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Use of pectinases produced by a new strain of *Aspergillus niger* for the enzymatic treatment of apple and blueberry juice

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ABSTRACT

In this work, pectinases-producing filamentous fungi were isolated with the aim of using their enzymes in the clarification of apple and blueberry juices. The experimental extract enzyme EE obtained in solid-state process with a strain identified as belonging to the species *Aspergillus niger* and designated LB23 was used for treating juices and compared with two commercial enzyme preparations: Pectinex®Clear (PC) for apple juice, and Pectinex®BE Color (PB) for blueberry juice. After the enzymatic treatment, the treated juices were evaluated with respect to parameters such as viscosity, turbidity, and degree of clarification, as well as antioxidant capacity, and total phenolic compound content. Considering all comparison criteria, the experimental preparation EE showed results statistically similar or superior to those obtained with the commercial enzyme preparations.

1. Introduction

Pectinolytic enzymes, also known as pectinases, can be produced in both solid-state (Fontana, Salvador, & Silveira, 2005) and submerged processes (Fontana, Polidoro, & Silveira, 2009) by selected fungi belonging to the genus *Aspergillus*. *Aspergillus niger* is the most used microorganism in large-scale production of pectinases for the fruit juice industry, since this fungus produces large amounts of the different enzymes belonging to this group (Grassin & Coutel, 2010).

Commercial pectinolytic enzymes are an important part of fruit juice technology almost since the beginning of the juice processing industry. Technical enzyme products have been used in the process of making fruit juices since the 1930s (Vasic-Racki, 2006). They are used to assist in the extraction and clarification of juices from many fruits (Abdullah, Sulaiman, Aroua, & Noor, 2007; Landbo, Karl, & Meyer, 2007; Pinelo, Zeuner, & Meyer, 2010; Sandri, Fontana, Barfknecht, & Silveira, 2011). Fruit juices are naturally cloudy especially due to presence of polysaccharides (pectin, cellulose, hemicelluloses and starch), proteins, tannins, and metal ions (Vaillant, Millan, Dornier, Decloux, & Reynes, 2001). The high concentration of pectin forms a colloidal dispersion, which is one of the main problems in clear fruit juices processing. Although the suspended pulp particles can be removed by filtration, the presence of pectin can make this difficult (Sulaiman, Sulaiman, & Liew, 1998).

Apple juice is one of the most popular juices in the world (Grimi, Mamouni, Lebovka, Vorobiev, & Vaxelaire, 2011). Several studies have shown that apples are an excellent source of phenolic compounds that could contribute to health due to their antioxidant properties (Kahle, Kraus, & Richling, 2005; Khanizadeh, Tsao, Rekika, Yang, & Deell, 2007; Proteggente et al., 2002). In Brazil, apple juice is the most produced among those whose industrial processing includes enzymatic clarification. This country is responsible by approximately 1.5% of the global production, and from this total 95% is produced in the southern region with about 30% of this volume processed to juice (Vieira et al., 2009).

Recently, new fruit cultures have aroused interest in Southern Brazil, e.g. blueberry, due to their high nutritional potential. Many reports have suggested that blueberries have various biological activities, including the prevention of urinary tract infections (Jepson & Craig, 2007), as well as antioxidative (Dulebohn et al., 2008) and anticancer activities (Seeram, 2008).

Due to the importance of these enzymes, studies aimed at isolating and selecting new strains of microorganisms for the potential use in industrial processes are of great relevance. In this context, the purpose of this study was to select pectinases-producing filamentous fungi, in especial those belonging to the genus *Aspergillus*, envisaging their application in operations characteristic of the fruit juice industry. In addition, we tested the efficiency of enzymes produced by the microbial isolates in the

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clarification of apple and blueberry juices and analyzed the polyphenol content and antioxidant activity of the treated juices.

2. Materials and methods

2.1. Microorganisms

In this study, 60 fungal isolates obtained from decomposing plant tissues were evaluated. The strains *A. niger* T0005007-2 (University of Salta, Argentina) and *Aspergillus oryzae* IPT 301 (Institute of Technological Research of São Paulo, Brazil) were used as controls of the pectinases production for solid-state (Fontana et al., 2005) and submerged (Fontana et al., 2009) processes, respectively. The control strains and isolates were maintained by periodic cultivation in glycerin-agar medium, at 30 °C for 5 days, and then stocked at 4 °C.

2.2. Fruits

Samples of apple (*Malus domestica*), Gala variety, and blueberry (*Vaccinium myrtillus*), Bluegem and Climax varieties, were harvested in farms in Caxias do Sul (RS), Brazil. The fruits were first selected and then carefully washed with tap water, before processing.

2.3. Media for identifying pectinolytic strains

The medium described by Hankin and Anagnostakis (1975) for isolation and identification of pectinolytic microorganisms has the following composition (g/L): citric pectin (CP Kelco SA, Brazil) 1.0; yeast extract, 0.05; agar, 1.5; triton X 100, 0.1; $(NH_4)_2SO_4$, 1×10^{-3}; KH_2PO_4, 2×10^{-3}; $FeSO_4 \cdot 7H_2O$, 1×10^{-4}; and Na_2HPO_4, 3×10^{-3}. The initial pH of the medium was adjusted to 4.0 with either NaOH 1 mol/L or H_2SO_4 1.5 mol/L. The media were autoclaved at 101.325 kPa for 20 min; then, about 20 mL of medium was distributed in Petri dishes previously sterilized at 170 °C for 3 h.

