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JULIANA DE FRANÇA SERPA

CASHEW APPLE BAGASSE LIGNIN AS SUPPORT FOR IMMOBILIZATION OF LIPASE B FROM *Candida antarctica*

FORTALEZA

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Tese apresentada ao Programa de Pós-Graduação em Engenharia Química da Universidade Federal do Ceará, como parte dos requisitos para obtenção do título de Doutor em Engenharia Química. Área de concentração: Processos Químicos e Bioquímicos.

Orientadora: Profª. Dra. Maria Valderez Ponte Rocha.

Coorientador: Prof. Dr. André Casimiro de Macedo.

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“The thing always happens that you really believe in; and the belief in a thing makes it happen.” (Frank Lloyd Wright).

RESUMO

O caju (*Anacardium occidentale* L.) é um pseudofruto tropical com um papel econômico destacado devido à exportação da castanha de caju. A indústria de suco de caju produz 15% (m/m) de bagaço, e esse material é composto de celulose, hemicelulose e lignina. Sua estrutura é altamente recalcitrante à biotransformação microbiana e enzimática, o que limita seu uso e torna sua conversão em produtos de valor agregado inviável economicamente. Assim, os pré-tratamentos são necessários para romper as estruturas recalcitrantes do material lignocelulósico para aumentar a digestibilidade do material antes da conversão em produto de valor agregado. Durante o pré-tratamento do bagaço de caju, a lignina é descartada e se torna um co-produto. No entanto, é uma matéria-prima potencial para a produção de diferentes materiais. Nesse contexto, esta pesquisa teve como objetivo extrair e caracterizar a lignina do CAB (Bagaço de Caju), para ser utilizado em um novo material. As condições de pré-tratamento para extração de lignina foram estudadas a partir de diferentes hidrolisados obtidos pelos pré-tratamentos com ácido diluído (A), ácido / alcalino (AA) e peróxido de hidrogênio alcalino (AHP). A porcentagem de extração da lignina dos hidrolisados obtidos pelos pré-tratamentos AA e AHP foram de 98,0% e 96,9%, respectivamente, atingindo alto rendimento de extração e ligninas com maior estabilidade térmica. A lignina AA foi escolhida para as próximas etapas deste estudo. Novos materiais compostos magnetita-lignina foram sintetizados usando lignina obtida do pré-tratamento AA subproduto, extraído do CAB e nanopartículas de magnetita (Fe_3O_4), bem conhecidas por suas propriedades não tóxicas e magnéticas. Esse material, denominado MNs/Lig, foi utilizado como suporte para a imobilização da lipase B de *Candida antarctica* (CAL-B), com o objetivo de obter um biocatalisador ativo e estável e com fácil recuperação do meio reacional. Os MNs/Lig suportes e os biocatalisadores produzidos foram caracterizados por Espectroscopia no Infravermelho por Transformada de Fourier (FTIR), Difração de raios-X (DRX), Magnetômetro de Amostra Vibratória (VSM), Termogravimetria (TGA), Microscopia Eletrônica de Varredura (MEV) e raios X dispersivos em energia espectroscopia (EDS). Os biocatalisadores preparados foram avaliados para a síntese de ésteres utilizando ácido oleico como substrato, avaliando o álcool da reação, álcool etílico (1: 1) e 2-etil-1-hexanol (1: 1). As conversões obtidas foram de 88,2% e 76,7%, utilizando 2-etil-1-hexanol e álcool etílico, respectivamente, avaliando 5 ciclos de reutilização e não foi observado perda de catalisador. Portanto, essa nova estratégia para obter um biocatalisador a partir de híbridos sintetizados (MNs / Lig) pode ser um veículo promissor para imobilização enzimática de lipases, além de ser considerado ambientalmente correto, visando seu uso em reações de interesse industrial.

Palavras-chave: Pré-tratamentos. Suporte. Biocatalisador. Imobilização enzimática.

ABSTRACT

Cashew apple (*Anacardium occidentale* L.) is a tropical pseudofruit with an outstanding economic role due to the cashew apple nut exportation. The industry of juice from cashew apple produces 15% (w/w) of bagasse, and this material is composed of cellullose, hemicelulose and lignin. Its structure is highly recalcitrant to microbial and enzymatic biotransformation, thus limiting its use and making its conversion into value-added products not economically feasible. So, the pretreatments are needed to disrupt the recalcitrant structures of the lignocellulosic material to increase the digestibility of the material prior to the conversion into value-added product. During the pretreatment of cashew apple bagasse, lignin is discarded and becomes a co-product. Though, it is a potential raw for production of different materials. In this context, this research aimed to extract and characterize lignin from CAB (Cashew Apple Bagasse), be used a new material. Pretreatment conditions for lignin extraction were studied from different hydrolysates obtained by diluted acid (A), acid/alkali (AA) and alkaline hydrogen peroxide (AHP) pretreatments. Lignin removals through AA and AHP pretreatments were 98.0% and 96.9%, respectively, achieving high extraction yield, and lignins with higher thermal stability. The lignina AA was chosen for the next steps of this study. Novel magnetite-lignin composite materials were synthesized using the by-product lignin from by AA pretreatment, extracted from CAB and nanoparticles of magnetite (Fe_3O_4), well-known for its nontoxicity and magnetic properties. This material, named MNs/Lig, was used as support for the immobilization of Lipase B from *Candida antarctica* (CAL-B), aiming to obtain an active and stable biocatalyst and with easy recovery of the reactional medium. The MNs/Lig supports and biocatalysts produced were characterized by Fourier Transform Infrared spectroscopy (FTIR), X-ray diffraction (XRD), Vibrating Sample Magnetometer (VSM), Thermogravimetric analysis (TGA), Scanning Electron Microscopy (SEM). The biocatalysts prepared were used in evaluated for the synthesis of esters using oleic acid as substrate and ethyl alcohol (1:1) and 2-ethyl-1-hexanol (1:1) as alcohol. The conversions obtained were 88.2% and 76.7% using 2-ethyl-1-hexanol and ethyl alcohol, respectively, being evaluating 5 cycles of reuse and it did not observe loss catalyst. Then, this new strategy to obtain a biocatalyst from synthesized hybrids (MNs/Lig) may be a promising carrier for enzymatic immobilization of lipases, in addition to being considered environmentally benign, aiming its use in reactions of industrial interest.

