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Article

A New Method for Immobilization of β-Galactosidase on Glass Fibre Rolls

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Abstract: The usability of glass fibers as immobilization support with a porous open structure has been investigated. We have developed a method to immobilize the enzyme β-galactosidase on special glass fiber rolls. The new method offers a simple, non-expensive and industrially applicable method. Glutaraldehyde was used as a non-specific cross-linking agent for the covalent binding of β-galactosidase on modified glass fibers. The efficiency of immobilization was tested with the known hydrolysis of lactose. All experiments were performed in a continuous laboratory reactor. The influence of the reaction temperature (20, 25 and 30 °C), the substrate flow rate (1, 2 and 3) mL/min and the *pH* of the reaction medium (6, 7 and 8) on the conversion was investigated. The reaction efficiency was monitored by measuring the glucose concentration with a spectrophotometer. High immobilization efficiency, enzyme activity and stability were obtained. The optimal reaction temperature, substrate flow rate and *pH* were found. The activity and stability of the enzyme entrapped on the glass fiber rolls remained almost unchanged during reuse, which is a good prospect for potential industrial applications.

Keywords: β-galactosidase; immobilization; glass fibers; lactose

1. Introduction

Lactose intolerance is the inability to digest a sugar called lactose, which is found in milk and dairy products. Nowadays, this inability is a serious problem. It is caused by a deficiency of the enzyme lactase or β-galactosidase in the digestive system, which leads to various digestive disorders. This can lead to various symptoms, including flatulence, diarrhea, nausea, intestinal bloating and abdominal cramps, and in some cases headaches. The severity of symptoms can vary depending on the amount of lactose consumed [1–3].

Lactose is less fermentable than other sugars and crystallizes at lower temperatures. This can lead to problems in the production of refrigerated dairy products. These problems can be solved by hydrolyzing lactose to glucose and galactose. Namely, lactose consists of the monosaccharides glucose and galactose [1]. The hydrolysis of lactose can be catalyzed by acids or enzymes. Enzymatic hydrolysis is preferred to acid hydrolysis as the process temperatures are lower and the catalyst is easier to separate from the reaction medium [2]. For the enzymatic catalysis of lactose hydrolysis, β-galactosidases are used. β-galactosidases can be isolated from microbial, animal and plant sources. β-galactosidases from microorganisms such as bacteria, molds, yeasts or fungi are available in relatively large quantities and at a reasonable price compared to β-galactosidases from animals or plants [1,3,4]. β-galactosidases are enzymes that convert lactose into glucose and galactose. They are often used to produce lactose-free dairy products for people suffering from lactose intolerance, also known as lactose malabsorption. Newborns usually have the ability to digest lactose, but this ability deteriorates with age because the older people are, the fewer lactase enzymes they have for hydrolyzing lactose. If the lactose is not hydrolyzed, it cannot be absorbed in the intestine and then becomes a substrate for the bacterial community, resulting in undesirable digestive disturbances. The prevalence of lactose intolerance varies according to people's geographical location. More than two thirds of the world's population are lactose intolerant. In a recent study, the global prevalence of

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lactose intolerance was estimated at 68%. On average, most people with lactose intolerance live in Asia (64%) and the fewest in Northern, Southern and Western Europe (28%). There are countries where the prevalence of lactose intolerance is very low (4%), such as Denmark and Ireland, and countries where almost every inhabitant is lactose intolerant, such as South Korea, Yemen and Ghana [5].

The dairy industry is involved in the processing of raw milk into various dairy drinks, fermented milk products, butter, different types of cheese, ice cream, etc., among other things. The production technologies in the dairy industry are adapted to obtain the desired product. Several different technological processes take place during the processing of milk into different types of cheese. During the production of cheese, a quantity of excess liquid is secreted, which is known as whey. The amount of whey secreted depends on the type of cheese and the water retained in the cheese grain, which affects the firmness of the cheese. Whey as a by-product is a waste with a high content of organic substances. A small proportion is used, otherwise it is thrown away. This poses a serious environmental problem. In order to reduce the amount of waste whey and the impact and pollution on the environment, new processes are being introduced to use whey for the production of value-added products. For example, an important use of β-galactosidase is to hydrolyze the lactose in whey. The hydrolyzed form of whey is sweeter, creamier and more biodegradable [1].

