. Ácido ferúlico/atividade antioxidante. Ácido ferúlico/fontes dietéticas. Ácido ferúlico/efeitos terapêuticos. Produtos naturais. Farmacognosia.

INTRODUCTION

Natural antioxidants exhibit therapeutic potential for a variety of diseases such as cancer, diabetes, and cardiovascular and neurodegenerative diseases (Kayahara *et al.*, 1999; Kim *et al.*, 2003; Soobrattee *et al.*, 2005) where free radicals play a key role in development (Prior *et al.*, 1998). Recently there has been increased public and scientific interest in employing natural antioxidants instead of synthetic antioxidants, due to their potential adverse effects on health which may include carcinogenicity (Ito *et al.*, 1983; Würtzen, 1990; Osawa *et al.*, 1990).

Antioxidants found in vegetables can act as sequesters, reducing agents, enzyme inhibitors, metal chelators or free radical scavengers (Wang *et al.*, 2000). Phenols are widely distributed in the plant kingdom and diet vegetables. There are found in significant concentrations in fruits, vegetables and beverages and

*Correspondence: F. M. Squina. Laboratório Nacional de Ciência e Tecnologia do Bioetanol (CTBE), Centro Nacional de Pesquisa em Energia e Materiais (CNPEM). Rua Giuseppe Máximo Scolfaro, 10.000, Guará - Distrito Barão Geraldo, 13083-970 – Campinas – SP, Brasil. E-mail: fabio.squina@bioetanol.org.br

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Simultaneous production of xylooligosaccharides and antioxidant compounds from sugarcane bagasse via enzymatic hydrolysis

F. Mandelli[a,b], L.B. Brenelli[a,c], R.F. Almeida[a], R. Goldbeck[a], L.D. Wolf[a], Z.B. Hoffmam[a,c], R. Ruller[a], G.J.M. Rocha[a], A.Z. Mercadante[b], F.M. Squina[a,*]

[a] Brazilian Bioethanol Science and Technology Laboratory (CTBE), Brazilian Center of Research in Energy and Materials (CNPEM), Campinas, Brazil
[b] Department of Food Science, Faculty of Food Engineering, University of Campinas (UNICAMP), Campinas, Brazil
[c] Institute of Biology, University of Campinas (UNICAMP), Campinas, Brazil

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ABSTRACT

Advances in industrial biotechnology offer potential opportunities for economic utilization of agro-industrial residues such as sugarcane bagasse, which is the major by-product of the sugarcane industry. Due to its abundant availability and despite the complex chemical composition, it can be considered an ideal substrate for microbial processes for the production of value-added products. In the present study we evaluated the enzymatic production of xylooligosaccharides (XOS) and antioxidant compounds from sugarcane bagasse using XynZ from *Clostridium thermocellum*, a naturally chimeric enzyme comprising activities of xylanase and feruloyl esterase along with a carbohydrate binding module (CBM6). In order to reveal the biotechnological potential of XynZ, the XOS released after enzymatic hydrolysis using different substrates were characterized by capillary electrophoresis and quantified by high performance anion exchange chromatography. In parallel, the antioxidant capacity related to the release of phenolic compounds was also determined. The results indicated noteworthy differences regarding the amount of XOS and antioxidant phenolic compounds produced, as well as the XOS profile, functions of the pre-treatment method employed. The ability of XynZ to simultaneously produce xylooligosaccharides, natural prebiotics, phenolic compounds and antioxidant molecules from natural substrates such as sugarcane bagasse demonstrated the biotechnological potential of this enzyme. Production of value-added products from agro-industrial residues is of great interest not only for advancement in the biofuel field, but also for pharmaceutical and food industries.

