TÉCNICO LISBOA

Investigation of enzyme interaction with biorefinery lignin

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To my amazing mother

and my beautiful family

“In the depth of winter I finally learned that

there was in me an invincible summer.”

Albert Camus

Preface

The work presented in this document was performed in the ambit of the Integrated MSc in Biological Engineering in Instituto Superior Técnico de Lisboa (IST), in order to obtain 30 ECTS.

The research was inserted in the BIOVALUE SPIR project and was carried at the Center for Bioprocess Engineering, Department of Chemical and Biochemical Engineering, at the Technical University of Denmark (DTU), from September of 2015 to February 2016, under the supervision of the Senior Researcher Henning Jørgensen and PhD student Demi Djajadi.

This report consists of a literature-based theoretical part related to the project and of the results obtained during the experimental work.

Agradecimentos

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Não imaginam o quanto eu me orgulho em ser vossa neta, sobrinha e filha.

Resumo

A chave para a produção de biocombustíveis e químicos a partir de biomassa é uma sacarificação eficiente capaz de converter os materiais lenhocelulósicos em açúcares. A ligação não-produtiva, que se crê dever à carga e hidrofobicidade da superfície da lenhina que, por sua vez, pode estar relacionado com a origem da biomassa e/ou pré-tratamento aplicado, limita a reutilização e a reciclagem de enzimas.

Neste trabalho foram estudadas as isotérmicas de adsorção de albumina de soro bovino (BSA), de uma mistura de enzimas comercial (CEM) e de uma Lacase em nove resíduos ricos em lenhina (sobrantes de milho; *Miscanthus x giganteus*; palha de trigo), após pré-tratamento hidrotérmico, hidrólise enzimática e remoção de proteína, para compreender o comportamento da ligação de enzimas durante a sacarificação.

A adsorção foi diferente entre severidades. Na mesma severidade, a mesma tendência foi verificada entre materiais. A maior adsorção de BSA foi obtida na palha de trigo de severidade média (136.3$mg_{proteina}/g_{EnzHR-P}$), enquanto de CEM e de Lacase foi no material de mais alta severidade dos sobrantes de milho (105.1$mg_{enzima}/g_{EnzHR-P}$ e 122.6$mg_{enzima}/g_{EnzHR-P}$, respectivamente). O ajuste ao modelo de Langmuir foi aplicado sem sucesso.

Um tratamento com Lacase foi realizado para estudar alterações na ligação das proteínas à lenhina. Após uma análise de variância, observaram-se diferenças estatísticas no decréscimo de adsorção de CEM no *Miscanthus* e BSA na palha de trigo, ambos tratados com Lacase e ácido 2,2'-azino-bis(3-etilbenzotriazolina-6-sulfónico).

Este trabalho contribui para uma melhor compreensão da influência da composição dos substratos na ligação das enzimas durante a sacarificação.

Palavras-chave:

Pré-tratamento hidrotérmico; resíduos ricos em lenhina; estudos de adsorção; Langmuir; tratamento com lacases

Abstract

The key for the production of biofuels and chemicals from biomass is an efficient enzymatic hydrolysis process to convert lignocellulosic plant cell walls to platform sugars. Non-productive binding limits the reuse or recycling of enzymes, and it is believed to occur due to lignin's surface charge and hydrophobicity, which could be related to the biomass origin and/or applied pretreatment.

This study investigated the adsorption isotherms of bovine serum albumin (BSA), a commercial enzyme mixture (CEM) and a Laccase on nine lignin-rich residues from hydrothermally pretreated feedstocks (corn stover; *Miscanthus x giganteus;* wheat straw), obtained after enzymatic hydrolysis and protein removal, to understand the protein binding behavior during the saccharification process.

Within the same feedstocks the adsorption was different between severities. Additionally, within the same severity, the same trend was verified between feedstocks. The highest adsorption of BSA was achieved in the medium severity wheat straw (136.3 $mg_{protein}/g_{EnzHR-P}$), while CEM bound more to highest severity residue of corn stover (105.1 $mg_{enzyme}/g_{EnzHR-P}$), as the Laccase (122.6 $mg_{enzyme}/g_{EnzHR-P}$). A fitting to the Langmuir adsorption isotherm model was unsuccessfully applied.

A Laccase treatment was performed to study modifications to the protein binding to lignin, and after an analysis of variance, a statistical difference was only verified in the decrease of adsorption of CEM in *Miscanthus* and BSA in wheat straw, both treated with Laccase and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid).

The results from this research can contribute to a better understanding of the influence of the substrates composition on the binding of enzymes during the hydrolysis process.

Keywords:

Hydrothermal pretreatment; lignin-rich residues; adsorption studies; Langmuir; laccase treatment

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Notation

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AFEX	Ammonia fiber explosion
ANOVA	Analysis of variance
AU	Aarhus University
BET	Brunauer–Emmett–Teller
BSA	Bovine serum albumin
CBM	Carbohydrate binding module
CBH	Cellobiohydrolase
CEM	Commercial enzyme mixture
DM	Dry matter
EG	Endoglucanase
EnzHR	Enzymatic hydrolysis residues
EnzHR-P	Enzymatic hydrolysis residues after protease treatment
GHG	Green house gases
HPI	N-hydroxyphthalimide
IBUS	Integrated Biomass Utilization System
IEA	International Energy Agency
LHW	Liquid hot water
LiP	Lignin peroxidase
MnP	Manganese peroxidase

NIR	Near-Infrared Spectroscopy
NREL	National Renewable Energy Laboratory
PEG	Polyethylene glycol
SIADEB	Ibero-American Society for the Development of Biorefineries
UV/Vis	Ultraviolet/Visible
VP	Versatile peroxidase

1. Introduction

1.1. The Biorefinery concept

Environmental, financial, economic and political concerns have been raised in the last decades due to the intensive consumption and dependence on fossil fuels, not only for energy but also for the chemical sector. The volatility associated to the prices and the fluctuating demand requires a robust and competitive system to meet this demand, an atom economy where every atom is valuable and utilized in the best possible manner. A transition from non-renewable carbon sources to renewable resources in an innovative Bio-based Economy is being more and more acknowledged as necessary: an economy where material wastes are minimized, Green House Gas (GHG) emissions are reduced and new bioproducts can replace their fossil equals.

The International Energy Agency (IEA) Bioenergy Task 42 Biorefineries defines biorefinery as "the sustainable processing of biomass into a spectrum of marketable products (food, feed, materials, and chemicals) and energy (fuels, power, heat)". Thus biorefinery can be a concept, a facility, a process, a plant or a cluster of facilities [1].

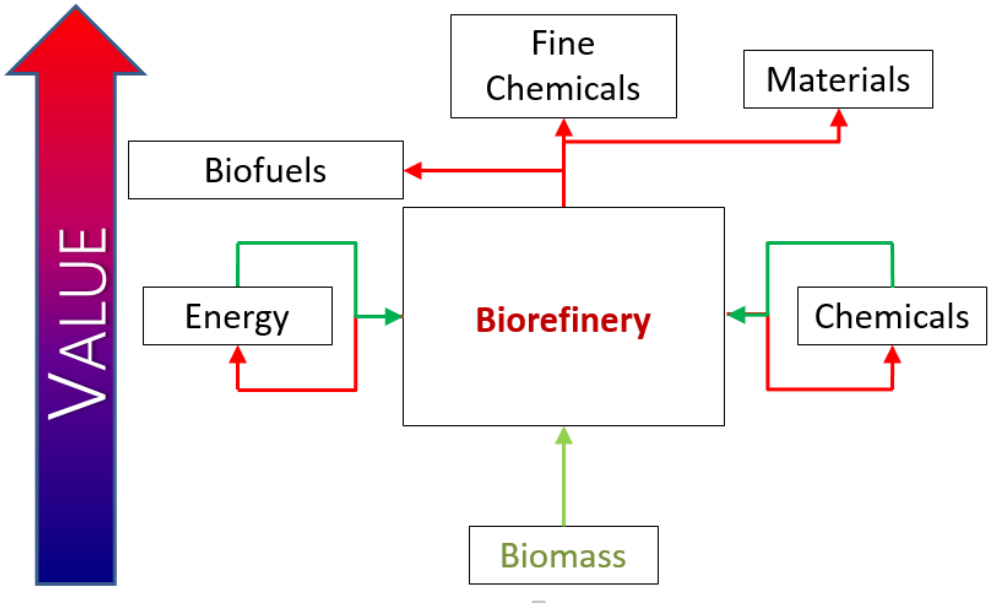

Figure 1 Scheme of the biorefinery concept. Adapted from SIADEB (2010).

Similar to the “petroleum refinery”, biorefinery uses abundant raw material composed mainly by renewable polysaccharides and lignin. After entering the plant and by multiple processes, the raw material is fractionated and converted into numerous different products (chemicals and transportation fuels, for example), while generating electricity and heat for its own use in the processes. This way, the value derived from the biomass feedstock can be maximized by taking advantage of the differences in biomass components [3,4]. The products obtained have also the advantage of being environmentally friendly and sustainable, while contributing for the decrease of the competition with food supplies and for the enhancement of rural economic development.

1.2. Lignocellulosic feedstock

Lignocellulosic biomass is seen as a promising base for biorefineries due to the fact that it is a renewable and sustainable feedstock, low cost and is available worldwide in a wide variety of species. It is the most abundant organic material in nature with an estimated annual production of 10-50 billion tons (dry mass weight) worldwide [5]. Being the involved quantities considerable and due to the high potential for bioconversion, a growing attention has been developed towards these materials. The valorization of lignocellulosic materials is achieved by recovering their polysaccharides in the form of fermentable sugars which may, for example, be used for the production of liquid fuel, solvents, protein mass for food and value added metabolites, such as vitamins (aspartic acid or glutamic acid), polyols (glycerol, xylitol, arabitol and sorbitol) or organic acids. Looking at the energy sector, the raising demand in the future will be covered by an active production of biomass crops, either by increasing productivity or by occupying marginal lands. Depending on the location and necessity, countries can choose different crops based on their climate, production capacity and aimed final products [6].

Based on their origin, lignocellulosic materials can be classified as forest residues (hardwoods like eucalyptus, oak and beech, or softwood, such as pine and fir and also residues from paper and wood industry); agricultural residues (straw, corn cobs, barks, stems, tree pruning); agro-industrial by-products (for example, sugarcane bagasse, brewery’s spent grain, fruit seeds and pulp); and urban waste (paper, cardboard and domestic waste mainly containing cellulose) [7].

1.2.1. Composition of lignocellulosic materials

The importance of the lignocellulosic materials is related to lignocellulose which is the major structural component of all plant cell walls. Lignocellulose is mainly composed by three macromolecular components:

cellulose and hemicellulose (polysaccharides) and lignin. The minor components are proteins, lipids, pectin, soluble sugars and minerals [8].

These components are associated in a hetero-matrix to different degrees and their relative composition depends on the species, origin, biological and genetic variations of each biomass [9]. On a weight basis, cellulose represents about 30–50% of the biomass, being the component in higher quantity, hemicellulose represents 20–40% and lignin 20-30% [10]. However, structural organization of the polymers in the plant cell wall consists of a microfibrilar cellulose skeleton surrounded by organized hemicelluloses, with lignin fillling the empty spaces in between. Extractives are found in cell lumen, cellular voids or channels [11].

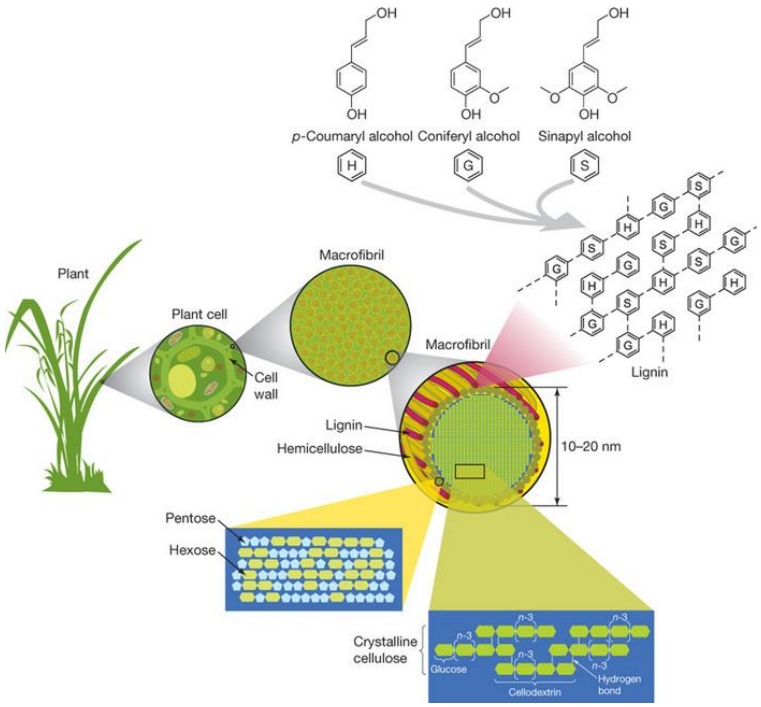

Figure 2 Structural organization of components in plant cell wall. © Nature

1.2.1.1. Cellulose

Cellulose is the main polymer present in plant cell wall, mostly in the secondary wall, responsible for structural support [8]. It consists of a long and linear chain of glucose units linked by β-1,4-glycosidic bonds, with the general chemical formula $(C_6H_{10}O_5)_n$, where "n" is the degree of polymerization ranging between 10 000 (wood) to 15 000 (native cotton) glucopyranose units. Consecutive glucose monomers are rotated 180 degrees, showing that the repeating unit of this polymer is the disaccharide, cellobiose [9,10].

Due to van der Waals interactions, and to intra- and intermolecular hydrogen bonds between oxygen of alternating glycosidic bond in one glucan chain and the primary hydroxyl groups at position 6 of glycosyl residues in another chain (side-by-side and top-down), cellulose chains bound with a precise spacing. This hold the chains together to form thin, flattened, rod-like structures that are referred to as microfibrils (Fengel & Wegener, 1984). These form the crystalline portion of cellulose, structure very recalcitrant to degradation. Aggregation of microfibrils form fibrils and aggregation of fibrils leads to cellulose fibres. Cellulose can also be classified as amorphous when the (1,4)-d-glucan chains are not bound together in an ordered structure, making it more susceptible to enzyme digestion [9,12,13].

Due to its structure, cellulose has high chemical resistance, being insoluble in most solvents, but can be chemically hydrolyzed by acids or enzymatically [9]. Its stability it is also due to the presence of other macromolecular components hemicellulose (linked mainly through hydrogen bonds) and lignin (besides hydrogen bonds, there is the contribution of stable lignin-carbohydrate complexes) [14].

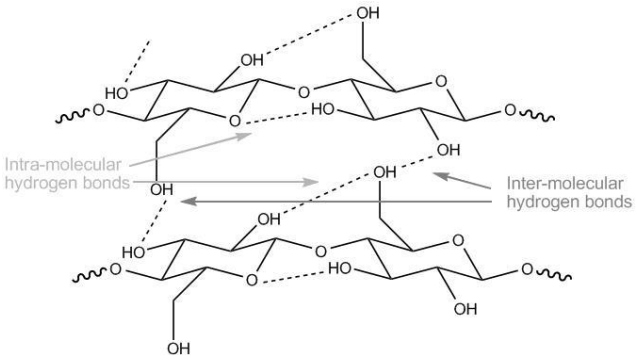

Figure 3 Chemical structure of cellulose.

1.2.1.2. Hemicellulose

Hemicelluloses are heteropolysaccharides present in the plant cell wall, responsible for support and cohesion that bounding covalently to lignin. These polymers are composed of short highly branched chains of different sugars and non-sugars: β-D-xylose and α-Ł-arabinose (pentoses, five-carbon sugars); α-D-galactose, β-D-glucose and β-D-mannose (hexoses, six-carbon sugars); α-D-glucuronic, α-D-galacturonic and α-D-4-O-methylgalacturonic acids (uronic acids). α-L-rhamnose and α-L-fucose can also be found in small amounts and the hydroxyl groups of sugars can be partially substituted with acetyl groups [14].

The most abundant hemicelluloses are xylans and glucomannans, with xylans being the most abundant with a variable composition, depending on the nature and source of each feedstock [15,16]. These are made up of a main backbone of xylose linked by β(1→4) bonds, where the structural units are often substituted at

positions C2 or C3 with arabinofuranosyl, 4-O-methylglucuronic acid, acetyl or phenolic substituents [17]. In Table 1 it is presented a small description of the polysaccharides that are present in grasses.

Although they are easier to hydrolyze than cellulose, hemicelluloses can contribute to the recalcitrance of cellulose and also to blocking the access of enzymes, derived from the complex branching and acetylation patterns of some structures [10]. These heteropolymers are soluble in alkaline solutions and easily hydrolyzed into monomers by acids, with the disadvantage of being less stable chemical and thermally when compared to cellulose. This fact may be imputed to its lack of crystallinity and low polymerization degree [11].

Table 1 Main type of polysaccharides present in the hemicellulose of grasses [18].

Biological origin	Polysaccharide type	Amount (%)	Units		
			Backbone	**Side Chains**	**Linkage**
Grasses	**Xyloglucan**	2-25	β- D -Glcp β - D -Xylp	β- D -Xylp	β-(1→4)
				β- D -Galp	α-(1→3)
				α-L-Araf	β- (1→2)
				α-L-Fucp	α-(1→2)
				Acetyl	α-(1→2)
	Arabinoglucuronoxylan	5-10	β - D -Xylp	4-O-Me-α-D-GlcpAβ-L-Araf	α-(1→2) α-(1→3)
	Glucuronoarabinoxylans	15-30	β -D-Xylp	α-L-Araf 4-O-Me-α-D-GlcpA Acetyl	α-(1→2) α-(1→3)

1.2.1.3. Lignin

Lignin is a complex, hydrophobic and aromatic heteropolymer. It is responsible for the plant structural support, impermeability, and resistance to oxidative stress and to microorganisms due to its hydrophobic nature and insolubility in water or aqueous solutions preventing access of organisms as well as degrading chemical agents [19]. It is the most abundant natural non-carbohydrate organic compound in fibrous materials responsible for the compact plant cell wall structure due to covalent bonds with hemicelluloses.

The polymer is composed of three phenolic monomers of phenyl propionic alcohol (monolignols) namely, P-coumaryl, coniferyl and sinapyl alcohol, linked through different types of ether and ester bonds, as of C-

C bonds. These monolignols are integrated into lignin in the form of p-hydroxyphenyl (H), guaiacil (G) and syringyl (S), respectively, bonded by C-O-C and C-C linkages. The relative amount of these compounds vary according with the species and the tissues of the lignocellulosic material. Most lignins also contain some esterified aromatic carboxylic acids (*p*-hydroxycinnamic – or *p*-coumaric – acids).

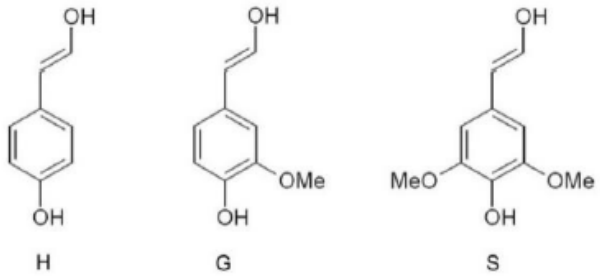

Figure 4 Lignin precursors: p-coumaryl alcohol (H); coniferyl alcohol (G); sinapyl alcohol (S).

Generally, softwoods contain more lignin than hardwoods and herbaceous plants (such as grasses), having the last ones the lowest contents of lignin [20]. Lignin contents generally vary from 24 to 33% in softwoods, being composed by more than 90% of coniferyl alcohol with the remaining being mainly p-coumaryl alcohol units. Hardwoods have a lignin content of 19 to 28% in temperate-zone, and from 26 to 35% in tropical hardwoods, and the ratios vary between coniferyl and sinapyl alcohol type of units. Grasses and agricultural residues are the materials with the lowest lignin content (10-30% and 3-15%, respectively). The content in lignin is affected by the age of the plant and varies also within the different structures of the plant [16,21].

From the biorefinery point of view, lignin has always been considered as an important barrier to polysaccharide utilization. The existence of strong C-C and C-O-C linkages hampers the chemical disruption. The combination of hemicellulose and lignin provides a protective sheath around the cellulose, which must be modified or removed before efficient hydrolysis of cellulose can occur [22]. Chang and Holtzapple (2000) showed that biomass digestibility is enhanced with increasing lignin removal. Lignin can be removed and biomass digestibility can be improved by pretreating the materials. This way, lignin can be extracted and/or altered due to changes in the chemical properties. As a consequence, the available surface area increases, as the accessibility of cellulolytic enzymes to cellulose.

Besides being a physical barrier, lignin is also a problem regarding enzyme's adsorption. During enzymatic hydrolysis, it is believe that non-productive enzyme binding occurs between cellulases and lignin, which limits the access of cellulases to cellulose, being a higher enzyme loading usually needed in hydrolysis. Enzymatic hydrolysis is also influenced by lignin's degradation, which form phenolic groups, responsible for deactivating or inhibiting cellulolytic enzymes [16].

Lignin modification has been studied with the intent of facilitating hydrolysis processes (especially for bioethanol production). Chen *et al.* (2006) worked on the genetic modification of the biosiynthetic pathway leading to a reduction of lignin formation, improving ethanol yield. As a disadvantage, the plant can lose its defenses, its natural protection/barrier [16].

Nowadays, there are processes that use lignin for energy production by combustion since it is considered a better fuel than other biomasses. This fact is related with the fact that when lignin arrives to the furnace is already presented in small particles derived from the bioethanol production processes, and also it does not contain contaminants (e.g. fouling alkaline metals) that could contribute for damaging the equipment [25]. From lignin it is also possible to obtain high value products, such as phenols, organic acids and vanillin, that can be produced recurring to organisms that can degrade these aromatic polymers [22].

1.2.2. Materials

In the present project, the three feedstocks were chosen because they have been studied as potential feedstock to biorefineries in Denmark. Corn stover and wheat straw are agricultural residues with a considerable amount produced per year in Denmark. *Miscanthus* (MS) has been studied as a possible energy crop to be planted due to its high productivity in cold climates.

1.2.2.1. Corn stover

Corn stover, a residue from corn (*Zea mays* L.) harvesting, is a renewable non-food agricultural feedstock used for production of biofuels, energy and other bioproducts. “Stover” is a generic term used to refer the parts of the corn plant that are usually not profitable, namely, the leaves, husks and cobs. Approximately, for each ton of corn grain produced, about one ton of corn stover is generated [26].

In the United States of America (USA), corn is the most widely planted crop with a productivity of approximately 5 500 ton/ha in 2013 (Ertl, 2013; FAOSTAT, 2015). It is usually harvested for animal feed or bedding, otherwise is left in the field to replace soil organic matter, nutrients and to minimize the effects of water and wind in soil erosion [30]. When harvested dry, it is typically packed in dense round bales or loose stacks, with a moisture content between 20-25% and stored outdoors. When is harvested wet, the moisture content is, approximately, 45% and it is preserved by ensiling [31].

