2016 7th International Conference on Biology, Environment and Chemistry
Volume 98 of IPCBEE (2016)
DOI: 10.7763/IPCBEE. 2016. V98. 9

A Low-Cost Support for Lipase Immobilization Produced from Sugarcane Bagasse

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Abstract. The biotechnology field has been gaining attention in academia and industry worldwide. Much of this interest is due to the use of enzymes as biocatalysts, considering their advantages, such as high selectivity and specificity. However, despite the numerous advantages of using biocatalysts, the difficulty of reusing them when not immobilized and their high cost create barriers for their use in industry. In this context, enzymes are commonly immobilized on solid supports to increase their thermal and operational stability, in addition to the recovery rate. However, many materials currently used as support are non-biodegradable, synthetic, or even toxic, causing numerous negative effects to the environment, as well as giving unwanted properties to reaction products. Therefore, there is a need for alternative materials that can be used as support and that have features like: biodegradability, renewability, low cost, and high availability. From this perspective, this study developed a low-cost support for lipase immobilization produced from sugarcane bagasse. The immobilized enzymes on our support had their activity compared to free enzymes. The results of this research indicate that enzymes were appropriately immobilized. Furthermore, the support favored a higher activity when compared to free enzymes.

Keywords: enzyme, immobilization, entrapment, biocatalysis.

1. Introduction

The use of enzymes or cells as substitutes to conventional chemical catalysts has been excelling in the field of biotechnology. According to Cardoso et al. [1], these substances are biocatalysts with excellent properties such as high selectivity and specificity. Such characteristics of enzymes allow satisfactory results in products of complex synthesis processes in ecologically sustainable conditions [2]. However, despite the numerous advantages of using biocatalysts, the difficulty of reusing them when not immobilized and their high cost create barriers for their use in industry [3].

In this context, enzymes are commonly immobilized on solid supports to increase their thermal and operational stability, in addition to the recovery rate. The methods available for immobilization may be divided into two groups: chemical methods, in which covalent bonds are formed; and physical methods, in which weak links between enzymes and the support are formed [4]. Among the methods that have been used are: adsorption on hydrophilic, hydrophobic or ionic areas, covalent attachment, and containment into polymer matrices [5].

However, many materials currently used as support are non-biodegradable, synthetic, or even toxic, causing numerous negative effects to the environment, as well as giving unwanted properties to reaction products - making them unsuitable for use. Therefore, there is a need for alternative materials that can be used as support and that have features like: biodegradability, renewability, low cost, and high availability [6].

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Under this perspective, this paper studies the use of sugarcane bagasse (or BC), a lignocellulosic material, as a low-cost support. Lignocellulosic materials have been gaining importance in the industry, mainly as support for immobilization of enzymes and cells, considering their high availability as an agricultural byproduct [7]. This project sought to immobilize enzymes lipase by methods of imprisonment, evaluating the rate of immobilization and enzyme activity.

2. Materials and Methods

2.1. Materials and Reagents

Magnetic stirrer; Distilled water; Bagasse of sugarcane (donation); Analytical Balance; Magnetic Bar; Glass rod; Beakers; Vacuum pump; Sodium carbonate; Sodium phosphate; Sodium caseinate; Coomassie brilliant blue G250; Aaron; Greenhouse; Ethanol 96%; n-hexane; Disposable syringe filters; PH tape; Buchner funnel; Glycerol; Sodium containing hydroxynometaline P.A.; Kitassato; Mill; Filter paper; PH meter bench; Automatic pipettor; Petri dishes; Syringe; 50 mM Sodium acetate buffer; Eppendorf tubes; Falcon Tubes; lipase enzymes extracted from *Candida rugosa* type VII; p-nitrophenyl palmitate; Bovine Albumin; UV spectrophotometer Shimadzu brand/VI. All reagents had a high degree of purity.

2.2. Methods

2.2.1. Support preparation

The Brutus sugarcane bagasse (BC) was washed in running water during 6 hours for the removal of impurities. Then it was added a solution of sodium hydroxide (NaOH) 2 M for the degradation of the lignin present in the cellulose fibers from bagasse, leaving them in this solution for 24 hours under agitation. After this period, bagasse fibers were washed thoroughly with running water until they present a pH close to neutral. Later, the washed BC was stowed in beakers of 600 ml and the fiber dried in an oven at 140 °c. After drying, the pomace was crushed in a grinder, provided by company Clariant, thus obtaining a particulate material, considerably increasing its contact surface. Finally, these particles were again submerged in NaOH solution (2 M), for a second extraction of lignin, for more 24 hours. After reaching neutral pH, the fibers were dried in vacuum and stowed in falcon tubes, for use in imprisonment.

