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Production and characteristics comparison of crude β-glucosidases produced by microorganisms *Thermoascus aurantiacus* e *Aureobasidium pullulans* in agricultural wastes

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ABSTRACT

The purified β-glucosidase of *Aureobasidium pullulans* ER-16 is one of more thermostable enzyme reported to date. Considering the unfeasibility of using purified enzyme for industrial application, it was interesting to analyze this property for the crude enzyme. Thermophilic fungus *Thermoascus aurantiacus* CBMAI-756 and mesophilic *A. pullulans* ER-16 were cultivated in different hemicellulosic materials on solid-state cultivation for β-glucosidase production. Wheat bran was most appropriate for β-glucosidase production by both microorganisms. *T. aurantiacus* exhibited maximum enzyme production (7.0 U/ml or 70 U/g) at 48–72 h and *A. pullulans* a maximum (1.3 U/ml or 13 U/g) at 120 h. Maximum activities were at 75 °C with optimum pH at 4.5 and 4.0, for *T. aurantiacus* and *A. pullulans*, respectively. *A. pullulans*'s β-glucosidase was more pH stable (4.5–10.0 against 4.5–8.0) and more thermostable (90% after 1 h at 75 °C against 85% after 1 h at 70 °C) than the enzyme from the thermophilic *T. aurantiacus*. The $t_{(1/2)}$ at 80 °C were 50 and 12.5 min for *A. pullulans* and *T. aurantiascus*, respectively. These data confirm the high thermostability of crude β-glucosidase from *A. pullulans*. Both β-glucosidases were strongly inhibited by glucose, but ethanol significantly increased the activity of the enzyme from *T. aurantiacus*.

1. Introduction

The enzymatic hydrolysis of cellulosic material into glucose involves the synergistic action of at least three different enzymes: endoglucanase or endo-β-1,4-glucanase (EC 3.2.1.4), exoglucanase or exo-cellobiohydrolase (EC 3.2.1.91), and β-1,4-glucosidase or cellobiase (EC 3.2.1.21). The first two are inhibited by cellobiose. β-Glucosidase is very important in the process because it hydrolyzes cellobiose and other cellodextrins into glucose, reducing the inhibitor effect of the cellobiose on the cellulases [1,2]. The β-glucosidase can also be used by the food industry to increase the bioavailability of the isoflavones in the human intestine, and by the beverage industry to improve the aromatic composition of juices and wines [3].

The difficulty in using the β-glucosidases in industrial process is its strong enzyme inhibition to glucose. The search for β-glucosidases insensitive to product inhibition, and of high-thermal stability, has increased recently. Enzymes with these characteristics would improve the process of saccharification of lignocellulosic materials [4]. An alternative to bypass the problem of inhibition, is to associate the cellulose's enzymatic hydrolysis to a simultaneous alcoholic fermentation, where the glucose will be microbiologically converted to ethanol [5,6]. Nevertheless, the enzymes in this process need to be relatively stable due to ethanol present in the reaction medium.

Most of the processes of industrial application of enzymes occur at high temperature, so the use of thermostable enzymes appears to be appropriated because they preserve their catalytic activity at high temperatures. A series of advantages such as faster reaction, decreased viscosity in processed fluid, increased solubility of the substrate, and reduced contamination risk by undesired organisms have been proposed for use of thermostable enzymes in biotechnology processes [7,8].

Previous works reported an expressive stability to pH and temperature of the purified β-glucosidases of the microorganisms *T. aurantiacus* e *A. pullulans* [2,5,9]. Leite et al. [10] compared the thermal characteristics of purified β-glucosidases of such microorganisms, they concluded that the enzyme produced by the

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mesophilic microorganism *A. pullulans* is more thermostable than that produced by the thermophilic *T. aurantiacus*. Nevertheless, the industrial utilization of purified enzymes is not economically justified. In this case, the use of a crude enzyme (enzyme preparation) is most adequate, bearing in mind the obviously lower commercial value for industrial use. The effective dosage of the crude enzyme costs about 1% of the cost of the pure enzymes. However, it is well established that the environment can affect the enzymes. Various components in the medium (such as different enzyme proteins, different inhibitor substances or salts) can affect the respective activities or thermostabilities. This fact, got us to study the behavior of these enzymes in its crude state. Furthermore, the production profile of the β-glucosidases by the microorganisms in different agricultural residues and their behavior in different concentrations of glucose and ethanol were also done.