Corn stover composition varies according to corn variety, planting site (weather, soil type, fertilization conditions, etc) and harvest year. The relative amount of different anatomical parts in the stover also affects the relative composition[32].

Figure 5 Corn stover ©. Source: http://www.biofuelsdigest.com/

Corn stover has been proposed as a feedstock for the biorefinery to produce bioethanol, for direct combustion or gasification to produce electricity and even for paper production as a supplemental fiber [31]. The constrains associated with using this or other agricultural feedstock in biorefinery are related with the harvesting method and logistics, and ultimately with the delivery costs, key issues that need to be evaluated to design an optimal supply chain. Also, regarding the average composition, like most herbaceous lignocellulosic feedstocks, corn stover contains relatively high levels of ash [26].

Although several companies are interested in pursuing corn stover as a feedstock, no biorefinery supply chains for this biomass have been substantially proven through successful multi-year operations yet. In that sense, significant opportunities to maximize the biorefining potential of corn stover regarding optimization of efficiencies and minimizing costs of its collection and supply remain to be achieved [26].

1.2.2.2. Miscanthus x giganteus

Miscanthus, commonly known as “Elephant Grass”, is a perennial rhizomatous grass originated from East Asia. It is widely spread across Asia and Europe, which caused a large genetic variability due to the different climates. It has a high lignin content and lignocellulosic fiber. There are several species of this plant, being the best known *Miscanthus sinensis* (better adapted to extreme climate conditions) and *Miscanthus x giganteus* (better adapted to temperate climates) [33]. Many studies consider this the new crop of choice due to its high yields and low energy input, thus having the higher potential for energy production [34].

Miscanthus x giganteus is a natural hybrid from *M. sacchariflorus* with *M. sinensis* [34]. It is a very resistant grass, with stalks diameter of up to 10 mm and can reach 2 meters high in the first year and around 4 meters

in the second year in Europe. This genotype reaches maturity in 2 to 3 years of plantation. Biomass productivity achieved is 10 to 30 t/ha.year, depending on the environment where is inserted. These values are limited by low winter temperatures (maximum -3.5ºC is tolerated by the specie) and, on the other hand, water depletion in hot summers.

Figure 6 Miscanthus x giganteus © Olena Kalinina, OPTIMISC–Project

This lignocellulosic feedstock is a C4 crop (C4 photosynthetic pathway), which is characterized by water-use efficiency and higher biomass production potential (in temperate climates). It has the advantage of growing fast in temperate climates and has a long life cycle (20 to 25 years), being able to be harvested every year [35]. It has some advantages when compared with other grasses. It is highly sustainable having reduced fertilizer and pesticides inputs, GHG emissions are reduced and can be grown on poor soils. It is also very resistant to plagues and to ecological adverse factors. One of the main problems associated with *Miscanthus* is related with the invasiveness associated with the development of perennial crops. This can be minimized genetically modification (non-flowering and hybrid sterile species) [33].

At the present, the main use of *Miscanthus x giganteus* biomass is combustion. These species was firstly introduced in Denmark in 1935 and, at the time, it was noticed that it had the capacity of developing with high productivity in low temperature environments (Lewandowski, 2000). In Europe, presently, *Miscanthus x giganteus* biomass is used with coal to produce electricity in the United Kingdom (UK), resulting in, approximately, 20 000 ha grown. Drax Coal Power Station, responsible for providing around 7% of the electrical power to the UK, has created a market for this biomass for co-combustion. Also, grants were established by the government to farmers, as fiscal incentives to power companies. Followed by the UK in

the production of this type of grass are Austria, Switzerland and Germany, using the biomass for thermal applications at smaller scales [33].

Miscanthus genotypes that can grow under harsh conditions, namely saline soils, have been identified in China (Dongying Agricultural Science Research Institute) [37].This constitutes a possibility of taking advantage of non-agricultural soils, also called marginal lands, and do added-value agriculture. Yet, little information is available about the quality of biomass from the different genotypes for the various uses.

1.2.2.3. Wheat straw

Wheat is an important annual rain-fed crop that generates a large amount of residues. The productivity of the cereal, in 2013, was superior to 3 250 ton/ha [29]. The annual production of straw is estimated to 1.3 ton per ton of wheat grains [38]. Since it is an abundant resource that is usually discarded, from a global perspective, it can be beneficial if a biorefinery uses wheat straw as feedstock.

This herbaceous crop is attractive to be used in biorefineries due to the fact that typically has low water content, being easy to store, and its soft material enables its transport in relatively high density form [39]. Theoretical ethanol yields from 31% to 84% were obtained in previous studies. The values vary depending on the origin of the wheat straw, the pretreatment applied, enzyme loading for the enzymatic hydrolysis and the yeast culture used for the fermentation [40]. The main applications of this residue are animal-feed [41], bedding and it is also used for energy and pulp production [42].

Figure 7 Wheat straw © Source: http://ocj.com/

The problem associated with the wheat straw is the amount of ash content. The content of alkali metals, as sodium and potassium, and in chlorine and silicon also pose a problem. When the biomass is incinerated, potassium and chlorine can cause a number of technical problems, such as corrosion of super heaters, slagging and fouling and deterioration of catalysts for NOx reduction [43].

In Denmark, since 1986 wheat straw has been used as a resource for energy production. It is implemented an efficient logistic system for the whole process until usage in the power plant (collection, storage and delivery). Direct contracts between farmers and the power company are made, being a favorable situation for both ends, while the power plant get the feedstock, the producer dispose of a waste while gets an income. The annual consumption of straw for heat and power production is 1.4 million tonnes, corresponding to, approximately, 25% of the straw produced, which contributes to 2-3% of total energy production in the country [43].

Table 2 Average composition of the raw feedstocks (corn stover, Miscanthus, wheat straw) from literature.

	Cellulose	Hemicellulose	Lignin
Corn stover[a]	31.9	25.7	13.3
***Miscanthus x giganteus*[b]**	51.2	25.3	12.3
Wheat straw[c]	36.5	22.5	17.5

[a]Tao *et al*., 2013
[b]Hodgson *et al*, 2011
[c]Talebnia *et al*., 2010

1.3. Biomass processing

1.3.1. Pretreatment processes

There are several ways to obtain bio-products, however, for the recovery of the different components, the first step is usually the materials fractionation. For example, the three major unit operations for the bioconversion of lignocellulosic biomass to ethanol are, in order, pretreatment, enzymatic hydrolysis and finally fermentation of the sugars. A pretreatment process is usually required to facilitate the digestibility of complex lignocellulosic biomasses into (fermentable) sugars and other several compounds [44]. The purpose of the pretreatments make structural changes to the lignocellulosic materials by increasing the surface area and the material's porosity. By modifying or removing lignin, partially polymerizing or removing hemicelluloses and disrupting the crystallinity structure of cellulose, cellulose and hemicellulose can be easily accessed and hydrolyzed into monomers by enzymes [44,45].

Due to the differences in the composition of each feedstock, it is yet not possible to define a pretreatment method suitable for all feedstocks. Thus the pretreatment must be chosen taking into account the effects on the polymers [46]**.** It must lead to an improvement of the hydrolysis of carbohydrates, and at the same time limit their loss or degradation, as the formation of degradation products can be inhibitory to the subsequent enzymatic hydrolysis and fermentation processes [21]**.** Avoidance of post-pretreatment processes (washing, neutralization or detoxification) and low energy demand are also desirable traits [46]**.**

The cost of the selected method has also to be considered, since it constitutes a significant share in the overall cost [46]**.** However, it is an important and sometimes critical step, since it has been proven the direct correlation between the removal of hemicelluloses and lignin and the digestibility of cellulose. In addition, still has potential for improvement in the process' efficiency and reduce costs [20,47]**.** Pretreatments can be physical (milling and griding), physico-chemical (steam explosion, hydrothermolysis, wet oxidation, etc), chemical (alkali, dilute acid, oxidizing agents and organic solvents), and biological processes [44,47]**.** To enhance the bio-digestibility they can be used solely or combined [48]**.**

1.3.1.1. Biological pretreatment

Biological treatment of lignocellulosic biomass has been used before to modify the materials in the paper and feed industry. Nowadays, it has been studied as a pretreatment for enhancing enzymatic saccharification for ethanol production [49]. This type of pretreatment is an environmental friendly process based in the action of microorganisms to degrade mainly hemicellulose and lignin. Cellulose can be also be degraded but in a smaller extent since it is more resistant. White, brown and soft-rot fungi are employed in the processes being white-rot fungi the most effective in delignifying lignocellulosic materials with the action of enzymes as peroxidases and laccases, for example [49]. This allows the compounds to be more accessible for hydrolysis and subsequent use (e.g., for bioethanol production).

On an industrial scale, disadvantages associated with this process are related with the rate of the treatment since is very slow when compared to others (residence time of 10 to 14 days), with is sensitivity due to the microorganisms growth conditions and the high cost of enzymes, factors not desirable in industrial processes [50].

1.3.1.2. Physical pretreatment

Physical pretreatments aim for size reduction of biomass to increase the accessible surface area (surface/volume ratio) and pore size of the material and reduce the crystallinity and degree of polymerization of cellulose present [47,51]**.** All these factors are responsible for the increase of the total hydrolysis yield of

the biomass that can go from 5 to 25% and the decrease of the digestion time by 23 to 59% (values vary from biomass to biomass and depend also of the type and duration of process used).It also makes the material handling easier during the following processes [21,48].

Mechanical comminution, extrusion and ultrasound pretreatments can be used to reduce the particle size [46]**.** In mechanical comminution a combination of processes as chipping, grinding or milling can be used depending on the final particle size needed. This type of process is energy demanding which cannot be economically feasible in a large scale. Extrusion resorts to an extruder were the biomass is heated, mixed and subjected to shear stress resulting in physical and chemical transformations that allow an easier access to the carbohydrates. Parameters such as screw speed and barrel temperature can be optimized in order to enhance the enzymatic digestibility by defibrillating, fibrillating and/or shortening the fibers [49]**.** In ultrasound pretreatment, the bubbles formed by the cavitation effect collapse resulting in the opening of the substrate structure, consequently enabling the enzymes access [46]**.**

Usually, the choice of the right method depends on the needed particle size for the following processing steps [21]**.**

1.3.1.3. Chemical pretreatment

The use of chemicals for delignification and/or removal of hemicelluloses, as for decreasing the degree of polymerization of cellulose has been studied extensively, not only to be applied in biomass but also in other industries such as pulp and paper [47].

The chemicals used for these processes can be oxidizing agents, alkali, acids and salts, for the fractions removal. While some organic acids can be used as catalysts (salicylic acid and acetylsalicylic acid or oxalic acid, for example), inorganic acids (HCl and H_2SO_4, for example) mixed in an organic or aqueous organic solvent mixture are also used to disrupt lignin and hemicellulose structure. Concentrated acids, due to the fact that they are corrosive and that after the process need to be recovered, are not desirable to be used since they turn the pretreatment more expensive [47].

1.3.1.4. Physico-chemical pretreatment

Physico-chemical pretreatments are a combination of both chemical and physical processes with the goal of altering lignin structure and solubilize hemicellulose. This allows, as previously stated, hydrolytic enzymes to access cellulose in further processes.

From the vast majority of pretreatment methods included in this category some are here referred: steam explosion, liquid hot water, ammonia fiber explosion (AFEX), wet oxidation, organosolv, CO_2 explosion and ionic liquids [47]. Since the physical and chemical properties of the materials are affected, these type of pretreatments depend on process conditions and solvents used.

1.3.1.4.1. Hydrothermal pretreatment

Hydrothermal pretreatments can be considered ecofriendly processes and have several advantages when compared to other methods. Since only water and lignocellulosic materials are used, these methods do not require chemicals, therefore there is no need for neutralization or recovering; depending on the pH and temperature condition, it is able to solubilize the hemicellulosic fraction to oligosaccharides and minimize the formation of sugar monomers and degradation products. Also as an advantage, regarding the economical aspect, the construction materials also have a lower price due to the risk of corrosion be much lower or non-existing [52,53].

Although during the hemicellulose hydrolysis acetic acid is released and is considered to function as a catalytic agent, the reaction rates of this type of processes are still slow, making the used temperature range to be usually high (190 to 230°C). This represents excessive energy costs associated with both the pretreatment and product recovery.

Nevertheless, the hydrothermal pretreatment is an expedite method for obtaining a solid fraction suitable for posterior enzymatic hydrolysis. One key factor is the setting of the pretreatment's severity, which has to ensure both the accessibility of the enzymes to the cellulose fibers and that hemicellulose is not degraded. After pretreatment, the fiber fraction (rich in C6 sugars, in the form of cellulose, and lignin) is usually separated from the liquid fraction (rich in hemicellulosic C5 sugars and easily extractable compounds, like inorganic salts) simply by pressing.

1.3.1.4.1.1. Integrated Biomass Utilization System (IBUS) process

The concept of IBUS was developed within the European project "Co-production of Bio-fuels" (December 2002 – March 2006) by the company DONG Energy (Denmark). The aim of this project was to develop a technology for the co-production of bioethanol and electricity from agricultural residues, such as wheat straw [54].

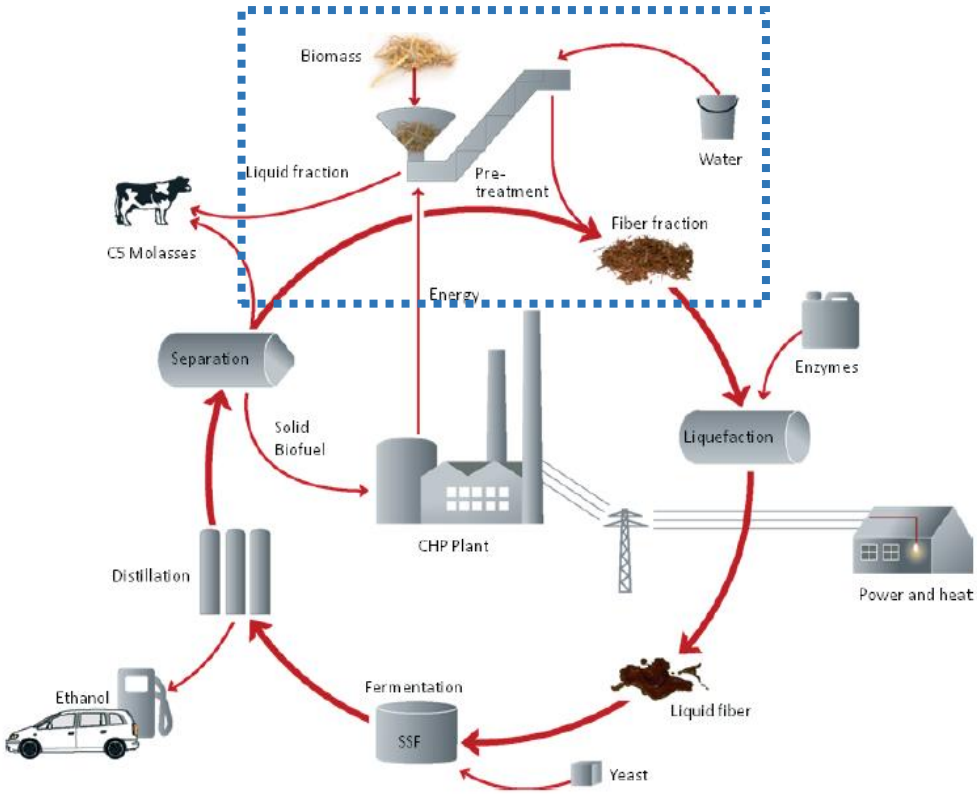

Figure 8 Process flow in the IBUS process at Inbicon. The mini-IBUS corresponds to the downscale of the first step of the process (blue dotted area). The fiber fraction represents the residues used for the adsorption studies, prior to the carbohydrates removal. Adapted from [25]

In this low energy-consuming process, wheat straw is converted into bioethanol, solid biofuel and feed (mainly xylan and xylose from the pretreatment). As advantages, it enables the use of feedstocks with high dry matter content (20-40% of lignocellulosic material); it is environmentally friendly, using only steam, water and enzymes; and it is also energy efficient by being integrated with a power plant [25]. This technology was further implemented at Inbicon Biomass Refinery (Kalundborg, Denmark) and it is currently used in projects in several countries like Brazil and China [55].

The IBUS comprises the 5 steps for the production of bioethanol represented in

Figure 8. In the pretreatment step the biomass, with high dry matter content, is treated in a continuous hydrothermal process with steam during a defined period of time, being washed at the end to remove potassium chloride, hemicellulose and some inhibitors that may have been produced. The temperature range of the defined isothermal period is between 180 and 200°C and the retention time is between 5 to 15 min [25].

For the development of the work presented, it was necessary to obtain solids with increased enzymatic digestibility and high lignin content. Therefore, the feedstocks used were only subjected to this phase, being recovered only the solid fraction after pressing. The washing step was not performed.

1.3.2. Enzymatic Hydrolysis

One of the main problems with conversion of biomass is the formation of degradation products. Biochemical conversion enables the hydrolysis of the polymers of carbohydrates into monomers maintaining the original structures, minimizing losses and avoiding generation of byproducts that could be inhibitory to further processes [56].

Although being considered as a sustainable technology for saccharification, is still a growing field due to constraints associated to enzymes specificity, sensibility, dosage and cost. Another limiting factor is related with the lignocellulosic complex matrix and the recalcitrance of each individual component, limiting the access to enzymes [10].

Biomass-converting enzymes are used to degrade the polysacharydes cellulose and hemicellulose into simple sugars that will be then fermented by microrganisms to be used for the synthesis of biofuel or valuable chemicals. Conversion of biomass by lignocellulose-degrading enzymes occurs by either hydrolytic reactions (cellulose and hemicellulose) or oxireductive reactions (mainly acting on lignin) [57].

An enzymatic cocktail is usually used to hydrolyze these polysaccharides to pentoses (xylose and arabinose) and hexoses (glucose, galactose and mannose). In most cases, enzymes have the highest performance when in the presence of different enzymes, beneficting from the synergysm. This can be observed as an effect on the specificity for the different compounds or regions of lignocellulose as well as a tool for diminishing inhibitory effects by components or degradation products [57]. The most common commercial enzyme cocktails used are produced by *Trichoderma reesei* fungus. The optimal conditions of the process occur in a range of temperatures between 40 and 50ºC at a pH 4.5-5.0 [10]. Although being more time consuming than, e.g., chemical treatments, the mild conditions needed for this process lead to less sugar degradation comparing to other methods, consequently achieving higher conversion yields [46].

The number of enzymes involved in cell-wall degradation are not exactly known, however, three main categories of enzymes are known to be necessary for the hydrolysis of the cell-wall fractions: cellulases, hemicellulases and lignin modifying and degrading enzymes [58].

1.3.2.1. Cellulases

From the biotechnological point of view, the importance of the enzymatic degradation of cellulose is based on the production of glucose for further fermentation.

According to the classical scheme cellulose is degraded by a system of cellulases composed by three components that work synergistically: endo-β-1,4 -glucanases (EC 3.2.1.4), cellobiohydrolase or exo-β-1,4-glucanases (EC 3.2.1.91 and 3.2.1.176) and β-glucosidase (EC 3.2.1.21). Endo-β-1,4- glucanases act on the polymers randomly cleaving glycosidic bonds by adding a water molecule in the cellulose polymer, generating new reducing and non-reducing chain ends where exo-acting enzymes will act and release cellobiose. These enzymes are also called cellobiohydrolases, are the most abundant proteins in natural and commercial enzyme mixtures. β-glucosidase catalyze the formation of glucose monomers by acting on the cellobiose formed by the other two classes of enzymes, avoiding or diminishing end-product inhibition [10,59].

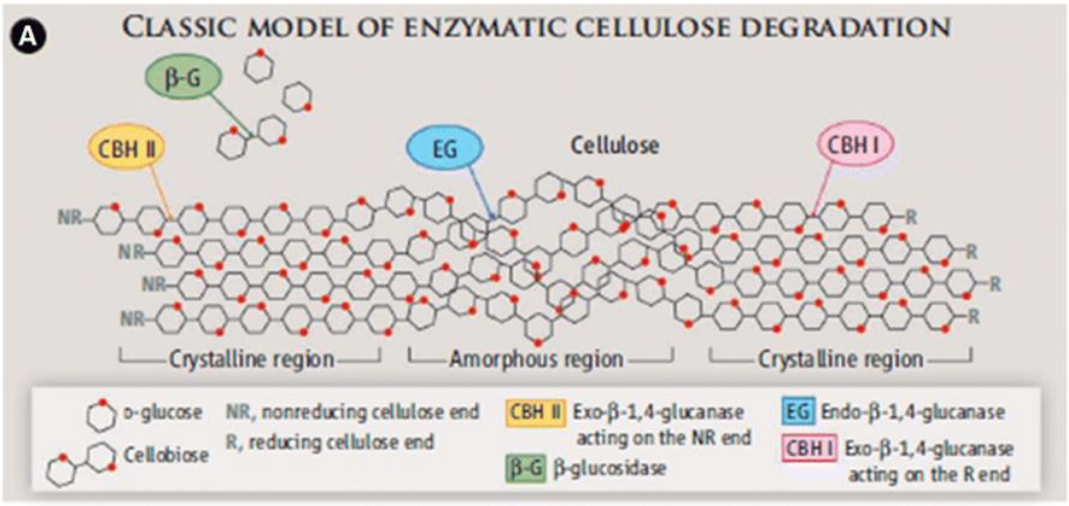

Figure 9 Cellulases: binding sites of each enzyme and respective hydrolysis products. ©American Chemical Society

Nowadays it is also known that some bacterial and fungal glycoside hydrolases have a multimodular architecture composed by a carbohydrate binding module (CBM), a linker and a catalytic domain. After binding to cellulose, the CBM helps its degradation by increasing its local concentration and promote a closer enzyme attachment and for a longer period of time to the recalcitrant material [59]. Natural cellulolytic enzyme systems may contain several enzymes (endo- and exo-acting) which vary the affinity to the form of cellulose where they act, for example, crystalline or amorphous cellulose. Such characteristic can be a consequence of variation in the presence of CBM [10].

Lignocellulolytic organisms (e.g. wood-degrading fungi and bacteria) produce specific enzymes that naturally degrade lignocellulose and enables it utilization as part of natural energy cycle and carbon cycle [57]. The fungus *Trichoderma reesei* has been greatly studied due to its cellulase system composed of, at least, five genetically different cellulases: two cellobiohydrolases (CBH I and II) and four endoglucanases (EG I, II, III, and V). The enzymes act in synergism during the hydrolysis of cellulose [60].

1.3.2.2. Hemicellulases

Hemicellulases are responsible for the hydrolysis of hemicelluloses, converting the heteropolysaccharydes monosaccharides, consequently allowing a better access to cellulose. These enzymes are either glycoside hydrolases or carbohydrate esterases (responsible for breaking hemicelluloses' side chains) [61]. The synergism is also applied between these enzymes and cellulases [57].

