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Production and immobilization of *Geotrichum candidum* lipase via physical adsorption on eco-friendly support: Characterization of the catalytic properties in hydrolysis and esterification reactions

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ABSTRACT

The present study reports the production of an extracellular lipase from *Geotrichum candidum* by submerged fermentation using cottonseed oil as inductor agent and its immobilization on poly-hydroxybutyrate (PHB) particles via physical adsorption. The catalytic properties of the biocatalyst prepared were determined in aqueous (hydrolysis of olive oil emulsion) and organic (ethyl linoleate synthesis) media. In the enzyme production, maximum hydrolytic activity of 22.91 IU/mL at 30 °C and pH 5.2 was reached after 48 h of cultivation. A single protein band with an apparent molecular mass of 65 kDa was detected by SDS-PAGE analysis. The biocatalyst prepared by offering 75 mL of crude enzymatic extract (without cells) per gram of support exhibited maximum hydrolytic activity of 404.4 ± 2.3 IU/g at 37 °C and pH 7.0, with a recovered activity percentage of around 40% and an immobilization yield of 59%. The optimal pH and temperature for both soluble and immobilized enzyme in the hydrolysis reaction was 8.0 and around 37–40 °C. The biocatalyst was more thermally stable than the crude enzymatic extract at 35 °C in 8 h (46.2% and 23.7%, respectively) and slightly more stable at 45 °C in 40 min (47.5% and 35.2%, respectively). In the esterification reaction, around 70% ester conversion was reached after 2 h of reaction under experimental conditions previously optimized by Central Composite Rotatable Design (CCRD). The biocatalyst retained 93% of its initial esterification activity after 6 successive cycles of esterification reaction. This biocatalyst is a promising one to catalyze reactions in aqueous and organic media.

1. Introduction

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) are enzymes that catalyze in vivo the hydrolysis of triglycerides to glycerol and free fatty acids [1,2]. In non-aqueous medium, they also catalyze esterification, transesterification and interesterification reactions [1–3]. Therefore, lipases can be applied in several industrial processes [1–4]. *Geotrichum candidum* has been described as a microorganism able to produce a multiple forms of several lipases [5–9] in the presence of an inducer in the culture medium. These lipases have different fatty acid specificities [5,7,9]. The most interesting lipases from *G. candidum* can hydrolyze triacylglycerols specifically at the positions containing unsaturated fatty acid at position *cis*-9 or *cis*-9,12 such as oleic or linoleic acids [7,9–13]. This type of specificity is not so common despite the large variety of the produced available lipases from microorganism and it enables the use of these lipases in the production of interesting fatty acids from the corresponding oils concentrated and also, may offer new enantioselective properties with relevance in fine and pharmaceutical chemistry [14–17].

The major limitation for application of the lipases in industrial process can be attributed to their highly cost. This can be determined by the amount of enzyme produced, type of purification process employed, its thermal stability and the possibility of reuse [18–21]. The immobilization of lipases on solid supports can be employed in order to minimize the cost of their application in industrial processes because it allows an easy separation of products at the end of the process and the biocatalyst can be

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reused if the enzyme is stable enough. Other advantages of the lipase immobilization can also be considered, as the possibility to improve enzyme features such as stability, activity and selectivity [22]. Moreover, the use of an immobilized enzyme allows to simplify the design of the reactor and greater improve the control of the reaction [23,24]. The immobilization of lipases by physical adsorption on hydrophobic supports has been widely reported [22,25–27]. In the presence of hydrophobic interfaces, lipases have a peculiar mechanism of action, known as interfacial activation [27], which facilitates access to the active site of the enzyme by substrates and results in enhanced activity [25–27].

In recent years, considerable efforts have been put into the studies of using hydrophobic natural and synthetic organic supports for lipase immobilization [28,29]. A promising hydrophobic support that can be used in lipase immobilization by adsorption is the poly-hydroxybutyrate (PHB). Its application as support for the immobilization of commercial lipases from several sources has been recently reported for the production of highly active biocatalysts in both aqueous and non-aqueous media [18,28,29].

The aim of this work was to prepare highly active biocatalysts of the lipase from *G. candidum* and analyze the prospects for its further application in ethyl linoleate synthesis by esterification in organic medium. The synthesis of this ester has special interest because the ester is stable, innocuous and it can be used in substitution of the linoleic acid, in food additives, drugs and cosmetics, since linoleic acid is easily oxidized and develops an unpleasant taste [30]. To our knowledge, this is the first report of the use of PHB particles as support to immobilize *G. candidum* lipase for further application in organic medium to catalyze ethyl linoleate synthesis. In the present investigation, the properties of the immobilized biocatalyst have also been determined such as the optimal pH, temperature, and thermal stability tests. The influence of different variables (concentration, temperature and molecular sieve concentration on the ester synthesis) was also verified in order to obtain the optimal experimental conditions using factorial design and response surface methodology analysis. This methodology has a great interest mainly when several variables may exhibit some interactions. Finally, the operational stability of the biocatalyst in this reaction was analyzed in 6 consecutive cycles of ethyl linoleate synthesis in heptane medium.

