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BIOTRANSFORMATION OF GREEN TEA BY TANNASE PRODUCED BY *Colletotrichum gloeosporioides* URM 7130

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ABSTRACT

Tannin Acyl Hydrolase is an enzyme that hydrolyzes esters and depside bonds of hydrolysable tannins. They are widely used in the food and beverage, pharmaceutical and chemical industries. The objective of this work was to evaluate the production of tannase by *Colletotrichum gloeosporioides* URM 7130, using submerged fermentation. At the same time, the enzymatic application was made in green tea and its effect was evaluated. For optimization were used Plackett-Burman and Doehlert designs. The total antioxidant activity total tannins and phenolics were evaluated. Results of enzymatic activity indicated 79.39 ± 0.33 U / mL. It was verified a reduction of 12.75% of the tannin content and it was possible to increase in 24.87% the phenolic compounds and 4.45% in the antioxidant activity. The results suggest that the green tea studied can be used as an alternative of consumption as functional foods, since it increased its antioxidant activity.

KEYWORDS

Antioxidant activity, Biotransformation, Gallic acid, Green tea and Tannin Acyl Hydrolase.

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INTRODUCTION

The Tannin acyl hydrolase (TAH, E.C: 3.1.1.20) is an enzyme that hydrolyzes esters and depside bonds of hydrolysable tannins such as tannic acid into glucose and gallic acid[1].

Tannase presents a wide application in the food, juice, beers, cosmetics, pharmaceutical and chemical industry. It is used in the stabilization of wine color, coffee-based soft drinks, effluent treatment in the leather industry and gallic acid production[1,2].

Gallic acid has a wide application in the chemical and pharmaceutical industries. It is extracted through the hydrolysis of tannic acid and used for syntheses of trimethoprim, pyrogallol and propyl

gallate. Trimethoprim is a drug widely used in the pharmaceutical industry as an antibacterial agent, pyrogallol is used as a preservative in the food industry and propyl gallate has the role of antioxidant in products containing fatty acids and oils[3,4]. Another great application is in the preparation of instant teas, since the addition of tanase in different stages of the industrial process decreases the turbidity and leads to the increase of the antioxidant capacity present in the same[5].

In living organisms, the function of antioxidants is to prevent radicals from damaging tissues and assisting cellular health, inhibiting the installation of pathogenesis linked to oxidative stress[6].

Certain organisms have the capacity to effect chemical modifications in compounds, denominating this process of biotransformation. In the case of filamentous fungi, it can occur using the whole organism (several sequential synthetic transformations) or through biocatalysis (reduced number of synthetic steps)[7].

The aim of the current study was to produce and apply tannase derived from *Colletotrichum gloeosporioides* URM 7130 into green tea in order to investigate the effect it has on total tannins, total phenols, as well as on the antioxidant activity.

MATERIAL AND METHODS

Microorganisms and conservation

The fungus *Colletotrichum gloeosporioides* URM 7130 was kept in PDA (Potato Dextrose Agar) medium wherein it was cultivated at pH 6.8. Next, it was incubated in Biochemical Oxygen Demand (B.O.D, Solab, SL-200/364, Piracicaba, Brazil) at 28 ± 2°C, for 10 days, before its use in the fermentation medium.

Tannase Production

The enzyme production was carried out through Submerged Fermentation using tannic acid as the only carbon source. The saline solution was composed of Czapeck-Dox medium (g / L: $NaNO_3$, 3.0; KCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; $K_2HPO_4 \cdot 3H_2O$, 1.3; yeast extract, 1.0). Subsequently, the container holding the mixture was autoclaved at 121°C, for 15 minutes. After the container cooled at room temperature, 0.45 μm of membrane-sterilized tannic acid (100 g / L) was added to it. Then, the content was inoculated with 2 mycelial discs (1.3 cm) and incubated at 30°C in Shaker (Solab, Refrigerada SL-221, Piracicaba, Brazil), for 168 hours/100 rpm.

