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Immobilization of enzymes on polymeric microparticles

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“Better to have tried and failed than to have never tried at all.”

- Sean-Paul Thomas

Acknowledgments

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Abstract

As society becomes increasingly aware of environmental problems and their consequences, and looks for “greener” options, the necessity of those same alternatives to become viable to be used in large scale increases. The consequences of uncontrolled use of non-renewable resources such as fossil fuels and the implementation of non-sustainable politics are starting to become more and more visible and worrying, painting an uncertain and hostile future for the next generations.

Taking this current scenario into account, the search for alternatives such as organic-based fuels created from biomass with low emissions has become increasingly more appealing, as a clean and sustainable solution to the high global energy demand. However, these alternatives still have high costs associated to their fabrication process, making them less competitive against conventional fuels.

One of the main goals of the work developed in this dissertation is to determine the viability of using chitosan magnetic microspheres, activated with a glutaraldehyde solution, as an active support to use with enzymes like *Viscozyme*, in the hope that their magnetic properties allow for easier handling. This is achieved by studying the factors that influence the sphere’s properties, optimizing their creation process and then immobilizing *Viscozyme* on their surface. Afterwards, the spheres are used in several hydrolysis reactions one after the other, in order to analyze their efficiency and operating stability.

Results show that magnetic microspheres are as viable as non-magnetic ones in terms of production and size manipulation when using a coaxial airflow bead generator system. Both types of microspheres also show similar results in terms of surface activation with glutaraldehyde and immobilization of *Viscozyme*. Results obtained from HPLC are inconclusive in terms of yield difference between free enzyme and immobilized enzyme, but also show no clear difference in terms of viability between both types of microspheres.

Keywords: Chitosan, microsphere, *Viscozyme*, biomass, hydrolysis, magnetic, immobilization

Resumo

À medida que a sociedade se apercebe cada vez mais da importância dos problemas ambientais e das suas consequências, e procura alternativas mais "verdes", também a necessidade de essas mesmas alternativas serem viáveis para uso em larga escala aumenta. Cada vez mais as consequências do uso descontrolado de recursos não-renováveis como combustíveis fósseis e implementação de políticas não-sustentáveis se tornam visíveis e mais preocupantes, prevendo-se assim um futuro incerto e hostil para as próximas gerações.

Tendo em conta este cenário atual, a procura de alternativas como combustíveis orgânicos à base de biomassa e de baixas emissões tem-se tornado cada vez mais apelativa como uma forma de responder às necessidades energéticas mundiais de forma sustentável e limpa. No entanto, estes combustíveis têm ainda um grande custo associado ao seu processo de fabrico, sendo esse o maior obstáculo corrente que impede a transição dos combustíveis tradicionais.

O trabalho desenvolvido nesta dissertação tem como objetivo principal determinar a viabilidade do uso de microesferas magnéticas de quitosano ativado com solução de glutaraldeído, como um suporte ativo do composto enzimático *Viscozyme*, na esperança de que as suas propriedades magnéticas facilitem o seu manuseamento. Tal é conseguido estudando os fatores que influenciam as propriedades das esferas e otimizar o seu processo de fabrico, e de seguida utilizar as esferas com enzima imobilizada em ciclos de reações de hidrólise e analisar a sua eficácia e estabilidade.

Os resultados obtidos demonstram que microesferas magnéticas são tão viáveis como as não-magnéticas em termos da sua produção e de manipulação dos seus parâmetros usando um "*coaxial airflow bead generator system*". Ambos os tipos de microesferas demonstram resultados semelhantes em termos de ativação de superfície utilizando glutaraldeído e em relação a imobilização de *Viscozyme*. Resultados obtidos pelo HPLC são inconclusivos em termos da diferença de rendimento entre enzima livre e enzima imobilizada, não se observando também uma diferença evidente nos valores de rendimento entre ambos os tipos de microesferas.

Palavras-Chave: Quitosano, microesfera, *Viscozyme*, biomassa, hidrólise, magnético, imobilização

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Glossary

Biomass - Organic materials, such as plant matter and manure, that have not become fossilized and are used as a fuel or energy source.

Hydrolysis - The reaction of water with another chemical compound to form two or more products, involving ionization of the water molecule and usually splitting the other compound. One example is the catalytic conversion of starch to glucose.

Bioconversion - The conversion of organic materials, such as plant or animal waste, into usable products or energy sources by biological processes or agents, such as certain microorganisms[1].

Immobilization - A type of technique used to attach an enzyme to an inert, insoluble material. This can provide increased resistance to changes in conditions such as pH or temperature. It also lets enzymes be held in place throughout the reaction.

Biocatalyst - a substance, usually an enzyme, that initiates and/or increases the rate of a biochemical reaction.

Van Der Waals forces - Relatively weak electric forces that attract neutral molecules to one another in gases, in liquefied and solidified gases, and in almost all organic liquids and solids.

Buffer - a solution containing either a weak acid and its salt or a weak base and its salt, which is resistant to changes in pH. Buffers are used to maintain a stable pH in a solution, as they can neutralize small quantities of additional acid of base[2].

Acronyms

LCB - Lignocellulosic Biomass;

RNA - Ribonucleic acid;

PBR - Packed Bed Reactors;

FBR - Fluidized Bed Reactors;

RPM - Rotations Per Minute;

BSA - Bovine Serum Albumin;

HPLC - High-Performance Liquid Chromatography;

1. State of the art

1.1 A general view

Biomass is organic material that originates from plants and animals, and it is classified as a renewable source of energy. Biomass can technically be described as organic matter that absorbed and stored energy from the sun; plants absorb the sun's radiation in a process called photosynthesis, as a way to create and store carbohydrates, such as sugars, cellulose and starches. In the last decades, the destination of biomass, especially as an agro-industrial residue, has become an important problem that has been getting more attention[3]. Accumulation of agro-industrial residues in the environment can cause serious ecological problems[3]. On the other hand, these kinds of rich carbohydrate materials can have economical value to different biotechnological processes, such as the microbial fermentative processes[3]. Biomass can either be burned directly or converted into liquid biofuels or biogas that can then be used as fuels. Biomass in the form of grasses, woods, crop residues and other biodegradable by-products from various industries offer an abundant, renewable and greenhouse-gas neutral source of organic compounds like cellulose, hemicellulose and lignocellulose, a material that can be converted in sugars which can be used to produce ethanol or other liquid fuels[4][5]. As the overall process can vary, the conversion of biomass to ethanol usually contains the following steps: pretreatment of feedstock, proceeded by hydrolysis, fermentation, and finally distillation of ethanol. One important draw of bioconversion using lignocellulose is the opportunity to include it into a bio-refinery, which can produce valuable and usable co-products, as well as enabling the use of wasted lignocellulose biomass for production of energy and fuels[4]. Despite these advantages, Lignocellulosic Biomass (LCB), due to its composition and structure of cellulose fibers (wrapped in a complex network of lignin and hemicellulose, referred to as the lignin-carbohydrate complex), is highly recalcitrant and difficult to modify. Because of this, several steps of pretreatments are usually needed to properly isolate each of its components, so that they can be of use[6].

The use of these substances as an alternative to fossil fuels has a considerable potential to change the current energy paradigm, economically, socially and environmentally. Non-renewable energy sources are currently being used as the source to approximately 87% of all energy used world-wide, with energy consumption increasing each year[7]. The consequences of a continued usage of fossil fuels can already be felt in several ways, such as climate changes due to increased greenhouse-gas emissions, increased concentration of toxic gases and particles in the lower atmosphere from vehicles and industrial emissions (smog), decrease of biodiversity and wildlife due to higher levels of pollution and habitat loss, or conflict over control, possession and distribution of said fossil fuels, around the world[7]. In order to realistically replace fossil fuels, the production cost of these liquid fuels must first become lower.

1.2 Enzymes

Enzymes are one of the key components in the conversion process of these organic by-products into more useful compounds like cellulose and fermentable monomers. The biological processes that occur within all living organisms are essentially chemical reactions, and most are regulated by enzymes. Without enzymes, many important reactions would not occur at a rate high enough to allow the formation and development of living organisms. Enzymes, like the commercially available *Viscozyme*, are biological macromolecules that can be used to catalyze specific chemical reactions, with each enzyme designed for a different reaction, working the same way as other catalysts (lowering the activation energy); their high specificity and variety comes from their complex three-dimensional structures. Enzymes were once thought to be proteins only, but the catalytic property of some nucleic acids, called ribozymes (or catalytic RNAs), has been demonstrated, refuting this idea[8]. The molecules upon which enzymes act are named *substrates* and the enzyme converts the *substrates* into different molecules, known as *products*. In terms of chemistry, enzymes work like any other catalyst and are not consumed in chemical reactions, nor do they alter the equilibrium of the reaction they catalyze. Despite their high efficiency and specificity, enzymes are fragile, and so they can only operate inside a very narrow window of factors, such as temperature and pH, without denaturing and losing their catalyzing properties[5]. Due to their high specificity, it can be advantageous to use a mix of different enzymes, depending on their intended use. *Viscozyme* is a multi-enzyme complex containing a wide range of carbohydrases including arabanase, cellulase, beta-glucanase, hemicellulase and xylanase. *Viscozyme* has a wide range of uses in industry, usually where useful products are being extracted from plant materials and in the processing of cereals and vegetables. It can enhance the availability of starch in fermentation by degrading the non-starch polysaccharides that are often bound to starch in plant materials. It can generally reduce the viscosity of materials derived from plants, hence possibly improving extraction yields. *Viscozyme* is also a widely used complex in other studies[9].

A possible solution to the high costs associated to production and usage of enzymes is immobilization. Enzymes usually lack long-term operational stability and are difficult to recover and reuse; by immobilizing them on a support/structure, these drawbacks can be overcome[7]. This is particularly important because of the high enzyme costs. In more recent years, a trend to use nanostructured materials as an effective support for the immobilization of enzymes can be noticed, based on the fact that due to their great surface/volume ratio, the surface area of nanomaterials can greatly improve the enzyme loading per unit mass of support[10].

