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Rich bioactive phenolic extract production by microbial biotransformation of Brazilian *Citrus* residues

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A B S T R A C T

Flavanones in *Citrus* are molecules that play an important role in antioxidant activities in nutraceutical products. Recent studies indicate that molecules of the simplest classes of phenolics have higher biological activity and absorption capacity. However, the molecules that have been shown to be very important bioactive compounds of *Citrus*, such as hesperetin, naringenin and ellagic acid, are found in trace concentrations in the fruit. An interesting environmentally friendly alternative that deserves attention regarding phenolic compound obtaining is the biotransformation of these molecules. The aim of this study was to develop a process of biotransformation of phenolics from Brazilian *Citrus* residues by solid-state fermentation with the microorganism *Paecilomyces variotii*. The optimized fermentation conditions were 10 g of *Citrus* residues (2.0 mm of substrate particle size), 20 mL distilled water, at 32 °C after 48 h of incubation. The development of this process has generated, simultaneously, an increase of 900, 1400 and 1330% of hesperetin, naringenin and ellagic acid concentration, respectively, and an increase of 73% of the antioxidant capacity. These results give strong evidence that microbial biotransformation does not only produce phenolic compounds but also compounds with high biological activity, such as hesperetin and naringenin.

Keywords: Agro-industrial residues; Solid-state fermentation; Tannase; *Paecilomyces variotii*; Flavanones; Antioxidant

1. Introduction

In recent years, *Citrus* flavonoids have gained much interest due to their chemoprotective effects. *Citrus* flavonoids exhibit antioxidant, antimicrobial, anticarcinogenic, antiviral, anti-allergic and anti-inflammatory activities. Through these benefits, there is interest in replacing synthetic food antioxidant substances with natural ones, which has fostered research on vegetable sources and the screening of waste materials aimed at identifying new and/or better antioxidant sources (Ferreres et al., 2012; Lin et al., 2012; Sergent et al., 2012; Tripoli et al., 2007).

There are many classes of flavonoids, flavanones being the most abundant group in *Citrus* fruits (Barros et al., 2012; Ferreira et al., 2013). Flavanones are highly present in plant species from the genus *Citrus*, abundant in the by-products, mostly in peels and pectinolytic material, accounting for 4–12% of the dry weight (Marín et al., 2007). The most prevalent flavanones in tissues and peels of *Citrus* fruits are naringin and hesperidin. Naringin exhibits many health benefits, including

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an ability to prevent cancer by suppression of carcinogenesis and inducing cell apoptosis (Meiyanto et al., 2012). Hesperidin has also been reported to reduce the proliferation of many cancer cells and also possesses an anti-inflammatory effect (Ferreira et al., 2013; Nazari et al., 2011; Park et al., 2008). The glycoside and aglycone forms of flavanones possess several different biological functions. Several studies have revealed that aglycones are superior to glycosides in various bioactivities, due to their effective absorption (Murakami et al., 2008). Only free flavonoids without a sugar molecule were thought to be able to pass through the intestine wall. Hydrolysis only occurs in the colon by microorganisms, which at the same time degrade flavonoids (Hollman and Katan, 1997). According to Nielsen et al. (2006) and Ohguchi et al. (2006), when free phenolics (hesperetin and naringenin) in *Citrus* residues are released from their glycosides (hesperidin and naringin), the photoprotective functionality of these phytochemicals can be improved.

Other important compounds found in *Citrus* fruits are hydroxybenzoic acids, however in lower concentrations than flavanones. Ellagic acid is a very important compound from this category and has a variety of benefits for anti-mutagenic, antimicrobial and antioxidant properties, as well as being an inhibitor of human immunodeficiency viruses (Nutan et al., 2013; Martins et al., 2011; Sepúlveda et al., 2011). The presence of ellagic acid in various functional commercial products is observed. Improving ellagic acid content in *Citrus* residues could provide an interesting source of this compound for the industry. Microbial degradation of tannins is highly documented, and most works report that the selective hydrolysis of galloyl groups from ellagitannins was catalyzed by tannase (Prasad et al., 2012).

