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Modelling of the high pressure–temperature effects on naringin hydrolysis based on response surface methodology

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Abstract

The aim of this study was the modelling, under high pressure, of naringin hydrolysis by naringinase. Response surface methodology (RSM) was used to compare the effects of the selected variables on the bioconversion under study. The combined action of temperature (13–61 °C) and pressure (80–216 MPa) on the catalytic activity of naringinase was investigated at pH 4.0 using naringin as the substrate. The choice of experimental domains resulted from preliminary studies.

Naringinase activity, for naringin hydrolysis at pH 4.0, could be described by a convex surface with a maximum of 0.13 mM min^{-1}, at 41 °C and 158 MPa. After 1 h of reaction time, reducing sugars production could also be described by a convex surface, with a maximum reducing sugars concentration of 8 mM at 38 °C and 168 MPa. The interaction temperature–pressure had a significant effect on both naringinase activity and reducing sugar formation after 1 h.

Under the optimized conditions, the naringin hydrolysis by naringinase was evaluated.

Keywords: High pressure–temperature effects; Naringin; Naringinase; RSM

1. Introduction

In recent years, biocatalysis offers a number of key advantages over chemical synthesis when working on complex molecules, advantages based on the chemo-, regio- and stereoselectivity of enzymes and conditions. The use of high pressure for the enzymatic synthesis of pharmacological molecules, namely flavonoids, is interesting. Flavonoids are a class of plant specific natural products involved in nodulation, UV protection and host defence in plants and various health-promoting effects in humans, such as antitumor, prevention of cardiovascular diseases, inhibition of enzymatic lipoperoxidation, and anti-inflammatory and antithrombotic effects (Di Carlo, Mascolo, Izzo, & Capasso, 1999). Flavonoids are potential chemopreventive agents, due to their activities as inhibitors of enzymes involved in the biotransformation of precarcinogens. Naringinase, an α-rhamnopyranosidase, hydrolyze naringin to naringenin which has important biological effects, such as anti-oxidant, anti-ulcer, anti-mutagenic, anti-inflammatory, anti-thrombotic, vasodilator, and anticancer effects, inhibiting the proliferation of breast cancer and delaying mammary tumorigenesis (Nishino, Ngao, Fujili, & Sugimura, 1983).

High pressure can be used to modulate both the stability and activity of several enzymes (Heremans & Smeller, 1998), can, also modify the catalytic behaviour of enzymes by changing the rate-limiting step or modulating the selectivity of the enzyme (Mozhaev, Heremans, Frank, Mansson, & Balny, 1996). It was found that pressure could

Abbreviations: CCRD, central composite rotatable design; CV, coefficient of variation; RSM, response surface methodology; R^2, determination coefficient (quadratic correlation coefficient); R^2_{adj}, adjusted determination coefficient; *P*, pressure; *T*, temperature.

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either activate or inhibit enzymatic activities depending on the proteins involved and conditions (Pedro et al., 2007; Vila-Real, Alfaia, Calado, & Ribeiro, 2007). These results underline the importance of investigating the influence of high pressure on a wide range of enzyme systems. In this work, the effects of high pressure and temperature on naringinase activity was investigated. Response surface methodology (RSM) is an efficient statistical technique for the modelling and optimization of multiple variables in order to predict the best performance conditions with a minimum number of experiments (Giovanni, 1983). This is a non-conventional approach that has been successfully used for the optimization of enzymatic reactions conditions (Ferreira-Dias, Correia, & da Fonseca, 2003; Ribeiro, Silveira, & Ferreira-Dias, 2003), medium composition (Ribeiro, Manha, & Brito, 2006; Kapat, Rakshit, & Panda, 1996; Montserrat, Inaki, François, Francesco, & Carles, 1993) and food preservation parameters (King, 1993). In this work, central composite rotatable design (CCRD) and RSM were used to compare the conjugated effects of temperature and pressure on naringin bioconversion by naringinase. The hydrolysis was carried out at high pressure (80–216 MPa) as a function of temperature (13–61 °C) in acetate buffer (0.02 M, pH 4.0), with 750 mg l^{-1} of naringin and 250 mg l^{-1} of naringinase.

