

Biotransformation of Pitanga Juice by Tannase from *Saccharomyces cerevisiae* CCMB 520

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Abstract

The pitanga (*Eugenia uniflora* L.) is a native species to Brazil and widely used by Brazilian industry, mainly in food, to juice, ice cream, soft drinks, jellies and liqueurs production. The fruit contains a high concentration of anthocyanins, flavonoids and carotenoids, which make it a promising source of antioxidant compounds. The objective of this work was to produce and purify tannase from *Saccharomyces cerevisiae* CCMB 520, to apply in the integral pitanga juice and to verify its physical and chemical effects. The tannase was produced under submerged fermentation in bench bioreactor. After the fermentation process the enzyme was partially purified. The partially purified tannase was applied in the integral pitanga juice using Doehlert statistical design. The effect of the enzymatic application was analyzed by means of phenolic compounds contents and antioxidant activity. Physical–chemical analyzes were carried out to investigate the Standard Identity and Quality of the juice. The best results for partial purification were obtained by ultrafiltration. After application, the total phenolics content was 4855 mg Eq. AG/L, and for the antioxidant activity was 952 μ MTrolox/L (69.41%). It has been found that it is possible by means of enzymatic treatment to improve the functional quality of the integral pitanga juice.

Keywords: antioxidant activity, bioconversion, *Eugenia uniflora* L., experimental design, tannin acyl hydrolase

1. Introduction

Tannin is a term widely used to characterize the second largest class of phenolic compounds, which, like the others, has the primordial and essential function of protecting plant tissues against attack by insects, fungi or bacteria. Tannins have a high molecular weight (500 to 3000 Da), are considered antioxidants and combine with cellulose and pectin, in addition to precipitating alkaloids and proteins [1]. These compounds occur naturally in a wide variety of vegetables, and can be found in the roots, leaves, fruits, seeds and barks. They are considered secondary metabolic products of great economic and ecological interest and have a wide value in the interactions between the plant and its ecosystem. Such compounds are responsible for the astringency of many fruits and vegetable products, due to the precipitation of salivary glycoproteins, which causes the loss of lubricating power [2, 3].

Classically, according to the chemical structure, tannins are classified into two groups: hydrolyzable and condensed. The current and most accepted classification divides the tannins into four groups (**Figure 1**): gallotannins, ellagitannins, condensed tannins and complex tannins [5]. Gallotannins are the simplest tannins and are formed by units of gallo or di-gallo esterified to a nucleus of glucose or other polyhydroxy alcohol. The molecules are usually composed of a glucose nucleus and 6 to 9 gallo groups. The most common is tannic acid [6]. Ellagitannins are esters of hexahydro-xidifenic acid (HHDP), and during its hydrolysis, the HHDP group dehydrates and spontaneously lactonizes to form ellagic acid. Condensed tannins are oligomeric and polymeric proanthocyanidins containing flavan-3-ol (catechin) or flavan-3,4-diol (leucoanthocyanins). The basic structure of complex tannins, on the other hand, consists of a unit of galotannin or ellagitannin and catechin [7, 8].

Hydrolyzable tannins can be easily hydrolyzed, either chemically or enzymatically. Tannin Acyl Hydrolase (TAH), also known as tannase (EC 3.1.1.20), is an enzyme capable of hydrolyzing tannins, leading to the release of glucose and gallic acid or ellagic acid [9]. Some are still able to perform a transesterification reaction for the production of propyl gallate [10].

TAH is a glycoprotein esterase formed predominantly by a gallic acid esterase and a depsidase. Tannase can be separated into two esterases, a specific esterase for aliphatic esters such as methyl gallate, and another depsidase that hydrolyzes depsidic bonds like m-digallic acid as shown [11]. However, the proportion between the two activities can vary according to the cultivation conditions [12]. Tannase is a biocatalyst produced by vegetables, animals, bacteria, filamentous fungi and yeast. Tannins of yeast are effective only in the decomposition of gallotannin, while bacterial and filamentous fungi are efficient in the hydrolysis of gallotannins and ellagitannins [13].

Tannase is versatile since it can be widely used in the food, pharmaceutical and chemical industries, and even in bioremediation [14]. Among the possible applications we can mention: preparation of instant teas [15], additive for animal feed [16, 17], production of gallic and ellagic acid [18, 19], synthesis of esters and effluent treatment [9, 20], beverage manufacturing (juices, beers and wines) [21] and clarification of juices [22, 23].

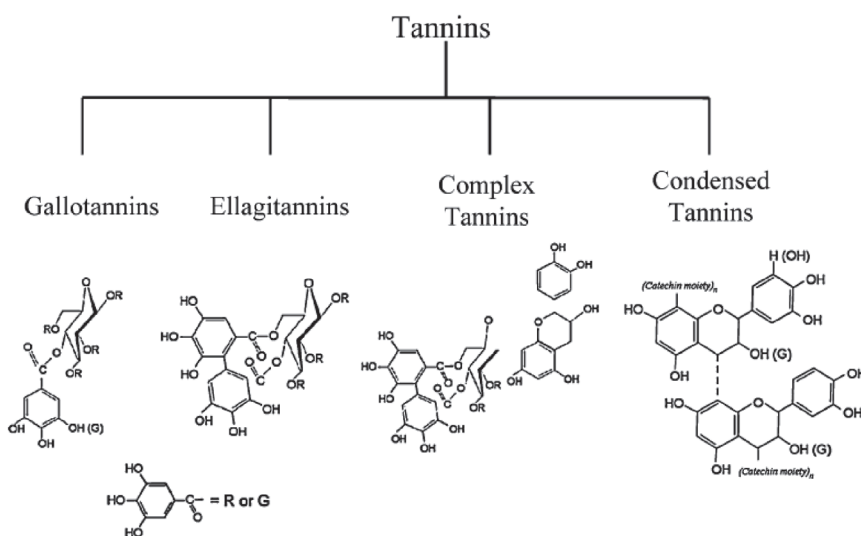


Figure 1.
Main chemical structures of the tannins [4].

