

Preparation of *Aspergillus niger* 426 naringinases for debittering citrus juice using of agro-industrial residues

Fernanda de Oliveira

Universidade Estadual de Londrina

Tereza Cristina Luque Castellane (✉ terezacastellane@gmail.com)

Universidade Estadual Paulista: Universidade Estadual Paulista Julio de Mesquita Filho

<https://orcid.org/0000-0002-7290-5314>

Marcelo Rodrigues de Melo

Universidade Estadual Paulista

João Batista Buzato

Universidade Estadual Paulista

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Abstract

Naringin (4,5,7-trihydroxy flavanone-7-rhamnoglucoside), considered as the main bitter component of grapefruit, requires the use of enzymes to reduce the level of this substance during juice processing. For this reason, it has been the focus of many studies. To increase the production of naringinase by *Aspergillus niger* cultivated in solid-state fermentation (SSF), it was verified whether the influence of agro-industrial residues as fermentation substrates and, finally, selected the best of the three inducers, or their mixtures to remove the bitterness of grapefruit juice. Cultivation with 2.3 g of grapefruit peel, 2.5 g of rice bran, and 5.2 g of wheat bran and medium supplementation with a mixture of naringin, rutin, and hesperidin in the concentration of (g / L): 2, 5, 4.5, and 3.0, respectively, leading to a maximum activity of 28 U / mL. The results indicate that the sequencing procedure, which allowed the definition of an optimal mixture of components, is a new way for microorganisms to have a high naringinase yield, in particular by SSF, since our data showed a 96% increase in the production of naringinase.

1. Introduction

Naringin (4,5,7-trihydroxy flavanone-7-rhamnoglucoside) is the major flavanone in grapefruit and it is responsible for bitter taste. The presence of bitterness, generally, has been the major limitation for the commercial acceptance of juices [1]. For this reason, naringinase (EC 3.2.1.40) has attracted increasing research attention due to its broad range of positive enzymatic hydrolysis is a possibility to overcome the bitterness and obtain compounds with improved biological activities.

Naringinase is a hydrolytic enzyme that contains the activities α -L-rhamnosidase (E. C. 3.2.1.2.3) and beta-glucosidase (EC 3.2.1.21). In the first stage of hydrolysis, the hydrolysis catalysis of naringin (4,5,7-trihydroxy flavanone 7-ramnoglucoside) occurs, in rhamnose and prunin. Being that, the prunin who is responsible for one third of the bitterness of the naringin. Second phase, prunin is hydrolyzed to tasteless naringenin (4,5,7-trihydroxy flavanone) and glucose [2, 3]. In the other stage, the enzyme β -glucosidase converts glucose from prunin and naringenin (4,5,7-trihydroxy flavanone) when releasing a D-glucose molecule [4]. There are many substrates like naringin, rutin, quercitrin, hesperidin, diosmin, and ter-phenyl glycosides, containing terminal α -rhamnose and β -glucose [5]. These products are the basic material for the synthesis of many substances.

It is commercially attractive due to its potential utility in citrus juices debittering, especially in grapefruits, and wine industries, reducing the intensity of its bitter taste. Naringinase, an important enzyme complex, also finds applications in the field of biotechnology, pharmaceuticals, and other industries, such as biotransformation steroids, antibiotics, and mainly glycolide hydrolysis, glycopeptides, flavonoid deglycosylation, and gelane depolymerization [6, 7]. Additionally, due to its medicinal properties, for example, improved signaling via, anti-inflammatory, anticancer also has great commercial importance in the medical area [8].

New screening methods for the potential production of naringinase by microorganisms have been described [9]. Naringinase can be obtained by fermenting various microorganisms, including especially *Aspergillus oryzae* 11250 [10], *Rhizopus stolonifer* [11], *Trichoderma longibrachiatum* ATCC18648 [12], among others. However, the fungus species *A. niger* is the preferred source of a variety of enzymes or expression system for foreign proteins. This species, several of which have a long history of safe use in the fermentation industry, is one of the most important sources of naringinase industrial use, such as for food industrial use mainly due to its safety characteristics and viable culture processes [13].

Several publications describe the improvement in naringinase production. For example, using an inductor [11], the presence of citrus peel, a source of naringin [2], or the optimization of the concentration of the substrates, carbon source or nitrogen sources [14] and temperature [15].

The industrial application of enzymes is growing exponentially, together with its wide application, it is necessary to increase the production of naringinase. In this case, optimizing the culture conditions, especially by adding enzyme inducers. This work aimed to define the concentration of three agro-industrial residues, among them grapefruit rind as a specific inducer, and three known inducers of naringinase (naringin, rutin, and hesperidin) by Experimental Mixture Design (EMD) using solid-state fermentation (SSF). This methodology is attractive because it can be applied to study the proportion of the components in a given culture medium, and can aid with the identification of relationships amongst these components during naringinase production.