2. Materials and methods

2.1. Materials

G. candidum strain NRRL Y-552 was kindly acquired from Fundação André Tosello (Campinas, SP, Brazil). Mesoporous PHB particles (average particle diameter of 75–90 μm, surface area of 17.1 m^2/g and porous diameter of 3.1 nm) were acquired from PHB Industrial (São Paulo, SP, Brazil). Olive oil (0.4% acidity) from Carbonell (Córdoba, Spain) was purchased at local market. Refined cottonseed oil was purchased from Campestre Indústria e Comércio de Óleos Vegetais (São Bernardo do Campo, SP, Brazil). Gum Arabic and anhydrous ethanol (purity > 99.5% m/m) were purchased from Synth® (São Paulo, Brazil), linoleic acid (purity ≥ 95% m/m) from Sigma–Aldrich Co. and molecular sieve UOP type 3 Å (form rod, and size 1/16 in) from Fluka Analytical (St. Louis, MO, USA). All other chemical reagents and solvents were supplied by Synth® and Vetec Química Ltd. (São Paulo, SP, Brazil).

2.2. Lipase production by submerged fermentation

The culture medium was kept at 4 °C on slants containing malt extract agar (MEA). MEA consisted of 2% (m/v) malt extract, 1.5% (m/v) agar and 0.5% (m/v) peptone. *G. candidum* was reactivated on Sabouraud Dextrose Agar (SDA) plates at 30 °C for 48 h. The inoculum medium was composed of 2% (m/v) peptone, 0.1% (m/v) yeast extract, 0.05% (m/v) each $MgSO_4$ and $NaNO_3$ and 1% (v/v) cottonseed oil. A colony with diameter of 5 mm each was removed from the SDA plate and inoculated in shaker flask of 1000 mL contained 100 mL of inoculum medium at 30 °C and 250 rpm for 24 h. A synthetic culture selective medium composed of 2% (m/v) peptone, 1.5% (v/v) cottonseed oil, 0.1% (m/v) yeast extract, 0.05% (m/v) each $MgSO_4$ and $NaNO_3$ was used. Enzyme production was carried out with 10% (v/v) inoculum of *G. candidum* in 1000 mL shaker flasks with 90 mL of culture medium at 30 °C and 250 rpm for 48 h. The pH was adjusted to 6.0 before sterilization with NaOH solution at 0.8 M. Samples were collected at different times to determine the catalytic activity of the enzyme on the hydrolysis of olive oil emulsion. After fermentation the suspension was filtered (Whatman filter paper 41) under vacuum and the resulting supernatant was used as crude enzymatic extract. Protein amount was determined according to methodology described by Bradford [31] by using bovine serum albumin (BSA) as standard.

2.3. Determination of hydrolytic activity

Hydrolytic activities (HA) of crude enzymatic extract and immobilized lipase were assayed by the hydrolysis of olive oil emulsion [32]. The substrate was prepared by mixing 50 mL of olive oil with 50 mL of Gum Arabic solution at 7% (m/v). The reaction mixture containing 5 mL of the emulsion, 4 mL of 100 mM sodium phosphate at pH 7.0 and 1 mL of crude enzymatic extract. However, the determination of hydrolytic activity for immobilized lipase was performed by using 5 mL of the emulsion, 5 mL of 100 mM sodium phosphate at pH 7.0 and 0.2 g of the biocatalysts prepared. The reaction systems were incubated at 37 °C by 5 min under continuous agitation in an orbital shaker (200 rpm). The reactions were stopped by adding 10 mL of commercial ethanol solution (minimum 95.0%, m/m) [29]. The liberated free fatty acids (FFA) were then titrated with 20 mM sodium hydroxide solution in the presence of phenolphthalein as indicator. The reaction blanks were made by adding 1 mL of culture medium for free enzyme and 0.2 g of PHB for immobilized lipase. One international unit (IU) of activity was defined as the amount of enzyme required to liberate 1 μmol of FFA per minute under the experimental conditions above described.

2.4. SDS-PAGE analysis

SDS-PAGE analysis was performed in the Mini-Protean II Dual-Slab Cell (BioRad, USA), according to the methodology previously described by Laemmli [33]. The analysis was performed by using 12% polyacrylamide for the stacking and resolving gels, respectively. Broad range molecular mass standards (Protein Ladder 10–220 kDa) from Life Technologies® were used. The gel was stained with Coomassie Brilliant Blue R-250.

2.5. Immobilization of G. candidum lipase on PHB particles

Initially, the support (1 g) was wetted incubated with 10 mL ethanol (95%, m/m) under static conditions for 30 min. Then, the ethanol treated PHB particles were thoroughly washed with distilled water to remove ethanol solution and fill the support pores with water, followed by washing with 5 mM buffer sodium phosphate at pH 7.0, and filtered (Whatman filter paper 41) under vacuum to eliminate the inter-particle water. The immobilization procedure on wet PHB particles was performed at different enzymatic crude extract: support volumes ratio. Thus, this ratio was varied from 5 mL to 105 mL of extract/g of support to determine the maximum immobilized loading lipase capacity. The suspensions were kept under continuous agitation in an orbital shaker