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Universidade Estadual Paulista
“Júlio de Mesquita Filho”

Faculdade de Ciências Farmacêuticas - FCF

Fernando Roberto Paz Cedeño

Imobilização de celulases e xilanases em óxido de grafeno magnetizado: avaliação cinética e potencial da hidrólise sobre bagaço de cana-de-açúcar

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Fernando Roberto Paz Cedeño

Thesis presented to the Graduate Program in Food and Nutrition to obtain the title of Doctor in Food and Nutrition.

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AUTOR: FERNANDO ROBERTO PAZ CEDEÑO

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Juan 16:23-24

RESUMO

O fracionamento de materiais lignocelulósicos é uma etapa fundamental para a utilização desses recursos na produção de bioprodutos de interesse comercial, como por exemplo, os biocombustíveis; no entanto, na etapa de hidrólise enzimática são encontrados problemas com a estabilidade de enzimas e seu custo. Uma estratégia para contornar este problema é a imobilização de enzimas em suportes sólidos com o objetivo principal de reutilizá-las. **Objetivo:** O objetivo deste trabalho foi estudar a imobilização de celulases e xilanases em óxido de grafeno magnético (OG-NPM) e aplicar o biocatalizador obtido na hidrólise de bagaço de cana-de-açúcar. **Métodos:** O bagaço de cana-de-açúcar foi pré-tratado em diferentes condições. As biomassas obtidas foram caracterizadas quimicamente e estruturalmente. O óxido de grafeno (OG) foi obtido pelo método tradicional de *Hummer's* e a adição de nanopartículas magnéticas foi realizada por co-precipitação de sais de ferro, obtendo o OG-NPM, o qual foi caracterizado estruturalmente. A imobilização de enzimas no OG-NPM foi realizada com os reagentes 1-etil-3-(3-dimetilaminopropil) carbodiimida e N-hidroxissuccinimida (NHS) obtendo-se o derivado (OG-NPM-Enz). O OG-NPM-Enz foi avaliado quanto à estabilidade térmica, de armazenamento, além dos efeitos da temperatura e pH na atividade enzimática. **Resultados:** O bagaço de cana-de-açúcar pré-tratado com sulfito-NaOH (SSB) apresentou maior remoção da lignina, mantendo-se a fração celulósica intacta. Além disso, o SSB apresentou a melhor resposta à hidrólise enzimática de celulose e xilana, em comparação aos bagaços submetidos a outros pré-tratamentos, atingindo conversões de 90%. O OG-NPM-Enz apresentou atividade relativa de endoglucanase, xilanase, β-glucosidase e β-xilosidase de 70%, 66%, 88%, e 70%, respectivamente, após 10 ciclos de atividade de seus respectivos substratos, resultando na maior frequência de *turnover* ($g.g^{-1}.h^{-1}$) quando comparado com reportados na literatura. O tempo de meia-vida ($t_{1/2}$) das enzimas imobilizadas foram superiores do que as enzimas em sua forma livre, com exceção de endoglucanase. Após 45 dias de armazenamento refrigerado, o OG-NPM-Enz apresentou atividades enzimáticas relativas superiores a 65% para todas as enzimas avaliadas. A taxa de hidrólise do SSB utilizando enzimas em suas formas livres foi maior quando comparado com as enzimas imobilizadas, porém, após 72h de hidrólise, as conversões de celulose e xilana em glicose e xilose, respetivamente, foram semelhantes com o uso de enzimas em suas formas livre ou imobilizada. O OG-NPM-Enz foi reutilizado com sucesso em vários ciclos de hidrólise do SSB, resultando em uma eficiência de aproximadamente 80% após o último ciclo. **Conclusão:** Os resultados mostram que a imobilização de celulases e xilanases melhora a estabilidade térmica das enzimas e o derivado obtido tem a capacidade de ser reutilizado na hidrólise do SSB, sendo, portanto, o OG-NPM-Enz um candidato potencial a ser aplicado, por exemplo, em processos de produção de bioetanol em escala piloto e industrial.

PALAVRAS-CHAVE: Material lignocelulósico; Pré-tratamentos; Hidrólise enzimática; Imobilização de enzimas; Óxido de grafeno; Nanopartículas magnéticas; Reutilização de derivado enzimático.

ABSTRACT

The fractioning of lignocellulosic materials is a fundamental step for the use of these resources in the production of bioproducts of commercial interest, such as biofuels; however, in the enzymatic hydrolysis stage, problems with enzyme stability and cost are encountered. An interesting strategy to overcome this problem is the immobilization of enzymes on solid supports with the aim of reusing them. **Objective:** The objective of this work was to study the immobilization of cellulases and xylanases in magnetic graphene oxide (GO-MNP) and to apply the biocatalyst obtained in the hydrolysis of sugarcane bagasse. **Methods:** The sugarcane bagasse was pre-treated under different conditions. The obtained biomasses were characterized chemically and structurally. Graphene oxide (GO) was obtained by the traditional Hummer's method and the addition of magnetic nanoparticles was carried out by co-precipitation of iron salts, obtaining the OG-NPM, which was structurally characterized. The immobilization of enzymes on GO-MNP was performed with the reagents 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide (NHS) obtaining the biocatalyst (GO-MNP-Enz). The GO-MNP-Enz was evaluated for thermal stability, storage, in addition to the effects of temperature and pH on enzyme activity. **Results:** The sugarcane bagasse pretreated with sulfite-NaOH (SSB) showed a greater removal of the lignin, keeping the cellulosic fraction intact. In addition, SSB showed the best response to enzymatic hydrolysis of cellulose and xylan, in comparison to sugarcane bagasse submitted to other pretreatments, reaching conversions of 90%. The GO-MNP-Enz showed relative activity of endoglucanase, xylanase, β-glucosidase and β-xylosidase of 70%, 66%, 88%, and 70%, respectively, after 10 hydrolysis cycles of their respective substrates, resulting in the greatest frequency turnover ($g.g^{-1}.h^{-1}$) when compared to those reported in the literature. The half-life ($t_{1/2}$) of immobilized enzymes was longer than the enzymes in their free form, with the exception of endoglucanase. After 45 days of cold storage, GO-MNP-Enz showed relative enzymatic activities greater than 65% for all evaluated enzymes. The hydrolysis rate of SSB using enzymes in their free forms was higher when compared to immobilized enzymes, however, after 72 h of hydrolysis, the conversions of cellulose and xylan into glucose and xylose were similar with the use of enzymes in their free or immobilized forms. GO-MNP-Enz was successfully reused in several cycles of SSB hydrolysis, resulting in an efficiency of approximately 80% in the last cycle. **Conclusion:** The results show that the immobilization of cellulases and xylanases improves the thermal stability of the enzymes and the biocatalyst obtained has the ability to be reused in the hydrolysis of the SSB, therefore, GO-MNP-Enz is a potential candidate to be applied, for example, in bioethanol production processes on a pilot and industrial scale.