As cellulases, an array of interdependent and synergistic hemicellulases are available such β-1,4-endoxylanase (for xylan degradation; EC 3.2.1.8), endoglucanases (xyloglucanases; EC 3.2.1.151) and β-glucosidases (for xyloglucan degradation; EC 3.2.1.21), and β-endomannanases (β-mannanases; EC 3.2.1.78) and β-mannosidases (for mannan degradation; EC 3.2.1.25).

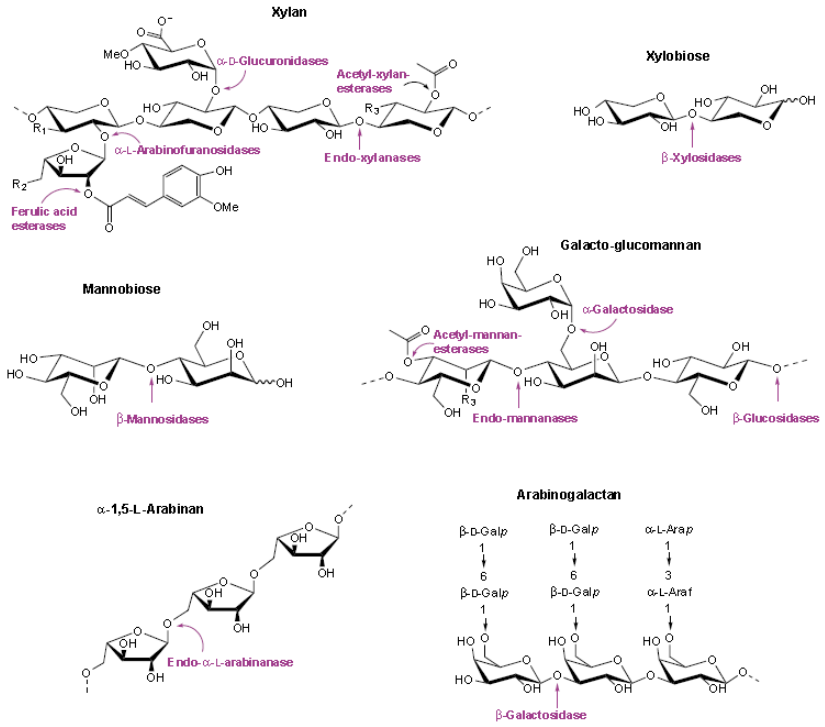

Figure 10 Examples of hemicellulases used in the market (represented in purple) and respective cleavage sites. © Liu (2016)

Depending on the strain and growth conditions, different cellulolytic microorganisms produce several cellulases and hemicellulases for effective lignocellulose degradation which can be valuable in industry. And, due to the diverse composition of hemicelluloses between feedstocks, different combinations can be necessary for the biomass proper conversion [57].

1.3.2.3. Laccases

A complex mixture with a concerted action of hydrolytic and oxidative enzymes, as well as accessory proteins, is needed for lignin degradation [59]. Several enzymes are known for acting on lignin, for instance, phenol oxidase (laccase) and heme peroxidases [lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP)]. In this work, the main focus is on laccases [63].

Laccases (benzenediol: oxygen oxidoreductase; EC 1.10.3.2.), also called phenol oxidases, are 60–70 kDa copper containing enzymes that can be found in plants, fungi and bacteria with different functions [63]. Laccases from plants are involved in the biosynthesis of lignin by inducing radical polymerization of the monolignols to the branched lignin network resulting in different bonds, while in wood-decaying fungi laccases are responsible for lignin degradation [64].

The most important laccase producers are white-rot fungi. These microorganisms delignify wood, resulting in the so-called ‘‘white rot’’ due to the laccases and enzymatic cocktail that they secrete, besides laccases and other ligninases, auxiliary enzymes that provide reduction equivalents for the peroxidases such as hydrogen peroxide [64,65].

1.4. Adsorption studies

Acquiring knowledge on adsorption of lignocellulolytic enzymes is of great importance in the progress of biomass hydrolysis processes. Studies have been developed to understand the adsorption phenomenon and the formation of enzyme-substrate complexes, aiming for the development of models that could describe the adsorption behavior. Purified monocomponent enzymes and commercial enzymes preparations have been used in feedstocks from different origins, subjected to different pretreatments, but the results have not been very fruitful, and have even been reported sometimes as contradictory [66].

During the research, several problems have emerged such as, for example: the unclear relationship between the hydrolysis rate and the adsorption of enzymes; the effect of temperature in the adsorption; the

unclear interaction between the enzymes and the substrate, if they have common or specific binding site, and also the effect of interaction of different enzymes that could be present during the hydrolysis process [66,67]. This last point derives from the fact that in industry pure enzymes are not used due to the high costs associated. Commercial mixtures are used, composed by different types of enzymes, in different ratios, that could interact and suffer from competitive inhibition [60].

Regarding the effect of temperature, adsorption isotherms have been determined usually in two different ranges of temperature. Some studies are performed at low temperatures (4-10ºC) in order to avoid hydrolysis of the material, thus interference in the final results. On the opposite side, some defend that, since hydrolysis has an optimum temperature between 40-50ºC, and if the study of adsorption of enzymes to the biomass that occurs during this process is the objective, this research needs to maintain the same range.

Concerning the substrate composition, more specifically the macromolecular components, enzymes are known to adsorb both to cellulose and to lignin. Binding of proteins to cellulose is desired and necessary in order to obtain the sugars, however it is possible that enzymes remain trapped in the porous surfaces of the material, or to occur loss of enzymes, consequence of unproductive binding to biomass in some degree, especially when binds to lignin [60,66]. Cellulases and hemicellulases are believed to adsorb to lignin due to hydrophobic interaction, but it is believed that different types of these enzymes have different affinities (e.g., enzymes without CBM seem to adsorb more to lignin) [68]. The identification of the group of enzymes that adsorb to each macrocomponent is still unknown as the type of binding that occurs on complex substrates containing both cellulose and lignin.

As a consequence, the irreversible binding to lignin reduces the hydrolysis rate representing higher costs to the process. In order to lower the costs, either lower enzyme loadings are used or enzymes need to be recycled. Recycling of 60% of the cellulolytic enzymes could have a major impact on the contribution of the enzymes to overall process costs [69]. Some processes have been developed in order to achieve an acceptable degree of recovery. One possibility is related with delignification processes where, during pretreatment, lignin is considerably reduced. Other possibilities are related with the use of specific components (proteins and peptides) that could bind to lignin, consequently avoiding enzyme's adsorption; enzyme immobilization onto a solid matrix ; use of chemicals like non-ionic surfactants (Tween 20 or Tween 80) or polyethylene glycol (PEG) [66,68].

1.4.1. Langmuir isotherm

The adsorption process is a phenomenon that occurs in the surface of an adsorbent solid material when this attracts a component, establishing connections via physical or chemical bonds. It depends on variables such as temperature, pressure, concentrations and deposition environments [70], and it is represented by

an adsorption isotherm that shows the equilibrium (the ratio between the adsorbed amount with the remaining in the solution) at a given temperature and pH (Figure 11). An equilibrium is observed when an adsorbate containing phase is in contact with the adsorbent for a given period of time, achieving a dynamic balance between the adsorbate in solution and the adsorbate in the interface [71].

Several equilibrium isotherm models have been used to describe the enzyme's adsorption to lignocellulosic materials, for example, Langmuir, Freundlich and Brunauer–Emmett–Teller (BET). One of the simplest and most direct methods to quantify adsorption is the Langmuir adsorption isotherm. The model was originally developed to describe the adsorption of gas species onto simple solid surfaces and is based on the following assumptions [72]:

- The adsorption sites in the surface are homogeneous;
- All sites are equivalent;
- Each adsorption site binds an individual molecule (monolayer);
- A dynamic reversible equilibrium is established;
- There are no interactions between adsorbate molecules on adjacent sites in order to alter their adsorption behavior.

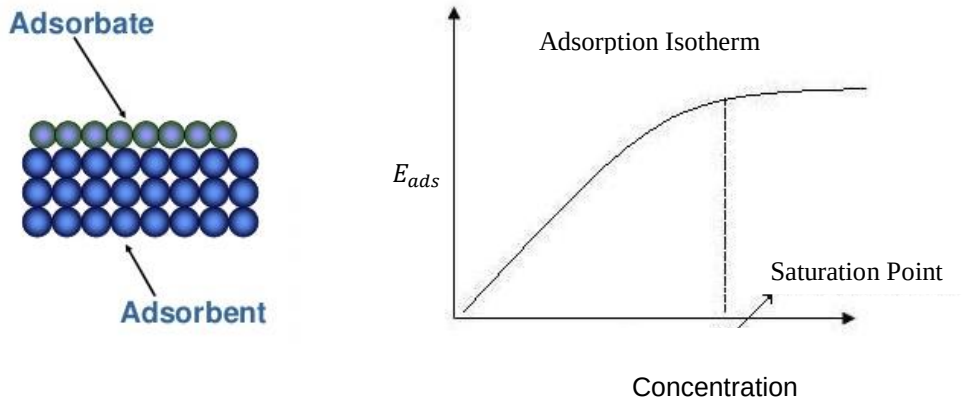

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Figure 11 Langmuir isotherm model. Representation of the monolayer described by the Langmuir model. The correspondent graphic is an adsorption isotherm, which enables a better understanding of the studied system.

Applied to proteins, the equilibrium can be represented by equation (1) and (2), where $[X]$ and $[S]$ represent the protein and the substrate's surface binding sites concentration, respectively, and $[XS]$ the concentration of protein at the surface of the substrate. The mathematical modeling is represented by the equation (3) and equation (4), where E_{ads} is the concentration of adsorbed protein ($mg_{protein}/g_{substrate}$), E_{max} the maximum

adsorbed enzymes ($mg_{protein}/g_{substrate}$), E_f the free enzyme in the supernatant after adsorption ($mg_{protein}/mL$) and, finally, K_p is the adsorption equilibrium constant ($mL/mg_{protein}$), a measurement for the adsorption affinity.

$$[X] + [S] \underset{Desorption}{\overset{Adsorption}{\rightleftharpoons}} [XS] \quad (1)$$

$$[X] + [S] \underset{K_{des}}{\overset{K_{ads}}{\rightleftharpoons}} [XS] \quad (2)$$

$$K_P = \frac{K_{ads}}{K_{des}} = \frac{[XS]}{[X][S]} \quad (3)$$

$$E_{ads} = E_{max} \frac{K_p E_f}{1 + K_p E_f} \quad (4)$$

Several studies in enzyme non-productive binding present this model since isotherm data from protein adsorption apparently fits reasonably to the model [72]. In this work, Langmuir was used to fit the experimental data in order to acquire some insight about the adsorption mechanism, trying to understand the role of lignocellulose surface properties as well as the degree of affinity of the three proteins in study.

1.5. Aim of studies

In the present work, the adsorption of three proteins on nine lignin-rich residues was studied. The three different proteins are a first model protein (bovine serum albumin - BSA), a commercial enzyme mixture (CEM) and Laccase.

The first objective was to perform a lignin isolation procedure to the nine pretreated feedstocks in order to obtain lignin-rich residues (with an expected composition of over 90% lignin). This consisted in an enzymatic hydrolysis and a protease treatment in order to remove protein that could have been stuck in the porous material.

Afterwards, the adsorption studies were performed using the same process conditions in order to compare the effects of the severity of a hydrothermal pretreatment. The amount of adsorbed protein was determined by measuring the amount of free protein in solution after the treatments using the ninhydrin assay, enabling the outline of the respective adsorption isotherms.

A non-linear curve fitting to the Langmuir model was performed to investigate the effect of lignin on the adsorption of the different proteins, obtaining the maximum adsorption capacity and affinity associated to each protein, in each studied substrate.

A Laccase treatment was later executed to two medium severity materials (*Miscanthus* and wheat straw) to test the hypothesis of enzymatic modification of the lignin hydrophobicity, and consequent modifications in adsorption.

The information gathered is expected to contribute to a better understanding of the influence of the substrates composition to the binding of enzymes during the hydrolysis process, in order to establish the feasibility of recovering and recycling the enzymes used for biomass processing and assess the cost effectiveness of the process.

1.6. Investigation strategy

To study the adsorption of enzymes to lignin from different feedstocks, a strategy was followed and it is represented in the following flow chart.

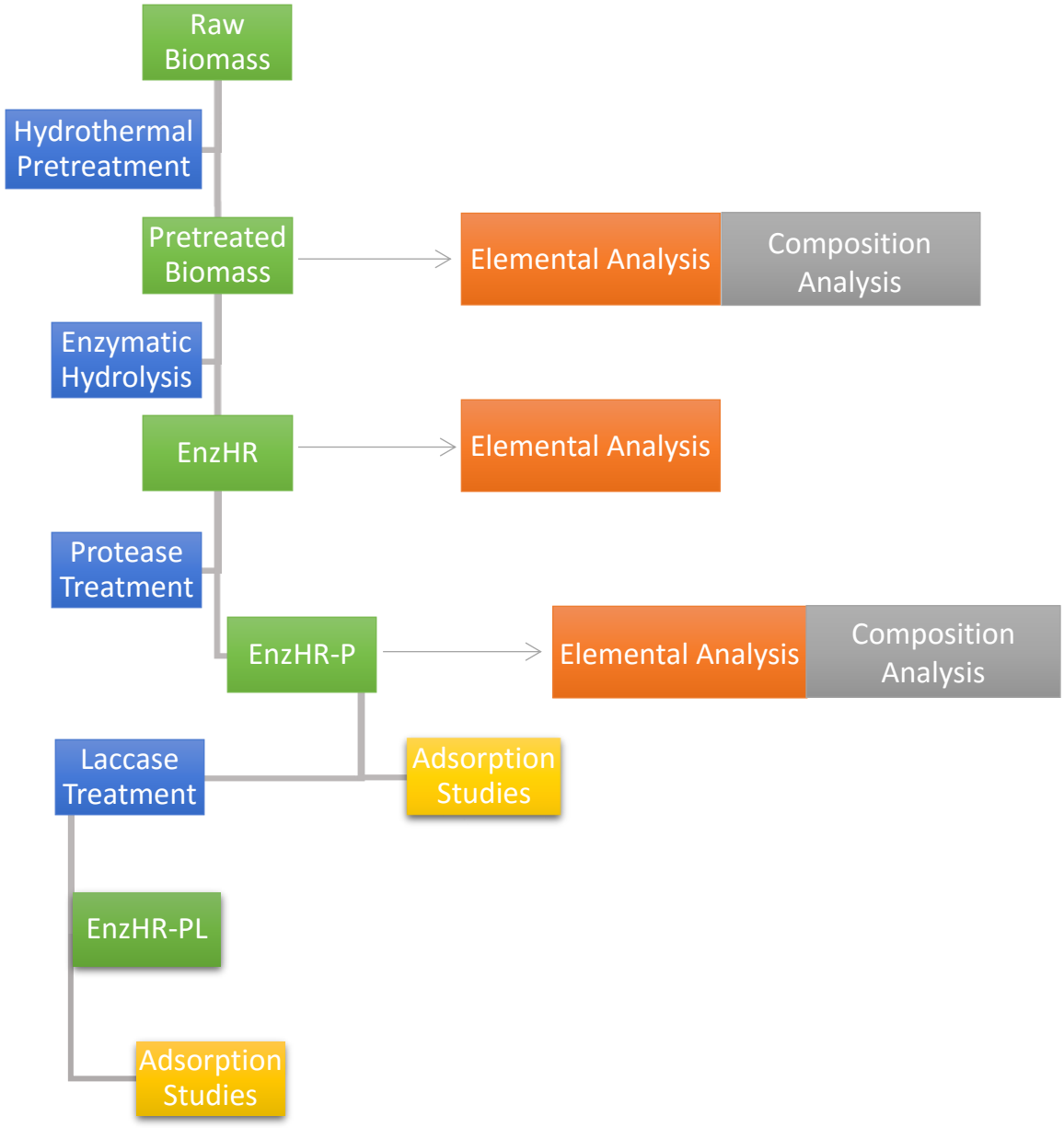

Figure 12 Schematic representation of the processes involved in the experimental setup. The blue shapes represent the processes used, the green shapes represent the evolution of the biomass after each process, the orange and grey shapes represent analytical methods used, and the yellow shapes represent the aim of the present work

2. Materials and methods

2.1. Materials

2.1.1. Raw materials

The materials used in this work were kindly provided by Søren Sommer Pedersen from Aarhus University (AU): Corn Stover (harvested at AU Jyndevad, autumn 2014); Wheat straw (*Triticum aestivum* L.) and *Miscanthus x giganteus* (harvested at AU Foulum, autumn 2014).

2.1.2. Hydrothermal pretreatment

In order to increase the cellulose digestibility a hydrothermal pretreatment was applied to the materials. A Liquid Hot Water pretreatment (LHW) was performed using the Mini-IBUS equipment at Center for BioProcess Engineering at DTU-Risø. Three different conditions were applied where the severity was increased in each treatment by changing the residence time and the temperature. The severity factor was calculated according to Overend and Chornet (1987) (see Appendix A).

After pretreatment, the biomass was pressed inside the reactor to obtain a solid fraction with around 35-40% dry matter (DM) and a liquid fraction. For each biomass and pretreatment condition, a minimum of three batches were done, each with a biomass loading of 1 kg DM. The solid fractions from all batches were mixed and immediately frozen at -20°C until use. Posteriorly they were subjected to a compositional analysis as described in section 2.5.

The employed conditions and the identification of the pretreated materials are presented in Table 3.

2.2. Lignin isolation

2.2.1. Size reduction

The unwashed pretreated materials were cut with a gardener's scissor for size reduction. Homogenization of the materials was obtained by introducing pieces smaller than 3 cm in a GRINDOMIX GM 200 knife mill (Retsch, Germany), for 1 min, at 7500 rpm. The final particle size was inferior to 300 µm (according to information from the manufacturer). The solids were frozen (-20°C) until further use.

Table 3 Conditions applied in the hydrothermal pretreatments and solid residues identification.

	Material code	Temperature (°C)	Pressure (bar)	Residence time (minutes)	Severity Factor
Corn Stover	**4CS**	190	12.6	10	3.65
	5CS	190	12.6	15	3.83
	6CS	195	14.0	15	3.97
Miscanthus	**8MS**	190	12.6	10	3.65
	9MS	190	12.6	15	3.83
	10MS	195	14.0	15	3.97
Wheat Straw	**14WS**	190	12.6	10	3.65
	15WS	190	12.6	15	3.83
	16WS	195	14.0	15	3.97

2.2.2. Enzymatic Hydrolysis

After pretreating the materials, the lignin of the feedstocks was isolated by removal of the carbohydrates content by enzymatic hydrolysis. First, the materials were thawed at room temperature and the moisture content was measured in order to establish the amount to add to the process. The DM content was obtained as described in section 4.3.

The enzyme cocktail was kindly provided by Novozymes A/S (Denmark). The enzymatic hydrolysis were performed in 2 L Erlenmeyer flasks with a solid loading of 7.5% (w/w) and an enzyme loading of 60 mg protein/g DM substrate, in 0.05 M sodium citrate buffer (pH 5.0). The experiments were performed in an orbital shaker incubator (INFORS HT Ecotron, Switzerland) at 150 rpm for 72h at 50°C. Every 24h, the Erlenmeyers' content was centrifuged for 10 min at 4000 rpm (Heraeus Multifuge 4KR, Thermo Scientific, USA) and the supernatants were removed and replaced with a new batch of buffer with the same initial enzyme loading.

Table 4 Dry matter content of pretreated biomasses before enzymatic hydrolysis.

Material code	DM content (%)
4CS	35.22
5CS	37.82
6CS	42.46
8MS	44.64
9MS	45.52
10MS	45.48
14WS	43.47
15WS	45.51
16WS	35.46

After 72h, the samples were centrifuged as previously described and 3 volumes of acidic water, MilliQ water (Synergy water purification system, Millipore, USA) previously adjusted with hydrochloric acid (HCl; Sigma-Aldrich, USA) to pH 2.5, were sequentially added to wash the solid residues through centrifugation at 4000 rpm for 5 min (Heraeus Multifuge 4KR, Thermo Scientific, USA). After the washings, the residues were filtered in a mesh and transferred to 50mL falcon tubes, frozen at -80°C overnight, freeze-dried (Scanvac CoolSafe, LaboGene, Denmark) and stored at room temperature. The obtained residues were analysed by quantitative acid hydrolysis, as described in section 2.5.

2.2.3. Protease treatment

During enzymatic hydrolysis, some of the enzymes can attach to the Enzymatic Hydrolysis Residues (EnzHR) that may affect the studies of enzyme's adsorption. A protease treatment was applied with a protease from *Bacillus licheniformis* (P4860), in order to remove the enzymes that attached to the lignin-rich residue after enzymatic hydrolysis.

The moisture content of the EnzHR stored at room temperature was previously measured as described in section 4.3. The materials were incubated at 50°C for 24h in 0.5 M $NaHCO_3/Na_2CO_3$ buffer (pH 9.6), in 500

mL Erlenmeyers, with a 5% (w/w) solid loading and protease P4860 (78.17 mg protein/g protease solution by Ninhydrin assay) with an enzyme loading of 20 mg protein/g DM EnzHR. The content of the flasks was then transferred to 50 mL Falcons and centrifuged at 4000 rpm for 10 min. The pellets were washed three times with 1 volume of MilliQ water previously adjusted with HCl to pH 2.5 each time, followed by centrifugation as described before. The remaining solids were frozen at -80°C overnight, freeze-dried (Scanvac CoolSafe, LaboGene, Denmark) and stored at room temperature.

Table 5 Identification of the enzymes used in the present project, with the activity and respective manufacturer's information.

Enzyme	Activity	Manufacturer
CEM	131.5 FPU/g	Novozymes, Denmark
Protease from Bacillus *licheniformis* (P4860)	2.4 U/g	Sigma-Aldrich, USA
Laccase	898 U/ml (based on syringaldehyde) 1372 U/ml (based on ABTS)	Novozymes, Denmark

2.3. Adsorption studies

The adsorption studies protocol was modified from Taherzadeh and Karimi [74].

The adsorption of a CEM, Laccase and BSA (Sigma-Aldrich, USA), was tested on two different lignin-rich hydrolysis residues: of the nine enzymatic hydrolysis residues after protease treatment (EnzHR-P) and of two materials (*Miscanthus* and wheat straw) of the medium severity pretreatment after protease and Laccase treatment.