2. Materials And Methods

2.1 Materials

Orange rind, grapefruit rind, rice bran, and wheat bran were used as solid substrates. The residues were provided by local agroindustry, then dried to 2% (w/w) humidity in an oven at 70 °C for 24 h and milled in a mill Wiley type at a particle size of approximately 2 mm.

Naringin, rutin, hesperidin, quercetin, citric acid, glucose, sucrose, xylose, and raffinose were purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade methanol and acetonitrile were products from Tedia Co. Inc. (Fairfield, OH). All other reagents were from analytical grade.

2.2 Microorganism

Aspergillus niger strain 426 was isolated from dried prunes and identified by the Institute of Food Technology (UNICAMP, Brazil). Stock cultures were stored at 4 °C on potato dextrose agar (PDA) slants. Microorganism activation was carried out in PDA plates at 28 °C for 3 days. The inoculum was prepared by harvesting the spores from 3-day-old cultures by adding 0.1% (v/v) Tween-80, and diluted to the desired spore concentration (1×10^9 spores/g substrate). The pour plate technique was used to calculate the number of spores.

2.3 Fermentation conditions

All cultivations were carried out in 250-mL Erlenmeyer flasks at a fixed total of 10 g of dry substrate. The substrate was moistened with a nutrient solution containing the following ingredients (g/L): $(\text{NH}_4)\text{H}_2\text{PO}_4$ (5.0), K_2HPO_4 (1.5), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5), KCl (0.5), yeast extract (5.0), and inducer (10.0). The pH was adjusted to 4.5 before sterilization. A volume equal to 10% (v/v) of inoculum was transferred to previously sterilized Erlenmeyer flasks containing 10 mL of nutrient solution with different components under investigation (orange rind, grapefruit rind, rice bran, and wheat bran) and incubated for 5 days at 28 °C with 97% (v/v) controlled atmospheric humidity. The moisture content was adjusted to 75% (v/v) by the addition of distilled water into the 250-mL Erlenmeyer flasks. With the fermentation flasks placed in an iced bath, the cell-free enzymatic extracts were recovered by adding 5 mL of sodium acetate buffer 50 mM, pH 4 and further mixing using a glass rod. Subsequently, the suspension was filtered and centrifuged 9000 g for 15 min at 4 °C and the clear, brown supernatant was used for subsequent analysis.

2.4 Substrate and inducer selection for naringinase production

In a first step, cultures were carried out in a 250-mL Erlenmeyer flask with 10 g of orange or grapefruit rind moistened with nutrient solution, but without the addition of inducers, to evaluate, between these two inducer substrates, which one was the best for naringinase production.

It was set up two EMD as a simplex centroid design (Table 1), with 2^{q-1} combinations of mixtures, where q , the number of components or variables whose sum is 1 or 100 %, to 3 components. First, grapefruit rind, rice bran, and wheat bran were used for substrate selection for naringinase production (EMD 1). Because the mixture space is a simplex, all design points must be at the vertices, on the edges or faces, or in the interior of a simplex [16]. Thus, runs with a single substrate, called linear blending (100%), 10 g of grapefruit rind, 10 g of rice bran, and 10 g of wheat bran was used. A special cubic model was used to represent the mean of the response variable as a function of the factors described in Equation 1: **see equation 1 in the supplementary files.**

where Y is the naringinase activity (U/mL), γ the estimated parameters, and x_i is the level of the independent variable. The statistical significance of the regression coefficients was determined by Fischer's test for analysis of variance (ANOVA) at a significance level of $p \leq 0.05$, and the extent of variance explained by each model was given by the determination coefficient R^2 . To minimize the error of ANOVA, the tests corresponding to the central point were repeated three times. Experimental and predicted values were compared to determine the validity of the models. The best mixture of substrates was used to study the ideal concentration of inducers naringin, rutin, and hesperidin from 5 to 25 g/L added in a nutrient solution.

In a second step, EMD 2 was also set up as a simplex centroid design (Table 1) and the components studied were naringin, rutin, and hesperidin at a fixed total concentration of 10 g/L. Control of each

condition was conducted without the addition of spores to the nutrient solution for the subsequent quantification of residual inducers. The remaining culture solution components were always the same, and the culture conditions were as described above. All the EMD design and analysis were performed using STATISTICA 7.0 software [17].