Enzyme extraction

After the fermentation period was finished, the resulting enzyme was extracted from the fermentation broth through filtration in qualitative filter paper (Whatman # 1) and centrifugation (Thermo Electron Led GMBH, Multifuge X1R, Kalkberg, Alemanha) at 10000 rpm, for 15 minutes, 4ºC, in order to get the extracellular enzyme. The supernatant, which was named crude enzymatic extract, was used in subsequent analytical determinations and application.

Tannase activity

Enzyme activity was estimated through the method modified[8] by using ethanolic rhodanine and tannic acid as substrate. The tannase activity unit (U / mL) was defined as the number of enzymes required to produce 1 μmol of gallic acid per minute, under assay conditions.

Protein content

Protein content was set according to the Bradford method[9]. Bovine serum albumin was used as standard.

Preparing the green tea

The tea extract was prepared by adding 25g raw tea into 200 mL of boiling distilled water and allowed to stand for 20 minutes and through filtration in qualitative filter paper (Whatman # 1)[10]. The extracted green tea was stored in a freezer and used in further enzyme application studies.

Treating the green tea with tannase

The statistical Doehlert design[11] using two variables - enzyme extract concentration (%, v/v) and enzyme application time (minutes) - was herein applied to investigate the best condition for tannin content reduction, as well as for phenolic compound content and antioxidant capacity increase. The enzyme extract concentration was assessed at three levels (2.0, 4.0 and 6.0%), whereas the application time was assessed at five levels (140, 150, 160, 170 and 180 minutes) - which are presented in their actual values and codified in Table No.1. The current study has used one control for each enzyme extract

percentage, wherein the extract was replaced by distilled water.

The system's behavior was explained through the following quadratic equation (Eq. (1)):

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{12} AB + \varepsilon \quad \text{(Eq. (1))}$$

Wherein Y is the predicted response; β_0 is the intercept; β_1 and β_2 are the linear coefficients; β_{11} and β_{22} are the quadratic coefficients; β_{12} is the interaction coefficient; A, B, A^2, B^2 and AB are the independent variables; and ε is the experimental error.

Each 10 mL of tea in Erlenmeyer flasks was added crude tannase at the proportions cited in Table No.1 and incubated in a shaker at 120 ± 1 rpm, 40°C. Soon after the enzymatic application was done, according to the pre-established time, the enzyme was denatured at 70°C, for 10 minutes.

The total tannins, phenolic compounds and antioxidant activity were evaluated before and after enzyme application.

Estimating the total tannin content

The total tannin content was estimated through the protein precipitation method[12] using tannic acid as standard. The results were presented as hydrolysis percentage.

Estimating the total phenols content

The total phenolic content was estimated according to the Folin-Ciocalteu method[13].

Estimating the antioxidant activity

The antioxidant activity was assessed through the DPPH (2, 2-diphenyl-1-picrylhydrazyl)[14].

Statistical analyses

The results were analyzed in the SISVAR software - Variance Analysis System[15] and the means were compared through the Scott-Knott test, at 5% probability level. In addition, an Analysis of Variance (ANOVA) using the Statistica 10.0 software (StatSoft, Inc., Tulsa, USA) was performed to indicate the variables with statistically significant effects ($p < 0.1$) and to fit the model to the experimental data. All assays were randomly performed.

RESULTS AND DISCUSSION

Tannase production

The present study is pioneer in reporting the tannase secreted by *Colletotrichum gloeosporioides* URM 7130. Crude tannase showed total and specific activity 79.39 ± 0.33 U / mL and 552.78 ± 6.99 U / mg, respectively.

The enzyme production is associated to the concentration of tannic acid which is added to the fermentation medium. This carbon source favors the rapid production of tannase which, in turn, cleaves tannins by providing continuous supply of carbon. According to[16,18] tannic acid plays the role of carbon source for the microorganism as well as the inducer of synthesis. In this way, the presence of tannic acid is essential for the synthesis of tannase.

Tannase production by *Aspergillus foetidus* (MTCC 3557) under submerged fermentation, after optimization using tannic acid as carbon source, were obtained enzyme activities from 15 to 30 U / mL[19]. Tannase production was evaluated by Riul *et al.* (2013)[20]. The highest level of tannase production (1.43 U/mL) by *A. phoenicis* was obtained in Khanna medium. Tannase produced by *C. gloeosporioides* URM 7130 it presented greater enzyme activity.