In the adsorption studies involving the EnzHR-P, different solutions of BSA (with a concentration range between 0.2-10 mg/mL) and solutions of the commercial cellulase mixture (0.1-5 mg/mL) were prepared with 50mM citric acid buffer (pH 5.0). 15 mg of solids were weighed into 2 mL low protein binding Eppendorf tubes corresponding to a final 1% solid loading. After addition of the enzyme solutions and mixing, the tubes were inserted in a tube rotator (Thermo Scientific, USA) and placed in an incubator (Lab-Therm, Adolf Kühner AG, Switzerland) at 50°C for 2h at 15 rpm. The liquid fraction was then separated by centrifugation,

for 1 min at 10 000 rpm, collected and stored in 1.5 mL Eppendorf tubes, in a cold environment (under 4°C) or freezed for further protein assay, as described in section 2.6.5. Controls lacking lignin or enzymes were used as references and background correction, respectively.

2.4. Laccase treatment

The Laccase treatment was performed on the EnzHR-P from medium severity *Miscanthus* and wheat straw to test the effect of Laccase treatments in the enzyme's adsorption to lignin. Six different conditions were tested: lignin with Laccase (L+lac), lignin with denatured Laccase (L+dlac), lignin with mediators 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and N-hydroxyphthalimide (HPI), L+lac+ABTS and L+lac+HPI, respectively, and lignin with Laccase (L+lac) and the same mediators.

A Laccase solution with a concentration of 0.2 mg/mL was also prepared for the treatments. Also, prior to the experiment, the mediators and a solution of denatured enzyme were prepared. For denaturing the enzymes, 2mL low protein binding Eppendorf tubes were filled with 2mL of Laccase solution and inserted in a thermomixer (Eppendorf, Germany) at 99°C for 20 min.

The Laccase treatment was performed in a thermomixer, in 1 mL low protein binding Eppendorf tubes with a solid loading 1% (m/v) in acetate buffer pH 5.0 at 50°C for 24h, 1250 rpm. After 24h, the samples were centrifuged at 10 000 rpm for 10 min, the supernatant was discarded and one volume of the same buffer was used for washing. The samples with ABTS and Lccase were washed 3 times due to the blue coloration. After vortex, the samples were centrifuged at 10 000 rpm for 10 min, the supernatant was discarded, and 1mL of protein solution (BSA or CEM), previously prepared in acetate buffer pH 5.0, with a concentration of 0.6 mg/mL was added to the pellets and the same conditions of the adsorption studies were applied, as described in section2.3.

Table 6 Identification of the samples used to study the Laccase effect on the lignin of EnzHR-P of medium severity Miscanthus and wheat straw.

EnzHR-P	Samples
L+B; L+ABTS; L+HPI; L+dlac	Controls
L+lac	Lignin treated with Laccase
L+lac+ABTS; L+lac+HPI	Lignin treated with Laccase + one of the mediators

2.5. Composition Analysis (Quantification of structural polysaccharides and Klason lignin)

The chemical composition of raw and pretreated materials and lignin-rich residues subjected to a protease treatment was determined using a modified protocol based on NREL/TP-510-42618 protocol [75]. The standards used were previously prepared with known concentrations of D-glucose, L-arabinose, D-galactose, D-xylose and D-mannose.

The acid hydrolysis was performed by adding 1.5 mL of sulphuric acid 72% (w/w) to the 60 ml borosilicate test tubes containing 0.15 g of dried solid samples. After stirring, the tubes where incubated in a water bath for 1h at 30°C and vortexed every 10 min. MiliQ water was then added diluting the samples' concentration of H_2SO_4 to 4% (w/w) and the tubes were autoclaved, together with the standards, for 1h at 121°C. After cooling down, the tubes were weighed and the content was filtered through porcelain filtering crucibles, previously burned in a muffle furnace at 575°C for 4h and weighed. The solid sample was then rinsed with 15-30 mL of warm MiliQ water and dried in an oven at 100°C to constant weight and then burned in a muffle furnace. The difference in weight is the amount of acid insoluble lignin. The acid soluble lignin was determined measuring the absorbance of the filtrate at 320 nm using Ultrospec 2100 pro UV/Vis Spectrophotometer (Amersham Biosciences, UK).

For the quantification of sugars, 5 mL of the acidic filtrate was neutralized in 15 mL centrifugation tubes with 0.20 g of calcium carbonate ($CaCO_3$). The samples were then centrifuged at 4000 rpm for 10 min, and the supernatants were collected. The analysis was performed as described in section 2.6.4 using a 2 g/L fucose solution as an internal standard.

For each sample the procedure was performed in triplicate.

2.6. Analytical methods

2.6.1. Determination of dry matter content

Dry matter content of the solid materials was determined using a HR83 Mettler-Toledo moisture analyzer (Switzerland) at 105°C.

2.6.2. Determination of total ash content

Total ash content was determined according to NREL/TP-510-42622 protocol [75]. Briefly, 0.5g of solid samples (moisture content was previously determined as described in section 4.3) were weighed in porcelain crucibles previously burned in a muffle at 575°C for at least 4h and tared and placed in a muffle furnace at 575°C for 4h. After cooling in a desiccator, the crucibles were weighed. The difference between the final weight of the crucibles and its tare is considered the ash content (calculations in annex).

Determinations were performed in duplicate for each sample.

2.6.3. Determination of protein content (Elemental Analysis)

The CHN-S elemental analysis was performed on the pretreated biomass, on the EnzHR obtained and on the protease treated lignin-rich residues of all of the materials in the study to indirectly evaluate the protein content by nitrogen analysis.

The samples were weighed in triplicate (1 to 1.5 mg) in a Sartorius MC 210 P high precision balance (Germany) into special tin containers for CHN-S analysis and folded. Elemental analysis was performed with a Euro EA 3000 element analyzer (Euro Vector Instruments & Software, Milan, Italy). Acetanilide (Euro Vector Instruments & Software, Milan, Italy) was used as a standard.

Table 7 Composition of the CHN-S standard acetanilide.

	C%	H%	N%	O%
Acetanilide	71.09	6.71	10.36	11.84

2.6.4. Quantification of monosaccharides by HPLC

The concentration of the monosaccharides glucose, xylose, arabinose, galactose and mannose in the liquid fractions were quantified by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC coupled with PAD) using a Dionex ICS-5000 system (DionexCorp ,Sunnyvale ,CA) equipped with a CarboPac PA1 analytical column (250x4 mm2) and a CarboPacPA1 guard column (250x4 mm2) operated at a flow rate of 1mL/min. Isocratic elution took place at 25°C, with water, for 30min. The column was then washed for 10min with 500 mM sodium hydroxyde (NaOH) and equilibrated with water for 10min. Detection was done by post-column addition of 0.5M NaOH at 0.5mL/min.

Standards of D-glucose, D-xylose, L-arabinose, D-galactose and D-mannose were used for quantification. Fucose was added as an internal standard to all standards and samples.

2.6.5. Quantification of total protein content (Ninhydrin Assay)

The amount of total protein in solution from the adsorption studies was quantified using a ninhydrin assay using BSA as standard.

In 2 mL screw cap tubes, 40 µL of each sample was mixed with 60 µL of 13.5 M NaOH to denature the proteins, vortexed, and autoclaved at 121°C for 20 min. After cooling off until room temperature, 100 µL of glacial acetic acid (Sigma-Aldrich, USA) was added for neutralization, vortexed, and 200 µL of 2% ninhydrin solution (Sigma-Aldrich, USA) was pipetted and, after vortex, the tubes were placed into a SW22 Julabo water bath (Germany) at 98 °C for 20 min. After cooling down to room temperature, the samples were diluted with 1 mL of ethanol 50% (v/v), mixed well and 300 µL liquid was added to a Nunc™ Microwell™ 96-well microplate (Thermo Scientific,USA). The absorbance was measured at a wavelength of 570 nm in an Infinite M200 Tecan 96-well microplate reader (Switzerland).

The standards concentration range was 0.5, 0.4, 0.3, 0.2 and 0.1 mg_{BSA}/mL.

2.6.6. Modelling of Langmuir adsorption isotherms

Nonlinear curve-fitting to the experimental data was performed with the OriginPro® v.9.0 software (OriginLab, USA), according to the model described in section 1.4.

2.6.7. Statistical analysis

Experimental errors are expressed as standard deviations displayed as vertical error lines when presenting the experimental data.

Also, analysis of variance (ANOVA) was performed to the data collected after the adsorption studies of the Laccase treated residues. The software used was JMP® (SAS, USA).

3. Results and discussion

Prior to study the adsorption of enzymes to biorefinery lignin, several procedures were necessary to obtain the lignin fraction with the highest degree of purity possible. The goal was to avoid interferences in the results due to adsorption to carbohydrates.

3.1. Chemical composition of the Pretreated Biomass

As discussed before, the composition of feedstocks can vary due to several factors such as their origin and the applied pretreatment. Preceding the lignin isolation, a quantitative acid hydrolysis was performed to access the relative composition in macromolecular components of the nine feedstocks obtained. The chemical composition of the pretreated materials is presented in Table 8.

Since the scope of this research was not on the pretreatment and the pretreated material was prepared for general use, a detailed analysis of the mass balance and recovery of the sugars is not included in this report.

All the materials show a variation in the composition of the solids between the different severities of treatments, with a clear decrease in the hemicellulose fraction from the lowest to the highest severity pretreatment. This decrease was expected since the applied pretreatment is the downscale of the process described in the work of Larsen *et al.* [25], where it was proven the existence of C5 sugars and acetic acid in the liquid fraction of pretreated wheat straw.

In the present work, less 53.98% of hemicellulosic sugars were obtained in the solids of corn stover from the highest severity when compared to the lowest. This effect was also verified in the work of Saha *et al.* [53], that underlined the effect of time and temperature in hemicellulose hydrolysis in corn stover separately, and concluded that the increasing of these parameters led to an easier enzymatic hydrolysis of the materials. Also, at 200°C, they confirmed the presence of acetic acid as a consequence of the deacetylation, among some sugar degradation products. Yang & Wyman [76] also tested hydrothermal pretreatments in different conditions (batch and flowthrough), and verified that in both cases, with the increase of Log R_O, there was an increase in xylan removal.

The same reduction was verified in *Miscanthus* and in wheat straw. In the available literature regarding wheat straw, Hansen *et al.* [77,78] achieved the same results when studied the structural modifications in industrial pretreated wheat straw from Inbicon (as referred before, the upscale equivalent of mini-IBUS,

where the pretreatments of this work were performed), in similar conditions (temperatures of 180-195°C, with a residence time of 12 min), and concluded that the pretreated wheat straw was fragmented, with a reduction of the particle size into individual cells and, recurring to compositional and infrared analyses, they proved hemicellulose removal during the process. For a severity factor of 4.02, they obtained 6.5% (± 0.5), which is similar to the percentage obtained in this work for the severity factor of 3.97. Also, Holopainen-Mantila *et al.* [79] with a similar hydrothermal process, performed in a fixed bed flow-through reactor, tested different severities and observed that the process increased solubilization of arabinoxylan in a temperature dependent manner. They noticed an increase in the glucose content and establish a connection with the decreasing amount of arabinose and xylose.

The hydrolysis of hemicelluloses as monomers and small weight oligomers in hydrothermal pretreatments occurs since water behaves like an acid due to the effect of the high temperatures (180-230°C) and pressures (2.4-2.8 MPa). The auto-ionization of water generates hydronium ions (H_3O^+), leading to hydrolysis and the loss of acetyl groups from hemicelluloses, that can enhance the acid-catalyzed reactions [79,80].

Table 8 Chemical composition of the solids of the three feedstocks after a hydrothermal pretreatment with three different severities in cellulose (measured as glucan), hemicellulose (measured as xylan, arabinan, galactan and mannan) and lignin (Klason lignin and acid soluble lignin).

	Material	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Ash (%)
Corn Stover	**4CS**	55,86	16,10	23,96	4,08
	5CS	58,85	12,61	23,66	4,88
	6CS	67,12	7,41	21,75	3,72
Miscanthus	**8MS**	53,71	12,57	32,59	1,14
	9MS	56,29	9,02	33,12	1,56
	10MS	56,84	5,26	36,23	1,68
Wheat Straw	**14WS**	53,93	15,93	28,78	1,36
	15WS	57,68	10,67	30,53	1,12
	16WS	61,34	7,27	30,36	1,03

Since there is a difference in the hemicellulosic sugars present in the solids of different severities, a rearrangement of the contribution of each fraction occurs, in other words, the relative amount of compounds

that is not hydrolyzed should increase as a results of other compounds being removed. This fact was not verified in some materials regarding the lignin content. In corn stover, lignin decreased 0.3% between the 4CS and the 5CS, and less than 2.0% between 5CS and 6CS, which could be evidence that the increase of temperature and time had an effect in the corn stover lignin, occurring some hydrolysis, in this case just noticeable in the medium and highest severity treatment. In 6CS, the effect seems to be more pronounced, meaning that the increase of temperature had more effect in the lignin of corn stover when compared to time. These results were also verified in literature where, e.g., Yang *et al.* [81] study the formation of hydrophobic microspheres in hydrothermal pretreatments of corn stem rind, and believed to be constituted of lignin polymers, highlighting that this phenomenon occurs at temperatures above 120°C and that the spheres can be detached or adsorbed into the solid, depending on temperature. These droplets seem to vary their surface, which led the researchers to believe that can exist a combination of lignin and carbohydrates in these microspheres formed [82].

In *Miscanthus,* an increase in the lignin percentage is noticed as the severity increases which could indicate that there was no removal of lignin due to the increase in the severity of the pretreatment, being just a consequence of the rearrangement of the contribution as referred above. A study performed also with a hydrothermal pretreatment, with a higher severity value (LogRo 4.1), observed that little cellulose and lignin degradation was caused during the process, which complies with the results of the present work [83].

In the wheat straw materials, the lignin content increases less than 1.0% from the 14WS to the 15WS and 16WS, since the values of these two materials are very similar (30.5% and 30.36%, respectively). As it was observed in the corn stover, the increase of the temperature was responsible for the decrease in the lignin content, while time did not had any effect since the amount of lignin is probably the same in 14WS and 15WS. These values support the hypothesis that during pretreatment of both corn stover and wheat straw the temperature exceeded the melting point of lignin (depending on the composition, from 120–200°C) and could have escaped from the cell wall matrix. Upon cooling, the lignin can deposit as droplets onto the remaining solid, that presents just a small difference in the content of the polymer. In the wheat straw case, Hansen et al. [77], with scanning electron microscopy (SEM), observed black lignin dots extracted during pretreatment and deposited during cooling, thus becoming increasingly concentrated on the surface.

The cellulose content of the solids after pretreatment, measured as glucan, is similar in each of the pretreated materials. Although there is a slight increase as the severity degree increases, it is related with the change in the proportions of the composition.

3.2. Lignin Isolation

To remove the carbohydrates, both the remaining hemicellulose and cellulose, an enzymatic hydrolysis was performed with a high enzyme loading of a commercial enzyme cocktail for 72h. To ensure a high hydrolysis rate and prevent product inhibition, a recurring removal and addition of fresh buffer and enzyme every 24h was done. From this process, lignin-rich EnzHR were obtained.

Taking into account that some of the enzymes could have adsorbed during this process to the materials, an additional treatment was needed to remove any proteins which could interfere with the subsequent studies by decreasing the binding affinity and by, probably, blocking the binding sites of the protein's catalytic domain. In literature, is common to find protease treatments that can be effective in digesting the bound enzymes, thus reducing the nitrogen content of the lignin-rich residues [84,85]. A protease treatment was applied to the EnzHR and an elemental analysis was performed on the pretreated biomass, on the EnzHR and EnzHR-P to obtain the corresponding nitrogen content.

3.2.1. Protease treatment

Protease removal has been previously performed using different conditions and different enzymes [86]. In the present work, in order to minimize any modification in the chemical and physical composition of the materials for the adsorption studies, it was imperative that the operational conditions were as similar as possible as the conditions of the enzymatic hydrolysis process. This treatment was performed in an orbital shaker at 50°C for 24h with the protease from *Bacillus licheniformis*, which, according to the manufacturer information, is stable at this specific temperature. After 24h it is expected that the amount of protein bound to the enzymatic residues has decreased as a result of the hydrolysis of the peptide bonds by the proteases. After centrifugation and disposing of the supernatant, a three-step wash with acidic water (pH 2.5) was performed to denature and remove the enzymes. The washing step is relevant for the reason that, if it is not efficient, some of the proteases can remain in the solids and influence the results of the adsorption studies. To ensure that no protease activity is retained in the residues, an additional denaturing step was performed. After the 24h, the samples were placed in a water bath at 100°C for 20 min, before centrifugation and the three-step wash. An elemental analysis of the EnzHR-P was performed in order to confirm the reduction in nitrogen content (Table 9).

From the obtained results, comparing to the pretreated materials, it was possible to observe that the nitrogen content increased significantly after the enzymatic hydrolysis, confirming the presence of adsorbed

enzymes to the EnzHR. This is consequence of the expected binding of the cellulases and hemicellulases from the CEM to the lignin-rich residues during the hydrolysis process.

Table 9 Nitrogen content of the solids obtained after pretreatment, enzymatic hydrolysis (EnzHR) and protease treatment (EnzHR-P).

%N	Pretreated Biomass	EnzHR	EnzHR-P
4CS	1.08 ± 0.11	2.64 ± 0.04	0.94 ± 0.08
5CS	1.06 ± 0.19	-	0.81 ± 0.09
6CS	0.84 ± 0.10	-	0.99 ± 0.08
8MS	1.22 ± 0.13	2.08 ± 0.24	0.59 ± 0.00
9MS	0.97 ± 0.04	-	0.73 ± 0.01
10MS	0.96 ± 0.05	-	0.93 ± 0.01
14WS	1.16 ± 0.18	2.47 ± 0.27	0.81 ± 0.14
15WS	0.81 ± 0.07	-	0.82 ± 0.01
16WS	0.84 ± 0.04	-	0.88 ± 0.04

After the protease treatment, a decrease in the nitrogen content is observed in the EnzHR-P, proving the efficiency of the treatment in removing the bound proteins. However, it can be noticed that some values are lower than the ones obtained for the initial materials, which can be related with the fact that the pretreated materials were not washed after the pretreatment, still carrying some soluble protein at the time of the elemental analysis.

3.2.2. Composition of the Enzymatic Hydrolysis Residues after Protease treatment (EnzHR-P)

To establish the effects of the severity of the pretreatments in the composition, the lignin-rich solids were subjected to a strong acid hydrolysis. This information was necessary to, afterwards, understand the relation between the residues composition and the enzymes' adsorption. The results obtained for the EnzHR-P of each feedstock are presented in Figure 13 to Figure 15.

As expected, all the EnzHR-P materials have high lignin content (over 57%), but did not reach the expected purity (around 90%). In the three feedstocks the tendency observed is the same, the higher the severity of the pretreatment, higher the lignin and lower the carbohydrates content. Comparing the pretreated materials with the EnzHR-P, it can be considered that the pretreatment and the enzymatic hydrolysis did not have a significant effect on lignin removal. On the opposite side, glucan content decreased considerably, being

possible to observe a decrease within materials with the increase of the severity of the treatment. As observed in section 3.1, this could be related with the physical disruption of lignocellulose during pretreatment, where a significant part of the hemicellulose was hydrolyzed, leaving the cellulose more exposed to the action of the enzymes, consequently, achieving higher digestibility [49].

From observing the results from each material, corn stover EnzHR-P has the highest ash content (8.00-12.0%) (Figure 13). This is probably due to the contaminant particles found in the raw biomass, mainly sands, from the harvest in the field that were not removed before pretreatment. Before enzymatic hydrolysis, the washing was also not performed, as above referred. During enzymatic hydrolysis, when the enzyme solution was replaced, part of the sand particles were removed, but a visible portion still remained.

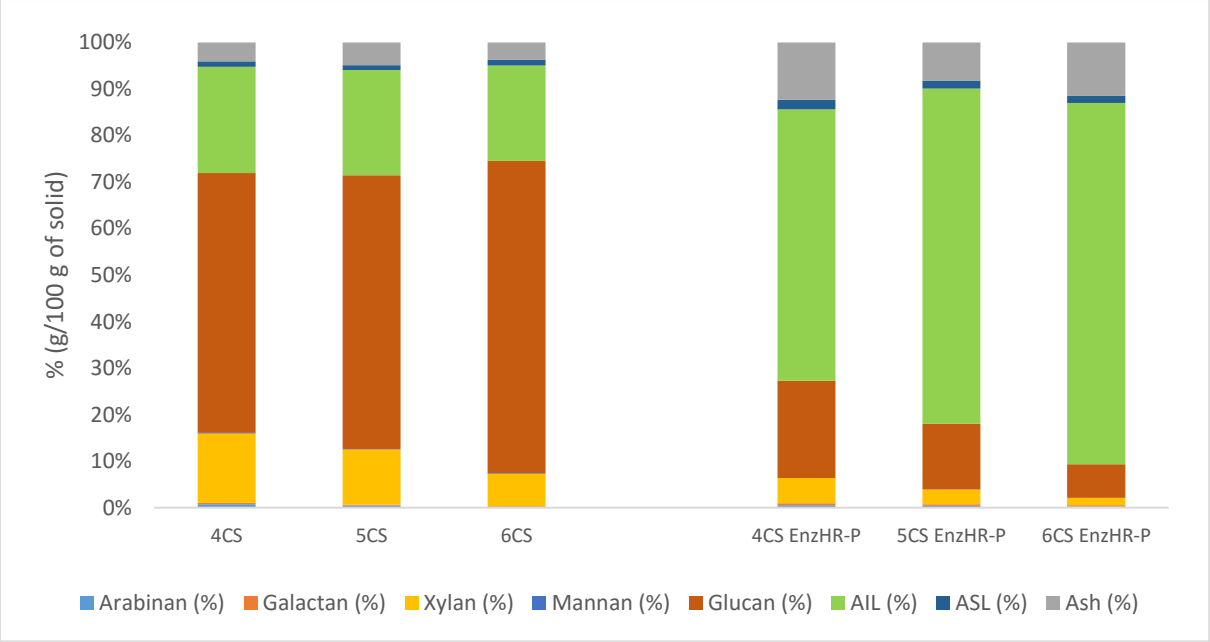

Figure 13 Chemical composition of the corn stover materials: on the right side the composition of the residues from the three pretreatment severities; and on the left side the composition of the corresponding EnzHR-P.

Miscanthus EnzHR-P (Figure 14) remained with the highest percentage of carbohydrates (39.6% for 8MS against 13.6% for 10MS). Since *Miscanthus* has more lignin in its composition, probably the sugars are not so available as in wheat straw or in corn stover, thus the higher amount of carbohydrates still available in the solids after the treatment.