2. Materials and methods

2.1. Materials

Naringin and naringinase (CAS Number 9068-31-9) were from Sigma Aldrich. The enzyme was kept at 0 °C. All other chemicals were of analytical grade and obtained from various sources.

2.2. Analytical methods

Total content of sugars was assayed by the 2,4-dinitrosalicylic acid (DNS) method (Miller, 1959). Standardization was obtained with different concentrations of an equimolar mixture of D-glucose and D-rhamnose.

Any contribution of thermal or pressure hydrolysis was rejected as no reducing sugars were observed after incubating the naringin solution at different pressures and temperatures.

Protein (naringinase) determination was carried out by the Bradford method.

2.3. High pressure apparatus and operation

The experimental apparatus for high hydrostatic pressure experiments was made up of a 400 MPa manual pump (Enerpac, model P228) and a high pressure vessel made of steel, according to the sketch in Fig. 1. The pressurization fluid was hydraulic oil (Enerpac HF 95 Y) and the pressure was controlled using a pressure gauge (Budenberg Gauge Co. Limited). The temperature was maintained constant to within ±0.1 °C by circulating thermostatted water.

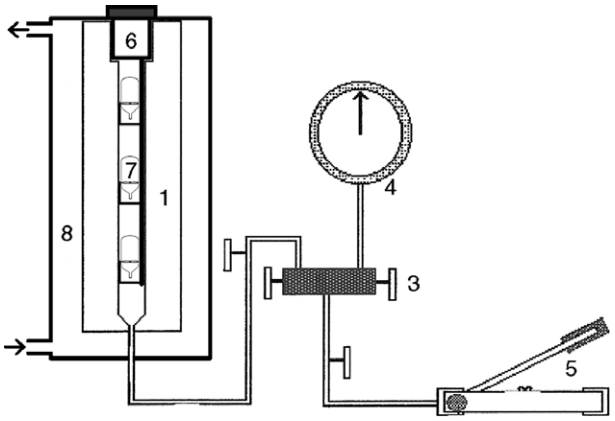

Fig. 1. High pressure apparatus: (1) high pressure vessel; (2) steel pipe; (3) valve; (4) pressure gauge; (5) manual pump; (6) vessel cap; (7) reaction cell; and (8) thermostatic bath.

2.4. Bioconversion studies

Naringin bioconversion studies were carried out in standard solutions of naringin (acetate buffer 0.02 M, pH 4.0), as a function of temperature and pressure, according to the experimental design followed (c.f. 2.5). The enzymatic reaction was conducted on cylindrical cells made of glass with an internal volume of 15 ml. Three cells were put simultaneously inside the high pressure vessel. The pressure was increased steadily within 1–3 min and maintained for different periods of time, respectively, 0, 5, 10, 15, 20, 30 and 60 min, in order to evaluate reducing sugars formation. Subsequently, the pressure was released within 1 min and the reaction was promptly stopped, lowering the temperature of the solutions above 0 °C. Each measurement required a new experiment because depressurization was necessary at the end of the incubation period under high pressure. Initial rates of naringin conversion (activity of naringinase) were calculated by linear regression on the data-points during the first reaction minutes (reducing sugars concentration *versus* time). Results were based on triplicate determinations.

2.5. Experimental design

The best reaction conditions (temperature and pressure) were established via response surface methodology (RSM). It consists of a set of mathematical and statistical methods developed for modelling phenomena and finding combinations of a number of experimental factors (variables) that will lead to optimum responses (Giovanni, 1983). With RSM, several variables are tested simultaneously with a minimum number of trials, according to special experimental designs, which elucidates interactions between variables (Giovanni, 1983). This is not an option with classical approaches. In addition, RSM has the advantage of being

less expensive and less time-consuming than the classical methods.

