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Surface response methodology for the optimization of lipase production under submerged fermentation by filamentous fungi

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ABSTRACT

A Plackett–Burman Factorial Design of 16 experiments was conducted to assess the influence of nine factors on the production of lipases by filamentous fungi. The factors investigated were bran type (used as the main carbon source), nitrogen source, nitrogen source concentration, inducer, inducer concentration, fungal strain (*Aspergillus niger* or *Aspergillus flavus* were selected as good lipase producers via submerged fermentation), pH and agitation. The concentration of the yeast extract and soybean oil and the pH had a significant effect ($p < 0.05$) on lipase production and were consecutively studied through a Full Factorial Design 2^3, with the concentration of yeast extract and pH being significant ($p < 0.05$). These variables were optimized using a central composite design, obtaining maximum lipolytic activities with the use of 45 g/L of yeast extract and pH 7.15. The statistical model showed a 94.12% correlation with the experimental data.

Introduction

Enzymes currently have various industrial applications.[1] The industrial market for enzymes continues to grow due to the development of new production technologies, the use of genetic engineering during production and emergence of new fields of application. The global enzyme market in 2007 was 2.3 billion US dollars and is expected to be 2.7 billion US dollars in 2012.[2] Among these enzymes, lipases are widely used. Their applications result from their ability to catalyze reactions, mainly the hydrolysis and inter- and transesterification

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of lipids, making these enzymes useful in the detergent,[3] medical[4] and food industries[5] as well as in the pharmaceutical industry as diagnostic tools, in cosmetics, in tea processing, in medical applications, as biosensors, in degreasing of leather and waste treatment.[6] Other applications include the maturation of cheeses,[7] the synthesis of aromas,[8] the production of lipids with high levels of unsaturated fatty acids[9] and methyl-esters of fatty acids (biodiesel).[10]

Industrial enzymes are produced primarily through submerged fermentation in batch and fed-batch cultures[1] using filamentous fungi. The most-cited genera for lipase production are *Aspergillus*, *Rhizopus*, *Penicillium*, *Mucor*, *Geotrichum* and *Fusarium*.[11,12] Submerged processes have some advantages over solid-state processes, such as higher homogeneity of the culture medium and more facility to control parameters like temperature and pH.[13]

Moreover, Mahadik et al.[14] mentioned that one of the disadvantages of solid state processes is the color of the fermented media, due to the presence of fungal spores, and other components remaining in the extract after the enzyme extraction process. However, submerged fermentation can present difficulty for the transfer of oxygen in liquid media, which is aggravated in the case of fungi due to the filamentous morphology of hyphae in liquid media.[1] Coradi et al.[15] mentioned that lipases have been produced by submerged fermentation because the recovery of extracellular enzymes and the determination of biomass are facilitated by being performed by simple filtration or centrifugation. However, solid-state fermentation can improve the use of agricultural waste and demands less water and energy.

Other factors, such as the types and concentrations of nutrients, pH, agitation and the presence and concentration of inducers can affect the productivity of these bioprocesses. Research that uses isolated microorganisms from new environments and that uses agro-industrial residues in the composition of media is needed to obtain high yields at lower costs.

The statistical optimization of processes has advantages compared to the classical practice of changing one variable at a time,[16,17] such as the lower number of experiments and the possibility of evaluating the interaction effects among variables. Numerous researchers have reported the use of these techniques for the production of lipases by microorganisms.[18–20] An efficient and widely used approach is the application of Plackett–Burman designs that allow efficient screening of key variables for further optimization in a rational way.[21,22] The aim of this work was to screen significant variables for lipase production through submerged fermentation and to optimize these variables through response surface methodology.

Materials and methods

Microorganisms and inoculum preparation

The fungi used in this study were two species of *Aspergillus* that were isolated from dairy effluent (isolate E-19) and soil contaminated with diesel oil (isolated O-8)[23] and that were previously selected as good producers of lipase via submerged fermentation.[24] Both isolates were submitted to genetic identification through Phred/Phrap and Consed, using the methodology cited by Smanioto et al.,[25] at the Center of Nuclear Energy in Agriculture (Cena), University of São Paulo (USP), Brazil.

Sequences were compared to 18S rRNA data obtained from GenBank (http://www.ncbi.nlm.nih.gov). The isolate E-19 was identified as *Aspergillus niger* strain DAOM (100% identity, GenBank accession number: KC545858.1), and the isolate O-8 was identified as *Aspergillus flavus* strain DAOM (99% identity, GenBank accession number: JN938987.1).

The microorganisms were kept in test tubes with PDA (potato-dextrose-agar) at 4 °C. The inoculum was prepared by inoculation of the fungi in Petri dishes containing 30 mL of solidified PDA medium and incubated at 30 °C for 5 days.

Culture medium and experimental apparatus

The culture medium was prepared with 10% (m/v) bran (wheat or soybean), which was boiled at 100 °C for 30 min. Afterwards, the medium was filtered, and the soluble extract was added to a10% (v/v) saline solution containing also the nitrogen source, inducer and distilled water to complete the final volume. The saline solution contained KH_2PO_4 (2 g/L), $MgSO_4$ (1 g/L) and trace solution (10 mL/L). The composition of the trace solution was $FeSO_4{\cdot}7H_2O$ (0.63 mg), $MnSO_4$ (0.01 mg), $ZnSO_4$ (0.62 mg)and distilled water up to a volume of 1 L.[26] The liquid medium was autoclaved, and the pH was adjusted to values pre-defined by the experimental design using solutions of 1.5 mol/L HCl or 1 mol/L NaOH.

The experiments were carried out in 300-mL Erlenmeyer flasks with 100 mL of medium. The inoculation was accomplished using 10 or 20 mm diameter circular areas containing spores grown in Petri dishes. The Erlenmeyer flasks, containing the inoculated culture medium, were incubated at 30 °C for 10 days in a shaker with a level of agitation pre-defined according to the experimental design. Aliquots (10 mL) were removed at 24 h, 48 h, 72 h and 96 h to measure lipolytic activity.

Experimental design

The optimization of lipase production by submerged fermentation was carried out using three sequential experimental designs. The first step aimed to assess the influence of nine variables on lipase production using a Plackett–Burman Design with 16 trials (1–16). The variables studied were bran type used as a carbon source (wheat bran or soybean bran), nitrogen source (sodium nitrate or yeast extract), nitrogen source concentration (10 or 30 g/L), inducer (soybean or olive oil), inducer concentration (10 or 30 g/L), culture medium pH (5 or 7), fungus strain used (*A. niger* E-19 or *A. flavus* O-8), inoculum diameter (fungal spores growth equal to 1 or 2 cm diameter in Petri dishes containing PDA) and agitation (120 or 160 rpm). Next, a 2^3 Full Factorial Design (FFD) (Trials 17–24) was carried out to study the influence of yeast extract concentration (YEC), soybean oil concentration (SOC) and pH on lipolytic activity. Subsequently, the pH and the concentration of yeast extract were optimized using a Central Composite Rotational Design (CCRD), including 4 factorial points, 4 axial points and 3 central points for evaluating the pure error for a