2.5 Naringinase Activity

The modified method of Thammawat et al. [18] was followed to assess naringinase activity. 1 mL of enzyme extract was added to 1 mL of 1% (w/v) naringin in an acetate buffer 0.1 M (pH 4.0). After incubation at 50 °C for 60 min, 0.1 mL was withdrawn. To this solution, 3.0 mL of diethylene glycol (90%, v/v) and 0.1 mL of (4.0 N) NaOH were added and incubated for 10 min at room temperature. The intensity of the yellow color produced was measured at 420 nm. One unit of naringinase activity is defined as the amount of enzyme that is required to hydrolyze 1 µmol of naringin per ml per minute, under the assay conditions.

2.6 Quantification of sugars

The extraction of soluble sugars from defatted substrates was performed according to the methodology proposed by Saravitz et al. [19] and filtrated through 0.45 µm filter (Millipore). When finished, the filtrate collects in the attached 1 mL Eppendorf tube. For the analysis of sugars, aliquots of 20 µL extracted samples were injected to HPLC (Model: Hitachi L-5000) using an Aminex HPX-87P column (300 x 7.8 mm) and eluted in a mobile phase of acetonitrile: water (75:25, v/v) in the following chromatographic conditions: 35 °C injection temperature, a flow rate of 1 mL/min in an HPLC system equipped with a RID detector (Shimadzu model RID - 10A). Each analysis was performed in triplicate. The total sugar level in each culture was determined using the phenol-sulfuric method described by Dubois et al. [20].

2.7 Quantification of citric acid from grapefruit rind

After extraction of soluble sugars from grapefruit rind, the material was resuspended in 500 µL of methanol. Citric acid was analyzed by reversed-phase chromatography (RP-HPLC) system equipped with a model SPD-M10A VP photodiode-array (PDA) detector (Shimadzu Scientific Instruments, Kyoto, Japan). The culture supernatant was filtered through 0.22 µm nylon filter. Citric acid was separated using RP-18 column. The mobile phase consisted of 0.1% (w/v) phosphoric acid with a flow rate of 1 mL/min. The detection was at 210 nm. It was identified and quantitated by comparing the retention time and peak area with solutions of pure citric acid.

2.8 Quantification of flavonoids

An aliquot (2 mL) of fermentation extracts was filtered through 0.45 µm Teflon membranes. Before the injection into the chromatographer column, 400 µL of the samples were added to 600 µL of methanol. An HPLC (Model: Hitachi L-5000) coupled with a Symmetry C₁₈ reverse-phase column (bonded C₁₈ ligands

on a high-purity base-deactivated silica) (4.6 × 150 mm, 3.5 μm) and a 2487 UV detector (Waters Corp., Milford, MA) was used to determine the concentrations of residual flavonoids. Following an injection of 20 μL of the reaction mixture, the column was eluted using a gradient elution at 35 °C and 0.4 mL/min. The mobile phase was composed of 11.4% methanol, 22.4% acetonitrile, and 62% purified deionized water. The target compounds were captured by a 2487 UV detector at 280 nm. The same methodology was used to quantify flavonoids impregnated in defatted grapefruit rind resuspended in 500 μL of methanol.

3. Results And Discussion

In previous shake-flasks experiments with *A. niger* 426 at 28 °C and pH 4.5, which were conducted on 10 g of orange or grapefruit rind added to a nutrient solution but without any addition of inducer, naringinase production was observed on both substrates. Maximal enzyme production was observed on grapefruit rind (1.92 ± 0.23 U/mL) against 1.05 ± 0.26 U/mL of activity when orange rind was tested. These results were supported by Mendoza-Cal et al. [21] who also yielded higher amounts of naringinase using grapefruit rind instead of orange rind as substrate. Puri et al. [2] reported that citrus peel powder contains high proportions of polyphenols, which act as inducers on naringinase production. The increased production of naringinase with *A. niger* on grapefruit rind is probably due to its very high naringin content and very low content of naringinase inhibitor compounds, such as monosaccharides when compared with orange rind [6, 22]. When the levels of monosaccharides decrease, naringinase synthesis is induced by naringin, leading to an increased naringinase production. This induction mechanism may help *A. niger* to degrade naringin to access further other nutrient supplies in the media, especially carbon [23]. Because of its high level of flavonoids, grapefruit rind was selected as one of the substrates for more in-depth studies of naringinase production. This is advantageous from an industrial point of view because naringinase can be produced cheaply by using agricultural waste.

3.1 Effect of substrate on naringinase production

The effect of the inclusion of naringin (10 g / L) was investigated. Since the variation in pH and temperature of the initial medium can have significant effects on the growth and production of naringinase by species of different fungi, including *Aspergillus*, we chose the acid pH 4.5, below or higher than this pH range, which can result in a drastic reduction in naringinase activity. Since the molecular charges and consequently the molecular interactions and functions are directly related to the pH of the medium; so any change in average pH affects many biological functions. Therefore, at 28 °C and pH 4.5, the time of 24 and 120 hours of SSF by *A. niger* showed greater production of naringinase, 22.4 ± 0.73 U / mL, respectively (Fig. 1). The central point was composed of a ternary mixture of 33.33% of each support substrate (grapefruit peel, rice bran, and wheat bran). After 120 h, 97% of the carbon source consumed in 5 mL of fermentation extract had been consumed and the enzyme formation was reduced. In this way, all of the following crops were carried out for only 5 days.