Despite these advantages when compared to normal enzymes, immobilized enzymes can get their overall activity reduced, which results in reduced efficiency in terms of how well they can catalyze a chemical reaction. There are several options in terms of enzyme immobilization, such as adsorption[11], covalent bonding[12], entrapment[13], cross-linking[14] and encapsulation[15]. Adsorption is a method where the enzyme is adsorbed to an external surface of the chosen support; because there is not a permanent chemical bond between the enzyme and the surface, only weak energetic

bond/interactions such as Van Der Waals forces stabilize the enzymes. The main advantage of the adsorption method is the fact that there is no “pore diffusion limitation” because the enzymes are immobilized externally on a support. Covalent Bonding is a method that involves the formation of covalent bonds between the enzymes and the support. Because of its simplicity, strong linkage of enzyme to the support and wide applicability, it is one of the most used methods of immobilization, even though chemical modifications of the enzyme used can lead to a loss of its functional conformation, decreasing its efficiency. Entrapment is a method that physically “traps” enzymes inside a porous matrix, stabilizing the enzymes with the matrix with covalent and non-covalent bonds. The form and type of matrix used depends on the enzyme being immobilized. In order to prevent enzyme loss, pore size can be controlled by adjusting the concentration of the polymer used. Entrapment is a cheap and fast method that has a reduced chance of conformational changes on enzymes, but it suffers from the possibility of enzyme leakage, especially with low molecular weight enzymes. Cross-linking is a method where enzymes are linked by covalent bonds between several groups of enzymes via polyfunctional reagents. Unlike other methods, there is not a support, or a matrix being used to immobilize enzymes. Even though it is a relatively cheap technique that is used in industrial applications, there is the possibility that the polyfunctional reagents used may modify the structure of the enzyme, which leads to the loss of its catalytic properties. Encapsulation is a method where immobilization of the enzymes is obtained by enclosing them inside a membrane capsule, made of a semi permeable material, like nitro-cellulose or nylon. In this method, effectiveness depends on how stable the enzymes are inside the capsule. This method is cheap and allows many enzymes to be immobilized inside its capsules, but it suffers from pore size limitation and because the membrane is not completely permeable, only small substrate molecules can cross it.

1.3 Chitosan

Chitosan is a widely used material for immobilization of enzymes .Chitosan has been used as a carrier for enzyme immobilization since the 70s[16]. This material is produced commercially by deacetylation of chitin[17], the structural element present in the exoskeleton of various crustaceans (such as crabs and shrimp) and also cell walls of some fungi. Chitosan has several commercial and biomedical uses. It can be used in agriculture as a seed treatment and as a biopesticide, helping plants fight off fungal infections. In winemaking, it can be used as a fining agent, while also helping prevent wine from spoiling. In medicine, it may be useful in bandages to reduce bleeding and as an antibacterial agent; it can also be used to create a drug-delivery system through the skin. Magnetic chitosan nanoparticles were also used for covalent immobilization of cellulase; different studies showed an improvement of overall enzyme stability under several unfavorable conditions (temperature, pH, storage), while also showing the possibility of multiple reuse of the biocatalyst[4]. In recent studies, protocols for enzyme immobilization on chitosan were developed, resulting in stable biocatalysts, for β-galactosidase, invertase and for chitinase[16]. Obtained from chitin, that is subjected

to N-deacetylation followed by purification procedures, the production of chitosan is an economically attractive use for crustacean shells[16]. Figure 1 is a visual representation of this process; it is however important to note that 100% acetylated polymer (chitin) and 100% deacetylated polymer (chitosan), as it's represented in this image, does not naturally occur. Chitin and chitosan are copolymers where acetylated and deacetylated units are both present. When molar fraction of deacetylated units is above 50%, it is considered to be chitosan, otherwise it is considered to be chitin.

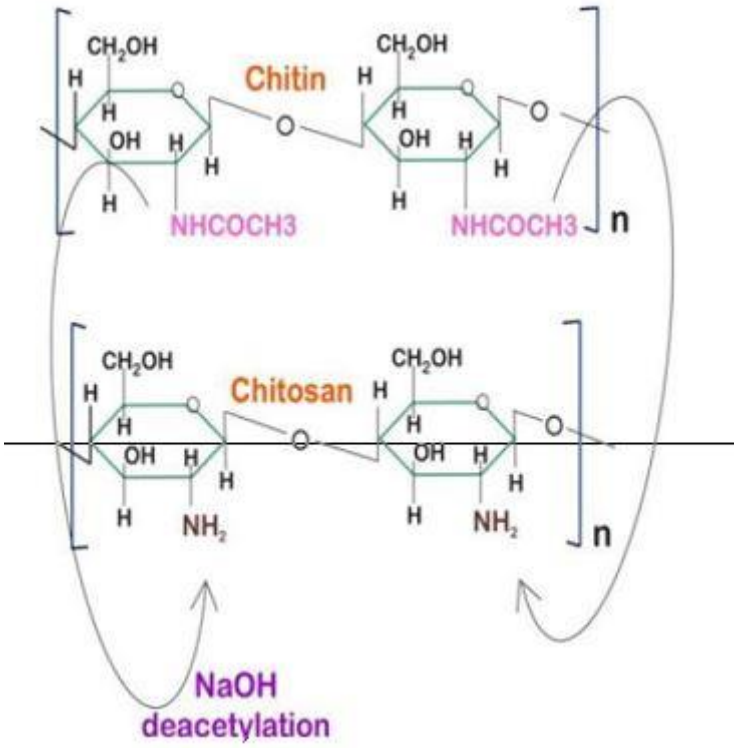

Fig 1 – Deacetylation of chitin into chitosan[17]

1.4 Packed Bed Reactors and Fluidized Bed Reactors

The presence of reactive functional groups, specifically hydroxyl and amino groups, for direct reactions and chemical modifications, results in hydrophilicity, high biocompatibility, good resistance to chemical degradation, non-toxicity, high affinity for proteins and ease of preparation in multiple forms; all these properties make chitosan an excellent choice as a support for covalent attachment of enzymes, being already used in Packed Bed Reactors (PBR) and in Fluidized Bed Reactors (FBR)[7]. Packed Bed Reactors (PBR) consists of a stack of catalyzing particles stuffed inside a tubular object, through which a reactant solution is pumped, improving contact between the reactant and the catalyst. This technique can suffer from poor temperature control,

which can lead to undesired thermal gradients, producing suboptimal results. An FBR consists of particles inside a container, which are supported by a porous substrate, known as a distributor. A liquid or gas passes through this substrate, at a high enough velocity to suspend the particles, changing their behavior as though they were a fluid, maximizing mixing and interaction between the liquid or gas and the particles. Unfortunately, current understanding of the actual behavior of the materials in a fluidized bed is still very limited, making it very difficult to predict and calculate, the complex mass and heat flows within the bed with accuracy.

1.5 Objectives

There are already several studies regarding the production of sphere-shaped surfaces of varying sizes using chitosan[18], with similar applications as the ones discussed[19], and others, like food processing[20]. Although there are many studies focused in immobilizing enzymes in different kinds of supports, most tend to focus on just one kind of enzyme, and study its effectiveness on a specific type of support, with a specific immobilizing technique and/or method, looking to either demonstrate how some factors may affect the enzyme's efficiency and longevity, such as the method used and/or materials[21].

Considering not only the high number of different enzymes and multi-enzyme complexes like *Viscozyme* that are already used and/or show some promise, but also the high number of different immobilization techniques and the different types of support, it becomes clear that there is still a lot to be studied in this field and therefore a lot of potential behind it, despite the efforts already made so far. Factors like temperature, pH and procedure time also play a role when trying to determine if a said enzyme or complex is viable when using a certain technique, or other important factors, like the size of the support used[20].

One of the main goals of this work is to successfully immobilize *Viscozyme* at the outer surface of chitosan microspheres (magnetic or non-magnetic) and analyze the resulting material in the hydrolysis of both a model polysaccharide, and biomass-hydrolysis liquor and compare the results afterwards. A methodology that leads to a preparation with high operational stability is also desirable in this study, emphasizing on the importance of its ability to use the microspheres repeatedly and with consistent results.

2. Materials and Methods

2.1 Chitosan microspheres

0,2 g of low molecular weight chitosan (CAS Number 9012-76-4) were slowly dissolved in 5 mL of acetic acid solution 2% v/v[22] and stirred until the solution was completely dissolved. Acetic acid solution was prepared by adding 0,2 mL of acetic acid (CAS Number 54-16-0) to 9,8 mL of distilled water. Then the solution was added to a bath of NaOH solution 10% v/v[22] drop-wise, using a syringe; NaOH bath was prepared by dissolving 20 g of NaOH pellets (Azkonobel) in 180 mL of distilled water. Standard parameters for the system used were: 3,5 mL/h syringe flow rate, 12,5 cm height from the NaOH solution and 3,0 L/min air flow rate. Parameter values were previously determined by testing their influence in shape and size of the chitosan microspheres, as seen in 3.1. The chosen values showed acceptable results in terms of sphere diameter and also a low standard deviation of diameter values.

The same procedure was used to create magnetic chitosan microspheres, by also adding 0.215 gram of $FeCl_2.4H_20$ (CAS Number 13478-10-9) and 0.59 gram of $FeCl_3.6H_20$ (CAS Number 7705-08-0) after adding and dissolving 1 gram of chitosan in 5 mL of acetic acid solution 2% v/v; the amount of $FeCl_3$ and $FeCl_2$ follow a 2:1 molar proportion in order to form magnetite[23]. The system parameters used, visible in table 1, were also the same as for the non-magnetic microspheres.

Table 1 – Parameter values used for sphere production.

Syringe flow rate (ml/h)	Height (cm)	Air flow rate (L/min)
3,5	12,5	3,0

The system used was a coaxial airflow induced dripping bead generator, model VAR J1 from Nisco[24][25]. The basic principle of the system is the use of a coaxial controllable air stream that “cuts” droplets from a needle tip, that fall into a bath of choice. This system is designed to create objects of small size (<1 mm diameter), although this may vary on the diameter of the syringe used. A visual representation of the production of the spheres can be seen in figure 2, as well as the system itself.

