Assessment of Catalytic Properties in Aqueous and Media of *Aspergillus Niger* Lipase Immobilized on Supports Vitreous

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1. Introdution

Lipases (glycerol ester hydrolases EC 3.1.1.3) are enzymes that are extremely versatile because of the large number of reactions, not necessarily esterification reactions, that they can catalyse. Lipases are produced by a widespread number of microorganisms, including bacteria [1], fungi, and yeasts [2]. In recent years, research on microbial lipases has increased because of their practical applications in industry, as the hydrolysis of fats, production of fatty acids and food additives, synthesis of esters and peptides, resolution of racemic mixtures, or additives in detergents [3,4]. However, factors controlling lipase synthesis and transport have been investigated only in a few cases. In *Aspergillus sp.* for example, the production of lipase has been shown to be strongly induced by triglycerides and detergents and not repressed by glucose or glycerol. On the other hand, long chain fatty acids, such as oleic acid, strongly inhibit lipase production.

Most industrial processes producing biodiesel use chemical methods. However, chemical processes in general require relatively high temperatures and the processing of raw materials which contain a high content of impurities is often prohibitively costly and complicated. The impurities mainly consist of moisture and free fatty acids, commonly found in cheap feedstock. The existence of these impurities causes the generation of many undesirable by-products (e.g., soap) when chemical processes are used. These by-products and glycerol are difficult to separate from the biodiesel and thus, chemical processes require additional procedures for the separation and purification of biodiesel. Therefore, enzymatic methods are preferred over chemical methods.

The aim of this work was to evaluate the efficiency of the immobilization support vitreous lipase produced in solid state fermentation by the filamentous fungi *Aspergillus niger* mutant 11T53A14.

2. Materials and Methods

Microorganism, maintenance and activation

Mutant strain of *Aspergillus niger* 11T53A14 (Couri & Farias, 1995) belonging to the culture collection of Embrapa Food Technology, and previously selected by Damaso et al. (2008), for lipase production. The strain was kept at-18°C and the soil turned into agar containing basic medium plus 2% (w/v) olive oil as carbon source and then transferred to medium ear of corn to produce conidia (Couri & Farias, 1995).

Inoculum preparation

The inoculum was prepared by adding 20 ml of 1% (v / v) Tween 80 per bottle of corn cob, filtering with gauze and seeking the suspension of conidia. The number of conidia/mL was determined by counting in a Neubauer chamber.

Lipase production in Erlenmeyer flask

The lipase production was conducted in 500 mL Erlenmeyer flasks containing 40g of culture medium composed of wheat bran 100g crushed (grain size $\leq$ 5 mm), mixed with 60 ml of 0.91% (w/v) ammonium sulfate solution pH 7.0 and 2% (w / w) of sludge from the refining of corn oil (Damaso et al., 2008). The medium was homogenized and sterilized at 1 atm for 15 minutes, and then inoculated with a suspension of 107 conidia/g of medium. After production of the enzyme at 32 ° C for 48 hours were added 2.5 ml sodium phosphate buffer pH 7.0 or this same buffer plus 1% polyethylene glycol per gram of fermented medium, which remained immersed for an hour, under stirring at 32°C.

Concentration of enzyme

For concentration of lipase was used Thermo Savant lyophilizer containing the refrigerated model RC 300.

Support

The supports used to the immobilization process for this study were silica with pore size of 270A° measured by adsorption of N_2 (ASAP 2000, Micrometrics).

Immobilization Techniques

The process of silanization of the support was done with 3-aminopropiltrietoxisilano (ATPS), according to the procedure: first the support was protonated with nitric acid solution 10% v / v for 30 minutes at room temperature at a rate of 30ml per gram of support. Then the supports were washed with the same acid solution with distilled water and acetone solution (25, 50, 75 and 100%) and then dried at 40 ° C for one hour. The silanization was performed with a solution of ATPS 0.5% v / v, pH 3.3 at 75 ° C for 3 hours at a rate of 30 ml of solution per gram of support. After this time, support was washed and dried. Activation with glutaraldehyde were performed using 2.5% (v/v) in 0.1M phosphate buffer pH 7.0 for one hour at 23°C at a rate of 12.5 mL per gram of support.

Immobilized enzyme

At pre-activated media was added a solution of lipase in 50 mM acetate buffer, pH 4.0 and incubated at 20 ° C for 24 hours. Throughout the process it was determined the enzyme activity of the supernatant.

Analytical determinations

Hydrolytic activities of the immobilized lipase were assayed by the olive oil emulsion method (11). The substrate was prepared by mixing 50 mL of olive oil with 50 mL of emulsification reagent. The reaction mixture consisting of 5 mL of the emulsion, 2 mL of 50 mM sodium citrate buffer (pH 4.0), and immobilized lipase (200 mg) was incubated for 5 min at 37°C. The reaction was stopped by adding 10 mL of acetone:ethanol:water solution (1:1:1). The liberated fatty acid was titrated with 0.02 M sodium hydroxide solution using phenolphthalein as an indicator. One unit of enzyme activity was defined as the amount of enzyme that produces 1 μmol of free fatty acid/min under the assay conditions (37°C, pH 7.0).

3. Results and Discussion

Production, extraction and concentration of lipase.

