From Wastepaper to sugars using immobilized enzymes

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Biotechnology

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Abstract

Lignocellulosic materials are widely dispersed through the environment and they are available in so many sources, in such large quantities that it would contribute heavily to our sustainability if we could use them to our benefit. The main focus of this thesis lies on the most valuable component of these materials – cellulose. By using cellulose-rich compounds, such as print paper, as substrate, a process was designed for the utilization of cellulolytic enzymes in a controlled environment to hydrolyze cellulose into glucose. Moreover, this work has demonstrated that these enzymes can be entrapped in a hydrogel support, which allows for their reusability.

A few sets of hydrogel particles (LentiKat®) with the enzymes immobilized separately (cellulase and β-glucosidase) were employed in the hydrolysis of several cellulose-rich substrates such as CMC, filter paper and print paper, revealing that the enzymes not only retained their hydrolytic abilities, but also achieved good glucose yields. In optimal conditions, the immobilized enzymes were able to convert 38% of cellulose present in the mixture, producing 1.9 g/L of glucose, while free enzymes were able to completely hydrolyze cellulose to glucose (5.3 g/L) in 23 hours. This demonstrates that the enzymes retain some hydrolytic capacity.

The same strategy was applied with higher volumes of media in an attempt to simulate a larger reactor with mechanical agitation. The ultimate goal would be to efficiently hydrolyze progressively larger amounts of cellulose for several runs, by recycling the biocatalyst.

KEYWORDS: Enzyme immobilization; Hydrogel particles; Entrapment; Cellulose hydrolysis; Reusable biocatalyst.

Resumo

Materiais lignocelulósicos estão amplamente disperses através do ambiente e estão disponíveis em tantas fontes e em tão grandes quantidades, que seria uma contribuição enorme para a sustentabilidade do planeta se de alguma forma pudessem ser aproveitadas. O foco principal desta tese consiste em utilizar compostos ricos em celulose, como resíduos de papel como substrato. Com esse intuito, foi desenvolvido um processo para utilização de enzimas celulolíticas, num ambiente controlado, para hidrolizar celulose a glucose. Para além disso, este trabalho demonstra que estes enzimas podem ser encapsulados em partículas de hidrogel, que promove a sua reciclagem, podendo ser re-utilizados.

Durante este trabalho, utilizando a tecnologia LentiKat®, este tipo de partículas foram produzidas para encapsular celulase e β-glucosidase, separadamente. Estas partículas foram posteriormente utilizadas na hidrólise de substratos ricos em celulose como, a CMC, papel de filtro e resíduos de papel, revelando, não só que as enzimas retêm a sua capacidade catalítica, mas também conseguiram gerar boas quantidades de glucose. Em condições óptimas, os enzimas imobilizados foram capazes de converter 38% da celulose presente no meio a glucose, produzindo 1.9 g/L da última. No entanto, o enzima livre foi capaz de hidrolizar totalmente a celulose presente no meio durante as 23 horas, resultando em 5.3 g/L de glucose.

Esta estratégia foi aplicada a maiores volumes de meio reacional numa tentativa de simular um reator maior com agitação mecânica. O objectivo final seria hidrolizar, de forma eficiente, quantidades progressivamente maiores de cellulose durante várias reações, reciclando o biocatalizador encapsulado.

PALAVRAS-CHAVE: Imobilização enzimática; Partículas de hidrogel; Encapsulamento; Hidrólise da cellulose; Biocatalisador reutilizável.

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List of Abbreviations

CMC – Microcrystalline cellulose

FP – Filter paper

PP – Print paper

PEG 600 – Poly (ethylene glycol) (~600 molecular weight)

DNS - 3,5-Dinitrosalicylic acid

PVA – Polyvinyl alcohol

Chapter 1 – Introduction

1.1 A bio-catalyst approach to Cellulose degradation

As it is well-known, cellulose holds in its constitution a very valuable component called glucose. Glucose is the monomer of the polysaccharidic chain of cellulose and has a fairly large broad spectrum of applications. In essence, this sugar is widely used for cell growth, working as a source of carbon. In a way, this product is not quite easy to extract from cellulose, mainly because it is usually associated with other components such as lignin and hemicellulose, which make up most lignocellulosic materials. The materials containing cellulose are usually recyclable and may vary in their cellulose content (will be discussed in higher depth in **Chapter 2**).

Cellulose poses as a great source of sugars that could be used in fermentative process to generate other products of relevant use in several areas. With this in mind, there is the inherent drive to degrade this compound in order to have a useful product that could have potential economic value. As will be mentioned throughout the work, there are several methods to degrade cellulose, such as chemical, mechanical and enzymatic (**Chapter 2**). In this particular experiment, the focus is enzymatic degradation of cellulose, using cellulolytic enzymes such as cellulase and β-glucosidase. Additionally, to facilitate the possible recovery of the product and also to ensure minimal enzyme expenses (since the enzymes are costly and are the main economic drawback of the process), the decision of immobilizing the enzymes in a hydrogel particle was more than necessary. The immobilization is a fairly simple process but does not come without several disadvantages that will be discussed in **Chapter 4**.

Using an enzymatic mixture to degrade a complex substrate always reveals itself an arduous task due to the number of variables that must be controlled to achieve optimal reaction conditions and outcome. In this experiment, some variables such as pH, temperature, agitation, concentrations of enzyme and substrate, type of substrate among others, were studied in an attempt to optimize the procedure and obtain the best sugar yield possible with the least amount of enzyme.

1.2 Large-scale hydrolysis of cellulose using immobilized biocatalysts

Considering the huge availability of cellulosic materials, mainly waste paper, the amount of cellulose that can serve as substrate is consequently very high as well. The potential economic advantage of having a large-scale process dedicated to produce sugar by degrading a fairly cheap substrate, such as cellulose, is irrefutably great. Although the knowledge necessary to apply the concept of a small reactor condition to an industrial scale is yet to be enough. Tune ups are always required when dealing with progressively higher volumes of reaction mixture and new variables appear, that must be controlled to ensure optimal conditions and, consequently, optimal yield and productivity.

The concept of immobilization, however, is necessary for the reaction of degrading cellulose to be economically viable because the amount of enzyme must increase proportionally with the amount of substrate. This would require incredibly high amounts of enzyme for every run of the reactor and thus. by having the enzymes immobilized in a surface or in a particle, these could eventually be recycled and used again on another reaction. In an economic perspective, this would significantly decrease the cost of the enzymes, since each reaction would not necessarily require a new batch of enzymatic solution.

Chapter 2 – Literature overview and State-of-the-art

2.1 Properties of Lignocellulosic Materials

Effective and economically supported conversion of lignocellulosic biomass is not only related to high sugar yields but also to simple, environmentally friendly and sustainable technological solutions.

Lignocellulosic biomass could be a valuable resource for the renewable energy industry and its potential has in fact been evaluated worldwide for several years now. Also, the effective conversion of biomass to electrical and heat energy has been shown elsewhere to have a considerable share in the total energy produce in those countries **[1]**.

Simultaneously, biomass-like resources, such as shrubs, grass, hay, straw and undefined agricultural waste could potentially replace energy crops as feedstock to produce biological-based fuel and gas, which currently occupy considerably large areas of agricultural fields that rest unavailable for food production.

Despite these well-known favorable points, owing to its highly complex lignocellulosic matrix consisting essentially of cellulose, hemicellulose and lignin, converting this resource directly into compounds that could be of higher value is not currently so easy (**Figure 1**). More specifically, to release fermentable sugars that could be of use in a vast range of applications, several methodologies are very important in a pretreatment stage of this resource **[2]**.

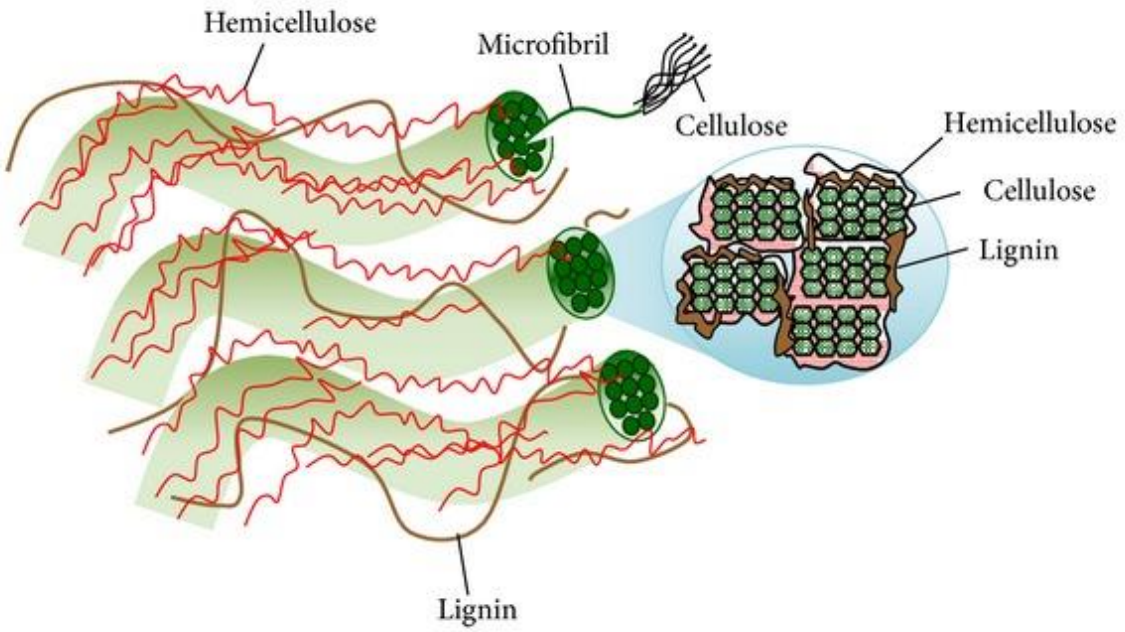

Figure 1 - Plant cell wall structure and microfibril cross-section. Adapted from Lee *et al.* (2014) [23].

Regarding the total yield of sugar extracted from this source, until recently this value went up to 99% which has been obtained by Ballesteros *et al.*, 2002. However, this value generally oscillates between 12% and 98%, and it depends significantly on the biomass source and treatment method used **[3,4]**. Regardless, there is an increased interest in optimizing laboratory techniques to obtain higher fermentable sugar yields and decreasing production costs for later application into a commercial scale.

Part of the lignocellulosic biomass consists of cellulose (40%, on average) which is the main source of fermentable sugars. The cellulose concentration varies from the biomass type studied, thus, affecting the product yields when a single pre-treatment/hydrolysis technique is used for various biomass sources **[5]**. However, this aspect can strongly affect both the cost and availability of the substrate, especially in the areas with a distinct seasonality and limited uniform feed material. Simultaneously, high productivity techniques, such as oxidative and physicochemical, producing sugar yields higher than 80% and, therefore, less affected by cellulose concentration, are mostly considered as too energy and labor consuming, environmentally unfriendly and might inhibit fermentation, thus, limiting their application in small to medium scale biofuel production platforms **[6]**.

2.2 Composition of Lignocellulosic Biomass

Essentially, lignocellulosic feedstocks are composed of three major polymers: cellulose, hemicellulose, and lignin as well as other minor components including proteins, extractives, and inorganic minerals. Cellulose, the main component of lignocellulosic biomass, is a linear homopolymer of glucose ($C_6H_{12}O_6$) units linked together in the form of D-anhydroglucopyranose units through β-(1,4)-glycosidic bonds (**Figure 2**). Typically, each cellulose molecule consists of 5000 to 10,000 units of glucose (depending on the degree of polymerization) **[7]**.

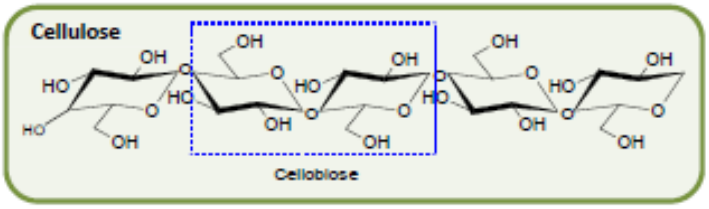

Figure 2 - Structure of Cellulose. Adapted from Solange I. Mussatto *et al.* (2016) [10].

The structure of cellulose comprises intra- and inter-molecular hydrogen bonds that lead to the formation of a rigid network of microfibrils that bond together to constitute fibrils and subsequently form cellulose fibers. These aggregates of cellulose molecules appear in either crystalline (highly ordered) or amorphous (less ordered) forms. Crystalline regions, which are harder to be hydrolyzed than amorphous regions, pose as a barrier to the enzymatic or chemical degradation of cellulose and it becomes insoluble in most solvents **[8]**.

In addition, hemicelluloses, the second most abundant component of lignocellulosic biomass, are a group of complex heterogeneous polysaccharides composed of 5-carbon sugars (xylose and arabinose), hexoses (glucose, mannose, and galactose), and some acids (acetic acid, D-glucuronic acid and D-galacturonic acid), with a degree of polymerization up to 200 units (**Figure 3**). They bind to cellulose microfibrils by hydrogen bonds and to lignin by covalent linkages. In opposition to cellulose, which differs only in crystallinity and degree of polymerization, hemicelluloses present variable and amorphous structures made up of short side chains along with a backbone chain that can be easily degraded by enzymatic hydrolysis or chemical treatments **[8].** The main polymer chain in hemicelluloses is usually composed of D-xylose (around 90%) and L-arabinose (around 10%).

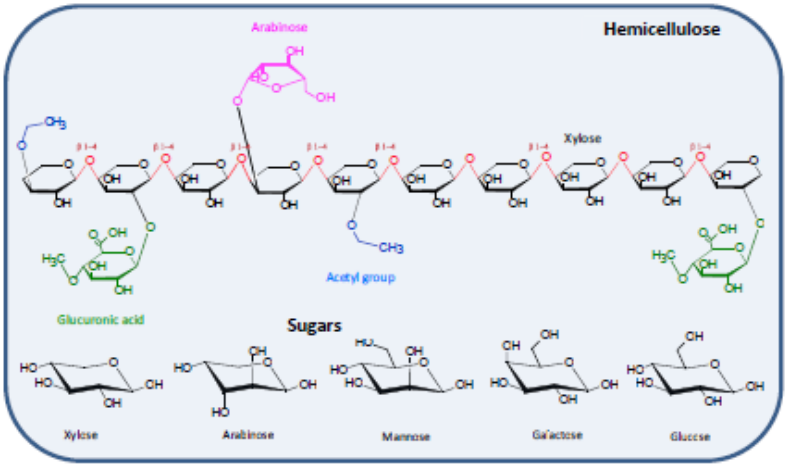

Figure 3 - Structure of Hemicellulose. Adapted from Solange I. Mussatto *et al.* (2016) **[10]**.

