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Production of methyl oleate with a lipase from an endophytic yeast isolated from castor leaves

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ABSTRACT

This work aims to optimize lipase production through experimental design and to immobilize these biocatalysts for methyl oleate synthesis. For that, it was applied a 2^{6-3} experimental design to optimize the lipase production, reaching the production of 25 U mL^{-1}. The enzymes produced by submerged fermentation were obtained from an endophytic yeast *Candida guillermondi* isolated from castor leaves (*Ricinus communis* L.). The obtained enzyme was partially purified and freeze-dried before the immobilization process using agarose and silica gel supports. The distribution of the enzyme on the silica gel was homogeneous and verified by atomic force microscopy. The free and the immobilized enzymes were evaluated by the methyl oleate synthesis in order to compare the efficiencies of the reaction processes. The immobilization process increased the conversion rates for the synthesis of methyl oleate in all immobilized enzymes experiments. In addition to the immobilization process ensuring greater efficiency, the immobilized enzyme could also be reused. Therefore, the enzyme used in this work is expected to be a good biocatalyst to be applied in ester synthesis in the food and biofuel industry or laboratorial applications.

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1. Introduction

Lipases (EC 3.1.1.3) are hydrolytic enzymes responsible for the catalysis of triacylglycerol hydrolysis reaction. In vitro they can perform several industrially useful reactions, such as esterification (Mohamed et al., 2011; Fernandes et al., 2007; Yucel, 2012). The microbial lipases, which are usually obtained from some species of bacteria and filamentous fungi, have an excellent potential for its use in several bioprocess due to their desirable characteristics such as: stability under organic solvent, they do not require cofactors and they can act over a large group of substrates (Jaeger and Reetz, 1998; Contesini et al., 2010).

Studies about enzymatic catalysis are usually performed under aqueous media, but when the substrates are hydrophobic and water is a reaction product then these kinds of media are not suitable, resulting in low yield (Fernandes et al., 2004). In this case, catalysis under organic medium emerges as an option because lipases usually have great stability under organic solvents, especially the ones with high apolarity. Beyond that, these solvents solubilize the majority of the reagents, facilitating the contact between the enzyme and the substrate and avoiding the inhibition by excess product due to the product dilution in the medium (Aragão et al., 2009; Hernandez-Rodriguez et al., 2009; Guan et al., 2010; Tan et al., 2010).

Esters are organic compounds largely explored due to their application in food industry (flavor esters, for example) and in bioenergy market (biodiesel). They can be obtained by chemical or enzymatic way. The enzymatic catalysis has been studied due its advantages such as its selectivity that leads to a purer product, since there is no secondary reaction. Besides, the catalyst and the product are easy to separate (Aragão et al., 2009; Sharma et al., 2001; Gamba, 2009).

Although this process has several advantages, it also has some limitations like the biocatalyst cost (Gamba, 2009). To lower the enzyme costs, some strategies can be applied like the use of agricultural and industrial residues as substrate for microbial production of these biocatalysts or else the immobilization of these molecules to increase the enzyme lifetime and stability (Fernandes et al., 2007; Rodrigues, 2009).

The immobilization methods target a weak or a covalent bound between the enzyme and the support. This process has several advantages since the enzyme gains different features like: kinetic properties changes, stability increase and insolubility

Abbreviations: AFM, atomic force microscopy; pNPP, p-nitrophenyl palmitate; PVA, poli(vinyl alcohol); SmF, submerged fermentation

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Nomenclature

U	unit of lipolytic activity, release of 1 μmol *p*-nitrophenol min^{-1}

(which makes the recovery in a continuous reactor possible) (Yucel, 2012; Tan et al., 2010; Dalla-Vecchia et al., 2004).

The ideal combination between support and enzyme is very particular, varying from one enzyme to another. To observe the process efficiency it is important – beyond the enzymatic activity recovery – the homogeneity of the enzyme distribution over the support. To observe it, scanning electron microscopy has commonly been used, but the atomic force microscopy (AFM) has been recently applied for showing a larger view of the system and for the easy sample treatment (Balasheva et al., 2001; Pancera, 2006).