KEYWORDS: Lignocellulosic material; Pretreatments; Enzymatic hydrolysis; Enzyme immobilization; Graphene oxide; Magnetic nanoparticles; Reuse of enzyme biocatalyst.

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SUMMARY

1. INTRODUCTION

Biofuels represent a topic of great global repercussion due to the current dependence on oil products for energy production and its consequence on the planet's climate changes. In this scenario, Brazil has an excellent opportunity to operate in new energy matrixes associated with the production of fuels and chemicals through renewable sources.

Sugarcane production in Brazil in the 2019/2020 period was around 643 million tons [1]. Sugarcane bagasse (SCB) is an abundant agricultural by-product from sugar-ethanol processing. SCB is usually burned / oxidized by plants for cogeneration of electricity. However, several plants do not have a thermoelectric plant (energy cogeneration) with the capacity to burn / oxidize all the SCB generated in the industry. Thus, excess SCB (around 20% of the amount) can be used for the production of different bioproducts from fractionation by chemical-enzymatic techniques. Several studies show that SCB is an economical and sustainable biomass for obtaining value-added products, such as bioethanol [2–6], biodiesel [7], biobutanol [8,9], biohydrogen [10–12], xylitol [13–16], citric acid [17], succinic acid [18], itaconic acid [19], lactic acid [20], butyric acid [21], gluconic acid [22], furfural [23], oligosaccharides [24–26], reducing sugars [27–29], among others.

Although SCB contains enough cellulose and hemicellulose to be used as a source of sugars for the production of bioproducts, such as bioethanol, this by-product is a highly recalcitrant lignocellulosic material and requires an efficient pretreatment step to guarantee a high conversion of cellulose to glucose, in a second step of enzymatic hydrolysis [30–32]. The recalcitrance

of lignocellulosic materials is related to several factors, including the close association of cellulose with hemicellulose and lignin in the cell wall, making it difficult for enzyme infiltration and action [33].

Biocatalysis processes have been applied in several sectors of biotechnology due to their high specificity, ease of production and conservation of the environment. The use of enzymes in industrial applications may be limited depending on their cost. In addition, maintaining the structural stability of some enzymes during any biochemical reaction is a major challenge [34]. According to Aragon (2013), the immobilization of enzymes in solid materials offers many advantages, among which the reuse of the enzyme, the separation of the product and the increase of enzymatic stability.

Graphene oxide (GO) is a highly versatile chemical platform due to the large surface area it offers, in addition to several chemical groups located on its surface. In this way, GO is a great support for immobilizing enzymes [36]. Recently, some work on GO magnetization, prior to its use as a support for enzyme immobilization, has been carried out, showing great potential for application [37,38].

Sugarcane Bagasse (SCB): main constituents and pre-treatments

Brazil, one of the largest agricultural producers in the world, generates significant amounts of biomass by-products in activities resulting from harvesting and processing products such as rice, cotton, sugar cane, corn and soybeans. Agricultural by-products come from the stage of cultivation of certain species, while agro-industrial by-products result from industrial

biomass processing [39]. Three different solid residues are produced in the processing of sugarcane: straw (during harvest), SCB and filter cake (in the ethanol process) [40,41]. Agro-industrial by-products, such as SCB, have a highly complex structure and chemical composition. The microstructure is linked to low molecular weight substances that include organic and inorganic substances. However, the macrostructure comprises macromolecules: cellulose, hemicellulose and lignin [42]. It is important to emphasize that the components of BCA are closely associated, in order to constitute the cell complex of plant biomass.

Cellulose

The cellulose structure can be classified into three organizational levels. The first is defined by the sequence of cellobiose residues linked by covalent bonds, forming the anhydroglycoside homopolymer that contains glycosidic bonds of the type β-D(1→4) (Figure 1.1), general formula $(C_6H_{10}O_5)n$.

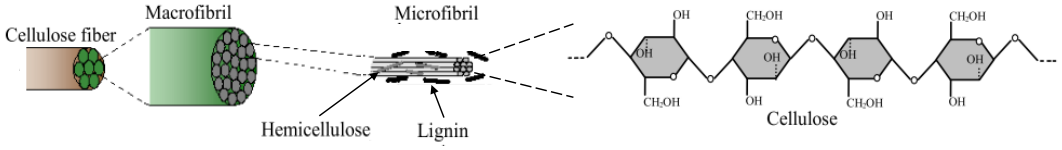

Figure 1.1. Cellulose molecule structure [43].

Figure 1.2 shows the second level that describes the molecular conformation, that is, the spatial organization of the repetitive units, being characterized by the lengths of the bonds and respective angles and by the intramolecular and intermolecular hydrogen bonds. The third level defines the association of molecules, forming aggregates with a given crystalline structure.

These aggregates provide high resistance to tension, making cellulose insoluble in water and in a large number of organic solvents. [44,45].

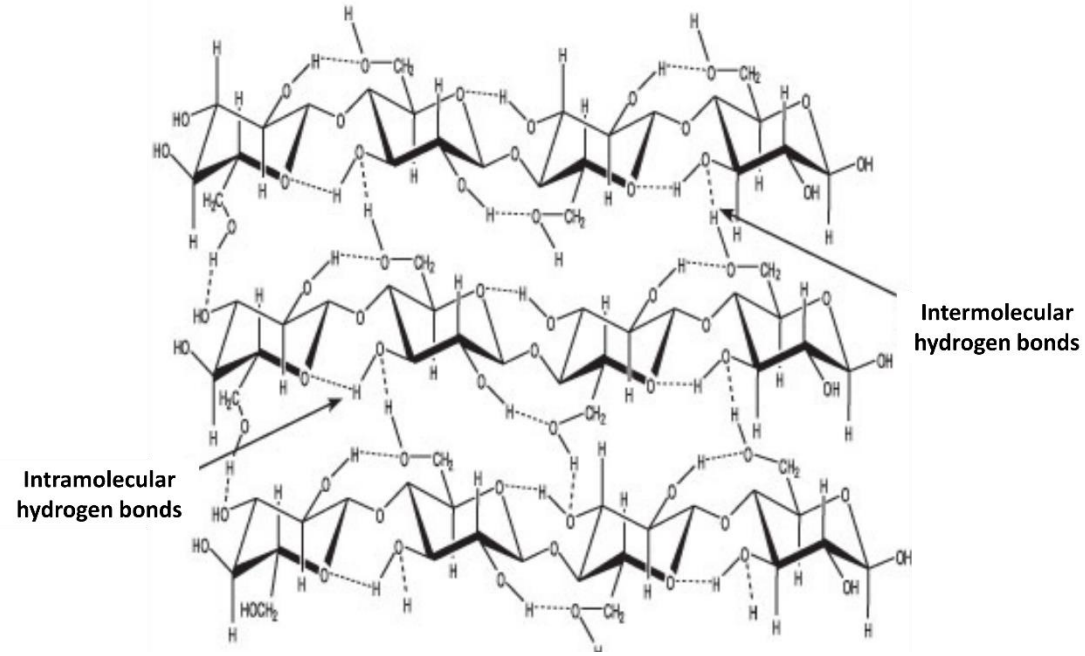

Figure 1.2. Representation of cellulose supramolecular hydrogen bonds [44].