There are basically two different ways of using β-galactosidases for the hydrolysis of lactose. Soluble, non-immobilized free enzymes are normally used in batch bioprocesses, while immobilized enzymes can be used in both batch and continuous bioreactors. With a suitable immobilization method, the activity and stability of the enzymes can be increased, which can reduce production costs, allow scale-up to industrial applications and enable successive multiple uses of the enzymes [2–5]. There are numerous immobilization techniques such as covalent bonding [6–8], physical adsorption [9], cross-linking [10–12], and microencapsulation or entrapment [4,5,13,14]. Which technique is used for the immobilization of enzymes depends on the respective application. However, it is important that the solid support has the desired characteristics, such as a large surface area, high porosity, non-toxicity, easy separation from the reaction medium and no leakage of the enzyme from the support [12,15,16].

Various supports, including polymeric fibrous materials, cotton fabric, chitosan, agarose, zeolite, nylon fibers, and κ-carrageenan have been successfully used for the immobilization of β-galactosidase [3,17–19]. In addition, some simple physical adsorption techniques have been used to improve the efficiency of the enzyme in lactose hydrolysis. Immobilization experiments of ß-galactosidase on zeolite pellets have been studied in fluidized bed columns [20]. However, several problems associated with these supports have also been reported. A common problem with the use of polymeric materials for enzyme immobilization is the lack of active sites on the polymer. Carrageenan has been used for immobilization of enzymes and cells using entrapment techniques. It is inexpensive but suffers from poor mechanical and thermal stability [21,22]. On the other hand, β-galactosidase from Aspergillus oryzae was immobilized by diazotization or condensation on nylon membranes grafted with glycidyl methacrylate. It has been found that immobilization by condensation strengthens the enzyme structure in contrast to immobilization by diazotization and confers higher resistance to temperature and acidic solutions to membranes prepared by the first method compared to those prepared by the second method [23]. In many cases, these supports are very expensive, mechanically poor in terms of stiffness, hardness and flexibility, and all susceptible to microbial attack [24,25]. The process conditions for immobilization should be mild enough so that the enzymes are not denatured during immobilization [26].

Various reactors have been used for the utilization of β-galactosidase. Reactors such as fluidized bed, hollow fiber, plug flow, capillary bed and rolling membrane reactors have all been used for lactose hydrolysis [3,27,28]. Membrane reactors have also been tested for this reaction and show lower enzymatic activity compared to the use of enzymes in batch reactors [29].

The aim of this work was to test a new method for immobilization of the enzyme β-galactosidase. As a porous support for enzyme immobilization, special industrial glass fibers were used, which usually occur as a residue in the industrial production of grinding plates. As this material is widely

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available and cheap, it could be used for specific immobilization procedures and further enzyme catalysis. The characterization of the solid support was carried out using an optical microscope, a scanning electron microscope, a thermogravimetric analyzer and by measuring the surface area and pore size. The efficiency of immobilization was tested by hydrolysis of lactose in a continuous reactor. The reactions were carried out at different temperatures, *pH* values of the substrate and flow rates. In addition, the reusability of the immobilized β-galactosidase enzyme was tested.

2. Materials and Methods

2.1. Substrates and Chemicals

Lactose 1-hydrate from Panreac Quimica S.A.U. was used as the substance for the hydrolysis study. Phosphate buffer was prepared with di-potassium hydrogen phosphate and potassium dihydrogen phosphate (from Emsure). A purified industrial liquid β-galactosidase enzyme was purchased from Sigma Aldrich. We purified the glass fibers with deionized water, ethanol ($w \geq 99\%$, Sigma Aldrich), and acetone ($w \geq 99.5\%$, Carlo Erba). Glutaraldehyde (GA, $w = 25\%$, Sigma Aldrich) served as a cross-linking agent for the amino group of the network linker 3-(triethoxysilyl)-propylamine (APTES, Merck). This enabled the covalent binding of the enzyme to the modified glass fibers support. The woven glass fibers (Figure 1) were produced by the company Swatycomet d.o.o. and used as a solid support for the immobilization of the enzyme β-galactosidase. This material is also used in the company for the production of grinding wheels for better cutting or grinding. Glass fibers are silicates made from a melt consisting of 70% SiO2 and 30% Al2O3. They have excellent tensile and compressive strength, temperature resistance, dimensional stability, low thermal conductivity and good fire resistance. Glucose ($w \geq 99.5\%$, Sigma Aldrich) was used for the preparation of standard solutions, which were used together with the glucose reagent GOD/PAP (Roche/Hitachi) to determine the concentration of glucose formed during the reaction and in the standard solutions. Albumin from bovine serum (BSA, Sigma Aldrich) and Commassie Brilliat Blue reagent (Sigma) were used for the determination of proteins.