The best conditions for the bioconversion of naringin at high pressure, with soluble naringinase, were established via RSM.

The response y is described by a polynomial equation as a function of the p independent variables, x_i, that is, $y = f(x_1, x_2, \ldots, x_p) + \varepsilon$, where ε represents the error observed in the response y; usually, the response is well modelled by a first or a second-order polynomial, representing a $(p+1)$-dimensional surface, i.e., the *response surface*. The parameters of these equations are usually unknown and, therefore, must be estimated from the experimental data by using the statistical principle of least squares. In second-order equations, the coefficients of the squared terms influence the direction of the curvature of the response surfaces. The designs most commonly used to fit first order models are the 2^p full factorial design. In addition to the 2^p points, a centre point (repeated several times) is frequently added to the designs. They are used to provide an estimation of the variance of the experimental error, which is assumed to be constant along the experimental domain. The contribution to the error variation is not only due to the experimental errors alone, but also to the lack of fit of the estimated model (Giovanni, 1983).

To fit second-order models, composite designs are usually followed. They consist of augmented 2^p factorial designs with star points (also called axial points) and center points. In our study, the experiments were carried out with a central composite rotatable design (CCRD) (Vuataz, 1986) as a function of temperature and pressure (Table 1). With central composite rotatable design, five levels for each factor were used, which allowed fit of first or second-order polynomials to the experimental data points. Therefore, curved surfaces can be fitted to the experimental data. Partial differentiation of these polynomial equations is used to find the optimum points, i.e. stationary points (Weisberg, 1985). However, the identification for each variable, on the regions corresponding to optimal responses, may be directly achieved by visual examination of the response surfaces and/or contour plots.

In all, 11 experiments were carried out in CCRD: four factorial points [coded levels as (+1) and (−1)]; four star points [coded as $(+\sqrt{2})$ and $(-\sqrt{2})$] and three centre points, coded as 0 (Table 2).

Table 1
Coded and decoded levels of the experimental factors used in central composite rotatable design

CCRD	Pressure (MPa)	Temperature (°C)
$-\sqrt{2}$	103.4	13
−1	120.0	20
0	160.0	37
+1	200.0	54
$+\sqrt{2}$	216.5	61

Table 2
Experimental data obtained for the optimization of temperature and pressure, on naringin hydrolysis

No.	Pressure (MPa)	Temperature (°C)	Activity (mM min^{-1})
1	120.0	20	0.0111
2	120.0	54	0.035
3	200.0	20	0.0202
4	200.0	54	0.0247
5	103.4	37	0.0233
6	216.5	37	0.0644
7	160.0	13	0.0054
8	160.0	61	0.0223
9	160.0	37	0.0813
10	160.0	37	0.0813
11	160.0	37	0.0813

2.6. Data analysis

The results of each CCRD were analyzed using the software "StatisticaTM", version 5, from Statsoft, USA. Both linear and quadratic effects of the two variables under study, as well as their interactions, on naringinase activity and reducing sugars formation, after 1 h of reaction, were calculated. Their significance was evaluated by analysis of variance. A surface, described by a second-order polynomial equation, was fitted to each set of experimental data points. First and second-order coefficients of the polynomial equations were generated by regression analysis. The fit of the models was evaluated by the determination coefficients (R^2) and adjusted R^2 (R^2_{adj}).

3. Results and discussion

Naringin hydrolysis, by naringinase, was carried out for 1 h, according to CCRD as a function of both the temperature (T) and pressure (P) (Table 2).

The significant effects of the temperature, pressure and interaction ($T \times P$) on the naringinase activity and reducing sugars formation, after 1 h, are shown in Table 3.