Hemicellulose

Hemicelluloses, also called polioses, are polysaccharides that make up the cell wall of plants, which occupy the second most renewable polymer in lignocellulosic materials, after cellulose. They represent a type of polysaccharide with a lower degree of polymerization (100-200 units) containing pendent groups. The complex structure contains xylose, glucose, mannose, galactose, arabinose, rhamnose, glucuronic acid and galacturonic acid as constituents, in varying amounts, depending on the plant source. [44,46–48]. Hemicelluloses are normally covalently linked to other components of the cell wall of lignocelluloses such as lignin and phenolic compounds [48].

The main hemicelluloses found in plants are xyloglucans (XyG), glucuronoarabinoxylans (GAX) and mannans (MN). Most plants have xyloglucan as the main hemicellulose. However, grasses have glucuronoarabinoxylans (GAX) as their main hemicellulose (Figure 1.3) [48], although they also have, in small proportions, xyloglucans and mannans. In addition to GAX, β-glucans are also found in sugarcane tissues [48,49].

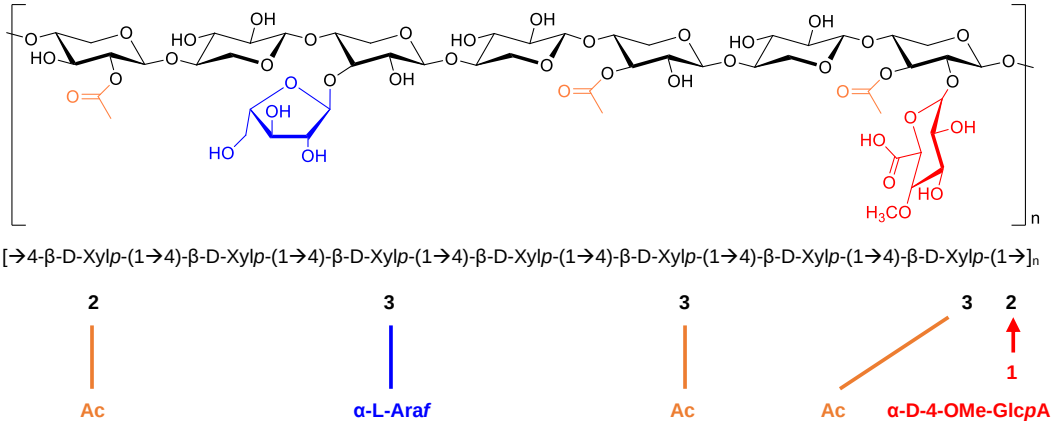

Figure 1.3. Schematic representation of the general structure of the glucuronoarabinoxylan molecule (GAX). Adapted from Ek et al. [50].

Lignin

Lignin is a complex amorphous macromolecule made up of phenylpropane units derived from three basic monomeric units: p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) that vary in quantity between species and according to the type of tissue in the cells. It is formed by the dehydrogenative polymerization of hydroxycinnamic alcohols (p-coumaryl, coniferyl and synaphyl). Because the polymerization process is random, the lignin macromolecule has a very complex structure, as shown in the model in Figure 1.4 [51].

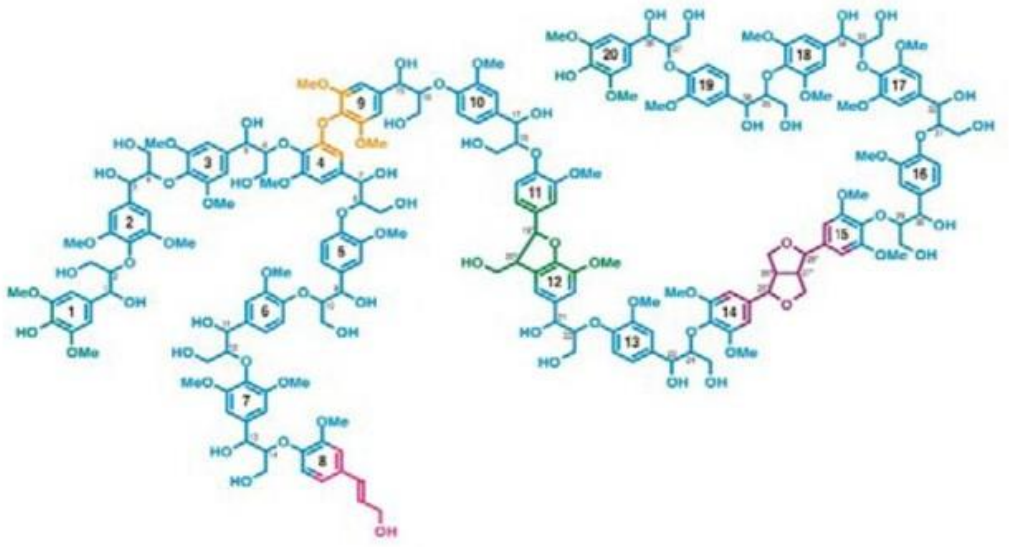

Figure 1.4. Proposed lignin structure model [51].

The structure of lignin is not homogeneous since it has amorphous regions and a globular structure [44,52,53]. The lignin structure hypothesis arises from the polymerization of phenyl radicals (β-O-4 ether bond, which are the most common bonds) formed on the cell wall by oxidative enzymes. Hardwood lignins are predominant in G and S units with traces of H units. Coniferous wood lignins (softwood) are mostly composed of G units [52,54], whereas monocotyledons or grasses (examples: corn straw, straw and SCB) incorporate equivalent amounts of G and S units, together with significantly higher amounts of H units [52].

Lignin in grasses is closely linked to hemicellulose through ferulic and p-cumaric acids. These phenolic acids are linked to the carbon 5 of an arabinose molecule, by an ester-type bond (Figure 1.15). Arabinose (pendant group) is coupled to hemicellulose (xylan) through an ether bond (Figure 1.5) [55].