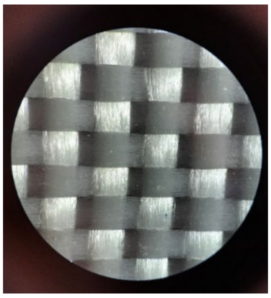

Figure 1. Part of the sheet of glass fibers at 40 x magnification under optical microscope.

2.2. Characterization of Glass Fibes

Optical microscopy, scanning electron microscopy, thermogravimetric analysis and N_2 adsorption/desorption techniques were used to characterize the glass fibers.

2.3. Immobilization

The immobilization procedure consisted of three main steps: preparation of the glass fibers, their purification and the immobilization of the β-galactosidase enzyme.

The β-galactosidase was immobilized on a 20 cm x 60 cm glass fiber sheet. After the glass fiber sheet was cut to the desired dimensions, it was dried in a dryer at 100 °C for 2 hours. The dried glass fiber sheet with a mass of 28 g was then folded three times on the long edge and placed in a flat square container. The glass fibers were cleaned with a mixture of 66 mL deionized water, 66 mL ethanol and 66 mL acetone to ensure that all impurities were removed. The glass fibers were shaken for 10 min at 25 min^{-1} in this cleaning solution. After the cleaning process, the glass fibers were rinsed with acetone.

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The cleaned glass fibers were then shaken with 200 mL of a 10 % *v/v* APTES solution in acetone for 10 min at 25 min^{-1}. After rinsing with acetone and additionally with phosphate buffer, they were treated with 200 mL of a 0.5% *v/v* solution of glutaraldehyde in phosphate buffer by shaking for 10 min at 25 min^{-1}. Then the glass fibers were rinsed with phosphate buffer. The last step in this procedure was the immobilization of the enzyme β-galactosidase on the prepared support. We prepared the solution of the enzyme by diluting 200 mL of β-galactosidase in 200 mL of phosphate buffer. This solution was then poured over the glass fibers and everything was shaken at 25 min^{-1} for 60 min. At the end, the glass fibers with the immobilized β-galactosidase were rinsed again with phosphate buffer to remove unbound enzyme. The sheet of glass fibers with immobilized ◎-galactosidase enzyme was rolled up, placed in the measuring cylinder filled with phosphate buffer and left in the refrigerator overnight.

2.4. Hydrolysis of Lactose by Immobilized β-Galactosidase

Lactase or β-galactosidase is an enzyme used for degradation (hydrolysis) of lactose. It catalyzes chemical reactions by converting lactose into two monosaccharides, glucose and galactose. The enzyme was covalently bound to a modified support – glass fiber rolls - as previously described. All experiments were carried out in a continuous catalytic laboratory reactor CEU from Armfield. The glass fiber roll containing the immobilized β-galactosidase enzyme was placed in the double-walled continuous flow reactor and then the reactor was filled with phosphate buffer at the desired *pH* such that all air bubbles were removed. The reactor contents were then preheated to the desired temperature. The individual experiment began by pumping the lactose solution through the reactor. The lactose solution was prepared by diluting an appropriate amount of lactose 1-hydrate in the phosphate buffer with the desired *pH* and a concentration of $c = 0.05$ mol/L to obtain a lactose solution with the concentration of $g = 6$ g/L. During the reaction, samples were taken at different time intervals for up to 4 hours. Each sample was treated at 100 °C for a few minutes to denature any enzymes that might leak from the glass fiber support into the sample. We performed the experiments at different temperatures of 20, 25 and 30 °C, substrate flow rates of 1, 2 and 3 mL/min and *pH* values of 6, 7 and 8. The conversion of lactose was determined by measuring the glucose concentration formed with a spectrophotometer at 510 nm.

