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Characterization of multienzyme solutions produced by solid-state fermentation of babassu cake, for use in cold hydrolysis of raw biomass

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ABSTRACT

The production of multienzyme solutions containing diverse hydrolytic enzymes has attracted increasing interest, because of their potential for simultaneously hydrolyzing different biological macromolecules. When these crude enzyme solutions are produced by solid-state fermentation (SSF) in agricultural byproducts, production costs are considerably lower than those of conventional submerged fermentation processes. Crude enzyme solutions containing mainly exo- and endoamylases, proteases, xylanases, and cellulases were produced under standardized procedures by means of SSF of babassu cake by *Aspergillus awamori* IOC-3914. Central composite designs were implemented and revealed optimal activities of the enzymes produced between pH 4.4–5.0 and 45–51 °C. Based on the application of a global desirability analysis on all the group of enzymes detected in the multienzyme solution, an overall optimal activity was observed at 53 °C and pH 4.7. These conditions were used for the separate hydrolysis of babassu cake and babassu flour, in order to evaluate the ability to simultaneously hydrolyze crude starch, cellulose, hemicelluloses, and protein. Under optimal global conditions, high levels of glucose, xylose, and free amino nitrogen (FAN) (up to 44, 19 and 0.75 g L^{-1}, respectively) were obtained. Finally, the use of Tween 80 was found to activate both exo- and endoamylases (up to 50% increase).

1. Introduction

Solid-state fermentation (SSF) based on the utilization of agricultural by-products is an excellent low-cost option for efficient enzyme production processes. This type of fermentation is carried out by culturing filamentous fungi on solid renewable resources such as cereals, oil cakes, fruit seeds, and various agricultural byproducts. The principal raw materials contained in agricultural resources are various macromolecules such as carbohydrates, protein, cellulose, hemicelluloses, and lignin. When filamentous fungi are cultured on agricultural raw materials, they produce different enzymes to hydrolyze the majority of available macromolecules. The sustainable utilization of agricultural byproducts necessitates the exploitation of all crude enzymes produced by SSF, in order to produce fermentation media enriched in directly assimilable sugars such as glucose [1,2], fructose [3,4], and xylose [5], as well as many other micro- and macronutrients such as amino acids, phosphorus, and minerals [6,7]. These small molecules are easily metabolized by microorganisms during subsequent submerged fermentations, to produce biofuels, microbial biopolymers, and a wide spectrum of other important biological metabolites.

An adequate feedstock which byproducts are commonly used for SSF is babassu (*Orbygnia phalerata*). The babassu palm is widely cultivated in northern and northeastern Brazil and in parts of southern Colombia [8]. The fruits of this palm are harvested as a renewable raw material for the production of biofuels such as ethanol and biodiesel [9]. Due to their composition, babassu fruits have potential industrial applications in the food, detergent, and cosmetic sectors [10]. Babassu cake is the solid residue derived from the process of oil extraction. This material has a high carbohydrate content (62%), composed principally of starch, cellulose, and hemicelluloses. Protein and residual lipid fractions in babassu cake comprise approximately 23 and 4.5% of the whole kernel, respectively [11]. As a result of these characteristics, when the filamentous fungus *Aspergillus awamori* is cultivated on babassu cake residues via SSF, a crude enzyme solution containing principally exo- and endoamylases, proteases, xylanases, and cellulases is produced

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[10]. Endoamylases are liquefying enzymes, composed mainly of α-amylases (EC 3.2.1.1), which catalyze the random hydrolysis of the internal α-1,4 linkages of amylose and amylopectin, releasing oligosaccharides of various lengths. Exoamylases are saccharifying enzymes consisting mostly of glucoamylases (EC 3.2.1.3), which catalyze the cleavage primarily of α-1,4 bonds at the chain terminals, releasing glucose as the main product. These enzymes can also catalyze the hydrolysis of α-1,6 linkages, at a slower rate. Xylanases and cellulases hydrolyze hemicelluloses and cellulose to generate various sugars, including both pentoses and hexoses. Proteases catalyze the hydrolysis of proteins, generating amino acids and short-chain peptides [2].

Protein hydrolysates can be used to replace commercial organic supplements during submerged microbial fermentation [2], avoiding the need to add commercial high-value nutrients such as casein or yeast extracts. In addition to their ability to generate various sources of nitrogen, proteolytic enzymes have the capacity to improve the performance of the hydrolytic enzymes mentioned above. Babassu cake, as well as many other agricultural by-products, contains various natural polymers that are intimately interlinked. The attack on one of these polymers by a specific group of enzymes is hindered by the presence of the other polymers. When several hydrolytic enzymes are present in a complex solution, a synergistic effect is produced, in which the various polymers are simultaneously hydrolyzed [12–15]. This synergistic effect is defined as the interaction of all enzymes, which improves the overall hydrolytic capability of the crude multienzyme solution.

