STARCH HYDROLYSIS BY IMMOBILIZED GLUCOAMYLASE ON 3D POLY-LACTIC ACID (PLA) CARRIERS IN BATCH REACTOR

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ABSTRACT

The aim of the present work was to immobilize a commercial fungal glucoamylase on 3D poly-lactic acid (PLA) carriers obtained by additive manufacturing and to use the immobilized enzyme in the hydrolysis of starch in a stirred tank reactor. The carriers were functionalized with amino groups using ethylenediamine (EDA), treated with $NaBH_4$ and then activated with glutaraldehyde. The covalent immobilization of glucoamylase was carried out at pH 10 with an enzyme load of 200 $mg \cdot g^{-1}$ of carrier. The immobilized enzyme was used in the reactor for starch hydrolysis and the maximum conversion of the substrate into glucose (95%) was observed at 12 h, indicating a better performance of the immobilized catalyst when compared to the soluble enzyme (maximum conversion of 88%, at 48 h). The operational stability of the immobilized glucoamylase was evaluated and it was possible to achieve 74% of starch conversion at 12 h, percentage that remained practically constant until the end of the experiment, at 48 h.

Keywords: Glucoamylase. Covalent immobilization. Polylactic acid. 3D printing. Batch starch hydrolysis.

1 INTRODUCTION

Microbial enzymes have been used in several industrial processes but there are still some challenges to be overcome such as the sensitivity of biocatalysts to the reaction conditions, the difficulty in separating the products and the impossibility of reuse. An interesting strategy to overcome these challenges is enzyme immobilization, the physical or chemical association of the enzyme with a solid support or matrix, insoluble in water and inert, through different techniques such as physical adsorption, covalent bonding, confinement or affinity. Such approach mainly aims to reuse the enzyme, but may also protect protein configuration in order to maintain and/or improve its activity and stability over several cycles of use[1].

The choice of support represents an important step for enzyme immobilization and the support must present characteristics such as stability, presence of active functional groups and must be economically viable[2]. Inorganic supports, such as silica, have long been used on an industrial scale as they have good thermal stability and high mechanical resistance. The use of organic supports, biopolymers from natural sources, is highly desirable since they are biocompatible, biodegradable and do not present toxicity[3].

Amylases stand out among industrial enzymes, being widely used in the food industry in the production of alcoholic beverages, fruit juices, breads, cakes and syrups with high glucose and fructose content. They are also used in the textile, detergent, paper and biofuel production industries, as well as in leather processing. They have been intensely studied since the first decade of the 19th century, currently representing around 30% of the global enzyme market. With the advancement of Biotechnology, the possibilities of amylases application have expanded, including the areas of Medical Chemistry, Pharmacology and Analytical[4].

Glucoamylase (1,4-α-D-glucanoglucohydrolase, EC 3.2.1.3) is one of the oldest and most widely used enzymes in the food industry, whose main application is the saccharification of starch or dextrins into glucose, an essential product for sereval fermentation processes in a wide variety of food and beverage industries. Also known as amyloglucosidase, it is an exoamylase produced mainly by fungi, which catalyzes the hydrolysis of α-1,4 glycosidic bonds from a non-reducing end of the substrate molecule. More recently, the potential of glucoamylases to saccharify starchy raw materials was studied with the aim of using the released glucose for bioethanol production. It is estimated that the glucoamylases market will reach 1.1 billion dollars in 2027, representing almost 10% of the industrial enzymes market[5].

Regarding the immobilization of glucoamylases, some studies mention the use of supports such as silica[6], magnetic nanocrystals[7], Fe_3O_4 nanocomposites and graphene oxide[8], polyaniline[9], among others.

Given the above, in the present work a commercial glucoamylase from *Aspergillus niger* was immobilized on polylactic acid 3D carriers printed by additive manufacturing, the derivatives were used in starch hydrolysis in a stirred tank reactor operating in batches and operational stability was evaluated in a new hydrolysis cycle (reuse).

2 MATERIAL & METHODS

2.1. Design of 3D PLA carriers

The Fused Filament Fabrication (FFF) technique using MakerBot Replicator + 3D printer was used to produce the 3D PLA carriers, from white PLA filament (3D Fila – Brazil). AutoCAD 2022 software (Student License, Autodesk, USA) was used for 3D modeling, while the MakerBot Print software (ver. 4.10.1.2056) was used for model slicing, and printing parameters were defined as follows: layer height 0.2 mm, extruder temperature 205 ºC, extrude retraction distance 0.6 mm, travel speed 150 mm/s. The carriers were designed with 6 mm high and 15 mm diameter and consisted of a mesh spaced 1 mm (Figure 1).

2.2. Functionalization and activation of PLA carriers

For functionalization, 35 g of carrier and 200 mL of EDA (Sigma-Aldrich) solution (2 mol·L^{-1} in isopropyl alcohol) were maintained under gentle stirring, for 120 min at room temperature. After functionalization, the carriers were left in contact with borohydride solution (0.057 g·g^{-1} carrier) for 4 h, under gentle stirring at room temperature in an open system, and then they were washed with sodium acetate buffer (0.1 mol·L^{-1}, pH 4.5) and plenty of distilled water. Functionalized carriers (1 g) were activated by immersion in 20 mL of glutaraldehyde solution (20% in a phosphate buffer 0.01 mol·L^{-1}, pH 7.5), for 15 hours. Then, carriers were rinsed with distilled water and dried using a vacuum pump.