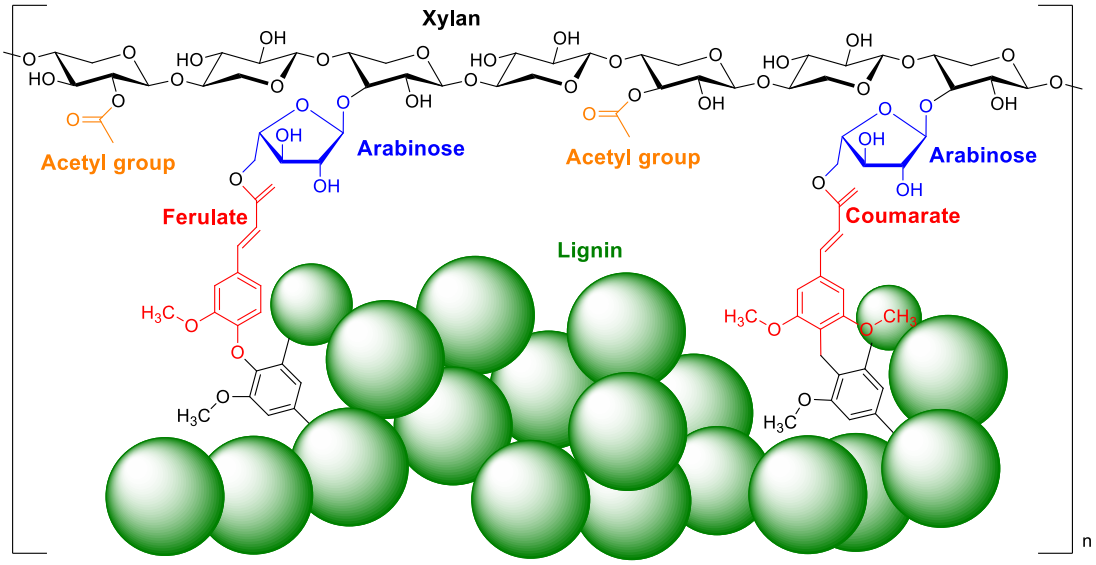

Figure 1.5. Scheme showing residues of ferulate and coumarate coupled to sugarcane bagasse lignin. Adapted from Hatfield et al. [55].

Lignin is connected to phenolic acids through propanoic chains on α-carbon, these bonds being ether-type. Phenolic acids are coupled to lignin via radical processes [55]. However, ferulic and p-cumáric acids are only coupled to monomers (coniferyl and synaphyl alcohols) and not to lignin oligomers, their main function being to act as nucleation points in the lignin biosynthesis in grasses [55]. Xu *et al*., [56] reported that BCA has approximately 1.2-1.3% ferulic acid and 1.6-1.8% p-cumaric acid.

Pretreatments

Pretreatment is one of the most important steps in the conversion of SCB, as it directly affects the efficiency of enzymatic hydrolysis. The main objective of pre-treatment is to cleave the recalcitrant structures of lignin in SCB making cellulose and hemicellulose more accessible to enzymes for efficient conversion into fermentable sugars [57,58]. The efficiency of lignin

removal depends on the type of pre-treatment used and the ideal conditions maintained during it. Pretreatments can be physical, chemical, biological or can be a combination of these methods. Although different pretreatments are available, developing pretreatment with or without little inhibitor formation is a challenge [59].

In the industrial sector, acid hydrolysis is considered the most prominent pre-treatment method. It is usually carried out with diluted solutions of mineral acids, organic acids and sulfur dioxide. The acid cleaves the glycosidic bonds of the polysaccharides which results in the formation of reducing sugars. The most commonly used acids are: sulfuric acid, nitric acid and hydrochloric acid [60].

Alkali-based pretreatments are also widely used to increase cellulose digestibility and remove lignin. Sodium hydroxide and potassium hydroxide are most commonly used, while some other alkalis, such as calcium hydroxide and ammonia, can also be used. This is a delignification process in which part of the lignin and hemicellulose are solubilized. The main reactions of the alkaline pretreatment are: abstraction of a proton from a free phenolic OH found in the aromatic rings of the lignin molecule resulting in the formation of a structure called methylene quinone, which favors the cleavage of the α-aryl ether bond and partial dissolution of lignin; cleavage of ester-like bonds located between hemicellulose and lignin, known as saponification, which leads to the separation of the lignin-carbohydrate complex, and the exposure of cellulose microfibrils increasing the enzymatic digestibility of cellulose [57,58].

Sulphonation of lignin by the action of bisulfite ions (HSO_3^-) is another widely used pretreatment. This process is usually conducted with sodium sulfite and can be in acidic or alkaline conditions. The reaction between a phenylpropane and bisulfite unit under acidic conditions proceeds through protonation of the hydroxyl group followed by the removal of water and addition of the bisulfite ion (Figure 1.6a). In neutral or alkaline conditions, the mechanism is analogous to the reaction in kraft pulping, through the formation of a methylene quinone intermediate followed by the addition of a sulfite ion to the α carbon, making the phenolic OH again protonated and the sulfonated α carbon. The chemical reaction proceeds with the cleavage of the β-aryl-ether bond, between the two aromatic rings culminating in the β-carbon sulfonation and lignin degradation (Figure 1.6b) [61].

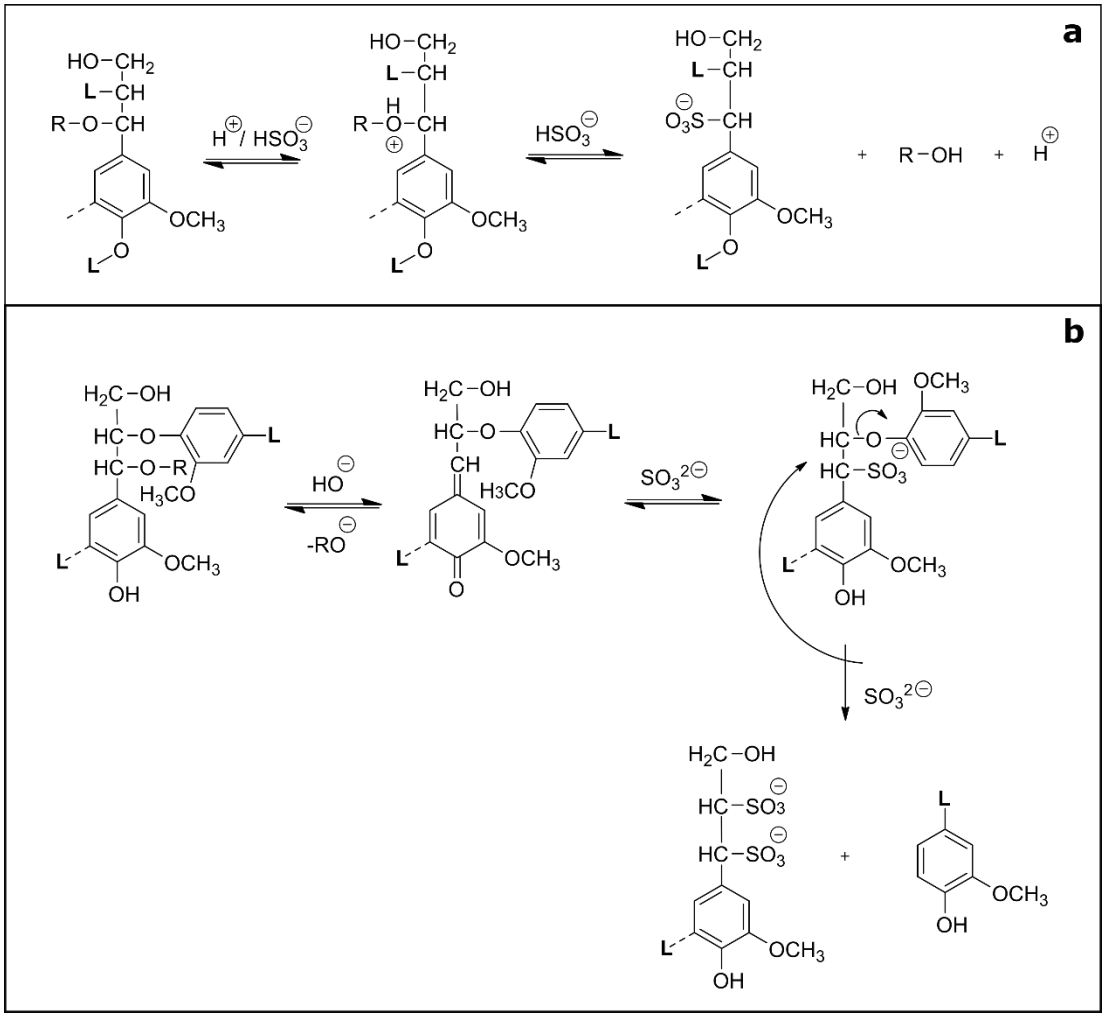

Figure 1.6. Mechanism of lignin sulfonation in pre-treatment in the presence of sulfite / bisulfite ions in (**a**) acidic and (**b**) alkaline conditions [61].