1. Introduction

Large amounts of waste are generated every year from the industrial processing of agricultural raw materials. Most of these wastes are used as animal feed or burned as an alternative for elimination. However, such wastes may be a potential source of many interesting compound, such as antioxidant compounds and xylooligosaccharides (Sultana et al., 2008; Akpinar et al., 2009). Plant biomass, like sugarcane bagasse, is a cheap, abundant and renewable raw material that can be employed for sustainable production of biofuel, bioenergy and several value added biomolecules (Jayapal et al., 2013; Gonçalves et al., 2012; Damásio et al., 2012). Currently, sugarcane is used worldwide as a feedstock for ethanol and sugar production. After sugarcane is milled for juice extraction, bagasse is obtained as a residue, which corresponds to about 25% of the total fresh weight and contains 60%–80% of carbohydrates (Betancur and Pereira, 2010). Sugarcane bagasse is composed of cellulose, hemicellulose, lignin, and small amounts of extractives and mineral salts (Rezende et al., 2011). The enzymatic conversion of lignocellulosic biomass could significantly improve bioethanol productivity and sustainability, but instead it is discarded as agricultural waste or burned for energy supply in sugar and ethanol mills (Pandey et al., 2000; Rocha et al., 2012). The close association and complexity of the carbohydrate–lignin complex is the main obstacle in bioconversion. In this context, much has been invested in technology to make this process economically feasible, such as the development of an efficient pre-treatment step and optimization of enzymatic cocktails for cell wall deconstruction.

The employment of enzymes in bioconversion processes provides advantages when compared to chemical processes, such as mild reaction conditions. Moreover, product specificity and waste minimization are also advantages that make the enzymatic process eco-friendly (Ruzzell, 1999). Xylanases (E.C. 3.2.1.8) are hemicellulases responsible for breaking down xylan, the major hemicellulosic component of plant cell walls, into short xylooligosaccharides by a

* Corresponding author at: Giuseppe Máximo Scolfaro Street, nº 10000, Campinas, SP 13083 970, Brazil. Tel.: +55 19 35183111; fax: +55 19 35183164.
E-mail address: fabio.squina@bioetanol.org.br (F.M. Squina).

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Acidification treatment of lignin from sugarcane bagasse results in fractions of reduced polydispersity and high free-radical scavenging capacity

Lívia Beatriz Brenelli[a,b], Fernanda Mandelli[a,c], Adriana Zerlotti Mercadante[c], George Jackson de Moraes Rocha[a], Silvana Aparecida Rocco[d], Aldo Felix Craievich[e], Adilson Roberto Gonçalves[a], Danilo da Cruz Centeno[f], Mario de Oliveira Neto[g], Fabio Marcio Squina[a,*]

[a] Laboratório Nacional de Ciência e Tecnologia do Bioetanol (CTBE), Centro Nacional de Pesquisa em Energia e Materiais (CNPEM), Caixa Postal 6192, CEP 13083-970 Campinas, São Paulo, Brazil
[b] Instituto de Biologia, Universidade de Campinas (UNICAMP), CEP 13083-862 Campinas, São Paulo, Brazil
[c] Departamento de Ciências de Alimentos, Faculdade de Engenharia de Alimentos, Universidade de Campinas (UNICAMP), CEP 13083-862 Campinas, São Paulo, Brazil
[d] Laboratório Nacional de Biociências (LNBio), Centro Nacional de Pesquisa em Energia e Materiais (CNPEM), Caixa Postal 6192, CEP 13083-970 Campinas, São Paulo, Brazil
[e] Instituto de Física, Universidade de São Paulo, CEP 05508-900 São Paulo, Brazil
[f] Centro de Ciências Naturais e Humanas (CCNH), Universidade Federal do ABC, CEP 09606-070 São Bernardo do Campo, São Paulo, Brazil
[g] Departamento de Física e Biofísica, Instituto de Biociências, Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP), CEP 18618-970 Botucatu, São Paulo, Brazil

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ABSTRACT

Lignin constitutes up to one-third of the material found in plant cell walls and is considered the second most abundant natural polymer in the world. Despite unique characteristics of lignin, it is mostly used for low-value commercial applications. In this study, lignin obtained after alkaline treatment of steam-exploded sugarcane bagasse was submitted to an acidification process. The soluble fractions produced at different pH values were comprehensively characterized and in vitro antioxidant capacity against reactive oxygen ($ROO^{\bullet}$ and H_2O_2) and nitrogen ($ONOO^-$) species was evaluated. The soluble fraction obtained at pH 2 exhibited the highest scavenging capacities against all species tested (10.2 ± 0.7 mmol Trolox equivalent g^{-1} for $ROO^{\bullet}$, IC_{50} = 14.9 μg mL^{-1} for H_2O_2 and IC_{50} ~ 2.3 μg mL^{-1} for $ONOO^-$) and the lowest polydispersity value (1.2) compared to others fractions. According to the SAXS data, the soluble fractions obtained at pH 4 and pH 2 consisted of small nanometer-sized discs and low molecular weight polyphenolic clusters, while soluble fractions obtained at high pH predominated wide lignin nanoparticles and larger aggregates. Mass spectroscopy analysis revealed the presence of phenolic and non-phenolic compounds, well-known as efficient antioxidants, which were identified in all soluble fractions. Collectively, our results provided further demonstration that acidification treatment is a promising strategy to upgrade heterogeneous lignin-enriched stream from sugarcane bagasse, such as preparations with homogeneous compositions and high antioxidant activity.