2.2.2. Lipase's immobilization

In an analytical balance, approximately 1 g of Sodium Caseinate was weighted, to which was added 1 ml of glycerol and 10 ml of water. This mixture was in constant stirring over a period of 1 hour. After complete homogenization, 6 ml of this solution were transferred to a beaker containing 3 g of BC and 12 mg of lipase and this mixture was stirred for 10 min and transferred to petri dish, and then expected the drying of the material. The same procedure was performed in the absence of the enzymes, which was used as a negative control support in experiments.

2.2.3. Enzymatic activity

The free and immobilized lipase activity was analyzed based on the work of Hung et al. [8]. All the analysis were double performed. It was 0.5% (m/v) of the substrate p-nitrophenyl palmitate (p-NPP) in differents solvents: n-hexane, ethanol, butanol and alcohol isopropyl. The reaction mixture consisted of 3 ml of substrate solution containing 3 ml 0.05 M phosphate buffer, pH. To the reaction medium, it was added 0.5 g of the supports, with the enzyme and control support, or 100 mg of free enzyme, under constant stirring at 30^{o} C. The reaction ended after 5 minutes of incubation by adding 1 ml of 0.5 M sodium carbonate at the rate of 1 ml of each sample. The hydrolysis of substrate by lipase increased the absorbance by 410nm. To determine the concentration of substrate hydrolysed, we used the extinction coefficient of 15.000 $M^{-1}.cm^{-1}$ to p-nitrophenyl palmitate [9]. A unit of activity was defined as the amount of enzyme required to 1 μmol/min hydrolysis of p-NPP under test conditions.

2.2.4. Analysis of the immobilization of enzymes on the support

Brackets showing enzymes and the negative control (no enzyme support) were transferred to different Erlenmeyer flasks in the presence of 30 ml of n-hexane or alcohol isopropyl solvents and agitated by 350 rpm for 60 min. Every 30 min, an aliquot was taken from each sample for analysis of protein release. The

dosage of protein was performed by the method of Bradford [10] using as standard the protein BSA (bovine albumin).

2.2.5. Washing and support storage.

After each enzymatic analysis, the supports were rinsed thoroughly with n- hexane and stored at 4° C.

3. Results and Discussion

3.1. Production Support for Enzyme Immobilization

The dry support showed good quality and thickness as well as satisfactory porosity for contact with the reaction medium. It was cut into cubes of 10 mm side for checking the setting of enzymes and enzymatic activity (Fig. 1).

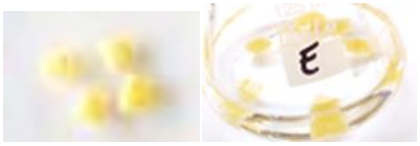

Fig. 1: The left dry BC brackets and directly supports in 30 ml of n-hexane solvent.

3.2. Enzymatic Activity

3.2.1. Activity analysis of free enzyme in different solvents

Before testing the activity of the immobilized enzyme on the support produced in this study, the activity of lipase of *Candida rugosa* in free form was analyzed in different organic solvents (Fig. 2). The analysis in different solvents had as objective the selection of the medium with higher enzymatic activity. The enzyme activity was calculated considering the increase in absorbance at 410 nm, which was due to the hydrolysis of the substrate.