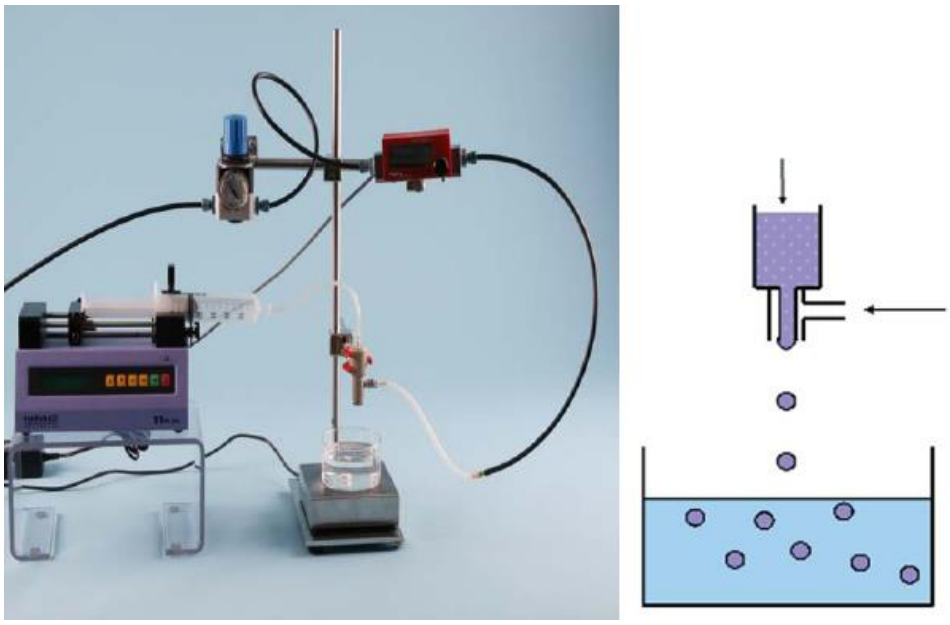

Fig 2 – Coaxial airflow induced dripping VAR J1 and a visual representation of microspheres being created[25].

2.2 Chitosan activation

0,2 g of previously prepared chitosan particles were incubated with 100 mL of a glutaraldehyde grade II, 25%(CAS Number 111-30-8) solution 4% v/v, prepared in 0.1 M sodium phosphate buffer at pH 7.0. Glutaraldehyde solution was prepared by mixing 4 mL of glutaraldehyde with 96 mL of sodium phosphate; buffer was prepared by mixing 42,3 mL of sodium phosphate monobasic (CAS Number 7558-80-7) with 57,7 mL of sodium phosphate dibasic (CAS Number 7558-79-4). Incubation was performed at room temperature, during 3 hours in an orbital shaker at 120 rpm, using an *Erlenmeyer* to increase contact area between particles and the solution. The activated support was then exhaustively washed with 50 mM sodium acetate buffer at pH 5.5 to remove excess glutaraldehyde[26]. The same protocol was used for both magnetic and non-magnetic chitosan microspheres.

Solution volume, glutaraldehyde volume concentration, type of buffer used, incubation time and temperature were chosen according to the methodology used from other similar studies[20][22], with not only sphere diameter/size of the microspheres produced, but also materials used being important factors that determined these parameters.

Table 2 – Chitosan activation parameters.

Solution volume (mL)	Glutaraldehyde concentration (%)	Type of buffer	Incubation time (h)
100	4	0.1 M sodium phosphate	3

2.3 Enzyme Immobilization

After glutaraldehyde activation, all chitosan particles (approximately 0,2 g) were incubated with an enzyme solution (125 µL of enzymatic solution (Sigma Aldrich Viscozyme®L) in 30 mL of 50 mM sodium acetate buffer at pH 5.5), during 3 hours at room temperature, stirred at low rpm's. After incubation, the particles were exhaustively and successively washed with approximately 350 mL of 50 mM sodium acetate buffer at pH 5.5, approximately 200 mL of 1 M sodium chloride and 200 mL of ethylene glycol 30% v/v solution, in order to remove unbound, ionically and hydrophobically bound enzyme molecules[26]. Ethylene glycol solution was prepared by mixing 60 mL of ethylene glycol (CAS Number 107-21-1) with 140 mL of distilled water. A sample of the solution was taken before washing and the washing liquids were collected to quantify protein using the Lowry method[26]. Like the chitosan activation protocol, magnetic and non-magnetic chitosan microspheres have the same protocol regarding this step. Parameter values can be seen in table 3:

Table 3 – Enzyme Immobilization parameter values.

Solution volume (mL)	Enzyme solution's volume (µL)	Type of buffer	Incubation time (h)
30	125	50 mM sodium acetate	3

2.4 Lowry Method

The Lowry Method, due to its simplicity, sensitivity and precision, is one of the most used procedure for the quantitative determination of protein[27][28]. The Lowry method is based on the Folin phenol reagent of Folin and Ciocalteu, the active constituent of which is phosphomolybdic-tungstic mixed acid[27]. This method was used to determine the percentage of enzyme that was immobilized on the activated surface of the microspheres. On the assumption that magnetic and non-magnetic microspheres shouldn't display significantly different results from each other, this protocol was only used on samples from one of the two groups, the magnetic microspheres, to avoid using unnecessary laboratory resources.

Two samples were prepared: one from the liquid medium the microspheres were in, after enzyme immobilization was complete but before washing (**A0**), and one from the liquid medium after washing (**A1**). 4 additional samples are prepared using BSA (Bovine Serum Albumin) with varying concentrations so that a calibration curve can be used to understand the obtained values.

After all samples (2 mL each) were ready, CFR (Complex-Forming Reagent) is prepared[28] and 1 mL is added to each sample, then stirred briefly in a vortex mixer and stand at room temperature for 10 min. 0.1 mL of Folin reagent[28] is then added to all samples, which are then stirred in a vortex mixer. Let the mixture stand at room temperature for a minimum of 30 min.

Samples were then taken to spectrometer set at 750 nm wavelength, to read their absorbance[28]. Values from the BSA (that has a standard 400 µg/mL solution) samples are then used to plot a standard curve of absorbance as a function of initial protein concentrations and used to determine the samples protein concentrations.

Table 4 – BSA samples. Concentration values used for standard curve plot

Sample	0	1	2	3	4
BSA (µL)	0	25	75	125	200
H_2O (µL)	200	175	125	75	0
Concentration (µg/mL)	0	50	150	250	400

2.5 Hydrolysis Reactions

After the immobilization process is completed, the chitosan microspheres are ready to be used as catalyzers in hydrolytic reactions. Different types of sugars were used in the reactions: arabinogalactan (CAS Number 9036-66-2), xylan (CAS Number 9014-63-5) and a solid extract containing mostly sugar oligomers, obtained by treating an agro-industrial byproduct with subcritical water (HCW.240 lyophilized, composed of 53% fructose, 29% glucose, 11% arabinose and 7% galactose, , as found by complete hydrolysis of the extract and full conversion of all sugar oligomers to monomers). In order to compare performances between free enzymes and immobilized ones in both magnetic and non-magnetic microspheres, 4 distinct group samples were prepared; one for free enzyme, 2 for immobilized enzyme (magnetic and non-magnetic chitosan microspheres) and a control group. Each group (except control group) had samples for the different types of sugars tested, with the free enzyme group having only one hydrolysis cycle, while immobilized enzyme groups were subjected to a total of 3 cycles in order to observe the effects of continued usage of the microspheres as biocatalysts.

Samples were taken from 20 mL Falcon conical centrifuge tubes, with a tube for each type of sugar and group, for a total of 10 tubes prepared for the first cycle, and 7 in each subsequent cycle, with sample volume being 1 mL each. 5 mL of sodium citrate, 5 mL of 50mM sodium acetate and 20 µL of sodium azide were added to each tube before adding either the free enzyme or the microspheres. 40 µL of *Viscozyme* was added to tubes belonging to the free enzyme group, with 150 mg of either Arabinogalactan, Xylan or HCW.240 to their respective tube. Approximately 0,1 g of microspheres (approximate equivalent of 40 µL of immobilized enzyme) were added to each of the tubes belonging to the immobilized enzyme groups, with also 150 mg of either Arabinogalactan, Xylan or HCW.240 to their respective tubes. Incubation time for the first cycle was 72 h, with samples being taken at T=0h, T=2h, T=20h, T=26h, T=48h and T=72 h. Incubation time for the other 2 cycles was 72h, with samples being taken at T=0h, T=1h, T=2h, T=3h, T=20h, T=24h, T= 48h and T=72h. All tubes were put in a controlled-atmosphere shaker with temperature set to 50º Celsius and stirred at low rpm's. After every hydrolysis reaction, all microspheres are carefully washed with 50mM sodium acetate to remove most excess sugars on their surface. The samples taken during hydrolysis are then filtered and prepared, so they can be analyzed using an HPLC (High-Performance Liquid Chromatography). HPLC is a technique used to separate, identify, and quantify each component in a given mixture, by pumping a pressurized liquid solvent that contains the sample mixture through a column, filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out of the column[29]. A representation of the different steps needed in HPLC can be seen in figure 2.

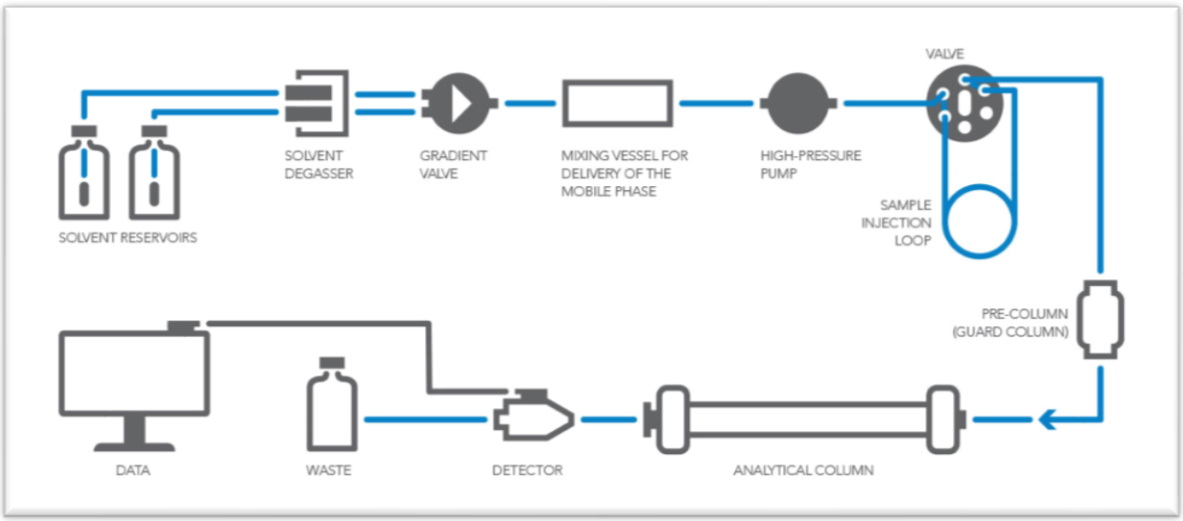

Fig 3 – Visual representation of all steps during an HPLC analysis[30]

Viscozyme is stabilized with sucrose, which is separated as glucose and fructose during enzymatic preparation; this means that all samples have an artificial amount of these sugars, observed in HPLC results. This means that even samples testing for xylan or arabinogalactan would also show some amount of glucose and fructose; all results showing in figure A2 have those amounts subtracted, calculated with average concentration values from control group.