The multienzyme synergistic effect would be even more important if it could be used under mild conditions, since in comparison to conventional methods involving starch gelatinization, the conversion of raw starch at low temperatures (named cold hydrolysis) can potentially reduce the energy requirements of the process [16,17]. In addition, the saccharification and fermentation stages could occur simultaneously, which would improve the overall yield and the productivity of the process [18].

With the idea of developing sustainable bioprocessing strategies in which the macromolecules (such as starch and proteins) present in agricultural byproducts are transformed into usable sugars and nutrients through a process of cold hydrolysis, the main objective of the present study was to determine an optimal combination for biomass hydrolysis, in terms of pH and temperature, where the multienzyme solution attains maximum synergy. Determination of this global optimum point would increase the simultaneous hydrolysis of carbohydrates and proteins, at the same time reducing wastewater production associated with conventional protein and carbohydrate extraction. A detailed characterization of the multienzyme solutions (in terms of operational hydrolytic parameters, i.e. pH and temperature) produced via SSF was carried out so as to apply these solutions to the hydrolysis of crude renewable sources under mild conditions. The development of these technologies can aid in developing bioprocesses that consume less energy than conventional processes.

2. Materials and methods

2.1. Raw materials

Babassu (*Orbygnia* sp.) cake (kernel residue) and babassu flour (mesocarp fraction) were kindly supplied by TOBASA Bioindustrial de Babaçu S.A. (Tocantinópolis, Brazil). The cake had a mean particle size of 923 μm (±7 μm S.D.), and the flour had a mean particle size of 155 μm (±3 μm S.D.), as estimated using a vibratory shaker (Viatest 76773, Kuhardt, Germany) coupled with sieves (Tyler 8-400 mesh). For SSF studies, the cake was dried, ground, and sieved to obtain particles smaller than Tyler mesh size 14 (<1.20 mm). Babassu cake and flour contain approximately (dry weight): 10 and 58% starch, 23 and 2.7% protein, and 23 and 11.6% fiber, respectively [9,11].

2.2. Microorganism and inoculum propagation

Solid-state fermentations were carried out with *A. awamori* IOC-3914, obtained from the culture collection of the Instituto Oswaldo Cruz (IOC; Rio de Janeiro, Brazil). The culture was maintained at 4 °C in starch agar medium containing (g L^{-1}): starch, 10; sodium nitrate, 3; monopotassium phosphate, 1; potassium chloride, 0.5; magnesium sulfate, 0.5; iron sulfate (III), 0.001; and agar, 20. A two-stage inoculum propagation strategy was implemented according to the methodology described by Castro [19]. First, fungal spores (approximately 1×10^7 spores mL^{-1}) of *A. awamori* from the maintenance medium were transferred to a solid starch medium and incubated for 7 d at 30 °C. Second, a fungal spore suspension of 2.5×10^5 spores mL^{-1} was incubated in liquid malt extract medium (Sigma–Aldrich, Brazil) for 28 h at 30 °C in an orbital shaker at 200 rpm.

2.3. Solid-state fermentation

Fungal spore suspensions produced from liquid malt extract medium were used to inoculate laboratory-scale tray bioreactors (5.83 mL, corresponding to a moisture content of 70%, wet weight) containing 2.5 g of babassu cake. The spore concentration used for inoculation was approximately 1×10^7 spores for each gram of cake. The trays were incubated in humidified chambers (95% air saturation) for 96 h at 30 °C. Enzyme extraction in aqueous solutions was carried out for 30 min, at a proportion of distilled water:solid of 10:1 (mL g^{-1}), 37 °C, and 200 rpm. Enzyme-rich aqueous extracts were separated from SSF solids via centrifugation for 20 min at 25 °C and 10,000 × *g*. The supernatants (multienzyme solutions) were used for the subsequent hydrolytic reactions.

2.4. Hydrolysis experiments

The hydrolytic capacity of the enzyme extracts was evaluated separately, by employing two different fractions of babassu: kernel residues (cake) and mesocarp (flour). Hydrolysis experiments were carried out for 24 h in a water bath with constant magnetic agitation, using 10% (w/w) raw material at three different temperatures (45, 53, and 60 °C) and two pH levels (4.7 and 5). Samples were taken at different times, and separated by centrifugation (11,000 × *g*). Glucose, xylose, and free amino nitrogen concentrations of the liquid samples were analyzed.

2.5. Assays

The groups of enzymes were not isolated individually from the crude enzyme solution. The activity of each group of enzymes was studied by varying the pH and temperature according to the experimental design described in the following section. The pH of the enzymatic extracts was adjusted by using two different buffer solutions, as proposed by McIlvaine [20].

It should be stressed that the assay methods were principally focused on describing both amylolytic enzymes (exo- and endo-acting). However, for the case of xylanases, cellulases and proteases, both exo and endo-acting enzymes were simultaneously analyzed. Detailed information on the method of calculating the enzyme activities is provided in a previous report [18]. For exoamylases activity calculation, 10 μL of extract were mixed for 10 min with 90 μL of 1% (w/v) soluble starch solution. Then, enzymes were inactivated by incubation for 5 min in a boiling water bath. The