Abbreviations: SCB, sugarcane bagasse; AT, alkaline treatment; SF, soluble fraction; ROS, reactive oxygen species; RNS, reactive nitrogen species; TPC, total phenolic compounds; LMW, low molecular weight; ORAC, oxygen radical absorbance capacity; FTIR, Fourier transform infrared spectroscopy; GPC, gel permeation chromatography; ^{1}H NMR, proton nuclear magnetic resonance; Mn, number-average molecular weight; Mw, weight-average molecular weight; SAXS, small-angle X-ray scattering; GC–MS, gas chromatography–mass spectrometry.

* Corresponding author at: Rua Giuseppe Máximo Scolfaro, 10.000, Polo de Alta Tecnologia, Caixa Postal 6170, Campinas, SP CEP: 13083-970, Brazil. Fax +55 19 35183104.
E-mail addresses: livia.paiva@bioetanol.com.br (L.B. Brenelli), fernanda.mandelli@bioetanol.org.br (F. Mandelli), azmercadante@gmail.com (A.Z. Mercadante), george.rocha@bioetanol.org.br (G.J.d.M. Rocha), silvana.rocco@lnbio.cnpem.br (S.A. Rocco), craievich@if.usp.br (A.F. Craievich), adilson.goncalves@bioetanol.org.br (A.R. Gonçalves), danilo.centeno@ufabc.edu.br (D.d.C. Centeno), mario.neto@ibb.unesp.br (M. de Oliveira Neto), fabio.squina@bioetanol.org.br (F.M. Squina).

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Effect of hemicellulolytic enzymes to improve sugarcane bagasse saccharification and xylooligosaccharides production

Rosana Goldbeck[a,b], Thiago A. Gonçalves[a,c], André R.L. Damásio[a,c], Lívia B. Brenelli[a,c], Lúcia D. Wolf[a], Douglas A.A. Paixão[b,c], George J.M. Rocha[a], Fabio M. Squina[a,*]

[a] Laboratório Nacional de Ciência e Tecnologia do Bioetanol (CTBE), Centro Nacional de Pesquisa em Energia e Materiais (CNPEM), Campinas, Brazil
[b] Departamento de Engenharia de Alimentos, Faculdade de Engenharia de Alimentos, Universidade de Campinas (UNICAMP), Campinas, Brazil
[c] Department of Biochemistry and Tissue Biology, Institute of Biology, University of Campinas (UNICAMP), Campinas, SP, Brazil

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ABSTRACT

Enzymatic hydrolysis of lignocellulosic biomass is limited by economic considerations arising from enzyme production costs and specific activities. The effect of six hemicellulases on raw sugarcane bagasse and two types of pretreated sugarcane bagasse was investigated using experimental designs. Our strategy was successful for developing more efficient and less expensive enzymatic mixture, and also revealed that hemicellulase mixtures with multiple activities could be less effective than expected. In this study, only two hemicelulases, the endo-1,4-xylanases (GH11) from *Penicillium funiculosum* (XynC1)(CAC15487) and the feruloyl esterase (CE1) from *Clostridium thermocellum* (CtFAE/ATCC27405), effectively breakdown hemicellulose from pretreated sugarcane bagasse (up to 65%), along with the production of xylooligosaccharides (XOS). Our results also demonstrated that GH11 and CE1 can improve biomass saccharification by cellulases. Treatment with these two enzymes followed by a commercial cellulase cocktail (Accellerase®1500) increased saccharification of pretreated lignocellulose by 24%. Collectively, our data contributes to the rational design of more efficient and less expensive enzyme mixtures, targeting the viable production of bioethanol and other biorefinery products.

1. Introduction

Enzymatic hydrolysis of pretreated lignocellulosic biomass is an ideal alternative for bioethanol production [1]. The pursuit of enzymatic cocktails with improved performance has prompted years of bio-prospecting, optimization of strains through genetic engineering and development of pretreatment strategies to mitigate inhibitory effects. The recent shift to comprehensive studies of purified proteins for developing more efficient and less expensive enzymatic mixture, reflects the evolution of this field of study [2].