Keywords: Pretreatments. Support. Biocatalyst. Enzymatic Immobilization.

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LIST OF ABBREVIATIONS AND ACRONYMS

CAB	Cashew Apple Bagasse
CAB-AHP	Cashew Apple Bagasse Pretreated by Alkaline Hydrogen Peroxide
CAB-H	Cashew Apple Bagasse Pretreated by Sulfuric Acid
CAB-HOH	Cashew Apple Bagasse Pretreated by acid/alkali
DA	Dilute acid
AA	Acid-alkali Pretreatments
AHP	Alkaline Hydrogen peroxide
AL	Lignin extracted for the pretreatment with acid diluted
AAL	Lignin extracted for the pretreatment with acid-alkali
PL	Lignin extracted for the pretreatment with alkaline hydrogen peroxide
SCL	Sugarcane Lignin
MNs	Magnetite Nanoparticles
Lig	Lignin from Cashew Apple Bagasse
MNs/Lig	Magnetite conjugated with lignin obtained by synthesis in ammonium hydroxide (NH_4OH);
MNs/Lig _NaID	Magnetite conjugated with lignin obtained by synthesis using a mixture of dioxane and sodium periodate (NaID)
MNs/Lig_CAL-B	CALB-lipase immobilized on magnetite conjugated with lignin
MNs/Lig_GA	Magnetite conjugated with lignin and actived with glutaraldehyde
MNs/Lig_GA_CALB	CALB-lipase immobilized on magnetite conjugated with lignin, actived with glutaraldehyde
MNs/Lig_GA_TRI_CALB	CALB-lipase immobilized on magnetite conjugated with lignin, actived with glutaraldehyde in the presence of 0.01 % (v/v) Triton X-100.

GA	Glutaraldehyde
ρ-NPB	*ρ*-Nitrophenyl Butyrate
XRD	X-ray diffraction
FTIR	Fourier Transform Infrared Spectroscopy
TGA	Thermogravimetric analysis
VSM	Vibrating Sample Magnetometer
SEM	Scanning electron microscopy
EDS	Energy dispersive x-ray spectroscopy
IY	Immobilization yield
At_T	Theoretical activity
At_B	Biocatalyst activity
$t_{1/2}$	Half-life time
CAL-B	Lipase B from *Candida antarctica*

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1 INTRODUCTION

Due to a constant search to reduce the environmental impacts caused by human action on nature, different researches are being developed for the reuse of agroindustrial residues as raw material, i.e cashew apple bagasse, mainly in bioprocesses (FERNÁNDEZ-RODRÍGUEZ et al., 2020; ALBUQUERQUE et al., 2015).

Cashew apple is a pseudofruit from the Northeastern region of Brazil with an outstanding economic role due to the cashew nut exportation. The industry of cashew apple juice produces 15% (w/w) of bagasse, representing one of the major waste from the Brazilian agribusiness (CORREIA et al., 2013; ROCHA et al., 2014; REIS et al., 2017). CAB is mainly composed of cellulose, hemicellulose and lignin that form a complex structure (REIS et al., 2017; CORREIA et al., 2013).

Lignin, found in around 10%-35% (w/w) of plants interms of dry weight and 40% in terms of energy, is one of the most abundant aromatic bio-polymer feedstock. It is still underutilized as a bio-based chemical and biofuel compared to cellulose and hemicellulose, although it has high potential (GILLET et al., 2017; SOONGPRASIT et al., 2020). Its structure is three-dimensional and consists of three phenol groups: p-hydroxyphenyl (H-unit), guaiacyl (G-unit), and syringyl (S-unit), which are derived from p-coumaryl alcohol,coniferyl alcohol, and sinapyl alcohol, respectively (LUPOI et al., 2015; SOONGPRASIT et al., 2020).

The lignin extracted during pretreatment has been a source of study in several works because it is a fibrous and quite resistant material (Li et al., 2015; Mohan et al., 2015). However, only an insignificant part is used in specialty products, the rest serves as fuel for thermal energy generation.

Currently, a wide variety of chemicals can be sustainably produced from the aromatic structures of lignin (SILVA et al., 2013). Due to its high molecular weight, lignin can be used to produce carbon fibers, polymer modifiers, adhesives and resins (FROLLINI and CASTELLAN, 2012). Also, lignin has antioxidant activity, due to presence of phenolic groups and benzylic hydrogens.

Lignin has been recently combined with magnetite (Fe_3O_4) in order functional hybrid nanomaterials or nanocomposites (KLAPISZEWSKI et al., 2019). In recent years, magnetite nanoparticles (MNs) have played a very important role in the field of nanotechnology. They have valuable and often exceptional properties which make them

suitable for use in many areas, including in and medicine (ULBRICH et al., 2016) and biology (MANIVASAGAN et al., 2016).