2.5. Analysis Procedure

By analyzing standard solutions of glucose with the concentrations $g = (0, 1, 2, 3 \text{ and } 4)$ g/L, the standard curve was obtained. The standard curve was used to determine the glucose concentrations in the samples. To the 990 mL of the glucose GOD/PAP reagent in the Eppendorf tube, 10 mL of the glucose standard solution or the sample solution was added, mixed thoroughly and thermostated at 35 °C for 10 min. The colored solution was transferred to the cuvette and then the absorbance was measured at 510 nm.

2.5. Bradford Analysis Procedure

The Bradford test was utilized for the determination of proteins. The standard solutions were prepared from albumin from bovine serum diluted in deionized water. The Bradford test was used to determine the amount of immobilized β-galactosidase enzyme from the difference in the concentration of proteins before and after the immobilization procedure. The concentrations of the proteins were determined by mixing the protein-containing solutions with Commassie Brilliat Blue reagent and measuring the absorbance of the resulting solutions at 595 nm using a spectrophotometer. The Bradford test was performed for each immobilization procedure and then the average value of the immobilized β-galactosidase enzyme was calculated. Thus, approximately 0.49 mg of β-galactosidase enzyme was immobilized on the single glass fiber sheet.

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3. Results and Discussion

3.1. Characterization of Glass Fibers

To characterize the glass fibers, we performed a scanning electron microscopy (SEM) analysis, which provides a complex image of the surface topography of the sample at high magnification. The SEM image of the glass fibers shown in Figure 2 was recorded with the Philips SEM XL series. We can see that a single fiber has a diameter of about 10 mm. We can see that the diameter of the glass fiber is the same over the entire length of the fiber. The length of the fibers is not important because we used the glass fiber sheets for the immobilization of the enzyme and later for the lactose hydrolysis reaction.

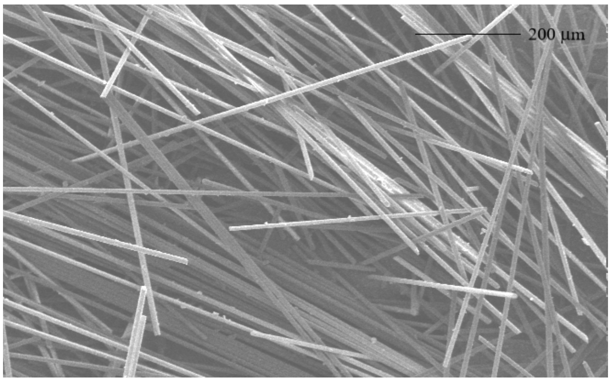

Figure 2. Scanning electron microscopy image of glass fibers (100 x magnification).

The thermogravimetric analysis of the glass fibers was carried out with the TGA 2 from Mettler Toledo. The analysis was performed in the temperature range from 30 to 800 °C with a heating rate of 10 °C/min in an air atmosphere. The results of the thermogravimetric analysis are presented in Figure 3.

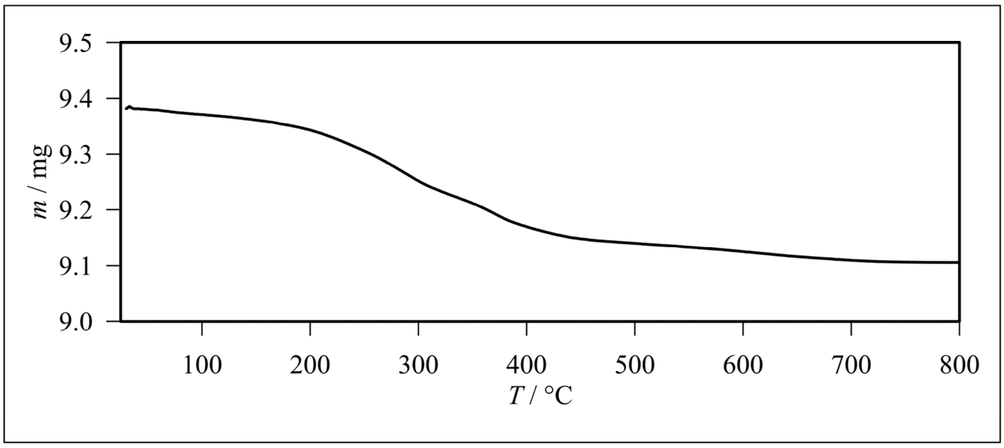

Figure 3. Thermogravimetric analysis results of glass fibers (TGA).