2. Materials and methods

2.1. Microorganisms

The yeast *Aureobasidium pullulans* ER-16 was isolated from orange juice industrial residues in Catanduva, São Paulo State, Brazil. The stock culture was preserved in potato dextrose agar (Oxoid) at 4 °C. The genus was identified by classical taxonomy based on physiological test and morphological observation, according to De Hoog [11] and by DNA-based methods. **PCR amplification and sequencing analyses**: Genomic DNA was extracted from cultures freshly grown in YMA medium according with Almeida [12]. DNA samples were diluted 1:750 in distilled water, and 5 μl aliquots were used as template in 25 μl PCR mixtures. The D1/D2 hypervariable regions of the large ribosomal DNA (rDNA) were first amplified using primers NL1 and NL4. The amplification protocol consisted in an initial step of 96 °C for 3 min, followed by 35 cycles of 96 °C for 30 s, 61 °C for 45 s and 72 °C for 1 min. The same primers were used in cycle sequencing reactions. Prior to sequencing, DNA fragments were purified using the GFX Band Purification Kit (Amersham Biosciences, Piscataway, NJ, USA). Both DNA strands were generated in an ABI Prism 377 DNA automated sequencer (Applied Biosystems Inc., Foster City, CA, USA), manually resolved and aligned through the software BioEdit Sequence Alignment Editor v. 7.0.5.3 [13]. The thermophilic fungus *Thermoascus aurantiacus* CBMAI-756 was isolated from decayed wood, Manaus, Amazonas State, Brazil. The fungus was grown on Sabouraud dextrose agar (Oxoid) for 2 days at 50 °C and the stock culture was maintained at 4 °C. The strain was identified and deposited in the Brazilian Collection of Microorganism of Industry and Environment (Coleção Brasileira de Microrganismos de Indústria e Meio Ambiente), CBMAI, at UNICAMP, Campinas, SP.

2.2. Inoculation

The fungus *T. aurantiacus* was cultivated in 125 ml Erlenmeyer flasks containing 20 ml of Sabouraud dextrose agar slope (Oxoid) for 48 h at a temperature of 50 °C. The yeast *A. pullulans* was cultivated in 125 ml Erlenmeyer flasks containing 20 ml of potato dextrose agar slope (Oxoid) for 48 h at a temperature of 28 °C. Microorganism suspension was obtained by softly scraping the culture medium surface using 25 ml of mineral solution [0.1% $(NH_4)_2SO_4$, 0.1% $MgSO_4{\cdot}7H_2O$, and 0.1% NH_4NO_3, w/v]. The respective culture means, solutions and materials used in this work stage were sterilized at 120 °C/30 min. The microorganisms inoculation in the substrates was carried out by transferring 5 ml of the suspension into Erlenmeyer flasks containing the previously prepared production medium.

2.3. Enzyme production

The microorganisms were cultivated for 96 h in five different types of substrates: wheat bran, soy bran, soy peel, corncob and corn straw and appraised for β-glucosidase production. All the substrates were previously washed with distilled water and dry at 50 °C for 48 h. In order to evaluate enzymatic production through cultivation process, the substrate that stimulated the largest enzyme production by microorganisms, was used in a new cultivation and samples were removed every 24 h throughout the period of 144 h.

2.4. Cultivation

The solid-state cultivation (SSC) was carried out in 500 ml Erlenmeyer flasks containing 5 g of substrate (ground to 2–3 mm size) mixed with a mineral solution (previously described) aiming at an initial humidity content of 60% and 75% for the *T. aurantiacus* and for the *A. pullulans*, respectively. Microorganism *T. aurantiacus* was cultivated at temperature of 50 °C, while the yeast *A. pullulans* was at 28 °C. For enzyme extraction, 50 ml of distilled water was added to each flask and stirred for 2 h on a rotary shaker at 80 rpm. The crude extract of solid cultivation was centrifuged (10,000 × g/20 min), and then the supernatant was used for enzyme activities assays.