Magnetite (Fe_3O_4) has been currently the most commonly used among the various nano-oxides exhibiting magnetic properties (WANG et al., 2016). This high level of interest of this substance is caused scale owing to their excellent magnetic properties, such as superparamagnetic behavior at room temperature, exceptional biocompatibility of their surfaces (ZHANG et al., 2012; FURLAN et al., 2019), low toxicity compared to both metals or other metal-oxide, high chemical stability, and the facility and low cost of the procedures available for their preparation (FIGUEROLA et al., 2010; FURLAN et al., 2019).

Recently, magnetic nanoparticles have attracted much attention an alternative support for enzyme immobilization (YONG et al., 2008; CHEN et al., 2009) and due to a substantial increase in their availability and versatility show to be very important support (SOUZA et al., 2017).

This possibility to obtain a new biocatalyst from synthesized hybrids (MNs/Lig) may be a promising carrier for enzymatic immobilization of lipases, in addition to being considered environmentally benign, aiming its use in reactions of industrial interest.

Large scale industrial application of enzymes is still a difficult process due to their considerably high cost, low stability difficult recovery and recycling. Moreover, it is difficult to separate them from the reaction system which limits its recovery and reuse (ALVES, et al., 2017; ADLERCREUTZ, 2013). However, the use of immobilized enzymes on a suitable support not only circumvents these problems but also has additional advantages: improve their activity, specificity, and stability and to facilitate the reuse of the biocatalysts (ALVES et al., 2017; FERNANDEZ-LAFUENTE, 2010).

In this context, the present work has as objective extraction of lignin from cashew apple bagasse (CAB), synthesize lignin with magnetite as a new material and to evaluate this material obtained as a support for immobilization of Lipase B from *Candida antarctica* (CAL-B).

1.1 Objectives

The main objective of the present work is the extraction of lignin from cashew apple bagasse (CAB), then use the lignin in the synthesis of a new magnetite_lignin compost and to evaluate this material obtained as a support for immobilization of Lipase B from *Candida antarctica* (CAL-B).

1.1.1 Specific Objectives

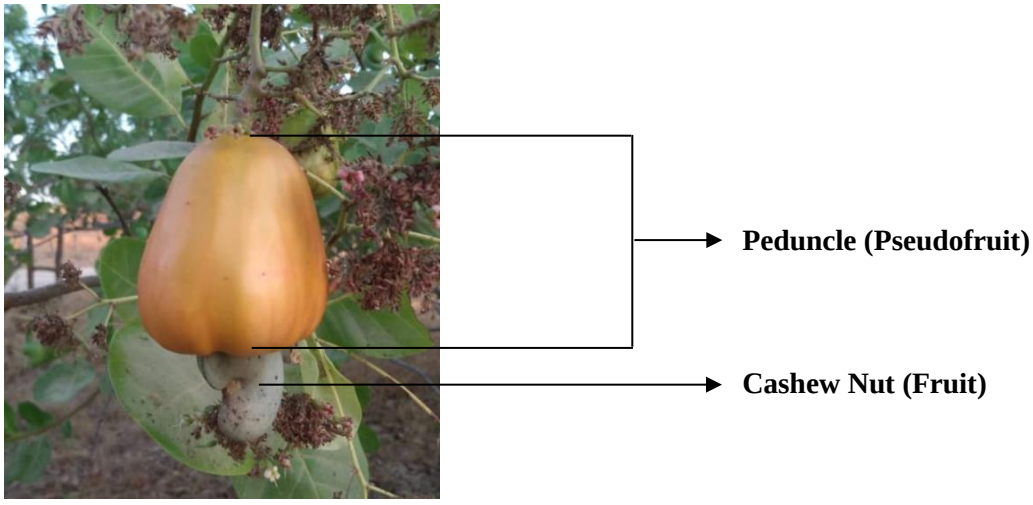
- To Extraction extract and to characterize of lignin from cashew apple bagasse (CAB);
- To synthesis of magnetite (Fe_3O_4) conjugated with lignin by two different procedure;
- To immobilize CAL-B lipase enzyme on magnetic nanoparticles conjugated with lignin (MNs/Lig);
- Characterization of the synthesized biocatalysts;
- Evaluation of the application conditions of the obtained biocatalysts.

2 LITERATURE REVIEW

2.1 Cashew apple

Cashew apple (*Anacardium occidentale L.*) is a native fruit from the tropical America and widely produced into 5 million hectares around the world, dispersed in several countries of South America, Africa, Asia and Central America, with cashew apple composed of cashew nut (fruit) and peduncle (pseudofruit) (Fig. 01). Cashew apple is a pseudofruit from the Northeastern region of Brazil with an outstanding economic role due to the cashew nut exportation. The industry of juice from cashew apple produce 15% (w/w) of bagasse (PADILHA et al., 2019; REIS et al., 2017), representing one of the major waste from the Brazilian agribusiness (CORREIA et al., 2013; ROCHA et al., 2014; REIS et al., 2017).

Figure 01- Cashew apple (*Anacardium occidentale* L.): peduncle and cashew nut



Source: Prepared by the author.

Cashew apple presents high vitamin C content, in average, equal to 269 mg/100 ml of juice, being this value five times higher than the level found in orange juice (CONTRERAS-CALDERÓN et al., 2011). It also contains niacin, riboflavin and

thiamine in addition to significant amount of minerals, such as copper, calcium, sodium, zinc, potassium, iron, phosphorous and magnesium (LOWOR and AGYENTE-BADU, 2009).