Ultimately, there is lignin, the main compound responsible for the recalcitrance of lignocellulosic biomass. This percentage of the feedstock is an amorphous three-dimensional polymer of phenylpropanoid units interconnected by different types of bonds (**Figure 4**) **[9]**. Lignin is also associated with hemicellulose and cellulose by covalent bonds through ester, ether, and glycosidic linkages. The lignin molecule encloses these polysaccharides, hampering their access.

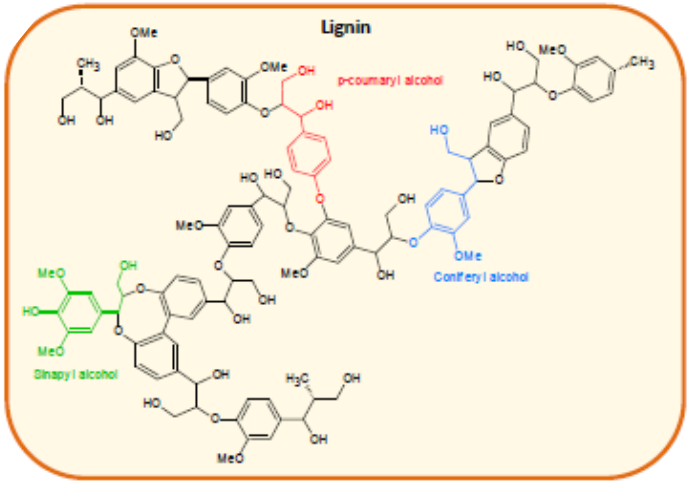

Figure 4 - Structure of Lignin. Adapted from Solange I. Mussatto et al. (2016) [10].

Also, in addition to the three main components of lignocellulosic materials previously mentioned, a minor fraction consisting of several components soluble in organic solvents (for example: ethanol, acetone, dichloromethane, and benzene) is also found in the biomass composition. These components (extractives), constitute a heterogeneous group that includes fatty acids, gums, waxes, resins, chlorophyll, terpenoids and other phenolic substances **[10]**.

Conclusively, the main constituents of the biomass can vary greatly among various sources (**Table 1**). Accurate measurements of the biomass constituents, essentially lignin and carbohydrates, are of great importance since they could assist tailored process designs for the maximum recovery of products from the raw materials.

Table 1 – Composition of a few common sources of biomass. Adapted from Sun, Y. *et al.* (2002) [14].

Lignocellulosic material	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Coastal Bermuda grass	25	35.7	6.4
Corn cobs	45	35	15
Cotton seed hairs	80-95	5-20	0
Grasses	25-40	35-50	10-30
Hardwood stem	40-55	24-40	18-25
Leaves	15-20	80-85	0
Newspaper	40-55	25-40	18-30
Nut shells	25-30	25-30	30-40
paper	85-99	0	0-15
Primary wastewater solids	8-15	NA	24-29
Softwood stem	45-50	25-35	25-35
Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
Sorted refuse	60	20	20
Swine waste	6	28	NA
Switchgrass	45	31.4	12.0
Waste papers from chemical pulps	60-70	10-20	5-10
Wheat straw	30	50	15

NA – Not Acknowledged

2.3 (Ligno)Cellulosic enzymes

The hydrolytic enzymes should be of desirable characteristics for their application in the hydrolysis of lignocelluloses. Some of the desirable characteristics of the enzymes include catalytic efficiency, thermal stability, adsorption, end-product inhibition resistance and shear inactivation **[11,12]**.

Unlike the acid hydrolysis, the enzymatic hydrolysis, still has not reached the industrial scale. Only few plants are available worldwide to investigate the process (pretreatment and bioconversion) at a pilot scale **[13]**.

2.3.1 Cellulolytic Capacity of Organisms

Different strategies for the cellulose degradation are used by the cellulase-producing microorganisms: aerobic bacteria and fungi secrete soluble extracellular enzymes known as *non-complexed cellulase systems*; anaerobic cellulolytic microorganisms produce *complexed cellulase systems*, called *cellulosomes* **[14]**.

In essence, a *non-complexed cellulase system* is a strategy used by many micro-organisms to degrade cellulose by secreting enzymes directly to the target area. One of the most fully investigated *non-complexed cellulase systems* is the *Trichoderma reesei* model. This saprobic fungus, known as an efficient producer of extracellular enzymes **[15]**. Its cellulase system includes two cellobiohydrolases, around seven endoglucanases and several β-glucosidases. Nevertheless, in *T. reesei* cellulases, the amount of β-glucosidase is not high enough for the efficient hydrolysis of cellulose into glucose. Consequently, the major product of hydrolysis is cellobiose. which is a dimer of glucose with strong inhibition toward endo- and exoglucanases. High concentration of cellobiose in the mixture significantly slows down the hydrolysis process **[16]**. By adding β-glucosidase (also known as cellobiase) to the media, the inhibitory effect of cellobiose can be significantly reduced. Particularly, this can be achieved by using co-culture systems **[17]**. Firstly, a chemical change occurs in the cellulose solid phase, in which the degree of polymerization of the molecules varies; a physical change also occurs in parallel with the chemical change, which relies on modifications in the accessible surface area of the target. This step involves essentially an *endoglucanase* (**Figure 5**). Secondly, the primary step of hydrolysis involves *cellobiohydrolases* and consists mainly of releasing soluble intermediates from the cellulose surface, being a slow process.

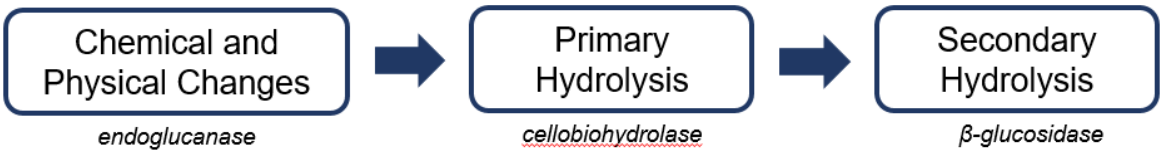

Figure 5 - Stages of the non-complexed cellulase system.

Lastly, secondary hydrolysis takes place and, like the previous stage, this one also consists of further hydrolyze the soluble cellulose into progressively lower molecular weight fractions, involving now β-glucosidase. Since the cellulose (soluble) is currently more accessible to the enzymes, this step is considerably faster than the previous.

On the other hand, *complexed cellulase systems* are utilized mainly by anaerobic bacteria and by a few fungi. Among bacterial species, *cellulosomes* are found in various clusters of the genus *Clostridium* **[13]**. These structures are protuberances produced on the cell wall of cellulolytic bacteria grown on cellulose-rich materials. They are essentially stable enzyme complexes tightly bound to the cell wall of the host bacteria but are, however, able to form a strong bond to cellulose. Additionally, these structures have two domains: *non-catalytic subunits* (*scaffoldings*) and *enzymatic subunits* (**Figure 6**). *Scaffoldings* are basically anchoring units with Cellulose Binding Domains (CBD) required for the consequent anchoring of enzymatic units that will proceed to degrade the substrate **[18]**.

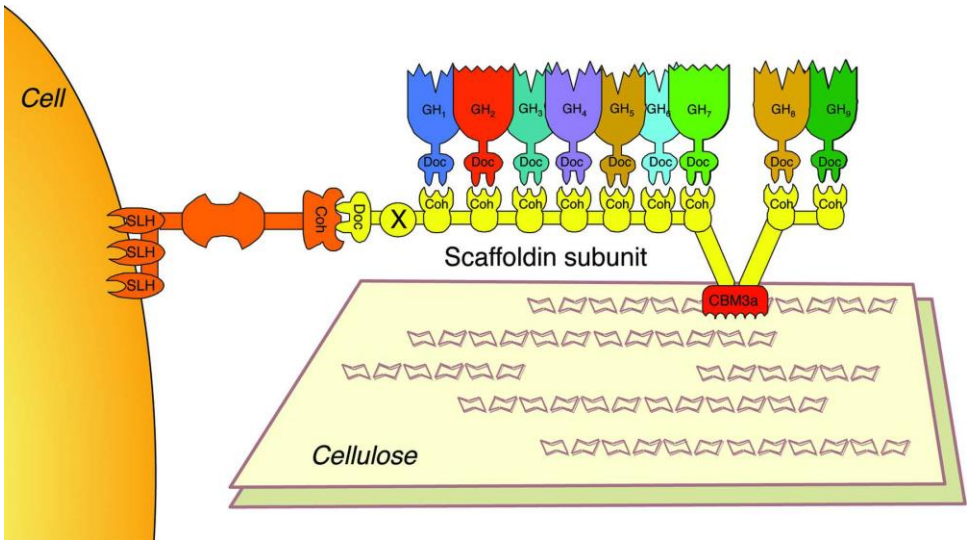

Figure 6 - Schematic representation of a cellulosome complex.

2.3.2 Commercially Available Enzyme Preparations

Most cellulase enzymes are relatively unstable at high temperatures. The maximum activity for most fungal cellulases and β-glucosidase occurs at roughly 50°C and a pH around 5 **[19]**. Generally, these enzymes lose about 60% of their activity in the temperature range 50–60°C and nearly lose all activity at 80°C **[20]**. Nonetheless, enzyme activity depends on the duration of hydrolysis and source of the enzymes **[13]**. Essentially, cellulases are very difficult to use for operations of long duration periods.

In addition, preparations of cellulases from a single organism may also not be highly efficient for the hydrolysis of different feedstocks. Consequently, the goal of companies that produce such enzymes has been to form cellulase cocktails by enzyme-assembly (meaning, a mixture of several enzymes) or to genetically engineer microorganisms to express the desired mixtures **[21]**. So that we can meet the future challenges, innovative bioprocesses for the production of new generation of enzymes are heavily required. As previously mentioned, conventional cellulases work within a range of temperature around 50°C and they are typically inactivated at temperatures above 60-70°C due to disorganization of their three-dimensional structures and subsequently by an irreversible denaturation **[22]**. Some opportunities of process improvement derive from the use of thermostable enzymes. The main companies that have currently made progress in this regard are *Genencor* and *Novozymes*® that have produced quite the arsenal of cellulose-degrading enzyme cocktails (**Table 2**).

Table 2 – Several cellulose-complex products and their respective function and Manufacturer.

Manufacturer	Product	Enzyme Activity
Genencor	Accelerase®1500	Cellulase complex (exoglucanase, endoglucanase, hemi-cellulase and β-glucosidase
	Accelerase®XP	enhances xylan and glucan conversion
	Accelerase®XC	contains hemicellulose and celullase activity
	Accelerase®BG	β-glucosidase
Novozymes	cellic Ctec®	Degrades a wide range of pretreated feedstocks
	cellic Htec®	

Biomass recalcitrance is, from the technological aspect, an issue that must be overcome and, pretreatment of the feedstock is the best and most studied methodology to achieve this goal. This problem is mainly caused by several factors such as (1) the lignin content and the protection of cellulose by lignin; (2) hemicellulose sheathing cellulose; (3) crystallinity, degree of polymerization, and low accessible surface area of cellulose; and (4) high fiber strength **[23]**.

Pretreatment decreases the influence of biomass recalcitrance by disrupting the biomass structure in order to make the cellulose fibers more accessible to the enzymes that will release sugars in the subsequent step of hydrolysis. Although it is one of the most expensive steps in the overall process of biomass conversion, it significantly impacts the environment **[10]**.

There is a wide range of alternatives which include physical, chemical, physicochemical, and biological methods that have been investigated and developed for biomass pretreatment, with the objective of modifying and/or removing hemicellulose and lignin, improving the access of the enzymes to the cellulose structure, Consequently, this increases the yield of fermentable sugars after enzymatic hydrolysis **[10]**.

2.4. Biomass Pretreatment with Acids

2.4.1 Acid Pretreatment: General Considerations

Biomass pretreatment with acids consists of the use of dilute or concentrated acid solutions to break the rigid structure of lignocellulosic materials. Throughout the process, the acid behaves as a catalyst, attacking intermolecular and intramolecular bonds among hemicellulose, cellulose, and lignin, proceeding to the hydrolysis of the carbohydrates present in the material structure, especially the hemicellulose **[10]**. Acid concentration is one of the most important variables affecting the efficiency of this whole process. Pretreatment of the biomass using acids, can be achieved using either a low acid concentration and high temperature or a high acid concentration and low temperature.

For instance, hemicellulose is the first constituent of the biomass structure to break down during this particular pretreatment, being hydrolyzed by dilute acids (<5% w/v) under moderate conditions of temperature (120-210 ^{o}C) while from cellulose low yields of glucose are obtained under the same conditions **[24,25]**. Nevertheless, pretreatment using high acid concentration (over 30% w/v) under moderate temperatures (<100 ^{o}C) allows to efficiently hydrolyze both the hemicellulose and cellulose fractions, providing high sugar yields **[26]**.

Several acid types, including mineral (sulfuric, phosphoric, hydrochloric, hydrofluoric, nitric, and formic acids) and organic such as maleic, oxalic, acetic, and fumaric acids, can be used in the pretreatment step of the biomass. Nonetheless, sulfuric acid has been the most extensively studied considering that it is inexpensive and highly effective **[10]**.

In addition, the solid loading or solid/liquid ratio is among the most important variables affecting the efficiency of the entire acid pretreatment process **[27,28]**. Using high solid loadings, meaning low volumes of liquid per gram of biomass, is something highly sought after, regarding the economy of the process because it opens the possibility to obtain a higher concentration of sugars in the resulting mixture, avoiding or, at least, minimizing the need of submitting the hydrolysate to a concentration step prior to using as a fermentation medium.

2.4.2 Dilute Acid Pretreatment

Process Technology and Main Effects on Biomass Structure

Nearly 80-90% of hemicellulose-derived sugars are usually recovered by dilute acid pretreatment **[29]**, while cellulose and lignin fractions remain almost intact in the solid material after pretreatment. Removal of the hemicellulose increases the material porosity and enhances the accessibility of enzymes to the cellulose in the residual solid. Additionally, although only a little lignin is dissolved during this process, its structure is disrupted, contributing to increase the digestibility of cellulose **[30]**.

Regarding its ability to selectively remove hemicellulose, pretreatment with dilute acid can be used as a first step in the overall process for lignocellulosic biomass fractioning into its three main components. Removing hemicellulose by dilute acid pretreatment, followed by alkali pretreatment to remove lignin, is an example of a strategy that can be applied to fraction biomass, resulting in a solid substrate containing relatively pure cellulose **[31]**. Usually, xylose, arabinose, and glucose are the main monosaccharides present in the resulting hydrolysate. However, the type of sugar varies according to the biomass that is processed, and its concentration in varies according to the conditions used for pretreatment.