3. Results and Discussion

3.1 Chitosan Microspheres

Because surface area is an important factor when activating the surface of the microspheres and when immobilizing enzymes, an experiment was done to try and optimize diameter value of microspheres. In order to save material and reduce costs, and assuming that composition differences between magnetic and non-magnetic microspheres does not affect the sphere's diameter during production, the experiment was conducted using only chitosan dissolved in 5 mL of acetic acid solution 2% v/v.

In order to observe how much each factor influences the sphere's diameter, a different experiment was made for each parameter, as seen in table 6, where only one parameter's value at a time was changed.

Table 5 – parameters tested for microspheres production. Values used were either smaller or higher from standard values used in the control group.

Experiment	1	2	3	4
Parameter	-	Flow	Air flow	Height
Parameter value	Standard	2,5 mL/h (smaller)	4,43 L/min (higher)	15,5 cm (higher)

Standard values correspond to: 3,5 mL/h flow, 3,0 L/min air flow and 12,5 cm height. Because one of the goals of this work is to maximize the efficiency of the immobilized enzyme on the surface of the chitosan microspheres, it is desirable to design the production of microspheres such that the diameter obtained is as low as possible, without compromising their applicability. Because of that and to avoid wasting resources, all parameter values were tested with either an increase or decrease in their values instead of both. Before these measurements were taken, a previous experiment was done to study and observe the immediate effects each parameter had on the diameter of the spheres and viability of the protocol and system used, in order to conclude how to proceed and change their values according to standard ones.

On table 7 the average and standard deviation values from the experiment groups are shown; a total of 20 samples from each experiment group were analyzed to calculate the following results. A visual comparison of the average values obtained can be seen in figure 4:

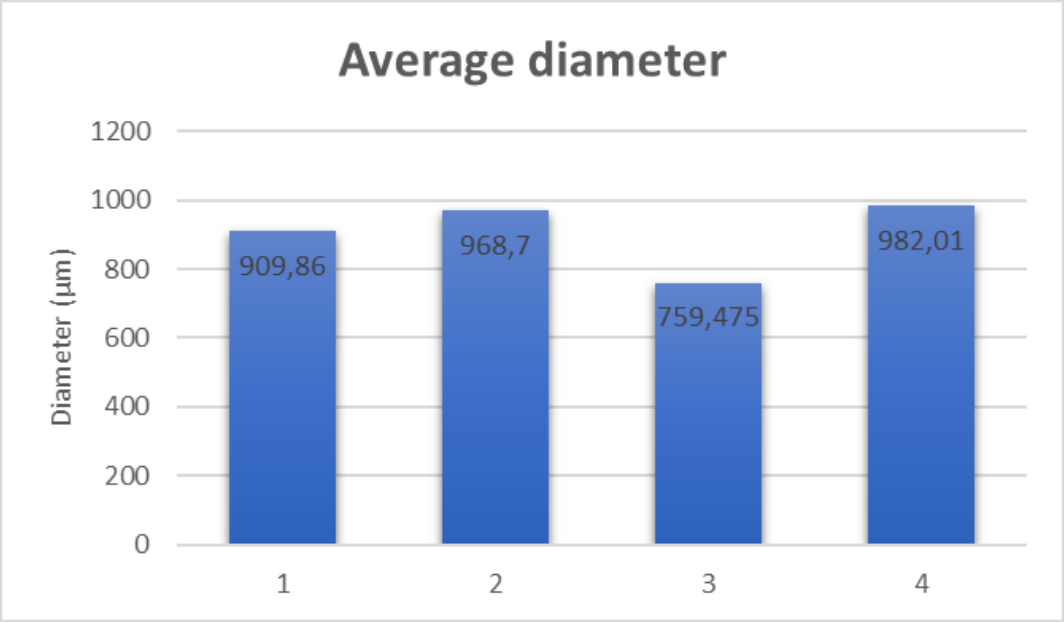

Fig 4 – Average diameters from all experiment groups: Control group (1), Flow (2), Air flow (3) and height (4).

Table 6 – Average and standard deviation values

	Control	Flow	Air flow	Height
Average (µm)	910	969	759	982
Standard Deviation (µm)	+/- 88	+/- 102	+/- 108	+/- 111
Sphere shape	regular	regular	regular	irregular

The values seen in Fig 4 were obtained by calculating the average diameter of 25 different microspheres each, created in all experiments. These values can be seen in table A1. Microsphere's diameters were measured using an optical microscope connected to a computer using connected to a digital camera. Measurements were done by hand using a measuring tool in the image processing software (Olympus stream Basic), meaning that the results obtained are not entirely accurate. Some of the microspheres also showed irregular shapes, which made it more difficult to identify and measure their actual diameter.

Looking at the average diameters obtained, it becomes apparent that the air flow value used during production carries the most influence, showing a decrease of approximately 150 µm when compared to control average, with a slight increase in standard deviation. On the other hand, the change (decrease) in flow parameter value shows similar results to control group, suggesting that the parameter has little influence on the microspheres diameter. Height also shows no considerable influence on the microspheres diameter, although it showed an additional problem during the experiment: It was observed that microspheres produced in experiment 4 showed increased size and abnormal shapes. This is possibly due to higher impact when entering contact with the liquid medium, which could result in a spreading effect and spheres displaying abnormal shapes, explaining the observed awkward diameter values, which can also explain the slightly higher standard deviation value obtained.

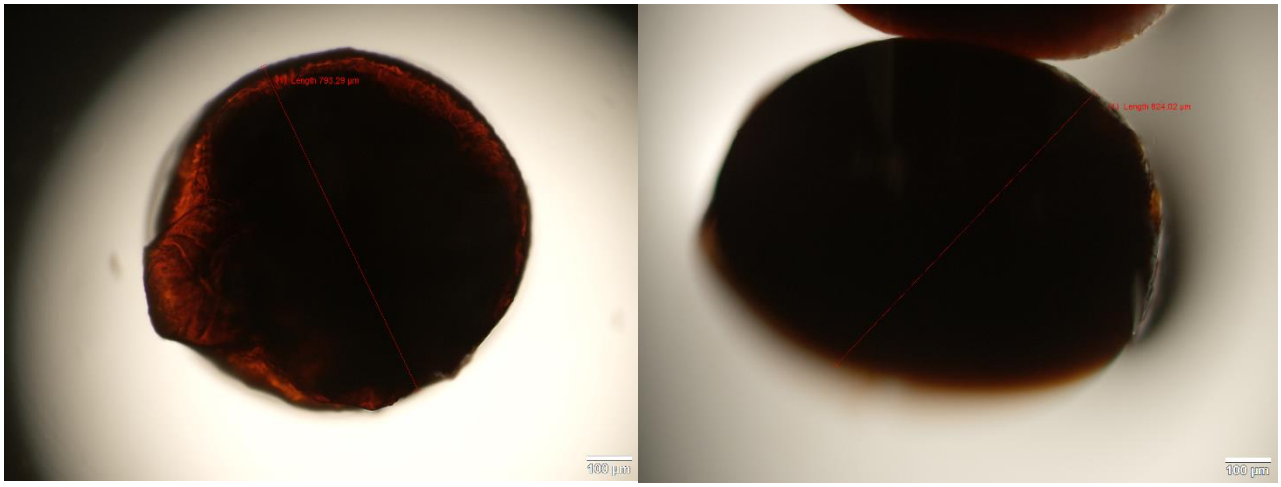

Fig 5 - Microsphere images from optic microscope. Image from the right is from control experiment (1) and image in the left is from height experiment (4) (scale: 100 µm)

Despite the good results obtained from the third experiment group, it was decided that the microspheres that would be used in the next steps would be the ones obtained from standard parameter values. Because one goal is being able to easily replicate the results from the system used (VARJ1) it is desirable to follow a protocol with a low standard deviation value; it is also valuable using microspheres with a similar size[18][20] to ones used in other studies, in order to not only compare protocols, but to compare results.

3.2 Protein quantification (Lowry Method)

Using the Lowry method, absorbance values were obtained through the use of a spectrophotometer. A calibration curve was made with the absorbance values obtained from BSA samples. Both results can be observed in table 8 and figure 6, respectively.