A high amount of cashew apple is annually processed to obtain cashew nuts and more than 80% of the fibrous peduncles (10-15 tons/1.0 nut ton) are discarded as an agricultural by-product after removing the nut. Although its rich nutritional composition, cashew apple utilization has been very limited due to certain disadvantages, such as high perishability and its unfavorable sensory characteristic (DAS and ARORA, 2017). However, in recent years, there has been an increasing trend towards more efficient utilization of agro-industrial residues, and some works have been conducted to evaluate alternatives to use this agricultural by-product as source for the production of various biomolecules and bio-based products, such as food derivatives, enzymes, biosurfactant, biopolymers, natural pigments and alcohol.

CAB is mainly composed by cellulose, hemicelluloses and lignin that form a complex and intricate structure (REIS et al., 2017; ROCHA et al., 2014; CORREIA et al., 2013; WANDERLEY et al., 2013). The following average proportion of cellulose, hemicellulose and lignin have been reported in the literature: 20-21% w/w; 10.20-16.30% and 33.60-35.30 % (w/w), respectively ROCHA et al., 2014; COSTA et al., 2015). Many agricultural wastes have been reported as potential sources of lignocellulosic material (REIS et al., 2017) such wheat straw (LOPES et al., 2013), sugarcane bagasse (PINHEIRO et al., 2017; JIANG et al., 2013), bamboo (XU, et al., 2019), in addition to the cashew apple bagasse (CAB), which seems to be a promising alternative.

In addition, the composition of cashew apple bagasse (CAB) points the raw material as an alternative and inexpensive lignocellulosic material product for obtaining value-added products, such as ethanol (RODRIGUES et al., 2016; ROCHA et al., 2011; RODRIGUES et al., 2011), xylitol (ROCHA et al., 2014; ALBUQUERQUE et al., 2015) carbohydrates (REIS et al., 2017) and other products such as enzymes (RODRIGUES et al., 2007).

In the works reported in the literature, cellulose and hemicleulose from cashew apple bagasse are the target molecules. Lignocellulosic materials are resistant to saccharification via enzymatic hydrolysis due to its complex structure and therefore, require pretreatment to improve their bioconversion (ZHAO et al., 2017). So, the pretreatments of lignocellulosic materials promote the removal of components that are

recalcitrant to increase the digestibility of the material prior to the conversion into product of interest. However, both the valorization of lignin and the economic aspects of this pretreatment are fundamental for production of different materials.

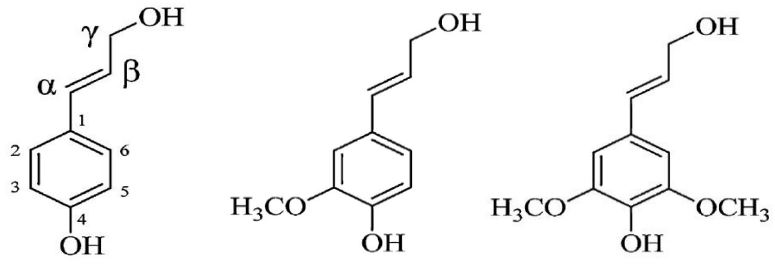
2.2 Lignin

Lignin is the second most abundant component of plant residues in terrestrial ecosystems (SUN et al., 2013; NOGUEIRA et al., 2019). It is can be obtained from various renewable raw materials, for example, wood, sugar cane, cedar trees or pine, and bagasse (CARVALHO et al., 2013).

In contrast to cellulose and hemicellulose, lignin is a complex macromolecule composed of phenolics (monolignols). Lignin is a biopolymer with high cross-linking of ether and carbon-carbon bonds that polymerizes 4-hydroxyphenylpropanoid monomers, whose formation is activated by laccases and/or peroxidases. It is three-dimensional and consists of three phenol groups which include: p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) (FENGEL and WEGENER, 1989).

It is a complex amorphous polyphenolic molecule (NOGUEIRA et al., 2019), with a structure composed of three different types of phenolic precursor units (coniferyl-, e.g. *p*-coumaryl- and synapil alcohols) (see Figure 2), which linked by carbon-carbon and ether bonds formed an irregular network biopolymer (SUN et al., 2013; BOERIU et al., 2004).

