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Potential use of different agroindustrial by-products as supports for fungal ellagitannase production under solid-state fermentation

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ABSTRACT

Ellagitannase is a novel enzyme responsible for biodegradation of ellagitannins and ellagic acid production. Ellagic acid is a bioactive compound with great potential in food, pharmaceutical and cosmetic industries. This work describes the ellagitannase enzyme production from partial purified ellagitannins as inducers by *Aspergillus niger* GH1 grown on solid-state fermentation. Solid-state fermentation was carried out on four different lignocellulosic materials (sugarcane bagasse, corn cobs, coconut husks and candelilla stalks) as matrix support and production of ellagitannase enzyme was evaluated. All lignocellulosic materials were characterized in terms of water absorption index and critical humidity point. The best lignocellulosic materials for ellagitannase production were sugarcane bagasse and corn cobs (1400 U L^{-1} and 1200 U L^{-1}, respectively). The lowest values were obtained with candelilla stalks (500 UL-1). The highest specific productivity was obtained with corn cobs (2.5 U mg^{-1} h^{-1}) which enable increase ellagitannase productivity up to 140 times. Corn cobs have great potential as support matrix for production of fungal ellagitannase in SSF.

Keywords: Ellagitannins; Ellagic acid; Biodegradation; Agro industrial by-products; *Aspergillus niger* GH1; Corn cobs

1. Introduction

Elagitannins are an important group of phytochemical compounds with great value in food, pharmaceutical and cosmetic industries. The ellagitannins (ETs) are water-soluble hydrolysable polyphenolic compounds (Wilson and Hagerman, 1990). These are considered secondary plant metabolites found in cytoplasm and cell vacuoles (Khadem and Marles, 2010). ETs presence has been principally described in leaves, stalks, husks of some fruits, flowers, etc. (Ascacio-Valdés et al., 2011). When ET's are exposed to acidic or basic strong conditions, the ester bounds are hydrolyzed and the hexahydroxydyphenic acid group (HHDP) is released, which spontaneously rearranged to form a stable and insoluble dilactone, commonly named ellagic acid (Aguilera-Carbó et al., 2008b; Gross, 2009)

Recent studies on sources and biological properties of ellagic acid showed its relevant bioactivity, such as antioxidant, anti-inflammatory, antiviral, antimicrobial, antimutagenic, antitumoral and anticarcinogenic, among others (Ascacio-Valdés et al., 2011). Ellagic acid is present in considerable amounts in cranberry (Vattem and Shetty, 2003), raspberry (Koponen et al., 2007) and pomegranate fruits (Aguilera-Carbó et al., 2008a; Robledo et al., 2008; Seeram et al., 2005). Several authors have reported the recovery of high levels of ETs with a high purity degree from pomegranate husks (Ascacio-Valdés et al., 2010; Robledo et al., 2008; Seeram et al., 2005).

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The studies on ETs hydrolysis have permitted established some control parameters of ellagic acid production using strong acids or bases, however this method generate high costs and high volumes of chemical wastes. Information about ETs biodegradation is scarce and confuse. However, in recent years some enzymatic studies have offered some clues to elucidate the degradation pathway of ETs. Action of some enzymes, such as β-glucosidase (Vattem and Shetty, 2002, 2003), tannase, polyphenol oxidase (Shi et al., 2005), cellulase (Huang et al., 2007), valonea tannin hydrolase (Huang et al., 2005) and ellagitannase (Aguilera-Carbó et al., 2009) has been reported.

Microbial hydrolysis of ETs using enzymes has been poorly evaluated in SSF, and the first reports hypothesized a synergistic action of different enzymes (Aguilera-Carbó et al., 2008b). Recently, Ascacio-Valdés et al. (2013) reported that *Aspergillus niger* GH1 was able to grown in solid state fermentation and produce the ellagitannase enzyme. The authors propose that the ester bonds among HHDP group and glycosides are degraded by ellagitannase which has high specificity, and this enzymatic activity allows the EA accumulation.

Solid-state fermentation (SSF) consists of microbial growth and product formation on surface and inside of a porous solid matrix, in absence or near absence of free water (Barrios-González, 2012). Substrate must contain enough moisture to allow microbial growth and metabolism, simulating natural growth conditions (Orzúa et al., 2009). Water availability in SSF is a critic limiting point which has an important influence on microbial growth and metabolism. This availability of water corresponds to water activity (a_W), a physico-chemical parameter defined as the relative humidity of the gaseous atmosphere in equilibrium with the substrate. Chemical composition and particle structure of the supports used in SSF have a determinant influence in the value of a_W which can range from 0.80 to 0.99 to permit an efficient fungal metabolism (Martins et al., 2011). A great variety of lignocellulosic materials (LM) have been tested as solid supports for SSF, including coffee by-products (Machado et al., 2012), rice bran and wheat bran (Khandeparkar and Bhosle, 2006); sugarcane bagasse and agave (Hernández-Salas et al., 2009; Pandey et al., 2000); mango peels (Buenrostro-Figueroa et al., 2010), grape skins (Botella et al., 2007; Rodríguez et al., 2010), cranberry pomace (Vattem and Shetty, 2003), pomegranate peels (Robledo et al., 2008); corn cobs (Mussatto et al., 2009b) and coconut husks (Orzúa et al., 2009) among others. Several of these by-products have been used as supports and/or substrates for production of metabolites of industrial importance, such as organic acids, antibiotics, pigments, flavor and aroma compounds, bioactive molecules and a great variety of enzymes (Martins et al., 2011). The aim of this study was produce ellagitannase enzyme by growing *Aspergillus niger* GH1 on SSF using different agroindustrial by products as supports and partially purified ellagitannins extracted from pomegranate peels as carbon source.