Table 1 summarizes the different combinations of grapefruit peel, rice bran, and wheat bran used for *A. niger* crops, and the maximum activities, in terms of naringinase for EMD 1. The maximum values of naringinase obtained in the experiments varied from 1.6 (run 1) to 22.6 U / mL (run 7), (Table 1).

The special cubic model was determined to estimate naringinase titers in terms of the concentrations of the components grapefruit rind (x_1), rice bran (x_2), and wheat bran (x_3) in the cultivation medium, as described by Eq. (2), in which terms with an asterisk are significant ($p < 0.05$). **See equation 2 in the supplementary files.**

A preliminary analysis of this data revealed the significance of the agro-industrial residues used as substrates for naringinase production. All linear blendings showed a statistically significant positive influence on naringinase activity titers since p -values were smaller than 0.05 for each of the components. As linear blending, wheat bran was significant in the highest level; which means that an increase of this substrate in the culture medium could improve the results. However, the quadratic term x_1x_2 (grapefruit rind and rice bran) was not significant, with $p > 0.05$. The most significant effect on naringinase activity was composed of a ternary mixture of each supporting substrate ($x_1x_2x_3$), followed by the quadratic term (x_1x_3). The determination coefficient for naringinase production was 99.96% for EMD 1. According to Silva et al. [24] values of $R^2 > 90\%$ are very good in the experimental design of bioprocesses.

Since the least favorable condition was grapefruit rind as a sole substrate (1.6 U/mL, Table 1) and due to the presence of citric acid in grapefruit juice, it was decided to study the effect of citric acid and sugars on naringinase activity. Norouzian et al. [25] reported that citric acid at 0.02 M non-competitively inhibited naringinase activity. Repression of naringinase activity by glucose and sucrose was also reported by Puri et al. [6], although these carbon sources supported excellent growth.

A quantitative HPLC analysis of substrates sugars found that grapefruit rind presented high amounts of glucose and sucrose (625.299 and 4.273 mg/g of dry substrate, respectively), (Table 2). Unlike wheat bran that presented the lowest amount of glucose (12.2 mg/g of the dry substrate). The number of sugars present in grapefruit rind might suggest a change in the metabolism of *A. niger* and a subsequent decrease in naringinase activity, this might also be due to citric acid amount 305.55 ± 0.172 mg/g of dry substrate, which represents 1.59 M present in 10 g of grapefruit rind, and could cause inhibition of naringinase biosynthesis. Although, grapefruit rind has a high content of naringin and rutin, 8.884 ± 0.464 and 5.503 ± 0.65 mg/g of dry substrate, respectively, which act as flavonoids inducers for naringinase production, so it can't be discarded as a substrate in the mixture.

The models used to describe the effects of each component on naringinase production (Eq. (2)) were used to generate the contour plots shown in Fig. 2a. These results revealed a broad region of elevated naringinase production. Using our data, a predictive analysis estimated the maximum naringinase activity to be 23.94 U/mL in cultures that contained 2.3 g of grapefruit rind, 2.5 g of rice bran, and 5.2 g of

wheat bran. Additional cultivation was performed to validate the proposed model and yielded a mean naringinase activity of 23.2 ± 0.02 U/mL. This result corresponded to 97% of the expected value, validating the effectiveness of the predictive model and confirming the substrate's proportions.

3.2 Comparison of different inducers on naringinase production

For a variety of applications, to increase the production of naringinase, an inducible enzyme, optimize the culture conditions, especially by performing the continuous or gradual addition of a type of inducer [26]. For the production of naringinase, the inducers reported are generally natural substrates or substrate analogs for the enzyme, such as naringin [6, 27], rutin [28] and hesperidin [29].

To evaluate the effect of flavonoid concentration on the production of naringinase by *A. niger*, the addition of naringin, rutin, and hesperidin in various concentrations was performed based on the best-selected condition of the EMD 1 culture. Naringinase activity was observed in all media (Fig. 3), maximum naringinase was produced with rutin (27.48 ± 1.23 U / mL) followed by naringin (23.45 ± 0.96 U / mL) and hesperidin (23.22 ± 1.12 U / mL) at a concentration of 10 g / L. The increase in the concentration of the inducers had no significant effect on the production of the enzyme. Custodio et al. [30] also reported that rutin (0.5%, w / v) was considered the most effective inducer of α -L-rhamnosidase production among quercitrin, naringin, naringenin, hesperetin, and hesperidin. On the other hand, Kumar et al. [28] reported a naringinase activity in different five media, that the enzyme naringinase had maximum production in naringin (7.48 IU / ml) while rutin (3.71 IU / ml). It is also worth mentioning that our products obtained in this work were much higher than the findings by Kumar et al. [28]. These same authors observed that with rutin and naringenin, naringinase was produced on the 8th day, but the enzyme activities were not distinguished on the 11th day.