The most widely used pretreatment compound has been dilute sulfuric acid since it is cheap and very effective when used at relatively low concentration (below 4% (w/v)). The main advantage is the high amount of sugars recovered from hemicellulose, which also has a positive effect on the subsequent step of hydrolysis considering the pretreated solid is enriched in cellulose, which is more accessible to the enzymes, requiring lower enzyme loadings (**Figure 7**).

Additionally, dilute acids are able to extract nutrients from biomass to the hydrolysate, which can be consumed by the microorganisms, reducing the need of adding nutrients to the culture media **[32-34]**. Ultimately, the main disadvantage is that the use of acids (even in low concentration), might involve problems related to corrosion of the equipment, requiring expensive materials for construction of the reactors, which consequently increases the overall costs. The use of high temperatures, the overall energy consumption, and the necessity of using small particle sizes (up to a few millimeters) also have an important impact in the overall costs of the process **[25]**.

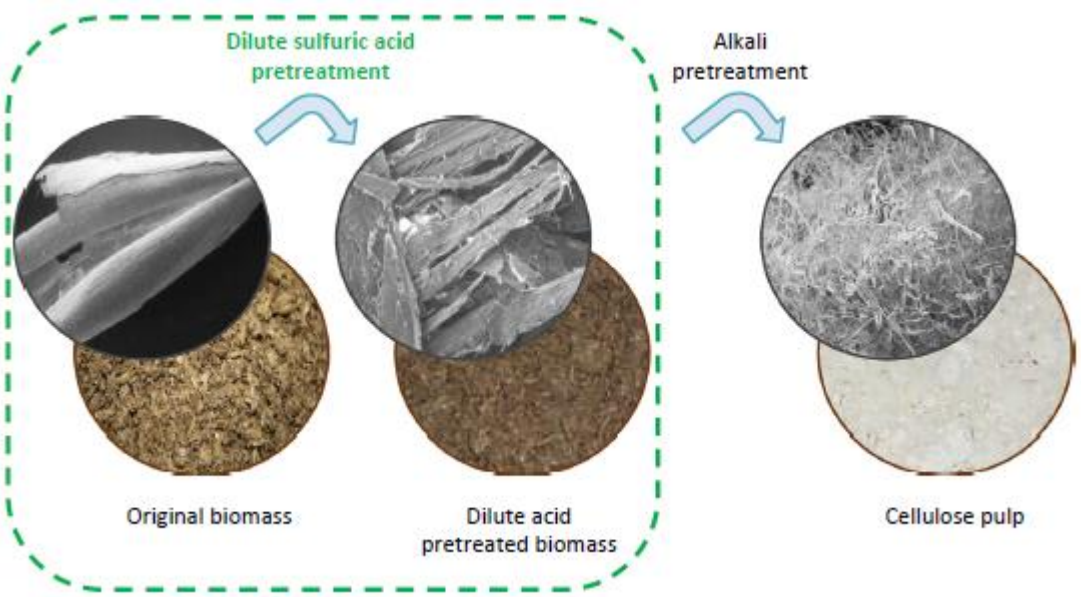

Figure 7 - Complete fractionation of brewer's spent grains using a two-stage pretreatment strategy. Extracted from Solange I. Mussatto et al. (2016) [10].

Lately, dilute acid pretreatment has been successfully applied to a wide variety of biomass feedstocks, including agricultural wastes, herbaceous crops, woods, and municipal solid wastes, among others. The results, in terms of sugar yields, greatly vary according to each biomass and the conditions used for pretreatment (**Table 3**).

Table 3 – Some dilute acid pretreatment assays applied to some feedstock biomass with their respective conditions and total sugar yields.

Biomass	[Sulfuric Acid] (%)	Temperature (ºC)	Reaction Time (min)	Sugar Yield (%)	Reference
Coastal Bermuda grass	1.2	140	30	94.0	**[35]**
Rice straw	1.0	160 to 180	1 to 5	83.0	**[36]**
Brewer's spent grains	1.25	120	17	74.8 to 88.7	**[27,31]**
Corn Stover	2.0	120	43	70.0 to 77.0	**[37]**

The results presented previously (**Table 3**) clearly show that different types of biomass require different conditions of pretreatment to be efficiently converted into sugars. Establishing the optimal pretreatment conditions is very important to maximize the sugar yield and also to minimize the formation of toxic compounds. Nevertheless, the optimal conditions depend on whether the goal is to maximize the sugar yield from hemicellulose or from cellulose after enzymatic hydrolysis of the pretreated solids or, regardless, to obtain maximum yields after both steps **[10]**.

2.4.3 Concentrated Acid Pretreatment

Process Technology and Main Effects on Biomass Structure

On the other hand, biomass pretreatment with concentrated acids is commonly performed using a high acid concentration (over 30% w/v), at ambient to moderate temperatures (<100 ^{o}C) and atmospheric pressures **[26]**. Under these conditions, the acid attacks the lignocellulosic structure and is able to promote the release of sugars from both the hemicellulose and cellulose fractions, providing high sugar yields. Concentrated solutions of sulfuric, nitric, hydrochloric, phosphoric, and trifluoroacetic acids could be used to treat lignocellulosic materials. Nonetheless, sulfuric is so far, the most commonly used acid in this method.

Essentially, the most relevant advantage of this pretreatment methodology is that it is very effective for cellulose hydrolysis, and a following step of enzymatic hydrolysis is usually not required as the acid itself hydrolyzes both the cellulose and hemicellulose fractions to fermentable sugars **[38]**.

On the other hand, since concentrated acids are highly toxic and corrosive, their use requires reactors made from expensive materials resistant to corrosion, making the pretreatment process expensive.

Moreover, owing to the high consumption of acid, its recovery and recycling after pretreatment are essential to make the process economically viable **[14]**. Acid recovery process is high-energy demanding **[26]**, but it is possible to recover the acid with elevated efficiency (up to 97%) **[39,49]**. If the acid is not recovered, then a large amount of neutralizing agent is required to adjust the pH of the liquid stream (hydrolysate) prior to using to be used in fermentation medium, which is also reflected in the cost of the process. Furthermore, a significant degradation of sugars with a consequent formation of fermentation inhibitors (furfural and hydroxymethylfurfural) might occur, requiring an additional detoxification step in order to reduce the concentration of these compounds in the hydrolysate (**Figure 8**).

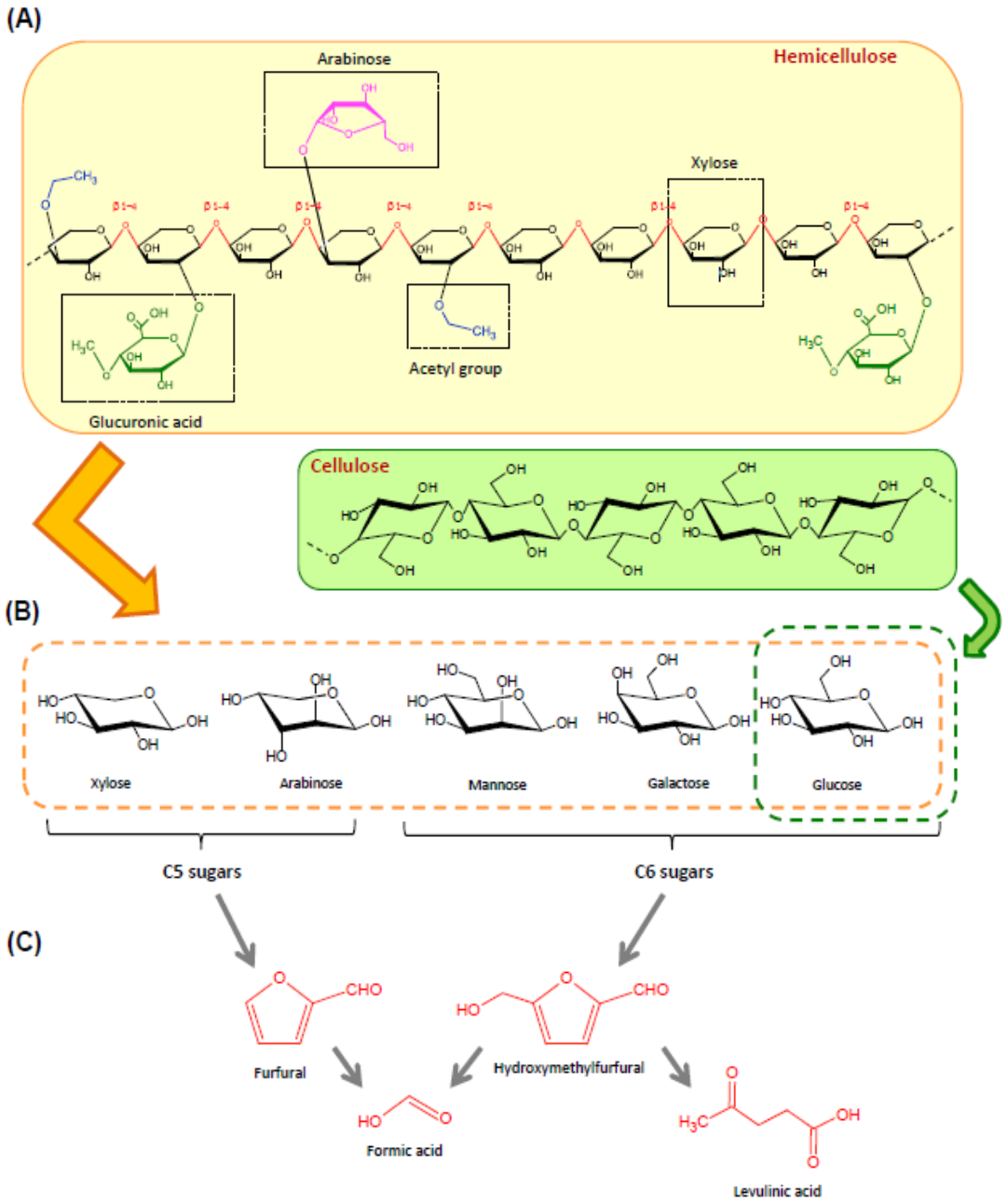

Figure 8 - (**A**) Structural representation of hemicellulose (with a xylan backbone, for example) and cellulose; (**B**) sugars that can be released from each structure during pretreatment; (**C**), potential toxic compounds that can be formed. Extracted from Solange I. Mussatto *et al.* (2016) [10].

2.4.4 Important topics for further development

Ultimately, using dilute and concentrated acids for biomass pretreatment have been subject to important improvements, both in technical aspects related to the maximization of sugar yields and in economic/environmental aspects. The pretreatment step is significantly responsible for the general costs regarding the biomass conversion chain. In order to decrease these costs, it is essential to develop a robust pretreatment method able to be efficiently performed with high solid loading while promoting minimal degradation of sugars.

2.5 Other Methodologies of Biomass Degradation

There are additional strategies to promote lignocellulosic biomass degradation in which the main goal, as previously mentioned is to make the cellulose more accessible to the enzymes that will be consequently in the hydrolysis stage further down the process chain **[10]**. These methods particularly include the removal of lignin and hemicellulose fractions so that the cellulose may be in a less crystalline form and with a higher accessible surface area. These include several strategies for either physical or chemical degradation of lignocellulosic biomaterials:

- **Mechanical** – include physical and mechanochemical processing of lignocellulosic raw material such as centrifugal mills and other grinding apparatuses that contribute to the de-lignification of the raw biomass.
- **Pyrolysis** – this reaction is defined as a controlled process that converts solids with a limited amount of water into a solid residue and volatiles under an inert atmosphere, by the action of heat.
- **Microwave-Induced Fractionation** – this technology focusses essentially on the solubilization of the feedstock (also using hydrothermal treatment) by using ionic conduction to disrupt chemical bonds within the polysaccharides that constitute the biomass.
- **Ultrasound** - The effective treatment of biomass with ultrasound is based on the principle of cavitation, which is described as the spontaneous formation, growth, and subsequent collapse of micro sized cavities/bubbles caused by the propagation of ultrasonic waves in liquid medium – the implosion of these bubbles produces high temperatures in their surroundings. This can help fraction the cellulose fibers.
- **Pulsed Electric Energy** (**PEE**) - this method impacts by inducing the loss of membrane barrier functions in the biomaterial – electroporation.
- **Acids**, - as previously mentioned, this method consists of a chemical pretreatment of the biomass which also includes some release of sugars.
- **Metal salts** - this strategy takes advantage of several salts in the form of chloride, phosphate, sulfate, nitrate, etc. The idea relies upon ionic dissociation of these compounds in water and their further disruption of the glycosidic bonds in the hemicellulose sugar chains.
- **Hydrotropic Technology** – hydrotropes are compounds containing both hydrophilic and hydrophobic functional groups. The mechanism of this pretreatment is the extraction of lignin from lignocellulosic biomass by improving its solubility in aqueous solutions.
- **Hydrothermal** (**Autohydrolysis**) - Liquid hot water is compressed and seeded to the lignocellulosic biomass. Using hydronium-catalyzed reactions (water reacting with water), these hydronium ions formed during water ionization lead to the depolymerization of hemicellulose by hydrolysis.

- **Steam Explosion** - It is a combination of thermal, chemical and mechanical phenomena that act on the biomass, solubilizing hemicellulose sugars, altering cellulose crystallinity, inducing lignin modifications and breaking lignocellulose fibers. Using heat and high-pressure steam (reaching temperatures of up to 260 ^{o}C) altering the physical-chemical properties of the biomass for further treatment.
- **Carbon Dioxide** - CO_2 is able to penetrate the lignocellulosic material structure, decreasing its crystallinity by disrupting the connections between cellulose and hemicellulose, yielding a substrate that is more easily accessible by enzymes or other pretreatment methodologies.
- **Ammonia** - Ammonia treatment results in the cleavage of complex linkages of lignin carbohydrate between lignin phenolic and hemicellulose side-chains that allow the removal of lignin and partially hemicellulose. Additionally, it also affects the crystallization of cellulose.
- **Sulfites** - Sulfite pretreatment to overcome the recalcitrance of lignocelluloses (SPORL) is able to dissolve a substantial amount of hemicellulose, depolymerizes cellulose, partially digests the biomass by promoting a highly sulfonated form of lignin. This strategy yields a biomass that is more readily digested by enzymes (although only at low dosages).