The application of tannase in juices rich in hydrolyzable tannins is done to decrease the concentration of these in this food matrix, since the high content of this compound is responsible for the appearance of turbidity, bitter taste and astringency, characteristics which are often undesirable. However, the hydrolysis of gallo-tannins causes nutritional and sensory changes in the juice, since with the release of the gallo group occurs a retarding effect on the oxidation of ascorbic acid, also increasing its antioxidant action [24, 25].

The pitanga (*Eugenia uniflora* L.), belonging to the Mirtaceae family, is native to Brazil, specifically in the South and Southeast regions, and has adapted favorably to the edaphoclimatic conditions of the Brazilian Northeast, mainly in the State of Pernambuco, with about 300 hectares cultivated [26]. It is widely used by the Brazilian industry for the production of juice, preparation of ice cream, soft drinks, jellies and liquors because it has a high economic potential, attracting the consumer for its high concentration of metabolites such as anthocyanins, flavonols and carotenoids, which make this fruit a promising source antioxidant compounds [26, 27]. The natural antioxidants present in the diet increase the resistance to damage caused by oxidation, thus presenting a significant impact on human health [27].

Based on this information, the tannase obtained from *Saccharomyces cerevisiae* CCMB 520 was applied in this study with purpose of biotransforming the integral pitanga juice polyphenols and, in this way, modifying their biological activity.

2. Material and methods

2.1 Reagents

Tannic acid, gallic acid, bovine serum albumin and rodhanine were purchased at Sigma Aldrich (Sigma Chemical Co., St. Louis, MO, USA). All other chemicals used in the experiment were of high-quality analytical grade.

2.2 Microorganism and its maintenance

The yeast species *Saccharomyces cerevisiae* CCMB 520 was kindly provided by the Culture Microorganisms Collection of Bahia (*Coleção de Cultura de Micro-organismos da Bahia* - CCMB), of State University of Feira de Santana, Bahia State, Brazil. The sample was kept on plates containing Yeast Malt (YM) and left to rest in YM medium, at pH 6.8, in order to be activated; subsequently, it was incubated in B.O.D at 28 °C for 48 hours.

2.3 Inoculum preparation

A 48-hours culture grown in YM medium (Merck, Darmstadt, Alemanha) was used to prepare the inoculum at pH 6.8 and 28 °C in B.O.D incubator (Cienlab, Campinas, Brazil). After the 48-hours period, culture fragments were inoculated in 0.85% saline solution to generate a suspension presenting optical density OD_{600nm}: 0.8 at 0.9.

2.4 Enzyme production and extracellular tannase obtainment

Enzyme production was performed in 7.5 L Bioreactor containing 2.5 L of submerged fermentation medium - Czapek-Dox broth (g/L) base: NaNO₃ (7.5), KCl (1.25), MgSO₄·7H₂O (1.25), FeSO₄·7H₂O (0.025), K₂HPO₄ 3H₂O (2.5), yeast extract (25) and tannic acid (150); media were sterilized at 121 °C for 15 minutes. Tannic

acid (sterilized through membrane 0.45 μm) and inoculum were added to the fermentation medium after the Bioreactor cooled down to room temperature. The initial pH, fermentation time, rotation and incubation temperature, of the fermentation process, were 7, 24 h, 112 rpm and 27 °C, respectively. The fermentation broth was centrifuged (Thermoelectron, Langenser, Germany) at 1000 rpm for 15 minutes at 4 °C. The supernatant was frozen at -20 °C and used for further tests.

2.5 Enzyme activity and protein content

Tannase activity was estimated by using ethanolic rhodanine and tannic acid as substrate [28]. The reaction medium consisted of 250 μL substrate (0.05%, w/v) in 0.05 mol/L citrate buffer (pH 5.0) and of 250 μL enzyme extract. The substrate and the enzyme extract remained in contact for 5 minutes at 30 °C. Enzyme reaction was stopped through the addition of 300 μL ethanolic rhodanine (0.667%, w/v). After spending 5 minutes at 30 °C, the reaction medium was added with 200 μL of 0.5 mol/L potassium hydroxide in order to form a chromogen violet staining. After five more minutes at 30 °C, the obtained volume of each reaction was diluted in 4 mL of distilled water. The control tubes (enzyme extract addition at the end of the reaction) were simultaneously used. After the samples were subjected to 10 more minutes at 30 °C, the experiment proceeded in spectrophotometer (Novainstruments, Piracicaba, Brazil) at 520 nm and molar extinction coefficient was 648.15 L/mol $\times$ cm. Tannase activity (U/mL) was expressed by the amount of enzyme required to produce 1 μmol of gallic acid per minute under assay conditions. Protein content was set according to the Bradford method [29]. Bovine serum albumin was used as standard. All tests were performed in triplicate and the mean values (different from <5%) were calculated.

