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# Efficient tannase production using Brazilian *citrus* residues and potential application for orange juice valorization

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ABSTRACT

Tannase is an interesting enzyme due to its capacity to hydrolyze galotannins and polyphenol compounds. Tannase from *Paecilomyces variotii* has been produced from different residues, such as wheat, castor bean cake and pomegranate, and has shown great potential in Brazilian *Citrus* residue. The process of tannase production using solid-state fermentation was optimized and activity increased tenfold. The tannase produced showed optimum activity at pH 5.0, with 70 °C and 80% stability between pH 4.0–6.5 and 20–60 °C. The enzyme was then applied to orange juice by flavanone hydrolysis to increase antioxidant and functional activity. It was concluded that aglycon flavanones present higher functional activity than glycosylated flavanones. The results demonstrate the benefits of producing tannase (from a low-cost process) for the food industry. The enzyme reacts under a range of temperatures and pH levels, and increases the biological effects of orange juice.



## 1. Introduction

The production of enzymes such as amylases, cellulases, pectinases and tannases by solid-state fermentation (SSF) has of late received increased attention since the product can be widely applied in the pharmaceutical and chemical industries due to its varied biological activities (antioxidant, anti-apoptotic, antibacterial, antiviral, analgesic etc.) (Dhillon et al., 2011; Madeira Jr. et al., 2013).

Tannin acyl hydrolase, commonly referred to as tannase (EC 3.1.1.20), is an enzyme that cleaves ester linkages in hydrolysable tannins to produce glucose and gallic acid (Battestin and Macedo, 2004). Tannase is an extracellular, inducible enzyme produced in the presence of tannic acid by fungi, bacteria and yeasts (Belmares et al., 2004). There are several potential industrial applications for tannase. However, due to its high production cost and our limited knowledge of its catalytic actions, it is currently only used in limited circumstances. Using tannase in feed preparations increases the bioavailability of nutrients by hydrolyzing phenolic (tannins) antinutritional factors (Belmares et al., 2004; Madeira Jr. et al., 2012).

The first step in the development of microbial enzyme production is lineage selection. Extracellular enzymes are ideal because they are easily extracted and do not require expensive extraction methods (Díaz et al., 2007). Solid-state fermentation provides several advantages over conventional enzyme production processes, and agro-industrial byproducts can be put to use and supplemented by tannic acid (Battestin and Macedo, 2004; Madeira Jr. et al., 2012). Adapting the necessities of SSF to optimize the process of fermentation parameters includes adding physico-chemical parameters. Once the basic fermentation parameters like fermentation period, moisture content, inoculum size, substrate concentration, pH, and temperature are optimized, it is necessary to increase product yields. Since the agrobased residues are generally ill-defined substrates, supplementation of various carbon and nitrogen sources, other organic substances such as fatty acids, alcohols, acids, and vitamins have proved to be beneficial in achieving higher product yields (Belmares et al., 2004; Díaz et al., 2007).

In this study, *Paecilomyces variotii* was used to produce tannase. The enzyme was biochemically characterized and applied to orange juice. Brazilian *Citrus* residues were used as a substrate for solid-state fermentation. *Citrus* residue is the residue product from the orange juice industry in Brazil, and is used as an ingredient in animal feed. In addition to enzymatic production, we analyzed the biochemical characterization and enzymatic biotransformation in *Citrus* residues and the changes it causes to the general composition of phenolic compounds.

## 2. Materials and methods

### 2.1. Materials

Hesperidin, hesperetin, naringin and naringenin were purchased from Sigma-Aldrich Corporation. *Citrus* residue was kindly

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donated by CP Kelco industry headquarters (Limeira, SP, Brazil) from subsequent juice extraction, the origin of a low quality residue with a low commercial value.

### 2.2. Microorganism and inoculum preparation

The *P. variotii* strain was isolated and selected as a source of tannase production. 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 Potato Dextrose Agar (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 $9 \times 10^6$ spores/ml (Madeira Jr. et al., 2012).

### 2.3. Fermentation process

*Citrus* residues were donated by CP Kelco industry headquarters (Limeira, SP, Brazil) in its dried state. 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 was established in previous studies on tannase production, and consisted of: 250 ml Erlenmeyer flasks, in which 10 g of the *Citrus* residue was added to 10 ml of distilled water (Madeira Jr. et al., 2012). After sterilization in autoclave, the flasks were inoculated with 1 ml of spore suspension ($9 \times 10^6$) and incubated at 30 °C at 90% relative humidity (Climate Chamber 420 CLD —Nova Etica, SP, Brazil) for up to 96 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 then filtered and centrifuged at 10,070 × *g* for 30 min at 4 °C (Centrifuge Beckman J2–21, Beckman-Colter, Inc., Fullerton, CA, USA). The supernatant was assayed for tannase activity.

### 2.4. Tannase assays

Tannase activity was evaluated according to Sharma et al. (2000) and adapted using tannic acid as a substrate. One unit of activity was defined as the amount of enzyme that released 1 μmol/min of gallic acid. Enzyme activity was expressed as total unit (U) per g of dry substrate (gds) of dry solid medium (based on the initial mass).