Hydrothermal pretreatment is a pretreatment method in which water as a liquid phase or vapor phase is used. There is no need to add catalyst and alleviates causes of corrosive problems. High-pressure water penetrates directly into biomass and cellulose, removing a substantial part of the hemicellulosic fraction, altering the structure of lignin, but removing very little of this fraction, which is a disadvantage for the efficiency of enzymatic hydrolysis of post-treatment cellulose. The solubilization of hemicelluloses is catalyzed by hydronium ions resulting from the cleavage of the acetyl group (substituting the hemicellulose chain) in acetic acid, in addition to the

autoionization of water. The formation of inhibitors (acetic acid, furfural acid, formic acid, among others) can be reduced by controlling the acidity and temperature of the pre-treatment [58,62].

Enzymes of commercial interest for conversion of lignocellulosic biomass

Cellulases

Cellulases are enzymes that catalyze the hydrolysis of glycosidic bonds of the β-1,4 type present in the cellulose molecule. Cellulases are enzymes applied in the industrial sector and play an important role in the global carbon cycle. They are very diverse in terms of their structure and mode of action. They can be produced by plants, fungi, some bacteria and some animals [63].

Cellulases are currently the third category of industrial enzyme most commercialized worldwide, due to its application in cotton processing, paper recycling, as detergent enzymes, in juice extraction and as additives for animal feed. However, cellulases could become the most commercialized enzymes in the world, if bioethanol, biobutanol or other biofuels obtained by fermenting sugars, become an important transport fuel [64]. Currently, industrial cellulases are almost all produced from fungi of the genus *Trichoderma, Humicola and Aspergillus* [65–68]. This is due to the ability of these organisms to produce extremely high amounts of cellulases with relatively high specific activity and the ability to genetically modify these strains to adjust the set of enzymes they produce, in order to provide an optimal activity for specific uses. [64].

There are three different types of cellulases: endocellulase (EC 3.2.1.4) (also known as endoglucanases), exoglucanase (EC 3.2.1.91) (also known as cellobiohydrolases) and cellobiase (EC 3.2.1.21) (also known as 1,4-β-D-glycosidases or β-glycosidase). All catalytic domains of endocellulases have an open active site and are able to bind to the inside of cellulose molecules and randomly cleave the inside of the chain. In contrast, all exoglucanases have their active sites located in a tunnel. There are two classes of exoglucanases: the first class cleaves the non-reducing end of a cellulose molecule, releasing cellobiose residues. All known exoglucanases in this class are in the GH-6 family. The exoglucanases of the second class cleave the reducing end of the cellulose chain, releasing cellobiose residues. Fungal exoglucanases in this class are in the GH-7 family, while bacterial exoglucanases are in the GH-48 family. [63]. Finally, celobiase is in charge of hydrolysing cellobiosis into glucose. For an efficient hydrolysis of cellulose, the synergistic action of cellulases is necessary (Figure 1.7).

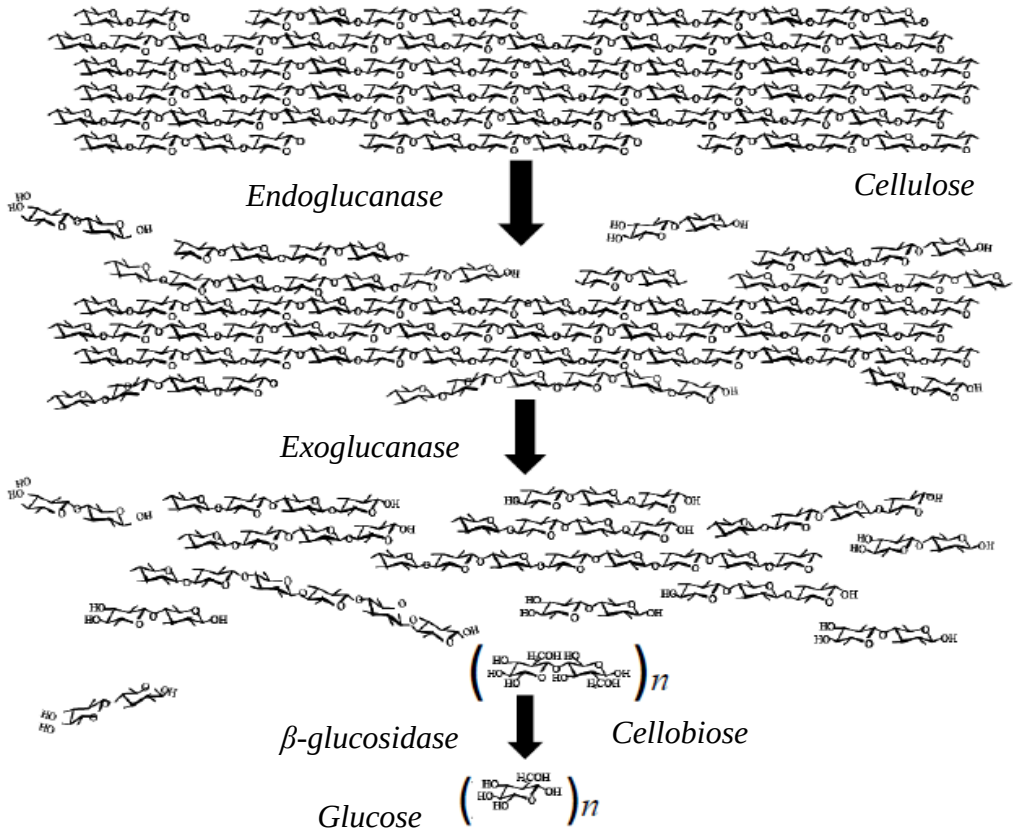

Figure 1.7. Mode of action of enzymes of the cellulolytic complex of *Trichoderma reesei* [69].