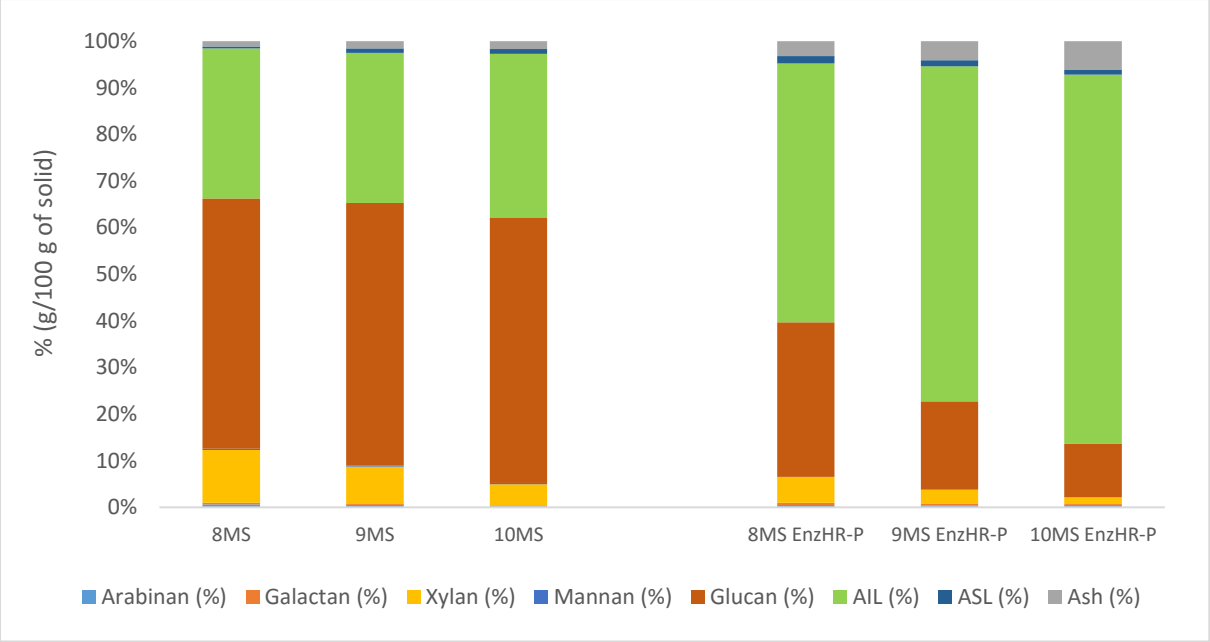

Figure 14 Chemical composition of the Miscanthus' materials: on the right side the composition of the residues from the three pretreatment severities; and on the left side the composition of the corresponding EnzHR-P.

The highest carbohydrate removal was observed in the wheat straw (Figure 15). A reduction of 75.1%, 86.7% and 91.4% of glucan content for EnzHR-P 14WS, 15WS and 16WS was obtained, a little higher than in other studies, e.g., Rodríguez-Zúñiga *et al.* [80] achieved 80.0% saccharification with a different CEM and lytic polysaccharide monooxygenase (LPMO) with wheat straw, hydrothermally pretreated with the lowest severity condition, but with a solid loading of 15.0%.

The final goal of achieving lignin-rich residue with over 90.0% purity was not accomplished, although 87.3% lignin content was achieved in 16WS. The amount of lignin did not vary much in this feedstock between severities, while in the other two, the difference is more visible. This could be related to the fact that *Miscanthus*, as above referred, has a highest lignin content in the pretreated materials. In corn stover, it can be derived from the fact that this feedstock is composed of more different parts of the plant, some being highly recalcitrant, not being so easily available for enzymes. Another relevant factor is the fact that the applied pretreatment was optimized for the wheat straw biomass, thus accomplishing higher yields for the removal of carbohydrates.

Since the composition of the enzymatic cocktail is unknown, besides cellulases, it can be inferred that some hemicellulases are also present since the xylan, arabinan and mannan content also decreased in the solids. In this analysis, concerning the EnzHR-P, the amount of mannan was considered null (the values obtained from the HPC analysis of the supernatants of the composition analysis were negligible).

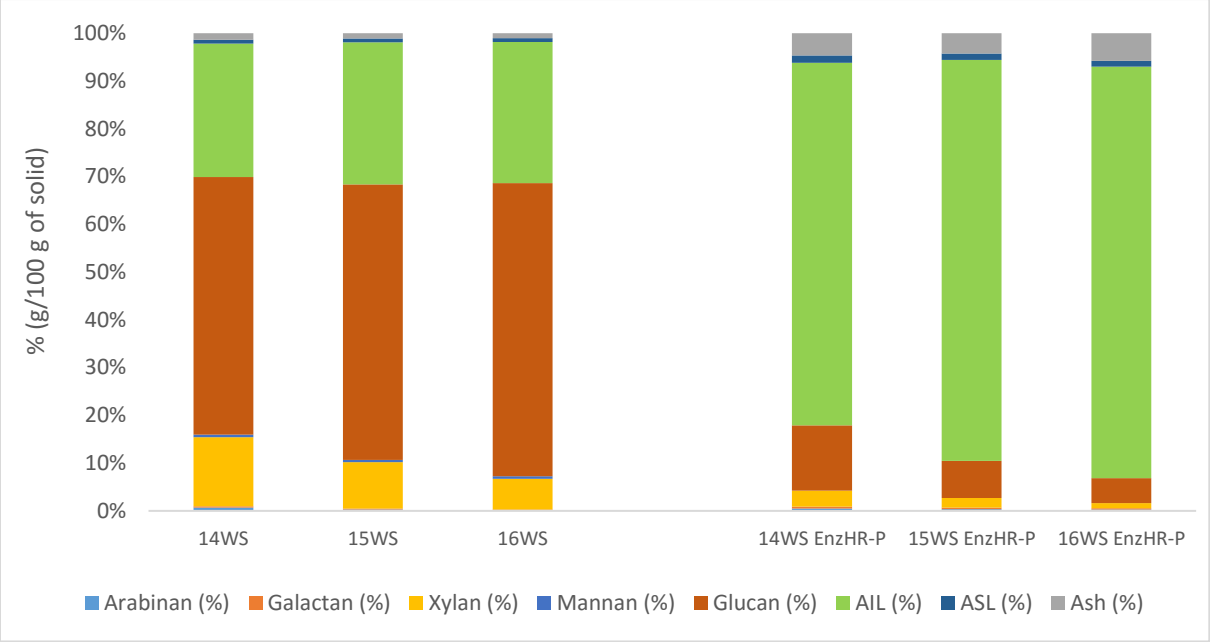

Figure 15 Chemical composition of the wheat straw materials: on the right side the composition of the residues from the three pretreatment severities; and on the left side the composition of the corresponding EnzHR-P.

Chemical composition influences the action of enzymes has seen above. However, it is important to note that the architecture of the cell wall matrix, which varies between plant species, tissues and plant maturity, can be affected by the pretreatment differently, influencing the affinity of cellulolytic enzymes. This is relevant when we work with feedstocks that are composed of different parts of the plants, such as agriculture residues (wheat straw, for example, is composed by more than 80% (w/w) of stems, in average, being the remaining leaves and nodes) [78].

3.3. Adsorption studies

The enzymatic hydrolysis process, used massively in biorefinery industries, depends on the different conditions which influence enzyme activity. One of the problems associated with this process, as referred, is the non-productive binding of the enzymes to the biomass, leading to a lower yield and to higher associated costs [60]. Some literature refers the lignin portion of the lignocellulosic materials as responsible for this inhibitory effect [68,85].

The main purpose of this work was to observe, and try to understand, the effects of the composition of different feedstocks, pretreated with three severities, on protein adsorption, namely BSA, CEM and Laccase.

Posteriorly, a Laccase treatment was performed to understand if these proteins can influence enzyme binding by modifying lignin.

The experiences were performed at 50ºC, simulating the enzymatic hydrolysis process, and they were performed for 2 hours since it is believed (and it is demonstrated in literature) that there is a decrease in the hydrolysis rate in this process after 10-60 minutes, effect attributed to the carbohydrates hydrolysis that are more available in the substrates [66].

Except for 4CS, 8MS and 14WS, all of the adsorption studies were performed in duplicate, with 7 points in a range of concentrations between 0.1 - 5.0 mg_{enzyme}/mL for CEM, and 0.2 - 10.0 mg_{enzyme}/mL for BSA and Laccase. A higher range of BSA concentrations was used due to the highest adsorption of this protein into the residues observed in preliminary tests. Regarding CEM, the chosen range for the study was smaller due to the high viscosity of the solution. Later in the work, it was also decided to test the adsorption of laccases to the residues, in order to understand if it can also bind to lignin and, although the original solution was also viscous, it was possible to dilute it and use the same range of concentrations as for BSA without compromising the results.

After the incubation period, the samples were centrifuged in order to separate the solids from the free enzymes in the supernatant. The protein content of the liquid was determined after hydrolysis of the proteins into amino acids by a ninhydrin assay, using BSA as a standard for the calibration curve of each experiment. The amount of protein adsorbed was calculated by difference between the ninhydrin measurement of the enzyme controls of each protein at the 7 concentrations and the measurements from the supernatants (see appendix). The ninhydrin assay was chosen since it is a spectrophotometric test that quantifies the total amount of amino acids that has been previously used for similar studies [87]. On the contrary to other methods, as for example the BCA assay, it does not suffer interferences in the readings derived from certain compounds such as reducing sugars in solution [88].

Adsorption isotherm curves were obtained for each EnzHR- P, representing the amount of enzyme adsorbed in the residues ($mg_{protein}/mg_{EnzHR-P}$) with the corresponding concentration of protein in solution added ($mg_{protein}$/mL), for each of the three proteins in study. In these experiments, it was expected to observe an equilibrium between the species in contact with the surface of the absorbent material and the species free in solution. Graphically, this is translated into a curve that reaches a plateau at a certain concentration, representative of the saturation point of the adsorbent's surface.

3.3.1. Effect of the composition of the pretreated feedstock

In the graphic representation of the results for the lowest severity pretreatment residues, it can be observed that the adsorption profile is different for each protein (Figure 16 to Figure 18). The described shape for Langmuir isotherms was only obtained for BSA (Figure 16). The three feedstocks seemed to follow the same trend by reaching a plateau, showing a possible equilibrium between adsorbed and desorbed proteins. However, 14WS has a clear difference and adsorbed more 41.1% than CS, and 32.5% than MS, probably due to the higher lignin content which can be associated with more surface area where BSA can bind. This hypothesis is supported also by the fact that CS and MS have nearly the same amount of lignin in their composition, and the isotherms have a very similar shape and reach a very close plateau.

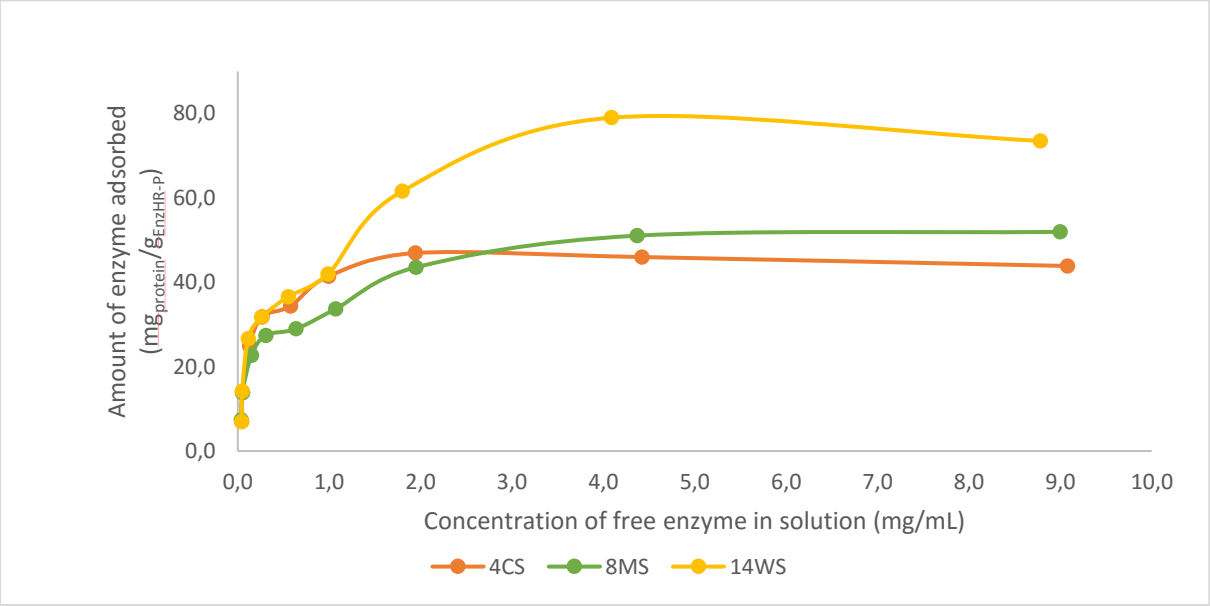

Figure 16 BSA adsorption isotherms obtained from the experimental data for the lowest severity EnzHR-P: 4CS; 8MS; 14WS.

Concerning the graphic representation for CEM (Figure 17), the expected isotherms were not obtained. Instead of a plateau or a positive slope, there is one point that shows a decrease in the adsorption at the second higher enzyme concentration studied. This can be related with the composition of the cocktail, where different enzymes in different proportions are found in the mixture (different cellulases and hemicellulases as referred after the observation of the enzymatic hydrolysis residues composition) that could have been binding at the same time, or competing for the same sites. Another assumption could be associated with the range of concentrations since the substrates did not seem to have reached a saturation point, which indicates that probably higher CEM concentrations could have been supported by the substrates.

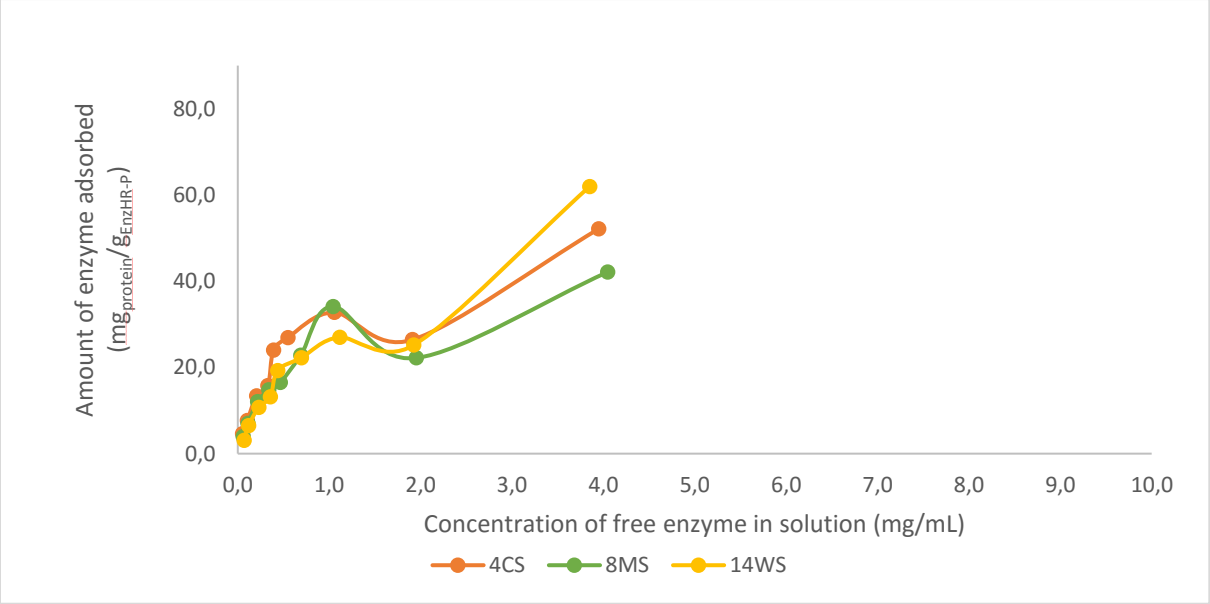

Figure 17 CEM adsorption isotherms obtained from the experimental data for the lowest severity EnzHR-P: 4CS; 8MS; 14WS

Proceeding with the observations, the three substrates seem to follow the same adsorption trend, except for the last point. It is known that both cellulases and hemicellulases that constitute the CEM bind to the respective polysaccharide, and also the relative amount of hemicellulose and cellulose that remain in the residues is not the same. This could lead to thinking that 14WS EnzHr-P had the highest amount of carbohydrates from the three residues, but, as observed in section 3.2.2, it is quite the opposite since the *Miscanthus* EnzHR-P is the residue with higher carbohydrate content and wheat straw EnzRH-P has the lowest. Such conflict in the adsorption behavior can be explained by the premise that these enzymes can bind and be retained by lignin.

The Laccase adsorption study relative to the lowest severity materials was performed with a lower range (Figure 18). As it can be observed, the curves seem to be increasing which confirms that the used range was not enough to reach saturation. Also, the three feedstocks seem to be following the same trend with no significant differences.

Relative to Figure 19, it is necessary to refer that the point of higher concentration stands out, probably due to an experimental error. For a better comparison the graphics have all the same scale which, due to the referred point, can be misleading by giving the impression that the increase of adsorption with the increment of concentration observed is not as prominent as it is in reality.

As observe before, higher the severity, lower the carbohydrates fraction and higher the lignin content in the EnzHR-P. Considering the curves in for BSA (Figure 19) and CEM (Figure 20) for the medium severity residues, there seems to be a constant increase and there is a display of a higher amount of protein being adsorbed for the same concentrations, which is consistent with the results where a higher amount of lignin

led to an increase in the amount of protein adsorbed. Additionally, it is visible the overlapping of the three adsorption isotherms of the different feedstocks regarding the three different enzymes. This can be related with the range of concentrations being short, not having enough protein that could bind to the different fractions of the substrates, and thus not exposing the related differences in the adsorption process, or the composition of the three residues is similar enough not to display major differences in the adsorption.

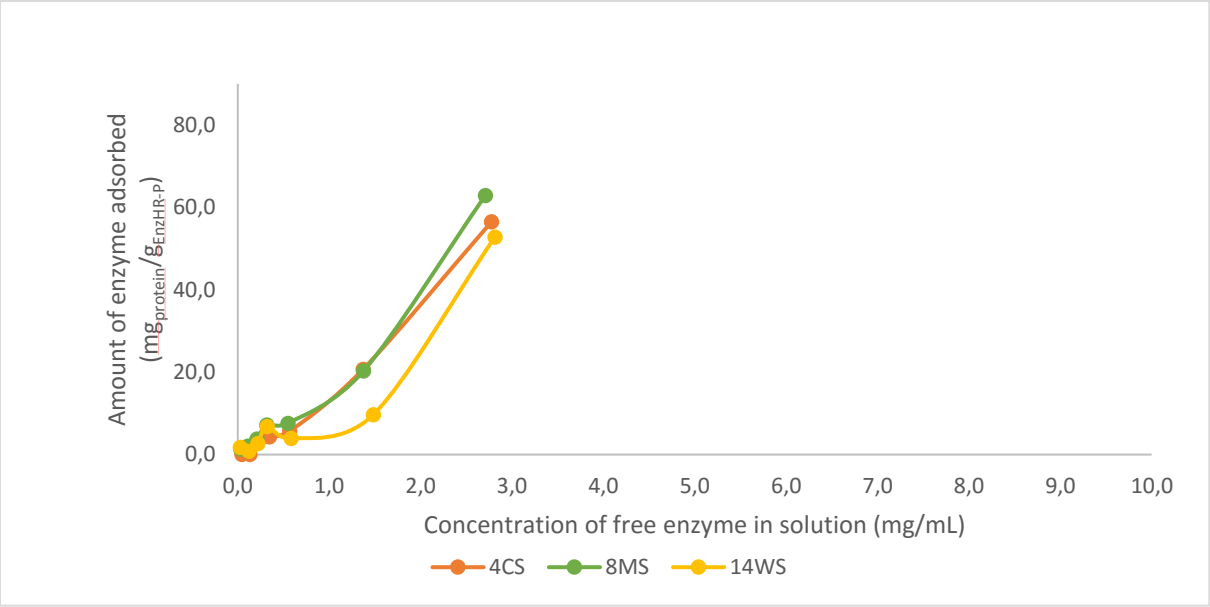

Figure 18 Laccase adsorption isotherms obtained from the experimental data for the lowest severity EnzHR-P: 4CS; 8MS; 14WS.

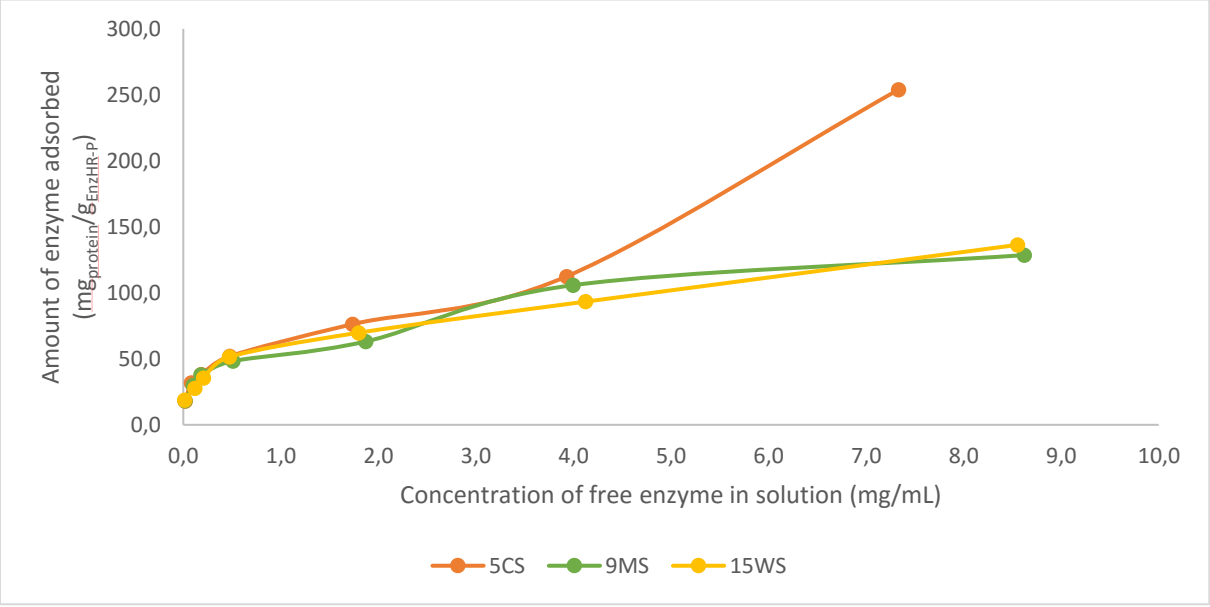

Figure 19 BSA adsorption isotherms obtained from the experimental data for the medium severity EnzHR-P: 5CS; 9MS; 15WS.