According to thermogravimetric analysis, the total loss of mass was less than 2.9%. Although the mass loss was really low, it was attributed to the loss of water at lower temperatures and probably some impurities at temperatures above 200°C. This is because the characterization was performed with the glass fibers as received without purification.

The N_2 adsorption/desorption technique was used to measure the surface area and pore size of the glass fibers. BET was performed with TriStar II from Micromeritics. The results are shown in Figure 4.

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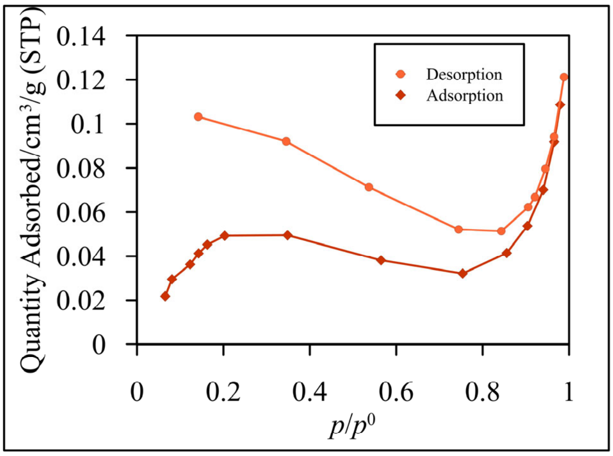

Figure 4. N_2 adsorption/desorption results of glass fibers (BET).

As we can see from the results of the BET measurement in Figure 4, the hysteresis loop was not fully completed. This is due to the very small surface area of the glass fibers. The BET surface area was determined to be 0.25 m2/g and the pore size 2.29 nm. Since the available surface area is small, we have to use a larger mass of the support for the immobilization of the enzyme compared to supports with a large surface area.

3.2. Hydrolysis of Lactose

Firstly, we tested the influence of GA and APTES concentration on the yield of immobilization of β-galactosidase and further on the hydrolysis of lactose conversion.

The immobilization procedure was performed with four different GA concentrations (φ_{GA} = 0.5, 1, 2, 5%), while the APTES concentration (φ_{APTES} = 10%) and the ◎ galactosidase enzyme concentrations were kept constant. The dynamic profiles of lactose conversion until steady state was reached are shown in Figure 5. The hydrolysis of lactose was carried out over a period of 4 hours and samples were taken every hour to analyze the amount of glucose formed. In this step, the experiments were carried out at a temperature of T = 30 °C and with a substrate flow rate of q_V = 3 mL/min. The lactose solutions were prepared with phosphate buffer at pH = 7 and a concentration of c = 0.05 mol/L.

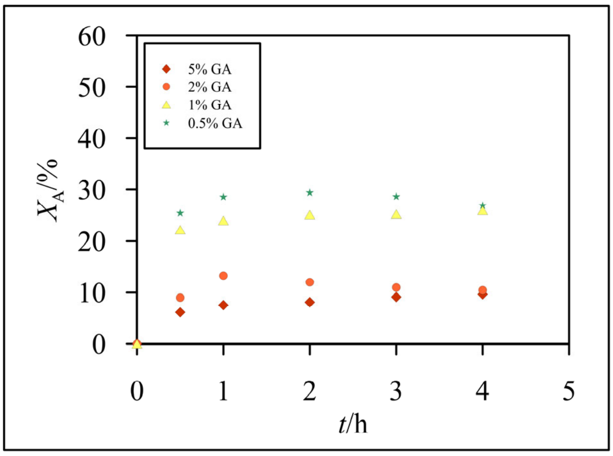

Figure 5. Conversion of lactose regarding residence time using different concentrations of GA (T = 30 °C, q_V = 3 mL/min, φ_{APTES} = 10 %).