Total tannins

Table No.2 indicates the tannase effect after application on the hydrolysable tannins present in green tea. There was reduction in the content of these compounds. In the experimental field evaluated, the greatest reductions in total tannins were obtained in assays 2 and 8, which were statistically superior to the other trials at the analyzed level.

The use of tannase in tannin reduction has been explored and can minimize production costs by industry. However, they are more commonly used in juice processing. The use in teas has not been verified in the literature on the reduction of these compounds. The initial investment to obtain clarified cashew juice with enzyme is lower than for juice clarified with gelatin[21]. This fact occurs due to the need for the additional filtration step. In the treatment with enzymes there is only one centrifugation step with the generation of solid residue rich in tannins, but due to the action of TAH, a significant fraction of the total tannin content (44.5%) was solubilized. In the conventional treatment there are two steps, the first to remove particulate matter already present and the

second to remove the precipitate resulting from the complexation of gelatin with the tannins. Thus, the conventional process presents a higher level of generation of a recalcitrant residue and a higher financial cost.

Total phenols

Several studies have shown that green tea has several medicinal properties, such as anticariogenic potential, against free radicals, allergies, inflammations, ulcers, viruses, tumors, for the prevention and treatment of obesity and its comorbidities. Inhibitory actions may also prevent platelet aggregation, reducing cardiovascular disease and thrombosis[22]. All beneficial actions may be related to the phytochemicals present in all parts of the plant. Among these compounds can be mentioned ellagic acid, gallic acid, quercetin, myricetin, isoquercitin, acetyl oleanolic acid, which are phenolic compounds in different concentrations and may be related to antioxidant activity and reduction of free radicals[23].

Table No.3 shows the gallic acid-equivalent results of total phenols, before and after the enzyme application. It can be verified that all the tests presented statistically significant difference ($p < 0.05$) in relation to their respective controls. However, assay 2 showed the highest content of total phenolic compounds. These results are in agreement with those presented in Table 2, since the enzyme degraded hydrolysable tannin present in green tea. After enzyme application it was possible to increase the phenolic compounds by 24.87% in the assay 2. Bastos *et. al.* (2007)[24] when evaluating the total phenolics of aqueous extracts of yerba mate and green tea obtained values of 7.73% and 7.15% in gallic acid equivalents, respectively. From the results obtained in this work, it can be affirmed that the enzyme application provided a significant increase of the total phenolic compounds present in the green tea, making advantageous its industrial use.

Antioxidant activity

Green tea has been extensively studied because of its properties, mainly related to the presence of its constituent principles, being flavonoids widely recognized for their antioxidant properties[25-27]. In general, antioxidant activity in the tea in question is related to the prevention of various diseases, including atherosclerosis, hepatic disease, obesity and various types of cancers[28,29].

An antioxidant can be defined as a substance that, at low concentrations, retards or prevents substrate oxidation (Halliwel *et al.,* 1995)[30]. The antioxidant capacity may be expressed through several parameters such as the peroxyl radical removal (ORAC - Oxygen Radical Absorbance Capacity, TRAP - Total reactive antioxidant potential), the metal reducing capacity (FRAP - ferric reducing antioxidant power, CUPRAC - cupric ion reducing antioxidant capacity), the organic radical removal capacity (ABTS - 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid, DPPH - peroxidation of 2,2-diphenyl-1-picrylhydrazyl), and the quantification of products formed during lipid peroxidation (TBARS, LDL oxidation, β-carotene co-oxidation)[31]. The assay 1 presented the highest total antioxidant activity (82.89%) (Table No.4).

The results obtained experimentally found for total antioxidant activity were evaluated through F Test (Fisher's Test) and Analysis of Variance (ANOVA (Table No.5). The regression was statistically significant and the lack of fit indicated a good agreement between the fitted model and the experimental data. The quality of the fit was also confirmed through coefficient of determination ($R^2 = 0.99$), and it implied that just 1% of the response variability was not explained by the model.

Figure No.1 shows that the time and concentration of the enzymatic extract, in its linear terms, were statistically significant for the total antioxidant activity, which presented negative and positive effects, respectively.