### 2.5. Optimization of fermentation parameters for tannase production

The fermentation parameters that had the greatest influence on tannase production were evaluated using two CCD (Central Composite Design) methodologies. The first design was run with the following physical parameters: particle size substrate (mm), water:substrate ratio (v:w) and temperature of incubation (°C). The water:substrate ratio was determined according to the maximum moisture absorption capacity of the orange pomace. The second design was carried out with the following nutritional parameters: ammonium sulfate concentration (w/w) and tannic acid concentration (w/w). The variables were coded, according to Eq. (2):

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (1)$$

Here, $x_i$ is the coded variable, $X_i$ is the natural variable of the nutrient factor, $X_0$ is the value of the natural variable at the center point, and $\Delta X_i$ is the step change value. The variables and levels are shown in Tables 1 and 2.

**Table 1**
Coded levels and real values (in parentheses) for the experimental design and results of CCD.

| Trial | Substrate particle size (mm) $X_1$ ($x_1$)[a] | Water:substrate ratio (v/w—%) $X_2$ ($x_2$)[a] | Temperature of incubation (°C) $X_3$ ($x_3$)[a] | Enzymatic activity (U/gds) Tannase |
|---|---|---|---|---|
| 1 | −1 (1.20) | −1 (8.00) | −1 (30) | 0.9234 |
| 2 | +1 (2.80) | −1 (8.00) | −1 (30) | 0.3010 |
| 3 | −1 (1.20) | +1 (17.00) | −1 (30) | 0.0454 |
| 4 | +1 (2.80) | +1 (17.00) | −1 (30) | 0.5371 |
| 5 | −1 (1.20) | −1 (8.00) | +1 (34.5) | 0.9416 |
| 6 | +1 (2.80) | −1 (8.00) | +1 (34.5) | 0.5150 |
| 7 | −1 (1.20) | +1 (17.00) | +1 (34.5) | 0.8203 |
| 8 | +1 (2.80) | +1 (17.00) | +1 (34.5) | 0.8468 |
| 9 | −1.68 (0.70) | 0 (12.50) | 0 (32) | 2.0826 |
| 10 | +1.68(3.35) | 0 (12.50) | 0 (32) | 0.5984 |
| 11 | 0 (2.00) | −1.68 (5.00) | 0 (32) | 0.8452 |
| 12 | 0 (2.00) | +1.68 (20.00) | 0 (32) | 0.4503 |
| 13 | 0 (2.00) | 0 (12.50) | −1.68 (28.5) | 0.4842 |
| 14 | 0 (2.00) | 0 (12.50) | +1.68 (36) | 0.1619 |
| 15 | 0 (2.00) | 0 (12.50) | 0 (32) | 1.3863 |
| 16 | 0 (2.00) | 0 (12.50) | 0 (32) | 1.3547 |
| 17 | 0 (2.00) | 0 (12.50) | 0 (32) | 1.3982 |

[a] $x_i$ is the coded value and $X_i$ is the actual value of the it independent variable. The conversion between $x_i$ and $X_i$ is described in Eq. (2).

**Table 2**
Coded levels (in parentheses) and actual values for the experimental design and results of the CCD.

| Trial | Tannic Acid (w/v%) $X_1$ ($x_1$)[a] | Ammonium Sulfate (w/v%) $X_2$ ($x_2$)[a] | Enzymatic activity (U/gds) Tannase |
|---|---|---|---|
| 1 | 2.2 (−1) | 1.5 (−1) | 10.4 |
| 2 | 7.5 (+1) | 1.5 (−1) | 12.4 |
| 3 | 2.2 (−1) | 8.5 (+1) | 9.3 |
| 4 | 7.5 (+1) | 8.5 (+1) | 11.6 |
| 5 | 0 (−1.41) | 5 (0) | 5.3 |
| 6 | 15 (+1.41) | 5 (0) | 11.2 |
| 7 | 12.8 (0) | 0 (−1.41) | 6.9 |
| 8 | 12.8 (0) | 10 (+1.41) | 9.6 |
| 9 | 12.8 (0) | 5 (0) | 16.5 |
| 10 | 12.8 (0) | 5 (0) | 16.2 |
| 11 | 12.8 (0) | 5 (0) | 15.1 |

[a] $x_i$ is the coded value and $X_i$ is the actual value of the *i*th independent variable. The conversion between $x_i$ and $X_i$ is described on Eq. (3).

The first experiment was defined as a full CCD methodology for 3 factors ($2^3$), consisting of 8 cubic points, 6 star points and 3 replicates at the center point, the details of which are presented in Table 1. The second experiment was defined as a full CCD methodology for 2 factors ($2^2$), consisting of 4 cubic points, 4 star points and 3 replicates at the center point, which served to estimate the experimental error and investigate the suitability of the proposed model, the details of which are presented in Table 2. The experimental results were fitted to a second-order polynomial function, and the Student *t*-test made it possible to check the statistical significance of the regression coefficients. Analysis of variance (ANOVA) was performed on the experimental data to evaluate the statistical significance of the model. The response model was expressed in terms of coded variables, ignoring the statistically non-significant terms.

### 2.6. Tannase biochemical characterization

After tannase production was optimized, the fermented material was added to 100 ml of 20 mM acetate buffer, at pH 5.0 and