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Optimization of the production and characterization of lipase from *Candida rugosa* and *Geotrichum candidum* in soybean molasses by submerged fermentation

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ABSTRACT

This present work describes the production and biochemical characterization of lipase by *Candida rugosa* and *Geotrichum candidum* in a culture supplemented with soybean molasses. After optimizing the fermentation times for both microorganisms, the effects of changing the soybean molasses concentration, the fermentative medium pH and the fermentation temperature were evaluated using the Central Composite Planning. When soybean molasses was used at a concentration of 200 g/L at 27 ± 1 °C and pH 3.5, the lipolytic activity measured in the broth was 12.3 U/mL after 12 h for *C. rugosa* and 11.48 U/mL after 24 h for *G. candidum*. The molecular masses were 38.3 kDa to *G. candidum* lipase and 59.7 kDa to *C. rugosa* lipase, determined by SDS-PAGE. The lipase from both microorganisms exhibited maximal hydrolytic activity at a temperature of 40 °C and were inhibited at pH 10.0. Using different concentration of *p*-nitrophenylbutyrate (*p*-NPB), the kinetic parameters were calculated, as follows: the Km of lipase from *G. candidum* was 465.44 μM and the Vmax 0.384 μmol/min; the Km and Vmax of lipase from *C. rugosa* were 129.21 μM and 0.034 μmol/min, respectively. Lipases activity were increased by metallic ions Mg^{2+} and Na^{+} and inhibited by metallic ion Cu^{3+}.

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1. Introduction

Triacylglycerol ester hydrolases (EC 3.1.1.3) are lipases (hydrolases) that catalyze the hydrolysis of long-chain triglycerides [1,2]. These enzymes act at the aqueous-organic interface to catalyze the hydrolysis of ester link-ages present in carboxylases, releasing organic acid glycerides and glycerol. Accordingly, lipases can catalyze esterification, transesterification or interesterification reactions [3,4]. These enzymes have great potential for bioenergetics applications, such as in the enzymatic transesterification of fats and oils in the presence of solvents in order to produce biodiesel [5].

Although lipases can be obtained from animal, plant and microbial sources [6,7], microbial lipases have been studied most intensively because of their versatile properties, such as stability at high temperatures, activity at a broad range of pH values, selectivity in racemic mixtures and ease of mass production [8,9]. However, the high cost of producing these enzymes limits their industrial use [10].

To obtain good results for the efficient production of lipases, the center of the submerged fermentation reaction must contain carbon sources, such as glucose, xylose, maltose, lactose, sucrose, and glycerol, among others, and the nitrogen sources and vitamins required for the metabolism of the microorganism [11,12].

Studies of lipase production in submerged fermentation systems involving different microorganisms, supplements and substrates

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could contribute to the search for ideal conditions for high yields of these enzymes, and thereby reduce the costs of the lipase production process on an industrial scale [13,14].

Soybean molasses is a byproduct that is generated from liquid evaporation during the drying of concentrated soy protein. Soybean molasses contains a high concentration of sugar, nitrogen, fatty acids, amino acids and minerals [15,16].

Treichel et al. [17] and Salihu et al. [18] report that the use of agro-industrial residues as substrates for lipase production significantly reduces the production costs associated with the substrate.

There are several varieties of lipase producing yeast; among then, *Candida rugosa* is a strong lipase producer [19,20] and *Geotrichum candidum* lipase acts more specifically on unreacted fatty acid double bonds [21]. Due to the significant value of these lipases, the production of these enzymes using soybean molasses as a substrate is a profitable alternative use for this agricultural byproduct. Thus, in this research work, we studied the lipase production from soybean molasses by *Candida rugosa* and *Geotrichum candidum* in a submerged fermentation process.

2. Materials and methods

2.1. Microorganisms

All of the fermentation experiments were conducted using cultures of *C. rugosa* and *G. candidum*, provided by Dr. Eliana Setsuko Kamimura of the College of Animal Science and Food Engineering of USP – Brazil.

The microorganisms were cultivated in Sabouraud agar, which is composed of 10 g/L bacteriological peptone, 40 g/L dextrose and 20 g/L bacteriological agar, incubated at 34 ± 1 °C for 48 h and subsequently maintained at 5 °C in a refrigerator.

2.2. Soybean molasses composition

The soybean molasses was obtained from the Selecta soybean processing company in Araguari/MG, Brazil. It was characterized by Romão et al. [22] who obtained the following results (average of three different samples): carbohydrates (glucose, fructose, sucrose, raffinose and stachyose) representing 60.75% of the dry mass of the molasses that is also composed of 6.44% proteins, 15.6% lipids and 7.88% ash.