Plant lignocellulosic biomass, a sustainable feedstock with minimal effect on agricultural food production, is mainly comprised of polymeric sugars (cellulose, hemicellulose and pectin) and polyphenolics (lignin) found within the plant cell wall [3]. While cellulose consists of linear chains of hundreds or thousands of glucose molecules, hemicellulose is a branched polymer consisting of a mixture of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose) and sugar acids. The composition of hemicellulose can vary significantly among plant species and even within the separate components of a single plant (leaves, stem and roots) [2].

Hemicellulose is typically found in large quantities in the plant cell wall (20–35% of natural lignocellulosic biomass) and the efficiency of its extraction and conversion is an important factor and determinant for economic viability of a biomass processing facility [2]. A diverse combination of enzymes is required for complete hemicellulose hydrolysis: endo and exoxylanases initiate the break down of cross-linked hemicelluloses generating xylooligosaccharides (XOS) of varying lengths, β-xylosidases are able to hydrolyze XOS into xylose; α-arabinofuranosidase hydrolyses arabinose units into both furanose and pyranose forms; α-glucuronisidase hydrolyses methyl glucuronic acid substitutes; while acetylxylan esterase and ferulic acid esterase hydrolyze acetyl groups substitutes and ferulic acid, respectively [4].

The economically viable production of biofuels via the enzymatic hydrolysis process is dependent on reduced production costs and enzymatic mixtures with increased specific activities (i.e. increased sugar production at reduced enzyme loadings). While the discovery and production of new enzymes through bioprospection, genetic engineering and strain improvements have

* Corresponding author at: Giuseppe Máximo Scolfaro Street, nº 10000, Campinas, SP 13083-970, Brazil.
E-mail address: fabio.squina@lnbr.cnpem.br (F.M. Squina).

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Expanding the Knowledge on Lignocellulolytic and Redox Enzymes of Worker and Soldier Castes from the Lower Termite *Coptotermes gestroi*

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French National Institute for Agricultural Research (INRA), France

***Correspondence:**
Fabio M. Squina
fabio.squina@bioetanol.org.br

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João P. L. Franco Cairo[1,2], Marcelo F. Carazzolle[3], Fidvia C. Leonardo[3,4], Luciana S. Mofatto[3,4], Livia B. Brenelli[1,2], Thiago A. Gonçalves[1,2], Cristiane A. Uchima[1], Rômênia R. Domingues[5], Thabata M. Alvarez[1], Robson Tramontina[1,2], Ramon O. Vidal[3], Fernando F. Costa[4], Ana M. Costa-Leonardo[6], Adriana F. Paes Lemo[3], Gonçalo A. G. Pereira[3] and Fabio M. Squina[1]

[1] Laboratório Nacional de Ciência e Tecnologia do Bioetanol (CTBE), Centro Nacional de Pesquisa em Energia e Materiais (CNPEM), Campinas, Brazil, [2] Departamento de Bioquímica e Biologia Tecidual, Universidade Estadual de Campinas (UNICAMP), Campinas, Brazil, [3] Laboratório de Genômica e Expressão, Universidade Estadual de Campinas (UNICAMP), Campinas, Brazil, [4] Centro de Hematologia e Hemoterapia (Hemocentro), Universidade Estadual de Campinas (UNICAMP), Campinas, Brazil, [5] Laboratório de Espectrometria de Massas, Laboratório Nacional de Biociências (LNBio), Centro Nacional de Pesquisa em Energia e Materiais (CNPEM), Campinas, Brazil, [6] Departamento de Biologia, Instituto de Biociências, Universidade Estadual Paulista (UNESP), Rio Claro, Brazil