Xylanases

Xylanases catalyze the hydrolysis of xylan, which is the second most abundant polysaccharide in hardwoods and grasses and an important component in plant cell walls. There are two enzymes, endoxylanase (EC 3.2.1.8) (also called xylanase) that hydrolyzes the inner part of the xylan chain and 1,4-β-D-xylosidase (EC 3.2.1.37) that hydrolyzes the xylobiosis dimer. These enzymes are produced mainly by microorganisms like bacteria and fungi [66,70–73]. Enzymes participate in the degradation of plant cell walls (together with other enzymes that hydrolyze polysaccharides) and also hydrolyze xylan during the germination of some seeds, for example, in malting

the barley grain. Xylanases can also be found in seaweed, protozoa, crustaceans, insects, snails and seeds of terrestrial plants [72].

The main industrial applications of xylanases are found in the biofuels, cellulose and paper, food and beverage and animal nutrition sectors [74–80]. Due to their biotechnological characteristics, xylanases are most often produced from microorganisms.

Enzyme immobilization

Biocatalysis processes have been widely applied in several sectors of biotechnology due to their high specificity, ease of production and conservation of the environment. The use of enzymes in large quantities is limited depending on the costs of these. In addition, maintaining the structural stability of some enzymes during a catalysis is a major challenge. In order to overcome these limitations, the immobilization of enzymes appears as a promising technique [34]. According to Aragon [35], for practical applications, the immobilization of microorganisms or enzymes in solid materials offers many advantages, among which the possibility of reusing the enzyme, easy separation of the product and increasing the stability of the enzyme. The term "immobilized enzymes" refers to enzymes that are physically confined or located in a specific region of space with retention of their catalytic activities and that can be used repeatedly and continuously [81]. Various materials can be used as a matrix or support system for enzymatic immobilization, usually inert polymers and inorganic materials. The ideal support matrix should have the following properties: inertia, stability, physical strength, ability to increase specificity / activity of the

enzyme, regenerability, ability to reduce product inhibition, ability to prevent unspecific adsorption and bacterial contamination, be inexpensive [34]. Most matrices have only some of the properties mentioned, therefore, the selection of the support matrix for enzyme immobilization should be chosen based on their properties and limitations.

The functions and properties of an immobilized enzyme depend on its own characteristics, the immobilization system and the support material [82]. The characteristic parameters of these three components collectively determine the properties of the immobilized enzyme system. Theoretically, an ideal immobilization method should provide the best immobilization yield, maintaining long-term activity and stability [83]. An enzymatic immobilization method can be based on a chemical reaction or physical adsorption [84]. There are different methods of enzyme immobilization and several factors that affect the performance of immobilized enzymes. The different methods of enzymatic immobilization are grouped as follows: physical adsorption, affinity adsorption, covalent bonding, trapping, encapsulation and cross-linking.

Covalent bonding, one of the most studied immobilization methods, involves the irreversible coupling of enzymes to an appropriate support. This method, therefore, is the most stable form of immobilization. There is a wide range of chemical bonding mechanisms and water-insoluble support materials that can be functionalized for covalent immobilization of enzymes. This wide range of options provides great flexibility in designing an immobilized enzyme system with specific desired properties. Physical parameters such as load distribution, hydrophobic / hydrophilic ratio and separation of the spacer arm

can be conveniently modified [84]. The covalent binding of enzymes depends heavily on the chemical properties of the support materials and on the natural or grafted functional groups in the enzyme molecules. Thus, to ensure efficient enzymatic recovery by covalent immobilization, the binding reaction conditions must not compromise the enzymatic activity. Despite the strong covalent bond, this type of immobilization has some disadvantages, for example, cost of the method and loss of enzyme activity due to enzymatic rigidity that can result from the multipoint bond [85,86].

Graphene oxide (GO)

Graphite oxidation has been studied for more than a century, with the study of B.C. Brodie in 1859 being available in the literature as the first example [87]. Typically, graphite reacts with strong oxidizing agents, such as potassium permanganate ($KMnO_4$) and concentrated sulfuric acid (H_2SO_4), followed by purification and exfoliation in water, which results in a yellow colloidal dispersion, as reported by Hummers [88]. Graphite oxide has a structure in the form of layers of atomic thickness similar to that of graphite, but the plane of the carbon atoms in graphite oxide is linked to several groups that contain oxygen, which not only expand the distance between the layers, but they also make them hydrophilic. These oxidized layers can be exfoliated in water or organic solvents under sonication. When the exfoliated material contains only one or a few layers of carbon atoms, such as graphene, this material is called graphene oxide (GO) [89]. Thus, GO is a single atomic layer material that comprises molecules of carbon, hydrogen and oxygen obtained

by oxidizing graphite crystals that are cheap and abundant, being dispersible in water and easy to process. (Figure 1.8). In addition, GO can be (partially) reduced to graphene by removing oxygen-containing groups. In this way, recovering a conjugated structure. The reduced GO can be considered a type of chemically derived graphene [90].

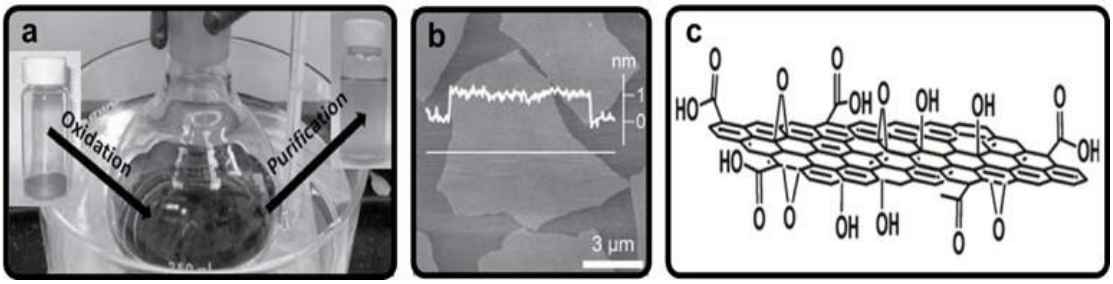

Figure 1.8. (a) Synthesis of graphene oxide (GO) (**b**) Atomic force microscopy of a GO sheet of the obtained colloidal dispersion. (**c**) Schematic model of the GO [36,91].

GO's abundance of chemical features makes it a highly versatile chemical platform for creating graphene-based materials. As shown in Figure 1.8b (the thickness profile of atomic force microscopy) the thickness of the layers of the GO is in the order of 1 nm, the thickness scale being typical of these molecules. On the other hand, the length of the GO molecule is on the order of micrometers, which is the typical length scale of colloidal particles. Therefore, depending on the length scale in question, the GO can be treated as a molecule or colloidal sheet [91].

Graphene oxide properties

One of the advantages of GO is its easy dispersibility in water and other organic solvents, as well as in different matrices, due to the presence of

oxygen molecules that act with functional groups. This property is very important when mixing GO with ceramic or polymeric matrices. The functionalization of GO can fundamentally change its physicochemical properties. The resulting chemically modified graphenes can potentially become more adaptable for many applications. There are many ways in which GO can be functionalized, depending on the desired application. For optoelectronics, bio-devices or drug-delivery systems, it is possible to replace amines with the organic covalent functionalization of graphene to increase the dispersibility of chemically modified graphenes in organic solvents [90].