To improve naringinase yield using citrus residues, Borkar et al. [15] studying the fermentative production of naringinase from *A. niger* van Tieghem MTCC 2425 with an inducer concentration of 14.9 g L^{-1} verified an ideal naringinase activity of 545.2 IU g^{-1} and concluded that the pH and the medium temperature act synergistically for the production of the enzyme, while the antagonistic behavior of the temperature and the concentration of the inducer at higher levels leads to a decrease in enzyme activity [15]. On the other hand, they obtained the activity of the enzyme can be completely reserved for up to 5 months at 4°C , same in the literature, naringinase from this species favored 45°C for naringinase activity [3]. However, researchers noted that scaling parameters vary significantly about microbial types, such as bacteria or fungi. About the fungus *Aspergillus niger*, studies that identify the most influential factors among the different media and process parameters during fermentation to produce naringinase are still scarce.

3.3 Effect of a mixture of inducers

To select the best mixture of inducers, fermentation experiments were performed based on the best condition selected from EMD 1 cultivation using 10 g/L of inducers concentration (Table 1, EMD 2). Maximum naringinase activities can be found in the central points that are composed of a ternary mixture of each inducer. According to the analysis of variance (ANOVA), the regression was statistically

significant ($p < 0.05$). The high value of the coefficient of determination ($R^2 = 0.994$) indicates that 99.4% of the variability of the responses can be explained by the model. The value of the adjusted determination coefficient (adjusted $R^2 = 0.977$) is also high, showing a high significance of the model.

In this way, the mathematical model representing naringinase production by adding inducers naringin (x_1), rutin (x_2) and hesperidin (x_3) in the experimental region considered here can be expressed as: **see equation 3 in the supplementary files.**

Similar to Eq. (2), Eq. (3) indicated that the most significant effect on naringinase activity was composed of a ternary mixture of each inducer, resulting in a naringinase activity of 28.16 U/mL (Table 1). Because $x_2 > x_3 > x_1$ we would conclude that rutin (x_2) increases naringinase production. Although all the linear terms, as well as the cubic term, had significant effects on the maximum attainable value of naringinase production, it can be seen that the effect of the quadratic term was not significant ($p > 0.05$). Furthermore, because x_2x_3 is negative, blending rutin and hesperidin would have an antagonistic effect, which means that the proportion of rutin in the blending should be greater than hesperidin. These results can provide a reference for inducer selection for similar studies in the future. To our knowledge, this is the first time that a mixture of inducers combined with the analysis of responses by Response Surface Methodology and optimization through a desirability function is used for producing naringinase.

According to Bokkenheuser et al. [31], rutin can be hydrolyzed to the monosaccharide 3-glucosylquercetin, which could be further hydrolyzed to the aglycone, quercetin by the β -D-glucosidase portion of naringinase.

A quantitative HPLC analysis of the fermentation broth revealed the presence of the inducer rutin in Controls 4-7, which represents cultivations without inoculation, and its aglycone quercetin in Runs 4-7 of the EMD 2 (Table 3). This indicates that *A. niger* successfully metabolized rutin. Additionally, rutin was detected at a concentration of 2.435 ± 0.422 mg/mL in Control 5 of EMD 2 even though it was not added. A possible explanation could be that rutin is coming from grapefruit rind, which has a rutin concentration of 5.503 ± 0.65 mg/g of the dry substrate (Table 2). This highlights the importance of agroindustrial residues application since this reduces the costs of production by the addition of flavonoids. This is advantageous on two levels, first because grapefruit rind is an inexpensive carbon source compared to other carbon sources and second its application on naringinase production could solve environmental problems resulting from grapefruit waste.

For better industry usage of this waste, it could be done a pre-treatment for monosaccharides inhibitor removal, but costs have to be taken into account so the process would remain financially advantageous.

The graph of the response surface (Fig. 2b) showed that maximum naringinase production could be achieved with a mixture of naringin, rutin, and hesperidin at a concentration of 2.5, 4.5, 3.0 g/L, respectively; furthermore, the inducer rutin would increase enzyme activity. The maximum predicted value was 27.86 U/mL. The mean value of the experimental validation of the optimized condition (28.10 ± 0.45

U/mL) was in excellent correlation with the predicted value, confirming the validity of the model. This activity is comparatively higher than those obtained by Petri et al. [32]. After medium optimization, these authors found a final α -L-rhamnosidase activity of 3.02 U/mL contained in 5 mL of enzymatic extract from SSF by *A. niger* 426.