The enzyme extract containing lipase was produced by mutant A. niger 11T53A14 in solid state fermentation after 48 h of culture. With the aim of assessing the stability of lipase during the process of concentration of the enzymes used two different types of solution for extraction of the enzyme: phosphate buffer pH 7.0 with 1% polyethylene glycol (PEG), stabilizing agent, and pure buffer. The lyophilization was carried out by a 80% reduction of the initial volume of extract. Table 1 shows the results of lipase activity in different conditions and harvested before and after concentration by lyophilization. According to these results, it is found that there was no loss of enzyme activity, demonstrating that the enzyme has a good stability during concentration and that the use of PEG stabilizing agent is not necessary to the process.

Table 1: Lipase activity during different conditions of extraction and concentration

Enzyme	Before lyophilization (U/mL)	After lyophilization (U/mL)
Extracted with buffer	24,6	148,0
Extracted with buffer + 1% PEG	23,6	120,0

Processes of Imobilization

The process used in this work it is to link covalently the enzyme to a support flint (silica), in which the support will have to suffer a silanization and activation due to the fact of not having the necessary reactive groups already available. The silanization process refers to the formation of a bridge in the organic support by reaction with 3-aminopropiltrietoxilano (ATPS), where the ethyl radical reacts with the ATPS silanol group support, creating an amine termination, which will activate the glutaraldehyde forming aldehyde group needed to bind to the enzyme. (Figure 1)
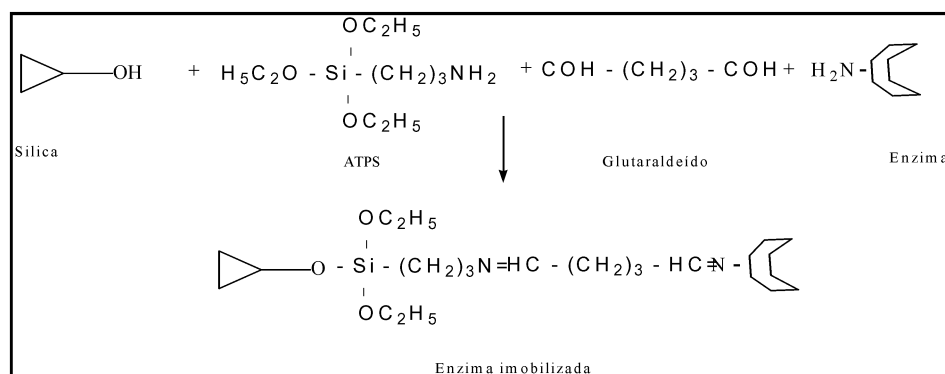

Figure 1: Scheme of immobilizing enzymes by covalent

For both enzymes were concentrated used a rapid decrease in activity in the supernatant, with an immobilization yield of around 90% (Figure 02). However, from further calculations it was observed that although both have shown a great reduction in the activity of the supernatant was recovered a difference in the activity of the derivatives obtained after the immobilization process, and 11.2% for lipase extracted only with buffer and 10.96% for lipase extracted with buffer more PEG (Table 02). This difference in values of the recovered activity of de derivatives generates can be attributed to problems of diffuse to the immobilized matrix, or the resistance to release the product outside of the matrix (Cabral et al, 2003, Gomes, 2006).
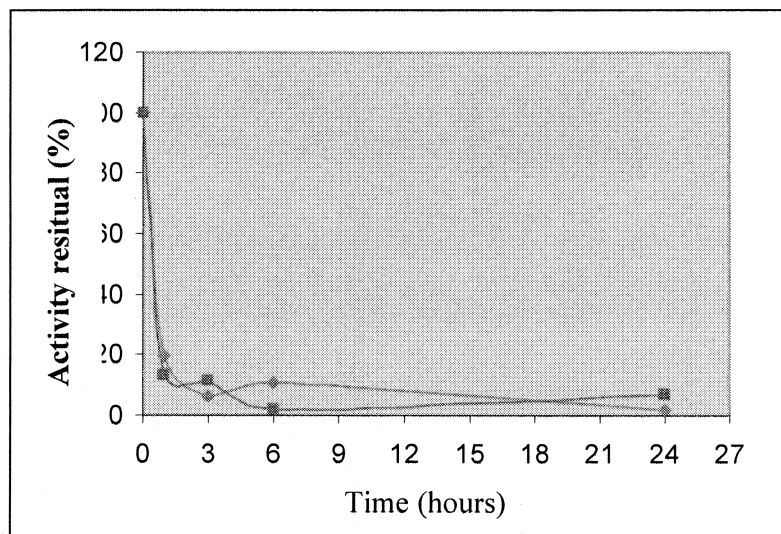
Figure 2: Residual activity of the supernatant , (●) Buffer and (■) PEG.

Table 2: Activity of immobilized lipase.

Activity	Enzyme + buffer	Enzyme + PEG
Transferred the supernatant to support (U/mL)	31,10	25,80
Derivative (U/g)	15,47	15,86
Recovered (%)	11,20	10,96

Just to see the changes caused in the properties of the original free lipase after immobilization procedures used in this study will be performed studies to determine the influence of pH and temperature on enzyme activity, estimated kinetic parameters and assess their potential use for biodiesel production processes.

4. Conclusion

From the results presented in this study we conclude that the addition of polyethylene glycol on the steps of extraction and concentration of the enzyme did not influence the activity of lipase lyophilized. This effect was also observed with the immobilized lipase, in which no significant differences among the activities of the derivatives generated with and without use of polyethylene glycol.

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