2.6 Enzyme Immobilization

Industrial implementation of biocatalysts is still in a relatively premature stage as compared to chemo catalysts, partly due to production and operation costs **[40]**. Immobilized enzymes can be defined as "enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, being able to be repeatedly and continuously used" **[41,42]**. Biocatalyst immobilization is a technique, which is able to improve whole cell or enzymatic performance as well as provide a broader spectrum of applications. This strategy, based on the fixation of the biocatalyst into or onto various materials, may thus increase robustness of the biocatalyst, allow its reusability, or improve the yield of a desired product. **[50]**

One example of enzyme immobilization is the isomerization of glucose to fructose by immobilized glucose isomerase, which is already processed industrially **[43]**. Regarding the economic aspects of enzyme immobilization, from producer's perspective, the cost of producing an immobilized form of an enzyme must also include a new application or offer some other benefit relative to the soluble form of the enzyme. The fact that immobilized enzymes can be re-utilized does not benefit the enzyme producer directly, but merely provides an incentive for customers to purchase an immobilized enzyme product.

As of today, there are only available a few articles on the immobilization of cellulase. This is highly influenced by the low solubility of cellulose – which would difficult even more its diffusion to the enzyme. In essence, some immobilization techniques, such as enzyme encapsulation, hamper the enzyme-substrate interaction. Immobilization of cellulases through covalent bonds appears to be the most suitable technique. Besides the enzyme stabilization, the covalent-immobilization allows the use of supported enzymes for several reaction cycles **[44]**.

2.6.1 Immobilization Strategies

To promote enzyme utilization in biotechnological processes, different methods to reduce its costs have been applied, being that immobilization is one of them. One of the most important aspects that must be taken into consideration upon the immobilization of enzymes is that these will most likely be affected by the chemical or physical properties of the immobilization support, depending on the nature of the latter. Usually, the immobilization method chosen imposes microenvironmental changes depending on the supporting matrix that affect the enzymes in terms of stability and kinetic properties. Furthermore, the surface in which the enzyme is immobilized must be able to maintain the tertiary structure of the enzyme by forming hydrogen or covalent bonds with the matrix. **[51]**

2.6.1-A Immobilization Techniques

The selection of an appropriate immobilization strategy is the one of the most crucial steps involved in the immobilization process as it will ultimately play the biggest role in determining the enzymatic activity and characteristics in the reaction. In essence, immobilization methods are divided in two classes – chemical and physical. Physical methods tend to consist of weaker, monocovalent interactions, such as hydrogen bonds, hydrophobic interactions, ionic binding of the enzyme with the support material, among other molecular interactions; or mechanical confinement of the enzyme units within the support. Chemical methods usually consist of the formation of covalent bonds between the enzyme and the support material or matrix. **[52]**

In addition, there are several techniques that allow for the immobilization of enzymes, such as adsorption, entrapment, covalent binding and cross-linking (Figure X).

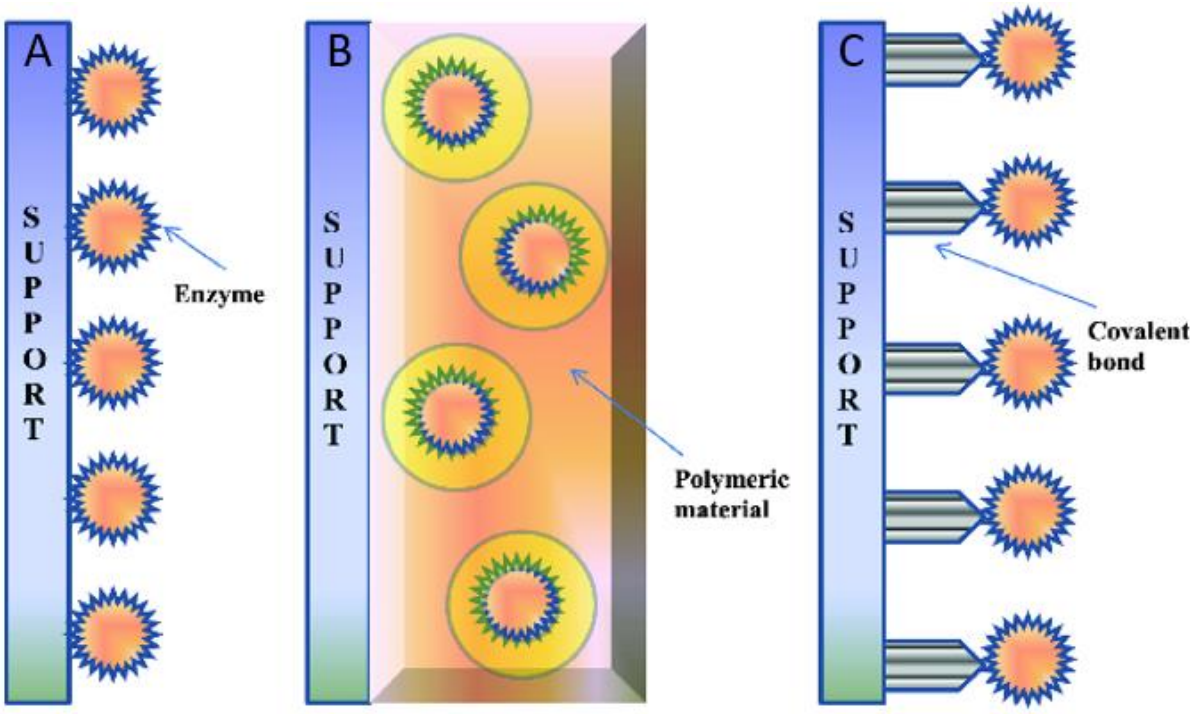

Figure 9 - Schematics of the three most common enzyme immobilization techniques: **(A)** physical adsorption, **(B)** entrapment and **(C)** covalent attachment/cross-linking. Adapted from Nur Royhaila Mohamad *et al.* [51]

Physical Adsorption

This straightforward method involves the physical absorption or attachment of the desired enzymes onto the support material. This procedure can occur through weak non-specific forces such as van der Waals, hydrophobic interactions and/or hydrogen bonds, while in ionic bonding, the enzymes are bound through salt linkages. The favourable aspect of this method is the reversibility of the process, meaning that the immobilized enzymes can be removed from the support under specific conditions. Such particularity is attractive because the enzyme activity may decay overtime and when that happens, the enzyme can be removed to allow for the regeneration of the support and subsequent reloading with new enzyme. In addition, this poses as an economic advantage, since the overall cost of the support is lower when compared to a situation when the support must be replaced upon loss of activity by the enzymes. **[51]**

However, since the interactions responsible for the attachment/absorption of the enzyme into the support are relatively weak and nonspecific, this method suffers from an important drawback – which is the possible leakage of protein from the support. This is a crucial aspect upon choosing the adequate immobilization method because, if the reaction mixture requires continuous mixing through, for example, mechanical stirring, the risk of a progressive loss of enzyme increases, due to the shear stress caused by the agitation. **[51]**

Entrapment

This method relies on the irreversible entrapment of the desired enzymes in a support or inside a matrix/fiber. This method of enzyme immobilization requires for the enzyme to be retained in a polymer while allowing for the free passage of substrate and products to pass through it. The procedure itself aims to recreate an optimal microenvironment that allows for the physical and chemical stability of the enzyme, since the latter does not interact chemically with the support. Additionally, entrapment partially solves the problem of protein leaching by being more resistant to abrasive mixing of the reaction media that could potentially detach the enzyme from the support. Since there are many support materials used in this technique, such as polymers, sol-gels, polymer/sol-gel composites and other inorganic materials, the microenvironment of the enzyme/matrix can be modulated according to the requirements and desired purpose of the immobilization. **[51]**

However, this methodology is no exception when it comes to potential drawbacks. For instance, its practical use is rather limited since the matrix tend to interfere with mass transfer, limiting the flow of substrate and product(s) inside the support. Furthermore, there is the possibility of enzyme leakage which, in this case, tends to occur when the pores of the support matrix are too wide. Other disadvantages include deactivation during immobilization, low loading capacity and the ratio of particle size to the support material pore size must be taken into consideration for the efficient usability of this method. **[53]**

Cross-linking

This technique of enzyme immobilization is irreversible, and it does not require a matrix support to prevent protein loss into the substrate solution. It is often named carrier-free immobilization since the enzyme acts as its own carrier and nearly pure enzyme is obtained, hence disregarding all aspects of a carrier (be it in entrapment or adsorption), mainly the dilution of activity owing to the addition of a large portion of non-catalytic material that ends up making a fairly large percentage of the enzyme-carrier complex (90 to 99%). The cross-linking owes its name to the formation of intermolecular cross-linkages between the enzyme molecules by means of multifunctional reagents. **[54]**

Generally, glutaraldehyde is the most commonly used cross-linking reagent used to perform this kind of enzyme immobilization technique, since it is less costly and is easily obtained in large quantities. The reaction usually consists of cross-linking free amino groups of lysine residues on the surface of enzyme molecules with oligomers or polymers of glutaraldehyde. Being a pH dependent reaction, it has associated risks regarding its efficiency and overall outcome. A well-known use for cross-linking immobilization is the cross-linking enzyme aggregates (CLEAs). **[55]**

Covalent-bonding

Another method for the irreversible immobilization of enzymes is by means of covalent bonding. Usually, the functional groups involved in the covalent binding of the enzyme are side chains of lysine, cysteine, aspartic and glutamic acids. It is very important that these functional group are not essentially for the catalytic activity of the enzyme, otherwise, its function might be compromised. Furthermore, the activity of the covalently bound enzyme depends on the size, shape and composition of the carrier material, nature of the coupling method and the specific conditions under which the procedure takes place. **[56]**

The coupling of the enzyme with the support can undergo in two different ways, depending on the active groups present in the molecular structure of the enzyme that would be immobilized. The reactive functional groups can be added to the support with no modifications, or the support can be modified in order to generate activated groups. Either way, it is expected that the reactive groups on the support will interact with the proteins. Depending on the strength of that interaction, the type and nature of the support matrix must be chosen accordingly. Generally, agarose, cellulose, poly (vinyl chloride), ion exchange resins and porous glass are the matrices of choice for such techniques. **[51]**

2.6.1-B Application of immobilized enzymes

Several methodologies have been applied in order to effectively immobilize enzymes. Researchers were previously able to use a number of immobilization strategies to immobilize the enzyme Glucose isomerase (GI). Those strategies include crosslinking of whole cell preparations (from glucose isomerase-expressing *Streptomyces sp.*) based on a heat-fixation technique in which the cells were subjected to elevated temperatures (60 to 80^{o}C) for short periods of time which led to a crosslinked matrix (denatured cellular proteins and other components) **[45]**. This strategy achieved a two-fold advantage over the non-immobilized form of the enzyme mainly because this immobilized GI could now be used in continuous bioreactor conditions such as plug flow reactors. In addition, this methodology was applied by the Clinton Corn Processing Company in 1967 to process corn-derived mixtures of glucose syrup to a mixture containing up to 42% fructose **[43]**. Additional chemical fixation techniques include organic acids as fixing agents, for example citrate and glutaraldehyde **[43]**.

Researchers from the University of Minho (Portugal) immobilized, in 2002, cellulases from *T. reesei* on *Eudragit L-100* (a polymer). They used the commercial enzyme cocktail *Celluclast® 1.5L* supplied by Novozymes. This methodology allowed improvements to the stability of the enzymes without loss of its specific activity. The adsorption of cellulases on this polymer lowered the enthalpy of denaturation, but slightly influenced the denaturation temperature **[46]**.

On the other, there are adsorption-based methods that essentially rely upon ionic adsorption of isolated enzyme units to resins that could be organic or inorganic. Overall, immobilization overcomes problems associated with whole cell-based methods, namely the unintentional presence of cellular components that might exert an inhibitory effect or even degrade the target enzyme **[43]**.

Recently, researchers have been able to immobilize cellulase in magnetic nanoparticles (MNPs). Currently, there is not an industrial applied process for high molecular weight cellulose hydrolysis with immobilized biocatalysts. The interesting factor about magnetized particles with enzymes covalently-attached is the ability to separate and collect the enzymes after one batch (for example). It has been demonstrated that an enzyme carrier system based on magnetic nanoparticles can be recycled and reused multiple times without loss of enzymatic activity **[40]**. One of the main difficulties of employing this technique is usually the incubation, which sometimes takes a long time and requires conditions that might result in the denaturation of the protein or in loss of activity. In addition to a magnetic particle-based apparatus, this system has also the possibility to be implemented in a magnetically stabilized bed reactor (MSBR), which is already used with magnetically-immobilized lipase. MSBR consists basically in high gradient magnetic separation for the recovery of the biocatalyst.

2.6.2 Re-usability of Immobilized Biocatalysts

Not only are enzymes expensive and difficult to reuse, they are short time span during which they are active, which makes their long-term storage difficult, further increasing costs and reducing industrial efficiency. Recently, researchers were able to immobilize a commercial sort of cellulases in both porous and non-porous silica in order to replicate the natural occurrence of *cellulosomes* in micro-organisms **[47]**. These *cellulosome*-like structures open the possibility of reusability of the enzymes, contributing to a process that is far more profitable and therefore economically attractive.

One possibility is immobilization of the enzyme and its repeated use. However, the material used must be able to hold the biocatalyst in the matrix and also ensure sufficient diffusion of the substrate and product. Moreover, the immobilization process has to maintain adequate enzyme activity compared to the free biocatalyst. **[50]**

Immobilization can significantly improve process economy but can also influence nutrient supply and product removal. These parameters are affected by diffusion limitations, which are closely connected to the shape, size, and structure of the carriers, as mentioned before.

2.7 Designing a Scalable Platform for Biomass Degradation

In view of the projected large-scale usage of cellulase enzymes in the bioconversion of cellulosic residues, there is a continuous renewed interest in search of novel sources of cellulases, which produce these enzymes with desired novel properties that cause the rapid hydrolysis of cellulose. Several research groups, including those from *National Renewable Energy Laboratory, Genencor, Novozymes®,* etc., have reported improvements in one or more of these properties, especially with respect to thermal stability **[9]**.

As previously mentioned, researchers have been exploring some new pathways leading to the immobilization and consequent reusability of lignocellulose-degrading enzymes. New methodologies arise very often than not, including the use of magnetic nanoparticles to create an industrially applicable bionano system for cellulose degradation. These are, however, only the initial steps towards an efficient and economically and environmentally sustainable strategy in this industry **[40]**.

One example of an industrially applicable immobilized enzyme system is the immobilized pectinase used in the food industry as an approach to overcome the negative impact of colloidal particles in fruit juice. A cross-flow reactor is used with enzymes immobilized on a membrane surface. This setup has been shown to offer several advantages for viscous substrate solution processing since the external limitations in diffusion are reduced by a high recycling flow rate of the enzyme **[43]**.

Chapter 3 - Materials and Methods

3.1 Materials

Reagents: Poly (ethylene glycol) (~600 molecular weight), 3,5-Dinitrosalicylic acid, Coomassie reagent (Bradford assay), Polyvinyl alcohol (LentiKat® solution), D-glucose, Microcrystalline cellulose - used with sodium acetate buffer 0.1 M as a 5 g/L solution, Filter paper (Whatmann 10 µm filters) – either in 5x5 mm shreds or pulverized, Print paper (Source: acid-free paper A4 Navigator Universal type from printed documents, only non-inked pieces used) – either in 5x5 mm shreds or pulverized, and Commercial solutions of cellulase - Novozymes® code NS22086 and β-glucosidase - Novozymes® code NS22118 (**Annex 1**).