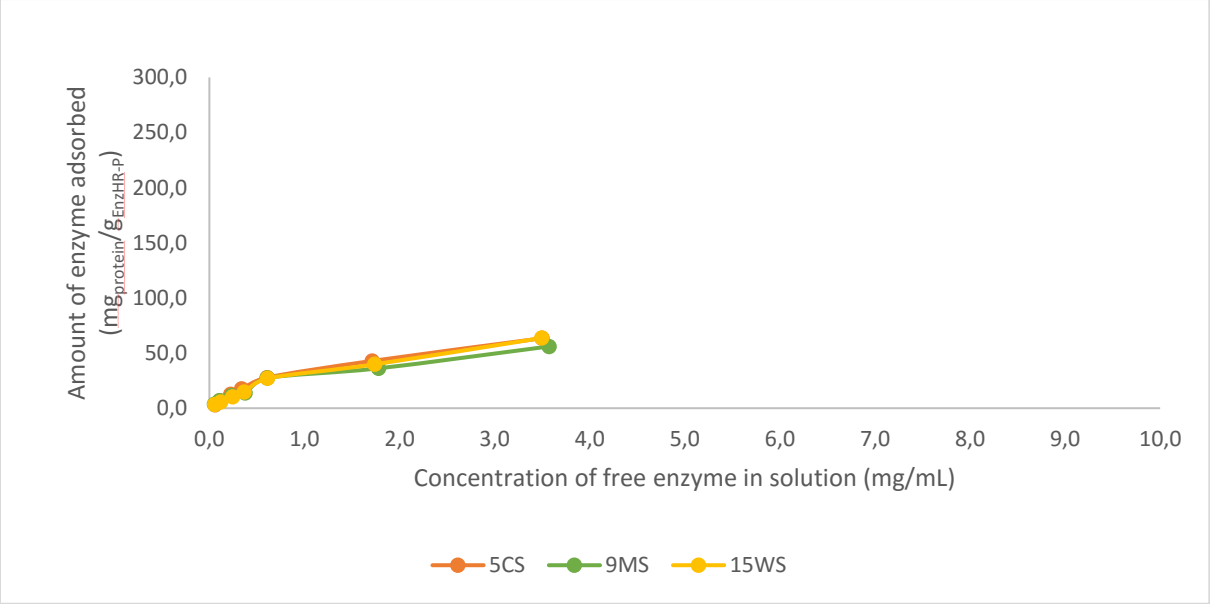

Figure 20 CEM adsorption isotherms obtained from the experimental data for the medium severity EnzHR-P: 5CS; 9MS; 15WS.

Relative to adsorption of Laccase in the medium severity residues, the increase is also noticed but there is a decrease in the amount of enzyme adsorbed for each concentration (Figure 21).

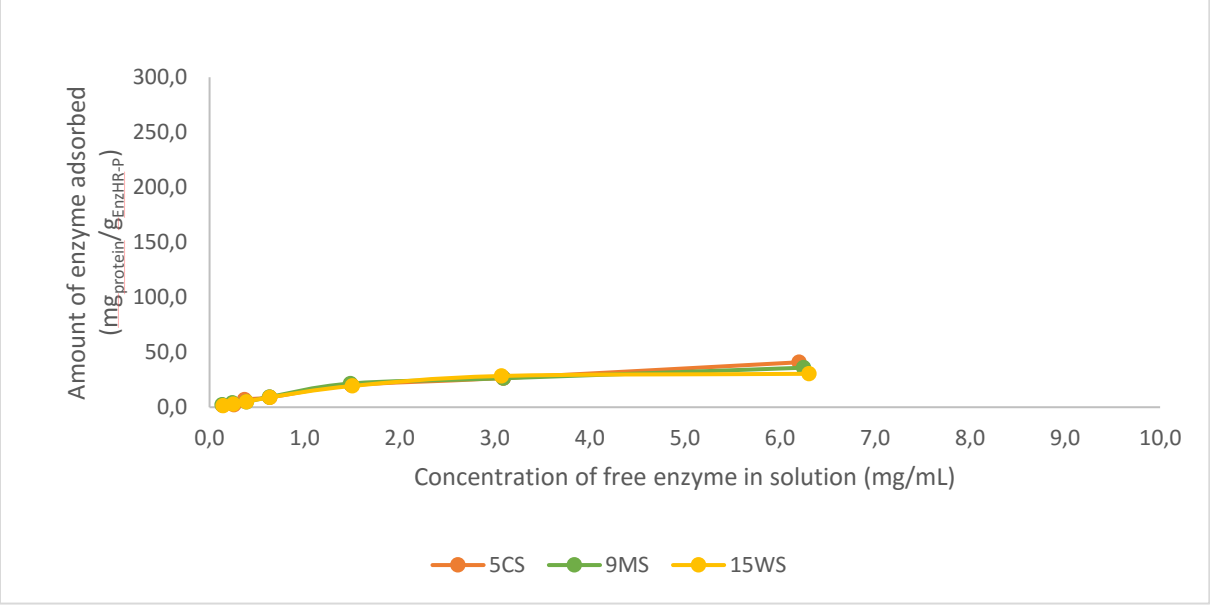

Figure 21 Laccase adsorption isotherms obtained from the experimental data for the medium severity EnzHR-P: 5CS; 9MS; 15WS.

Concerning the highest severity residues, the observed tendency for the CEM is the same as the obtained in the medium severity residues (increasing adsorption with increasing enzyme solution concentration), while there is clear differences in the BSA and Laccase isotherms.

Looking more closely to the BSA chart (Figure 22), the curves of the three EnzHR-P seem to follow the same trend except for the point of highest concentration. However, it is necessary to highlight that the corresponding point of the 10MS is associated with an error relative to the blank of the residues used in the adsorption study (the respective duplicates presented a higher amount of protein in the supernatant than usual, possibly related with some kind of contamination), leading to the false idea that a higher amount of enzyme was adsorbed. In reality, the point should be very close to the point of higher concentration of 6CS. Taking this into account, the adsorption of BSA in corn stover and *Miscanthus* is very similar.

BSA adsorption seems to reach a plateau in the wheat straw EnzHR-P (16WS), while still increases in the corn stover and in the *Miscanthus* residues. This could be explained by the composition of the materials and the effect of the severity of the pretreatment. As described in section 3.2.2, the lignin content in the wheat straw is higher than in the other two feedstocks and is similar between pretreatment severities (11.3% of difference between the lowest and the highest severity). This explains the higher adsorption of BSA in the lowest and medium severity residues (not accounting for the anomalous value of 5CS previously referred). However, in the highest severity morphological and structural changes could have occurred during pretreatment, explaining why BSA bonded less and reached an apparent saturation. On the contrary, a considerable increase is noticeable in the lignin content of corn stover and *Miscanthus* between severities but, at the same time, the residues from these two feedstocks have the highest relative amounts of carbohydrates, which can explain not reaching a plateau in the adsorption with these concentrations. It can also explain the lower amount of protein adsorbed at each concentration when compared with the residues from medium severity.

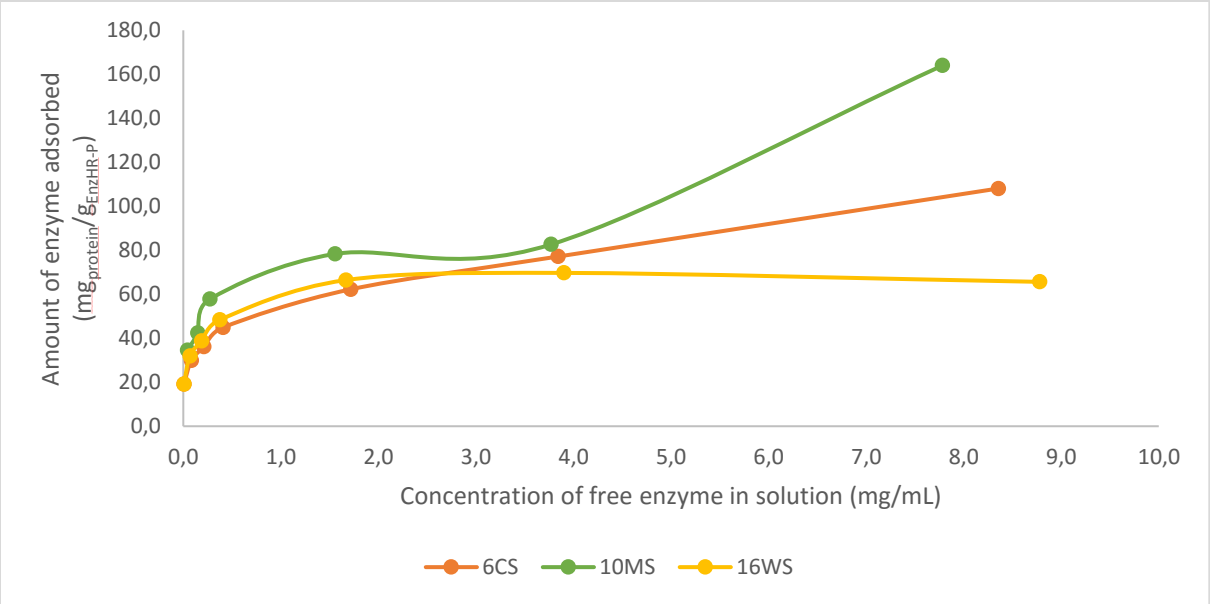

Figure 22 BSA adsorption isotherms obtained from the experimental data for the highest severity EnzHR-P: 6CS; 10MS; 16WS.

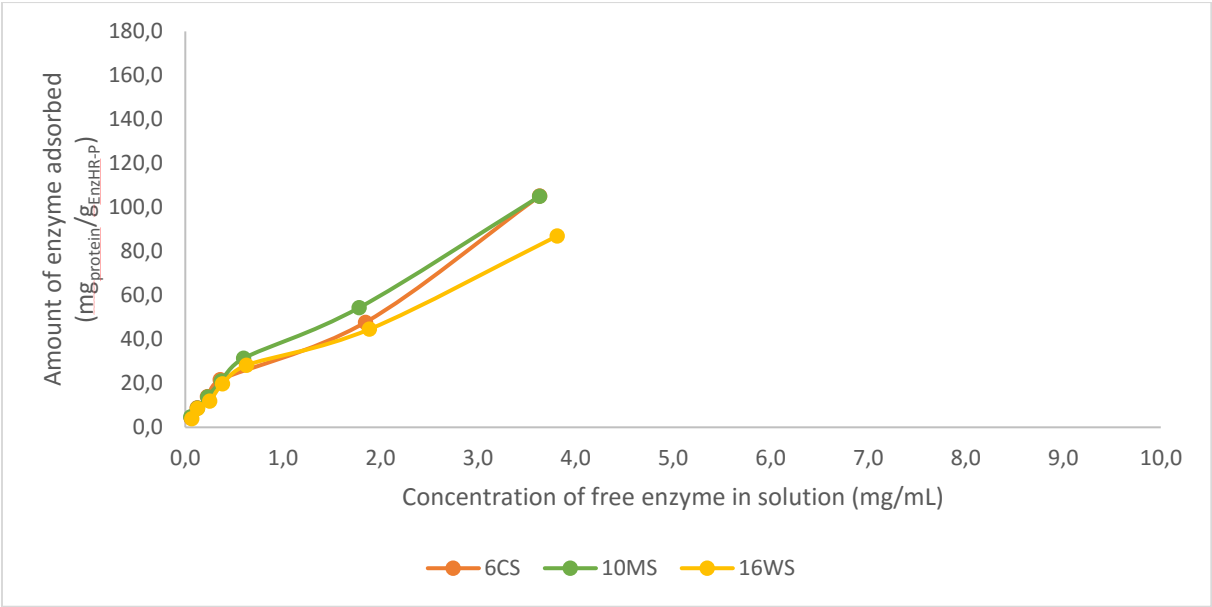

Figure 23 CEM adsorption isotherms obtained from the experimental data for the highest severity EnzHR-P: 6CS; 10MS; 16WS.

In the adsorption of Laccase (Figure 24), the binding of the enzymes in the three materials seem to have the same behavior although wheat straw adsorbed less. Again it could be related with modifications in the lignin derived from the severity of the pretreatment.

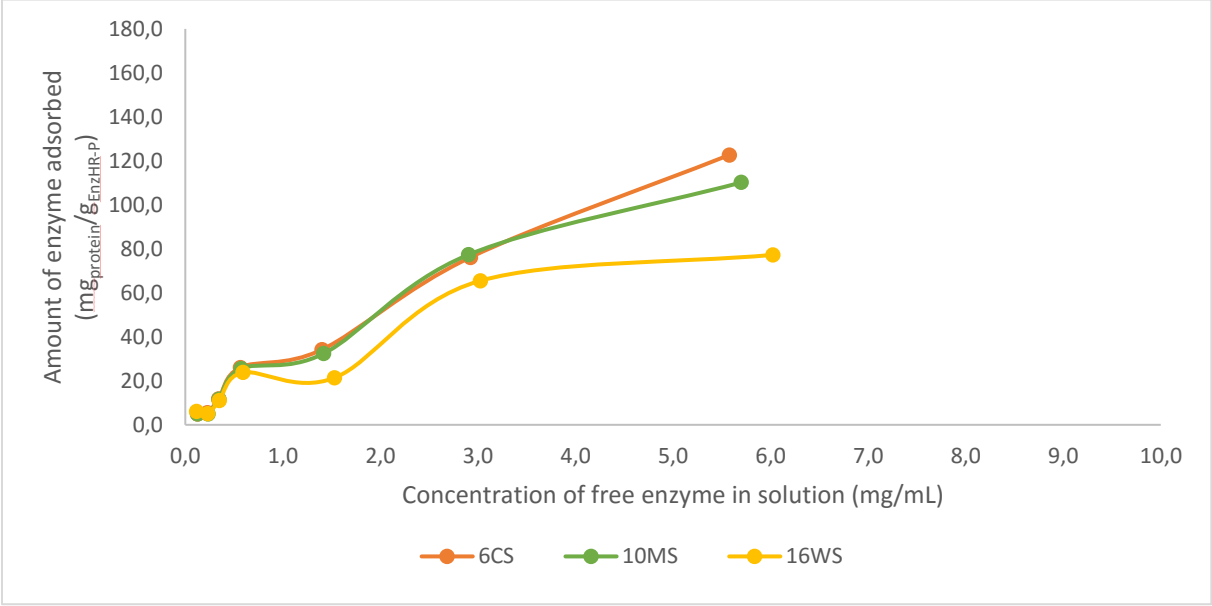

Figure 24 Laccase adsorption isotherms obtained from the experimental data for the highest severity EnzHR-P: 6CS; 10MS; 16WS.

From the figures above, it is possible to report a tendency for the proteins to have a more similar adsorption behavior as the severity of the pretreatment increases. The trend of the isotherms of the three proteins seemed to become more alike, and in the case of *Miscanthus* and corn stover, they almost overlapped, probably due to the composition of the two materials being very similar. A slight difference was observed in the adsorption behavior in the wheat straw, probably related with modifications caused by the severity of the pretreatments in the lignin's structure, as previously stated.

3.3.2. Effect of the severity of the pretreatment

Although the effect of the severity of the pretreatment is closely related with the effect of the composition of the feedstock, it is important to observe the result from this perspective in order to determine which pretreatment contributed to a lower adsorption of enzymes in each feedstock.

Regarding the adsorption of BSA (Figure 25 to Figure 27), the higher values of protein adsorbed were obtained with the medium severity EnzHR-P of the three feedstocks (128.6 $mg_{protein}/g_{EnzHR-P}$ for 9MS; and 136.3 $mg_{protein}/g_{EnzHR-P}$ for 15WS). The materials that adsorbed less were the lowest severity residues, with the exception of wheat straw where the highest severity residues had a lower amount of protein adsorbed, although with very similar results. Regarding the total amount of protein adsorbed in these residues, due to the higher lignin content, the wheat straw EnzHR-P adsorbed more (65.6 $mg_{protein}/g_{EnzHR-P}$) when compared to corn stover (43.9 $mg_{protein}/g_{EnzHR-P}$) and *Miscanthus* (52.0 $mg_{protein}/g_{EnzHR-P}$).

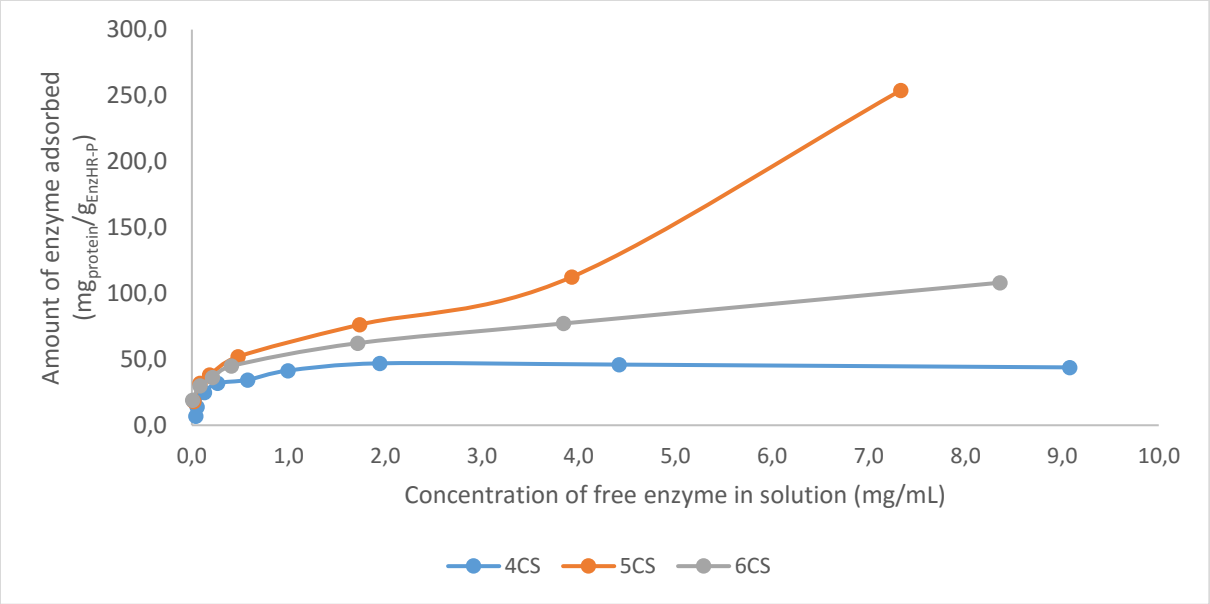

Figure 25 BSA adsorption isotherms obtained from the experimental data for the EnzHR-P of corn stover: 4CS; 5CS; 6CS.

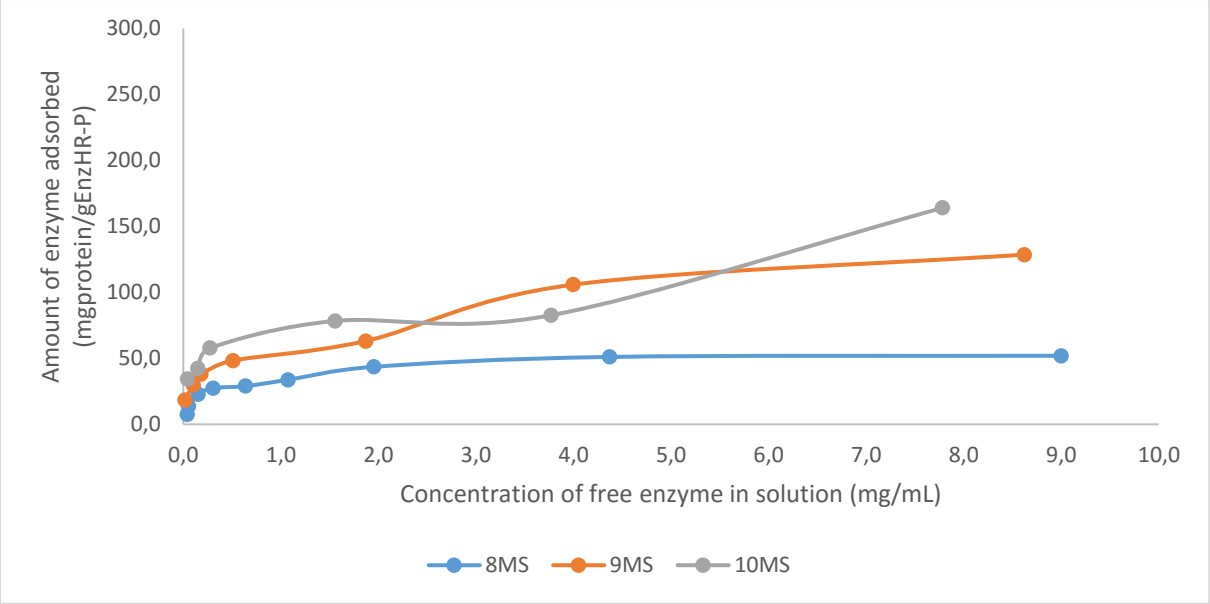

Figure 26 BSA adsorption isotherms obtained from the experimental data for the EnzHR-P of Miscanthus: 8MS; 9MS; 10MS.

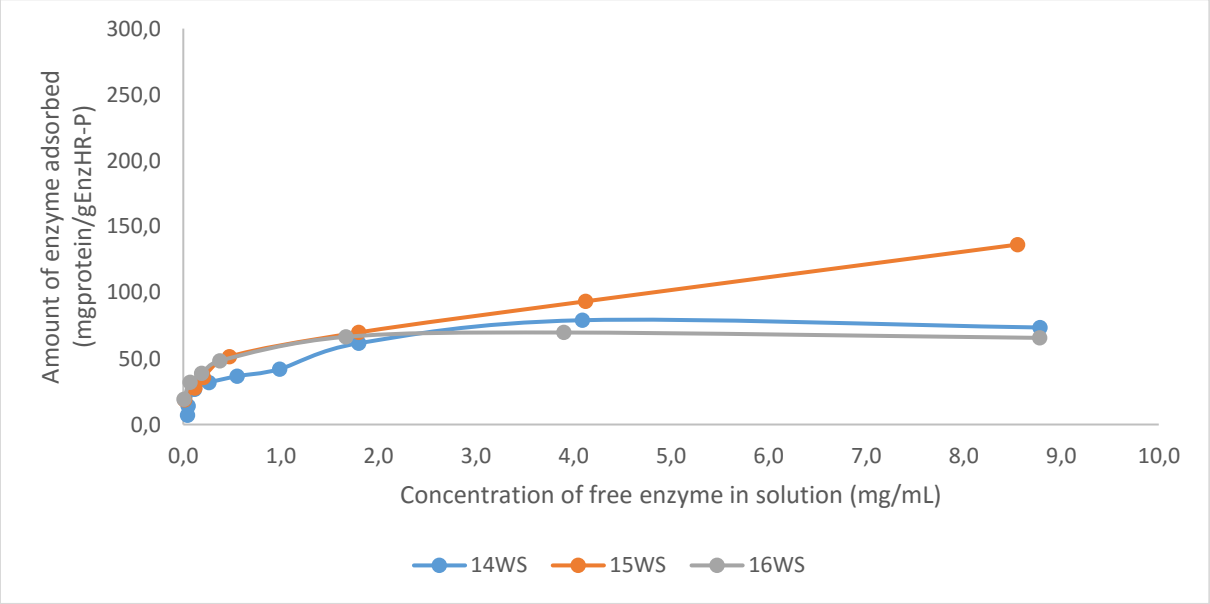

Figure 27 BSA adsorption isotherms obtained from the experimental data for the EnzHR-P of wheat straw: 14WS; 15WS; 16WS.