2.6 Partial purification by different methods

2.6.1 Ammonium sulphate precipitation

The crude enzyme extract was fractioned by ammonium sulphate precipitation at percentage saturation ranged of 0–20, 20–40, 40–60, 60–80% (w/v), respectively [30]. At each saturation, the solution was left to stand for 2 hours. The sample was dialyzed against distilled water for 4 hours at 4 °C and the precipitate was collected by centrifugation (5000 rpm for 20 minutes at 4 °C). The precipitates were solubilized in 0.04 mol L⁻¹ sodium citrate buffer (pH 5) and subjected to analysis of enzyme activity and total protein as previously described.

2.6.2 Ultrafiltration membrane (30 KDa)

The crude culture filtrate (10 mL) was added to the membrane and subsequently centrifuged at 4000 rpm for 60 minutes at 4 °C, and then the retained and permeated material were collected. The volumes obtained were separately reconstituted to the initial volume (10 mL). Soon afterwards, enzyme activity and total protein tests were performed as previously described.

2.6.3 Ethanol precipitation

The fractional precipitation followed the methodology from [31] with modifications. The solvent was cooled to a temperature of 0 °C and then added dropwise to the crude extract until you reach the desired concentrations of the same (50 to 90%, v/v). The mixture remained in contact for 1 hour at a temperature of -18 °C. After

this period, the reaction medium was centrifuged at 10,000 rpm for 20 minutes at 4 °C. The precipitate was resuspended in 0.04 M sodium citrate buffer, pH 5.0, in the same volume of crude extract added during the precipitation process. Soon afterwards, enzyme activity and total protein tests were performed as previously described. After partial purification, tannase was used in the bioconversion of integral pitanga juice.

2.7 Preparation of integral pitanga juice

The pitanga fruits (*Eugenia uniflora* L., 2000 g) were harvested in the orchard that is located near the Federal Institute of Education, Science and Technology of Pernambuco, Campus Barreiros, Brazil. They were collected between March and April, selected and cleaned in chlorinated water at 50 ppm for 15 minutes. Then were carried out, rinsing, pulp removal and crushing in an industrial blender. The integral pitanga juice was sifted and stored under freezing for further studies on the application of the enzyme.

2.8 Enzymatic biotransformation

The statistical Doehlert [32] using two variables – partially purified tannase concentration (% v/v) and application time (minutes) - was herein applied to investigate the best condition for antioxidant capacity increase. The enzyme extract concentration was assessed at three levels (4.5, 6.0 and 7.5%), whereas the application time was assessed at five levels (160, 180, 200, 220 and 240 minutes), which are presented in their actual values and codified in **Table 1**.

For each percentage of partially purified tannase, a control was performed, exchanging it for distilled water.

System behavior was explained through the following quadratic equation (Eq. (1)):

$$Y = \beta_0 + \beta_1A + \beta_2B + \beta_3C + \beta_{11}A^2 + \beta_{22}B^2 + \beta_{33}C^2 + \beta_{12}AB + \beta_{13}AC + \beta_{23}BC + \varepsilon \quad (1)$$

Wherein: Y = experimental response, β_0 intercept, β_1 , β_2 , β_3 = linear coefficients, β_{11} , β_{22} , β_{33} = quadratic coefficients, β_{12} , β_{13} , β_{23} = interaction coefficients, A, B, C = independent variables, and ε = experimental error.

Experiment	Partially purified tannase (% v/v)	Application time (minutes)
1	7.5 (0.866)	180 (−0.5)
2	7.5 (0.866)	220 (0.5)
3	6.0 (0)	160 (−1.0)
4	6.0 (0)	200 (0)
5	6.0 (0)	200 (0)
6	6.0 (0)	200 (0)
7	6.0 (0)	240 (1.0)
8	4.5 (− 0.866)	180 (−0.5)
9	4.5 (− 0.866)	220 (0.5)

Table 1.
Doehlert matrix (real and coded) used to optimize tannase application in the bioconversion of integral Pitanga juice.

Each 10 mL of pitanga juice in Erlenmeyer flasks was added partially purified tannase at the proportions cited in **Table 1** and incubated in a shaker at 120 ± 1 rpm at 30 °C, optimal temperature of the tannase from *Saccharomyces cerevisiae* CCMB 520 [33]. After the enzymatic application was done, according to the pre-established time, the enzyme was denatured at 70 °C, for 10 minutes.

2.9 Physico-chemical analysis of the pitanga juice

The physical–chemical evaluation is necessary since bioconversion cannot influence the loss of quality with respect to the pre-established minimum standards for the Standard of Identity and Quality of a specific product, in this case the integral pitanga juice.

2.9.1 pH

The pH was determined directly in the same with the aid of a previously calibrated pHmeter, after filtration [34].

2.9.2 Total soluble solids (°Brix)

Total Soluble Solids (°Brix) was determined by a Reichert digital refractometer by dropping two drops of the sample onto the surface of the properly calibrated apparatus.

2.9.3 Total acidity

A 2.5 mL sample of pitanga juice was previously homogenized and filtered in 100 mL Erlenmeyer flasks, afterwards it was diluted in 25 mL of distilled water and then stirred. Soon after, the electrode was introduced into the solution and then it was titrated with Sodium hydroxide solution (0.1 N) until the pH remained between 8.2 and 8.4 [34]. The potentiometer was previously calibrated before the analysis with pH 4 and 7 buffer solutions.

2.10 Total phenolics

The total phenolic content was estimated according to the Folin–Ciocalteu method [35].

2.11 Antioxidant activity

The antioxidant activity was assessed through the DPPH (2, 2-diphenyl-1-picrylhydrazyl) method [36].

