From Wastepaper to sugars using immobilized enzymes

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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. Overall, the potentialities of this lignocellulosic materials vary greatly among different types of biomass. This variability also promotes the development of novel strategies to approach the potential of reusing this material for the benefit of humankind. The main focus of this thesis lies on the most valuable component of these materials – cellulose. There are a group of biocatalysts called cellulases that are able to degrade such compound, converting it to sugars such as glucose. By using cellulose-rich compounds, such as waste paper, as substrate, a process was designed for the utilization of these 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 waste paper, revealing that the enzymes not only retained their hydrolytic abilities, but also achieved good glucose yields. 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.

1. Introduction

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]**. 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. 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]**. 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. Typically, each cellulose molecule consists of 5000 to 10,000 units of glucose (depending on the degree of polymerization) **[3]**. 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 **[4]**. In this work, the main objective was to hydrolyze this polymer into fermentable sugars (glucose) via enzymatic hydrolysis, using commercial enzymes – cellulase and β-glucosidase, supplied by Novozymes®.

The hydrolytic enzymes should be of desirable characteristics for their application in the hydrolysis of cellulose-rich substrates. Some of the desirable characteristics of the enzymes include catalytic efficiency, thermal stability, adsorption, end-product inhibition resistance and shear inactivation **[5,6]**.

Cellulase-producing organisms use different strategies to hydrolyze cellulose. Aerobic bacteria and fungi secrete soluble extracellular enzymes known as *non-complexed cellulase systems*, while anaerobic cellulolytic microorganisms produce *complexed cellulase systems*, often referred to as *cellulosomes* **[7]**. Most cellulase-systems comprehend several enzymes such as endo- and exo-glucanases, cellobiohydrolase and β-glucosidase, to allow for a progressive hydrolysis of the long chains of cellulose **[8]**.

The scope of this project was to develop a strategy to hydrolyze cellulose-rich substrates via enzymatic hydrolysis. However, one of the key factors was the immobilization of the enzymes so that they could be recycled and re-used in another reaction, contributing to a more economically efficient process.

To promote enzyme utilization in biotechnological processes, different methods to reduce its costs have been applied, being that immobilization is one of them **[9]**.There are several methods for enzyme immobilization, namely, physical adsorption of the enzymes to a support material; entrapment in a polymeric matrix (most of the times, irreversibly) – which was the method chosen in the experimental work; by cross-linking, sometimes forming aggregates of enzyme units (CLEAs); and covalent-bonding to a carrier material **[9, 10, 11]**.

The enzymes were entrapped in a hydrogel matrix (LentiKat®) of poly(vinyl) alcohol and subsequently used to hydrolyze cellulose-rich substrates such as print paper and filter paper, resulting in the production of glucose.

2. Materials and Methods

2.1 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.

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).

2.3 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. However, two methods were used: the plate method and the Poly (ethylene glycol) method (PEG). The plate method consisted in dropping small droplets of the enzyme (either cellulase or β-glucosidase, separately) + PVA solution in a plate to dry at room temperature, hence forming flat lenticular particles; the PEG method was very similar, however, the enzyme + PVA solution in a solution of PEG 600, in which the particles would solidify instantly upon the formation of hydrogen bonds, resulting in spherical/lenticular particles.

2.4 Reactor operation

Enzymatic hydrolysis was usually performed inside small vessels with 15 mL of useful volume, with either orbital or magnetic stirring. The reaction mixture consisted of enzyme (cellulase, β-glucosidase or both), a buffer (usually sodium acetate 10 mmolar) at pH 5 and a cellulosic substrate – microcrystalline cellulose (CMC), filter paper (FP) or print paper (PP). In addition, a larger reactor (100 mL useful volume) was used to test the influence of mechanical agitation in the hydrolytic performance of the enzymes.

2.5 Quantification of glucose

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

2.6 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.

3. Results and Discussion

3.1 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. The conditions chosen regarding the product specifications (Novozymes®) were: 50ºC and pH=5.0, herein described as standard conditions.

3.1.1 Optimizing enzymatic hydrolysis

Figure 1 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. 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.