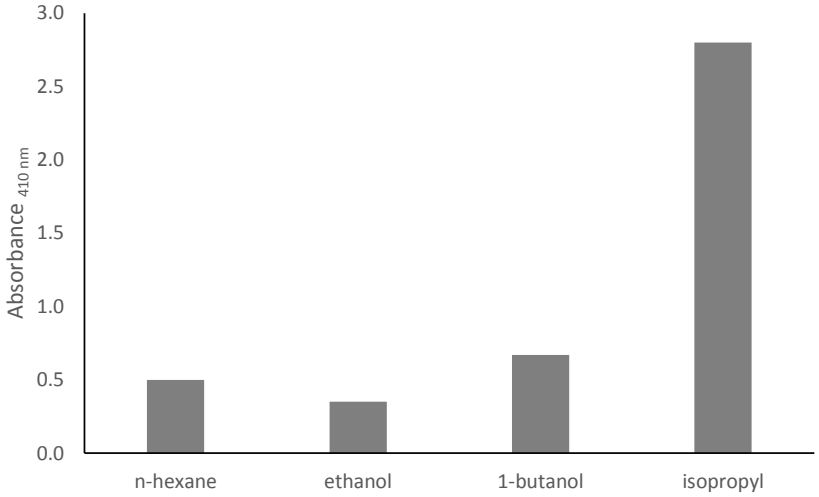

Fig. 2. Free enzyme activity in different solvents. Increase of absorbance due to the hydrolysis of the substrate by the lipase

As we observed a much greater activity of the lipase in the medium containing isopropanol (Fig. 2), three times greater than in 1-butanol, the former solvent was selected for characterization of enzymatic activity of free and immobilized enzymes.

3.2.2. Comparison of enzyme activity: free enzyme *versus* immobilized enzyme alcohol isopropyl.

The extinction coefficient of 15000 $M^{-1}.cm^{-1}$ for p- nitrophenyl palmitate was used to determine the concentration of hydrolyzed substrate. A unit of activity was defined as the amount of enzyme necessary for the hydrolysis of 1 micromol / min p- NPP under the test conditions.

Table I: Analysis of the enzymatic activity to support produced from bagasse sugarcane and sodium caseinate.

Type	Protein Quantity (mg)	Enzimatic activity μmol.min^{-1}	Specific activity (Ea) μmol.min^{-1}.mg^{-1}of protein	Relative specific activity RSA % 1st use
Lipase in support	0.3	3.02	10.06	214
Free enzyme	5	23.4	4.68	100

While Table I presents a highest enzyme activity for the free enzyme, the specific activity in which the enzyme activity is divided by the amount of enzyme present in the medium must be considered. And so, by comparing the specific activity, it may be found a higher activity for the immobilized enzyme. The relative specific activity is also a way to compare the catalytic efficiency, which is considered 100% of the free enzyme activity.

A very important characteristic to be considered in developing a new support is to reuse.The support was used for two more times to verify the stability and preservation of enzyme activity. These data are shown in Table II.

Table II: Analysis of catalytic efficiency upon reuse of the support

Type	Ea 1st use	RSA % 1st use	Ea 2nd use	RSA % 2 st use	Ea 3rd use	RSA % 1st use
Lipase in support	10.06	214	8.51	218	8,03	243
Free enzyme	4,68	100	3.9	100	3.3	100

Comparing the specific activities, we can conclude that the immobilized enzyme had an activity recovered over 100% in the three assays using the same support. Mendes et al. [11] tested many immobilizing supports for lipase obtaining recovered activity ranging from 78% to 21%. Chiou in Wu [4] obtained a recovered activity of 389% in a support produced from chitosan.

3.3. Analysis of the Release of the Enzymes

After immersing the substrates in the solvent, 1 ml aliquot was removed from the medium to verify the release of enzymes. As sodium caseinate used in the entrapment of enzymes is also a derived protein, a negative control is produced by preparing a support fixed under the same conditions but without adding lipases. This control is very important because the quantification of proteins to check the release of lipase may be interference from caseinate, if this substance is also produced detaching the support.

Whereas the control has no enzymes, proteins dosage value for this sample is related to the release of caseinate. However, to estimate the release of immobilizing support enzymes containing lipases, the value of the dosage found for the control was subtracted dosing support lipase. After dosing protein, it was found that the value for the medium was similar to the control, indicating no release of the enzyme to the support. This result corroborates those obtained for the enzymatic activity of support that did not show reduced activity after reuse.

4. Conclusions

It was found a higher enzyme activity for *Candida rugosa* lipase type VII in isopropyl alcohol. The quantification of proteins in the support medium after incubation with immobilized enzymes showed entrapment of lipases, since the results obtained were similar to the negative control. The immobilized enzyme showed a higher specific activity (average RSA 225%, considering the three analysis) when compared to the free enzyme (RSA of 100%). The results indicate that the support produced using low cost

bagasse sugarcane and sodium caseinate was able to retain lipases favoring an increase of enzyme activity when compared to free enzymes.

5. Acknowledgements

We would like to thanks to the Scientific Initiation scholarship program of the Federal Institute of Sao Paulo, the PIBIFSP, as well as CNPq for supporting this research.

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