The DPPH *radical scavenging* activity was calculated according to the equation (Eq.(2)) below:

$$\text{DPPH (\%)} = [(A_o - A_1/A_o)] \cdot 100. \quad (2)$$

Where A_o corresponded to the absorbance of the negative control, and A_1 to the absorbance in the presence of the compound (sample and Trolox). Trolox was the positive control.

2.12 Statistical analysis

The results were analyzed in the SISVAR software - Variance Analysis System [37] and the means were compared through the Scott-Knott test at 5% probability level. In addition, the results were assessed through Analysis of Variance (ANOVA) in the Statistica Version 10.0 software (StatSoft, Inc., Tulsa, USA) [38] to find the variables presenting statistically significant effects on enzyme application ($p < 0.05$), as well as the model fitting the experimental data. All assays were performed in random order.

3. Results and discussion

3.1 Partial purification

As can be seen in **Table 2**, after the precipitation with ammonium sulphate, it was not possible to recover the activity of the enzymatic extract in the fractions of 0–20 and 60–80%. In the other fractions, it was not possible to obtain a considerable purification factor (greater than 1). Thus, it was found that the use of ammonium sulphate as a precipitating agent was not efficient in the precipitation of the target protein (tannase), since this salt may have caused the denaturation of the enzymes, under the experimental conditions evaluated.

In the precipitation using ethanol, it was found that in the 50 to 70% saturation it was not possible to verify enzymatic activity and in the concentrations of 80 and 90% a reduction in it. In purification, the most desirable is that the proteins/contaminants are decreased and the activity of the target protein is concentrated or not decreased. The use of organic solvents as a precipitating agent may have negatively influenced the activity of the enzyme, as already demonstrated by several authors [39–41]. The ethanol and ammonium sulphate might have caused denaturation through a conformational change in the enzyme tertiary structure.

Stage	VA (U/mL)	TP (mg/mL)	SA (U/mg)	PF
Crude extract	3.17	0.60	5.23	1.00
Retained (30 KDa)	21.080	0.67	31.66	6.040 a
Permeate (30 KDa)	19.56	0.67	29.010	5.54 a
Ammonium sulphate (0–20%)	—	—	—	—
Ammonium sulphate (20–40%)	2.41	0.54	4.46	0.85
Ammonium sulphate (40–60%)	1.10	0.80	1.30	0.24
Ammonium sulphate (60–80%)	—	—	—	—
Ethanol (50%)	—	—	—	—
Ethanol (60%)	—	—	—	—
Ethanol (70%)	—	—	—	—
Ethanol (80%)	0.19	0.28	0.66	0.085
Ethanol (90%)	0.27	0.37	0.72	0.093

VA – Volumetric activity; TP – Total protein; AE – Specific activity; PF – Purification factor. The experiments were performed in triplicate and the mean $\pm$ standard deviation values were presented. Values followed by the same letter did not statistically differ in the Scott-Knott test at 5% probability.

Table 2.
 Partial purification of tannase from *S. cerevisiae* CCMB 520.

In reference [42], tannase was obtained and purified from *Aspergillus niger*. and The precipitation method using ammonium sulphate (50–70%) resulted in a purification factor of 4.89. Whereas in reference [43], after partial purification of tannase obtained from *Aspergillus niger* MTCC 2425, through precipitation with ammonium sulphate (75%) were obtained a purification factor around 1.4. In reference [44] tannase from *Aspergillus nomius* GWA5 was purified after three steps, using acetone and two chromatographic processes and the authors obtained the following purification factors: 1.59 (acetone fraction), 3.21 (molecular exclusion) and 4.48 (ion exchange).

After carrying out the 30 kDa membrane separation process, was possible to verify a higher degree of compaction, resulting from the internal encrustation caused by smaller particles that were adsorbed on the tube walls, thus providing a result that characterized a partial purification (factor of purification above 1), with no statistically significant difference between the two fractions obtained (retained and permeated).

3.2 Biotransformation of integral Pitanga juice by partially purified tannase from *Saccharomyces cerevisiae* CCMB 520

3.2.1 Physico-chemical analysis

The physical–chemical results are shown in **Table 3** and the Standard of Identity and Quality for the pitanga juice are in **Table 4**. The samples of the integral pitanga juice before and after partially purified tannase application comply with the standards required by current Brazilian legislation [45].

Samples	pH	Total Soluble Solids (°Brix)	Total acidity (g/100 g, citric acid)
0	3.40 a	11.85 a	1.67 a
1	3.40 a	12.10 a	1.67 a
2	3.40 a	12.55 a	1.57 a
3	3.40 a	12.25 a	1.55 a
4	3.30 a	12.00 a	1.62 a
5	3.40 a	10.35 a	1.38 a
6	3.20 a	12.20 a	1.66 a
7	3.40 a	12.20 a	1.73 a
8	3.40 a	11.70 a	1.74 a
9	3.40 a	12.40 a	1.70 a

Sample 0: before application; Samples 1 to 9: after application. Values followed by the same letter did not statistically differ in the Scott-Knott test at 5% probability.

Table 3.

Physico-chemical parameters of integral Pitanga juice before and after application of partially purified tannase from *Saccharomyces cerevisiae* CCMB 520.

Legislation (BRAZIL, 2016)	Minimum	Maximum
pH	2.50	3.40
Total Soluble Solids (°Brix)	6.00	—
Total acidity (g/100 g, citric acid)	0.92	—

Table 4.

Standard of identity and quality for Pitanga juice.

From the data, we can evidence that the tannase application in integral pitanga juice did not change the evaluated parameters, indicating that it would be within the pre-established national standards.