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. 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 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

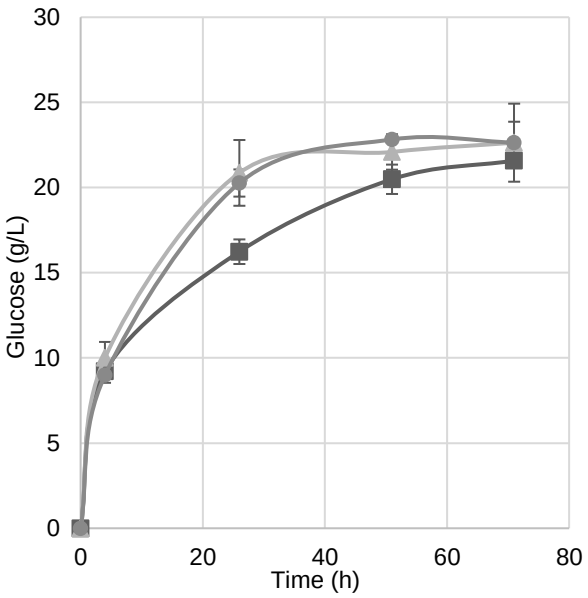

Figure 1 - Comparison between three different buffer solutions at standard conditions (50 ºC and pH 5): (■) Acetate buffer 10 mmolar; (▲) Tap water and (●) Distilled Water, to evaluate the production of glucose by the free cellulase and β-glucosidase in a flask reactor with orbital shaking, using FP as a substrate.

Importance of controlling the pH

Figure 2 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. 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. 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).

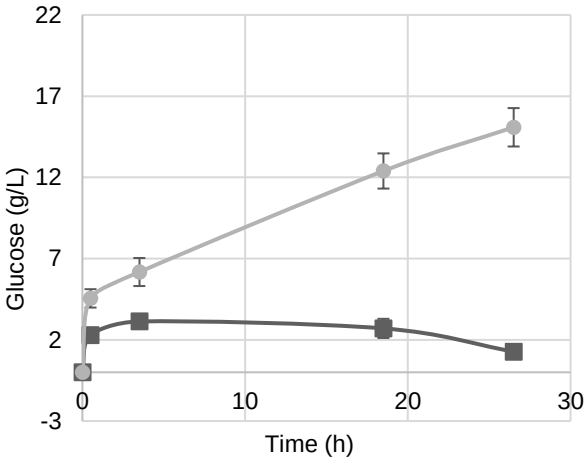

Figure 2 - Comparison between two conditions using acetate buffer (●) with pH adjustment and (■) without pH adjustment after addition of PP, to evaluate the production of glucose by the free cellulase and β-glucosidase in a flask reactor with orbital shaking at 140 rpm. PP was added in a shredded form.

A second assay was performed to compare the way the substrate was added to the mixture (**Figure 3**).

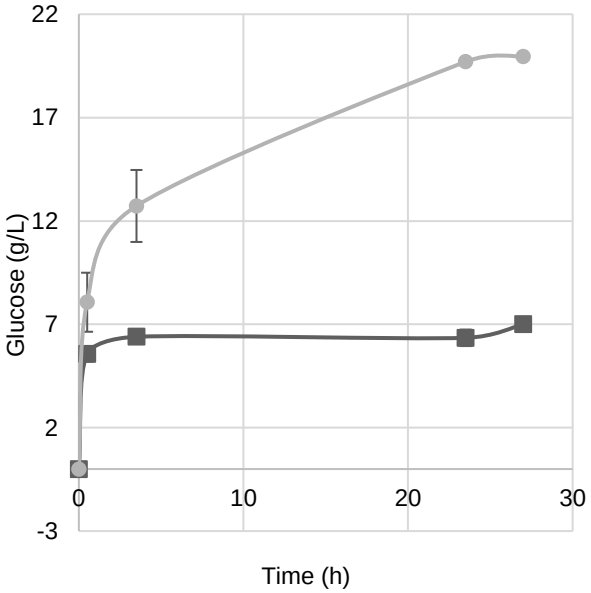

Figure 3 - Comparison between two conditions using acetate buffer (●) with pH adjustment and (■) without pH adjustment after addition of PP, to evaluate the production of glucose by the free cellulase and β-glucosidase in a flask reactor with orbital shaking at 140 rpm. PP was added in a pulverized form.

Figure 2 and **3** differ in the form the substrate was added, shredded pieces and pulverized, respectively.

The results represented in **Figure 3** reveal both a higher yield and productivity, when compared to the previous study (**Figure 2**) using this pulverized form of the print paper. For the same period of time, there is a higher yield of glucose whenever the availability of cellulose increases, indicating that the form in which it is added to the mixture influences the efficiency of the reaction.