Table 7 - BSA and samples absorbance values

	0	1	2	3	4	A0	A1
Concentration µg/mL	0	50	150	250	400		
Absorbance	0,022	0,071	0,183	0,308	0,423	0,079	0,005

In order to determine the concentration of samples, a calibration curve was plotted with these values:

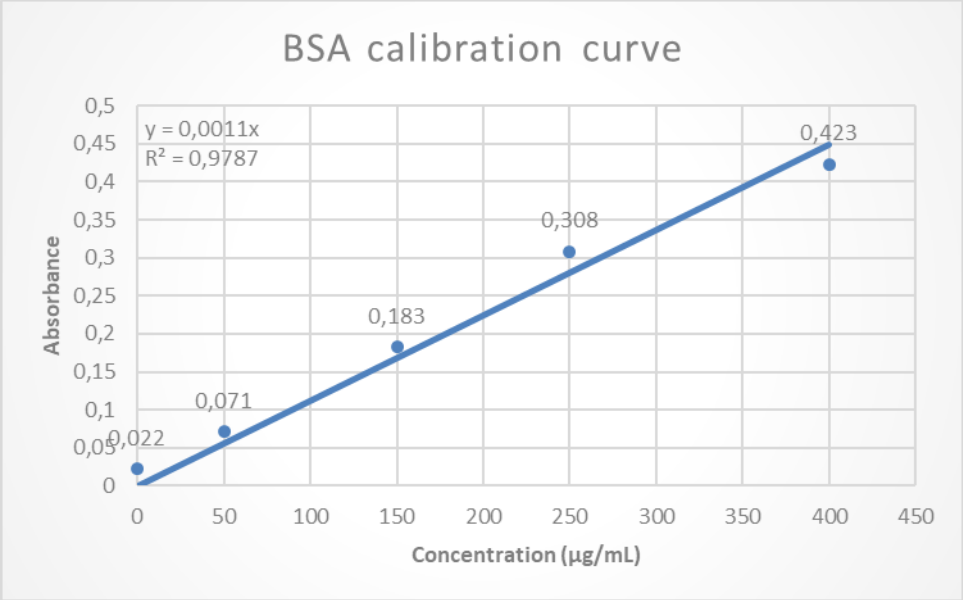

Fig 6 - Plot curve from BSA absorbance values

After determining a linear fit, the following equation is the result:

$$Y = 0{,}0011x \text{ (eq. 1)}$$

Through equation 1 we can calculate protein concentration from the samples absorbance values:

$$\boldsymbol{A0}_{conc} = \frac{0{,}079}{0{,}001} = 52{,}6\ \mu g/mL$$

$$\boldsymbol{A1}_{conc} = \frac{0{,}004}{0{,}001} = 4\ \mu g/mL$$

In immobilization protocol, microspheres are in contact with 125 µL of enzymes, mixed with 30 mL of buffer solution and that *Viscozyme* has a density of 1,21 g/mL[31]; It is also known that only 7% of the dry weight of *Viscozyme* is composed by enzymes.

During washing procedure, approximately 750 mL of washing waste was used to clean the microspheres.

*1,21*0,125 = 0,15125 g = 151250 µg (Total Viscozyme weight)*

*(151250*7)/100 = 10587,5 µg (Total enzyme weight)*

*52,6*30 = 1578 µg*

*4*750 = 3000 µg*

$$\%_{loss} = \frac{(1578+3000)*100}{10587,5} = 43,2\%$$ (eq. 2)

Using equation 2, it is determined that the percentage of enzymes that were not immobilized was about 43,2%, which means that approximately 67% of enzymes were successfully immobilized on the activated surface of the microspheres. It is important to note that the time interval between activating the surface of the microspheres and immobilizing enzymes should be as low as possible, otherwise immobilization percentages can be affected, resulting in lower percentages.

3.3 Hydrolysis reactions results

Before starting hydrolysis reactions, a new batch of both magnetic and non-magnetic microspheres were produced using the same protocols. After surface activation and enzyme immobilization, all done within a short time interval, immobilization percentage was calculated using the Lowry method. Using the results from the spectrophotometer, it can be seen in figures A2 and A3 their respective BSA calibration curve plots and trend lines. BSA curves were done for both types of microspheres and using the same concentrations and number of points as previously. 2 samples from their liquid medium after immobilization before and after washing, for both magnetic (A0, A1) and non-magnetic (B0, B1) were analyzed. Using the same methods to calculate the enzyme loss % previously, the results from both sample groups can be seen in table 9. Cleaning liquids used for both samples were approximately 700 mL.

Table 8 - Loss percentage of magnetic and non-magnetic samples

	A0	A1	B0	B1
Absorbance	0,031	0,005	0,047	0,009
Concentration (µg/mL)	31	5	31,3	6
Loss (%)	8,7	33,1	8,9	39,7

Results show that magnetic particles immobilized approximately 59% of enzyme and non-magnetic microspheres immobilized approximately 52% of all enzymes during enzyme immobilization protocol.

Results from the hydrolysis reactions samples can be seen in figure A2, in annexes. Table 9 gives the designations used to identify the matrices hydrolyzed.

Table 9 – Hydrolysis reaction sample designation.

	arabinogalactan	HCW.240	Xylan	Control
Magnetic particles	A	C	E	G
Non-magnetic particles	A’	C’	E’	
Free enzyme	B	D	F	

HCW.240 is a solid extract obtained from an agro-industrial byproduct.

Because yield calculations only take into account the initial and final values of the reaction, the second and third cycle samples were only measured at the beginning and at the end of their hydrolysis reactions. This was also done in order to lower the needed number of samples sent to HPLC for analysis. Due to a technical error during HPLC analysis, no values were obtained for samples E and E’ from the first cycle, making yield calculations impossible for these samples.

Because there are no replicas for each experiment, the values obtained are susceptible to measuring variables from HPLC analysis. The fact that the amount of immobilized enzyme in the microspheres is overall lower than the 40 μL used in the free enzyme samples, means that yield values from immobilized enzyme are expected to be lower by a certain percentage, compared to free enzyme. Concentration values for yield calculations can be seen in table 10:

Table 10 - Sugar concentrations from all cycles for all samples (mg/L)

Arabinose	A	A'	B	C	C'	D	E	E'	F
1º Cycle	44,33	65,65	322,428	5,17	-	153,8955			-
2º Cycle	-	-		55,6	6,82				
3º Cycle	-	-		-	-				
Galactose	**A**	**A'**	**B**	**C**	**C'**	**D**	**E**	**E'**	**F**
1º Cycle	210,09	138,16	-	12,63	0,31	122,13			-
2º Cycle	-	-		-	-				
3º Cycle	-	-		-	-				
Glucose	**A**	**A'**	**B**	**C**	**C'**	**D**	**E**	**E'**	**F**
1º Cycle			-	67,64	47,28	245,58			118,94
2º Cycle				664,14	102,72				
3º Cycle				-	-				
Xylose	**A**	**A'**	**B**	**C**	**C'**	**D**	**E**	**E'**	**F**
1º Cycle			-	-	-	124,33	-	-	2987,814
2º Cycle				-	5,609		882,35	-	
3º Cycle				-	-		4372,72	-	
Frutose	**A**	**A'**	**B**	**C**	**C'**	**D**	**E**	**E'**	**F**
1º Cycle			-	-	-	35,14			20,14
2º Cycle				432,61	59,18				
3º Cycle				-	-				

Blank areas in B, D and F columns represent the fact that free enzyme sample groups were not used for both the second and third hydrolysis cycles. Missing concentration values are a consequence of the irregular values obtained through HPLC analysis seen in figure A2. The values shown in table 10 are of all sugar concentrations that were pertinent to measure for each sample group (for example, A, A' and B concentration values are of arabinose and galactose values from figure A2, meaning there are no values of glucose, xylose and fructose for these sample groups). Missing concentration values are from sample values where the final concentration is lower than initial one, or in the case of sample groups E and E' in the first cycle, they were caused by technical error during HPLC analysis.

Because most sample values obtained through HPLC analysis show irregular concentration values, it is impossible to not only accurately determine the total yield % after 3 cycles for any sample group of immobilized enzymes, but also compare those results to their respective free enzyme yield %.

Enzyme specific activity was calculated for the first cycle; second and third cycles provide too few valid results to calculate enzyme specific activity, so they were not used for these calculations. Values shown in table 11 are in mg/h/mg (mg of sugar per hour per milligram of enzyme).

$$A_{enzyme} = \frac{C*V}{t*m} \quad \text{(eq. 3)}$$

Using equation 3, specific enzyme activity (A_{enzyme}) can be calculated. "*C*" represents the average concentration difference between final concentration and initial concentration values, "*V*" represents the liquid volume used during hydrolysis reactions (15 mL), "*t*" represents the amount of time the hydrolysis reaction took (despite immobilized samples in the first cycle had a total duration of 96h, because there is little difference in terms of results for the results obtained for T=72h, the value used is 72h, also due to free enzyme reaction samples only have been tested for 72h) and "*m*" represents the total enzyme mass present in 40 μL of *Viscozyme* (0,0484 mg). specific enzyme activity can be seen in table 11:

Table 11 – Specific enzyme activity values for the first hydrolysis reaction cycle

	Arabinose	Galactose	Glucose	Fructose
Specific enzyme activity (mg/h/mg)	1,025	0,675	0,785	0,119

Specific enzyme activity value for xylan is missing due to missing sample values from xylan-testing groups from the first cycle of hydrolysis reactions.

3.4 Microspheres morphology after hydrolysis

After hydrolysis reactions, both magnetic and non-magnetic microspheres were again visually analyzed with an optical microscope, in order to confirm whether the microspheres maintained their overall shape and size after hydrolysis reactions, by measuring their diameter. 25 samples were taken for each type of sugar and microsphere, forming a total of 6 sample groups, as seen in Fig A3, in annexes. In Figures 9 and 10, average values calculated from all sample groups, as well as the average value from control group previously calculated can be seen and its correspondent average percentage diameter loss. It is assumed that the initial diameter values (before hydrolysis) from all experiment groups averaged near the control group value.

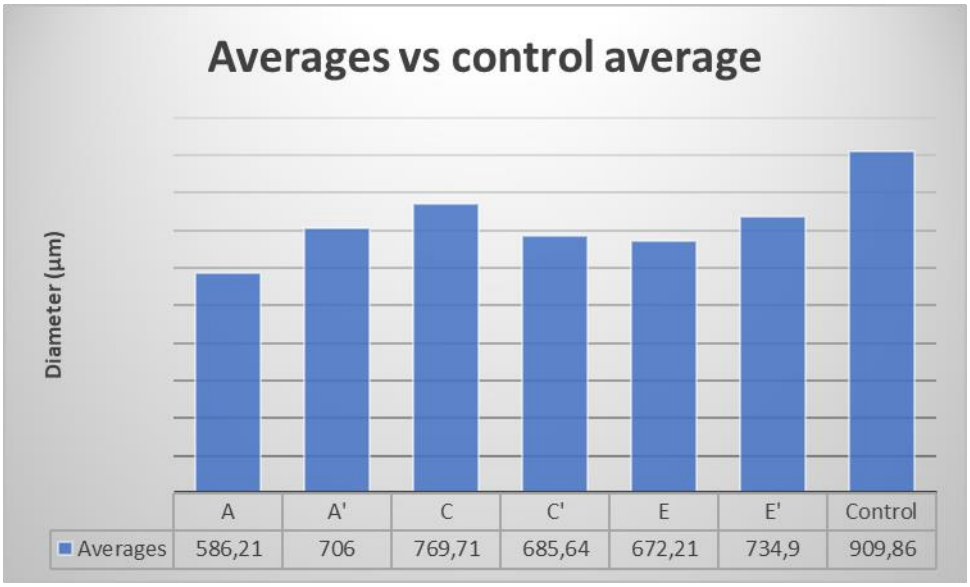

Fig 7 - Average values comparison between all sample groups and control

As seen in figure 7, the microspheres measured do show a decrease in diameter compared to their supposedly initial diameter value, equal to the control group average value. This decrease might be a consequence of the constant motion/movement of the microspheres for an extended period of time on all three hydrolysis reactions, while also being in a high temperature environment (50ºC) during said reactions. Because some amount of sodium azide is mixed in the same medium where hydrolysis reactions take place, it is safe to assume that the size decrease is not related to any bacterial activity that might consume the microspheres.