Figure 5 shows that the steady state was reached after 1 hour and that the highest conversion of lactose (30%) was achieved with GA at a concentration of φ_{GA} = 0.5%.

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We continued with the experiments by changing the concentrations of APTES using the previously determined optimal concentration of GA (φ_{GA} = 0.5%). Figure 6 shows that the highest lactose conversion (30%) was achieved with APTES at a concentration of φ_{APTES} = 10%.

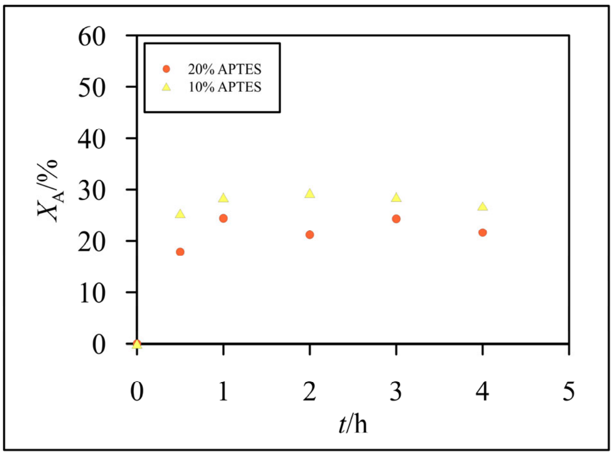

Figure 6. Conversion of lactose regarding residence time using different concentrations of APTES (T = 30 °C, q_V = 3 mL/min, φ_{GA} = 0,5 %).

The optimal concentration of APTES for successful β-galactosidase immobilization varies [30,31]. Therefore, we tested the immobilization procedure with APTES at concentrations of φ_{APTES} = 10% and 20%. Since a higher concentration only decreased the immobilization efficiency (Figure 6), we continued the study with the previously reported value.

After determining the optimal immobilization conditions (φ_{GA} = 0.5% and φ_{APTES} = 10%), we investigated the influence of substrate flow rate, reaction temperature and pH on lactose conversion.

The influence of the lactose flow rate (1, 2, 3 mL/min) on the final conversion is shown in Figure 7. These experiments were performed at a temperature of T = 20 °C. The reaction of lactose hydrolysis was carried out for 4 hours. As expected, the lowest lactose flow rate, q_V = 1 mL/min, results in the highest conversion of lactose, namely 38.7%. At the two higher flow rates, the conversion was almost the same, 26.8%.

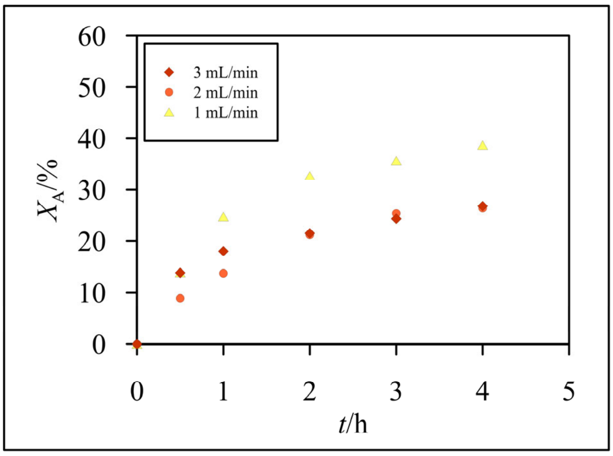

Figure 7. Conversion of lactose regarding residence time at different flow rates (T = 20 °C).

In the next step, we repeated the experiments at 25 °C. Again, we investigated the effects of the flow rate of the lactose solution on the reaction conversion – Figure 8.

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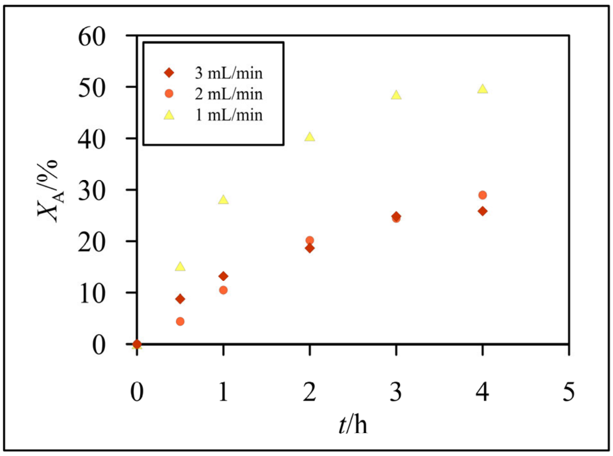

Figure 8. Conversion of lactose regarding residence time at different flow rates (T = 25 °C).