Surface modification of graphene oxide

Covalent and non-covalent functionalization are the two approaches used for surface modification of carbon materials. In covalent functionalization, functional groups containing oxygen, including carboxylic groups at the extremities and epoxy / hydroxyl groups at the basal plane, can be inserted to alter the surface functionality of the GO. In addition, isocyanate treatment can be used to reduce the hydrophobicity of GO by forming amide and carbamate esters in the carboxyl and hydroxyl groups, respectively. Consequently, the isocyanate modified GO rapidly forms a stable dispersion in polar aprotic solvents, culminating in exfoliated graphene films with a thickness of 1 nm. This dispersion also facilitates the intimate mixing of the GO film with matrix polymers, providing a new synthesis path to form graphene nanocomposites and polymers [90]. Figure 1.9 shows the functionalization of graphene and its derivatives with avidin-biotin, peptides, nucleic acids, proteins, aptamers, small

molecules, bacteria and cells through physical adsorption or chemical conjugation. Functionalized graphene biosystems with unique properties have been used to build biological platforms, biosensors and bio-devices [92].

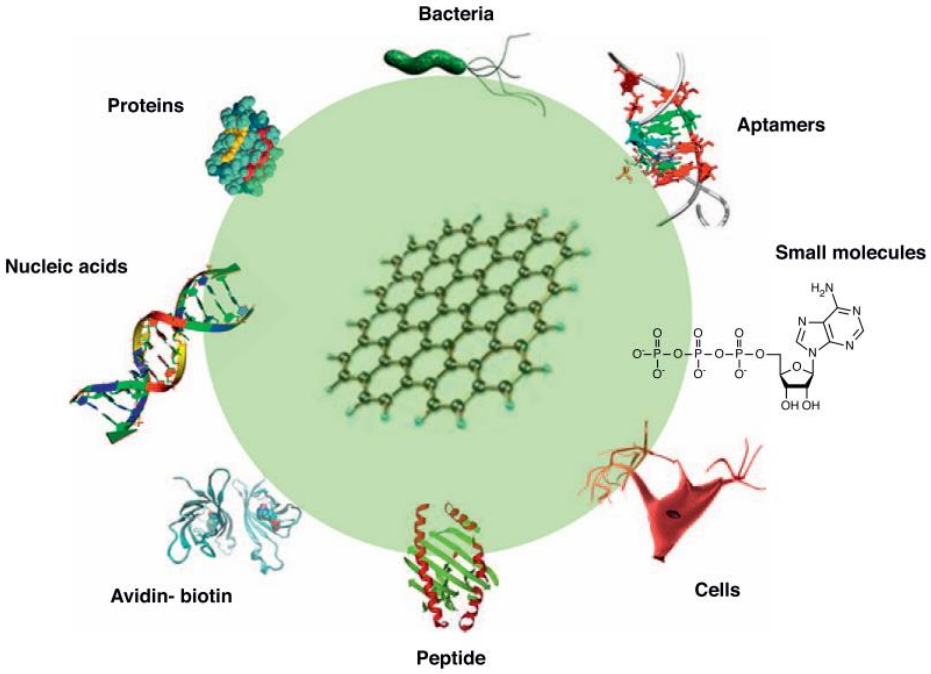

Figure 1.9. Graphene functionalized with avidin-biotin, peptides, nucleic acids, proteins, aptamers, small molecules, bacteria and cells through physical adsorption or chemical conjugation [92].

Graphene and OG have been linked with several proteins, which results in biosystems with unique properties. It was verified that the horseradish peroxidase and lysozyme can be spontaneously immobilized in an individual GO film, as it is enriched with oxygen-containing groups, which allows immobilizing enzymes without surface modifications or coupling reagents [36].

Magnetization of graphene oxide

Since the discovery of the first magnetic nanoparticles, several types of applications have been developed in drug administration [93], detection

methods [94], medicine and catalysis [95–97]. Magnetic nanoparticles (MNP) can be formed from iron oxide III (Fe_3O_4) and iron oxide II (Fe_2O_3). These nanomaterials are famous for their magnetic characteristic which, in recovery, can be quickly separated from the media by an external magnet [37]. Doustkhah and Rostamnia [37] showed that Fe_3O_4 nanoparticles can be bonded to the surface of the OG by coprecipitating Fe^{2+} and Fe^{3+} in alkaline solution over the addition of ammonia at 80 °C (Figure 1.10).

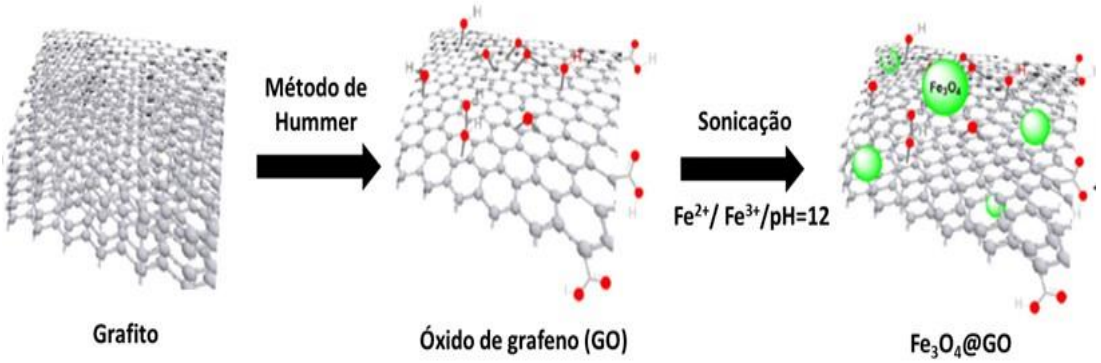

Figure 1.10. Schematic route for the synthesis of graphene oxide with magnetic nanoparticles (GO-MNP) [37].

Immobilization of enzymes in graphene oxide as a support

The incredibly large specific surface area (accessible from both sides), the abundant functional groups containing oxygen, such as epoxy, hydroxyl and carboxyl groups and the high solubility in water make GO a very promising material for many applications [98,99]. However, to date, few studies on the binding of biomacromolecules, such as enzymes, to GO have been reported in the literature. [36].

Zhang [36] et al*.,* demonstrated that GO films can be used as supports to immobilize the horseradish peroxidase enzyme. The functional groups on

the surface of the GO cause the enzyme immobilization to occur quickly through electrostatic interaction without making any previous modifications to the material. The flat surface of the GO allows to see the immobilized enzyme “in situ” using atomic force microscopy, as shown in Figure 1.11.