No work was found in the bibliographic review that used lipase secreted by endophytic yeast from castor leaves. Therefore, this study uses enzymes from the endophytic yeast *Candida guillermondi* isolated from castor leaves (*Ricinus communis*, L.) and produced by submerged fermentation. The objective was to optimize the lipase production through experimental design and immobilize these biocatalysts to apply them in the methyl oleate synthesis.

2. Materials and methods

2.1. Microorganism and inoculum preparation

The yeast strain (*Candida guillermondi*) used in this work was isolated from castor leaves by the biotechnologist Jean Figueiredo, from the Tuiuti University of Paraná. The strain was kept in an inclined tube containing potato dextrose agar and transferred to a new tube each 15 days for its maintenance.

The yeast was inoculated in a medium containing 15 g L^{-1} of brewer yeast extract and 10 g L^{-1} of sucrose. The flask was incubated in a shaker at 30 °C under 180 rpm stirring for 48 h. The cell concentration was measured with a Neubauer counting chamber.

2.2. Lipase production optimization by 2^{6-3} experimental design

The submerged fermentation (SmF) was chosen for the enzyme production. The fermentation was performed in 250 mL Erlenmeyer flasks containing 150 mL of fermentative medium, kept in a shaker at 30 °C under 180 rpm stirring for 72 h. All these media were solubilized in 25 mmol L^{-1} phosphate buffer (pH 6.5).

To optimize the production, a 2^{6-3} experimental design was conducted. Experimental design is a set of tests established with statistical criteria, in order to determine the influence of several variables on the results of a given process. This analysis also aims to optimize the process parameters in a short time, because it employs a small number of experiments (Salihu et al., 2011). The variables chosen for the experimental design were the components of the cultivation medium: Tween 80, glycerol, soybean oil, manganese sulfate, brewer yeast extract and ammonium sulfate. The variables levels (minimum, central point and maximum level) can be viewed in Table 1.

All media were sterilized in autoclave and once reached the room temperature 10^9 cells were inoculated. The chosen response variable was the lipolytic activity in U mL^{-1} of each fermented medium.

For interpretation and analysis of the experiments was assumed the value of $p < 0.05$. The interpretation of the *p*-value allows to evaluate the probability of the results obtained have been generated randomly or by the variables studied. In the case of the effects have been caused by the variables, these results are considered significant at a confidence level of 95%. Analyses of the effects generated by the variables and their *p*-values were calculated using the software Statistica 7.0.

2.3. Enzymatic experiment

The enzymatic activity, of the fermented media was determined by the pNPP (p-nitrophenyl palmitate) hydrolysis method, described by Ghori et al. (2011) adapted to this work. A 100 μL sample of the fermented medium was incubated for 5 min at 30 °C with 800 μL of 0.25% PVA [poly(vinyl alcohol)] solution in pH 6.5 phosphate buffer and 100 μL of 8 mmol L^{-1} pNPP solution in isopropanol. After the incubation time, the reaction was stopped by 500 μL of 3 mol L^{-1} HCl solution, then the mixture was centrifuged and 500 μL of the supernatant was added to 1 mL of 2 mol L^{-1} NaOH solution. The absorbance was measured in a spectrophotometer under 410 nm. One unit of enzymatic activity was defined as the release of 1 μmol of p-nitrophenol per minute.

2.4. Partial purification of the enzyme

After fermentation, the enzyme was partially purified by solid ammonium sulfate precipitation. The precipitation was performed on ice using the saturation levels of 50, 60, 70 and 80%. In the ranges of 50–70% irrelevant lipolytic activities were recovered, and then discarded. The enzyme precipitated in the range of saturation of 80% showed higher lipolytic activity and, therefore, was used in this study. The precipitant was suspended with a minimal amount

Table 1
Minimum and maximum levels and range of the six independent variables used in 2^{6-3} experimental design for lipase production by *Candida guillermondi*.

Independent variables	Levels[a] and ranges		
	Minimum level (−1)	Central point (0)	Maximum level(+1)
Tween 80 (g L^{-1})	10	30	50
Glycerol (g L^{-1})	10	30	50
Soybean oil (g L^{-1})	30	60	90
Manganese sulfate (g L^{-1})	0.1	0.2	0.3
Brewer yeast extract (g L^{-1})	10	30	50
Ammonium sulfate (g L^{-1})	10	30	50

[a] Minimum and maximum levels correspond to lower and higher concentrations of the variables used in the experimental design.

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