2.3. Immobilization of commercial glucoamylase

Immobilization of commercial glucoamylase (Sigma -Aldrich A7095) Awas performed using 1 g of carrier in 10 mL of aqueous phase composed of glucoamylase (200 mg·g^{-1} of carrier) and bicarbonate buffer (0.5 mol·L^{-1}, pH 10.0), at 25 ºC, under 80 rpm, for 24 h. The carriers were washed with distilled water and McIlvaine buffer (pH 5.5, 0.1 mol·L^{-1}), dried using a vacuum pump and assayed for glucoamylase activity.

2.4. Glucoamylase activity and protein quantification

The reaction mixture consisted of 1.8 mL of substrate solution, 200 µL of Mc Ilvaine buffer (0.1 mol·L^{-1}, pH 4.93) and the immobilized glucoamylase. The reaction was performed at 50 °C, for 10 min, and then stopped in an ice bath. Quantification of released glucose was performed by adding 500 µL of DNS reagent to 500 µL of the hydrolysate. The mixtures were then brought to a boiling point of 100 °C for 5 minutes, cooled in an ice bath, and added to 4 mL of distilled water. The absorbance values were measured using a UV spectrophotometer at 540 nm, as described by Miller (1959)[10]. One unit of glucoamylase activity was defined as the amount of enzyme required to release 1 µmol glucose·min^{-1}, under assay conditions, and the enzyme activity is expressed as U per gram of support. Protein was quantified as described by Bradford[11] based on an analytical curve using bovine serum albumin.

2.5. Enzymatic hydrolysis of starch on stirred tank batch reactor

Starch enzymatic hydrolysis assays were performed in stirred tank batch reactor (BSTR) (4 cm diameter, 6.5 cm height, 81.64 mL total volume) using a 1% (w/v - dry weight basis) solution of potato starch in Mc Ilvaine buffer (0.1 mol·L^{-1}, pH 5.5) at 50 °C and enzyme load of 37.5 or 133.33 U.g^{-1} of substrate (dry mass) for free and immobilized glucoamylase, respectively, to a final reaction volume of 70 mL. The assays were performed in duplicates and samples were collected at 0, 6, 12, 24 and 48 h. After 48 h, for the experiments of reusing immobilized glucoamylase, they were removed, washed with buffer and then used in a new hydrolysis reaction of the same duration and under the same condition.

2.6. Analysis of starch hydrolysis products by high-performance liquid chromatography (HPLC)

Glucose was quantified using an HPLC system equipped with a BIO-RAD Aminex HPX – 42A chromatographic column (300 x 7.8 mm) operating at 80 °C. Ultrapure water served as the mobile phase at a flow rate of 0.6 mL.min^{-1}, and a sample volume of 20 µL was utilized. A refractive index detector operating at 60 °C was also employed. Chromatographic analysis of analytical curves. For glucose and maltose were conducted using analytical-grade standards dried with phosphorus pentoxide and vacuum. Starch conversion was calculated using Eq 1:

$$\delta=((\text{glucose} \times f)/n)*100 \quad \text{(Eq. 1)}$$

where δ = Starch conversion into glucose (%); glucose = mol of glucose; *f* = hydrolysis factor of 0.9 for starch (mol.mol^{-1}); n = initial mol of starch.

3 RESULTS & DISCUSSION

3.1. Starch enzymatic hydrolysis

Figure 2 illustrates the conversion of starch into glucose by both immobilized and soluble glucoamylase, based on the hydrolysis experiment conducted in a batch stirred tank reactor. The conversion percentage remained above 90% from 6 hours of reaction until the end of the reaction when the immobilized enzyme was used, indicating that its sustained activity under the reaction conditions. In contrast, the percentage of substrate conversion by the soluble enzyme was approximately 28% lower after 6 hours of reaction. The soluble glucoamylase achieved the highest conversion rate of 88.56% after 12 h of reaction.

The reuse of the immobilized catalyst was evaluated and in the first 6 h of the reaction it afforded higher conversion percentage (68.37%) than that obtained by soluble glucoamylase (62.70%). After this time, conversion rates remained relatively constant until the completion of the 48 h reaction (74.11 to 77.06%).

Figure 1. CAD-designed poly(lactic acid) carriers used to immobilize commercial glucoamylase..

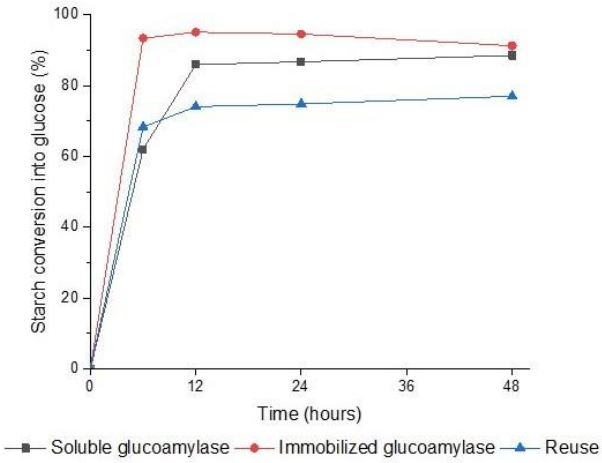

Figure 2. Conversion of starch into glucose by soluble and immobilized glucoamylase.

4 CONCLUSION

The results demonstrate that PLA carriers hold great potential as immobilization carrier for glucoamylase and that the resulting derivatives can be successfully used for starch hydrolysis in enzymatic reactors. This strategy enables biocatalyst reuse, still with a good percentage of substrate conversion into product, which is not possible with enzymes in their soluble form.

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