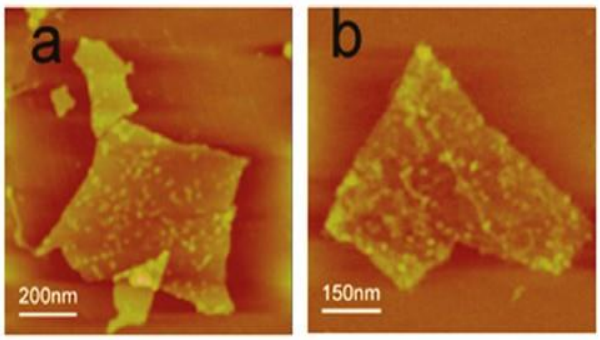

Figure 1.11. Atomic force microscopy images of horseradish peroxidase linked to graphene oxide with (**a**) low enzyme load and (**b**) high enzyme load. [36].

Heidarizadeh [38] et al., covalently immobilized porcine pancreas lipase on the surface of magnetically separable GO (GO-MNP), functionalizing its surface using (3-aminopropyl) triethoxysilane (APTES) and carbon disulfide (CS_2), as shown in Figure 1.12.

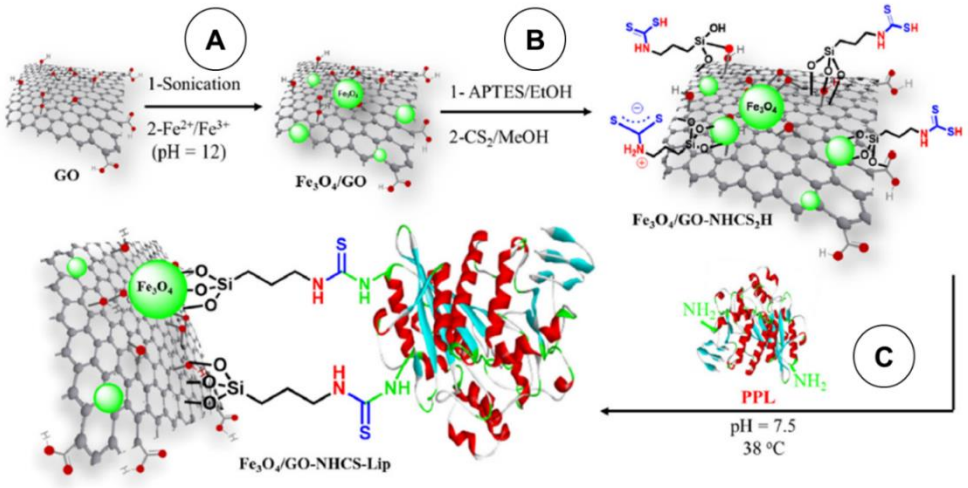

Figure 1.12. (A) Magnetization procedure and **(B)** functionalization of graphene oxide. **(C)** Lipase immobilization [38].

OBJECTIVE

The main objective of this study was to evaluate the immobilization of cellulases and xylanases in graphene oxide decorated with magnetic nanoparticles (GO-MNP) and to study the potential of hydrolysis of pre-treated sugarcane bagasse (SCB). A seguir apresentamos os objetivos específicos que foram estabelecidos para atingir a meta do estudo:

- Evaluate the effect of different pretreatments on chemical composition of sugarcane bagasse (SCB);
- Assess the effect of pretreatments in response to enzymatic hydrolysis of SCB;
- Evaluate the synthesis of magnetized graphene oxide (GO);
- Examine the structure of magnetic graphene oxide (GO-MNP);
- Assess the immobilization of cellulose and xylan enzymes on the surface of the GO-MNP;
- Determine the stability parameters of previously immobilized cellulases and xylanases;
- Evaluate the reuse of immobilized enzymes through hydrolysis of specific substrates for cellulases and xylanases;
- Assess the enzymatic hydrolysis of cellulose and xylan from SCB pretreated and the potential for reuse of the biocatalyst obtained;

2. CHAPTER 1

Evaluation of the effects of different chemical pretreatments in sugarcane bagasse on the response of enzymatic hydrolysis in batch systems subject to high mass loads

Article published in October 2020 in Renewable Energy.

DOI: 10.1016/j.renene.2020.10.092

3. CHAPTER 2

Magnetic graphene oxide as a platform for the immobilization of cellulases and xylanases: ultrastructural characterization and assessment of lignocellulosic biomass hydrolysis

Article published in September 2020 in Renewable Energy

DOI: 10.1016/j.renene.2020.09.059

4. CAPÍTULO 3

Stability of Cellic CTec2 enzymatic preparation immobilized onto magnetic graphene oxide: Assessment of hydrolysis of pretreated sugarcane bagasse

This manuscript is in the final preparation phase (final review of the discussion and English language).

5. FINAL CONSIDERATIONS

The sugarcane bagasse (SCB) was subjected to several pretreatments, of which it was possible to identify that the pretreatment with sulfite-NaOH was the one that promoted the greatest removal of lignin, considerably reducing the recalcitrance of the cellulosic fraction and hemicellulose of SCB. This allowed the enzymatic hydrolysis of SCB pretreated with sulfite-NaOH to result in better performance, both for enzymes in their free and immobilized forms.

It was possible to synthesize and characterize magnetic graphene oxide (GO-MNP) and immobilize cellulases and xylanases from the enzymatic preparation Cellic CTec 2. The biocatalyst showed a stable behavior and maintained high relative activity after 10 cycles of hydrolysis with specific substrate for endoglucanase, xylanase, β-glycosidase and β-xylosidase, with a turnover frequency higher than that reported in the literature. In addition, immobilization favored the half-life ($t_{1/2}$) of the enzymes when evaluated in their immobilized form.

The biocatalyst proved to be efficient for the hydrolysis of SCB pretreated with sulfite-NaOH, and after 4 cycles of hydrolysis, the efficiency was maintained at approximately 80%. However, when the hydrolysis of SCB pretreated with sodium chlorite was evaluated, the efficiency decreased to approximately 30%, after 4 cycles. This highlights the importance of pre-treatment for enzymatic hydrolysis.

Thus, the immobilization of cellulases and xylanases in GO-MNP by the method described in this study, proved to be technically feasible, in addition to

improving the thermal stability of the enzymes and promoting high hydrolysis efficiency after several cycles.

Collaborations

During the development of this research project, the doctoral student did an internship at the MackGraphe Graphene and Nanomaterials Research Center at Mackenzie Presbyterian University under the supervision of Professor Dr. Ricardo Keitel Donato. In addition, the doctoral student carried out an internship abroad, which allowed to expand collaboration with the research group on hybrid materials of the Institute of Chemical Technology of the Universitat Politècnica de València (ITQ-UPV). This led to the signing of a collaboration agreement between UNESP and the ITQ-UPV published in the *Diário Oficial do Estado de São Paulo* on December 22, 2020 (*Convênio 2100.0243-TA*).

Finally, it is noteworthy that, besides the published articles, this study generated the request of an application for patent entitled: *Processo de imobilização simultânea de celulases e xilanases sobre óxido de grafeno magnético e uso do mesmo*. The request was made on a joint basis between UNESP and ITQ-UPV. The request received a positive patentability opinion by the UNESP Innovation Agency (*Agência de Inovação* - AUIN) (code: 19CI202) and currently awaiting deposit at the National Institute of Industrial Property (*Instituto Nacional da Propriedade Industrial* - INPI).

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