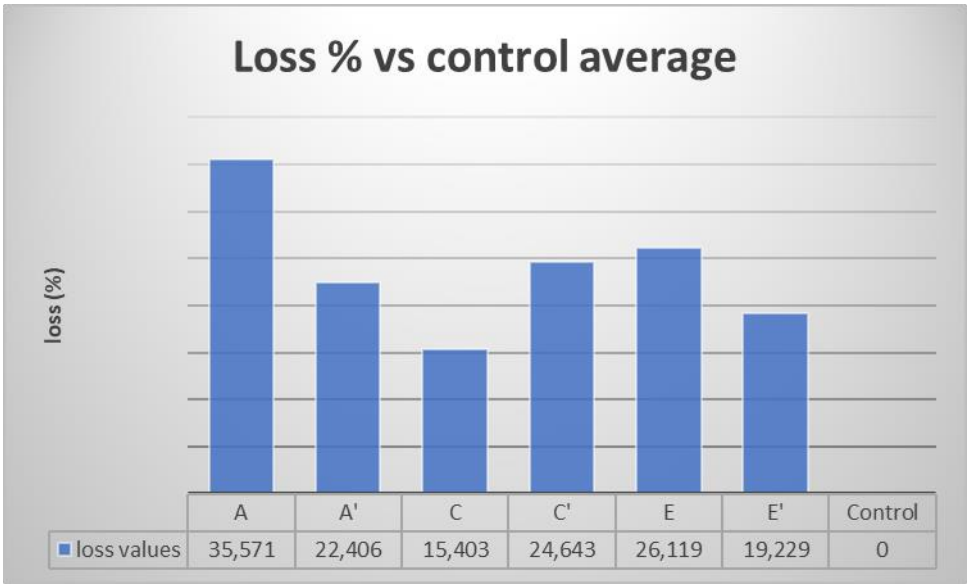

Fig 8 – Average diameter loss % compared to control group

By comparing the new average diameter values with the control group, it is possible to calculate the estimated percentage loss of diameter of each experiment group, as seen in figure 8. Overall, all experiment groups, aside from A and C, show an average of approximately 24% loss in terms of size compared to their presumed initial sizes, before hydrolysis reactions. Magnetic and non-magnetic microspheres show only a little difference in size loss, averaging about 26% for magnetic microspheres and about 22% for non-magnetic microspheres. With only a difference of approximately 4% in average size loss, it is safe to assume that the difference in composition of both microspheres should not influence the percentage of size loss during hydrolysis.

Because the microspheres analyzed displayed not only a change in size, but also shape to some extent, some diameters were more complicated to be correctly measured, which means the values shown might not be entirely accurate and would require a more extensive statistical analysis. In figure 11, the physical degradation on the surface of microspheres is clearly visible.

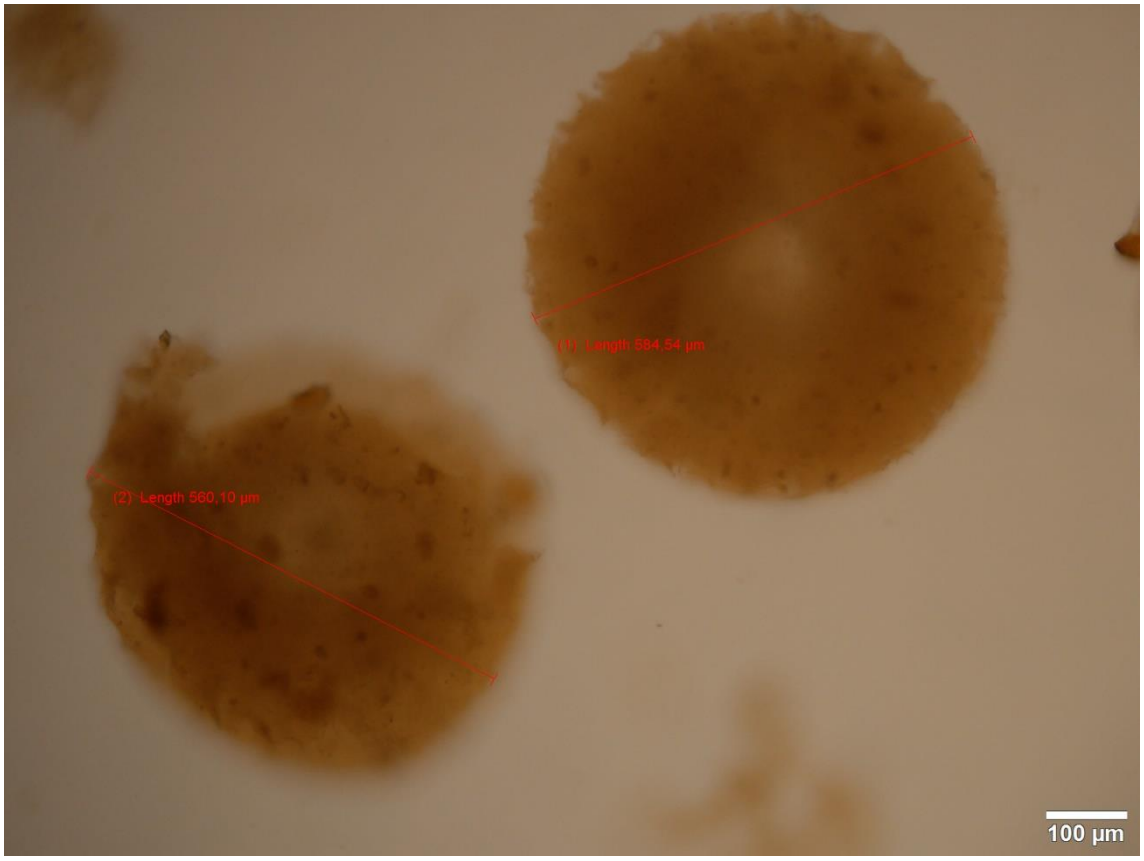

Fig 9 – Image of microspheres from experiment group "A" after hydrolysis reactions

4. Conclusions and future prospects

Results from testing the coaxial airflow system parameters show that mainly through manipulating the airflow used during operation, one can considerably influence the size of the microsphere being produced, as long as the dripping is occurring at a proper height from the bath. On the other hand, in the case of excess height, the desired spherical shape is compromised while also increasing standard deviation values, making the process less desirable. While flow value used seemed to show little influence in the overall size of the microspheres, it is important to note that due to the nature of the system used, it is not advised to go lower than the values used, as the chitosan mixtures, both magnetic and non-magnetic, have a higher viscosity than water, creating the risk of clogging the system; this risk also increases when using needles with lower diameters for the purpose of obtaining smaller particles. From results and observations made during the procedures, it can be concluded that there is no considerable difference in viability in terms of production of magnetic microspheres, when compared to non-magnetic ones. Size values obtained through this method show smaller values (~1 mm diameter or less) and less size dispersion than similar studies[18], reporting sizes of 2-5 mm diameter.

It is important to note that all diameter measures were manually done with a digital measuring tool, meaning that there is a certain error associated with it, and that the number of samples for each experiment was also relatively low; ideally, a more extensive statistical study, with a design of experiment on how each factor influences the shape and size of the microspheres, paired with a more accurate way to measure the microspheres would achieve more solid conclusions.

Looking at the results obtained from the Lowry method, surface activation procedure through the use of glutaraldehyde solution and immobilization procedure, were reasonably effective at immobilizing *Viscozyme* on the surface of the microspheres, although lower than results from other similar studies[16]. Results from the immobilization of *Viscozyme* in both magnetic and non-magnetic microspheres used in hydrolysis reactions show no significant difference in terms of the quantity (%) of immobilized enzymes between them, which indicates that magnetic microspheres are as viable as non-magnetic ones in terms of percentage of immobilized enzyme. Some more experiment with immobilization protocol factors, such as amount of enzyme put into contact with microspheres, incubation time and temperature could be useful in order to obtain a better % of immobilized enzyme.

HPLC results from all 3 hydrolysis reactions, due to both technical errors (missing sample values) and non-valid results for yield calculations, are inconclusive in terms of the difference in overall efficiency between free enzyme and both types of immobilized enzyme as biocatalysts. These results may be a consequence of a suboptimal hydrolysis reaction protocol, or also imperfect surface cleaning between hydrolysis cycles. Other possible explanations are enzyme denaturation, but also the degradation of the surface

of the microspheres that was observed after hydrolysis reactions, which might have been a consequence of the hydrolysis reaction setup used. Loss of dynamic viscosity from chitosan could also explain the degradation observed after hydrolysis reactions. This loss of dynamic viscosity might be a consequence of the temperature used during hydrolysis[32]. There was a previous failed attempt of starting hydrolysis cycles with magnetic microspheres, where the spheres showed a total loss of structural integrity after only one cycle, invalidating HPLC results; these results were initially thought to be caused by the fact that the spheres used were already weakened, due to have been produced more than a week before starting hydrolysis.

For the future of this project, it would be interesting to determine the maximum amount of enzyme that can be immobilized on the surface of a single microsphere of a given size, enabling an easier comparison between an amount of free enzyme and a group of microspheres with immobilized enzyme. This would allow for a more accurate yield value comparison between an equal amount of free enzyme and immobilized enzyme using the HPLC results from the hydrolysis reactions. A more extensive statistical study would also be interesting, with more parallel hydrolysis reactions comparing free enzyme and immobilized enzyme, in order to get more reliable data. Alternative ways of setting up an appropriate environment for the hydrolysis reactions that does not require as much motion, to avoid collisions between microspheres, could also be a valid way to determine whether the observed degradation of the microspheres was indeed a consequence of the setup used or not; assuming it was, it is also advisable to do so in order to have a better perspective at exactly how much immobilized enzyme yield % lowers after each cycle of hydrolysis reaction.