Figure No.2 shows the response surface and contour curves concerning the relation between enzyme application time and enzyme extract concentration. The figure indicates that by decreasing the levels of enzyme application time and increasing the concentration of enzyme extract, the total antioxidant activity increases. The response surface showed that the best response could be obtained when the enzyme extract is applied at 6.93% concentration for 141.30 minutes, which resulted in 83.04% of total antioxidant activity.

The antioxidant activity of the tea has been evaluated by several researches[29,32,33]. Cao *et al.* (1996)[32] using the ORAC method, found that green tea and black tea have higher antioxidant activity against peroxidic radicals than some vegetables (garlic, spinach and Brussels sprouts). Using the FRAP method Langley-Evans (2000)[29] found a higher total antioxidant capacity in green tea (92.1%) when compared to black tea (28.8%).Giada (2006)[34] using the DPPH method, showed that black tea had higher antioxidant activity (91.26%) when compared to green tea (89.91%). Hong *et al.* (2013)[35] observed an increase in the concentration of gallic acid, (-)-epigalocatechin and (-)-epicatechin after the use of tannase in green tea extract. In a study developed by Lu and Chen (2008)[36], the authors verified an increase in antioxidant and chelating activity when compared to untreated tea, after using tannase in green tea.

From the results obtained, it is necessary to apply the tannase to the functional improvement of green tea.

Table No.1: Doehlert design matrix used in the enzymatic treatment of green tea

S.No	Enzyme extract (%, v/v)	Enzyme application time (minutes)
1	6.0 (0.866)	150 (-0.5)
2	6.0 (0.866)	170 (0.5)
3	4.0 (0)	140 (-1.0)
4 C	4.0 (0)	160 (0)
5 C	4.0 (0)	160 (0)
6 C	4.0 (0)	160 (0)
7	4.0 (0)	180 (1.0)
8	2.0 (- 0.866)	150 (-0.5)
9	2.0 (- 0.866)	170 (0.5)

C: Central point.

Table No.2: Doehlert matrix results after the application of the enzyme extract containing *Colletotrichum gloeosporioides* URM 7130 tannase to reduce tannins in the green tea

S.No	Experimental tannin reduction (%)	Predicted tannin reduction (%)
1	7.77 ± 2.14 c	6.41
2	11.40 ± 5.16 a	12.75
3	2.50 ± 1.72 e	3.85
4 C	5.30 ± 0.66 d	5.56
5 C	5.60 ± 0.59 d	5.56
6 C	5.80 ± 2.45 d	5.56
7	9.89 ± 1.14 b	8.54
8	11.08 ± 2.63 a	9.72
9	6.72 ± 0.27 c	8.07

C: Central point. Means followed by different letters in the column differ from each other, according to the Scott-Knott Test, at 5% probability level.

Table No.3: Doehlert matrix results for the total phenolic content in green tea before and after the application of enzyme extract containing *Colletotrichum gloeosporioides* URM 7130 tannase

S.No	Total experimental phenols (mg of EAG/L)/gallic acid increase (%)	Total predicted phenols (mg of EAG/L)/gallic acid increase (%)
1	633 ± 5.77/12.43 b	648
2	703 ± 17.32/24.87 a	688
3	623 ± 23.09/11.65 c	608
4 C	613 ± 5.77/9.86 c	623
5 C	623 ± 33.05/11.65 b	623
6 C	633 ± 11.55/13.44 b	623
7	643 ± 0.00/15.23 d	658
8	593 ± 17.33/5.06 b	608
9	633 ± 17.33/12.43 b	618
Before application		
$C_{2,0}$ %	563 ± 17.33 e	-
$C_{4,0}$ %	558 ± 21.21 e	-
$C_{6,0}$ %	563 ± 11.55 e	-

C: Central point. Means followed by different letters in the column differ from each other, according to the Scott-Knott Test, at 5% probability level.