2.3. Submerged fermentation

The study of lipase production from soybean molasses was performed in two steps. First, preliminary tests were performed to compare the results with those obtained by Ref. [20]. The medium contained soybean molasses at a concentration of 200 g/L and an initial pH of 6.15.

After the preliminary tests, Central Composite Planning (CCP) was performed with three replicates of the central point to evaluate the effects of varying the soybean molasses concentration, initial pH and fermentation temperature.

Soybean molasses was used at concentrations of 100 g/L, 150 g/L, 200 g/L, 250 g/L, 300 g/L and 350 g/L, and the pH was adjusted with 0.1 M sodium hydroxide and 0.1 M phosphoric acid.

The microorganisms were inoculated using an alkaline platinum rebound in 250 mL bench scale reactor (flasks) containing 50 mL of each tested medium and placed under stirring in a rotating incubator at 130 rpm and 25 °C. *C. rugosa* were grown for 12 h and *G. candidum* were grown for 24 h.

2.4. Central composite planning

Central Composite Planning (CCP) was used to study the effects of the concentration of soybean molasses, initial pH of the medium and fermentation temperature on the lipases production by evaluating the response of enzymatic activity. Levels $-\alpha$ and $+\alpha$ represent the extremes of the variables (chosen by preliminary tests results). These values were selected as follows: soybean molasses concentrations from 100 g/L ($-\alpha$) to 300 g/L ($+\alpha$); pH levels from 2.5 ($-\alpha$) to 3.5 ($+\alpha$), and temperatures from 25 °C ($-\alpha$) to 35 °C ($+\alpha$), where α is the distance in units between an axial point (also called star point) and the central point calculated in the software STATISTICA 7. The selection of tracks from the above variables was based on results obtained in preliminary tests.

2.5. Lipase centrifugation and membrane dialyzing

After each fermentation, the broth was centrifuged (12,000*g* for 20 min), and the supernatant was retained for dialyzing for 24 h with three changes of dialyzer medium (distilled water) at 4 °C in membrane Dialysis Tubing, size 5 inf. diameter 24/32″ – 19.0 mm: 30 M and 12–14,000 Da.

2.6. Purification of lipases

To purify the lipases of *C. rugosa* and *G. candidum* from another contaminant protein (e.g. proteases and esterases) the lipase preparations were adsorbed on octyl-sepharose following the protocols as previously described [23]. In order to desorb the enzymes, the adsorbed lipases were washed with Triton X-100 in 5 mM sodium phosphate buffer at pH 7 and 4 °C (different Triton X-100 concentrations were necessary for the two lipases studied: 0.5% for *C. rugosa* lipase and 0.05% for *G. candidum* lipase).

2.7. Determination of molecular weight using sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE)

SDS-PAGE was conducted to determine the purity (after the purification steps) and molecular weight of the enzymes using 5% (w/v) stacking and 12% (w/v) separating gels. The samples were prepared by mixing the purified enzymes (v/v) with MiliQ water containing 10 mM Tris-HCl (pH 8.0), 2.5% sodium dodecyl sulphate (SDS), 10% glycerol, 5% β-mercaptoethanol and 0.002% bromophenol blue. The samples were heated at 100 °C for 5 min before electrophoresis. After electrophoresis, the gels were stained with 0.25% Coomassie Brilliant Blue R-250 in 45% ethanol/10% acetic acid. The molecular weights of the purified lipases were estimated using GE Healthcare low-molecular-weight calibration kit as the marker.

2.8. Protein identification by mass spectrometry (MS)

The observed bands were analysed by MS at the Center of Molecular Biology Severo Ochoa, CBM-CSIC in Universidad Autónoma de Madrid, plataforma en red de proteómica Carlos III (Proteored ISCIII). After drying, gel bands were destained in acetonitrile:water (1:1) and digested on site with sequencing-grade trypsin (Promega, Madison, WI), according to Shevchenko et al. [24]. The desalted protein digest was dried, resuspended in 10 μL 0.1% formic acid and analysed by RP-LC–MS/MS in an Easy-nLC II system coupled to an ion trap LTQ-Orbitrap-Velos-Pro mass spectrometer (Thermo Scientific). The peptides were concentrated (on-line) by reverse phase chromatography using a 0.1 mm × 20 mm C18 RP precolumn (Acclaim PepMap® 100, nanoViper, Thermo Scientific) and then separated using a 0.075 mm × 250 mm C18 RP column (Acclaim

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