Results from analyzing the morphology of the microspheres used in the hydrolysis reactions show that microspheres have indeed lost some percentage of their volume, with an average of about 24% across all experiment groups. This loss of volume might have resulted from the inherent movement imposed on the falcons where the reactions took place, in order to maximize contact between the activated surfaces of the microspheres and the testing component; this might have led to collisions and attrition between the spheres themselves, or even undissolved sugar at the early stages of the reactions. Another factor that might have helped expedite this process was the relatively high temperature used in the protocol (50ºC)[32], needed to make sure the hydrolysis reactions occurred; it is important to note that there is no direct evidence of this, and evidence that this level of degradation could occur solely from the high temperature used.

Although there is not enough evidence by itself, it is plausible to conclude that the observed degradation of microspheres influenced the yield values obtained after the first hydrolysis cycle, as the attrition likely degraded the activated surface of the microspheres, which ends up affecting the immobilized enzymes and the yield values calculated from the HPLC results.

5. References

[1] "Bioconversion | Definition of Bioconversion by Merriam-Webster." [Online]. Available: https://www.merriam-webster.com/dictionary/bioconversion. [Accessed: 14-Jan-2019].

[2] R. Scorpio, *Fundamentals of acids, bases, buffers and their application to biochemical systems*. Kendall/Hunt Pub. Co, 2000.

[3] S. Park, J. C. Gildersleeve, O. Blixt, and I. Shin, "Carbohydrates from biomass: Sources and Transformation by microbial enzymes," *Carbohydrates - Compr. Stud. Glycobiol. Glycotechnol.*, no. figure 1, pp. 441–458, 2012.

[4] M. Ungurean, C. Paul, and F. Peter, "Cellulase immobilized by sol-gel entrapment for efficient hydrolysis of cellulose," *Bioprocess Biosyst. Eng.*, vol. 36, no. 10, pp. 1327–1338, 2013.

[5] S. T. Merino and J. Cherry, "Progress and challenges in enzyme development for biomass utilization," *Adv. Biochem. Eng. Biotechnol.*, vol. 108, no. June, pp. 95–120, 2007.

[6] A. A. Shamsuri and D. K. Abdullah, "Ionic Liquids: Preparations and Limitations," *Makara, Sains*, vol. 14, no. 2, pp. 101–106, 2010.

[7] J. V. Madeira Junior, C. B. Teixeira, and G. A. Macedo, "Biotransformation and bioconversion of phenolic compounds obtainment: an overview," *Crit. Rev. Biotechnol.*, vol. 35, no. 1, pp. 75–81, 2015.

[8] P. G. Higgs and N. Lehman, "The RNA World: Molecular cooperation at the origins of life," *Nat. Rev. Genet.*, vol. 16, no. 1, pp. 7–17, 2015.

[9] X. Guan and H. Yao, "Optimization of Viscozyme L-assisted extraction of oat bran protein using response surface methodology," *Food Chem.*, vol. 106, no. 1, pp. 345–351, 2008.

[10] T. Dai, N. Miletić, K. Loos, M. Elbahri, and V. Abetz, "Electrospinning of poly[acrylonitrile-co-(glycidyl methacrylate)] nanofibrous mats for the immobilization of candida antarctica lipase B," *Macromol. Chem. Phys.*, vol. 212, no. 4, pp. 319–327, 2011.

[11] T. Jesionowski, J. Zdarta, and B. Krajewska, "Enzyme immobilization by adsorption: A review," *Adsorption*, vol. 20, no. 5–6, pp. 801–821, 2014.

[12] Z. Zhou, F. Piepenbreier, V. R. R. Marthala, K. Karbacher, and M. Hartmann, "Immobilization of lipase in cage-type mesoporous organosilicas via covalent bonding and crosslinking," *Catal. Today*, vol. 243, no. C, pp. 173–183, 2015.

[13] M. M. Barsan, M. David, M. Florescu, L. Ţugulea, and C. M. A. Brett, "A new self-assembled layer-by-layer glucose biosensor based on chitosan biopolymer entrapped enzyme with nitrogen doped graphene," *Bioelectrochemistry*, vol. 99, pp. 46–52, 2014.

[14] J. D. Cui and S. R. Jia, "Optimization protocols and improved strategies of cross-linked enzyme aggregates technology: Current development and future

challenges," *Crit. Rev. Biotechnol.*, vol. 35, no. 1, pp. 15–28, 2015.

[15] K. Liang, C. J. Coghlan, S. G. Bell, C. Doonan, and P. Falcaro, "Enzyme encapsulation in zeolitic imidazolate frameworks: A comparison between controlled co-precipitation and biomimetic mineralisation," *Chem. Commun.*, vol. 52, no. 3, pp. 473–476, 2015.

[16] A. S. G. Lorenzoni, L. F. Aydos, M. P. Klein, R. C. Rodrigues, and P. F. Hertz, "Fructooligosaccharides synthesis by highly stable immobilized β-fructofuranosidase from Aspergillus aculeatus," *Carbohydr. Polym.*, vol. 103, no. 1, pp. 193–197, 2014.

[17] S. Africa, "HHS Public Access," vol. 4, no. 11, pp. 411–427, 2017.

[18] M. P. Klein *et al.*, "Effect of the support size on the properties of β-galactosidase immobilized on chitosan: Advantages and disadvantages of macro and nanoparticles," *Biomacromolecules*, vol. 13, no. 8, pp. 2456–2464, 2012.

[19] P. L. Bombeck, V. Khatri, F. M. Mouelhi, D. Montplaisir, A. Richel, and M. Beauregard, "Biotechnology for Biofuels Predicting the most appropriate wood biomass for selected industrial applications : comparison of wood , pulping , and enzymatic treatments using fluorescent - tagged carbohydrate - binding modules," *Biotechnol. Biofuels*, pp. 1–14, 2017.

[20] M. P. Klein *et al.*, "Chitosan crosslinked with genipin as support matrix for application in food process: Support characterization and β- d -galactosidase immobilization," *Carbohydr. Polym.*, vol. 137, pp. 184–190, 2016.

[21] A. Manrich, A. Komesu, W. S. Adriano, P. W. Tardioli, and R. L. C. Giordano, "Immobilization and stabilization of xylanase by multipoint covalent attachment on agarose and on chitosan supports," *Appl. Biochem. Biotechnol.*, vol. 161, no. 1–8, pp. 455–467, 2010.

[22] K. Pospiskova and I. Safarik, "Low-cost, easy-to-prepare magnetic chitosan microparticles for enzymes immobilization," *Carbohydr. Polym.*, vol. 96, no. 2, pp. 545–548, 2013.

[23] A. I. Ash, S. H. Units, and A. Total, "Product specification," *Build. Res. Inf.*, vol. 21, no. 1, pp. 21–22, 1993.

[24] "Nisco Modular Microencapsulation Units - Var j1." [Online]. Available: http://www.nisco.ch/var_j1.htm. [Accessed: 14-Sep-2018].

[25] "Nisco Modular Microencapsulation Units -Principle of Coaxial Airflow Induced Dripping VAR J1." [Online]. Available: http://www.nisco.ch/working_principle_coaxial_air_flow_driven.htm. [Accessed: 14-Sep-2018].

[26] D. R. Figueira, "Valorization of agro-industrial waste through chemical and microbiological approaches," 2017.

[27] G. L. Peterson, "Review of the folin phenol protein quantitation method of lowry, rosebrough, farr and randall," *Anal. Biochem.*, vol. 100, no. 2, pp. 201–220, 1979.

[28] J. H. Waterborg and H. R. Matthews, "Chapter 1 Method for Quantitation,"

Notes, vol. 1, no. 173, pp. 7–9, 1984.

[29] F. Gerber, M. Krummen, H. Potgeter, A. Roth, C. Siffrin, and C. Spoendlin, "Practical aspects of fast reversed-phase high-performance liquid chromatography using 3 μm particle packed columns and monolithic columns in pharmaceutical development and production working under current good manufacturing practice," *J. Chromatogr. A*, vol. 1036, no. 2, pp. 127–133, 2004.

[30] "High-performance liquid chromatography (HPLC)." [Online]. Available: https://www.tudelft.nl/citg/over-faculteit/afdelingen/watermanagement/research/waterlab/equipment/equipment/high-performance-liquid-chromatography-hplc/. [Accessed: 15-Sep-2018].

[31] P. D. Sheet, "Viscozyme ®," 2012.

[32] B. Martini, S. Dimida, E. De Benedetto, M. Madaghiele, and C. Demitri, "Study on the degradation of chitosan slurries," *Results Phys.*, vol. 6, pp. 728–729, 2016.