At a temperature of T = 25 °C and a flow rate of q_V = 1 mL/min, a conversion of up to 50% was reached (Figure 8). At lower flow rates, the conversions were again similar and around 29%.

Regardless of the temperature, we observed that at low flow rates (q_V = 1 mL/min) the shape of the glass fiber roll apparently allowed good contact between substrate and immobilized enzyme, while at higher flow rates some of the substrate flowed through the small hole in the middle of the roll, where there was less contact with the enzyme and consequently conversions was noticeable lower.

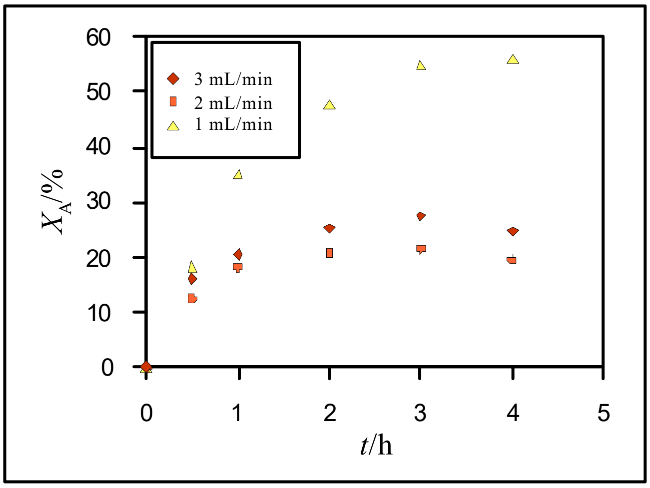

Figure 9. Conversion of lactose regarding residence time at different flow rates (T = 30 °C).

Figure 9 shows that at 30 °C the highest conversion of lactose is 56% at the lowest flow rate and more than 30% lower at the two higher flow rates. The difference in conversion of lactose of about 5% was observed between 2 and 3 mL/min only at the highest temperature of 30 °C, while the profiles of lactose conversion at the two highest flow rates are practically the same at the two lower temperatures (20 and 25 °C). The difference is probably more pronounced at higher temperatures, which is due to the higher reaction rate.

If we compare the conversions of lactose at the same flow rate as a function of temperature (Figures 7–9), we see that within this temperature range, the conversion of lactose increases with increasing temperature. We could probably expect a higher conversion if we further decreased the flow rate of lactose. However, for our study we only tested the efficiency of immobilization at three selected flow rates. The optimal temperature range for the hydrolysis of lactose is between 25 and 50 °C and depends on whether the enzyme is free or immobilized [1,15,32].

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In order to determine the optimal values of the most important process parameters for the lactose hydrolysis reaction, we also investigated the influence of the *pH* of the phosphate buffer used for the preparation of the lactose solutions. We performed the hydrolysis of lactose at three *pH* values of the substrate: 6, 7 and 8. Figure 10 shows the conversion profiles during the reaction until steady state was reached for different *pH* values of the lactose solution.

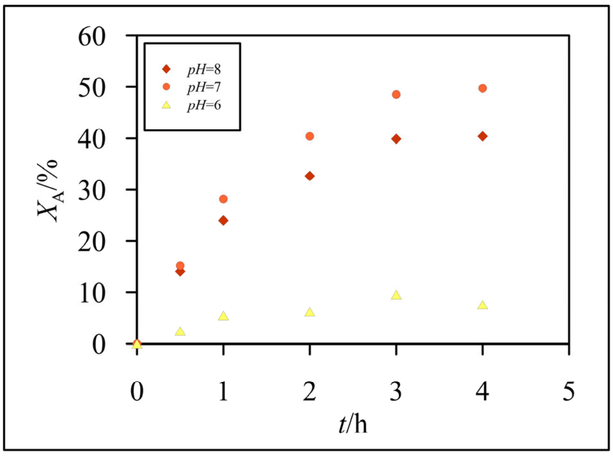

Figure 10. Conversion of lactose regarding residence time at different *pH* of the substrate (T = 25 °C, q_V = 1 mL/min, φ_{APTES} = 10 %, φ_{GA} = 0,5 %).