Buffer Solutions: Citrate buffer 0.1 Molar (pH 5), Sodium acetate buffer 0.1 M (pH 5), Distilled water (pH 5) and Tap water (pH 5). – both adjusted with hydrochloric acid (0.1 M) and Sodium hydroxide (0.1 M)

Equipment: Incubators with orbital shaking (Agitorp 160E, ARALAB Equipamentos de Laboratório), Incubators with platforms for magnetic agitation (Selecta P with Thermo Scientific variomag telesystem magnetic agitator and LERTOMAT H, B. Braun), Spectrophotometer (Spectra Max Plus 384), 15 mL Flasks for enzymatic hydrolysis, 100 mL reactor with heating jacket (LAUDA E100, Ecoline Staredition), Mechanical agitator (Heidolph RZR1), Centrifuge (Sartorius 1-15P, Sigma, rotor 12000 rpm) and Blender - to obtain paper shreds.

3.2 Immobilization in Magnetic Microparticles (MMP)

Prior to the immobilization of cellulase and β-glucosidase in hydrogel spheroid particles, another strategy was used in an attempt to achieve a solid immobilized bio-catalyst that could be easily recovered from the media by means of a magnet. For this purpose, a set of magnetized iron microparticles was used as a support to carry the process of immobilization of the enzymes. The microparticles were delivered to us previously magnetized. The procedure was followed as described by Keziban Can *et al.* **[57]**

3.3 Production of hydrogel particles

For the immobilization of cellulase and β-glucosidase, the method utilized was through occlusion which consisted in the entrapment of the enzymes in a hydrogel particle of spherical/lenticular shape made of polyvinyl alcohol – using the patented LentiKat® technology. This method allows for the easy separation of the particles with the bio-catalyst from the media, making it also very easy to re-utilize. The method itself requires only the enzymatic solution and the commercial PVA solution but there is a procedure that also requires heating and cooling the LentiKat solution to 90-100 ^{o}C and then let it cool down to around 40 ^{o}C to avoid denaturation of the enzymes upon mixing.

To produce the hydrogel particles, two methods were applied – the plate method and the PEG method. Both result in hydrogel particles of lenticular/spheroidal shape, however, the procedure using poly (ethylene glycol) ensures a more homogeneous distribution of the proteins in the individual particle, along with a more spherical shape. Worth noting that both enzymes, cellulase and β-glucosidase, were immobilized separately. Nevertheless, the concept behind the formation of the gel particles is the same for both methods, which is the formation of hydrogen bonds.

3.3.1 Plate method

Figure 10 shows a schematic representation of the method employed to produce a set of hydrogel particles with the enzyme entrapped. In this methodology, the droplets of PVA with the enzyme are left to dry in a petri dish at room temperature, hence the term “plate”.

The procedure was performed according to a series of steps: Heating the PVA solution to 90-100 ºC in a water bath and after achieving a homogeneous solution, it was left to cool down until it was at around 40 ºC. The cooling was followed by the addition of 10 mL of PVA solution to a heated vessel with 2 mL the enzyme solution (also at 40 ºC to avoid premature solidification). The solution was mixed until a homogeneity was achieved and with the aid of a syringe, small droplets were dropped in a petri dish. After utilizing most of the solution, the droplets were left to dry at room temperature and followed by storage at 4 ºC in a sodium acetate buffer solution 0.1 M at pH 5.

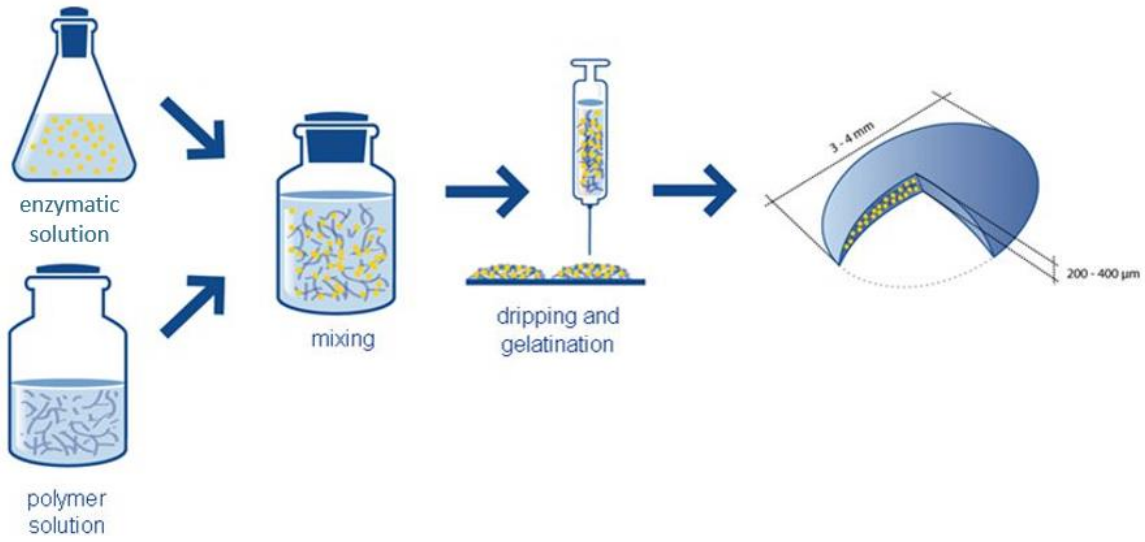

Figure 10 - Schematic representation of the work flow to produce enzymes entrapped in hydrogel particles using the plate method. Adapted from Lentikat’s Biotechnologies®.

3.3.2 PEG method

Similarly, **Figure 11** shows another schematic representation of the method employed to produce a set of enzymes entrapped in hydrogel particles. In this instance, the hydrogel particles were formed upon contact with the poly (ethylene glycol).

The procedure was performed according to a series of steps: Heating the PVA solution to 90-100 ºC in a water bath and after achieving a homogeneous solution, it was left to cool down until it was at around 40 ºC. The cooling was followed by the addition of 10 mL of PVA solution to a heated vessel with 2 mL the enzyme solution (also at 40 ºC to avoid premature solidification). The solution was mixed until a homogeneity was achieved and with the aid of a syringe or electric pump, small droplets were dropped in a vessel containing a PEG solution. As the droplets fell into the PEG solution they instantly solidify, resulting in spheroid/lenticular shaped particles. After exhausting the PVA-enzyme mixture, the particles formed were left in the PEG solution for 2 hours. After that time, they were washed 3 time with sodium acetate buffer (0.1 M at pH 5) to remove excess PEG, followed by storage at 4 ºC in a sodium acetate buffer solution 0.1 M at pH 5.

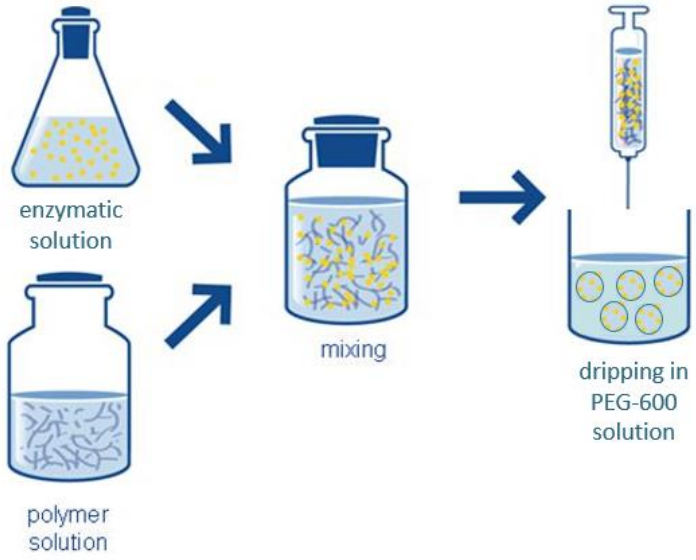

Figure 11 - Schematic representation of the work flow to produce enzymes entrapped in hydrogel particles using the PEG method. Adapted from Lentikat's Biotechnologies®.

3.4 Reactor operation

Enzymatic hydrolysis was usually performed inside small vessels with 15 mL of useful volume herein described as small flask reactors. Occasionally, a reactor with a useful volume of 100 mL with mechanical stirring and a heating jacket to provide the appropriate temperature, herein described as stirred reactor, was also used. Both types operated under agitation and defined conditions of temperature, pH, substrate concentration and enzyme concentration.

3.4.1 Small flask reactors

To the small vessels either a given volume of was added the enzyme solution (when in the free enzyme condition) or a given amount of the gel particles (when in the immobilized enzyme condition), the substrate and the buffer solution, were added. These flasks would usually remain fixed in the platform inside the incubator for the entire duration of the assay and were only briefly removed for sampling. The flasks were under agitation by either an orbital shaking platform inside the incubator or a magnet bar inside the flask which was on top of a magnetic platform. In either case, the agitation was constant throughout the experiment, only stopped occasionally and briefly for sampling. The total volume of the reaction media in these reactors was typically 5 mL, unless specified otherwise.

3.4.2 Stirred reactor with heating jacket

In order to simulate a large reactor for the hydrolysis of cellulose-rich substrates, a larger vessel with a useful volume of 100 mL, was utilized. The conditions were standard, 50 ^{o}C and pH=5 but the orbital shaking/magnetic agitation was replaced with mechanical stirring aiming to recreate a real reactor. Although the concentration of substrate remained the same, the concentration of enzyme was higher, and the total volume was shifted to 40 mL.

3.5 Quantification of glucose

The quantification of glucose in the mixture was performed using the DNS method (3,5-Dinitrosalicylic acid) – Miller (1959). This method allows for the quantification of reducing sugars that are formed during the hydrolysis of cellulose.

The DNS reaction was performed on 96-deep well microplates and every sample was either duplicated or triplicated and the values obtained were an average of each well corresponding to the same sample. Each well contained 100 µL of the sample (either diluted or not) and 100 µL of DNS reagent. The positive control used was a 5 g/L solution of D-glucose and the negative control used was a solution of distilled water.

The reaction takes place in a water bath for 100ºC for the duration of 5 minutes. After the reaction, 500 µL of distilled water at room temperature are added to the mixture and it is left to settle for a couple of minutes. The sample must be diluted to fit in the linearity range of absorbance values, so that the Lambert-beer law can be applied. Finally, 200 µL of each well are collected and transferred to a 96-shadowwell reading microplate (shallow wells) which is placed inside the microtiter plate reader. Readings are performed at the wavelength of 540 nm.

3.5.1 Sampling of glucose

Throughout each hydrolysis, samples were taken to evaluate the progress of the reaction. The number of samples taken varies from 5 to 10, including an initial sample to account for the reducing sugars present in the mixture that do not result from enzymatic hydrolysis, to have an accurate representation of the time course of the reaction while ensuring that the total volume of the reaction medium does not change significantly. Maintaining the ratio substrate-enzyme is of extreme importance because a considerable reduction in reaction volume would interfere with the hydrodynamics of the mixture, mainly in mass transfer between liquid and gaseous phase., Upon retrieving a sample from the immobilized enzyme conditions, the volume sampled does not contain enzyme since the latter is trapped inside the gel particles, however it remains important that the reaction volume does not change drastically. The sampling of the reaction often required 100-300 µL, depending on the condition.

In addition, there was always a duplicate of every flask reactor (and sometimes triplicate) to ensure reliability of the results. The samples taken were often diluted, especially in the case of the free enzyme conditions since the initial concentration of sugars is usually above the concentration threshold of the test (5 mg/mL), in which case said dilution would be performed with distilled water.

3.6 Quantification of protein

The quantification of protein, mainly enzyme, was performed using the Bradford assay for low concentrations of protein. Such test is designed to detect protein concentrations between 1 µg/mL and 25 µg/mL. The calibration curve was obtained with bovine serum albumin (BSA). The reaction took place at room temperature for 10 minutes in the absence of light in a 96-shallow well microplate with 150 µL of the sample and 150 µL of the Coomassie reagent. After the reaction is complete, the microplates were placed in the microtiter plate reader and read at the wavelength of 595 nm.

Chapter 4 - Results and Discussion

4.1 Optimizing enzymatic hydrolysis

The degradation of cellulose-rich waste is rather complex since it is very much dependent on the quality of the substrate, mainly the fraction of cellulose it contains. For instance, microcrystalline cellulose (CMC) was heavily used throughout this work and is nearly pure cellulose (in crystalline form) – around 99%. Other substrates used were Print paper (PP) and filter paper (FP) which have 85-99% and 99% cellulose in their constitution, respectively. The hydrolytic capacity of cellulases is highly influenced by the conditions of the solution such as the concentration of substrate, pH and temperature.

Despite all the factors influencing the activity of the enzymes, these were however provided as a commercial solution by Novozymes®. As mentioned previously, the enzymes used are the ones described henceforth – cellulase and β-glucosidase.

In addition, the product specification also states the concentration of the enzymatic solutions, however, since it was a broad interval (115-230 mg/mL for cellulase and similar for β-glucosidase), these values were estimated using the Bradford method referred in **Chapter 3**, which was about 99.4 mg of enzyme per mL of solution.

According to the supplier, Novozymes®, cellulase and β-glucosidase reach optimal activity at temperatures between 45-50ºC and 45-70ºC, respectively. In addition, the optimal pH required for maximum hydrolytic activity is 5.0-5.5 for cellulase and 2.5-6.5 for β-glucosidase (**Annex 1**). These were the values taken into consideration upon planning the first set of hydrolytic runs. The conditions chosen regarding the product specifications were: 50ºC and pH=5.0, herein described as standard conditions. Any occasional change to these specifications will be pointed out in the text or in the captions of the results that contain said modification.

As addressed previously, different substrates return different concentration of cellulose in their constitution. The idea of having a relatively large reactor which can take up as much substrate as possible in order to maximize the yield of the hydrolysis, is highly influenced by the efficiency of the mixing. Ultimately, the reactor would be able to process large quantities of cellulose-rich materials such as several types of biomass – **Chapter 2**, **Table 1**. With that concept in mind, the setup of some assays included substrates that are relatively more difficult to hydrolyze due to lower cellulose content and/or that may have been subjected to a pre-treatment, which is the example of print paper. Such pre-treatments may or may not alter the pH of the material, making it difficult for the buffer to maintain the appropriate level of pH for the optimal activity of the enzymes.