2. Materials and methods

2.1. Materials preparation and physico-chemical characterization

The agro industrial by-products used in this study were collected from different Mexican agricultural regions and included: sugarcane (*Saccharis officinalis*) bagasse (SB), corn (*Zea mays*) cobs (CC), coconut (*Cocos nucifera*) husk (CH) and candelilla (*Euphorbia antisyphilitica*) stalks (CS). All of them were grinded up to particle size of 0.85 mm of diameter. Before use, the matrix supports were pre-treated by boiling during 10 min and washed three times with distilled water. Support was dried at 60 °C until constant weight is reached (Mussatto et al., 2009b). Physical and chemical tests consisted of water absorption index (WAI) (Orzúa et al., 2009), critical humidity point (CHP) (Mussatto et al., 2009a) and packing density (PD) determination (Santomaso et al., 2003). For WAI determination, the sample (1.5 g) was placed in 50 mL centrifuge tube and 15 mL of distilled water was added. The sample was stirred for 1 min at room temperature (25 °C) and centrifuged at 3000 g for 10 min. The supernatant was discarded, and the WAI was calculated from the weight of the remaining gel and expressed as g gel/g dry weight. The CHP was estimated by adding 1 g of sample in a thermo-balance at 120 °C for 60 min. PD was calculated by placing 10 g of sample in standard graduated cylinders and clamped to a shaker and vertically agitated until no change in volume during 5 min was observed.

To be used as matrix support, the materials were pretreated by boiling during 10 min, washed three times with distilled water, and subsequently dried at 60 °C for 24–48 h (Mussatto et al., 2009a). Prior to use, all LM were autoclaved at 121 °C for 15 min.

2.2. Fungal strain and cell culture

Aspergillus niger GH1 strain (Food Research Department Collection, Universidad Autonoma de Coahuila, Mexico) was used. The strain has been previously isolated, characterized and identified (Cruz-Hernández et al., 2005), highlighting their ability to degrade ellagitannins (Robledo et al., 2008; Sepúlveda et al., 2012). The strain was maintained at −40 °C in glycerol-skimmed milk. Spores of *A. niger* GH1 were activated in potato dextrose agar (PDA-Bioxon) medium at 30 °C for five days. The culture spores were harvested with sterile solution of 0.01% Tween-80 and counted in a Neubauer® chamber.

2.3. SSF conditions

Ellagitannase production experiments were performed in 60 mL sterile columns (100% polypropylene) considered as bioreactor, which were aseptically packed an homogeneous mixture containing the following fermentable mass: 3 g of each support (SB, CC, CH and CS) was mixed with 7 mL of Pontecorvo culture medium (Aguilera-Carbó et al., 2009) with the following composition ($g\,L^{-1}$): $NaNO_3$ (6.0), KH_2PO_4 (1.52), KCl (0.52), $MgSO_4{\cdot}7H_2O$ (0.52), $ZnSO_4$ (0.001), $FeCl_3$ (0.85) and trace metals ($1\,mL\,L^{-1}$). The trace metals solution contained ($mg\,L^{-1}$) $Na_2B_4O_7{\cdot}10H_2O$ (10.0), $MnCl_2{\cdot}4H_2O$ (50.0), $Na_2MoO_4{\cdot}2H_2O$ (50.0) and $CuSO_4{\cdot}5H_2O$ (250.0). Pomegranate husk ellagitannins (PHE) supplied by the Bioprocess Laboratory of the Food Research Department (School of Chemistry, Universidad Autonoma de Coahuila, Mexico) were used as carbon source and ellagitannase inducer. The medium pH was adjusted to 9 and then autoclaved ($1.1\,kg/m^3$, 121 °C) for 15 min. PHE ($30\,g\,L^{-1}$) were added to the culture broth when the temperature was between 35–40 °C. Final pH was 6.5. The fermentable mass was aseptically inoculated with 2×10^7 spores/g of support. The SSF was carried out at 30 °C for 32 h (Ascacio-Valdés et al., 2013). Forced air was not supplemented for aeration of column bioreactor. Enzymatic extract (EE) was obtained by adding 7 mL of 50 mM citrates buffer pH 5 to each reactor. Fermented material was compressed and