2.5. Enzymatic activity

β-Glucosidase was assayed using 50 μL of appropriately diluted culture filtrate, 250 μL of sodium acetate buffer (100 mM, pH 5.0), 250 μL *p*-nitrophenyl-β-D-glucopyranoside (PNPG) (4 mM, Sigma). After incubation at 60 °C for 10 min, the reaction was interrupted by adding 2 mL of sodium carbonate 2 M, and the color was measured at 410 nm. One unit of β-glucosidase corresponds to the amount of enzyme that releases 1 μM nitrophenol per min in the reaction mixture [2].

2.6. Physico-chemical characteristics

For optimum pH, β-glucosidase activity was measured over a pH range of 3.0–8.0 in McIlvaine buffer 100 mM, using PNPG, 2 mM as substrate. Then, tests were carried out at the pH producing maximum activity to find the optimal temperature. For optimum temperature, β-glucosidase activities were determined using PNPG, 2 mM as substrate over a temperature range of 25–90 °C as controlled by a circulation bath water. For pH stability, enzymes were stored for 24 h at room temperature diluted 10-fold with McIlvaine buffer 100 mM (pH 3.0–8.0), Tris–HCl 100 mM (8.0–9.0) and glycine–NaOH 100 mM (9.0–11). The remaining β-glucosidase activity was measured under standard conditions at optimum pH and temperature. For the thermostability, enzymes were tested by incubating the enzymes, without substrate, for 1 h at acetate buffer 100 mM pH 5.0, over a temperature range of 35–90 °C as controlled by a circulation bath water. After incubation, aliquots were withdrawn and cooled on ice before assaying for residual activities at optimum pH and temperature.

2.7. Determination of thermoinactivation constant (K_d) and half-life ($t_{1/2}$)

The kinetics were measured as described by Tomazic and Klibanov [14], with minor modifications. The concentrations of protein in enzyme solutions were adjusted to the same level (0.5 mg/ml) by dilutions with 100 mM acetate buffer pH 5.0, and then the time course of thermoinactivation of β-glucosidases was measured by incubating 1 U/mg of protein of the respective enzymes, at indicated temperatures, taken in tightly rubber-capped small glass tubes. Periodically, by using a syringe with needle, samples were withdrawn, cooled to 25 °C and stored at this temperature for 24 h before assaying for remaining activity. This time of 24 h ensure that the thermal inactivation is irreversible; if there is refolding mechanism in the protein the initial activity is recouped for all assayed temperatures. For the *T. aurantiacus* enzyme, temperatures of preincubation and removal were: at 80 °C, every 6 min for 60 min; at 82.5 °C, every 1.5 min for 9 min and at 85 °C, every 1 min for 5 min. For the *A. pullulans* enzyme, temperatures of preincubation and removal were: at 80 °C, every 10 min for 90 min; at 82.5 °C, every 6 min for 36 min and at 85 °C, every 3 min for 12 min. The half-life ($t_{1/2}$) of enzymes was defined as the time at which, after 80 °C incubating temperature, enzyme activity was 50% of the original activity. The $t_{(1/2)}$ was also determined according to Eq. (1). Thermoinactivation rate coefficients (k_d) were determined by linear regression from the plot of natural logarithm of residual activity (Ln ra) versus the time of preincubation at the indicated temperatures at which enzymes were thermally inactivated by the first order rate constant. k_d was the absolute value of the slope in the regression line.

$$t_{(1/2)} = \frac{0.693}{k_d} \quad (1)$$

2.8. Effect of glucose and ethanol on β-glucosidase activities

Enzymatic activity was quantified with the addition of glucose and ethanol, in different concentrations, in the reaction mixture. The assays were carried out in 100 mM sodium acetate buffer pH 4.5, at 60 °C and PNPG (2 mM) was used as enzyme substrate.

3. Results and discussion

3.1. Microorganism identification

On basis of morphology and nutritional physiology the thermophilic fungus was identified as *T. aurantiacus* CBMAI-756. On the basis of morphology, nutritional physiology, ribosomal DNA D1/D2 sequencing, the mesophilic microorganism was identified as *A. pullulans* ER-16. This strain included typical black colonies and color variants. Because of the polymorphism of this fungus, molecular techniques [13] were also carried out aiming to confirm the results.

3.2. Enzyme production

After 96 h of cultivation, the enzymes were extracted for addition of 50 ml of distilled water in each Erlenmeyer flasks. These