3.2.2 Total phenolics

Through the results obtained for the total phenolic contents, presented here in **Table 5**, we can infer that in all tests these compounds increased when compared to their respective controls. The assay 8 (4.5% and 180 minutes) stood out statistically significantly among the others, reaching 3630 mg Eq. AG/L (285.59 mg/100 g).

The phenolic compounds are substances involved in the prevention processes of chronic diseases, including diabetes, cancer, heart disease and Alzheimer's, and knowledge about their presence in different fruit can contribute to the development of production, consumption, rural diversification and income generation [46].

In [47] after evaluating phenolic compounds in red pitanga found levels around 257 mg/100 g. Whereas in [45] found levels of 95.90 mg/100 g for the pitanga hydroalcoholic extract.

The results obtained experimentally for total phenolics were evaluated through F Test (Fisher's Test) and Analysis of Variance (ANOVA) (**Table 6**). The regression

Assay	Total phenolics (mg Eq. AG/L)
After application	
1	3630.00 ± 106,066 d
2	4230.00 ± 318,20 b
3	4142.50 ± 53,033 b
4	3555.00 ± 141,42 d
5	3842.50 ± 53,033 c
6	3567.50 ± 159,099 d
7	4317.50 ± 88,39 b
8	4855.00 ± 35,36 a
9	3955.00 ± 106.066 c
Controls (white)	
C ₁	2655.00 ± 70.71 f
C ₂	2467.50 ± 17.68 f
C ₃	2630.00 ± 35.36 f
C ₄	2830.00 ± 35.36 f
C ₅	2467.50 ± 17.68 f
C ₆	2642.50 ± 194.45 f
C ₇	3205.00 ± 176.78 e
C ₈	3567.50 ± 123.74 d
C ₉	3242.50 ± 17.68 e
Before application	2663.33 ± 115.47 f

The experiments were performed in triplicate and the mean ± standard deviation values were presented. Values followed by the same letter did not statistically differ in the Scott-Knott test at 5% probability.

Table 5.
 Doehlert matrix results for total phenolics in Pitanga juice before and after application of partially purified tannase from *Saccharomyces cerevisiae* CCMB 520.

Variation Source	Sum of squares	Degree of freedom	Mean square	Fcal	Ftab
Regression ^a	1324618	5	264923.60	9.18	9.01
Residual	86563	3	28854.14		
Lack of Fit	33750	1	33750	1.28	18.51
Pure Error	52813	2	26406.25		
Total	1411181				

^aStatistically significant at 95% confidence interval. Fcal – calculated F value; Ftab – tabulated F value. R² = 0.94.

Table 6.
Analysis of variance applied to the data shown in **Table 5**.

was statistically significant (Fcal 9.18 > 9.01 Ftab) and the lack of fit indicated a good agreement (Fcal 1.28 < 18.51 Ftab) between the fitted model and the experimental data. Furthermore, the quality of the fit was also confirmed through coefficient of determination (R² = 0.94), and it implied that just 6% of the response variability was not explained by the model.

The model equation after regression, for the increase of phenolic compounds, was obtained (Eq. (3)):

$$\begin{aligned} \text{Total phenolics (mgEQAG/L)} = & 39442.50 \cdot (\pm 5459.69) - 4545.83 \cdot EE \cdot (\pm 805.25) \\ & + 161.11 \cdot EE^2 \cdot (\pm 49.45) - 217.19 \cdot T \cdot (\pm 40.56) \\ & + 0.36 \cdot T^2 \cdot (\pm 0.093) + 12.29 \cdot EE \cdot T \cdot (\pm 2.71) \end{aligned} \quad (3)$$

From the **Figure 2**, we found that only the interaction (positive effect) was statistically significant in the experimental field studied. **Figure 3** shows the response surface and contour curves obtained as a function of enzyme application time and tannase concentration, where it indicated that the increase in the variables under study increased the phenolic compounds. While, by decreasing the two variables, there was also an increase in phenolic compounds. This result can be seen in the positive interaction term obtained in Eq. (3) and **Figure 2**.

3.2.3 Antioxidant activity

Studies have shown that the consumption of fruits and vegetables reduces the risk of chronic diseases such as cancer, cardiovascular diseases and stroke [48]. This may be due to the presence of several secondary metabolites, these being related to various biological activities, including antioxidant activity.

The results of the total antioxidant activity are shown in **Table 7**, where it can be seen that test 8 (69.41%), as well as for phenolics (**Table 5**), was the one that presented values statistically superior to the other tests. We also found that all tests in the presence of the enzyme were superior to their respective controls. This demonstrates that the tannase from *S. cerevisiae* CCMB 520 acted on the compounds present in the integral pitanga juice, biotransforming them and increasing their biological activity.

The results obtained experimentally for the total antioxidant activity were evaluated by Test F and ANOVA (**Table 8**). The regression was statistically significant (Fcal 20.61 > 9.01 Ftab) and the lack of fit indicated a good agreement (Fcal 10.33 < 18.51 Ftab) between the adjusted model and the experimental data. The fit of the