Multiple regression coefficients, obtained by employing a least squares technique to predict a quadratic polynomial model for naringinase activity and reducing sugars formation after 1 h, are summarized in Table 4. Examination of these coefficients with the t-test indicated that, in naringin-

Table 3
Effects and respective significance levels (p) of pressure (P) and temperature (T) on naringinase activity and reducing sugars formation after 1 h of naringin hydrolysis

Variables	Naringinase activity (mM.min^{-1})	$[\text{Reducing sugars}]_{1h}$ (mM)
P (linear term)	0.00294	0.343^{***}
P (quadratic term)	-0.0457^{**}	-0.831^{*}
T (linear term)	0.0179	0.232
T (quadratic term)	-0.0657^{***}	-2.20^{**}
$P \times T$	−0.0192	0.0225

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Table 4
Second-order model equations for the response surfaces fitted to the experimental data points, as a function of temperature (T) and pressure (P), respectively, coefficient of determination, R^2 and R^2_{adj} and coefficient of variation, CV %

Model equations	R^2	R^2_{adj}	CV %
$\text{Activity} = -0.549 - 1.4 \times 10^{-7}(P)^2 + 0.0005(P) + 0.00011(T)^2 + 0.0112(T) - 1.4 \times 10^{-6}(P) \times (T)$	0.929	0.858	0.80
$[\text{Reducing sugars}]_{1h} = 9.634 - 2.6 \times 10^{-6}(P)^2 + 0.00868(P) + 0.0038(T)^2 + 0.285(T) + 1.65 \times 10^{-6}(P) \times (T)$	0.897	0.830	3.05

ase activity, quadratic terms of pressure and temperature, were highly significant, $p < 0.01$ and $p < 0.001$, respectively (Table 3). Linear and quadratic terms of pressure in reducing sugars formation, in naringin hydrolysis, were significant, respectively, $p < 0.001$ and $p < 0.05$, and the quadratic term of temperature was significant ($p < 0.01$) (Tables 3 and 4). There were no significant interactions between the variables tested ($p \geqslant 0.05$). A negative interaction, pressure–temperature ($P \times T$) indicates that higher naringinase activities are obtained at higher pressure and at moderate temperatures.

The contributions of linear and quadratic terms to the model were 7.4% and 81.3%, respectively, for naringinase activity and 4.3% and 85.4% for reducing sugars formation.