Termites are considered one of the most efficient decomposers of lignocelluloses on Earth due to their ability to produce, along with its microbial symbionts, a repertoire of carbohydrate-active enzymes (CAZymes). Recently, a set of Pro-oxidant, Antioxidant, and Detoxification enzymes (PAD) were also correlated with the metabolism of carbohydrates and lignin in termites. The lower termite *Coptotermes gestroi* is considered the main urban pest in Brazil, causing damage to wood constructions. Recently, analysis of the enzymatic repertoire of *C. gestroi* unveiled the presence of different CAZymes. Because the gene profile of CAZy/PAD enzymes endogenously synthesized by *C. gestroi* and also by their symbiotic protists remains unclear, the aim of this study was to explore the eukaryotic repertoire of these enzymes in worker and soldier castes of *C. gestroi*. Our findings showed that worker and soldier castes present similar repertoires of CAZy/PAD enzymes, and also confirmed that endo-glucanases (GH9) and beta-glucosidases (GH1) were the most important glycoside hydrolase families related to lignocellulose degradation in both castes. Classical cellulases such as exo-glucanases (GH7) and endo-glucanases (GH5 and GH45), as well as classical xylanases (GH10 and GH11), were found in both castes only taxonomically related to protists, highlighting the importance of symbiosis in *C. gestroi*. Moreover, our analysis revealed the presence

Annex 2

Institutional Biosafety Committee (IBC) Application Form

Formulário de aprovação da comissão interna de biossegurança (CIBIO)

Uso exclusivo da CIBio:

Número de projeto / processo: FMS 3.2

Formulário de encaminhamento de projetos de pesquisa para análise pela CIBio - Comissão Interna de Biossegurança do CNPEM – Centro Nacional de Pesquisa em Energia e Materiais

Título do projeto: Caracterização química e capacidade antioxidante de frações de lignina extraídas de bagaço de cana-de-açúcar

Pesquisador responsável: Dr. Fabio Marcio Squina

Experimentador: Lívia Beatriz Brenelli de Paiva

Nível do treinamento do experimentador: [ ]-Iniciação científica, [ ]-mestrado, [x]-doutorado, [ ]-doutorado direto, [ ]-pós-doutorado, [ ]-nível técnico, [ ]-outro, especifique:_____

Resumo do projeto: Considerada uma macromolécula formada de unidades de fenilpropano, a lignina apresenta uma conformação tridimensional e amorfa, representando de 20% a 30% do total dos lignocelulósicos. Quando fragmentada, a lignina gera compostos fenólicos e polímeros com diferentes propriedades e funcionalidades que podem ser empregados nas mais diversas indústrias. Considerando a tendência mundial do desenvolvimento sustentável, o emprego de moléculas antioxidantes derivadas da lignina na cosmética, em alimentos e no uso terapêutico, representa uma interessante forma de conversão de um sub-resíduo agroindustrial sem valor em produtos com alto valor agregado. Nossos resultados preliminares mostraram que a lignina extraída do bagaço de cana é uma fonte adequada para obtenção de moléculas antioxidantes. Desta forma, o presente projeto nível Doutorado tem como objetivo estudar as propriedades dos compostos fenólicos presentes em frações de lignina obtidas do bagaço de cana a fim de aproveitar o potencial antioxidante que esses compostos exibem. O plano estratégico deste trabalho se inicia com a fragmentação de lignina via rota química e enzimática, caracterização das frações obtidas através de técnicas experimentais e determinação da capacidade antioxidante destas frações através de métodos descritos na literatura. Por fim, as análises estatísticas multivariadas correlacionarão a composição das frações de lignina com as capacidades antioxidantes e posteriormente será preparado uma emulsão não-iônica das frações que apresentarem maiores capacidades antioxidantes a fim de avaliar a estabilidade das moléculas.

A CIBio analisou este projeto em reunião realizada no dia: 17.1.2013.

Parecer final: [X]-projeto aprovado, [ ]-projeto recusado, [ ]-projeto com deficiências, favor comentários anexo.

Presidente da CIBio – CNPEM-LNBio
Jörg Kobarg

Membro da CIBio – CNPEM -LNBio
Prof. Dr. Celso Eduardo Benedetti

Membro da CIBio – CNPEM -LNBio
Andrea Balan

Membro da CIBio da CNPEM -CTBE
Fabio Squina

Membro da CIBio da CNPEM -CTBE
Sindelia Freitas Azzoni

Membro da CIBio da CNPEM -CTBE
Roberto Ruller

Annex 3

Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada **APLICAÇÃO DE ABORDAGENS QUÍMICAS E BIOLÓGICAS PARA VALORIZAÇÃO DA LIGNINA**, não infringem os dispositivos da Lei n.° 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 25 de Agosto de 2017

Assinatura : _____
Nome do(a) autor(a): **Lívia Beatriz Brenelli de Paiva**
RG n.° 43.502.349-04

Assinatura : _____
Nome do(a) orientador(a): **Fabio Marcio Squina**
RG n.° 209817-77