Figure 2 - The three main precursors (monolignols) of lignin molecule

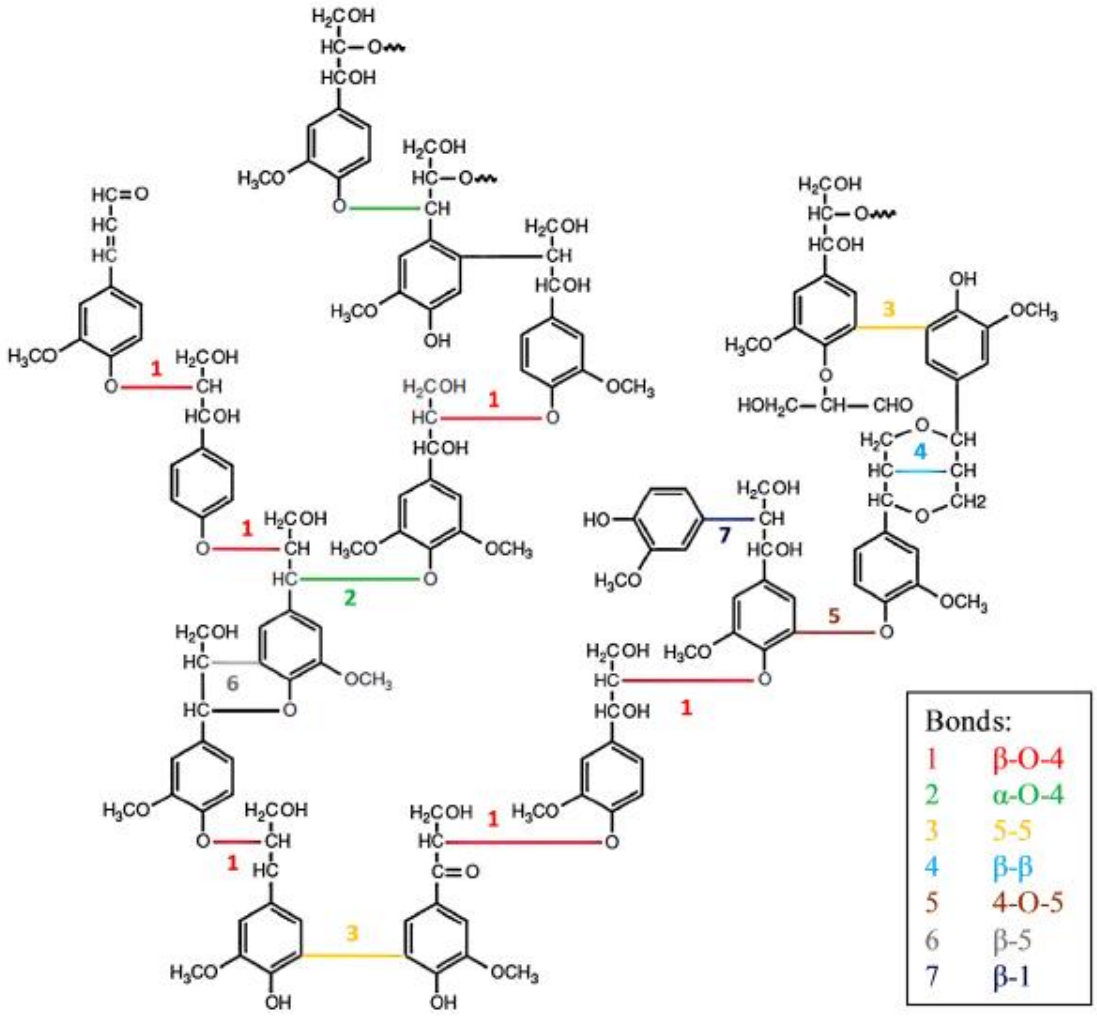


p-coumaryl alcohol **coniferyl alcohol** **sinapyl alcohol**

Source: WINDEISEN and WEGENER, 2012.

Adding strength and structure to the cell walls, the lignin plays a major role in woody plants, controlling fluid flow and protecting against biochemical stresses by inhibiting enzymatic degradation of other components (LAURICHESSE and AVÉROUS, 2014). The monolignols units are linked together via radical coupling reactions during the biological lignification process, to form a complex three-dimensional molecular architecture (Figure 3) that contains a great variety of bonds withtypically around 50% B-O-4 ether linkages (RALPH et al., 2004; CHEN and SARKANEN, 2003).

Figure 3 - Main linkages in a softwood lignin



Source: WINDEISEN and WEGENER, 2012.

As one of the most widespread biopolymers in the world, lignin has many advantages, because it is a waste material that is produced in large amounts in the paper and pulp industry, the renewable nature indicates that the lignin resources will never be depleted and the biopolymer is harmless to live organisms (KLAPISZEWSKI et al., 2019).

The lignin extracted during pretreatment has been a source of study in reports literatures because it is a fibrous and quite resistant material (LI et al., 2015). Due to its high molecular weight (LI et al., 2015), lignin can be used to produce carbon fibers, polymer modifiers, adhesives, bioactive compounds and resins (PADILHA et al., 2019). Also, lignin has antioxidant activity, because of the presence of phenolic groups and benzylic hydrogens.

To obtain the of lignin are applied different treatment methods. These mainly include physical-chemical treatments (e.g., steam explosion with SO_2, liquid hot water, ammoniun fiber explosion and microwave pretreatments) (ROCHA et al., 2011; RODRIGUES et al., 2011), treatments chemical (e.g., alkali, acid, ozonolysis, organosolv and ionic liquids), physical treatments (mechanical and extrusion), and alkaline hydrogen peroxide treatments (KARAGÖZ et al., 2012; CORREIA et al., 2015).

Natural lignin is a pale yellow or colourless but on treatment with acid or alkali, its color changes to dark brown or brown. The range of monolignol content in plant sources yields plurality in both the chemical and the physical properties of the resulting lignin materials. Molecular masses of isolated lignin are in the range 1000 – 20,000 $g.mol^{-1}$, but the degree of polymerization in nature is difficult to estimate since contains numerous types of subunits which repeat randomly and lignin is consistently fragmented during extraction (DAVIN et al., 2008).

Lignin has degradable property and in common practice, oxidation and hydrogenation are the two most common techniques used to degrade lignin. It has many other properties such as antioxidant, high thermal stability, antimicrobial behavior and biodegradability and, adhesive properties and relative abundance. Lignin shows the properties of the additives, blending and dust dispersant (MAHMOOD et al., 2016).

2.2.1 Extraction of lignin

To extract lignin from lignocellulosic material, it must undergo to a treatment, due to their recalcitrant structure, showing a great barrier to the fractionation (separation) of the biomass components (cellulose, hemicellulose and lignin) (KIM et al., 2001).

The isolation processes can be achieved using mechanical energy and/or chemical, although the former is preferred as damage to the fibers is minimized in this case. Commercial pulping and bleaching processes use alkalis, acids, organic solvents or biological agents that attack the lignin, causing its degradation and dissolution, thereby enabling separation of the cellulose fibers from the lignin (HON, 1996).