4. Conclusions

In this work, the first agro-industrial waste was added to a standard medium for SSF, and the best result of the mixture of substrates was used in the second stage of the process. According to a statistical mixing project, the ternary mixture of each solid substrate (grapefruit peel, rice bran, and wheat bran) was also combined with a ternary mixture of inductors (naringin, rutin, and hesperidin) obtained better activity values of naringinase. This sequential procedure allowed the definition of an optimal mixture of components resulting in a 96% increase in the production of naringinase concerning the orange peel as a tested substrate without the addition of inductors. The results suggest that the proportion of these components can be applied for further investigation, as a lower-cost alternative, adding economic value to waste and by-products and, on the other hand, solving the problem of its disposal, in the production of enzymes, specifically naringinase. Although the grapefruit peel decreases the production of naringinase, it cannot be discarded as a carbon source, due to the large number of flavonoids impregnated in this substrate.

Declarations

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Author information

Department of Biochemistry and Biotechnology, State University of Londrina, 86051-980, Londrina, Paraná, Brazil.

Fernanda de Oliveira, Marcelo Rodrigues de Melo and João Batista Buzato.

Departamento de Tecnologia, UNESP - Univ Estadual Paulista, Faculdade de Ciências Agrárias e Veterinárias, Rod. Prof. Paulo Donato Castellane km 5, CEP 14884-900, Jaboticabal, SP, Brasil.

Tereza Cristina Luque Castellane

Authors' Contributions

Fernanda de Oliveira: research conceptualization, data curation, investigation and methodology, writing;
Tereza Cristina Luque Castellane: HPLC investigation and methodology, critically revised the manuscript;

Marcelo Rodrigues de Melo and João Batista Buzato: research conceptualization and supervision.

Corresponding authors

Correspondence to Tereza Cristina Luque Castellane and Fernanda de Oliveira.

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Ethics declarations

Conflict of interest

Fernanda de Oliveira, Tereza Cristina Luque Castellane, Marcelo Rodrigues de Melo, and João Batista Buzato declare that they have no conflict of interest.

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Tables

Table 1. Summary of naringinase maximum values for the *A. niger* according to EMD, using components grapefruit rind, rice bran, and wheat bran at a total amount of 10 g for EMD 1, and inducers (g/L) naringin, rutin and hesperidin at a total concentration of 10 g/L for EMD 2. EMD 2 was performed based on the best condition selected from EMD 1. Experiments were run using SSF for 5 days at 28 °C, with initial pH 4.5.

Run number	Coded mixture (x_1 ; x_2 ; x_3)**	Original mixture* (x_1 ; x_2 ; x_3)**	EMD 1 naringinase activity (U/mL)	EMD 2 naringinase activity (U/mL)
1	(1; 0; 0)	(10.0; 0; 0)	1.60	23.56
2	(0; 1; 0)	(0; 10.0; 0)	17.40	25.5
3	(0; 0; 1)	(0; 0; 10.0)	18.30	26.82
4	(5; 5; 0)	(5.0; 5.0; 0)	8.70	24.56
5	(5; 0; 5)	(5.0; 0; 5.0)	20.60	25.95
6	(0; 5; 5)	(0; 5.0; 5.0)	20.10	24.89
7	(3.3; 3.3; 3.3)	(3.3; 3.3; 3.3)	22.60	28.16
8	(3.3; 3.3; 3.3)	(3.3; 3.3; 3.3)	22.00	27.67
9	(3.3; 3.3; 3.3)	(3.3; 3.3; 3.3)	22.30	27.92

*total amount of 10 g of substrate for EMD 1 and total concentration of 10 g/L for EMD 2

** x_1 grapefruit rind, x_2 rice bran, x_3 wheat bran for EMD 1 and x_1 naringin, x_2 rutin, x_3 hesperidin for EMD 2

Table 2. Quantitative HPLC analysis for quantification of sugars, citric acid and flavonoids.

Compound (mg/g of dry substrate)	Grapefruit rind	Rice bran	Wheat bran
Glucose	625.299 ± 4.202	64.126 ± 8.741	12.173 ± 0.565
Sucrose	4.273 ± 0.046	0.275 ± 0.033	0.267 ± 0.021
Xylose	ND	45.039 ± 4.504	ND
Raffinose	ND	*	*
Citric acid	305.55 ± 0.172	ND	ND
Naringin	8.884 ± 0.464	ND	ND
Rutin	5.503 ± 0.65	ND	ND

All procedures were performed in triplicate. Mean values (\pm standard deviation)

ND = Not detected

**Values below the limit of the method*

Table 3. Quantitative HPLC analysis for quantification of flavonoids in tests of EMD 2 (Table 1).