6. Annexes

1 – Microsphere diameter values obtained with VARJ1

Control (µm)	flow rate (µm)	air flow rate (µm)	Height (µm)
999,39	991,77	733,76	959,84
862,02	944,9	668,49	824,02
863,26	996,25	745,31	941,81
858,5	1059,54	731,97	1048,86
794,15	1151,56	701,5	991,06
990,14	942,28	690,86	954,16
862,32	969,01	717,75	946,28
932,37	1125,49	760,05	1263,75
925,63	939,4	758,9	1045,52
967,33	1020,54	846,34	950,49
903,42	1116,11	835,23	1127,31
887,08	1088,88	1006,92	1036,12
952,03	1024,13	848,56	901,48
944,75	893,6	982,37	1022,31
916,3	968,39	832,87	988,21
1147,06	935,67	850,11	1196,03
962,95	877,68	1008,22	975,81
793,29	760,64	751,93	1229,71
800,89	844,96	911,05	961,24
779,94	850,5	663,84	955,09

Fig A1 - Diameter values from the experiences, with control group using standard parameter values

2 – Calibration Curves for magnetic and non-magnetic Microspheres

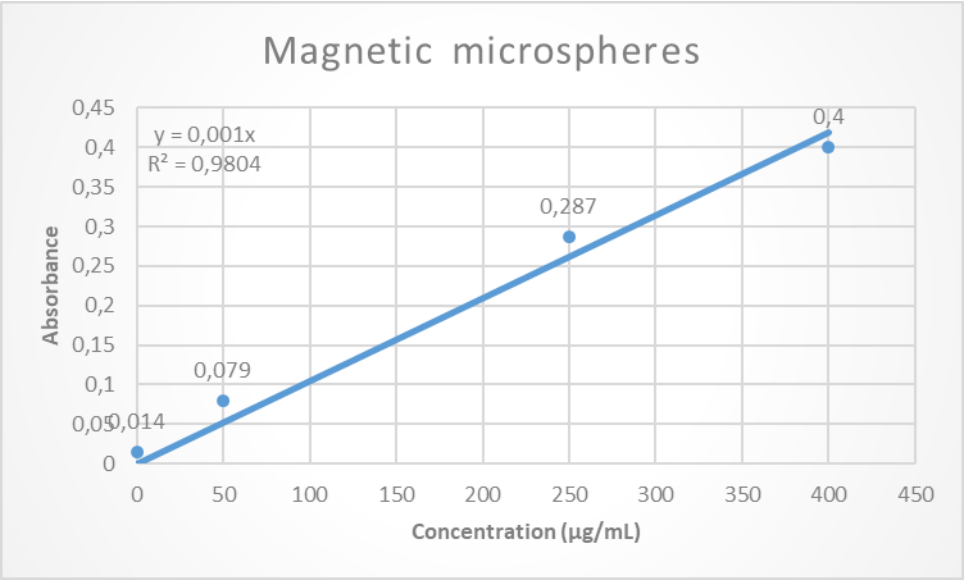

Fig A2 – Magnetic microspheres calibration curve

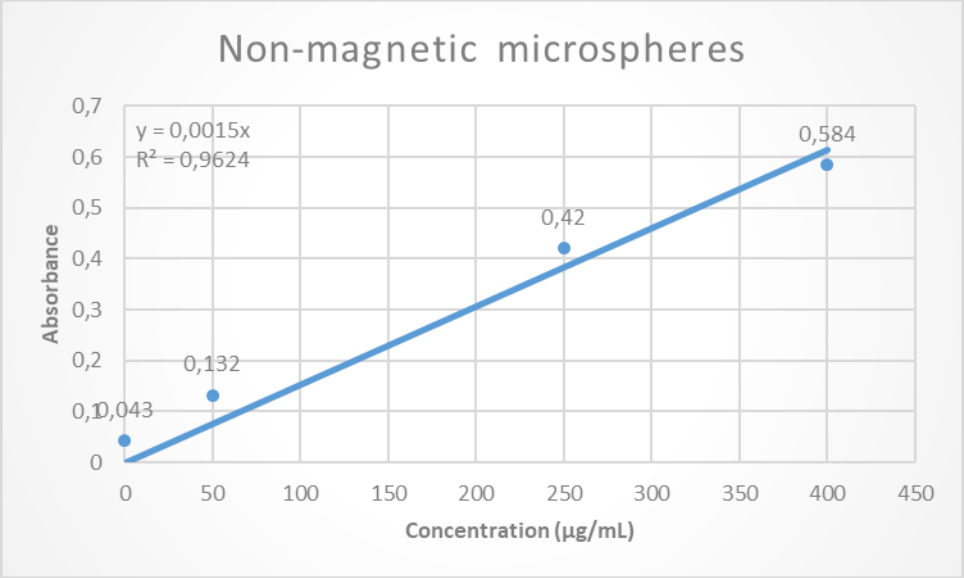

Fig A3 – Non-magnetic microspheres calibration curve

3 - Hydrolysis reactions samples from HPLC

In these tables, separated by each type of sugar identified and measured by the HPLC method, we can see the values for the concentrations for each sample, taken at at T=0h up to T=72h. All values shown represent the final concentration value subtracted by initial concentration value, for yield calculation purposes:

Table A1 – Arabinose samples results

Arabinose	A	A'	B	C	C'	D	E	E'	F
T=0h	39,97	0	41,8125	70,06	75,15	250,682			196,0995
T=1h	64,71	64,11		71,8	73,4				
T=2h	66,41	64,96	35,979	74,6	83,1	262,043			206,4555
T=3h	64,75	64,37		76,72	76,1				
T=20h	69,18	63,7	267,8625	79,94	78,4	284,797			41,582
T=24h	67,33	63	287,695	78,51	76,71	308,6625			42,38
T=48h	68,39	65,21	339,993	72,83	63,48	376,4545			N/A
T=72h			364,2405			404,5775			49,673
T=96h	84,3	65,65		75,23	69,14				
T=0h	22,86	24,48		27,72	20,9		0	0	
T=72h	13,93	13,24		83,32	27,72		0	0	
T=0h	34,07	32,44		44,34	45,53		0	0	
T=72h	13,24	2,77		34,43	24,01		0	0	

Table A2 – Galactose samples results

Galactose	A	A'	B	C	C'	D	E	E'	F
T=0h	92,47	0	122,427	26,48	13,18	158,9355			1175,04
T=1h	135,66	123,36		27,97	12,49				
T=2h	141,36	128,58	143,0695	27,37	16,73	187,1825			1148,108
T=3h	145,99	121,43		31,56	9,48				
T=20h	181,35	132,63	0	32,95	16,79	213,4455			149,33
T=24h	187,81	127,76	0	33,11	10,33	237,872			150,3555
T=48h	221,8	134,6	0	35,4	9,53	281,198			N/A
T=72h			0			281,0655			172,206
T=96h	302,56	138,16		39,11	13,49				
T=0h	32,01483	31,61483		0	0		0	0	
T=72h	17,42483	16,68483		0	0		0	0	
T=0h	88,51	84,52		0	0		0	0	
T=72h	52,66	27,25		0	0		0	0	

Table 3 – Glucose sample results

Glucose	A	A'	B	C	C'	D	E	E'	F
T=0h			352,3826	614,4126	708,1026	621,8326			98,95262
T=1h				654,9826	676,7026				
T=2h			134,3126	671,8226	808,8626	602,1426			81,73262
T=3h				669,7526	670,0426				
T=20h			86,72262	749,7826	765,1526	709,9323			192,7026
T=24h			72,93262	754,9426	689,4426	778,0326			158,5426
T=48h			126,9826	677,3926	651,8426	767,3926			N/A
T=72h			194,5626			867,4126			217,8926
T=96h				682,0526	755,3826				
T=0h	0	0		261,7126	204,7126		0	0	
T=72h	0	0		925,8526	307,4326		0	0	
T=0h	0	0		421,1026	436,9826		0	0	
T=72h	0	0		322,7926	247,9626		0	0	

Table 4 – Xylose sample results

Xylan	A	A'	B	C	C'	D	E	E'	F
T=0h			579,35	0	11,299	233,29			15,586
T=1h				0	9,709				
T=2h			617,12	0	20,689	182,258			17,324
T=3h				0	0				
T=20h			22,368	0	16,039	266,1			1352,24
T=24h			22,464	0	0	286,17			1384,8
T=48h			23,874	0	0	316,42			N/A
T=72h			25,856			357,62			3003,4
T=96h				0	0				
T=0h	0	0		0	0		13677,41	14633,76	
T=72h	0	0		51,079	5,609		14559,76	14319,11	
T=0h	0	0		0	6,579		12131,41	17761,56	
T=72h	0	0		0	0		16504,13	14913,51	

Table 5 – Fructose sample results

Frutose	A	A'	B	C	C'	D	E	E'	F
T=0h			255,8565	701,3465	777,6765	1143,417			93,10648
T=1h				691,5265	752,7665				
T=2h			53,16648	672,6565	887,1365	269,7865			78,30648
T=3h				674,5565	792,9165				
T=20h			108,1265	704,5765	741,8765	1111,617			47,41648
T=24h			78,76648	699,8665	705,7065	1149,137			14,45648
T=48h			144,1665	602,3865	725,0865	1090,197			N/A
T=72h			190,9565			1178,557			113,2465
T=96h				599,0965	727,2265				
T=0h	0	0		89,89648	37,82648		0	0	
T=72h	0	0		522,5065	97,00648		0	0	
T=0h	0	0		199,8665	213,3165		0	0	
T=72h	0	0		112,9865	61,11648		0	0	

The color scheme used represents the different hydrolysis cycles. Green is used to represent the first cycle, yellow represents the second cycle and orange represents the third. Blacked out areas are values not measured in purpose due to not being needed (for example, measuring the concentration of xylan in samples with only arabinose and galactose). Greyed out areas are values that are missing due to HPLC technical errors. Values depicted in bold were used for yield calculations.

4 - Sample groups from hydrolysis reactions for microscopic analysis

Diameter values from microspheres from hydrolysis reactions, displayed in fig A4, were measured in μm.

A	A'	C	C'	E	E'
501,73	785,3	656,22	672,71	613,28	777,84
549,64	641,16	828	618,44	702,14	698,6
519,08	757,32	924,2	676,36	607,18	700,34
626,48	910,46	735,58	696,5	595,75	735,04
552,97	842,6	906,85	751,25	704,34	746,83
577,33	731,57	929,37	661,38	624,18	616,56
559,71	691,78	752,76	683,94	813,17	647,28
662,03	702,31	786,14	630,86	608,1	682,77
520,91	688,78	712,02	694,59	650,82	728,19
570,83	871,94	587,42	506,17	685,48	651,95
648,36	672,48	785,44	681,26	641,72	1031,15
599,83	658,02	784,49	864,56	627,75	806,2
481,64	683,84	933,63	727,98	704,34	732,66
581,93	692,76	622,46	711,49	602,97	776,32
617,56	714,12	894,04	666,97	615,31	721,61
520,88	676,66	672,96	713,67	681,39	822,63
519,01	625,68	812,55	662,04	687,67	593,21
605,55	729,33	630,37	656,5	683,05	609,66
663,5	687,27	837,5	816,84	637,85	784,1
706,01	720,16	715,03	685,06	798,91	731,5
679,06	659,43	789,34	774,65	710,02	714,82
661,47	618,51	676,7	851,39	740,35	689,93
585,08	677,45	838,95	743,06	702,07	759,89
560,1	678,84	671,36	532,22	628,03	756,42
584,54	532,41	759,48	461,32	739,51	856,72

Fig A4 – Diameter values from all microsphere groups after hydrolysis