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Passion fruit peel as novel substrate for enhanced β-glucosidases production by *Penicillium verruculosum*: Potential of the crude extract for biomass hydrolysis

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ABSTRACT

β-Glucosidases show great potential as catalysts for various biotechnology processes including biomass hydrolysis for bioethanol production. In this study, response surface methodology was used to evaluate the effect of some variables on β-glucosidase production by *Penicillium verruculosum* using passion fruit peel as substrate, and on hydrolysis of this process residue with *P. verruculosum* crude extract, by applying a full factorial central composite design. Process optimization resulted in a 5.7 fold increase in β-glucosidase activity. The enzymes were more active at 65 °C, pH 4.5, remaining stable at 55 and 60 °C and over a broad pH range. *P. verruculosum* crude extract hydrolyzed passion fruit peel with glucose yield of 45.54%. This article provides, for the first time, the production of remarkable yields of β-glucosidase and the achievement of expressive levels of glucose through the use of passion fruit peel, an abundant and inexpensive agro-industrial residue.

1. Introduction

Cellulose is the most abundant renewable carbon source on the planet. It is composed of D-glucose units linked by β-1,4 glycosidic bonds [1]. While this polysaccharide is a highly promising polymer for renewable energy generation, its usefulness is limited by the high cost and low efficiency of cellulases [2,3].

Cellulases are the third most abundant industrial enzyme. These enzymes are attracted attention due to its role in cotton processing, paper recycling, detergent formulation and juice extraction [4]. In recent years, they have been further exploited for agricultural biotechnology and bioenergy generation [5]. Cellulases are comprised of three separate enzymes: exoglucanase, endoglucanase, and β-glucosidase, which act synergistically to hydrolyze cellulose [6]. Among the cellulose degrading enzymes, β-glucosidases are the most essential for efficient hydrolysis of cellulosic biomass. Specifically, they help to relieve inhibition of the cellobiohydrolases and endoglucanases by reducing cellobiose accumulation [7]. While

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many microorganisms are capable of degrading and utilizing cellulose as a carbon and energy source, their β-glucosidases are generally produced in relatively low levels [8].

Enzymatic conversion of lignocellulosic biomass will be a key to unlock the future bioenergy. To date, the cost of the cellulase cocktails considerably influences the viability of lignocellulosic materials conversion into fuels and other chemicals [9]. To overcome this issue, recent studies have focused on increasing the efficiency of biomass-degrading enzyme production by identifying novel microbial strains [10,11], development of recombination techniques to obtain cellulases hyperproducing microorganisms [12–14] and more efficient fermentation techniques [6,14–16].

In an effort to decrease the cost of enzyme production, studies have more recently focused on the use of agro-industrial waste in the fermentation process [4]. These agro-industrial by-products are relatively low-cost and their use can help to reduce the environmental impact associated with their disposal [17]. Passion fruit waste is a by-product of the processing industry and consists of seeds and peels. This lignocellulosic material is rich in carbohydrates, including pectin. It is estimated that the residue from the production of passion fruit juice reaches 52% of the amount of processed fruits [18]. Brazil is the world leader in the production of yellow passion fruit, with volumes reaching 776,097 tonnes in 2012 [19]. As a consequence, a large quantity of passion fruit waste is generated. Like many other agro-industrial by-products, passion fruit waste has low commercial value and its deposition on a large scale may result in a negative environmental impact. Currently, its main use in Brazil is as a supplement to animal feed, which presents several transport and storage problems due its high moisture content. In recent years, some workers have proposed alternative uses for this waste, such as pectin production [20] and in the biosorption of dyes [21]. However, industrial passion fruit by-products still remain relatively unused [18].

Among the 80 strains of fungi isolated from Brazilian soil at the Ecological Station of Juréia-Itatins, SP, in the Atlantic Forest region, *Penicillium verruculosum* attracted significant attention for producing β-glucosidase activity in the presence of passion fruit peel. In the present study, we explored the optimum fungal growth conditions for the production of β-glucosidase by *P. verruculosum*, using passion fruit peel as substrate. In addition, we further characterized the enzymes that were produced, and examined the potential application for *P. verruculosum* β-glucosidases in the saccharification of pre-treated passion fruit peel.

2. Material and methods

2.1. Substrate preparation

Passion fruit (*Passiflora edulis*) peel used in this study was kindly provided by a local industry juice. The mature fruits (full yellow color) were harvested directly from the plants cultivated in a private farm in Corumbataí do Sul, PR, Brazil (Latitude: 24.0603 and Longitude 52.0712), in 2013. After passion fruit pulp extraction, the peels were air-dried and stored at room temperature until utilization. After that, the substrate was prepared by exhaustive washing with distilled water. The passion fruit peel was then dried at 80 °C for 24–48 h and milled (35 mesh).

2.2. Microorganism and culture conditions

P. verruculosum was isolated from the Atlantic forest at the Ecologic Station Juréia-Itatins, located in São Paulo State, Brazil. It was previously identified by conventional macroscopic and microscopic morphological examination. The strain belongs to the Culture Collection of the Environmental Studies Center – CES/UNESP, Brazil (accession number PVJ54) and has been deposited in the Culture Collection of the Federal University of Pernambuco, Brazil, under accession number 6713.

The fungus was grown on Vogel's solid medium [22] containing (g dm^{-3}) glucose 15.0 and agar 15.0, at 28 °C, for 7 days for conidia production. Submerged fermentation was carried out in 125 cm^3 flasks containing 25 cm^3 of Vogel's liquid medium, pH 6.5 and inoculated with 1.0 cm^3 spore suspension (1.0×10^7 spores cm^{-3}). The medium composition and culture conditions were varied according to experimental design. Following incubation, they were vacuum filtered and the filtrate was assayed for extracellular β-glucosidase activity.

2.3. Enzymatic activity determination

β-Glucosidase activity was determined by measuring the amount of *p*-nitrophenol released from *p*-nitrophenyl-β-D-glucopyranoside (pNPG) at 405 nm. A 0.2 cm^3 solution of 5 mol m^{-3} pNPG was preincubated for 5 min in McIlvane buffer pH 5.0, at 50 °C and 0.3 mL of appropriately diluted enzyme sample was then added. After 5 min incubation, the enzymatic activity was stopped by adding 2 cm^3 of a 2 kmol m^{-3} Na_2CO_3 solution. The extinction coefficient for *p*-nitrophenol under these conditions was 18,100 kmol m^{-3} cm^{-1}. One unit of enzyme activity was defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol per cm^3, per min of reaction.

Cellobiohydrolase and endoglucanase activities were mensuared using avicel and carboxymethyl cellulose (CMC) as substrates, respectively, according to the standard conditions described by Ghose [23] Reducing sugar released during enzymatic reactions were quantified based on their reducing power towards 3,5-dinitrosalicylic acid (DNS) [24], using glucose as standard for calibration curves. The enzyme activities described above were defined as the amount of enzyme required to liberate 1 μmol of reducing sugar per cm^3, per min of reaction, under the assay conditions. All enzyme assays were performed in triplicate and results are presented as mean values.

2.4. Optimization of β-glucosidase production

Response surface methodology (RSM) was used to optimize the submerged fermentation process for enhanced β-glucosidase production by *P. verruculosum*. A 2^3 full factorial central composite rotary design (CCRD) with three factors and five levels including six axial points and three central points was used for fitting a second order response surface.