4.1.1 Improving reaction conditions

Figure 12 depicts the behavior of the free enzymes over 71 hours of hydrolysis in different liquid environments (buffered and non-buffered), all at standard conditions. The concentration of cellulase and β-glucosidase was 0.99 mg/mL and 0.099 mg/mL, respectively. Further evaluation points to the fact that the hydrolysis might be performed using “low-cost” conditions such as the buffer which, most likely, is to be replaced after every single run. All three conditions were able to completely hydrolyze the cellulose present in the media, resulting in 20 mg/mL of glucose, which equals the concentration of filter paper added to the mixture. Although the requirements for the values of pH are quite strict and crucial for the optimal performance of cellulase, there is the possibility of using a cheap water solution. Nevertheless, this information must be subject to further studies since there are other factors influencing the overall hydrolytic capacity of the enzyme, such as contact with other types of substrate. Print paper, as previously mentioned, is usually subjected to acid/base treatment and may interfere with the pH of the media - in such cases, the need for a competent buffer is inevitable for the stabilization of the reaction and, consequently, the optimal performance of the enzyme.

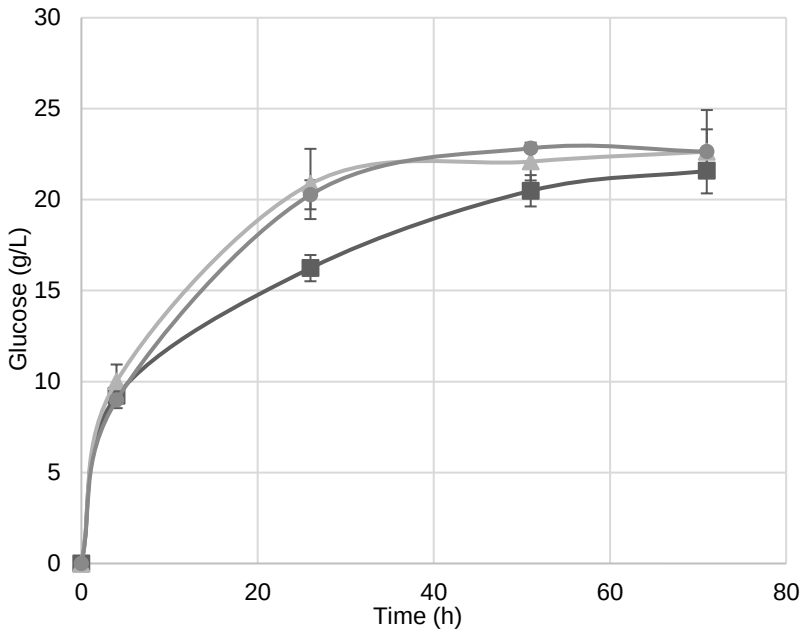

Figure 12 - Comparison between three different buffer solutions at standard conditions (50 ºC and pH 5): (■) Acetate buffer 10 mmolar; (▲) Tap water and (●) Distilled Water, to evaluate the production of glucose by the free cellulase and β-glucosidase in a flask reactor with orbital shaking, using FP as a substrate.

4.1.1-A Importance of controlling the pH

Regarding the importance of the buffer solution and having a stable pH for the reaction to take place, a couple of assays were designed to evaluate whether or not these parameters would have a significant impact on enzymatic activity. **Figure 14** compares two conditions to evaluate the consequences of adjusting or not the pH of a 10 mmolar acetate buffer (initially at pH 5.0) after the addition of the substrate. A concentration of print paper of around 20 mg/mL was used and, in addition, cellulase and β-glucosidase with concentrations of 0.99 mg/mL and 0.099 mg/mL, respectively. Print paper heavily influenced the final pH of the mixture, raising it to 6.1. Meanwhile, in the second condition the pH was adjusted after the addition of the substrate, lowering it back to 5.0, which are, essentially, the standard conditions. The noteworthy impact on the pH value was mainly due to a very high concentration of substrate - approximately 20 mg/mL.

After 26.5 hours of hydrolysis, the differences between both conditions were substantial. This difference represents the necessity of having a stable pH for the optimal activity of the enzymes, especially cellulase, which is able to tolerate a smaller range of pH values (5.0-5.5).

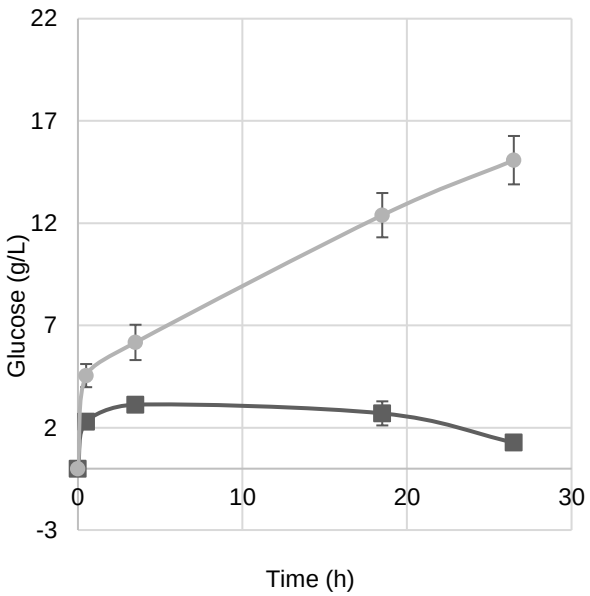

Figure 14 - Comparison between two conditions using acetate buffer (●) with pH adjustment and (■) without pH adjustment after addition of PP, to evaluate the production of glucose by the free cellulase and β-glucosidase in a flask reactor with orbital shaking at 140 rpm. PP was added in a shredded form.

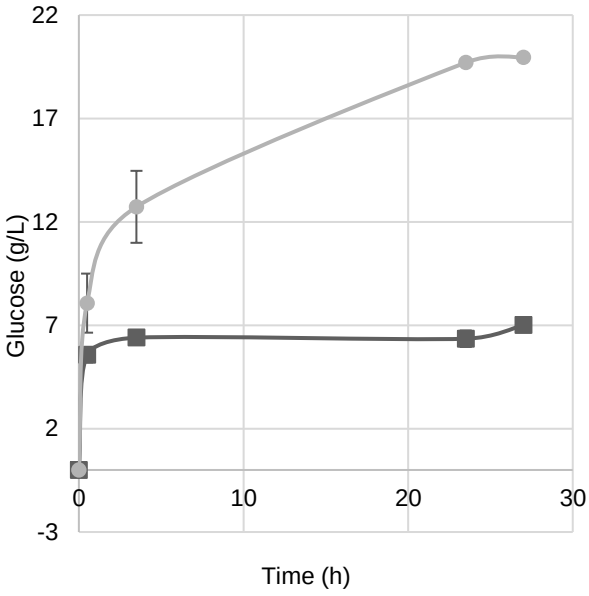

Figure 13 - Comparison between two conditions using acetate buffer (●) with pH adjustment and (■) without pH adjustment after addition of PP, to evaluate the production of glucose by the free cellulase and β-glucosidase in a flask reactor with orbital shaking at 140 rpm. PP was added in a pulverized form.

Furthermore, a second assay was performed to compare the way the substrate was added to the mixture. On the previous experiment (**Figure 14**), the print paper was added as small shredded pieces, while on the following, the substrate was added in a pulverized form, to maximize the surface of contact between cellulose and the enzymes. The results represented in **Figure 13** reveal both a higher yield and productivity, when compared to the previous study using this pulverized form of the print paper. The fact that, for the same period of time, there is a higher yield of glucose whenever the availability of cellulose increases, indicates that the availability of the substrate and hence, the form in which it is added to the mixture, influences the efficiency of the reaction.

Nevertheless, not adjusting the pH after the addition of the substrate remains as a setback since it goes up to 6.4, which is still non-optimal for cellulase activity. The concentrations of substrate, cellulase and β-glucosidase are the same as **Figure 12**. After 27 hours of hydrolysis, the final glucose concentration in both conditions was 19.9 mg/mL and 7.0 mg/mL, with and without pH adjustment, respectively. For this reason, the substrates used henceforth were more carefully selected so they would not alter the pH. This would result in another variable that could influence the results, since the enzymes would be operating at suboptimal conditions.

4.1.1-B Hydrolysis affected by structural parameters

Following the idea that agitation of the mixture is of vast importance to optimize the reaction inside the flask reactors, another set of experiments was designed. **Figure 16** represents the comparison between two conditions in which the flask reactors were either horizontal or vertical inside the incubation chamber. The idea was to evaluate whether the area of mass transfer was important for the efficiency of the reaction. Moreover, address the possibility of having multiple small flasks working in parallel without the need of mechanical agitation, which would be ideal in this sort of reaction due to the robust substrates. Additionally, another study was performed in parallel to address how the substrate was added to the mixture (shredded pieces or pulverized) and its influence in the overall enzymatic performance – **Figure 15**.

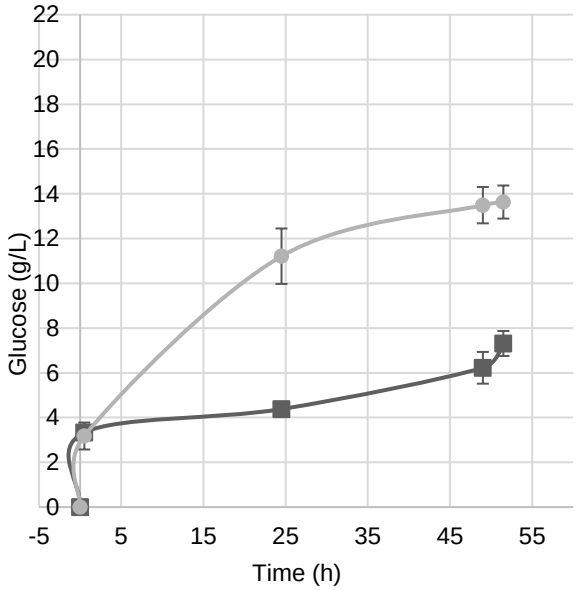

Figure 16 - Comparison between two orientations of the flask reactor: (●) vertical and (■) horizontal, to evaluate the production of glucose by the free cellulase and β-glucosidase in a flask reactor with orbital shaking at 140 rpm. FP was added in a shredded form.

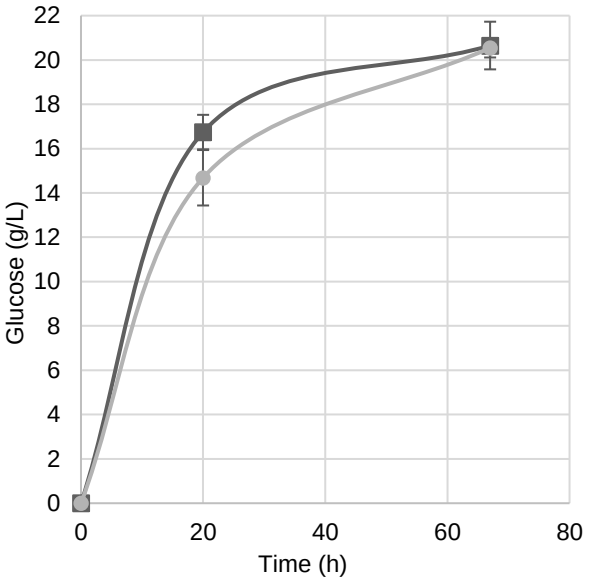

Figure 15 - Comparison between two orientations of the flask reactor: (●) vertical and (■) horizontal, to evaluate the production of glucose by the free cellulase and β-glucosidase in a flask reactor with orbital shaking at 140 rpm. FP was added in a pulverized form.

Data provided in **Figure 16** is useful enough to enlighten about the influence that the reactor and all the structural parameters have on the hydrolytic performance of the enzymes. After 51.5 hours of reaction, the final yield of glucose in the flask positioned horizontally i.e. with less agitation of the mixture, was around 7.3 mg/mL, while the one positioned vertically and hence with greater agitation, reached 13.6 mg/mL. This difference can be attributed solely to the disposition of the flask reactors since they were all at standard conditions and with the same concentration of cellulase and β-glucosidase – 0.99 mg/mL and 0.099 mg/mL, respectively. It is clearer that, in addition to its low solubility, the availability of cellulose greatly impacts the final yield of glucose.

Another study performed using the same reasoning, complemented the previous one by replacing the shredded pieces with a pulverized form of the filter paper. Results displayed in **Figure 15** add useful information to the study by revealing that after 67 hours of reaction, both conditions had converted all available cellulose to glucose – 20.6 mg/mL in the horizontal reactors and 20.5 mg/mL in the vertical reactors. It seems clear, that the nature of the cellulosic substrate to the enzymes is more impactful for the progress of the reaction than the orientation of the reactor, as the former is the key issue for enhancing the availability of cellulose to the enzyme.

4.1.2 Other factors influencing cellulose degradation

As mentioned in Chapter 1, cellulose appears in two molecular forms, depending on the material – crystalline or highly ordered and amorphous or less ordered. The former poses as the greater barrier against enzymatic hydrolysis due to its low solubility in most solvents while the latter is far more accessible to enzymes making it possible to efficiently hydrolyze to sugars. However, there is also another aspect of the reaction that is equally as important as crystallinity of cellulose, which is the intermediary product cellobiose. In the composition of the commercial enzyme solution by Novozymes®, there is also a fraction of sugars that include glucose and cellobiose, among others. Which means that, in theory, by increasing the concentration of cellulase used to hydrolyze a sample of cellulose, the overall efficiency of the enzyme will decrease by influence of cellobiose, that acts as an inhibitor.

The recommended dosage of cellulase, stated by the manufacturer is therefore 1-5% (w/w) which was followed throughout most of the experimental work – approximately 5 mg of protein to hydrolyze 100 mg of cellulose-rich substrate, being the equivalent to 5% (w/w). In addition, β-glucosidase, when added, was in 0.5% (w/w) – approximately 0.5 mg of protein for the same 100 mg of substrate. In essence, the recommended dosage was established to avoid non-enzymatic interactions, made possible by a concentration of solids that is too high. The main chemical interaction interfering with cellulase, apart from pH of the mixture, is the inhibitory action employed by cellobiose.

4.1.2-Effect of cellulase concentration in the mixture

The inhibitory phenomena inherent to cellulase activity was not studied in depth. However, the concentration of the enzyme in the mixture was tested for further interactions that might improve the overall reaction. Using the manufacturer's recommended dosage of 1-5% (w/w) as a standard concentration, it was decided to test higher concentrations for possibly higher yields of glucose, or to speed up the hydrolysis of the substrate. **Figure 18** represents the results obtained from testing three different concentrations of cellulase with a 5 mg/mL sample of CMC. After 22 hours of reaction, the yield of glucose was 2.9 mg/mL, 2.3 mg/mL and 1.9 mg/mL for the cellulase concentrations of 0.99 mg/mL, 2.98 mg/mL and 4.97 mg/mL, respectively and correspond to approximately 5%, 15% and 25% of the total cellulose content of the mixture, respectively.