Table No.4: Doehlert matrix results for total antioxidant activity in green tea before and after the application of enzyme extract containing *Colletotrichum gloeosporioides* URM 7130 tannase

S.No	Experimental Antioxidant activity	Predicted Antioxidant activity
1	82.89 ± 0.71 a	82.83
2	81.45 ± 0.36 b	81.52
3	81.88 ± 0.24 b	81.94
4 C	81.80 ± 0,12 b	81.59
5 C	81.38 ± 0.24 b	81.59
6	79.78 ± 0.12 d	79.73
7	80.12 ± 0.83 c	80.06
8	79.11 ± 1.07 d	79.17
Before application		
$C_{2,0\ \%}$	80,79 ± 0.12 c	-
$C_{4,0\ \%}$	80,87 ± 0.00 c	-
$C_{6,0\ \%}$	79,36 ± 0.00 d	-

C: Central point. Means followed by different letters in the column differ from each other, according to the Scott-Knott Test, at 5% probability level.

Table No.5: Analysis of variance applied to the data shown in Table 4

S.No	Variation source	Sum of squares	Degrees of freedom	Quadratic mean	Fcal	Ftab
1	Regression	11.020	5	2.20	40.00	9.29
2	Residue	0.11	2	0.055		
3	Lack of fit	0.019	1	0.019	0.22	39.90
4	Pure error	0.088	1	0.088		
5	Total	11.13				

Fcal – Calculated F; Ftab – Tabulated F (Confidence level: 90 %). R^2 = 0, 99.

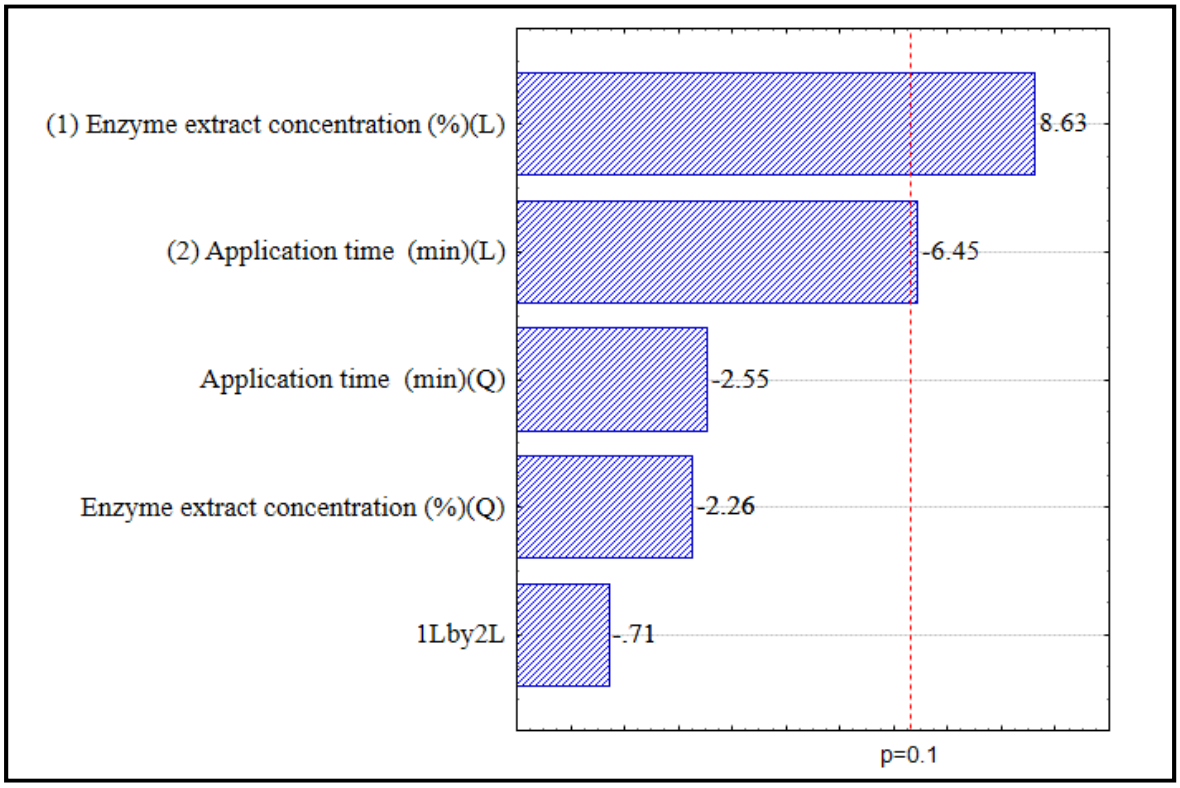

Figure No.1: Pareto chart for the effects of the variables on the total antioxidant activity of green tea, according to statistical planning of the Doehlert design