Several methods (pretreatments have been presented in the literature for the availability of lignin, highlighting treatments with acid (H_2SO_4), and with alkalis (NaOH) (CORREIA et al., 2013; ROCHA et al., 2011). Once the cellulose is separated, the lignin-rich residue is generally burnt or discarded, disregarding a more profitable exploitation of precious aromatic photosynthates (MATSUSHITA et al., 2001).

2.2.2 Lignin characterization studies

Methods of analysis of the chemical structure such as ultraviolet, infrared, ultraviolet or visible spectrometry, thermogravimetric analysis, chromatography or magnetic resonance, are also used with lignin. But they require greater care in interpreting the results because of their structural complexity when used with lignin.

The important topic to be considered in the characterization of lignin is the thermal decomposition and can be evaluated through thermogravimetric analysis (TGA). Lignin degradation is a complex process considered where thermal decomposition takes place over a wide temperature range because the various oxygen-based functional groups have different thermal stability (LAURICHESSE and AVÉROUS, 2014).

The infrared spectroscopy of the extracted lignin is a widely used technique for the qualitative characterization of lignin and its derivatives and can be used as an instrument to understand the structure and chemical groups altered, removed and/or added to it (Ramesh et al., 2004). However, there are difficulties regarding the interpretation of the infrared spectra of lignin caused by the influence of some factors, such as modifications introduced in the process of separation and structural heterogeneity.

Li et al. (2021) synthesized lignin grafted poly (ε-caprolactone) (lignin-*g*-PCL) copolymers via ring-opening polymerization of ε-caprolactone with different types of lignins of varying botanical sources and lignin extraction methods (Kraft and ethanol organosolv pulping). The structure and thermal properties of the lignin-*g*-PCL were investigated using Fourier-transform infrared spectroscopy (FTIR), 2D heteronuclear single quantum correlation (HSQC) NMR, 31P nuclear magnetic resonance (NMR), differential scanning calorimetry (DSC) AND gel permeation chromatography (GPC). They found that all the technical lignins were reactive to the copolymerization reaction regardless of their plant source and isolation methods. The molecular weights of the synthesized lignin-*g*-PCL copolymers were positively correlated with the content of aliphatic lignin hydroxyls, suggesting that the copolymerization reaction tends to occur preferentially at the aliphatic hydroxyls rather than the phenolic hydroxyls of lignin. The thermal behavior of lignin-*g*-PCL copolymers varied depending on the lignin feedstocks employed in the copolymerization reaction.

2.2.3 Current applications of lignin

Continuing technological progress means that scientists are constantly finding new solutions that make use of lignin and its derivatives (EVSTIGNEYEV et al., 2004). Lignin research and its applications have been going on for decades. Many studies have reported about the possibility to use lignin as high value product (AGRAWAL et al., 2014; LUO and ABU-OMAR, 2017) in various sectors, such as food, cosmetics, pharmaceuticals, chemicals and textiles.

The complexity and richness of its functional groups makes it attractive for converting into a variety of value added products like high performance carbon fiber, bio-oil, vanillin, and phenolic resin to name a few (BAJUA et al., 2019). Over the years lignin has been predominantly burnt as fuel for heat and power. Less than 2% of the available lignin was sold, primarily in the formulation of dispersants, adhesives and surfactants (BAJUA et al., 2019).

However, in the last decade lignin-based research and new product development has picked significant momentum due to the bio-refinery concept as aging pulp and paper mills need to diversify their products portfolio to maintain their vitality (BAJUA et al., 2019; LUO and ABU-OMAR, 2017.

Many studies on the application of lignin as an support for immobilizing lipase have been conducted in recent years. Zdarta, et al (2015), for example, demonstrated that a lignin composite could potentially serve as a lipase-immobilizing support and Zhang et al. 2014 revealed that lignin could be used as an activator to increase the activity of α-amylase and lipase.
However, these applications have not reached the industrial scale yet. (BAJUA et al., 2019).

2.3 Enzyme Biocatalysts

Enzymes are recognized as green catalysts that act in many reactions (ALI et al., 2017; LI et al., 2018). The use of enzymes as catalysts have been widely studied in recent decades and is a very interesting means for the development of the sustainable industrial chemistry: they are very selective, specific and capable to display a very high activity under very mild experimental (FERNANDEZ-LAFUENTE et al., 2009; ALVES et al., 2017).

In the drive towards green, biocatalysis affords both and sustainable technology, and it is being widely applied in the production of pharmaceuticals, commodity chemicals, and polymers (HOSSEINI et al., 2019; SHELDON and WOODLEY, 2017). It offers significant benefits for biologically mediated chemical reactions, a biodegradable catalyst, and environmentally acceptable solvent and mild reaction conditions (physiological pH and temperature) (SHELDON and RANTWIJK, 2004).

Lipases are among the groups of enzymes that stand out for the variety of reactions catalyzed in organic systems due to the high stability in these environments e with low water content, further solubility of organic substrates (BONAZZA et al., 2017; KORDEL et al., 1991).

2.3.1 Lipases

Lipases (triacylglycerol ester acylhydrolases EC 3.1.1.3) are one of the most used industrial enzymes that catalyze the hydrolysis of triacylglycerols (oils and fats) to glycerol and free fatty acids at the water/oil interface (DUARTE et al., 2016; BONAZZA et al., 2017; ALVES et al, 2017). These enzymes can also catalyze esterification,

transesterification, thiotransesterification, interesterification, oximolysis and aminolysis reactions in non-aqueous media (MENDES; DE CASTRO; GIORDANO, 2014; ALVES, et al, 2017).