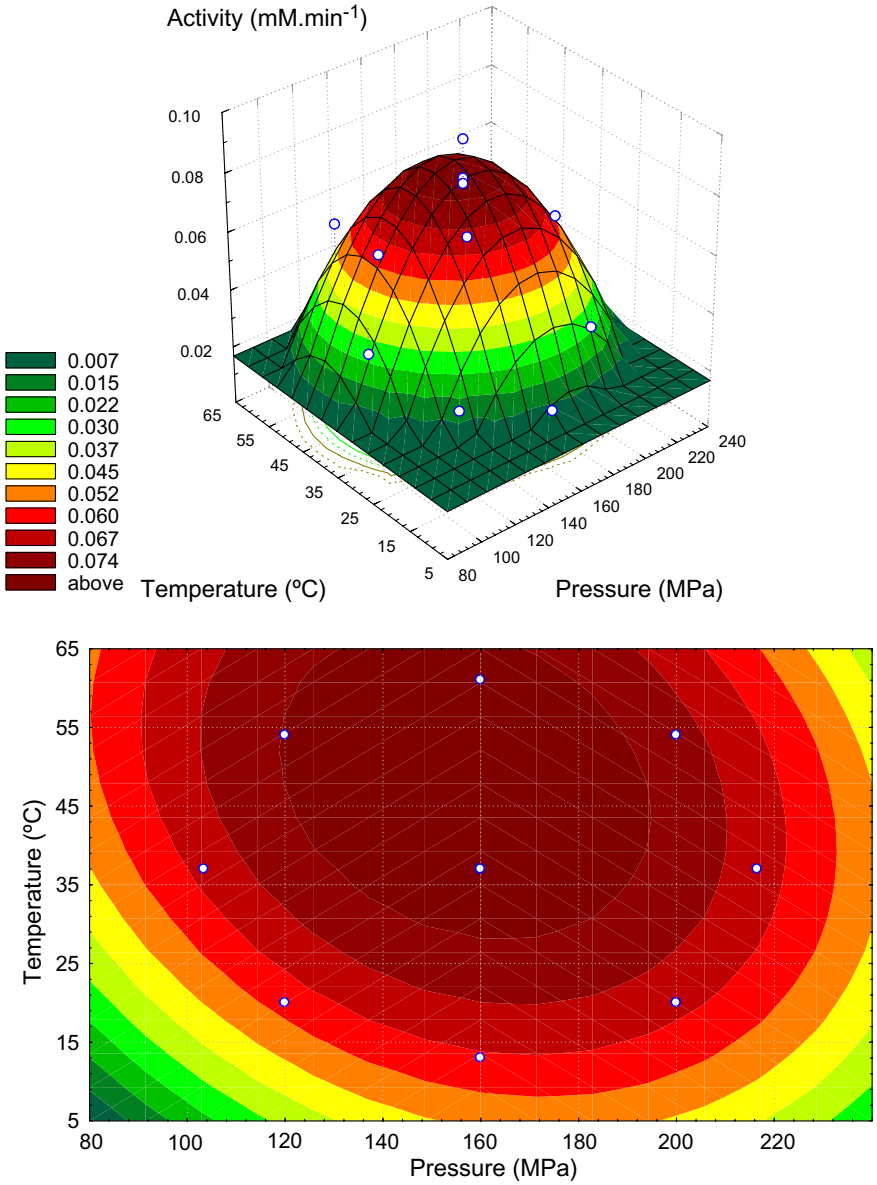

Fig. 2. Response surface and respective contour plot, fitted to the experimental data points, corresponding to naringinase activity, as a function of temperature (°C) and pressure (MPa). Bioconversion runs were carried out in acetate buffer 0.02 M, pH 4, with 750 mg l^{-1} of naringin and 250 mg l^{-1} of naringinase.

The multiple regression analysis performed to fit the second order polynomial equations to the experimental data points (Table 4), can be described by response surfaces (Figs. 2 and 3). In the design of these models, the significance effects ($p < 0.05$) and those having a confidence interval smaller than the value of the effect, or smaller than the standard deviation (data not shown), were considered. In fact, these last effects have a lower probability, but their values are not small enough to be neglected.

Figs. 2 and 3 show the relationship between independent and dependent variables in three-dimensional representations of the response surfaces for naringinase activity and reducing sugars formation after 1 h of reaction time. Convex surfaces (Figs. 2 and 3) were obtained for both the naringinase activity and reducing sugars formation after 1 h. The high values of R^2 and R^2_{adj} of these models (Table 4) show a close agreement between the experimental results and the theoretical values predicted by these models (Vuataz, 1986). The adjusted coefficients of determination in naringinase hydrolysis, respectively for naringinase activity ($R^2_{adj} = 0.86$) and reducing formation after 1 h of reaction time ($R^2_{adj} = 0.83$), implied that 86% and 83%, respectively,