2.4. Media for enzyme production

The medium used in the solid-state process was defined by Fontana et al. (2005), according to the following composition (per 100 g): wheat bran (Moinho Nordeste, Brazil), 36.6 g; citric pectin (CP Kelco S.A., Brazil), 6.0 g; salt solution, 36.6 mL. The formulation of salt solution (g/L) was: $(NH_4)_2SO_4$, 4.0; $MgSO_4$, 1.0; KH_2PO_4, 2.0; $FeSO_4 \cdot H_2O$, 6.3×10^{-4}; $ZnSO_4$, 6.3×10^{-4}; and $MnSO_4$, 1.0×10^{-5}. The initial moisture of the media was adjusted to 63 g/100 g by adding distilled water, and the media were autoclaved at 121 °C for 20 min.

The medium used in the submerged process was defined by Malvessi and Silveira (2004), and had the following composition (g/L): wheat bran (Nordeste, Brazil), 40; citric pectin (CP Kelco S.A., Brazil), 20; yeast extract, 0.05; $(NH_4)_2SO_4$, 5; $MgSO_4$, 0.5; KH_2PO_4, 2.5; $FeSO_4 \cdot 7H_2O$, 6.3×10^{-5}; $ZnSO_4$, 6.2×10^{-5}; and $MnSO_4$, 1×10^{-6}. The initial pH of the medium was adjusted to 4.0 with either NaOH 1 mol/L or H_2SO_4 1.5 mol/L. The media were autoclaved at 121 °C for 20 min.

2.5. Selection and identification of pectinolytic strains

Screening tests of the pectinolytic isolates were performed according to Hankin and Anagnostakis (1975). Conidial suspensions of different isolates were spread on Petri dishes containing medium with pectin to obtain counts of about 10^6 spores/mL. After 48 h of incubation, the formation of halos, due to degradation of the pectin of the medium by total pectinases produced by the fungi, was revealed by adding a solution 1 g/100 mL of cetyltrimethylammonium bromide (CTAB) to the Petri dishes. The microbial isolates were compared based on the ratio between the diameters of the pectin degradation halos and of the colony itself.

From the results of the screening tests, the best producing isolate was identified by conventional taxonomy at the Andre Tosello Foundation (Campinas, Brazil), a reference laboratory for microbial identification in Brazil. The identification tests applied based on the analysis of differential characteristics of morphology, physiology, and biochemical metabolism.

2.6. Preparation of the enzyme extract in the solid state process

Beakers (100 mL) with 12 g of medium were used to obtain the enzyme extract in the solid-state process. The flasks were inoculated with fungi spores in order to have an initial concentration of 1×10^7 spores and they were incubated at 30 °C in a humidity-saturated environment for 72 h. The extraction of the enzymes obtained in the solid-state process was carried out by suspending 2.7 g of solid medium in 15 mL of distilled water (pH 4), using 125 mL flasks under reciprocal agitation of 200 rpm, at 30 °C. The solutions obtained were then centrifuged for 10 min at 5000× *g*. The supernatant was stored at 4 °C for use in subsequent tests (Fontana et al., 2005).

Erlenmeyer flasks (500 mL) with 100 mL of substrate were used to obtain the enzyme extracts by submerged process. The flasks were inoculated with fungi spores in order to have an initial concentration of 1×10^7 spores/L of medium and they were incubated at 28 °C and 200 rpm, in a reciprocal shaker (B. Braun Biotech model BIOSTAT® B, Germany). After 96 h, the media were centrifuged for 10 min at 5000× g, and the supernatant being stored at 4 °C for further tests (Malvessi & Silveira, 2004).

The best pH and temperature for total pectinases activity (TPA) of the enzyme extract produced in solid-state cultivation by the chosen fungal isolate (EE) were determined for the experimental extract. The reaction pH was assayed using different buffer solutions – sodium biphthalate (pH 3.0; 4.0; 5.0) and phosphate (pH 6.0; 7.0) – at 30 °C for 30 min. The reaction temperature was compared in a range of 20–80 °C at pH 4.0.

2.7. Apple and blueberry juices preparation

Apple juice was produced by centrifugation (Walita, Brazil). Blueberry juice was produced by liquefying equal amounts of Bluegem and Climax varieties using an industrial blender (Metvisa, Brazil) and then filtering the juice through a 10-mesh sieve.

Clarification of the apple and blueberry juices with the experimental enzyme extract EE was evaluated and compared with the results obtained with two commercial preparations (Novozymes Latin America Ltd, Brazil): Pectinex®Clear (PC) for the apple juice, and Pectinex®BE Color (PB) for the blueberry juice. According to Novozymes Latin America Ltd, Pectinex®Clear and Pectinex®BE Color has been specifically developed to aid in processing of apple and red berries, respectively. These commercial enzyme preparations are obtained from cultures of *A. niger* and *Aspergillus aculeatus* and contain high activities of pectin lyase and polygalacturonase. The experimental enzyme extracts, as well as the commercial preparations, were diluted to 10 units (U) of total pectinases (TPA) per mL as previously standardized by Sandri et al. (2011). This enzyme/juice ratio is within the range recommended by the manufacturer for the use of PC and PB. The clarification assays were performed in a thermostatic bath (B. Braun Biotech model Certomat WR, Germany), at 40 or 50 °C, for a reaction time of 60 min. The samples were then cooled in an ice bath to stop the reaction and centrifuged at 1000× *g* and 20 °C for 10 min. The supernatant was filtered through Whatman n° 1 paper to remove any suspended