From all three conversion profiles in Figure 10, we can see that a steady state was reached after about 3 hours. The highest conversion of lactose (50%) was obtained at *pH* = 7, while the conversion of lactose was slightly lower at *pH* = 6 (40%) and less than 10% at *pH* = 8. As can be read in the literature, the optimal *pH* of lactose hydrolysis using the ⊚ galactosidase enzyme varies depending on the origin of the b-galactosidase, the type of enzyme (free or immobilized) and the substrate used [32–34]. The optimal *pH* for the immobilized b -galactosidase on the Cu-trimesic acid support was found at *pH* = 7 [33],], on the polymer-coated magnetic nanoparticles at pH = 6[15] and for the free b-galactosidase entrapped in calcium alginate at *pH* = 7 [1].

After obtaining the optimal process conditions for the immobilization and hydrolysis reaction, we investigated the possibilities of reusing the immobilized β-galactosidase enzyme. The immobilized enzyme was used repeatedly for 3 cycles - Figure 11.

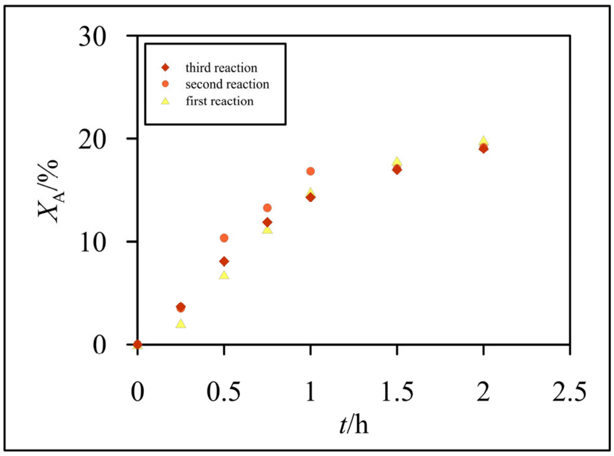

Figure 11. Conversion of lactose regarding residence time after immobilized β-galactosidase enzyme reuse (T = 30 °C, q_V = 1 mL/min).

The highest conversion of lactose that was reached after 2 hours was around 20%. Figure 11 shows that immobilization of the β-galactosidase enzyme on the glass fiber rolls preserves enzyme activity and stability. The same roll was reused for three consecutive lactose hydrolysis reactions with

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almost unchanged conversion, which certainly indicates high immobilization efficiency and practically no leakage of the enzyme from the support during the reaction and in between the reactions cleaning of the glass fiber rolls.

4. Conclusions

The main objective of this study was to investigate the possibility of using industrial glass fibers as a matrix for the immobilization of the enzyme β-galactosidase. Originally, this material is used in the production of cutting disks and coated abrasives. This residue is cheap and widely available. On the other hand, the enzymatic hydrolysis of sugars by the enzyme β-galactosidase, which produces glucose and galactose monosaccharides, is becoming an important biotechnological process with application in the dairy industry.

The enzyme β-galactosidase was immobilized on the sheets of glass fibers rolled into a cylindrical shape. We determined the optimal conditions for immobilization: pH of the phosphate buffer, $pH = 7$, concentration of APTES, $\varphi_{APTES} = 10\%$, and concentration of glutaraldehyde, $\varphi_{GA} = 0.5\%$. The efficiency of immobilization was tested using the known hydrolysis of lactose. Conversions of up to 55% were obtained, which confirms the good activity of the immobilized biocatalyst. The shape of the glass fiber rolls allows good contact between the substrate and the immobilized enzyme at a low flow rate, q_V = 1 mL/min, and a temperature of T = 30 °C. At higher flow rates, the substrate flowed through the small hole in the center of the roll, resulting in lower conversion.

We also checked the activity and stability of the enzyme immobilized on the glass fiber rolls during its reuse, which remained almost unchanged after three reactions. Our results show a great perspective for possible industrial applications.

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