As it has been mentioned before, the composition of CEM is unknown, however the adsorption isotherms obtained present a very similar profile between feedstocks, never reaching a visible plateau, but decreasing the adsorption capacity after 0.6 mg_{CEM}/mL in the three feedstocks. From Figure 28 to Figure 30 it is possible to observe that the highest adsorption values were obtained in all the materials pretreated with the highest severity, namely, the materials with higher lignin content (105.1 $mg_{enzyme}/g_{EnzHR-P}$ for 6CS; and 87.0

$mg_{enzyme}/g_{EnzHR-P}$ for 16WS). This confirms the inversely proportional relation observed in the binding of cellulolytic enzymes used in enzymatic hydrolysis and the amount of carbohydrates present: the lower the amount of carbohydrates, higher the adsorption of proteins to the substrates, most likely due to unproductive binding to a greater surface of lignin. Another explanation could be related with the structure of the highest severity residues being more complicated, and an entrapment of the enzymes can occur translated into higher adsorption values.

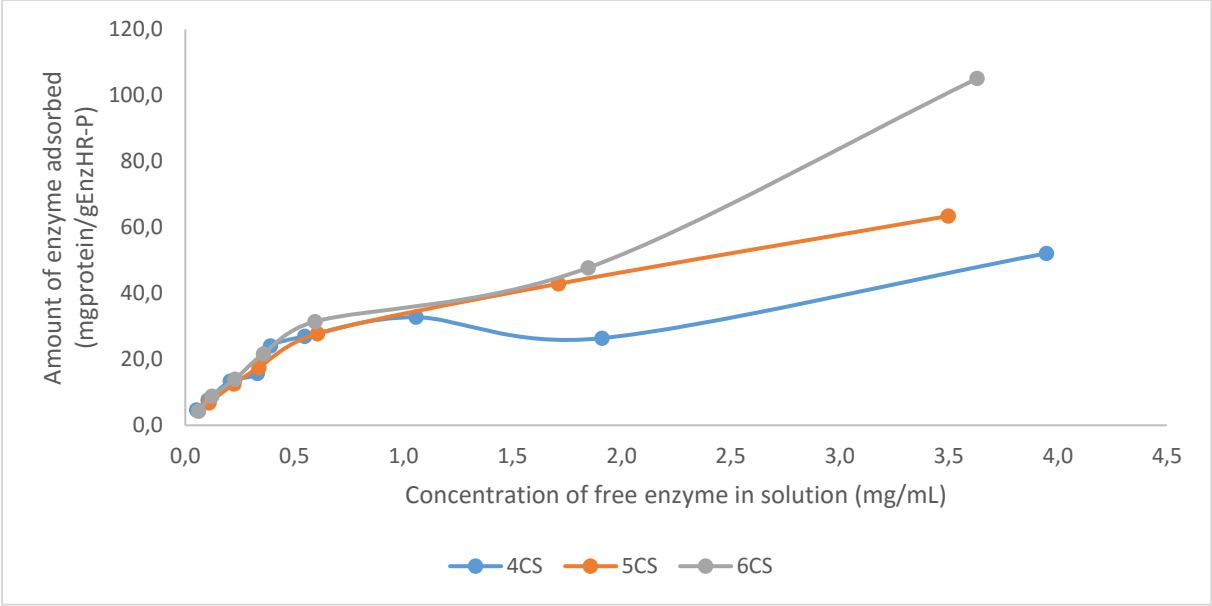

Figure 28 CEM adsorption isotherms obtained from the experimental data for the EnzHR-P of corn stover: 4CS; 5CS; 6CS.

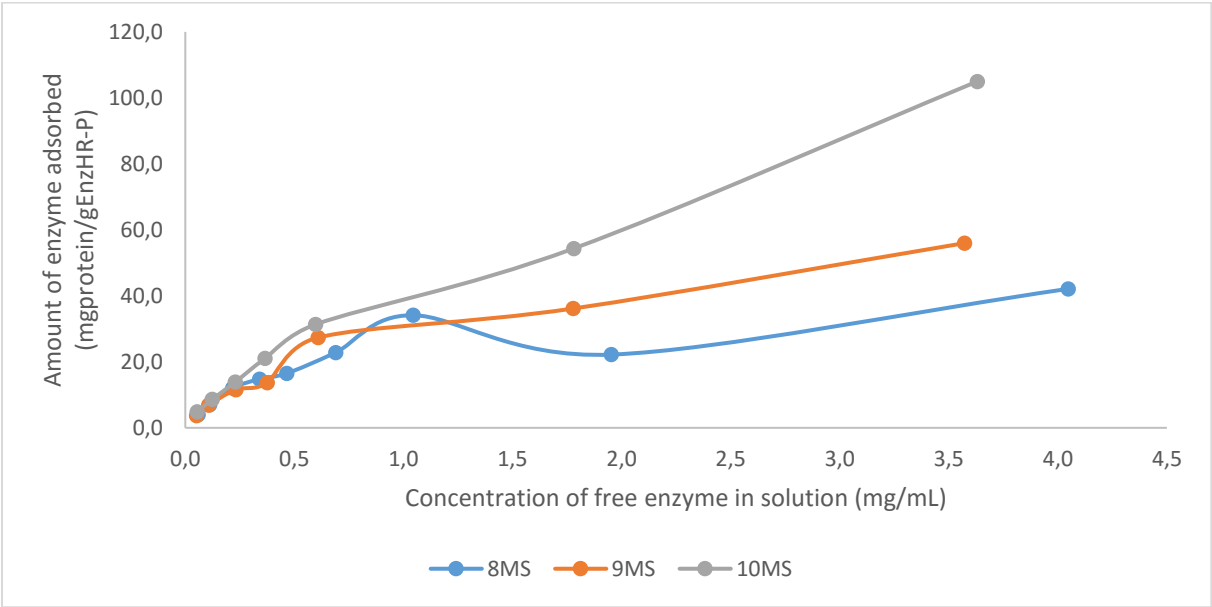

Figure 29 CEM adsorption isotherms obtained from the experimental data for the EnzHR-P of Miscanthus: 8MS; 9MS; 10MS.

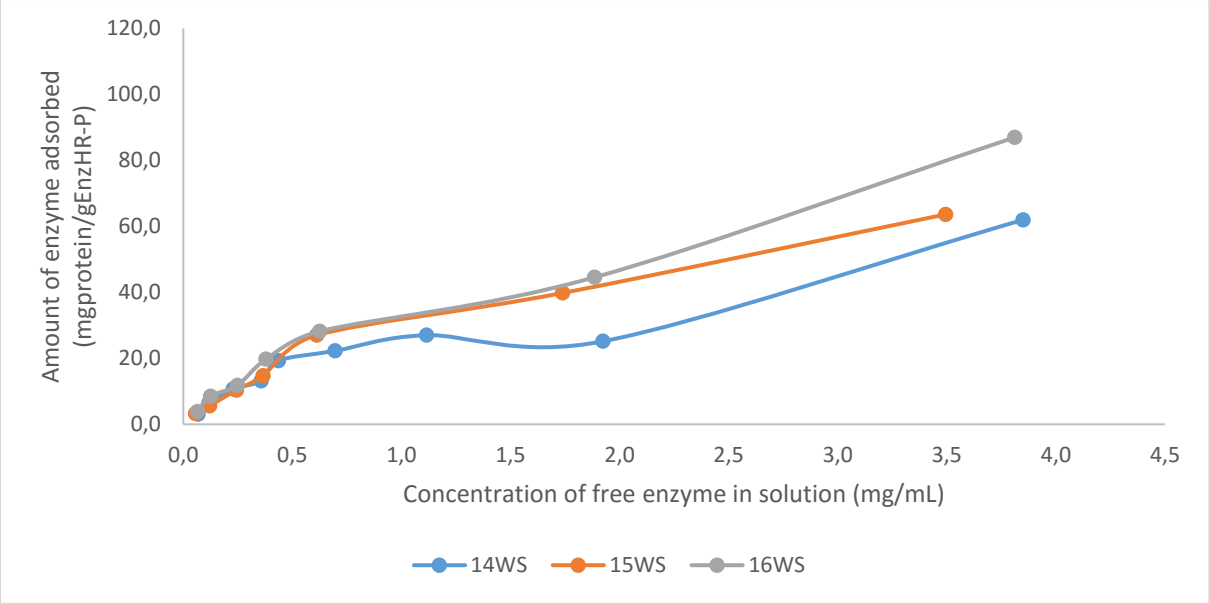

Figure 30 CEM adsorption isotherms obtained from the experimental data for the EnzHR-P of wheat straw: 14WS; 15WS; 16WS.

The lowest values of bound enzymes from CEM were surprisingly obtained for the lowest severity EnzHR-P, where there is the highest carbohydrate fraction where these enzymes are expected to adsorb. However, this observation can indicate that the enzymes present in the CEM could have been modify in order not to adsorb to carbohydrates unproductively. The lowest adsorption values of CEM were obtained for the *Miscanthus* 8MS EnzHR-P (42.1 $mg_{enzyme}/g_{EnzHR-P}$).

The adsorption of Laccase showed that more enzymes bound to the highest severity EnzHR-P of the three feedstocks, and bound less to the medium severity residues (Figure 31 to Figure 33). Comparing the effect of the feedstocks, these results are inconclusive since the lowest adsorption was observed in the wheat straw (that even reached an apparent plateau in the medium severity residues - Figure 33), the substrate with higher lignin content in all EnzHR-P.

A trend between feedstocks is confirmed when comparing the adsorption phenomenon in the EnzHR-P of three different pretreatment severities for the same protein. In general, while the concentration of the protein solution increases, the percentage of protein adsorbed decreases, thus the concentration of free protein in the supernatants also increases. This is observed in all feedstocks only differing the proportion of the increase. However, at low concentrations, the protein determination was difficult for BSA, for example, especially in the lowest severity residues, where more values were off than for the other substrates. When looking to the values obtained for the highest severity, the values of protein adsorbed seem to increase through the range of concentrations as expected, and decrease the overall adsorption. Besides the fact that probably the fractionation of the biomass affected the lignin composition, this can also be related with the porosity of the material. The major removal of carbohydrates in the highest severity residues during pretreatment process could have created gaps in the structure that could have later collapsed due to the

several treatments applied, thus reducing the available binding sites. At the same time it could present a larger lignin surface than the lower severity residues.

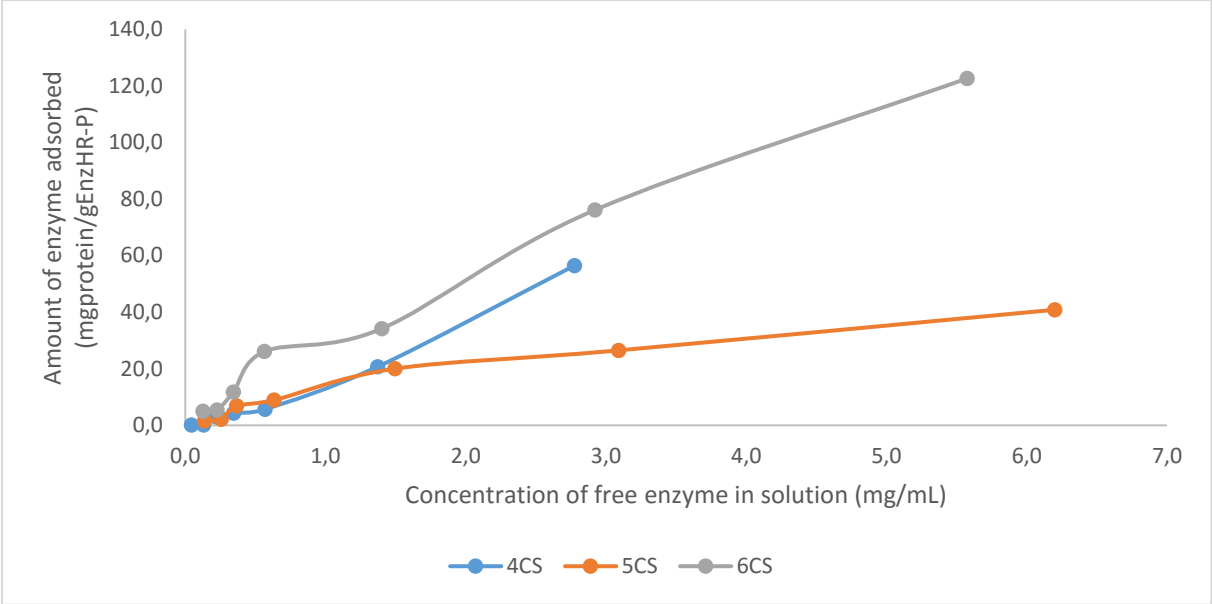

Figure 31 Laccase adsorption isotherms obtained from the experimental data for the EnzHR-P of corn stover: 4CS; 5CS; 6CS.

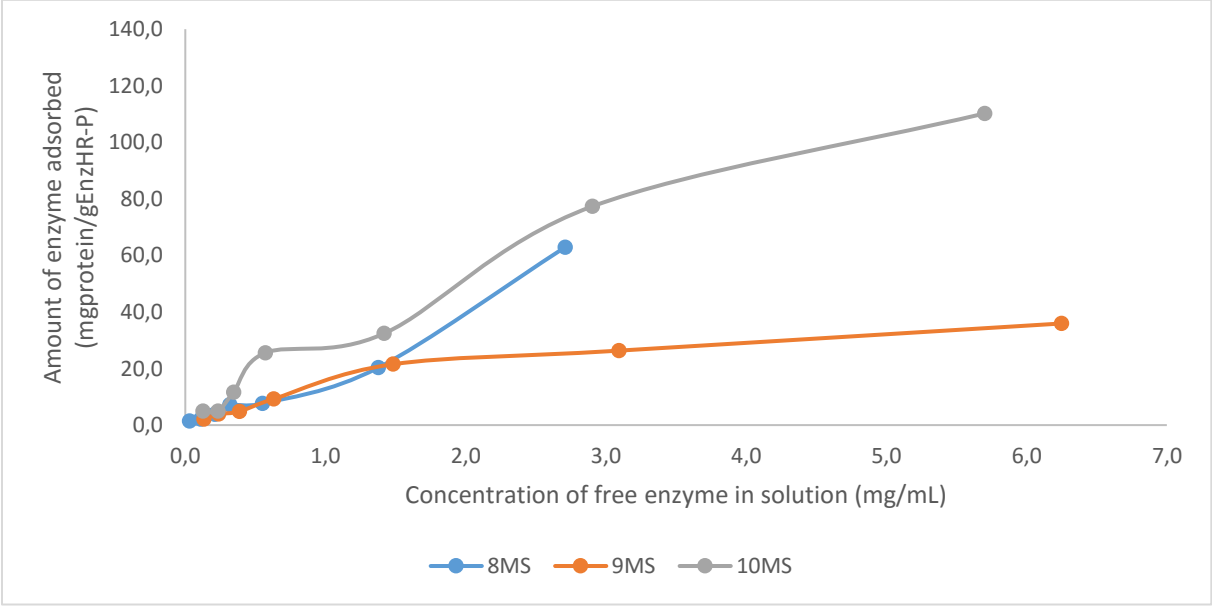

Figure 32 Laccase adsorption isotherms obtained from the experimental data for the EnzHR-P of Miscanthus: 8MS; 9MS; 10MS.

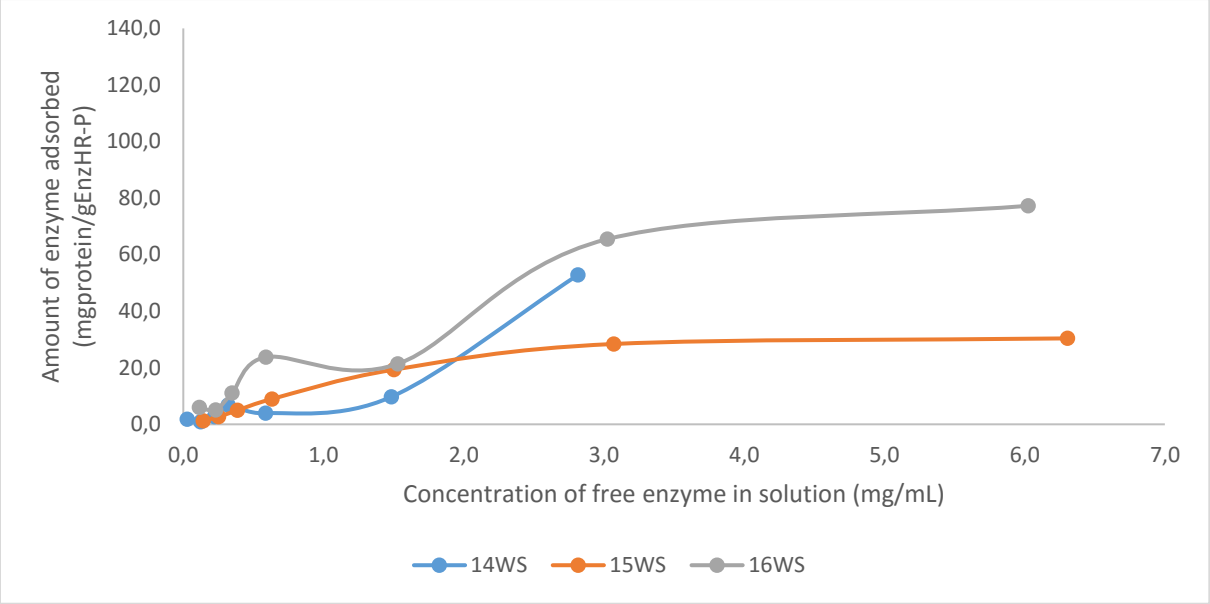

Figure 33 Laccase adsorption isotherms obtained from the experimental data for the EnzHR-P of wheat straw: 14WS; 15WS; 16WS.

Specifically concerning the proteins, in general, BSA was more adsorbed than CEM and Laccase. This can be related to the higher affinity of BSA to lignin. Previous studies have shown that BSA irreversibly bonds to lignin binding sites and even enhances cellulose hydrolysis by preventing non-productive binding of cellulolytic enzymes by binding first to lignin [82,89]. Also, an increase of bound enzymes is observed with CEM with the severity increase, showing that not only the carbohydrates fraction of the biomass is responsible for the adsorption of these catalysts. The adsorption of Laccase presented a different behavior relative to the effect of the pretreatment severity, but it is possible to observe a more similar behavior in the feedstock with higher lignin content, wheat straw.

3.3.3. Analysis of the adsorption at a specific protein concentration

To test if the proteins adsorption between these nine materials was significantly different, an analysis of variance was performed to the percentage of protein adsorbed at 0.6 $mg_{protein}$/mL. The concentration was chosen taking into account the final concentration of CEM that was used in the enzymatic hydrolysis of the substrates.

An analysis of variance was performed to assess if the adsorption results had a considerable difference between them. This statistical test allows a comparison for differences of means to determine whether the mean difference between specific pairs of groups are statistically significant and to estimate by how much they are different. Letters are attributed to each and if two samples are not statistically different, the same letter is given to each. The more apart the letters are, more different are the pairs.

In Figure 34 is presented the percentage of enzyme adsorbed for the concentration of 0.6 $mg_{protein}$/mL, as the result for the analysis of variance. In this figure it is possible to confirm that the adsorption of BSA (letter **A**), CEM (letter **B**) and Laccase (letter **C**) is statistically different between the proteins, as observed in the previous section. Also, regarding the binding of BSA within the same feedstock, a significant difference is observed between severities. Between the materials this is not verified, except in the higher severity residues (6CS; 10MS; 16WS). CEM had statistically different results between the materials from the medium severity (Figure 20), but the feedstocks at different severities are not significantly different (Figure 28 to Figure 30). In the adsorption of Laccase, significant difference is observed between the binding in the highest severity materials and the other severity materials. However, there is not a significant difference between the different feedstocks at that severity (Figure 24).

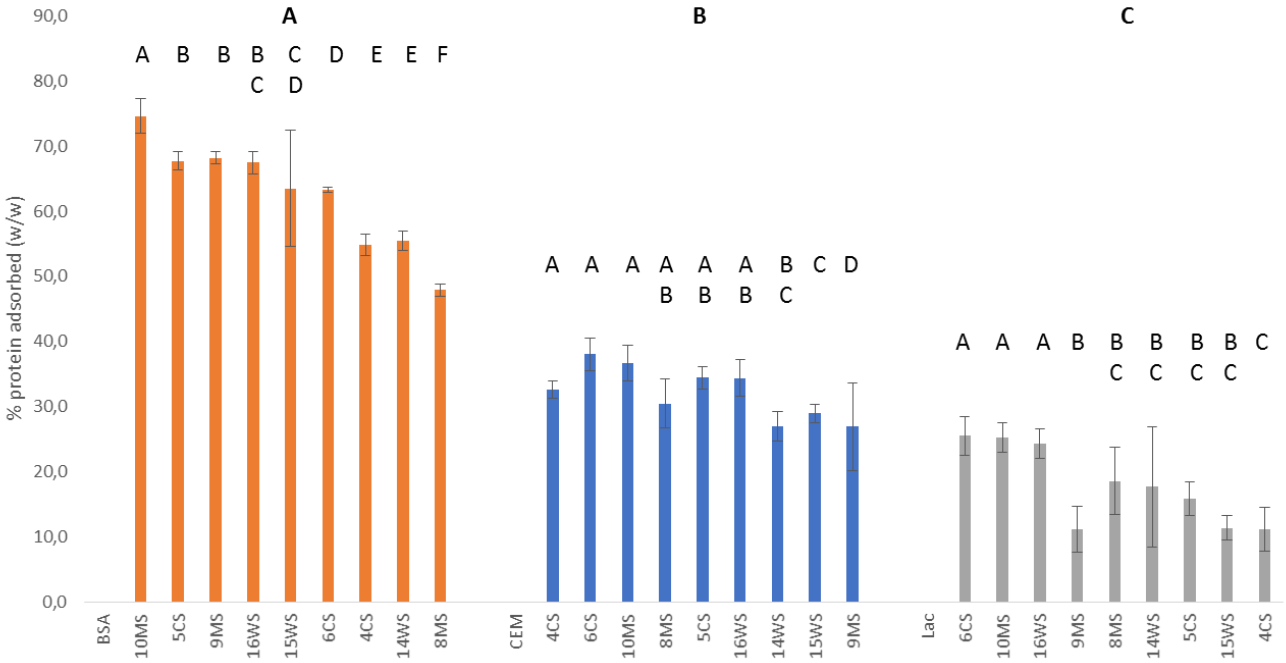

Figure 34 Graphical representation of the percentage of protein adsorbed using a protein solution with 0.6 mg/mL of concentration. The letters A – F represent the results from the analysis of variance.