Run number	Original mixture ($x_1; x_2; x_3$)**	Rutin (mg/mL)	Quercetin (mg/mL)
Control 4	(5.0; 5.0; 0)	4.071 ± 0.784	ND
Control 5	(5.0; 0; 5.0)	2.435 ± 0.422	ND
Control 6	(0; 5.0; 5.0)	5.080 ± 1.427	ND
Control 7	(3.3; 3.3; 3.3)	2.499 ± 0.472	ND
Run 4	(5.0; 5.0; 0)	*	1.873 ± 4.950
Run 5	(5.0; 0; 5.0)	*	1.578 ± 10.382
Run 6	(0; 5.0; 5.0)	*	2.634 ± 0.017
Run 7	(3.3; 3.3; 3.3)	*	1.905 ± 1.208

All procedures were performed in triplicate. Mean values (\pm standard deviation)

ND = Not detected

*** x_1 naringin, x_2 rutin and x_3 hesperidin*

**Values below the limit of the method*

Figures

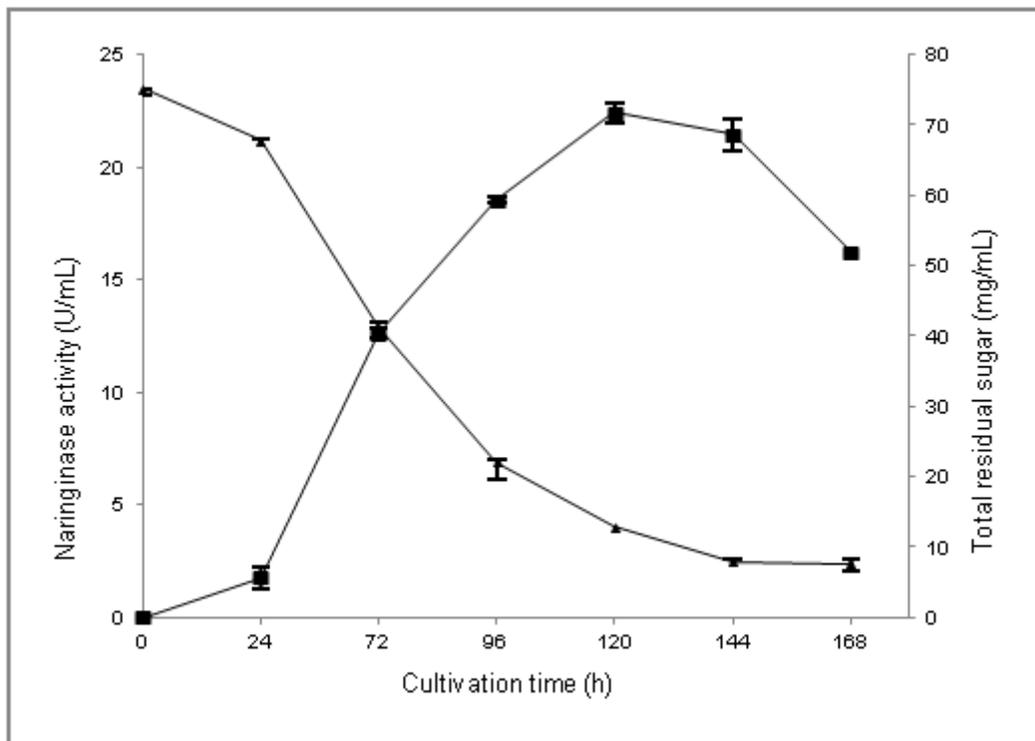


Figure 1

Kinetics of the central point of EMD 1 for naringinase production by *A. niger* at 28 °C, pH 4.5 and naringin (10 g/L) as inducer. Each data point represents the mean of triplicate determinations \pm SD.