Hydrolysis affected by structural parameters

Figure 4 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.

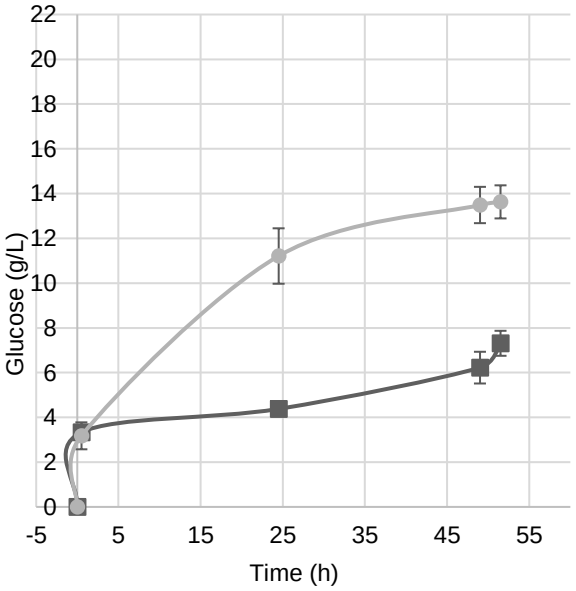

Figure 4 - Comparison between two orientations of the flask reactor: (●) vertical and (■) horizontal, to evaluate the production of glucose by the free cellulase and β-glucosidase in a flask reactor with orbital shaking at 140 rpm. FP was added in a shredded form.

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.

3.1.2 Other factors influencing cellulose degradation

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. It was decided to test higher concentrations of enzyme for possibly higher yields of glucose, or to speed up the hydrolysis of the substrate. **Figure 5** 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

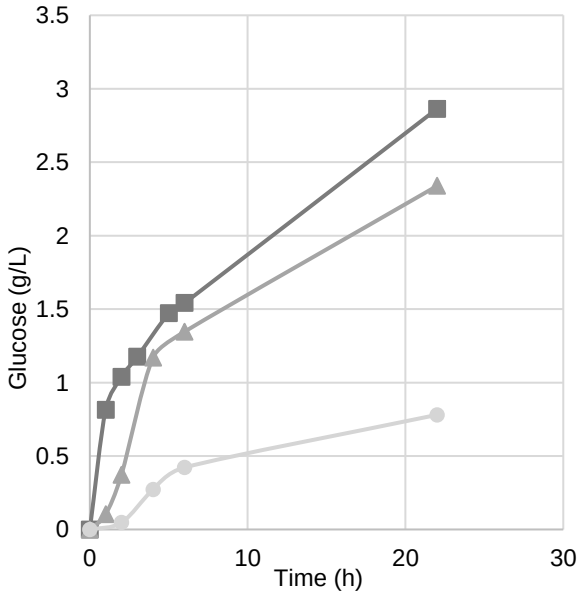

Figure 5 - Comparison between three different cellulase concentrations: (■) 0.99 mg/mL; (▲) 2.98 mg/mL and (●) 4.97 mg/mL, to evaluate the production of glucose in a flask reactor with orbital shaking at 140 rpm, using CMC as a substrate.

3.2 Hydrolytic capacity of Immobilized Cellulosic enzymes

3.2.1 Immobilization of Cellulase in LentiKat Particles

Both methodologies (plate and PEG methods of immobilization) 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 6**). 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.

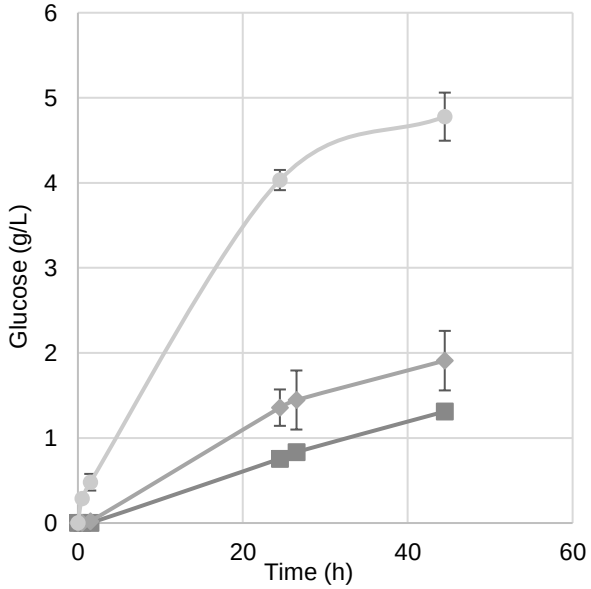

Figure 6 - Comparison between three different setups: (●) Free enzyme; (■) LentiKat Cellulase (PEG) and (♦) LentiKat Cellulase (plate), to evaluate the production of glucose with both methods of immobilization, in a flask reactor with orbital shaking at 140 rpm. Substrate used was CMC 5g/L.