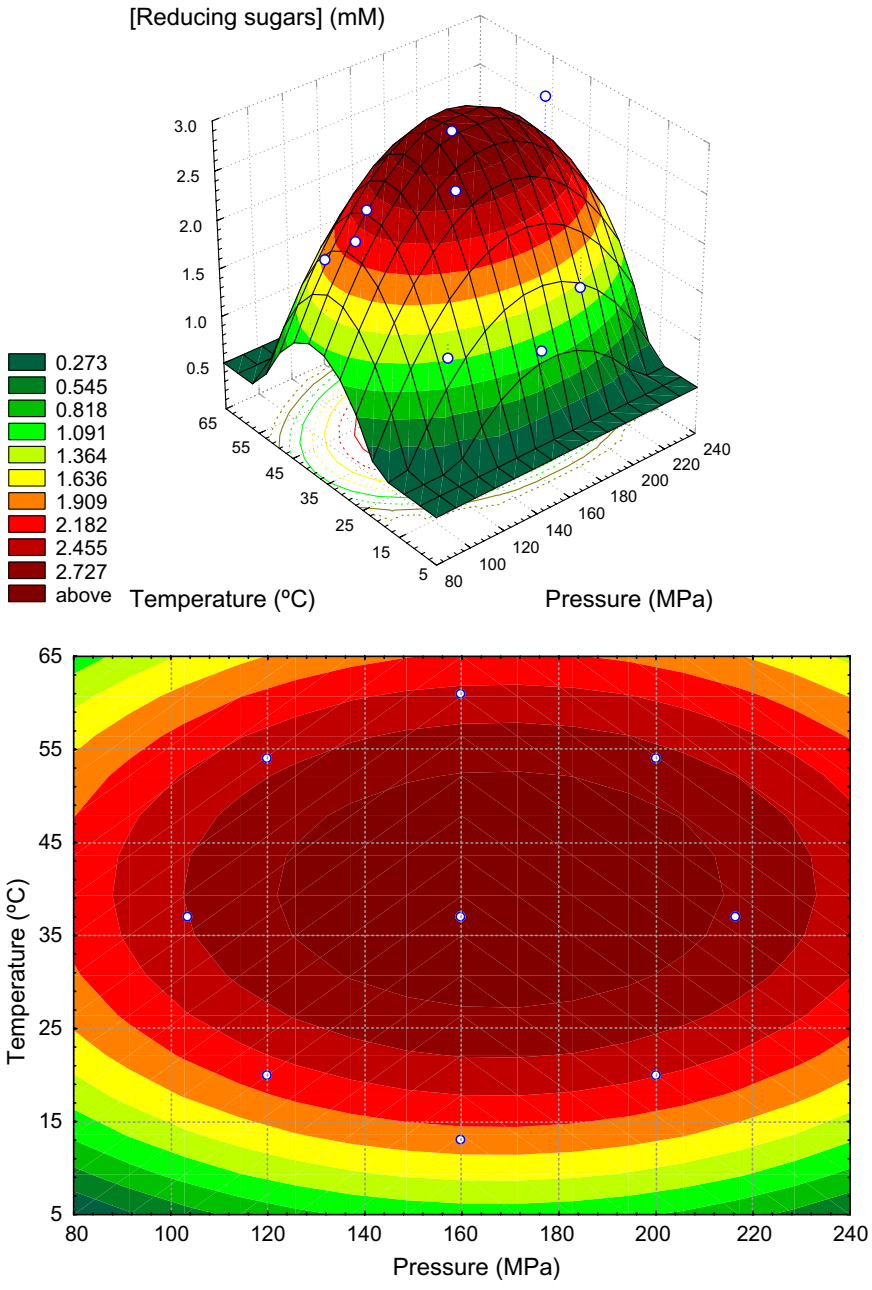

Fig. 3. Response surface and respective contour plot, fitted to the experimental data points, corresponding to reducing sugars production after 1 h of reaction time, as a function of temperature (°C) and pressure (MPa). Bioconversion runs were carried out in acetate buffer 0.02 M, pH 4, with 750 mg l^{-1} of naringin and 250 mg l^{-1} of naringinase.

Table 5
Analysis of variance (ANOVA) for second order polynomial model fitted to response surface

Source	D.F.[a]	Sum of squares	Mean square	*F*-value
Naringinase activity				
Lack of fit[b]	3	4.39×10^{-4}	1.46×10^{-4}	1.57[c]
Pure error	2	1.86×10^{-4}	9.31×10^{-5}	
Total error	5	8.78×10^{-3}		
[Reducing sugars]$_{1h}$				
Lack of fit sum of squares	5	0.804	0.201	20.17[c]
Pure error	2	0.018	0.009	
Total error	7	7.99		

[a] Degrees of freedom.
[b] Lack of fit, sum of squares (SS) = total SS − pure error SS.
[c] Not significant.

of the variations could be explained by the fitted models. A coefficient of variation (CV) less than 5% indicated that the model was reproducible, namely for naringinase activity (CV = 0.8%) and for reducing sugars formation after 1 h of reaction time (CV = 3%) (Table 3).