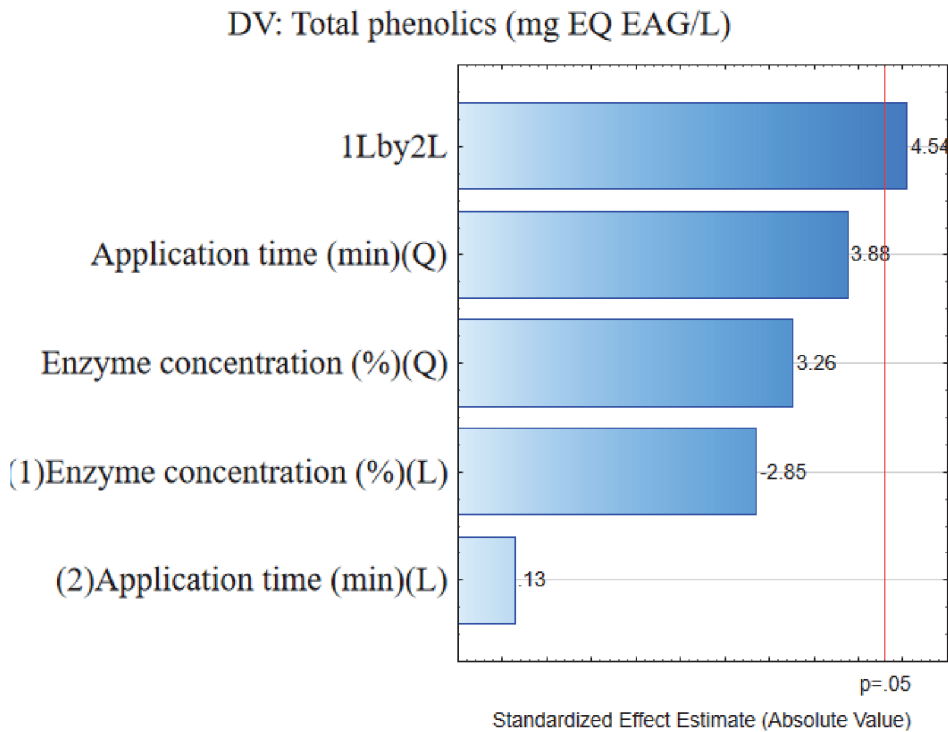


Figure 2.
Pareto chart for the effects of the variables on the total phenolic content of Pitanga juice, according to statistical planning of the Doehlert design.

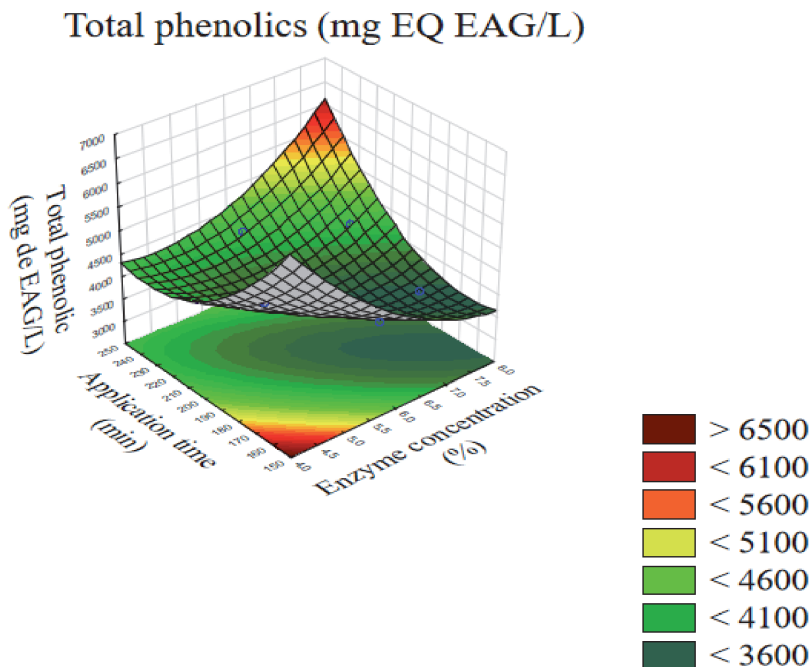


Figure 3.
Response surface and contour plot to total phenolic content, according to the Doehlert design. The three-dimensional plot shows partially purified tannase concentration and application time.

Assay	Antioxidant activity – DPPH (%)	Antioxidant activity – μ MTrolox/L
After application		
1	57.56 $\pm$ 1.78 d	757.00 $\pm$ 35.56
2	64.96 $\pm$ 6.091 b	803.67 $\pm$ 40.069
3	61.34 $\pm$ 0.59 c	835.33 $\pm$ 4.71
4	64.71 $\pm$ 1.54 b	892.00 $\pm$ 29.63
5	65.55 $\pm$ 0.48 b	915.33 $\pm$ 9.43
6	64.71 $\pm$ 1.90 b	863.67 $\pm$ 11.79
7	62.35 $\pm$ 1.19 c	852.00 $\pm$ 23.57
8	69.41 $\pm$ 1.43 a	952.00 $\pm$ 28.28
9	59.16 $\pm$ 0.71 d	778.67 $\pm$ 14.14
Controls (white)		
C ₁	50.00 $\pm$ 1.31 e	607.00 $\pm$ 25.93
C ₂	42.017 $\pm$ 2.38 f	448.67 $\pm$ 47.14
C ₃	42,10 $\pm$ 1.31 f	450.33 $\pm$ 25.93
C ₄	45.46 $\pm$ 2.97 f	517.00 $\pm$ 58.93
C ₅	42.27 $\pm$ 0.12 f	453.67 $\pm$ 2.36
C ₆	41.76 $\pm$ 0.59 f	443.67 $\pm$ 11.79
C ₇	50.42 $\pm$ 1.90 e	615.33 $\pm$ 37.71
C ₈	49.07 $\pm$ 0.71 e	588.67 $\pm$ 14.14
C ₉	46.97 $\pm$ 1.54 e	547.00 $\pm$ 30.64
Before application		
	51.26 $\pm$ 2.38 e	632.00 $\pm$ 47.14

The experiments were performed in triplicate and the mean $\pm$ standard deviation values were presented. Values followed by the same letter did not statistically differ in the Scott-Knott test at 5% probability.