3.3.4. Langmuir adsorption isotherms

Recurring to the software OriginPro® version 9.0, a non-linear fitting of the results was made to the Langmuir adsorption model. The models presented in this work describe how the adsorbed amount of a protein depends on the concentration of the substance in solution and, recurring to the parameters maximum adsorbed protein E_{max} (mg/g of lignin), and the coefficient related with the affinity between the adsorbate and the adsorbant, K_p(mL/$mg_{protein}$), it is believe that is possible to investigate and comprehend the interaction between the enzymes and the substrates (e.g., comprehend which substrate adsorbs more could

lead to understand the amount of enzyme that needs to be spent in the saccharification process). The values of the parameters from the model, the associated standard deviation and the correlation factor obtained from the respective curve fitting are represented in Table 10.

Table 10 Langmuir parameters: maximum protein adsorbed (E_{max}), and protein affinity (K_p) obtained by non-linear curve fitting to the Langmuir isotherm model, as the respective associated standard deviations ($\sigma_{E_{max}}$; σ_{K_P}) and correlation values (R^2).

			BSA		
EnzHR-P	E_{max} **($mg_{protein}/g_{EnzHR-P}$)**	$\sigma_{E_{max}}$	K_p **(mL/ $mg_{protein}$)**	σ_{K_P}	**R^2**
4CS	468E-1	155E-2	723E-2	113E-2	97.0E-2
5CS	333E1	178E2	1.00E-2	6.00E-2	87.0E-2
6CS	942E-1	123E-1	256E-2	138 E-2	76.0E-2
8MS	500E-1	333E-2	378E-2	108 E-2	90.0E-2
9MS	127E0	192E-1	120E-2	68.0E-2	82.0E-2
10MS	125E0	237E-1	289E-2	240E-2	62.0E-2
14WS	783E-1	658E-2	214E-2	67.0E-2	91.0E-2
15WS	127E0	180E-1	124E-2	65.0E-2	84.0E-2
16WS	675E-1	507E-2	104E-1	418 E-2	83.0E-2
			CEM		
EnzHR-P	E_{max} **($mg_{protein}/g_{EnzHR-P}$)**	$\sigma_{E_{max}}$	K_p **(mL/ $mg_{protein}$)**	σ_{K_P}	**R^2**
4CS	509E-1	75.0E-1	166E-2	65.0E-2	84.0E-2
5CS	857E-1	71.1E-1	72.0E-2	13.0E-2	98.0E-2
6CS	323E0	229E0	13.0E-2	12.0E-2	94.0E-2
8MS	431E-1	67.2E-1	160 E-2	67.0E-2	82.0E-2
9MS	734E-1	93.3E-1	74.0E-2	22.0E-2	96.0E-2
10MS	242E0	728E-1	20.0E-2	9.00E-2	97.0E-2
14WS	100E0	339E-1	33.0E-2	19.0E-2	85.0E-2
15WS	967E0	123E-1	50.0E-2	13.0E-2	98.0E-2
16WS	173E0	531E-1	24.0E-2	12.0E-2	96.0E-2
			Lac		
EnzHR-P	E_{max} **($mg_{protein}/g_{EnzHR-P}$)**	$\sigma_{E_{max}}$	K_p **(mL/ $mg_{protein}$)**	σ_{K_P}	**R^2**
4CS	243E3	2.97E8	7.78E-5	10.0E-2	96.0E-2
5CS	659E-1	77.9E-1	25.0E-2	6.00E-2	99.0E-2
6CS	395E0	126E0	8.00E-2	3.00E-2	99.0E-2
8MS	271E3	3.90E8	7.82E-5	11.0E-2	95.0E-2
9MS	503E-1	47.0E-1	40.0E-2	8.00E-2	98.0E-2
10MS	256E0	644E-1	14.0E-2	5.00E-2	98.0E-2
14WS	674E3	6.14E9	2.35E-5	21.0E-2	83.0E-2
15WS	433E-1	50.3E-1	47.0E-2	13.0E-2	97.0E-2
16WS	140E0	466E-1	22.0E-2	13.0E-2	92.0E-2

Although, in some cases, a good correlation value was obtained, only in a few samples it was observed a good fitting with reasonable values for the parameters (accordingly with the adsorption isotherms obtained before). This happened in the samples where a plateau was reached (discussed in section 3.3.1). As the values of adsorbed enzyme per gram of substrate tend to still increase in the highest severity, the curve did not fit the model and considerably high values for the standard deviations were obtained. A probable explanation could be related with the fact that the basic assumptions of this model are not fulfilled. The residues used do not have a uniform surface due to the presence of different amounts of carbohydrates, for example. Also, there is no proof that, in case of CEM, the different enzymes do not interact while adsorbed. As referred in the theoretical part, competitive binding can occur between enzymes. In the work of Medve *et al.* [60], while studying the adsorption of pure cellulases, could not fit the curves of the Langmuir model to the experimental data, also observing low correlation factors.

However, an analysis of tendencies can be made and compared with the results from the adsorption isotherms in section 3.3.1 and 3.3.2. In the adsorption of CEM it is noticed that with the increase of severity, an increase of the maximum amount of protein that can bound also increased, which agrees with the results of the obtained adsorption curves. The decrease in the values of the affinity constant can derive from changes in the chemical structure of lignin (lower number of binding sites).

The results from the model for the adsorption of Laccase seem to demonstrate the same trend as observed before. There is a decrease in the binding of the proteins from the lowest to the medium severity, followed by an increase in the highest severity residues. The standard deviation associated with the values of the lowest severity are unreasonable, confirming the unfitting of the model.

With corn stover high associated standard deviations were also obtained because it did not fit the model well, but still presents the same trend, except for 16WS, which agrees with the result obtained in the previous graphs, where we have less adsorption in the wheat straw residue.

3.3.5. Effect of the Laccase treatment

Several studies defend that laccases have the ability of changing lignin structure, thus influencing the enzyme binding [90]. They also state that Laccases have different behaviors when in solution with other chemicals, namely, mediators.

In order to determine the effect caused by laccases to the adsorption of proteins, a test was prepared where the effect of Laccase and the effect of Laccase with two different mediators (ABTS and HPI) was tested. Also, controls were used in order to account for any effect from the lignin and from the lignin with the mediators. A sample test with denatured Laccase at 100°C for 20 min was performed to understand if only the protein's presence affects the adsorption process. The residues used were the medium severity

Micanthus x giganteus and wheat straw, since the first represents an energy crop and the second an agricultural residue.

The different Laccase treatments were applied in acidic conditions, at 50°C for 24h in a thermomixer at 1250 rpm. After, the samples were centrifuged and one volume of buffer was used to wash the pellets, except the samples with Laccase and ABTS that were washed with three volumes due to the blue coloration from the oxidization of the mediator in the presence of the enzyme. After, a solution of a fixed concentration (0.6 mg/mL) of BSA and CEM was added to the samples to test the adsorption of the proteins as described before. After performing a ninhydrin assay to the supernatants, the results obtained are represented in Figure 35 and Figure 36, as the results for the statistical analysis of variance.

In both figures it is possible to confirm that there is no significant difference between the controls, since there is an overlap of the attributed letters (samples divided between letters A and B), which was expected since there was no Laccase added in order to cause any difference in the lignin. Also, it is important to notice that the denatured Laccase did not affected the adsorption, which means that probably just stayed in solution during the treatment.

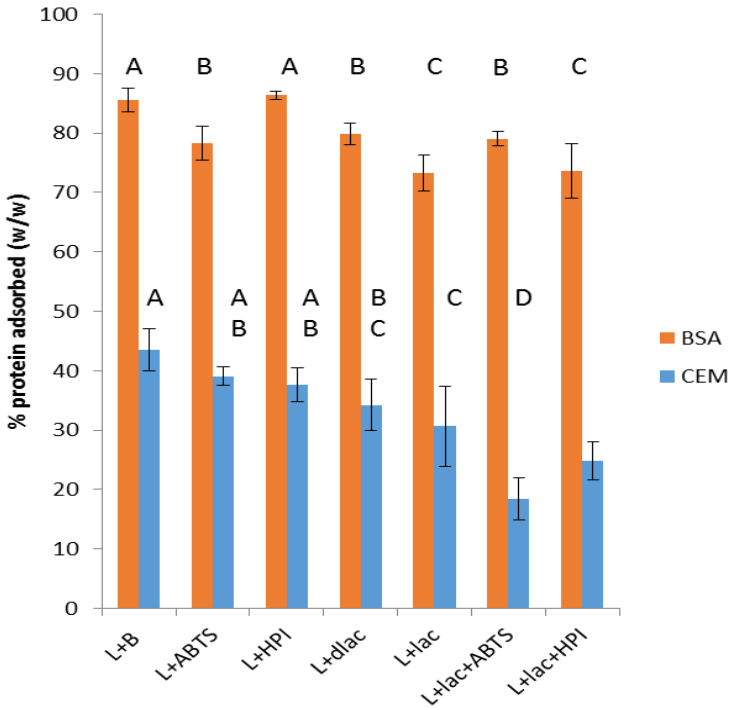

Figure 35 Representation of the amount of BSA (orange) and CEM (blue) adsorbed after a Laccase treatment on 9MS. The results are represented as percentage of enzyme adsorbed from the enzyme solutions of 0.6 mg/mL added.

The results relative to the Laccase treated samples also showed no major difference in both materials, except for the adsorption of BSA in 9MS, and CEM in 15WS, both samples treated with Laccase and ABTS. Both exhibited a lower amount of protein bound. This could indicate that Laccase with ABTS can have an effect on the lignin, modifying it so other proteins could bind less, which could be appealing in terms of enzyme recovery processes. However, since the difference is small and with a considerable error associated, it would need further investigation to assess the viability of the treatment.

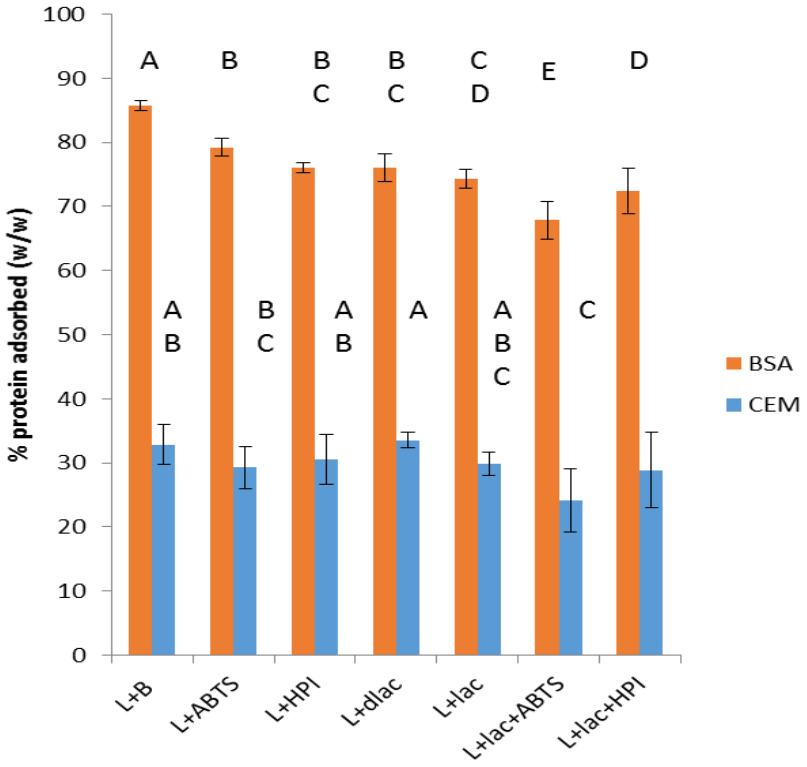

Figure 36 Graphical representation of the amount of BSA (orange) and CEM (blue) adsorbed after a Laccase treatment on 15WS. The results are represented as percentage of enzyme adsorbed from the enzyme solutions of 0.6 mg/mL added.

4. Conclusions and future prospects

Considerable progress has been made in the scientific world, in order to understand the underlying mechanisms responsible for the non-productive adsorption of enzymes onto lignocellulosic materials. The work presented here was performed to investigate the effect of lignin in enzymatic processes, more specifically, in the enzyme adsorption to the substrates. Three different feedstocks, with high potential for biorefinery in Denmark, but also worldwide, subjected to hydrothermal pretreatment with steam injection, each using three different severities (Log R_O 3.65, 3.83 and 3.97), were studied in order to understand the contribution of the pretreatment in the adsorption phenomenon.

Aiming to study the adsorption onto lignin, an extensive enzymatic hydrolysis, using a commercial enzyme mixture, was performed aiming to obtain lignin-rich residues. The highest lignin purity achieved was 87.3% with wheat straw pretreated with the highest severity. The adsorption studies were performed in all the residues obtained (EnzHR) and, prior to the studies, a CHN-S analysis was performed. CHN-S analysis confirmed the presence of higher protein content after enzymatic hydrolysis, clearly showing the existence of adsorbed enzymes derived from the enzymatic process. After protease treatment, another analysis showed the reduction in nitrogen content in the EnZHR-P, demonstrating the efficiency of these enzymes.

With all the EnzHR-P, the adsorption of three proteins was tested, namely, BSA, CEM and Laccase. Different responses were obtained in each feedstock and in each severity. Comparing between severities for the adsorption of CEM and Laccase, all the feedstocks had higher adsorption of protein in the higher severity residues, which could either be related with entrapment of the enzymes in the residues structure, and/or with the fact that these enzymes can be bonding irreversibly to lignin. Surprisingly, the lower amount of CEM adsorbed was obtained with the lowest severity residues (8MS with 42.1 $mg_{enzyme}/g_{EnzHR-P}$), since these residues still present a considerable carbohydrate content. For Laccase, the lowest adsorption was obtained in the medium severity residues (15WS with 30.4 $mg_{enzyme}/g_{EnzHR-P}$), the opposite behavior of what it was observed in BSA adsorption (highest protein adsorption for 15WS of 136.3 $mg_{protein}/g_{EnzHR-P}$). Since both proteins are known to bind to lignin, it is possible to admit that some modification could have happened that caused a different behavior from what it was expected. The effect of the severity of the pretreatment and consequent lignin melting and condensation, could have altered the structure of the polymer.

Looking at the effects between different feedstocks at the same conditions, the adsorption of each protein followed a similar trend as the severity of the pretreatment increased. The trend of the isotherms of the three proteins seemed to become more alike, and in the case of *Miscanthus* and corn stover, they almost overlapped, probably due to the composition of the two materials being very similar.

It is important to point that when the adsorption of the proteins was tested at the fixed concentration of protein used in the saccharification process, the percentage of enzyme adsorbed was not significantly different between all the materials, probably related to the not so different composition. From this, it would be easily infer that there is a concentration where the amount of bound enzyme would be the same for every grass feedstock, which is known to be not true. These results should be further investigated since a conclusion cannot be drawn as these results derived from a 2h experiment in EnzHR-P, and the enzymatic hydrolysis is usually performed for 72h.

A fitting of the experimental data to the Langmuir isotherm model was proposed and performed, unsuccessfully. Some of the parameters obtained were associated with high errors and low correlation values, demonstrating that this model is not appropriate for modelling the adsorption of the studied proteins in the mentioned pretreated materials.

Also the effect of a Laccase treatment was studied, revealing that, in these materials, only Laccase associated with the mediator ABTS can probably modify the lignin fraction and have any significant effect in the adsorption phenomenon.

As future prospects, it would be helpful for a better understanding of these results to:

- use a larger range of concentrations and, at the same time as the protein amount is measured, measure the activity. Specially regarding CEM, a measurement of the proteins' activity in the solids after the adsorption studies should also be performed to differentiate binding from unproductive binding;
- study the adsorption with individual enzymes would help to understand if there is any synergy between enzymes that affect the binding;
- similarly, test the adsorption of the proteins to lignins from different pretreatments to comparison;
- study the effect of the laccase treatment in protein adsorption to lignin with different concentrations, since in this work only one concentration was tested;
- Use other models to fit the experimental data.

Further investigation needs to be done in order to understand why the adsorption occurs, at a molecular level. Also, the study of alternatives to avoid the enzyme binding, as surfactants (e.g. BSA, PEG, Tween 20) or enzyme immobilization, needs to be thoroughly accessed.

The conclusions of this work could help to further understand the role of lignin in the reduction of adsorption of cellulases on substrates and, in a near future, contribute to the reduction of costs of these processes by reusing or recycling these biocatalysts.

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Appendix

A - Combined Severity Factor (Log *R*o)

The severity factor proposed by Overend and Chornet [73], was calculated by the following expression:

$$\text{Log } R_o = \int_o^t \exp\left(\frac{T(t) - 100}{w}\right) dt \quad (5)$$

In this formulae T represents the temperature (ºC); t represents time (min); and w is an empirical parameter characteristic of the process with a value of 14.75

B - Analytical methods

Moisture content

The moisture content (%) of the samples was calculated using the following expression

$$\text{Moisture } (\%) = \frac{\text{wet sample weight (g)} - dry\ sample\ weight\ (g)}{\text{wet sample weight (g)}} \times 100 \quad (6)$$

Ash content

The ash content (%) of the samples was calculated using the following expression

$$\text{Ash } (\%) = \frac{\text{Ash weight (g)}}{\text{dry sample weight (g)}} \times 100 \quad (7)$$

Quantification of structural polysaccharides and lignin in solid samples

Concentrations of glucose, xylose, arabinose and galactose in the liquors resulting from the quantitative acid hydrolysis of the pretreated materials and EnzHR-P were used for the calculation of glucan, xylan, arabinan and galactan content (%), respectively. The acid-insoluble residue, was quantified as Klason lignin (KL) after correction for the ash content.

$$Glcn = F_1 \times \frac{100}{1005} \times \frac{162}{180} \times Glc \times \frac{Wsol}{A} \quad (8)$$

$$Xyln = F_2 \times \frac{100}{1005} \times \frac{132}{150} \times Xyl \times \frac{Wsol}{A} \quad (9)$$

$$Aran = F_3 \times \frac{100}{1005} \times \frac{132}{150} \times Ara \times \frac{Wsol}{A} \quad (10)$$

$$Galn = F_4 \times \frac{100}{1005} \times \frac{162}{180} \times Gal \times \frac{Wsol}{A} \quad (11)$$

$$KL = \frac{AIS - Ash}{A} \times 100 \quad (12)$$

- Glcn, Xyln, Aran, Galn and KL are the concentrations of glucan, xylan, arabinan, galactan and Klason lignin (g/100 g of dry solid) respectively;
- Glc, Xyl, Ara and Gal are the concentrations of glucose, xylose, arabinose, and galactose in liquors (g/L), respectively;
- The terms $\frac{162}{180}$, $\frac{132}{150}$ are stoichiometric conversion factors of monomers into polysaccharides;
- F_1, F_2, F_3, F_4 is the correction factor accounting for sugar degradation (1.04 for glucose and galactan; and 1.09 for xylose and arabinose);
- W_{sol} and A are the weights of the solution and dried sample used in the test, respectively (g);
- AIS and Ash are the weight of the acid-insoluble residue of the sample and its ash content, respectively (g).

C - Chemical composition of the Enzymatic Hydrolysis Residues after Protease treatment (EnzHR-P)

The following table presents the composition analysis of the EnzHR-P.

Table 11 Chemical composition obtained by quantitative acid hydrolysis of the EnzHR-P.

		Cellulose		Hemicellulose			Lignin			
Biomass		**Glucan (%)**	**Xylan (%)**	**Arabinan (%)**	**Galactan (%)**	**Mannan (%)**	**AIL (%)**	**ASL (%)**	**Ash (%)**	**Total (%)**
Corn Stover	**4CS**	**20.89** ± 0.55	**5.44** ± 0.22	**0.56 ±** 0.00	**0.39** ± 0.02	**0.00** ± 0.00	**58.32** ± 1.30	**2.05** ± 0.16	**12.34** ± 0.49	100
	5CS	**14.17** ± 0.05	**3.14** ± 0.08	**0.43** ± 0.00	**0.33** ± 0.02	**0.00** ± 0.00	**72.01** ± 0.90	**1.73** ± 0.05	**8.20** ± 0.01	100
	6CS	**7.20** ± 0.20	**1.58** ± 0.01	**0.28** ± 0.00	**0.28** ± 0.00	**0.00** ± 0.00	**77.67** ± 1.58	**1.47** ± 0.05	**11.51** ± 0.51	100
Miscanthus	**8MS**	**33.13** ± 0.70	**5.56** ± 0.09	**0.52** ± 0.01	**0.46** ± 0.00	**0.00** ± 0.00	**55.56** ± 0.70	**1.60** ± 0.02	**3.17** ± 0.14	100
	9MS	**18.94** ± 0.56	**2.98** ± 0.11	**0.39** ± 0.02	**0.44** ± 0.02	**0.00** ± 0.00	**71.89** ± 0.82	**1.28** ± 0.06	**4.07** ± 0.07	100
	10MS	**11.51** ± 0.47	**1.40** ± 0.03	**0.29** ± 0.02	**0.43** ± 0.00	**0.00** ± 0.00	**79.16** ± 0.32	**1.14** ± 0.06	**6.06** ± 0.62	100
Wheat Straw	**14WS**	**13.67** ± 0.56	**3.34** ± 0.13	**0.49** ± 0.02	**0.38** ± 0.01	**0.00** ± 0.00	**75.90** ± 0.72	**1.56** ± 0.09	**4.65** ± 0.32	100
	15WS	**7.77** ± 0.03	**1.97** ± 0.04	**0.37** ± 0.03	**0.32** ± 0.02	**0.00** ± 0.00	**83.99** ± 0.08	**1.29** ± 0.02	**4.28** ± 0.09	100
	16WS	**5.23** ± 0.09	**1.07** ± 0.03	**0.28** ± 0.01	**0.28** ± 0.00	**0.00** ± 0.00	**86.16** ± 0.99	**1.16** ± 0.04	**5.81** ± 0.37	100

D – Adsorption studies calculations

In each adsorption study performed, a ninhydrin assay was performed in order to establish the concentrations of the free enzyme in solution. With each ninhydrin assay, a calibration curve was prepared. Due to the sensitivity of the method, the set of defined BSA solutions of known concentration had to be in the same process conditions as the samples to avoid interferences. The calibration curves are not here presented since they are numerous and specific to each ninhydrin process performed.

After calculating the concentrations of free enzyme in solution ($mg_{protein}$/mL), in order to obtain the graphical representation of the adsorption isotherms, the value of enzyme adsorbed per gram of residue was needed. The calculations used are presented in the equations 13 to 15.

$$m_{free\ enzyme} = C_{free\ enzyme} \times volume_{enzyme\ solution} \quad (13)$$

$$m_{enzyme\ adsorbed} = m_{\ enzyme\ in\ initial\ solution} - m_{free\ enzyme} \quad (14)$$

$$m_{mg\ enzyme_{adsorbed} \cdot gsolids} = \frac{m_{\ enzyme\ in\ initial\ solution}}{m_{\ weighed\ solids}} \quad (15)$$