Upon comparing the three conditions, one can attribute the higher concentrations of cellulase to be the cause of lower yields of glucose. However, according to the manufacturer, only 10 to 20% of the enzyme kit is actual protein, so the remaining 80 to 90% would contain most of the sugars described. **Figure 17** illustrates this relation by comparing the concentration of enzyme with the initial concentration of glucose.

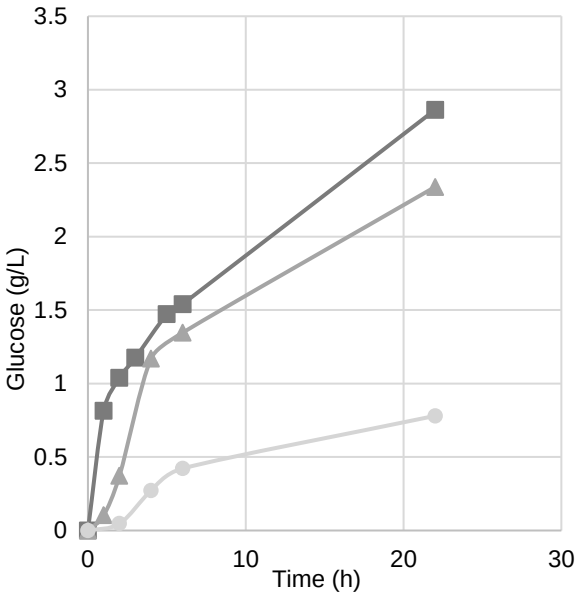

Figure 18 - Comparison between three different cellulase concentrations: (■) 0.99 mg/mL; (▲) 2.98 mg/mL and (●) 4.97 mg/mL, to evaluate the production of glucose in a flask reactor with orbital shaking at 140 rpm, using CMC as a substrate.

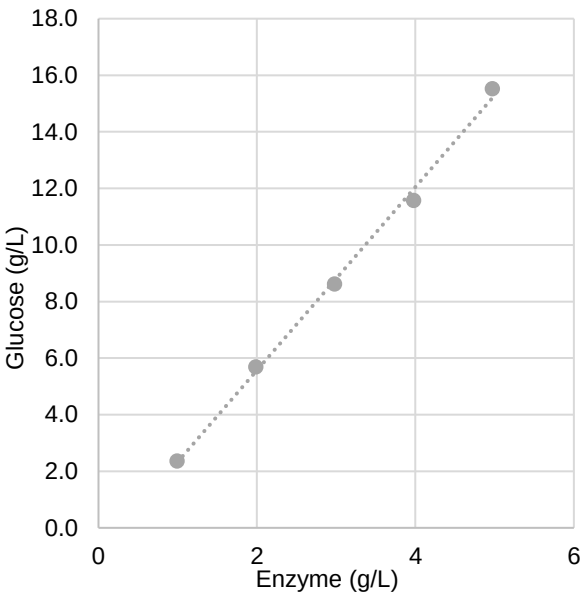

Figure 17 - (●) Relation between enzyme concentration (Free Cellulase) and concentration of glucose in flask reactors with 5 mL of a 5 g/L solution of CMC with acetate buffer.

The concentrations of enzyme tested for glucose were 0.99 mg/mL, 2.98 mg/mL and 4.97 mg/mL. The concentration of glucose is linearly related to the concentration of enzyme, making it quite possible for the concentration of cellobiose to increase as well. As previously stated, the inhibitory effect of cellobiose would be greater when using higher concentrations of cellulase, which is corroborated by the data presented in **Figure 18**.

4.2 Hydrolytic capacity of Immobilized Cellulosic enzymes

Based on a sustainable perspective, this process could only be of major significance if the materials used could be somehow re-utilized or, at most, be of the lowest possible cost. The materials used are mostly paper residues or other cellulose-rich compounds that are inherently cheap. Since the source of the raw substrates are of such low cost, the bio-catalyst should also be of lower cost, to make the procedure more sustainable from an environmental and economical point of view.

The bio-catalysts tested in this work are, as mentioned previously, a commercial cocktail of cellulose-degrading enzymes, namely cellulase and β-glucosidase. Starting from a state where the enzymes were mixed directly with the substrate in small flask reactors, the main goal was to eventually immobilize them in a gel-like particle that could eventually be recycled after every hydrolytic run. This factor would contribute to using less enzyme per amount of substrate than a free enzyme methodology, that should be replenished after every hydrolytic run.

4.2.1 Immobilization of Cellulase in LentiKat Particles

Initially, the plan was to immobilize the enzymes in magnetic micro-particles (MMP) so that their recovery from the media post-hydrolysis was achieved using a magnetic apparatus. Ideally, the procedure would require a magnet strong enough to attract the particles in a relatively big reactor. Such immobilization technique was attempted in the early stages of the work but to no avail, probably due to inactivation of the enzyme during the incubation with the particles. Therefore, another strategy was implemented, which consisted of an immobilization procedure through entrapment in hydrogel particles – LentiKat (**Chapter 3**).

Using the commercial form of Poly-vinyl alcohol mixed with the enzymatic solution, the formation of gel-like particles was achieved by means of depositing small droplets of the mixture into a petri dish and left to dry at room temperature. Although this method was efficient, the shape of the particles was rather flat in comparison to the second method. Additionally, other method was attempted, which consisted of mixing the poly-vinyl alcohol solution with the enzymatic solution and dropping the mixture in Poly (ethylene glycol) (PEG-600). This second procedure allowed for the formation of near-spherical particles that were slightly more robust than those obtained using the plate method. Unfortunately, performing a protein detection assay such as Bradford to calculate the immobilization efficiency was not feasible since the PEG 600 solution is detected as a positive, being completely useless to quantify the protein present in the supernatant after the formation of the particles take place. Moreover, BCA or Lowry assays would also yield low-confidence results due to the media in which the enzymes are contained. Overall, spectrophotometric methods would not yield trustworthy results that could eventually be reproduced.

Both methodologies were quite efficient in maintaining the enzymatic activity of the bio-catalysts, although they revealed slightly less hydrolytic activity in comparison to the free enzymes (**Figure 19**). Worth noting that the concentration of enzyme utilized was not the same in both conditions (0.99 mg/mL of free cellulase and 1.33 mg/mL of immobilized cellulase), however, in practical terms, the concentration of immobilized cellulase molecules could be misleading since the immobilization efficiency could not be calculated due to lack of data. In essence, the solution of PEG might have contained a percentage of the enzyme solution (that was not immobilized in the particles) and because of that, the theoretical value might be slightly off. However, the concentration of enzyme lost in the immobilization could be estimated if PEG was not detected in protein-detecting assays (such as Bradford). Moreover, the specific activity could be estimated using a reaction mixture with substrate and the PEG solution with the non-immobilized enzyme, but this could not be achieved. The reason behind this technical difficulty lies in the inaccuracy of spectrophotometric methods in the quantification of by-products in a complex reaction media such as this.

Furthermore, the differences between both immobilization strategies were analyzed relatively to the glucose formed during the hydrolytic runs and compared against a flask reactor with free enzyme. In this set of hydrolysis, free enzyme remains with the highest glucose concentration after 44.5 hours of reaction, with 4.8 mg/mL. Both methods of immobilization had a lower yield, however positive – 1.3 mg/mL and 1.9 mg/mL for PEG and plate methods, respectively.

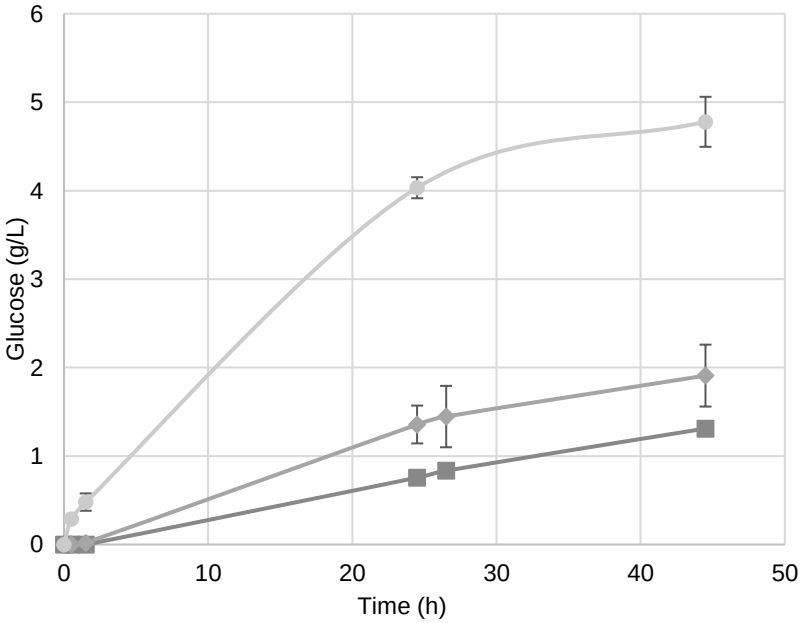

Figure 19 - Comparison between three different setups: (●) Free enzyme; (■) LentiKat Cellulase (PEG) and (♦) LentiKat Cellulase (plate), to evaluate the production of glucose with both methods of immobilization, in a flask reactor with orbital shaking at 140 rpm. Substrate used was CMC 5g/L.

4.2.2 Further evaluation of the Immobilized Enzymes

Throughout the rest of the lab work, the immobilization using PEG was the chosen methodology because it is more reliable regarding the shape of the particles. The reaction was rather influenced by the presence of cellobiose in the media in the case of free enzyme, which was attenuated by the presence of β-glucosidase. To allow for the hydrolysis of this by-product, β-glucosidase was also immobilized using the same PEG method. **Figure 21** shows that the immobilized form of the biocatalyst is less affected by the presence of cellobiose than the its free enzyme counterpart. This is partially due to the smaller initial concentration of sugars (which include glucose, cellobiose and other sugars) present in the reaction mixture which contains the immobilized enzyme.

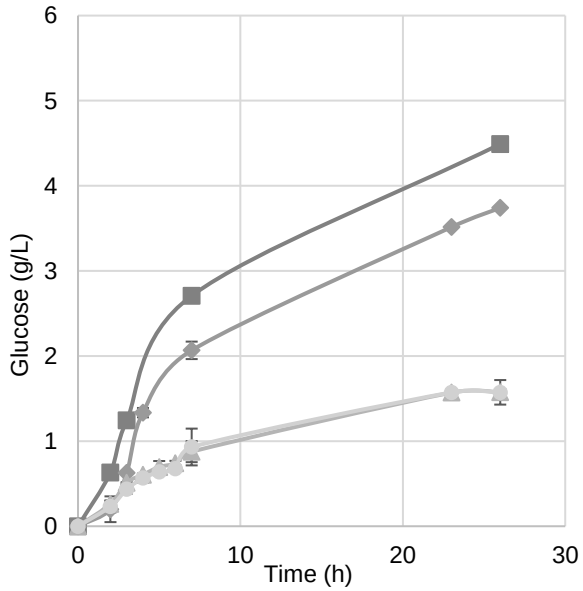

Figure 21 - Comparison between four different setups: (●) Immobilized cellulase; (▲) Immobilized cellulase and β-glucosidase; (♦) Free cellulase and (■) Free cellulase and β-glucosidase, to evaluate the importance of β-glucosidase present in the mixture regarding the overall glucose yield from the hydrolysis of CMC. Reaction took place in flask reactors at standard conditions and orbital mixing at 140 rpm.

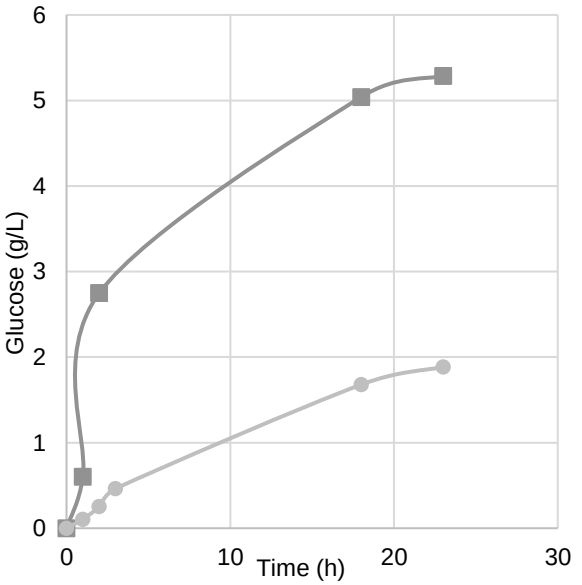

Figure 20 - Comparison between two different setups: (●) Immobilized cellulase and β-glucosidase and (■) Free cellulase and β-glucosidase. Reaction took place in flask reactors at standard conditions and orbital mixing at 140 rpm, but the total volume of the mixture was doubled (10 mL). Glucose resulted from the hydrolysis of CMC.

The data reveals that, although the immobilized form of the enzymes shows less activity compared to the free enzyme, it behaves similarly whether in the presence or absence of β-glucosidase. On one hand, this shows that inhibitory compounds interact less with the immobilized enzyme or it is less affected by them. On the other hand, the reduced activity also reveals that some enzyme units might have their active site occluded due to the particle's structure, being unable to perform their hydrolytic activity. Data on **Figure 20** shows that during the same period (23 hours), the free enzymes condition managed to hydrolyze all the cellulose present in the media (5.3 g/L), while the immobilized enzymes condition only reaches 38% of the total glucose potential (1.9 g/L).

Nevertheless, it is worth noting that although the glucose yield seems very low for the immobilized enzymes setup, the concentration of free enzyme was greater than that of immobilized enzymes, being the former 2.98 mg/mL and 0.298 mg/mL - for cellulase and β-glucosidase, respectively, and the latter, 1.33 mg/mL and 0.133 mg/mL. Although the activity was substantially lower, the most important fact is that there was in fact hydrolytic activity and despite having lower solubility, the CMC was able to penetrate the particle's pores in order to access the enzyme units.

4.2.3 Testing Immobilized Enzymes with different substrates

As previously stated, an enzymatic approach to degrading cellulosic materials is very complicated, mainly due to the accessibility of the cellulose to the enzymes. In this project, there was no involvement of lignocellulosic biomass, in which case, the approach would have to include some sort of pre-treatment to facilitate the accessibility to the cellulose such as the pre-treatments briefly discussed in **Chapter 1**. As an attempt to test the immobilized enzymes with a substrate of more difficult access and robustness, filter paper (FP) and print paper (PP) were used as a source of cellulose.