Lipases are produced in high yields by several plants, animal tissues and microbial organisms (ALVES et al., 2017). They are widely used as biocatalysts in hydrolysis and synthesis reactions because of their excellent properties such as regioselectivity, stereoselectivity and chemoselectivity (ROMERO et al., 2018), in both academic and industrial levels due to its wide availability in nature and low cost (FONSECA et al., 2015).

Ferreira et al. (2019) optimized the free fatty acid production by enzymatic hydrolysis of cottonseed, olive and palm kernel oils in stirred-tank reactors using a lipase from *Geotrichum candidum* (GCL-I). Thermal stability tests and thermodynamic studies were also performed. O GCL-I exibiu a maior atividade na hidrólise de óleos vegetais, ricos em ácidos graxos insaturados (sementes de algodão e azeite).

Gama et al. (2019) proposed a novel support (Phenyl–SiO_2) via functionalization of rice husk silica with triethoxy(phenyl)silane and this functionalized support was used to immobilize lipase from *Thermomyces lanuginosus* (TLL) by physical adsorption via hydrophobic interactions. The authors reported a maximum conversion of 92% after 330 min to synthesize cetyl oleate by esterification.

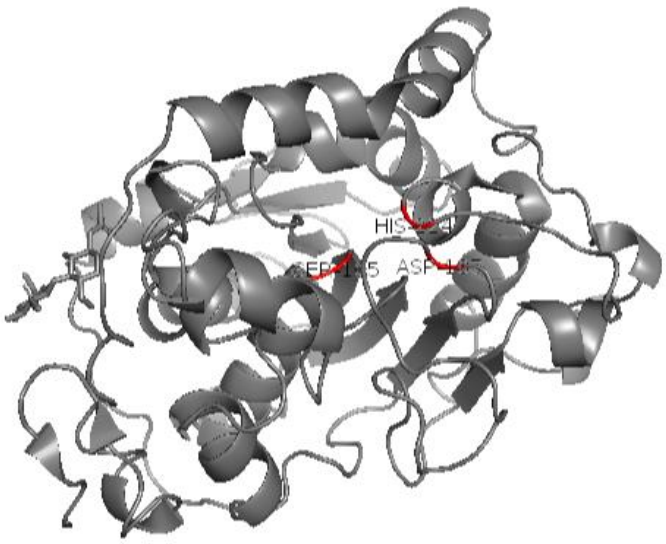
2.3.2 Lipase B from Candida antarctica

Lipase B from *Candida antarctica* (CAL-B) is commercially known as Novozym® 435, where it is immobilized on a macroporous acrylic resin (HOCK et al., 2018). It has a wide range of alkaline pH (7.0 to 10.0) in which it remains stable, but its optimum pH is 7.0 (UPPENBERG et al., 1994). It has a globular structure and consists of 317 amino acid residues, an isoelectric point (pI) of 6.0 and a molecular mass of 33 kDa (HOCK et al., 2018; UPPENBERG et al., 1994). The catalytic triad is formed by Ser105, His224 and Asp187 (Figure 4) (HOCK et al., 2018). The CAL-B surface is divided into patches that have a hydrophilic nature at the back of the enzyme and a predominant hydrophobic nature near the lipid binding site, allowing an orientation at water-lipid interfaces (HOCK et al., 2018; BASSO et al., 2007).

The CAL-B does not efficiently hydrolyse triglycerides, unlike other lipases, but it is preferred in a wide range of applications replacing industrial synthetic processes due to its stereo and enantioselectivity, thermal stability, resistance to organic solvents, and high efficiency (TANASKOVIĆ et al., 2017; RODRIGUES et al., 2008; IDRIS and BUKHARI, 2012).

CAL-B has been used through BASF to produce chiral compounds, such as the herbicide Dimethenamide-P, which was previously made chemically. The use of the immobilized enzyme has provided significant advantages over a chemical process, such as the possibility to use equimolar concentration of substrates, obtain an enantiomeric excess > 99%, use relatively low temperatures (< 60 °C) in organic solvent, obtain a single enantiomer instead of the racemate as in the chemical process (BALKENHOHL et al., 1996).

Figure 4 – CAL-B 3D Structure. Schematic representation of amino acids residues on CALB. Catalytic triad of the active site (Ser 105, Asp 187, His 224). The structure was taken from the Protein Data Bank (PDB) using PyMOL Educational. The PDB code for CAL-B is 1TCA



Source: Own author (2020).

Bourkaib et al., (2019) studied the *Candida antarctica* B lipase (CAL-B) immobilized on purified and functionalized multiwalled carbon nanotubes (MWCNTs). Were investigated Both immobilization routes, covalent bonding and physical adsorption. The enzyme loadings reached were significant: around 16 wt. % and 21 wt.% for non-covalent and covalent immobilization, respectively. Thus, it was shown that a fully green enzymatic process can be achieved with these prepared CAL-B@MWCNT biocatalyst.

2.3.3 Immobilization of enzymes

The application of enzymes on a large scale is still a difficult process due to their considerably high cost, low stability, and difficult recovery and recycling (GONG et al., 2017). Moreover, it is difficult to separate them from the reaction system which limits its recovery and reuse (ALVES, et al., 2017). However, the use of immobilized enzymes on a suitable support can be minimize these problems and also can improve their activity, specificity, and stability and to facilitate the reuse of the biocatalysts (ALVES et al., 2017).