The analysis of variance (ANOVA) for a second order polynomial model fitted to the response surface is given in Table 5. The *F*-value for lack of fit is not significant, so the second order model, for naringinase activity, was appropriate for the description of the response surface (Table 5, Figs. 2 and 3).

The ANOVA for the two response variables (Table 6) indicated that the model developed for naringinase activity during naringin hydrolysis was adequate, with the quadratic term showing a highly significant effect (<0.5%).

From statistical analysis (Table 3), both temperature and pressure are important factors in naringin hydrolysis, because they affect naringinase activity and reducing sugars formation significantly ($p < 0.05$).

Since the models have shown lack of fit to be insignificant, the response surfaces (Figs. 2 and 3) were sufficiently explained by the regression equations. The regression models allowed prediction of the effects of the two parameters, pressure and temperature, on naringin hydrolysis. Moreover, the response and contour plots generated for naringinase activity (Fig. 2) showed that pressure and temperature influenced the response in a quadratic manner. Further, in reducing sugars formation after 1 h of reaction time, the response also increased in a quadratic manner with pressure and temperature (Fig. 3).

Table 6
Analysis of variance (ANOVA) of the regression parameters for response surface models[a]

Regression	D.F.[b]	Sum of squares	R^2	*F*-value
Naringinase activity				
Linear	2	6.53×10^{-4}	0.074	7.02
Quadratic	2	9.00×10^{-3}	0.813	31.68[c]
Cross product	2	3.69×10^{-4}	0.042	3.96
Total	6	1.00×10^{-2}	0.929	1.51
[Reducing sugars]$_{1h}$				
Linear	2	0.342	0.043	11.86
Quadratic	2	7.746	0.854	10.79[c]
Cross product	2	5.06×10^{-4}	6.00×10^{-5}	0.056
Total model	6	8.089	0.897	20.25[c]

[a] R^2 = coefficient of determination.
[b] Degrees of freedom.
[c] Significant at 0.5% level.

Table 7
Optimal pressure and temperature for the hydrolysis of naringin by naringinase, according to surface response methodology and experimental data

	Surface response	Experimental
Pressure (MPa)	158	160
Temperature (°C)	41	40
Activity (mM min^{-1})	0.13	0.11

The enzyme showed activity optima within the tested ranges. Table 7 compares the predicted maximum activity conditions and values with those obtained experimentally. These experimental values were reasonably close to the predicted values, confirming the validity and adequacy of the models. A maximum naringinase activity of 0.13 mM min^{-1} is expected at 41 °C and 158 MPa, while a maximum reducing sugars concentration, after 1 h of reaction, of 8 mM is expected at 38 °C and 168 MPa.

4. Conclusions

The modelling of the hydrolysis of naringin to naringenin, catalyzed by naringinase, was attempted by RSM. The reaction was well described by second-order models.

Naringin hydrolysis by naringinase in model solutions can be represented by a convex surface, described by 2nd order polynomials (Figs. 2 and 3 and Table 4).

Temperature and pressure showed a significant (quadratic) effect on naringinase activity (Fig. 2 and Table 3). The interaction temperature–pressure had a significant effect on naringin hydrolysis, for both naringinase activity and reducing sugar formed after 1 h of reaction time (Table 3). A maximum naringinase activity of 0.13 mM min^{-1} is expected at 41 °C and 158 MPa, while a maximum reducing sugars concentration of 8 mM is expected at 38 °C and 168 MPa. The high values of R^2 and R^2_{adj} of the models show close agreement between the experimental results and the theoretical values predicted by these models.

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