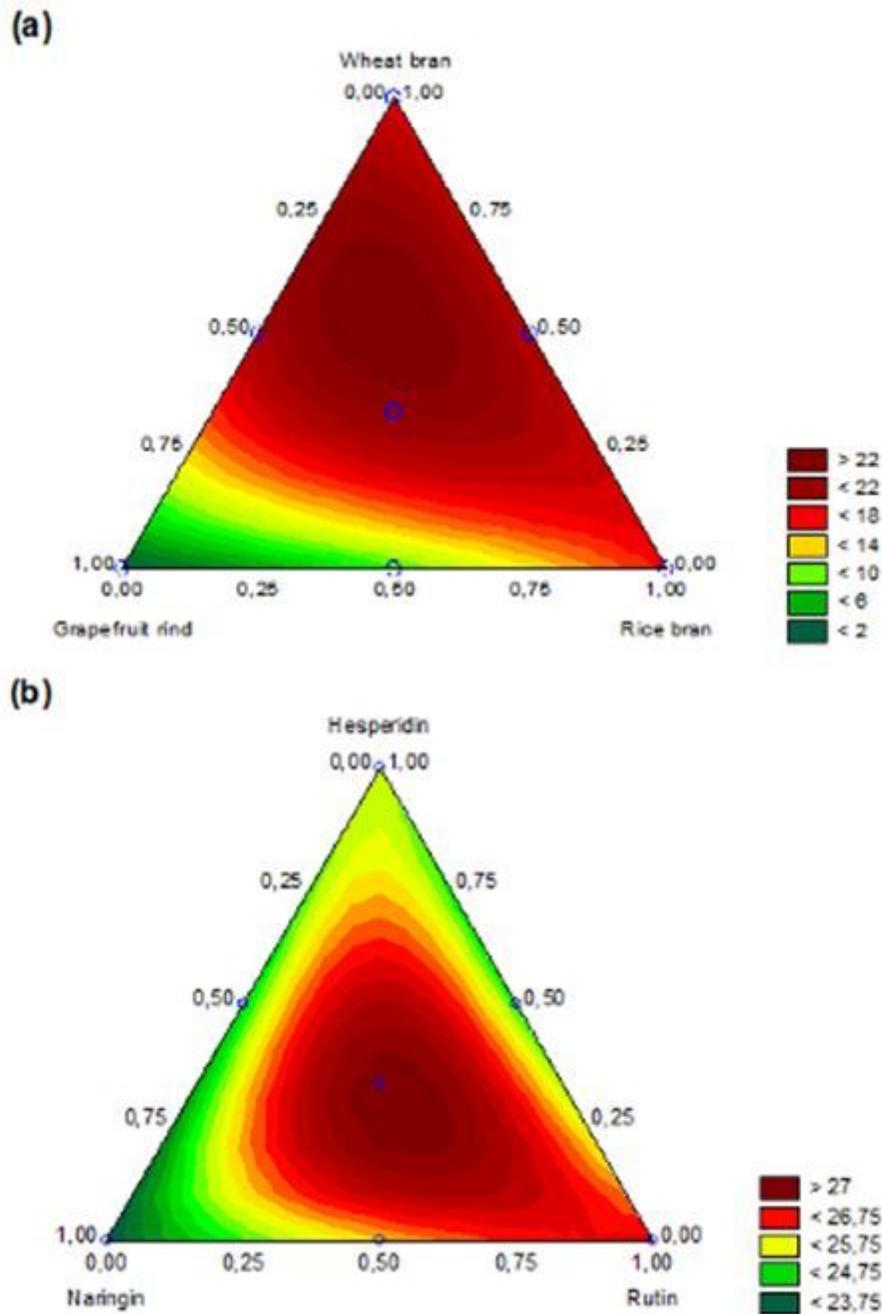


Figure 2

Contour plots of naringinase responses for *A. niger* cultivation using the culture medium components, (a): (10 g) grapefruit rind, rice bran and wheat bran; and (b): (10 g/L) naringin, rutin and hesperidin. Fermentation experiments were run using SSF for 5 days at 28 °C, with initial pH 4.5.

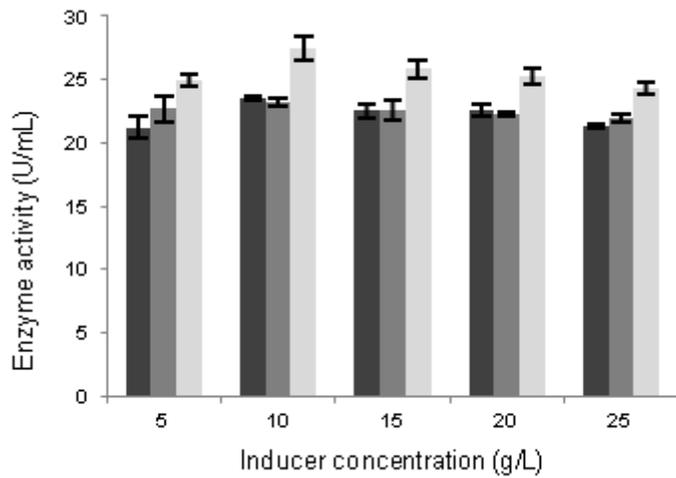


Figure 3

Effect of different inducers (■ naringin, ■ hesperidin and ■ rutin) on naringinase produced by *A. niger* 426 using SSF for 5 days at 28 °C, with initial pH 4.5. Fermentation experiments were performed based on the best condition selected from the first EMD cultivation. Each data point represents the mean of triplicate determinations \pm SD.