3.2.2 Testing Immobilized Enzymes with different substrates

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. 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 7**).

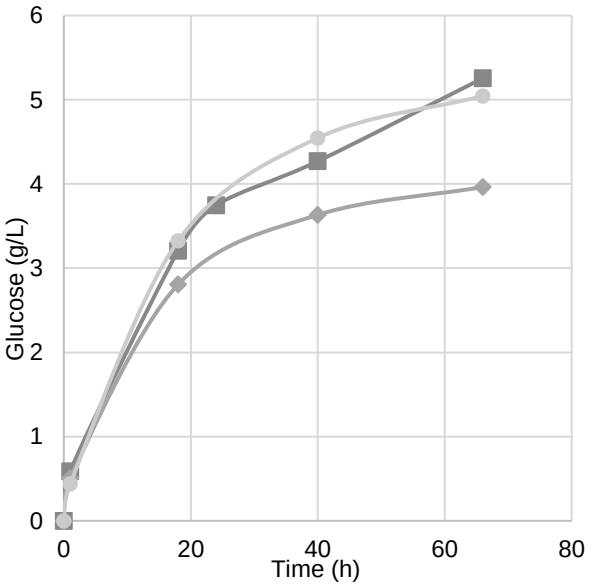

Figure 7 - Glucose Formation using Immobilized Enzymes (Cellulase and β-Glucosidase) in a Flask Reactor at standard conditions with Magnetic Stirring at 265 rpm. (●) Filter paper; (♦) Waste paper and (■) Microcrystalline cellulose.

As depicted, 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.

3.3 Scale up

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. 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 (**Figure 8**).

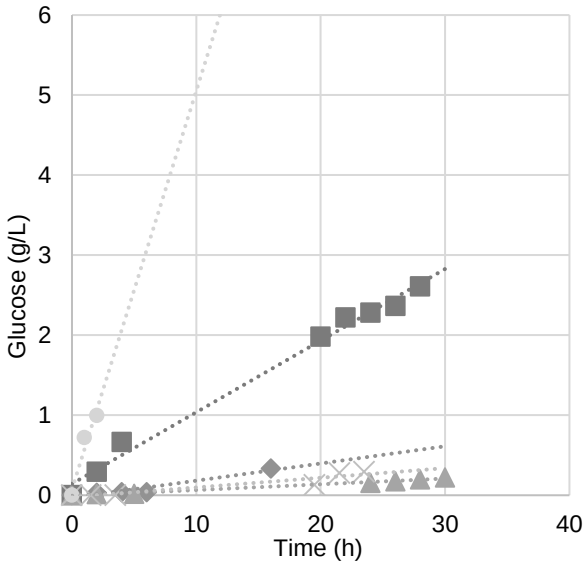

Figure 8 - Comparison between five different stirring speeds: (●) 55 rpm; (■) 96 rpm; (X) 184 rpm; (♦) 375 rpm and (▲) 527 rpm, that were tested to optimize the production of glucose by the immobilized enzymes (cellulase and β-glucosidase) in a heated reactor with Mechanical stirring. Data for 55 rpm was not sufficient, so a projection line was added to help estimate the evolution of the reaction.

In the reaction mixture displayed in the five cases of **Figure 8**, 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 assay revealed that a lower agitation speed is preferred over a higher agitation rate. By having a lower agitation rate, it allows for the cellulose molecules to enter the particle's pores and be available for enzymatic hydrolysis. 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 8**. However, a broader sample would be necessary to make assumptions with higher fidelity.

3.3.1 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 8** – 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. These results reveal the importance of controlling the shear stress that is put into the particles with the increased agitation rate.

4. Conclusions and Future perspectives

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 the pH. 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, more studies are required regarding the immobilization, possibly a microscopic analysis of the particle's pores, to determine if they are in fact appropriate and allow for the free passage of substrate and products, which could eventually be improved. 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. 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.

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