Currently, phenolic compounds are obtained by chemical synthesis or extraction. An interesting environmentally friendly alternative that deserves attention regarding phenolic compound production is the solid-state fermentation (Madeira et al., 2013; Banerjee et al., 2012; Martins et al., 2011). The current bioprocess (SSF) has many advantages, such as high concentration, product stability and growth of microorganisms in non-water soluble substrates, the process is usually cheaper with higher productivity than submerged fermentation (Barrios-González, 2012). The SSF on *Citrus* residue using the *Paecilomyces variotii* strain was initially developed by this research group for the production of tannase enzyme. The potential of this enzyme to produce more bioactive forms of the polyphenol molecule extract from vegetables was studied in previous works (Madeira et al., 2012). Georgetti et al. (2009) evaluated the biotransformation of polyphenol glycosides from soybeans to form non-glycosides through solid-state fermentation by *Aspergillus awamori*. The conversion of the glycoside to the form of phenolic non-glycoside was accompanied by production of the enzyme β-glucosidase. The non-glycoside form presents a greater number of free hydroxyl groups in regard to glycoside, thus increasing their biological activity. The microbial biotransformation of phenolic compounds seems to be a promising way to increase the concentration of phenolics with high biological potential.

The present work aimed to optimize some important parameters of this fermentation process in order to produce some phenolic compounds with high bioactivity, such as naringenin, hesperetin and ellagic acid, from *Citrus* residue. These have no viable source of extraction so far, being present in very low concentration in vegetables; however, literature shows their functional potential is increasing more and more every day.

2. Materials and methods

2.1. Materials

Hesperidin, hesperetin, naringin, naringenin, ellagic acid, 2,2′-azobis(2-methylpropionamidine) (97%) (AAPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Potato Dextrose Agar (PDA) were purchased from Sigma–Aldrich Co. Fluorescein was purchased from ECIBRA, and Trolox® (97%) was purchased from ACROS Organics. *Citrus* residue (from 5 different cultivars: *Citrus latifolia*, *Citrus sinensis* Hamlin, Valencia, Pera riu and Pera Natal) was kindly donated by CP Kelco industry headquarters (Limeira, SP, Brazil) from juice and pectin extraction, giving origin to a residue of low quality and commercial value.

2.2. Microorganism and inoculum preparation

The *P. variotii* strain was isolated and selected to be the tannase producer and grew on different agro-industrial residues such as castor bean cake, wheat bran and *Citrus* residue (Battestin and Macedo, 2004; Madeira et al., 2011, 2012). The fungus strain was deposited at the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI) under the number 1157. The *P. variotii* was preserved in PDA medium slants and refrigerated at 4 °C with Vaseline. For sporulation, the fungal strain was inoculated on plates containing PDA medium and incubated at 30 °C for 3 days. The spores were suspended in distilled water at a concentration of 9×10^6 spores/mL (Madeira et al., 2011).

2.3. Culture condition for phenolic compound production

Citrus residue was donated by CP Kelco industry headquarters (Limeira, SP, Brazil) as a dried residue. The residue was ground in a knife mill (Philips, RI 1725) and separated in a sieve shaker (Mesh 10, particle size under 1.86 mm).

The initial fermentation medium established in a previous work (Madeira et al., 2012) for tannase production consisted of the following: 250 mL Erlenmeyer flasks, in which 10 g of the *Citrus* residue was added to 10 mL of distilled water. After sterilization in an autoclave, the flasks were inoculated with 1 mL of spore suspension (9×10^6) and incubated at 30 °C at 90% relative humidity (Climate Chamber 420 CLD – Nova Etica, SP, Brazil) for up to 120 h.

After the incubation period, the tannase extraction was performed by adding 50 mL of acetate buffer (pH 5.5, 0.02 M) to 5 g of fermented substrate. The solution was shaken at 200 rpm for 1 h, and filtered and centrifuged at 10,070 × *g* for 30 min at 4 °C (Centrifuge Beckman J2-21, Beckman-Coulter, Inc., Fullerton, CA, USA). The supernatant was assayed for tannase activity. The phenolic compound extraction was performed by adding 25 mL of methanol 70%, to 1 g of fermented residue. The solution was treated in ultrasonic (Unique UltraSonic Cleaner model USC-1800A) at 40 kHz for 30 min, after being shaken at 200 rpm for 30 min and then passed through a 0.45 μm filter. The filtered extract was assayed for identification and quantification of phenolic compounds by HPLC-DAD (High Pressure Liquid Chromatography-Diode Array Detector).