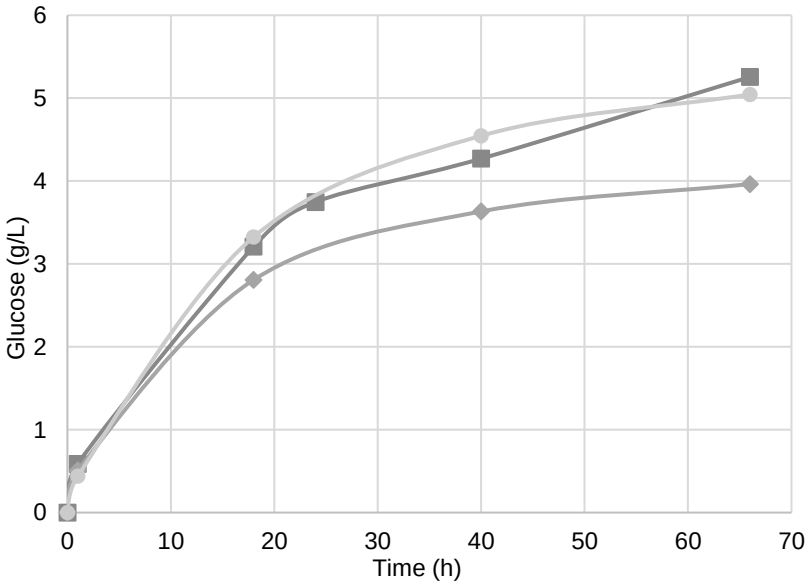

Figure 22 - Glucose Formation using Immobilized Enzymes (Cellulase and β-Glucosidase) in a Flask Reactor at standard conditions with Magnetic Stirring at 265 rpm. (●) Filter paper; (♦) Print paper and (■) Microcrystalline cellulose.

As presented in **Figure 22**, all cellulose was consumed by the immobilized enzymes – both cellulase and β-glucosidase, after 66 hours of incubation time. In these conditions, the concentration of cellulase and β-glucosidase used were 1.33 mg/mL and 0.133 mg/mL, respectively. Microcrystalline cellulose and filter paper seem to have roughly the same outcome, regarding enzymatic hydrolysis, having the former a slightly higher yield of glucose (5.17 mg/mL) than the latter (5.0 mg/mL). This difference is obviously due to the different content of cellulose that each substrate has but, nevertheless, the fact that filter paper is more compact poses an obstacle for enzymatic hydrolysis mainly due to low accessibility. However, this is not observed in the results, which points to the notion that the pores in the particles are wide enough for the free passage of cellulose. This last aspect is also favored by the magnetic stirring and also to the higher agitation rate.

4.3 Scale up

As previously stated, the main importance of a process designed to degrade cellulosic compounds into usable/fermentable sugars is the ability to hydrolyze high volumes of feed, making it more efficient. Although, small-scale reactors allow us to study the interaction between the immobilized enzymes and the substrate in higher detail, a larger scale unit will ultimately open the possibility of improving the procedure, since it studies its overall performance.

In order to simulate a large-scale process, the reaction mixture, including the substrate solution and the enzymes immobilized in LentiKat particles were introduced in a heated reactor with mechanical stirring. The main parameters involved in the preliminary studies was the speed of agitation and the protein leakage, since it would ultimately influence the accessibility of the enzymes to the cellulose and the re-usability of the particles (**Figure 23**).

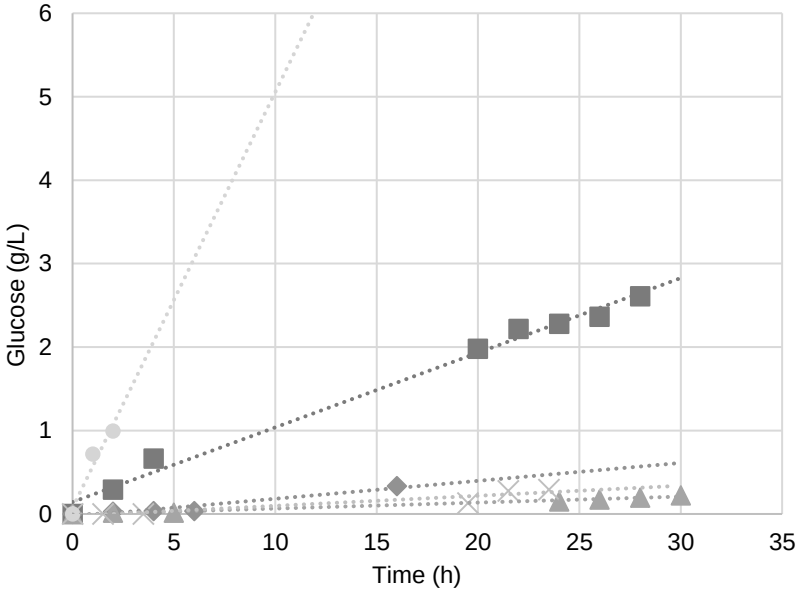

Figure 23 - Comparison between five different stirring speeds: (●) 55 rpm; (■) 96 rpm; (X) 184 rpm; (♦) 375 rpm and (▲) 527 rpm, that were tested to optimize the production of glucose by the immobilized enzymes (cellulase and β-glucosidase) in a heated reactor with Mechanical stirring. Data for 55 rpm was not sufficient, so a projection line was added to help estimate the evolution of the reaction.

Although the hydrolysis of cellulose-rich substrates by the cellulolytic enzymes in study is rather slow, the reactions could not be prolonged for more than a day due to intense evaporating that influenced the concentration of glucose. Using a completely sealed reactor that would not be subject to this influence, the reaction would most likely have the same tendency as those discussed in **4.2.3**.

In the reaction mixture displayed in the five cases of **Figure 23**, a 5 g/L CMC solution was used as substrate for the immobilized enzymes - cellulase and β-glucosidase, with concentrations of 1.33 mg/mL and 0.133 mg/mL, respectively. The experiment revealed that a lower agitation speed is preferred over a higher agitation rate. This is easily explained by the fact that the enzyme units are immobilized through occlusion and therefore the substrate needs to have an easy access inside the particle for the reaction to occur. This can only be achieved by having a lower agitation rate to allow for the cellulose molecules to enter the particle's pores and be available for enzymatic hydrolysis.

On the other hand, the reaction mixture cannot be completely idle, otherwise the cellulose, which has already low solubility, will deposit on the bottom of the reactor, yielding minimal or absent sugar conversion. The agitation rate of 55 rpm was the most efficient since the maximum sugar yield would be achieved after roughly 10 hours, according to the projection line on **Figure 24**. However, a broader sample would be necessary to make assumptions with higher fidelity.

4.3.1 Protein loss associated with mechanical stirring

As inevitable as it is, the lifetime of an immobilized bio-catalyst is limited to a certain number of utilizations. Although, the exact number of utilizations was not estimated, protein quantification was performed after a few hydrolytic runs to address the amount of enzyme present in the supernatant. On average, each hydrolytic run presented in **Figure 23** – four agitation rates: 96, 184, 375 and 527 rpm, had a protein loss of around 0.17 mg/mL corresponding to 1% of the total protein present in the LentiKat particles, although the agitation rate of 527 rpm had the highest loss of protein – 0.2 mg/mL corresponding to 1.2% of the total concentration of protein inside the reactor.

Chapter 5 – Conclusions

Enzymatic hydrolysis of complex substrates is of great difficulty, especially due to all the factors that may interfere with the enzymes aside from the main reaction, such as substrate-induced pH shift that must to be countered by adjusting the pH of the mixture after its addition. As thoroughly tested throughout this experimental work, pH is of valuable importance for establishing optimal conditions for enzymatic hydrolysis (especially cellulase, since it has lower tolerance for pH variance). This could be achieved more easily by utilizing a stronger buffer solution (with higher molarity). That is yet to be studied since the implications that it has on the progress of the reaction are not solely due to the pH value but also to the nature of the buffer solution.

Relatively to the enzyme immobilization, there is a slight drawback, which is the failure to efficiently quantify the protein present in the hydrogel particles that could potentially aid in the accurate measurement of the appropriate quantities of immobilized catalyst to use. In addition, the immobilization procedure is inherently difficult, due to the uncertain aspects of enzyme immobilization, such as pore size. Further studies are required regarding that issue. Another challenge is the scale-up of the process, which could improve if the evaporation issue was resolved – the evaporation of the water in the mixture influences the accurate measurement of glucose, leading to less trustworthy results.

Overall, the performance of the immobilized form of the enzymes was relatively good, meaning that despite the uncertainty of the analytic methods to quantify the immobilization efficiency, the particles were able to efficiently hydrolyze cellulose into glucose. The strategies applied in this study revealed that these immobilized enzymes are a practical use for the conversion of cellulose to glucose, since they are able to retain their hydrolytic activity

In conclusion, other factors influencing this efficiency is yet to be explored, however, the results presented here are a good way of propelling the knowledge of enzyme immobilization a step forward towards their industrial application, which would be the final objective of this study, if it was not for the limited amount of time.

Chapter 6 – Future perspectives

A biocatalyst-based process must undergo several stages to ensure its economic viability, such as its production (and possible engineering to optimize its performance), its application in the reaction and the subsequent recovery of the desired product (**Figure 24**). Future studies should focus on hydrolysis of high loadings of cellulosic or even lignocellulosic biomass and examine the enzymatic performance using immobilized cellulase and β-glucosidase. So far in this work, the hydrolysis experiments on cellulosic substrates have only been conducted using low substrate loadings, however, in an industrial process it is important to obtain higher yields of glucose and, consequently, higher loadings of cellulose are necessary.

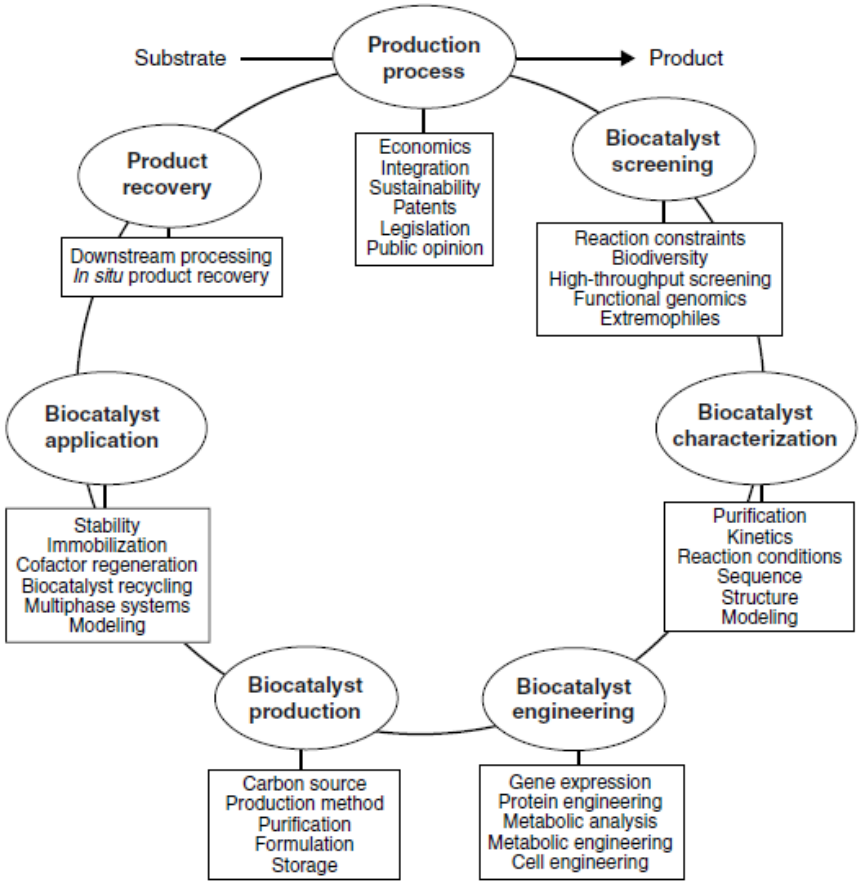

Figure 24 – Schematic representation of the development of a biocatalyst-based process. Extracted from Jan B van Beilen *et al.* [58]

Like any project regarding a biocatalyst, its application and subsequent recovery of the product, there is inevitable complications associated. For instance, the preliminary steps of developing optimal conditions to maximize enzymatic activity and production of the desired compound are usually designed for a low-volume reaction mixture. However, these conditions may need to be tuned to accommodate other variables that come into play when the process is submitted to a scale-up.

Regarding the downstream processing of the product of the reaction, which was not part of the experimental work presented in this thesis, there are important factors to consider, such as the recovery of the glucose and their subsequent processing and characterization. There is also one important factor that may play a crucial role in the whole process which is the recovery and subsequent storage of the particles with the immobilized enzymes, which may require special storage conditions such as a defined solution designed to protect the active site of the enzymes and therefore prevent their loss of activity. Ultimately, the useful lifetime and mechanical properties of the hydrogel matrix must be taken into consideration so that it does not influence negatively the performance of the biocatalyst.

In a broader view, the possible uses for the glucose produced through the hydrolysis of cellulose should be considered. Fermentative micro-organisms have already been used, using glucose with the same origin, to produce bio-ethanol which, consequently, has potential use as a bio-fuel.

Chapter 7 – Bibliographic References

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Chapter 8 – Annexes

Annex 1 – Novozymes® Cellulosic Ethanol Enzyme Kit specifications

Enzyme classification	Activity[1]	Density[2] (g/ml)	pH	Temperature (°C)	Dosage[3] (% w/w (TS))
NS22086 Cellulase complex	1,000 BHU(2)/g	1.15	5.0–5.5	45–50	1–5%
NS22083 Xylanase	2,500 FXU-S/g	1.09	4.5–6.0	35–55	0.05–0.25%
NS22118 β-glucosidase	250 CBU/g	1.2	2.5–6.5	45–70	0.2–0.6%
NS22119 Enzyme complex	100 FBG/g (~ 13,700 PGU/g)	1.19	4.5–6.0	25–55	0.05–0.4%
NS22002 Hemicellulase	45 FBG/g (~ 470 FXU/g)	1.20	5.0–6.5	40–60	0.4–2%
NS22035 Glucoamylase	750 AGU/g	1.15	4.5–5.5	60–70	0.01–0.06%

Table 2. Enzyme activity, density, pH, temperature, and recommended dosage.

1) EGU = Endo-Glucanase Unit, CBU = CelloBiase Unit, FBG = Fungal Beta-Glucanase Unit, PGU = PolyGalacturonase Unit, FXU-S = Fungal Xylanase Unit, and AGU = AmyloGlucosidase Unit. See Appendix A for further information on activity units.

2) Density values are approximate.

3) The required dosage is heavily dependent on feedstock type, pretreatment technology, and processing conditions. Enzyme dosage requirements may therefore vary significantly.