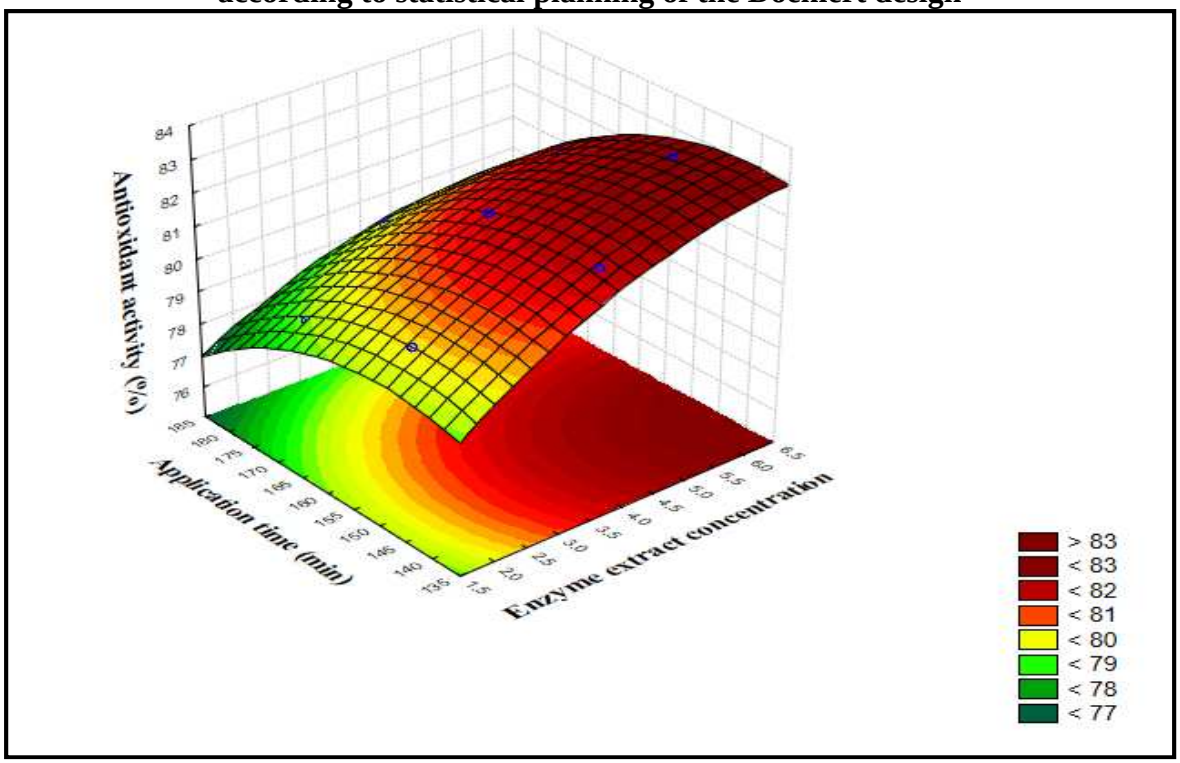

Figure No.2: Response surface and contour curves of total antioxidant activity considering the interaction between enzyme application time and enzyme extract concentration

CONCLUSION

The results obtained in the present work show the promising potential of the tannase obtained from the endophytic fungus *C. gloeosporioides* URM 7130. In this work the application of the tannase in the biotransformation of the green tea was done. There was a reduction of 12.75% in total tannin content, a 24.87% increase in total phenolics and a 4.45% increase in total antioxidant activity.

It has been found that the tannase produced by *C. gloeosporioides* URM 7130 can be used in biotechnological applications. Green tea obtained after enzymatic application can be used as an alternative for consumption as a functional food once its total antioxidant activity has increased.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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