Table 7.
Doehlert matrix results before and after the application of partially purified tannase from *Saccharomyces cerevisiae* CCMB 520.

Variation Source	Sum of squares	Degree of freedom	Mean square	Fcal	Ftab
Regression ^a	101.01	5	20.20	20.61	9.01
Residual	2.95	3	0.98		
Lack of Fit	2.48	1	2.48	10.33	18.51
Pure Error	0.47	2	0.24		
Total	103.96				

^aStatistically significant at 95% confidence interval. Fcal – calculated F value; Ftab – tabulated F value. $R^2 = 0.97$.

Table 8.
Analysis of variance applied to the data shown in **Table 7**.

model was measured by the coefficient of determination (R^2), which had a value of 0.97 suggesting that 97% of the total variation in residual antioxidant activity was explained by the adjusted model. It is worth mentioning that this is the first report

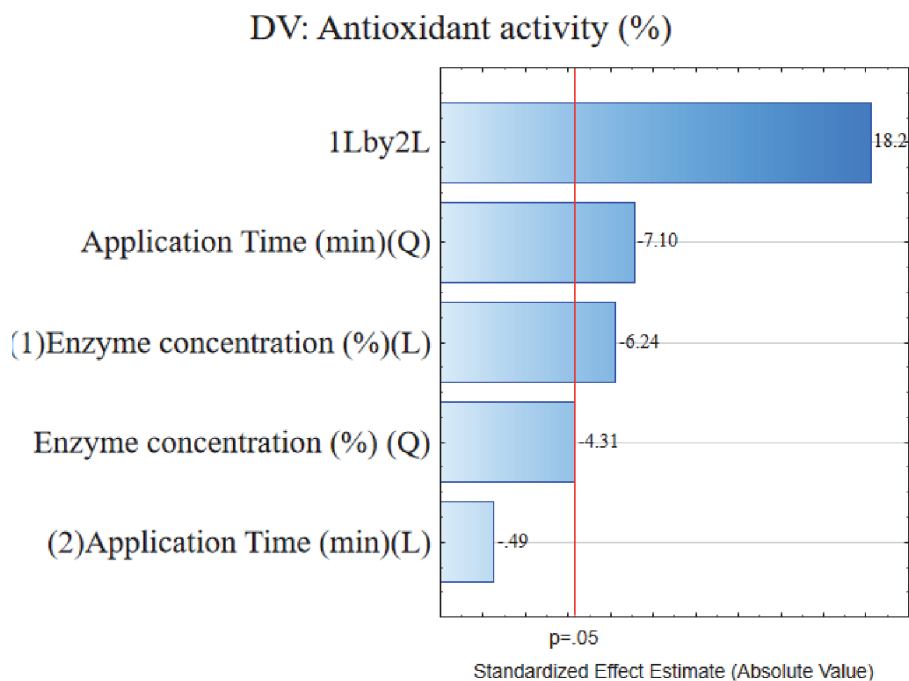


Figure 4.
Pareto chart for the effects of the variables on the total antioxidant activity of Pitanga juice, according to statistical planning of the Doehlert design.

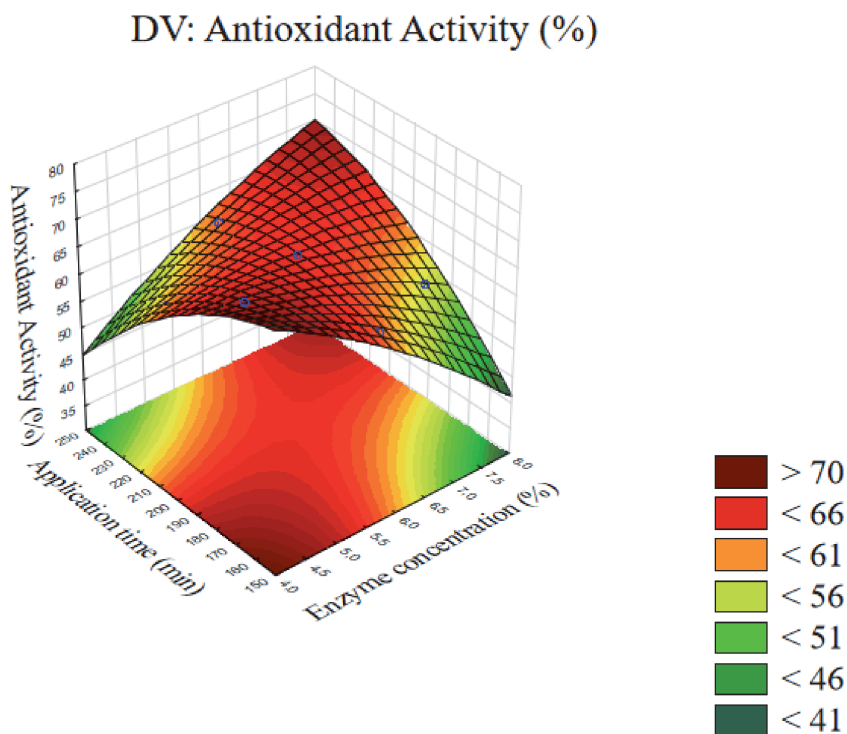


Figure 5.
Response surface and contour plot to total antioxidant activity, according to the Doehlert design. The three-dimensional plot shows partially purified tannase concentration and application time.

on the application of tannase in integral pitanga juice and its effect on total antioxidant activity and phenolic contents.