The use of immobilized enzymes is now a routine process for the manufacture of many industrial products in the pharmaceutical, chemical and food industry. Some enzymes, such as lipases, are naturally robust and efficient, can be used for the production of many different molecules and have a wide range of industrial applications thanks to their broad selectivity (BASSO and SERBAN, 2019). For enzyme immobilization there is a large number of materials and methods (BILAL et al., 2018). It is important that their choice is carefully justified and considered taking into account the catalytic process and the specifics of the pair of enzyme-carrier components (ZAITSEV et al., 2019).

Usually, after the catalytic process, the immobilized enzymes onto solid supports can be facile removal and it will be possible to reuse for many times, and this will contribute to the reduction of the cost of industrial process (WAHBA et al., 2017; ELNASAR et al., 2010).

Different studies were carried out to evaluate the best support and immobilization strategy of lipase. For example, chitosan activated with divinyl sulfone was evaluated as a heterofunctional support for lipase B from *Candida antarctica* immobilization by Pinheiro et al. (2019).

2.4 Magnetic nanoparticles (Fe_3O_4)

Recently, magnetic nanoparticles have attracted much attention an alternative support material for enzyme immobilization (YONG et al., 2008; CHEN et al., 2009), and due to a substantial increase in their availability and versatility show to be a very important support (SOUZA et al., 2017).

The enzymes can be easily separated from the reaction medium, when they are immobilized on magnetic nanoparticles stored, and reused with reliable results (KALRA et al., 2001; PASHANGEH et al., 2017). Thus, this procedure offers a simple technique for separating and reusing enzymes longer than the use of free enzymes in the reaction (PASHANGEH et al., 2017).

It is advantageous the use of iron-based catalyst systems because iron is a naturally-occurring, abundant compound, nontoxic, readily renewable sustainable and environmentally safe. Some forms of iron oxide facilitating the removal of reactants due its magnetic properties (ARANTES et al., 2017; LUO & ZHANG, 2009).

Iron oxides, in particular magnetite, with the molecular formula Fe_3O_4 and a dark-colored, represent the magnetic particles that are most commonly associated with a polymer matrix in a nanometric scale owing to their excellent magnetic properties, such as superparamagnetic behavior at room temperature, exceptional biocompatibility of their surfaces (ZHANG et al., 2012; FURLAN et al., 2019), low toxicity compared to both metals or other metal-oxide, high chemical stability, and the facility and low cost of the procedures available for their preparation (FIGUEROLA et al., 2010; FURLAN et al., 2019).

Lima et al. (2016) studied mono and heterofunctionalized silica magnetic microparticles (SMMPs) synthetized for immobilization of lipase B from *Candida antarctica* (CAL-B). These supports allowed the immobilization of CAL-B by hydrophobic adsorption or hydrophobic/covalent linkages, achieving immobilization yield of 88% and recovered activities of 128% and 59%, respectively. The performance of the magnetic biocatalysts was evaluated in the synthesis of xylose fatty acid esters (laurate or oleate) in tert-butyl alcohol medium, yielding around 60% conversion after 48 h under optimized conditions (xylose/fatty acid molar ratio of 1:0.2, 55 ◦C, and activity load of 37.5 U/g). The magnetic biocatalyst was used in 10 reaction cycles of 48 h at 46 ◦C maintaining high xylose conversions.

Candida antarctica Lipase B (CAL-B) immobilized onto iron magnetic nanoparticles was evaluated by Souza et al., (2017) as biocatalyst for the synthesis of flavor esters. Methyl and ethyl butyrate were synthesized by esterification of butyric acid with methanol and ethanol, respectively, in a medium containing solvent. The maximum conversions of methyl butyrate and ethyl butyrate were higher than 90 %. The synthesis of flavor esters was also conducted by using Novozym® 435, a commercial catalyst, for comparison purposes.

Monteiro et al., (2019) studied lipase A from *Candida antarctica* (CALA) immobilized by covalent bonding on magnetic nanoparticles coated with chitosan and activated with glutaraldehyde (CALA-MNP), (immobilization parameters: 84.1% $\pm$ 1.0 for immobilization yield and 208.0 $\pm$ 3.0 U/g $\pm$1.1 for derivative activity). The immobilized biocatalyst showed a half-life 8–11 times higher than that of the soluble enzyme at pH 5–9. The immobilized enzyme was more active than the free enzyme at all studied pH values, except pH 7.

4.5 CONCLUSION

The synthesis of a nanomagnetite composite with lignin extracted from cashew apple bagasse was efficiently attained via a simple method that can potentially enable the upscaling process for industrial applications of this material. The investigated magnetite-lignin composite demonstrated a high thermal stability and good magnetic properties. CAL-B immobilized on MNs/Lig-activated glutaraldehyde was the most stable ($t_{1/2}$ > 480 min) among those studied. Under the evaluated conditions, conversions of 88.2% of ethyl oleate and 76.7% of 2-ethylhexyl oleate were reached after 24 h of reaction performed in a solvent-free system by MNs/Lig_Tri_CALB. The biocatalyst prepared in this study also exhibited satisfactory reusability in esterification reaction cycles for the five cycles evaluated. Therefore, the new strategy of obtaining a new biocatalyst from a synthesized composite (MNs/Lig) may be a promising route for the enzymatic immobilization of lipases, in addition to being considered environmentally benign. It shows promising use in a variety of reactions of industrial interest, such as in the synthesis of biolubricant through solvent-free reaction. The immobilized lipase could be easily recovered by using an external magnetic field, allowing for the recycling of the biocatalyst for five times, with no significant loss of enzymatic activity.

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