The second order equation (Eq. (4)) that describes the experimental data is presented:

$$\begin{aligned} \text{TAA (\%)} = & 146.71 \cdot (\pm 16.29) - 22.79 \cdot \text{EE} \cdot (\pm 2.40) - 0.64 \cdot \text{EE}^2 \cdot (\pm 0.15) - 0.10 \\ & \cdot T \cdot (\pm 0.12) - 0.0020 \cdot T \cdot (\pm 0.12) - 0.0020 \cdot T^2 \cdot (\pm 0.00028) + 0.15 \\ & \cdot \text{EE} \cdot T \cdot (\pm 0.0081) \end{aligned} \quad (4)$$

From **Figure 4**, it appears that the time in its linear term was not statistically significant in the experimental field studied.

Wherein: TAA = Total antioxidant activity.

The **Figure 5** illustrates the response surface and contour curves regarding the relationship between application time and tannase concentration. Corroborating with the data obtained for phenolic compounds, it was found that increasing or decreasing the independent variables, together, increases the response variable.

In reference [49] was evaluated samples of aqueous, ethyl acetate and butanolic extracts from pitanga fruits, where the author observed total antioxidant activity in the highest concentration (1000 µg / mL): 35.6, 86.1 and 88.7%, respectively.

Several patent filings have demonstrated the application of tannase in juices with the aim of increasing antioxidant activity. The Indiana patent application 613/KOL/2005, in [50], which describes a 37% increase in gallic acid content and an 8% increase in antioxidant activity after tannase application in the pomegranate juice. The Brazilian patent application BR 10 2015 001163–6, in reference [51] describes a total antioxidant activity of 98.20%. The results obtained by other researchers corroborate those presented in the present study. In this work, an increase in antioxidant activity of around 18.15% was possible.

Considering that the antioxidant activity is largely attributed to the presence of phenolic compounds, Pearson's correlation was calculated to verify the existence of a relationship between the two independent variables. The **Figure 6** illustrates a

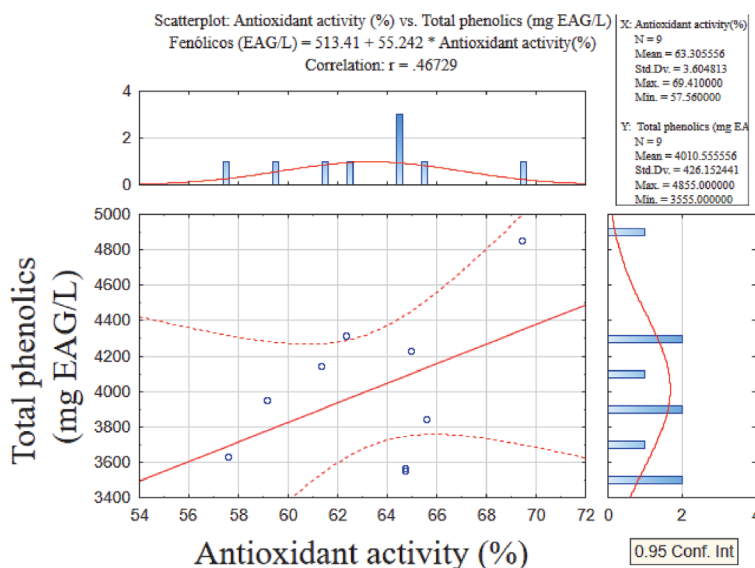


Figure 6.

Pearson's correlation between antioxidant activity and total phenolics.

moderate positive correlation between variables, by increasing phenolic compounds, antioxidant activity is increased.

The use of tannase for the release of phenolic antioxidants has become interesting for various types of food matrix. This is because most of them can release the phenolic compounds without requiring a pre-treatment such as the action of the pectinase or cellulase, or variation in temperature or pH [52]. The biotransformation of bioactive compounds is also an interesting alternative that deserves attention, since it precludes the use of toxic compounds such as organic solvents in the extraction. In these processes, bioactive compounds are obtained from natural sources by microorganisms through their secondary metabolism or by exogenous enzymatic action [53, 54]. According to [55], the bioconversion by enzyme as well as whole cell biocatalyst has tremendous importance in industry owing to escalated yields, low impurity profiles, environmental safety, and process reproducibility.

The values found after tannase application, in relation to phenolic compounds and antioxidant activity, were due to the conversion of substances present in integral pitanga juice. These data demonstrate the action of tannase obtained from *S. cerevisiae* CCMB 520 in the biotransformation of this food matrix, suggesting that the enzyme has biotechnological potential in the production of foods with better nutraceutical properties.

4. Conclusions

This is the first work to report application of tannase in integral pitanga juice. The purpose of the present study was to produce and apply tannase obtained from *S. cerevisiae* CCMB 520. From the results presented, we found that is possible, through enzymatic treatment, to increase the functional quality of integral pitanga juice, once there was an increase in total antioxidant activity, which is associated with an increase in total phenolic compounds.

The results suggest that the partially purified tannase of *Saccharomyces cerevisiae* CCMB 520 can potentially be used for industrial biotechnological application, as in the biotransformation of juices, to obtain a product with greater biological activity (functional property). It is worth mentioning that after the application of partially purified tannase, the juice remained with its physico-chemical characteristics within the Standard of Identity and Quality, according to the current legislation.

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Conflict of interest

The authors declare no conflict of interest.

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